obacunone, limonin, nobiletin and silymarin, present in edible plants against AOM-induced colon tumorigenesis is described above. All these compounds are antioxidants. In general, plants are complicated mixtures of numerous chemicals, and interactions with their components may affect the effectiveness of an antioxidant. The effectiveness of the tested compounds as *in vivo* antioxidants has been reported, but the metabolic pathways and actions of naturally occurring antioxidative compounds are not clear. Flavonoid compounds, which are widely distributed in the plant kingdom and occur in considerable quantities, show a wide range of pharmacological activities other than their antioxidative properties. These compounds have been used to treat various pathological conditions, including allergies, inflammation and diabetes. There is accumulating experimental data, including this report, showing their antitumor activities: their chemopreventive potential, however, has not been fully proven clinically. Their behavior and fate should be investigated *in vivo*. As reported, commonly consumed foods contain non-nutritive compounds capable of inhibiting CRC in an animal model. The diet provides a rich abundance of these compounds, which have the ability to intervene in all phases of carcinogenesis. Mechanisms of action include effects on Phase I and Phase II enzymes activities, interception of DNA mutating agents and influences on cell proliferation and oncogene activation. Each of these mechanisms have been studied in isolation. In order to explain reduced risk for cancer in populations with a greater reliance on fruits and vegetables in the daily diet, future research should focus on potential combinations of foods and the protective components within them[156].

The association of certain malignancies with chronic inflammation has been recognized for many years[157]. The link between inflammation and subsequent malignancy in visceral sites is known. Examples include large bowel cancer after ulcerative colitis or Crohn's disease[157]. Central to the concept of inflammation and cancer is the finding that chronic irritation of the squamous or glandular epithelium results in migration of inflammatory cells to the injured site by a mechanism dependent on neutrophil adhesion molecules. These cells, stimulated to produce reactive oxygen species (including superoxide radicals, NO and/or hydroxy radicals) via a respiratory burst and NADPH activation, can function as facilitators in the process of carcinogenesis. There is convincing evidence from animal model systems that prolonged exposure of cells to these products of activated oxygen can result in cell injury and play a role in several stages of carcinogenesis[155,158,159]. Using our own model of colitis-related mouse colon carcinogenesis[155,160] (Fig. 6), several non-nutritive compounds have been demonstrated to suppress colonic epithelial malignancies in the inflamed colons of mice[161]. Recently, upregulation of COX-2, but not COX-1, gene expression was reported in human colorectal neoplasms[162]. New drugs, specific for inhibition of COX-2, may provide effective tumor prevention with reduced side effects[163,164]. The elevation of COX-2 expression can protect intestinal epithelial cells from apoptosis[165]. Certain COX-2 inhibitors can induce apoptosis[166] and inhibit tumor angiogenesis[167]. More recently, synthetic antioxidants have been reported to reduce COX-2 expression, PG production and proliferation of CRC cells[168]. This suggests that COX-2 may provide a new chemopreventive target in colorectal malignancies[164,169], if there are natural products from edible plants that are specific inhibitors of COX-2 expression.

From the evidence mentioned above, our search for chemopreventives against CRC focuses on several flavonoids and some other compounds possessing certain biological activities, including anti-inflammatory and/or antioxidative properties present in foods. Approximately 2,000 individual members of the flavonoid class have been described, and flavonoids are consumed in rather large amounts through dietary vegetables and fruits.

Future Prospects

An important component of the chemopreventive agent development research is the identification and characterization of intermediate biomarkers[76,170] that may serve as surrogate end points for cancer incidence reduction in chemoprevention clinical trials. This type of effort is critical to the progress of chemoprevention and potential for cost-effective development of chemopreventive research.

ACFs were first reported in rodents injected with AOM by Bird in 1987[171] and similar lesions were characterized in humans in 1991[90] and 1994[172] by Pretlow; since then, the AOM-induced ACF model has been the most widely used animal model system for evaluating natural and synthetic chemicals[70,71,77]. The growth dynamics, morphological and molecular features of ACFs support the contention that they are putative preneoplastic lesions. For instance, ACFs have a hyperproliferative, hyperplastic or dysplastic cryptal cells, and their sizes increase with time[173,174]. The nuclear atypia observed in some ACFs are similar to those seen in the crypts of adenocarcinomas in the colons[173]. Furthermore, identification of dysplasia and monoclonality strongly links this lesion to neoplastic progression[175]. Recently, two new types of early lesion that progress into colon neoplasms have been described in the colon. Yamada *et al.*[176] identified new possible precursor lesions, β-catenin accumulated crypts (BCAC), for colon carcinoma in whole-mount preparations of the colons of rats exposed to AOM using an immunohistochemical method. These lesions are different from ACFs in terms of their morphology and location. In the lesions, nuclear accumulation of β-catenin is more prominent than that observed in ACFs[176]. Cell proliferation activity estimated by counting the number of AgNORs/nucleus in the lesions is also greater than in ACFs[177]. In addition, Cademi *et al.*[178–180] identified mucin-depleted foci (MDF) in unsectioned colon stained with high iron diamine-alcian blue (HID-AB). These newly described lesions are not yet well characterized, and we do not know if BCACs and MDFs are related lesions. It is interesting to note that

BCAC, like MDF, have a low production of mucins and are thought to be premalignant lesions rather then preneoplastic lesions. A recent review article described the significance of these three lesions (ACF, BCAC and MDF) in colon carcinogenesis[181]. Since ACFs are widely accepted as a reliable end point in experimental colon carcinogenesis, this study reports the effects of herbal supplements on the "classical" ACF. We should thus estimate the reported chemopreventive efficacy of non-nutritives in edible plants using both ACFs and these new lesions as biological markers for colon carcinogenesis in future studies. Since the ligands for PPARs can inhibit AOM-induced ACFs, which weakly express PPARγ[182], we are now searching for natural compounds that act as ligands for PPARs[51,58,59]. In the near future, we would like to provide promising non-nutritive compounds (including citrus compounds, auraptene and nobiletin) with less toxicity from edible Asian plants[105,183,184] for use in clinical CRC chemoprevention trials. Furthermore, new compounds with more effective chemopreventive effects can be synthesized from the non-nutritive compounds, including collinin[161,185], in edible plants if a small amount of the parent compound can be isolated. Development of prodrugs that can easily reach target tissues and exert their biological activity greater than the parent chemicals at the sites is also important for cancer chemoprevention strategies[154]. Additionally, low doses of combinations of known non-nutritive chemopreventive agents can be considered to obtain a pronounced chemopreventive effect against CRC development in the future[156].

Epidemiological studies have shown that obesity and diabetes might be risk factors for CRC development[186]. An animal study using *db/db* mice, which have obese and diabetic phenotypes because of disruption of the leptin receptor, demonstrated that these types of mice are highly susceptible to colon carcinogenesis[187]. Citrus unshiu segment membrane (CUSM) contains fiber, flavonoids and pectin, but its biological activity is unknown. Therefore, we conducted a short-term experiment to determine whether dietary CUSM affects the development of AOM-induced ACFs and BCACs in the colons of C57BL/KsJ-*db/db* mice[188]. Male *db/db* mice were given subcutaneous injections of AOM (15 mg/kg body weight) once a week for 5 weeks. From one week after the last dosing of AOM, they received a diet containing 200, 1,000, or 5,000 ppm CUSM for 7 weeks. At week 12, dietary administration of CUSM caused a reduction in the frequency of ACFs (53–59% reduction). In addition, the number of BCACs was lowered by treatment with CUSM (29–62% reduction). Furthermore, pathological alterations (fibrosis) in the liver that resembled a metabolic disorder, non-alcoholic steatohepatitis[189] (NASH), were reduced by feeding with CUSM. NASH may cause fibrosis, cirrhosis and premature death resulting from liver failure in some cases. Its prevalence is increasing, and it is probably underestimated as a cause of cirrhosis and/or liver cell cancer. The need to determine an effective treatment is clear and urgent using an animal model of NASH[190]. Our data may indicate that CUSM is useful for reducing the risk of colon carcinogenesis in obesity and diabetes.

Finally, we should note the unexplained discrepancies between the results of different animal carcinogenesis assays and the exact mechanisms of carcinogenesis. Also, we should explain why so many apparently different protective agents are able to prevent experimental CRC[70,71,74–77,79]. Although several studies using the AOM rat model have demonstrated that diets expected to increase serum triglycerides and glucose are associated with indicators of risk of neoplasm in the colon, direct associations have yet to be demonstrated because of lack of these measures in previous studies. Thus, further investigations are needed to determine whether dietary non-nutritive compounds affect these serum biomarkers that influence the risk of CRC through systemic effects involving the circulation and through luminal effects.

Conclusion

In conclusion, certain flavonoids and other substances with biological activity, including antioxidative and/or anti-inflammatory properties, that are present in edible plants, including vegetables and fruits, can exert chemopreventive action on rat colon carcinogenesis as shown herein. However, more work needs to be done to better understand the underlying mechanism(s) of action and to confirm their safety for use in humans. Since plants are complex mixtures of chemicals, the potential for finding new chemopreventive agents in plants is high. Studies are underway to identify new compounds in edible plants with chemopreventive potential. In order to screen chemopreventive agents based on different mechanisms, a new *in vitro* co-culture model[191] and microarray analysis[192] might be useful. The effects of these agents on colon carcinogenesis should be carefully studied to assist the discovery and development of new chemopreventive agents and to understand carcinogenesis mechanisms. Our goal is to develop chemopreventive agents that are effective in decreasing the risk of CRC in general and/or in high-risk populations. Even if this strategy is only partially successful, it would have a significant impact on reduction of CRC mortality.

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References

1. Block G, Patterson B, and Subar A. Fruit, vegetables, and cancer prevention: a review of epidemiological evidence. Nutr Cancer. **18**: 1–29. 1992.
2. Steinmetz KA and Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc. **96**: 1027–1039. 1996.
3. Reddy BS. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environ. Mol. Mutagen. **44**: 26–35. 2004.
4. Weisburger JH. Causes, relevant mechanisms, and prevention of large bowel cancer. Semin Oncol. **18**: 316–336. 1991.
5. Hirayama T. Diet and cancer. Nutr Cancer. **1**: 67–81. 1979.
6. Steinmetz KA and Potter JD. Vegetable, fruits, and cancer. I. Epidemiology. Cancer Causes Control. **2**: 325–357. 1991.
7. Steinmetz KA and Potter JD. Vegetable, fruits, and cancer. II. Mechanisms. Cancer Causes Control. **2**: 427–442. 1991.
8. Tanaka T. Effect of diet on human carcinogenesis. Crit Rev Oncol/Hematol. **25**: 73–95. 1997.
9. Giovannucci E, Rimm EB, Stampfer MJ, Colditz GA, Ascherio A, and Willett WC. Intake of fat, meat, and fiber in relation to risk of colon cancer in men. Cancer Res. **54**: 2390–2397. 1994.
10. Weisburger JH. Mechanisms of action of antioxidants as exemplified in vegetables, tomatoes and tea. Food Chem Toxicol. **37**: 943–948. 1999.
11. Schatzkin A, Lanza E, Corle D, Lance P, Iber F, Caan B, Shike M, Weissfeld J, Burt R, Cooper MR, Kikendall JW, and Cahill J. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. N Engl J Med. **342**: 1149–1155. 2000.
12. Alberts DS, Martinez ME, Roe DJ, Guillen-Rodriguez JM, Marshall JR, van Leeuwen JB, Reid ME, Ritenbaugh C, Vargas PA, Bhattacharyya AB, Earnest DL, and Sampliner RE. Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. N Engl J Med. **342**: 1156–1162. 2000.
13. Tsubono Y, Nishino Y, Komatsu S, Hsieh CC, Kanemura S, Tsuji I, Nakatsuka H, Fukao A, Satoh H, and Hisamichi S. Green tea and the risk of gastric cancer in Japan. N Engl J Med. **344**: 632–636. 2001.
14. Micozzi MS. Foods, micronutrients, and reduction of human cancer. In: Nutrition and Cancer Prevention: Investigating the Role of Micronutrients, TE Moon, and MS Micozzi (eds). Marcel Dekker, Inc., New York. 213–242. 1989.
15. Tanaka T, Kawamori T, Ohnishi M, Makita H, Mori H, Satoh K, and Hara A. Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. Carcinogenesis. **16**: 2957–2963. 1995.
16. Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, and Hara A. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. Cancer Res. **55**: 4059–4064..1995.
17. Tanaka T, Morishita Y, Suzui M, Kojima T, Okumura A, and Mori H. Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin. Carcinogenesis. **15**: 15–19. 1994.
18. Weisburger JH, Reddy BS, Rose DP, Cohen LA, Kendall ME, and Wynder EL. Protective mechanisms of dietary fibers in nutritional carcinogenesis. Basic Life Sci. **61**: 45–64. 1993.
19. Angres G, and Beth M. Effects of dietary constituents on carcinogenesis in different tumor models. An overview from 1975 to 1988. In: Cancer and Nutrition, RB Alfin-Slater and D Kritchesvsky (eds). Plenum Press, New York. 337–485. 1991.
20. Wattenberg LW. Inhibition of neoplasia by minor dietary constituents. Cancer Res. **43**: 2448s–2453s. 1983.
21. Wattenberg LW. Chemoprevention of cancer. Cancer Res. **45**: 1–8. 1985.
22. Huang M-T, Osawa T, Ho C-T, and Rosen RT (eds). Food Phytochemicals for Cancer Prevention I and II. Fruits and Vegetables. American Chemical Society, Washington, DC. 1994.
23. Formica JV and Regelson W. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol. **33**: 1061–1080. 1995.
24. Pierpoint WS. Flavonoids in the human diet. Prog Clin Biol Res. **213**: 125–140. 1986.
25. Hertog MG, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarienen M, Simic BS, Toshima H, Feskens EJM, Hollman PCH, and Katan MB. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch Intern Med. **155**: 381–386. 1995.
26. Mori H, Tanaka T, Shima H, Kuniyasu T, and Takahashi M. Inhibitory effect of chlorogenic acid on methylazoxymethanol acetate-induced carcinogenesis in large intestine and liver of hamsters. Cancer Lett. **30**: 49–54. 1986.
27. Tanaka T, Shinoda T, Yoshimi N, Niwa K, Iwata H, and Mori H. Inhibitory effect of magnesium hydroxide on methylazoxymethanol acetate-induced large bowel carcinogenesis in male F344 rats. Carcinogenesis. **10**: 613–616. 1989.
28. Kawamori T, Tanaka T, Hara A, Yamahara J, and Mori H. Modifying effects of naturally occurring products on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. Cancer Res. **55**: 1277–1282. 1995.
29. Kawamori T, Tanaka T, Hirose Y, Ohnishi M, and Mori H. Inhibitory effects of d-limonene on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. Carcinogenesis. **17**: 369–372. 1996.
30. Kawamori T, Tanaka T, Kojima T, Suzui M, Ohnishi M, and Mori H. Suppression of azoxymethane-induced rat colon aberrant crypt foci by dietary protocatechuic acid. Jpn J Cancer Res. **85**: 686–691. 1994.
31. Onogi N, Okuno M, Komaki C, Moriwaki H, Kawamori T, Tanaka T, Mori H, and Muto Y. Suppressing effect of perilla oil on azoxymethane-induced foci of colonic aberrant crypts in rats. Carcinogenesis. **17**: 1291–1296. 1996.
32. Komaki C, Okuno M, Onogi N, Moriwaki H, Kawamori T, Tanaka T, Mori H, and Muto Y. Synergistic suppression of azoxymethane-induced foci of colonic aberrant crypts by the combination of beta-carotene and perilla oil in rats. Carcinogenesis. **17**: 1897–1901. 1996.
33. Tanaka T, Makita H, Kawabata K, Mori H, Kakumoto M, Satoh K, Hara A, Sumida T, Tanaka T, and Ogawa H.

Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. Carcinogenesis. **18**: 957–965. 1997.
34. Tanaka T, Makita H, Kawamori T, Kawabata K, Mori H, Murakami A, Satoh K, Hara A, Ohigashi H, and Koshimizu K. A xanthine oxidase inhibitor 1'-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci. Carcinogenesis. **18**: 1113–1118. 1997.
35. Tanaka T, Kawabata K, Kakumoto M, Makita H, Hara A, Mori H, Satoh K, Hara A, Murakami A, Kuki W, Takahashi Y, Yonei H, Koshimizu K, and Ohigashi H. *Citrus* auraptene inhibits chemically induced colonic aberrant crypt foci in male F344 rats. Carcinogenesis. **18**: 2155–2161. 1997.
36. Morishita Y, Yoshimi N, Kawabata K, Matsunaga K, Sugie S, Tanaka T, and Mori H. Regressive effects of various chemopreventive agents on azoxymethane-induced aberrant crypt foci in the rat colon. Jpn J Cancer Res. **88**: 815–820. 1997.
37. Tanaka T, Kawabata K, Kakumoto M, Makita H, Matsunaga K, Mori H, Satoh K, Hara A, Murakami A, Koshimizu K, and Ohigashi H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 1'-acetoxychavicol acetate. Jpn J Cancer Res. **88**: 821–830. 1997.
38. Sugie S, Okamoto K, Rahman KM, Tanaka T, Kawai K, Yamahara J, and Mori H. Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats. Cancer Lett. **127**: 177–183. 1998.
39. Tanaka T, Kawabata K, Kakumoto M, Hara A, Murakami A, Kuki W, Takahashi Y, Yonei H, Maeda M, Ota T, Odashima S, Yamane T, Koshimizu K, and Ohigashi H. *Citrus* auraptene exerts dose-dependent chemopreventive activity in rat large bowel tumorigenesis: the inhibition correlates with suppression of cell proliferation and lipid peroxidation and with induction of phase II drug-metabolizing enzymes. Cancer Res. **58**: 2550–2556. 1998.
40. Kohno H, Maeda M, Honjo S, Murakami M, Shimada R, Masuda S, Sumida T, Azuma Y, Ogawa H, and Tanaka T. Prevention of colonic preneoplastic lesions by the beta-cryptoxanthin and hesperidin rich powder prepared from *Citrus Unshiu* Marc. juice in male F344 rats. J Toxicol Pathol. **12**: 209–215. 1999.
41. Tanaka T, Kawabata K, Kakumoto M, Makita H, Ushida J, Honjo S, Hara A, Tsuda H, and Mori H. Modifying effects of a flavonoid morin on azoxymethane-induced large bowel tumorigenesis in rats. Carcinogenesis. **20**: 1477–1484. 1999.
42. Kawabata K, Tanaka T, Murakami T, Okada T, Murai H, Yamamoto T, Hara A, Shimizu M, Yamada Y, Matsunaga K, Kuno T, Yoshimi N, Sugie S, and Mori H. Dietary prevention of azoxymethane-induced colon carcinogenesis with rice-germ in F344 rats. Carcinogenesis. **20**: 2109–2115. 1999.
43. Tanaka T, Kawabata K, Honjo S, Kohno H, Murakami M, Shimada R, Matsunaga K, Yamada Y, and Shimizu M. Inhibition of azoxymethane-induced aberrant crypt foci in rats by natural compounds, caffeine, quercetin and morin. Oncol Rep. **6**: 1333–1340. 1999.
44. Kawabata K, Yamamoto T, Hara A, Shimizu M, Yamada Y, Matsunaga K, Tanaka T, and Mori H. Modifying effects of ferulic acid on azoxymethane-induced colon carcinogenesis in F344 rats. Cancer Lett. **157**: 15–21. 2000.
45. Tanaka T, Kohno H, Murakami M, Shimada R, Kagami S, Sumida T, Azuma Y, and Ogawa H. Suppression of azoxymethane-induced colon carcinogenesis in male F344 rats by mandarin juices in beta-cryptoxanthin and hesperidin. Int J Cancer. **88**: 146–150. 2000.
46. Tanaka T, Kohno H, Shimada R, Kagami S, Yamaguchi F, Kataoka S, Ariga T, Murakami A, Koshimizu K, and Ohigashi H. Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats. Carcinogenesis. **21**: 1183–1189. 2000.
47. Tanaka T, Maeda M, Kohno H, Murakami M, Kagami S, Miyake M, and Wada K. Inhibition of azoxymethane-induced colon carcinogenesis in male F344 rats by the citrus limonoids obacunone and limonin. Carcinogenesis. **22**: 193–198. 2001.
48. Yoshitani SI, Tanaka T, Kohno H, and Takashima S. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by dietary capsaicin and rotenone. Int J Oncol. **19**: 929–939. 2001.
49. Kohno H, Yoshitani S, Tsukio Y, Murakami A, Koshimizu K, Yano M, Tokuda H, Nishino H, Ohigashi H, and Tanaka T. Dietary administration of citrus nobiletin inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Life Sci. **69**: 901–913. 2001.
50. Tanaka T, Shimizu M, Kohno H, Yoshitani S, Tsukio Y, Murakami A, Safitri R, Takahashi D, Yamamoto K, Koshimizu K, Ohigashi H, and Mori H. Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary zerumbone isolated from *Zingiber zerumbet*. Life Sci. **69**: 1935–1945. 2001.
51. Kohno H, Suzuki R, Noguchi R, Hosokawa M, Miyashita K, and Tanaka T. Dietary conjugated linolenic acid inhibits azoxymethane-induced colonic aberrant crypt foci in rats. Jpn J Cancer Res. **93**: 133–142. 2002.
52. Kohno H, Yamaguchi K, Taima M, and Tanaka T. Prevention of colonic aberrant crypt foci by dietary feeding of chalcone and 2-hydroxychalcone in male F344 rats. J Toxicol Pathol. **15**: 137–143. 2002.
53. Kohno H, Maeda M, Tanino M, Tsukio Y, Ueda N, Wada K, Sugie S, Mori H, and Tanaka T. A bitter diterpenoid furanolactone columbin from *Calumbae Radix* inhibits azoxymethane-induced rat colon carcinogenesis. Cancer Lett. **183**: 131–139. 2002.
54. Kohno H, Tanaka T, Kawabata K, Hirose Y, Sugie S, Tsuda H, and Mori H. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Int J Cancer. **101**: 461–468. 2002.
55. Shimoji Y, Sugie S, Kohno H, Tanaka T, Nanda K, Tamura Y, Nishikawa Y, Hayashi R, Uenakai K, and Ohigashi H. Extract of vinegar "Kurosu" from unpolished rice inhibits the development of colonic aberrant crypt foci induced by azoxymethane. J Exp Clin Cancer Res. **22**: 591–597. 2003.
56. Shimoji Y, Kohno H, Nanda K, Nishikawa Y, Ohigashi H, Uenakai K, and Tanaka T. Extract of Kurosu, a vinegar from unpolished rice, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Nutr Cancer. **49**: 170–173. 2004.
57. Suzuki R, Kohno H, Sugie S, Okada T, and Tanaka T. Preventive effects of powdered broccoli sprout on azoxymethane-induced rat colonic aberrant crypt foci. J Toxicol Pathol. **17**: 119–126. 2004.
58. Kohno H, Suzuki R, Yasui Y, Hosokawa M, Miyashita K,

and Tanaka T. Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically induced colon carcinogenesis in rats. Cancer Sci. **95**: 481–486. 2004.
59. Kohno H, Yasui Y, Suzuki R, Hosokawa M, Miyashita K, and Tanaka T. Dietary seed oil rich in conjugated linolenic acid from bitter melon inhibits azoxymethane-induced rat colon carcinogenesis through elevation of colonic PPARgamma expression and alteration of lipid composition. Int J Cancer. **110**: 896–901. 2004.
60. Suzuki R, Kohno H, Murakami A, Koshimizu K, Ohigashi H, Yano M, Tokuda H, Nishino H, and Tanaka T. Citrus nobiletin inhibits azoxymethane-induced large bowel carcinogenesis in rats. Biofactors. **22**: 111–114. 2004.
61. Suzuki R, Kohno H, Sugie S, Sasaki K, Yoshimura T, Wada K, and Tanaka T. Preventive effects of extract of leaves of ginkgo (*Ginkgo biloba*) and its component bilobalide on azoxymethane-induced colonic aberrant crypt foci in rats. Cancer Lett. **210**: 159–169. 2004.
62. Wynder E and Gori G. Contribution of the environment to cancer incidence: an epidemiologic exercise. J Natl Cancer Inst. **58**: 825–832. 1977.
63. Muller M and Kersten S. Nutrigenomics: goals and strategies. Nat Rev Genet. **4**: 315–322. 2003.
64. Riboli E and Norat T. Epidemiologic evidence of the protective effect of fruit and vegetables on cancer risk. Am J Clin Nutr. **78** (3 Suppl.): 559S–569S. 2003.
65. Temple N. Fruits, vegetables, and cancer prevention trials. J Natl Cancer Inst. **91**: 1164. 1999.
66. Gate L, Paul J, Ba G, Tew K, and Tapiero H. Oxidative stress induced in pathologies: the role of antioxidants. Biomed Pharmacother. **53**: 169–180. 1999.
67. Block KI. Antioxidants and cancer therapy: furthering the debate. Integr Cancer Ther. **3**: 342–348. 2004.
68. Torres M and Forman HR. Redox signaling and the MAP kinase pathways. Biofactors. **17**: 287–296. 2003.
69. Palou A, Serra F, and Pico C. General aspects on the assessment of functional foods in the European Union. Eur J Clin Nutr. **57** (Suppl. 1): S12–S17. 2003.
70. Corpet DE and Tache S. Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer. **43**: 1–21. 2002.
71. Corpet DE and Pierre F. Point: From animal models to prevention of colon cancer. Systematic review of chemoprevention in min mice and choice of the model system. Cancer Epidemiol Biomarkers Prev. **12**: 391–400. 2003.
72. Tanaka T. Cancer chemoprevention. Cancer J. **5**: 11–16. 1992.
73. Tanaka T, Kojima T, Suzui M, and Mori H. Chemoprevention of colon carcinogenesis by the natural product of a simple phenolic compound protocatechuic acid: suppressing effects on tumor development and biomarkers expression of colon carcinogenesis. Cancer Res. **53**: 3908–3913. 1993.
74. Tanaka T. Cancer chemoprevention by natural products (Review). Oncol Rep. **1**: 1139–1155. 1994.
75. Tanaka T and Mori H. Inhibition of colon carcinogenesis by non-nutritive constituents in foods. J Toxicol Pathol. **9**: 139–149. 1996.
76. Tanaka T. Chemoprevention of human cancer: biology and therapy. Crit Rev Oncol/Hematol. **25**: 139–174. 1997.
77. Tanaka T, Kohno H, and Mori H. Chemoprevention of colon carcinogenesiss by dietary non-nutritive compounds. Asian Pac J Cancer Prev. **2**: 165–177. 2001.
78. Corpet DE and Pierre F. How good are rodent models of carcinogenesis in predicting efficacy in humans? A systematic review and meta-analysis of colon chemoprevention in rats, mice and men. Eur J Cancer Prev. **41**: 1911–1922. 2005.
79. Tsuda H, Ohshima Y, Nomoto H, Fujita K, Matsuda E, Iigo M, Takasuka N, and Moore MA. Cancer prevention by natural compounds. Drug Metab Pharmacokinet. **19**: 245–263. 2004.
80. Shirley BW. Flavonoid biosynthesis: 'new' functions for an 'old' pathway. Trends Plant Sci. **1**: 377–382. 1996.
81. McCue P and Shetty K. Health benefits of soy isoflavonoids and strategies for enhancement: a review. Crit Rev Food Sci Nutr. **44**: 361–367. 2004.
82. Hoensch HP and Kirch W. Potential role of flavonoids in the prevention of intestinal neoplasia: a review of their mode of action and their clinical perspectives. Int J Gastrointest Cancer. **35**: 187–195. 2005.
83. Webb AL and McCullough ML. Dietary lignans: potential role in cancer prevention. N Engl J Med. **51**: 117–131. 2005.
84. Sarkar FH and Li Y. Soy isoflavones and cancer prevention. Cancer Invest. **21**: 744–757. 2003.
85. Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res. **428**: 305–327. 1999.
86. Lambert J and Yang C. Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. Mutat Res. 523/**524**: 201–208. 2003.
87. Lai PK and Roy J. Antimicrobial and chemopreventive properties of herbs and spices. Curr Med Chem. **11**: 1451–1460. 2004.
88. Pereira MA, Barnes LH, Rassman VL, Kelloff GV, and Steele VE. Use of azoxymethane-induced foci of aberrant crypts in rat colon to identify potential cancer chemopreventive agents. Carcinogenesis. **15**: 1049–1054. 1994.
89. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett. **93**: 55–71. 1995.
90. Pretlow TP, Barrow BJ, Ashton WS, O'Riordan MA, Pretlow TG, Jurcisek JA, and Stellato TA. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. Cancer Res. **51**: 1564–1567. 1991.
91. Tsukamoto T, Fukami H, Yamanaka S, Yamaguchi A, Nakanishi H, Sakai H, Aoki I, and Tatematsu M. Hexosaminidase-altered aberrant crypts, carrying decreased hexosaminidase alpha and beta subunit mRNAs, in colon of 1,2-dimethylhydrazine-treated rats. Jpn J Cancer Res. **92**: 109–118. 2001.
92. Yamashita N, Minamoto T, Onda M, and Esumi H. Increased cell proliferation of azoxymethane-induced aberrant crypt foci of rat colon. Jpn J Cancer Res. **85**: 692–698. 1994.
93. Pretlow TP, Cheter C, and O'Riordan MA. Aberrant crypt foci and colon tumors in F344 rats have similar increases in proliferative activity. Int J Cancer. **56**: 599–602. 1994.
94. Ochiai M, Watanabe M, Nakanishi M, Taguchi A, Sugimura T, and Nakagama H. Differential staining of dysplastic aberrant crypt foci in the colon facilitates prediction of carcinogenic potentials of chemicals in rats. Cancer Lett.

220: 67–74. 2005.
95. Zheng Y, Kramer PM, Olson G, Lubet RA, Steele V, Kelloff GJ, and Pereira MA. Prevention by retinoids of azoxymethane-induced tumors and aberrant crypt foci and their modulation of cell proliferation in the colon of rats. Carcinogenesis. **18**: 2119–2125. 1997.
96. Li H, Kramer PM, Lubet RA, Steele VE, Kelloff GJ, and Pereira MA. Effect of calcium on azoxymethane-induced aberrant crypt foci and cell proliferation in the colon of rats. Cancer Lett. **124**: 39–46. 1998.
97. Wang A, Yoshimi N, Tanaka T, and Mori H. Inhibitory effects of magnesium hydroxide on c-*myc* expression and cell proliferation induced by methylazoxymethanol acetate in rat colon. Cancer Lett. **75**: 73–78. 1993.
98. Cohen SM and Ellwein LB. Cell proliferation and carcinogenesis. Science. **249**: 1007–1011. 1990.
99. Pegg AE. Polyamine metabolism and its importance in neoplastic growth as a target for chemotherapy. Cancer Res. **48**: 759–774. 1988.
100. Luk GD, Hamilton SR, Yang P, Smith JA, O'Ceallaigh D, McAvinchey D, and Hyland J. Kinetic changes in mucosal ornithine decarboxylase activity during azoxymethane-induced colonic carcinogenesis in the rat. Cancer Res. **46**: 4449–4452. 1986.
101. LaMuraglia GM, Lacaine F, and Malt RA. High ornithine decarboxylase activity and polyamine levels in human colorectal neoplasia. Ann Surg. **204**: 89–93. 1986.
102. Wilkinson JI and Clapper ML. Detoxication enzymes and chemoprevention. Proc Soc Exp Biol Med. **216**: 192–200. 1997.
103. Szarka CE, Pfeiffer GR, Hum ST, Everley LC, Balshem AM, Moore DF, Litwin S, Goosenberg EB, Frucht H, Engstrom PF, and Clapper ML. Glutathine S-transferase activity and glutathione S-transferase m expression in subjects with risk for colorectal cancer. Cancer Res. **55**: 2789–2793. 1995.
104. Buch SH and Burks TF. Capsaicin: hot new pharmacological tool. Trends Pharmacol Sci. **4**: 84–87. 1983.
105. Yun T-K. Update from Asia: Asian study on cancer chemoprevention. Ann NY Acad Sci. **889**: 157–192. 1999.
106. Tominaga M and Julius D. Capsaicin receptor in the pain pathway. Jpn J Pharmacol. **83**: 20–24. 2000.
107. Cunningham ML, Soliman MS, Badr MZ, and Matthews HB. Rotenone, an anticarcinogen, inhibits cellular proliferation but not peroxisome proliferation in mouse liver. Cancer Lett. **95**: 93–97. 1995.
108. Hansen WH, Davis KJ, and Fitzhugh OG. Chronic toxicity of cube. Toxicol Appli Pharmacol. **7**: 535–542. 1965.
109. Udeani GO, Gerhauser C, Thomas CF, Moon RC, Kosmeder JW, Kinghorn AD, Moriarty RM, and Pezzuto JM. Cancer chemopreventive activity mediated by deguelin, a naturally occurring rotenoid. Cancer Res. **57**: 3424–3428. 1997.
110. Gerhauser C, Mar W, Le eSK, Suh N, Luo Y, Kosmeder J, Luyengi L, Fong HH, Kinghorn AD, Moriarty RM, Mehta RG, Constantinou A, Moon RC, and Pezzuto JM. Rotenoids mediate potent cancer chemopreventive activity through transcriptional regulation of ornithine decarboxylase. Nat Med. **1**: 260–266. 1995.
111. Gerhauser C, Lee SK, Kosmeder JW, Moriarty RM, Hamel E, Mehta RG, Moon RC, and Pezzuto JM. Regulation of ornithine decarboxylase induction by deguelin, a natural product cancer chemopreventive agent. Cancer Res. **57**: 3429–3435. 1997.
112. Pretlow TP, O'Riordan MA, Somich GA, Amini SB, and Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. Carcinogenesis. **13**: 1509–1512. 1992.
113. Betarbet R, Sherer TB, MacKenzie G, Garcia-Osuna M, Panov AV, and Greenamyre JT. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. **3**: 1301–1306. 2000.
114. Fong CH, Hasegawa S, Herman Z, and Ou P. Limonoid glucosides in commercial juices. J Food Sci. **54**: 1505–1506. 1989.
115. Miller EG, Porter JL, Binnie WH, Guo IY, and Hasegawa S. Further studies on the anticancer activity of citrus limonoids. J Agric Food Chem. **52**: 4908–4912. 2004.
116. Dictor M, Ehinger M, Mertens F, Akervall J, and Wennerberg J. Abnormal cell cycle regulation in malignancy. Am J Clin Pathol. **112** (1 Suppl. 1): S40–S52. 1999.
117. Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. Cancer Res. **48**: 235–245. 1988.
118. Wang QS, Papanikolaou A, Sabourin CL, and Rosenberg DW. Altered expression of cyclin D1 and cyclin-dependent kinase 4 in azoxymethane-induced mouse colon tumorigenesis. Carcinogenesis. **19**: 2001–2006. 1998.
119. Montanari A, Chen J, and Widmer W. Citrus flavonoids: a review of past biological activity against disease. Discovery of new flavonoids from Dancy tangerine cold pressed peel oil solids and leaves. Adv Expe Med Biol. **439**: 103–116. 1998.
120. Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G, and Middleton EJ. Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma *in vitro*. Cancer Lett. **56**: 147–152. 1991.
121. Wall ME, Wani MC, Manikumar G, Abraham P, Taylor H, Hughes TJ, Warner J, and McGivney R. Plant antimutagenic agents, 2. Flavonoids. J Nat Prod. **51**: 1084–1091. 1988.
122. Bennett A, Civier A, Hensby CN, Melhuish PB, and Stamford IF. Measurement of arachidonate and its metabolites extracted from human normal and malignant gastrointestinal tissues. Gut. **28**: 315–318. 1987.
123. Cuendet M and Pezzuto JM. The role of cyclooxygenase and lipoxygenase in cancer chemoprevention. Drug Metab Drug Interact. **17**: 109–157. 2000.
124. Steele VE, Holmes CA, Hawk ET, Kopelovich L, Lubet RA, Crowell JA, Sigman CC, and Kelloff GJ. Lipoxygenase inhibitors as potential cancer chemopreventives. Cancer Epidemiol Biomarkers Prev. **8**: 467–483. 1999.
125. Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S, and Ohigashi H. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. Cancer Res. **60**: 5059–5066. 2000.
126. Minagawa A, Otani Y, Kubota T, Wada N, Furukawa T, Kumai K, Kameyama K, Okada Y, Fujii M, Yano M, Sato T, Ito A, and Kitajima M. The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice. Jpn J Cancer Res. **92**: 1322–1328.

2001.
127. Sato T, Koike L, Miyata Y, Hirata M, Mimaki Y, Sashida Y, Yano M, and Ito A. Inhibition of activator protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinases-1 and -9 in human fibrosarcoma HT-1080 cells. Cancer Res. **62**: 1025–1029. 2002.
128. Tang M, Ogawa K, Asamoto M, Hokaiwado N, Seeni A, Suzuki S, Takahashi S, Tanaka T, Ichikawa K, and Shirai T. Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of the prostate (TRAP) and human prostate carcinoma cells. Cancer Sci. **98**: 471–477. 2007.
129. Murakami A and Ohigashi H. Cancer-preventive anti-oxidants that attenuate free radical generation by inflammatory cells. Biol Chem. **387**: 387–392. 2006.
130. Valenzuela A, Guerra R, and Videla LA. Antioxidant properties of the flavonoids silybin and (+)-cyanidanol-3: comparison with butylated hydroxyanisole and butylated hydroxytoluene. Planta Med. **52**: 438–440. 1986.
131. Fiebrich F and Koch H. Silymarin, an inhibitor of lipoxygenase. Experientia. **35**: 1548–1560. 1979.
132. Fiebrich F and Koch H. Silymarin, an inhibitor of prostaglandin synthetase. Experientia. **35**: 1550–1552. 1979.
133. Comoglio A, Leonarduzzi G, Carini R, Busolin D, Basaga H, Albano E, Tomasi A, Poli G, Morazzoni P, and Magistretti MJ. Studies on the antioxidant and free radical scavenging properties of IdB 1016 a new flavanolignan complex. Free Rad Res Commun. **11**: 109–115. 1990.
134. Katiyar SK, Korman NJ, Mukhtar H, and Agarwal R. Protective effects of silymarin against photocarcinogenesis in a mouse skin model. J Natl Cancer Inst. **89**: 556–566. 1997.
135. Lahiri-Chatterjee M, Katiyar SK, Mohan RR, and Agarwal R. A flavonoid antioxidant, silymarin, affords exceptionally high protection against tumor promotion in the SENCAR mouse skin tumorigenesis model. Cancer Res. **59**: 622–632. 1999.
136. Agarwal R, Agarwal C, Ichikawa H, Singh RP, and Aggarwal BB. Anticancer potential of silymarin: from bench to bed side. Anticancer Res. **26**: 4457–4498. 2006.
137. Sheu SY, Lai CH, and Chiang HC. Inhibition of xanthine oxidase by purpurogallin and silymarin group. Anticancer Res. **18**: 263–267. 1998.
138. Zi X, Grasso AW, Kung HJ, and Agarwal R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res. **58**: 1920–1929. 1998.
139. Gershbein LL. Action of dietary trypsin, pressed coffee oil, silymarin and iron salt on 1,2-dimethylhydrazine tumorigenesis by gavage. Anticancer Res. **14**: 1113–1116. 1994.
140. Zi X, Feyes DK, and Agarwal R. Anticarcinogenic effect of a flavonoid antioxidant, silymarin, in human breast cancer cells MDA-MB 468: induction of G1 arrest through an increase in Cip1/p21 concomitant with a decrease in kinase activity of cyclin-dependent kinases and associated cyclins. Clin Cancer Res. **4**: 1055–1064. 1998.
141. Vinh PQ, Sugie S, Tanaka T, Hara A, Yamada Y, Katayama M, Deguchi T, and Mori H. Chemopreventive effects of a flavonoid antioxidant silymarin on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder carcinogenesis in male ICR mice. Jpn J Cancer Res. **93**: 42–49. 2002.
142. Kohno H, Suzuki R, Sugie S, Tsuda H, and Tanaka T. Dietary supplementation with silymarin inhibits 3,2'-dimethyl-4-aminobiphenyl-induced prostate carcinogenesis in male F344 rats. Clin Cancer Res. **11**: 4962–4967. 2005.
143. Sohn OS, Ishizaki H, Yang CS, and Fiala ES. Metabolism of azoxymethane, methylazoxymethanol and N-nitrosodimethylamine by cytochrome P450IIE1. Carcinogenesis. **12**: 127–131. 1991.
144. Miguez MP, Anundi I, Sainz-Pardo LA, and Lindros KO. Hepatoprotective mechanism of silymarin: no evidence for involvement of cytochrome P450 2E1. Chem-Biol Interact. **91**: 51–63. 1994.
145. Reddy BS, Rao CV, Rivenson A, and Kelloff G. Chemoprevention of colon carcinogenesis by organosulfur compounds. Cancer Res. **53**: 3493–3498. 1993.
146. Lin HJ, Probst-Hensch NM, Louie AD, Kau IH, Witte JS, Ingles SA, Frankl HD, Lee ER, and Haile RW. Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas. Cancer Epidemiol Biomarkers Prev. **7**: 647–652. 1998.
147. Deep G and Agarwal R. Chemopreventive efficacy of silymarin in skin and prostate cancer. Integr Cancer Ther. **6**: 130–145. 2007.
148. Kohno H, Taima M, Sumida T, Azuma Y, Ogawa H, and Tanaka T. Inhibitory effect of mandarin juice rich in beta-cryptoxanthin and hesperidin on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced pulmonary tumorigenesis in mice. Cancer Lett. **174**: 141–150. 2001.
149. Elliott S and Brimacombe J. The medicinal plants of Gunung Leuser National Park, Indonesia. J Ethnopharmacol. **19**: 285–317. 1987.
150. Murakami A, Takahashi M, Jiwajinda S, Koshimizu K, and 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. **63**: 1811–1812. 1999.
151. Murakami A, Takahashi D, Kinoshita T, Koshimizu K, Kim H, Yoshihiro A, Nakamura Y, Jiwajinda S, Terao J, and 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. **23**: 795–802. 2002.
152. Suzuki R, Kohno H, Sugie S, Yamaguchi F, Kataoka S, Ariga T, and Tanaka T. A polyisoprenylated benzophenone garcinol inhibits azoxymethane-induced rat colon carcinogenesis. In: Carcinogenesis and Modification of Carcinogenesis 2005, T Tanaka, and H Tsuda (eds). Research Signpost, Kerala, India. 187–198. 2005.
153. Han BS, Park CB, Takasuka N, Naito A, Sekine K, Nomura E, Taniguchi H, Tsuno T, and Tsuda H. A ferulic acid derivative, 3-(4'-geranyloxy-3-methoxyphenyl)-2-propenoate, as a new candidate chemopreventive agent for colon carcinogenesis in the rat. Jpn J Cancer Res. **92**: 404–409. 2001.

154. Miyamoto S, Epifano F, Curini M, Genovese S, Suzuki R, and Tanaka T. A novel prodrug of ferulic acid suppresses colitis-related colon carcinogenesis in mice. Clin Cancer Res. **13**: in press. 2007.
155. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, and Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci. **94**: 965–973. 2003.
156. Reddy BS. Strategies for colon cancer prevention: combination of chemopreventive agents. Subcell. Biochem. **42**: 213–225. 2007.
157. Gordon LI and Weitzman SA. Inflammation and cancer. Cancer J. **6**: 2257–2261. 1993.
158. Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, and Wakabayashi K. 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**: 229–238. 2005.
159. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, Sakano K, Takahashi M, and Wakabayashi K. 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. in press. 2005.
160. Suzuki R, Miyamoto S, Yasui Y, Sugie S, and Tanaka T. Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate. BMC Cancer. **7**: 84. 2007.
161. Kohno H, Suzuki R, Curini M, Epifano F, Maltese F, Gonzales SP, and Tanaka T. Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice. Int J Cancer. **118**: 2936–2942. 2006.
162. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, and Dubios RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology. **107**: 1183–1188. 1994.
163. Reddy BS, Rao CV, and Seibert K. Evaluation of cyclooxygenase-2 inhibitor for potential chemopreventive properties in colon carcinogenesis. Cancer Res. **56**: 4566–4569. 1996.
164. Wakabayashi K. NSAIDs as cancer preventive agents. Asian Pac J Cancer Prev. **1**: 97–113. 2000.
165. Tsuji M and DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase-2. Cell. **83**: 493–501. 1995.
166. Hara A, Yoshimi N, Niwa M, Ino N, and Mori H. Apoptosis induced by NS-398, a selective cyclooxygenase-2 inhibitor, in human colorectal cancer cell lines. Jpn J Cancer Res. **88**: 600–604. 1997.
167. Tsuji M, Kawano S, Tsuji S, Sawaoka H, Hori M, and DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell. **93**: 705–716. 1998.
168. Chinery R, Beauchamp D, Shyr Y, Kirkland SC, Coffey RJ, and Morrow JD. Antioxidants reduce cyclooxygenase-2 expression, prostaglandin production, and proliferation in colorectal cancer cells. Cancer Res. **58**: 2323–2327. 1998.
169. Rustgi AK. Cyclooxygenase-2: The future is now. Nat Med. **4**: 773–774. 1998.
170. Armstrong WB and Taylor TH. Can a marker be a surrogate for development of cancer, and would we know it if it exists? Recent Results Cancer Res. **166**: 99–112. 2005.
171. Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett. **37**: 147–151. 1987.
172. Pretlow TP, Roukhadze EV, O'Riordan MA, Chan JC, Amini SB, and Stellato TA. Carcinoembryonic antigen in human colonic aberrant crypt foci. Gastroenterology. **107**: 1719–1725. 1994.
173. McLellan EA, Medline A, and Bird RP. Dose response and proliferative characteristics of aberrant crypt foci: putative preneoplastic lesions in rat colon. Carcinogenesis. **12**: 2093–2098. 1991.
174. McLellan EA, Medline A, and Bird RP. Sequential analyses of the growth and morphological characteristics of aberrant crypt foci: putative preneoplastic lesions. Cancer Res. **51**: 5270–5274. 1991.
175. Siu IM, Robinson DR, Schwartz S, Kung HJ, Pretlow TG, Petersen RB, and Pretlow TP. The identification of monoclonality in human aberrant crypt foci. Cancer Res. **59**: 63–66. 1999.
176. Yamada Y, Yoshimi N, Hirose Y, Kawabata K, Matsunaga K, Suzui M, Hara A, and Mori H. Frequent beta-*catenin* gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. Cancer Res. **60**: 3323–3327. 2000.
177. Yamada Y, Yoshimi N, Hirose Y, Matsunaga K, Katayama M, Sakata K, Shimizu M, Kuno T, and Mori H. Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, probable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. Cancer Res. **61**: 1874–1878. 2001.
178. Caderni G, Femia AP, Giannini A, Favuzza A, Luceri C, Salvadori M, and Dolara P. Identification of mucin-depleted foci in the unsectioned colon of azoxymethane-treated rats: correlation with carcinogenesis. Cancer Res. **63**: 2388–2392. 2003.
179. Femia AP, Dolara P, and Caderni G. Mucin-depleted foci (MDF) in the colon of rats treated with azoxymethane (AOM) are useful biomarkers for colon carcinogenesis. Carcinogenesis. **25**: 277–281. 2004.
180. Femia AP, Bendinelli B, Giannini A, Salvadori M, Pinzani P, Dolara P, and Caderni G. Mucin-depleted foci have beta-catenin gene mutations, altered expression of its protein, and are dose- and time-dependent in the colon of 1,2-dimethylhydrazine-treated rats. Int J Cancer. **116**: 9–15. 2005.
181. Mori H, Hata K, Yamada Y, Kuno T, and Hara A. Significance and role of early-lessions in experimental colorectal carcinogenesis. Chem-Biol Interact. **155**: 1–9. 2005.
182. Tanaka T, Kohno H, Yoshitani S, Takashima S, Okumura A, Murakami A, and Hosokawa M. Ligands for peroxisome proliferator-activated receptor a and g inhibit chemically-induced colitis and formation of aberrant crypt foci in rats. Cancer Res. **61**: 2424–2428. 2001.
183. Tanaka T (eds). Tanaka's Cyclopedia of Edible Plants of the World. Keigaku Pub. Co., Ltd., Tokyo. 1976.

184. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat. Rev. Cancer. **3**: 768–780. 2003.
185. Curini M, Epifano F, Maltese F, Marcotullio MC, Tubaro A, Altinier G, Gonzales SP, and Rodriguez JC. Synthesis and anti-inflammatory activity of natural and semisynthetic geranyloxycoumarins. Bioorg Med Chem Lett. **14**: 2241–2243. 2004.
186. McTiernan A. Obesity and cancer: the risks, science, and potential management strategies. Oncology (Williston Park). **19**: 871–881. 2005.
187. Hirose Y, Hata K, Kuno T, Yoshida K, Sakata K, Yamada Y, Tanaka T, Reddy BS, and Mori H. Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-*db/db* mice. Carcinogenesis. **25**: 821–825. 2004.
188. Suzuki R, Kohno H, Yasui Y, Hata K, Sugie S, Miyamoto S, Sugawara K, Sumida T, Hirose Y, and Tanaka T. Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male *db/db* mice. Int J Cancer. **120**: 252–258. 2007.
189. Pessayre D and Fromenty B. NASH: a mitochondrial disease. J Hepatol. **42**: 928–940. 2005.
190. Sahai A, Malladi P, Pan X, R. P, Melin-Aldana H, Green RM, and Whitington PF. Obese and diabetic *db/db* mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. Am J Physiol Gastrointest Liver Physiol. **287**: G1035–G1043. 2004.
191. Mace K, Offord EA, Harris CC, and Pfeifer AMA. Development of *in vitro* models for cellular and molecular studies in toxicology and chemoprevention. Arch Toxicol. **20** (Suppl.): 227–236. 1998.
192. Hokaiwado N, Asamoto M, Tsujimura K, Hirota T, Ichihara T, Satoh T, and Shirai T. Rapid analysis of gene expression changes caused by liver carcinogens and chemopreventive agents using a newly developed three-dimensional microarray system. Cancer Sci. **95**: 123–130. 2004.

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Original

Lack of Enhancing Effect of Lauric Acid on the Development of Aberrant Crypt Foci in Male ICR Mice Treated with Azoxymethane and Dextran Sodium Sulfate

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Abstract: The effect of lauric acid (LA), which is reported to induce cyclooxygenase (COX)-2 expression in macrophage cells (RAW 264.7) on the development of aberrant crypt foci (ACF), putative precursor lesions of colonic adenocarcinoma, was investigated in an inflammation-related mouse colon carcinogenesis model treated with azoxymethane (AOM) and dextran sulfate sodium (DSS). To induce ACF, male ICR mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight) and then followed by 1% DSS in drinking water for one week, starting one week after dosing of AOM (AOM/DSS group). The AOM/DSS/LA group was fed with a diet containing 1% LA for 7 weeks, starting one week after the cessation of DSS administration. Other groups included the AOM/LA group given AOM and 1% LA diet for 9 weeks, the DSS/LA group given DSS and the diet with 1% LA, the AOM group that received AOM alone, the DSS group given DSS alone in drinking water, the LA group fed with 1% LA-containing diet alone, and the untreated group. At week 10 (end of the study), the frequency of ACF did not significantly differ between the AOM/DSS group (7.4 ± 3.0) and the AOM/DSS/LA group (8.4 ± 5.0). The value was extremely low in the AOM/LA group (1.0 ± 1.0) and in the AOM alone group (2.4 ± 2.7). No ACF developed in other groups. Our findings suggest that dietary LA did not influence the occurrence of ACF in the AOM/DSS-induced mouse colon tumorigenesis, indicating a lack of LA enhancing effects on the early phase of inflammation-related mouse colon carcinogenesis. (J Toxicol Pathol 2007; **20**: 93–100)

Key words: lauric acid, aberrant crypt foci, inflammation, azoxymethane, dextran sulfate sodium, mice

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer deaths in the Western countries. Globally, CRC accounted for about 1 million new cases in 2002 (9.4% of the world) and the mortality rate is about one half that of incidence (about 529,000 deaths in 2002)[1]. The incidence of CRC is particularly high in Canada and Australia where there is high consumption of red and processed meat[2]. In contrast, Mediterranean countries have lower rates of CRC when compared with other Western countries[3]. The low incidence rates might be due to diet[4], because the consumption of fruits, vegetables, fish and olive oil is quite high in Mediterranean countries. In 1969, Wynder *et al.*[5] first suggested that patients with CRC have a high caloric intake in the form of fats, and that dietary fats may be involved in the pathogenesis of CRC development. Since their innovative case-control study, a number of epidemiological studies have implicated dietary fat in the etiology of CRC[6]. In most industrialized societies, CRC has a high incidence among both women and men. In Japan, CRC incidence has particularly increased since the end of World War II with an increase in dietary fat intake. Some of the inconsistencies in findings on dietary fats may relate to the fact that they are generally assessed in accordance with their quantity (total fat), origin (animal or vegetable) or type (saturated, monounsaturated or polyunsaturated)[7–9]. However, on the basis of results reported from a number of studies conducted in different countries, there is sufficient evidence to suggest that certain fatty acids play a role in CRC occurrence. Currently, some epidemiological studies have indicated that higher concentrations of butyric acid and eicosanopentaenoic acid (EPA) provide protection against CRC[10–12]. These fatty acids induce apoptosis in colonic cancer cells[13,14]. In contrast, specific fatty acids that increase

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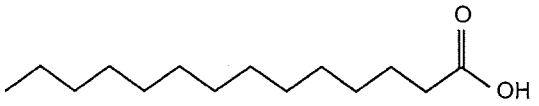

$C_{12}H_{24}O_2$ MW 200.32

Fig. 1. Chemical structure of lauric acid (LA) that is the main constituent of triacylglycerol contained in coconut oil, coconut milk, and palm tree oil. LA is also present in butter and lard.

the risk of CRC are unclear. Recently, a high fat intake was reported to be associated with an increased risk for inflammatory bowel disease (IBD), such as ulcerative colitis (UC)[15] and Crohn's disease (CD)[16]. CRC is one of the complications of both diseases[17].

Lauric acid (LA, $C_{12}H_{24}O_2$, Fig. 1), also called *n*-dodecanoic acid is a medium chain fatty acid, which forms monolaurin in the human or animal body. The highest content of LA is found in a mother's breast milk and coconut oil[18]. LA occurs as the glyceride in many vegetable fats, especially coconut oil and laurel oil and is used chiefly in the manufacture of soaps, detergents, cosmetics and lauryl alcohol. It is also used in food additives and insecticides. LA was recently reported to have antiviral[19] and antibacterial[20,21] actions, and is able to destroy lipid-coated viruses including human immunodeficiency, herpes, cytomegalovirus and influenza viruses. More recently, interesting reports describing that LA induces the expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), both of which are involved in colon carcinogenesis[22–25], through toll-like receptor 4 in mouse macrophage 264.7 cells have been published[26,27]. Over-expression of both enzymes was also immunohistochemically observed in the inflamed colon of a colitis-related mouse colon carcinogenesis model treated with azoxymethane (AOM) and dextran sodium sulfate (DSS)[28].

Recently, many studies have reported that several non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, suppress the development of chemically-induced colon carcinomas in rats[29–32]. In addition, clinical trials have demonstrated that a NSAID, sulindac, suppresses adenomas in patients with familial adenomatous polyposis[33]. Nimesulide, a selective inhibitor of COX-2, suppresses the formation of aberrant crypt foci (ACF), a putative precancerous lesion of the colon cancer[27,34–36], induced by a colon carcinogen AOM in rats[37], and is able to inhibit the occurrence of colonic adenocarcinoma induced by AOM/DSS in mice[38]. Similarly, iNOS, a generator of cellular nitric oxide is also overexpressed in colon tumors[39,40]. These results suggest that COX-2 and iNOS play important role in the incidence and development of colon cancer.

In the current study, we investigated whether LA promotes the occurrence of ACF in an inflammation-related mouse colon carcinogenesis model[41]. Also, we investigated whether LA-albumin complex induces COX-2 expression in RAW264.7 cells (a murine macrophage-like cell line) as previously described[26]. RAW264.7 cells are known to be useful for examining the production of inflammatory mediators, including cytokines, prostaglandin E_2, COX-2 and iNOS, after inflammatory stimuli[42].

Materials and Methods

Chemicals and reagents

A colonic carcinogen, AOM, was purchased from Sigma-Aldrich K.K. (Tokyo, Japan). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). LA was obtained from Wako Pure Chemicals (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Bovine fetal serum albumin (BSA) and lipopolysaccaride (LPS) were purchased from Sigma-Aldrich K.K. Enhanced chemiluminescence western blot detection kits and reagents were purchased from Amersham Pharmacia Biotech. (Buckinghamshire, UK).

Animals, drinking water and diet

Male Crlj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo, Japan) aged 5 weeks were used in this study. They were maintained at the Animal Facility of Kanazawa Medical University according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (5 mice/cage) with free access to drinking water (tap water) and basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2°C). They were quarantined for the first 7 days after arrival, and then divided into experimental and control groups. DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v) every day. Experimental diet containing LA (1%, w/w) was prepared every week by mixing with powdered basal diet CRF-1. The dose of LA was selected, based on a report by DeLany *et al.*[43]

Experimental procedure

Thirty-six male ICR mice were divided into eight groups (Fig. 2). Groups 1 (n=5), 2 (n=5), 3 (n=5) and 5 (n=4) were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Group 1 was then given a one-week exposure of 1% DSS in drinking water, and was given no further treatment. Group 2 was given AOM and DSS, as per group 1. Then, mice of group 2 were fed a diet containing 1% LA for 7 weeks, starting one week after the cessation of DSS administration. Animals of group 3 were given a diet containing 1% LA for 9 weeks, starting one week after the AOM injection. Group 4 (n=4) was given 1% DSS in drinking water and a diet containing 1% LA alone for 7 weeks. Groups 5, 6 (n=4) and 7 (n=5) were treated with AOM alone, 1% DSS alone and 1% LA-containing diet alone, respectively. Group 8 (n=4) served as an untreated control. At week 10, all mice were sacrificed under ether

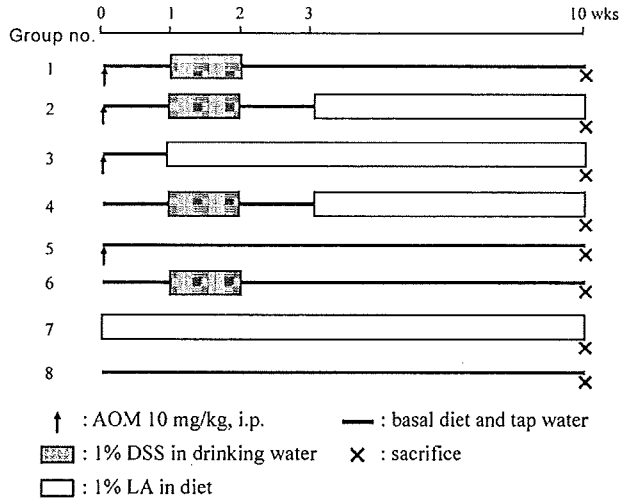

Fig. 2. Experimental protocol.

anesthesia to assess the occurrence of colonic ACF. They underwent careful necropsy, with emphasis on the colon, liver, kidney, lung and heart. All grossly abnormal lesions in any tissue, and the organs such as liver, kidney, lung and heart were fixed in 10% buffered formalin solution for histopathology and COX-2 immunohistochemistry.

Determination of ACF

The number of ACF per colon was determined according to the method described in our previous report[44]. At necropsy, the length (from the ileocecal junction to the anal verge) of the large bowel was measured. The colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. Colons were cut and fixed in 10% buffered formalin for at least 24 h. Fixed colons were dipped in a 0.5% solution of methylene blue in distilled water for 20 s, and placed on a microscope slide for counting ACF. After counting ACF, colons were routinely processed for histopathology.

Histopatholgy and COX-2 immunohistochemistry

Tissues, including colon, were examined on hematoxylin and eosin-stained sections. Colitis with or without ulceration (inflammation score) was also evaluated on hematoxylin and eosin-stained sections, according to the following grading system[45]: 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 entire crypts with severe inflammation in the mucosa, but with retention of the surface epithelium; and grade 4, loss of entire crypts and surface epithelium with severe inflammation in the mucosa, muscularis propria and submucosa.

Immunohistochemistry for COX-2 was carried out with 4-μm thick, paraffin-embedded sections. Anti-COX-2 mouse monoclonal antibody (diluted 1:200, Transduction Laboratories) was used as the primary antibody. To reduce the non-specific staining of mouse tissues by the mouse antibodies, a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Inc., Burlingame, CA, USA) was applied. Staining was performed using a LSAB KIT or DAKO EnVision kit (DAKO, Glostrup, Denmark) or Vectastain Elite ABC Kit (Vector Laboratories). At the last step, the sections were counter-stained with hematoxylin. As a negative control, the primary antibody was omitted.

Cell culture

RAW264.7 cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured in DMEM containing 10% FBS, L-glutamine (330 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2/air environment. Cells (2×10^6) were plated in a 60-mm dish and cultured for an additional 18 h to allow the number of cells to approximately double. Cells were maintained in serum-poor (0.25% FBS) medium for another 18 h prior to the treatment with LA.

Preparation of LA-albumin complexes

LA was solubilized in ethanol or combined with fatty acid-free and low endotoxin BSA at a molar ratio of 10:1 (fatty acid : albumin) in serum-poor medium (0.25% FBS). Fatty acid-albumin complex solution was freshly prepared prior to each experiment.

Western blotting

For western blot analysis, 2×10^6 cells were lysed in lysis buffer [protease inhibitor, phosphatase inhibitor, 10 mM Tris, 1% sodium dodecyl sulfate (SDS), 1 mM sodium vanadate (V)]. Protein concentration was determined using a DC protein assay (Bio-Rad Laboratories Ltd., Kyoto, Japan), with γ-globulin used as the standard. Denatured proteins were separated using SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred to PVDF membranes. After blocking overnight at 4°C in Block Ace (Dainippon Pharmaceutical, Osaka, Japan), the membranes were incubated with the primary goat polyclonal antibody against COX-2 (SC-1745, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution and the primary goat polyclonal antibody against β-actin protein (SC-1615, Santa Cruz Biotehcnology) at 1:1000 dilution. Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (AMI3404, Biosource International, Camarillo, CA, USA) at a dilution of 1:1000 for 2 h at room temparature. The blots were developed using an ECL western blotting detection reagent (Amersham Biosciences, Buckinghamshire, UK). The intensity of each band was analyzed using NIH Image.

Statistical analysis

Where applicable, data were analyzed using one-way ANOVA with the Bonferroni correction (GraphPad Instat

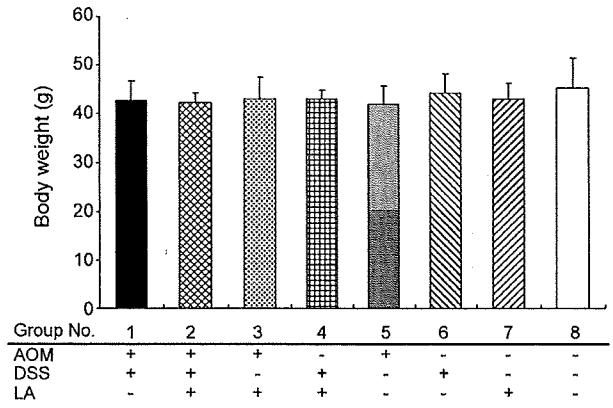

Fig. 3. Body weights (g, mean ± SD) of each group.

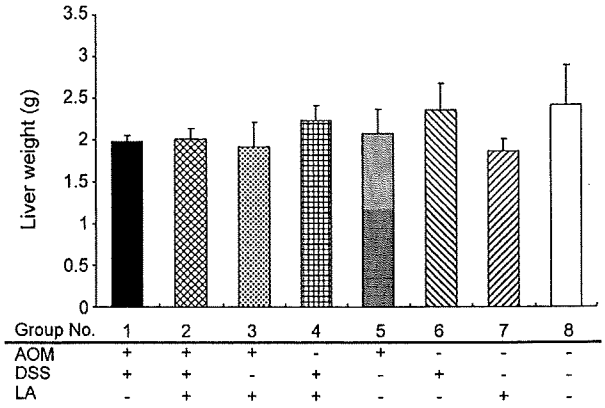

Fig. 4. Liver weights (g, mean ± SD) of each group.

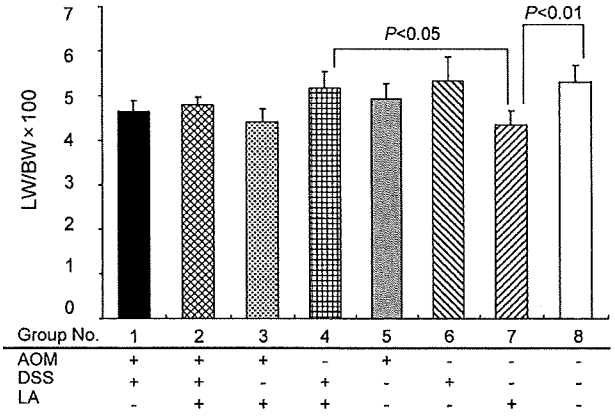

Fig. 5. Relative liver weights (g, liver weight/g body weight, mean ± SD) of each group.

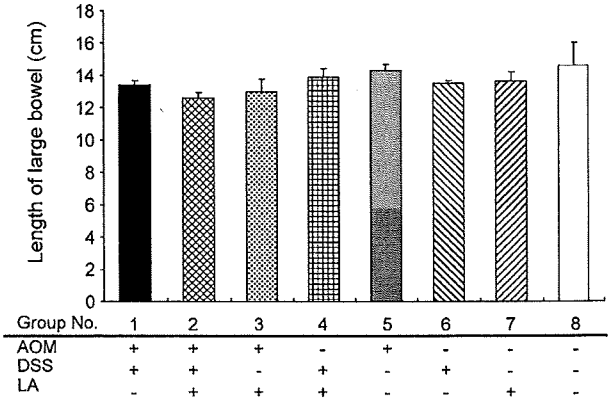

Fig. 6. Length of large bowels (cm, mean ± SD) of each group.

version 3.05, GraphPad Softwear, San Diego, CA, USA) and $P<0.05$ as the criterion of significance.

Results

General observation

Bloody stool was observed in some mice in groups 1, 2, 4 and 6, when they were given DSS. There were no significant changes of weight gains of mice in any group during the study (data not shown). Mean body, liver and relative liver (g liver weight/100 g body weight) weights at sacrifice are shown in Figs. 3, 4 and 5, respectively. Although there were no significant differences in the body and liver weight among the groups (Figs. 3 and 4), differences of the relative liver weights between groups 4 and 7 ($P<0.05$) and groups 7 and 8 ($P<0.01$) were statistically significant (Fig. 5). No significant differences were noted among the groups in the length of the large bowel (Fig. 6).

Frequency of ACF

ACF (Fig. 7a and 7b) developed in mice of groups 1, 2, 3 and 5. They were mostly small ACF consisting of 1–3 aberrant crypts (Fig. 7a). Only a few large ACF consisting of 4 or more aberrant crypts (Fig. 7b) in groups 1 (2 large ACF) and 2 (1 large ACF) were seen. As summarized in Fig. 8, the numbers of ACF per colon in groups 1, 2, 3 and 5 were relatively low, with predominance in group 2. However, the mean numbers of ACF per colon did not significantly differ between groups 1 and 2. The value of group 3 was much lower than those of groups 1 ($P<0.01$) and 2 ($P<0.001$), and was smaller than that of group 5, but without statistical significance.

Histopathology, colonic inflammation score and COX-2 immunohistochemistry

There were no significant alterations in the histopathologies of the liver, kidney, lung, and heart among the groups. In the colon, inflammation with or without mucosal ulcer was observed in mice of groups 1, 2, 4, and 6. The order of inflammation score in the groups was as follows: group 2 (2.80 ± 0.84) > group 1 (2.60 ± 0.89) > group 4 (1.75 ± 0.50), group 6 (1.75 ± 0.96) > group 3 (0.40 ± 0.55) > group 5 (0.25 ± 0.50) > group 7 (0.20 ± 0.45) >

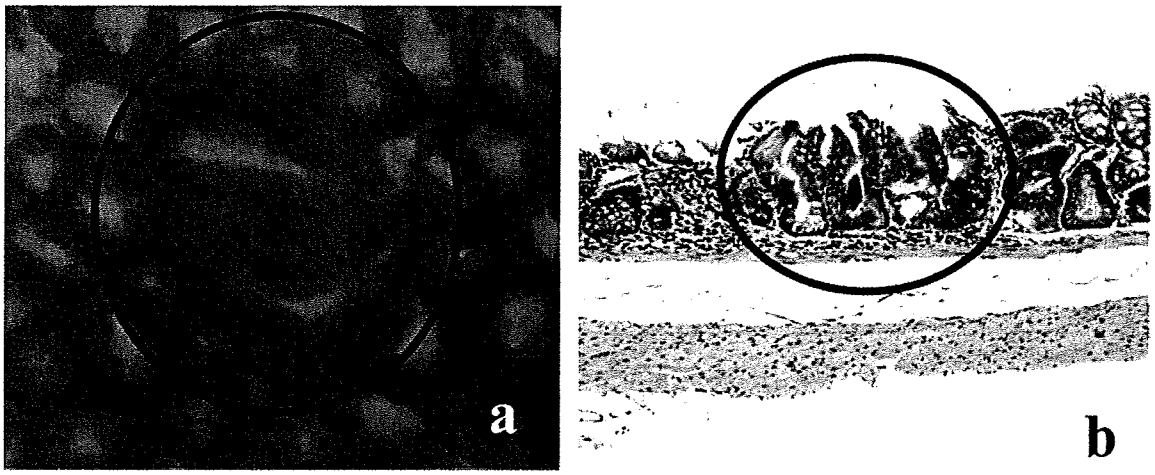

Fig. 7. (a) A small ACF consisting of 2 aberrant crypts and (b) a large ACF consisting of 5 aberrant crypts from a mouse that received AOM and 1% DSS. (a) Methylene blue stain. (b) Hematoxylin and eosin stain. Original magnification, (a) and (b) ×20.

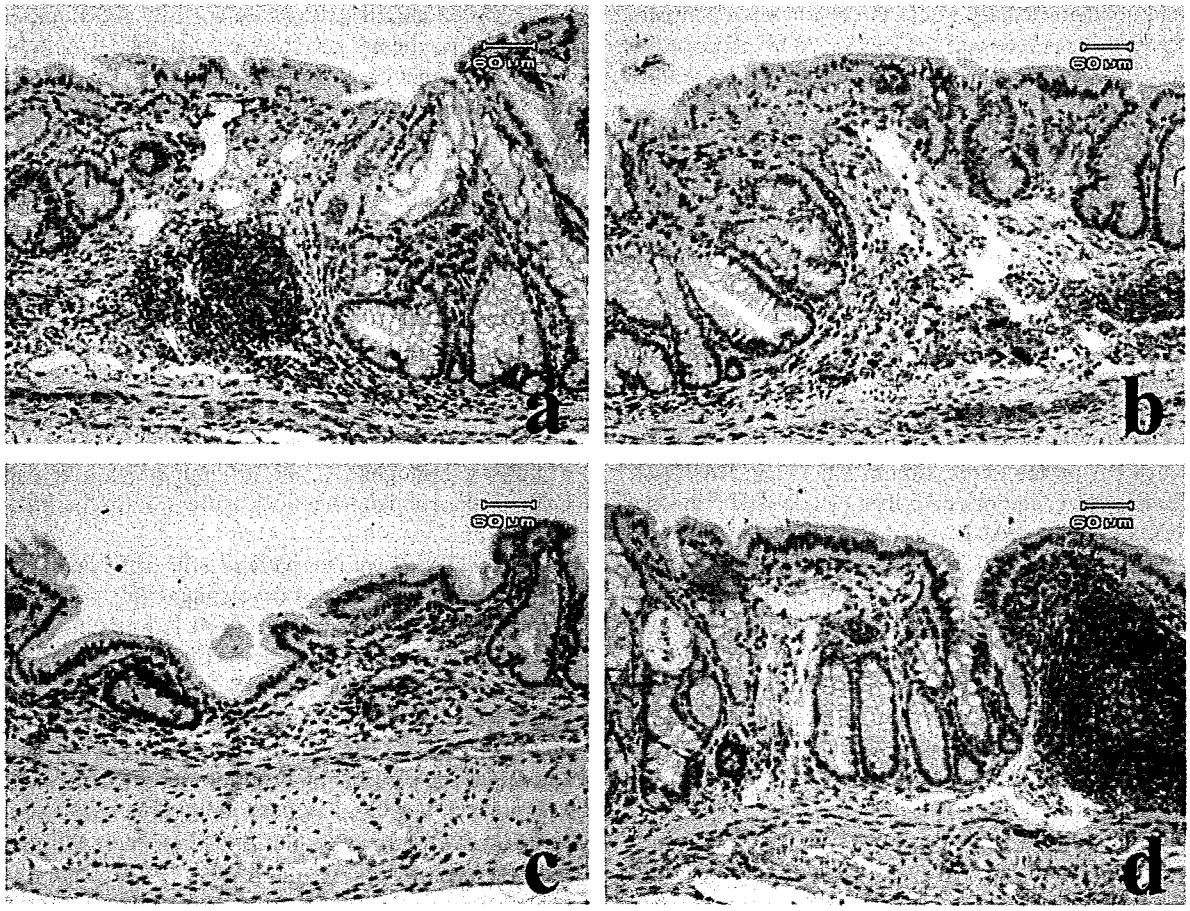

Fig. 9. COX-2 immunohistochemistry of the non-lesional area of mouse colons from each of groups 1 (a), 2 (b), 4 (c), and 6 (d). Bars inserted are 60 μm.

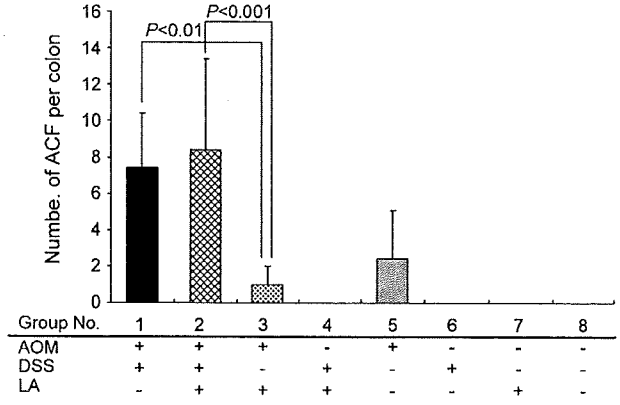

Fig. 8. The mean numbers of ACF per colon in each group.

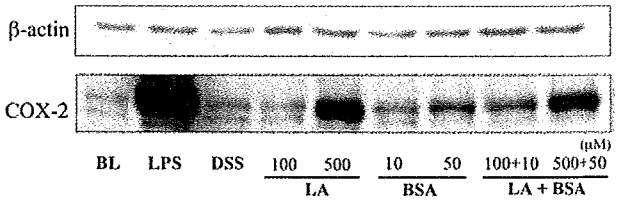

Fig. 10. A complex of a high dose of lauric acid (LA) and BSA induced COX-2 expression. RAW 264.7 cells maintained in serum poor (0.25%) medium were treated with the indicated concentrations of LA solubilized with BSA at a molar ratio of 10:1 (fatty acid : BSA). After 11 hours, cell lysates were analyzed by COX-2 or β-actin immunoblot. Lane 1, cells treated in medium alone; lane 2, cells treated with LPS alone; lane 3, cells treated with DSS alone; lanes 4 and 5, cells treated with LA without BSA; lanes 6 and 7, cells treated with BSA without LA; and lanes 8 and 9, cells treated with LA in medium with BSA.

group 8 (0 ± 0). Significant differences were observed between groups 1 and 5 ($P<0.001$), groups 2 and 3 ($P<0.001$), groups 2 and 4 ($P<0.001$), groups 4 and 7 ($P<0.05$), and groups 6 and 8 ($P<0.05$). However, dietary LA did not significantly affect the colon when compared with the mice that received AOM/DSS, AOM alone or DSS alone. Also, the inflammation score of mice treated with LA alone was low.

COX-2 immunoreactivity was strongly positive reaction in the infiltrated inflammatory cells and the endothelium of small blood vessels in the lamina propria of the non-lesional colonic mucosa of mice that had received DSS (groups 1, 2, 4, and 6; Fig. 9), but in groups 3, 5, 7 and 8 it was very weak (data not shown).

Expression of COX-2 in RAW264.7 cells teated with LA-albumin compex

Since we did not observe a modifying effect of LA on AOM/DSS-induced colon carcinogenesis, we tested the effect of LA on the expression of COX-2 in RAW264.7 cells, under the conditions described by Lee *et al.*[26] in which complexes with 10 μM LA-1 μM BSA, 50 μM LA-5 μM BSA or 100 μM LA-10 μM BSA induced COX-2 protein expression in RAW 264.7 cells. We did not observe over-expression of COX-2 in RAW264.7 cells treated with complexes with 10 μM LA-1 μM BSA, 50 μM LA-5 μM BSA or 100 μM LA-10 μM BSA (data not shown), but the complexes with 500 μM LA + 50 μM BSA and 500 μM LA alone induced COX-2 expression (Fig. 10).

Discussion

In the current study, 1% LA feeding after exposure to AOM and DSS did not significantly enhance ACF formation, suggesting no synergistic effects of LA with DSS in inflammation-related mouse colon carcinogenesis. Since LA has been reported to induce expression of inflammatory marker gene products such as COX-2, inducible nitric oxide and interleukin (IL)-1α in mouse macrophage 264.7 cells, we expected treatment with LA to enhance ACF formation induced by AOM and DSS. However, our findings suggest no modifying effect of dietary LA in inflammation-related mouse colon carcinogenesis induced by AOM and DSS. Interestingly, dietary LA (group 3: AOM + LA) lowered ACF formation induced by AOM when compared to group 5 (AOM alone), but the difference between ACF formation in these two groups was not significant.

Lee *et al.*[26] previously reported that even a low dose of LA (10 mM) and BSA (1 mM) complex can up-regulated COX-2 expression in RAW 264.7 cells. In our *in vitro* study we were not able to reproduce their results, but a high dose of the complex, which contained 500 μM LA and 50 μM BSA, induced COX-2 expression. Moreover 500 μM LA alone also induced the expression. While *in vitro* experimental conditions are not relevant to *in vivo* experiments, data from our ACF assay may indicate that dietary LA at a dose of 1% does not influence the early phase of colon carcinogenesis, the inflammation score and the COX-2 immunohistochemical expression in the inflamed colon.

It is well known that patients with IBD, both UC and CD, are at increased risk of developing CRC. Furthermore, activated transcription factor NFκB is found in inflamed mucosal biopsies of patients with IBD[46]. NFκB can stimulate iNOS to generate NO and COX-2 to generate prostanoids that have proinflammatory and carcinogenic effects[47]. COX-2 is over-expressed in about 90% of colorectal adenocarcinomas and in 40–90% of colorectal adenomas[48,49]. COX-2 expression is thus observed at an early stage of colorectal tumor development and in most tumors, either benign or malignant. Animal experiments have been shown the direct evidence of the important contribution of COX-2 in colorectal tumor development. Indeed, treatment with selective COX-2 inhibitors in animal models of familial adenomatous polyposis (FAP) significantly reduced the number of polyps[50,51]. Selective COX-2 inhibitors also reduce tumor incidence and multiplicity induced by AOM and DSS[38]. These findings

confirm COX-2 plays an important role in colorectal carcinogenesis.

LA was the most efficient at inducing COX-2 expression in mouse macrophage RAW264.7 cells among a group of saturated fatty acids tested (C8:0–C18:0)[26]. However our results described here suggest that LA has no promoting effect on AOM/DSS- and AOM-induced ACF formation in mice. Also, LA feeding, at a dose of 1% in diet, did not cause severe inflammation and elevation of COX-2 expression in the mouse colon. A recent review article reports that COX-2 expression might not be the driving force for the development from inflammation to cancer, but rather plays an enhancement role in cancer development from chronic inflammation[52]. DeLany *et al.*[43] found that LA, a medium-chain fatty acid, is the most highly oxidized fatty acid, and the unsaturated fatty acids and the long-chain saturated fatty acids are the least oxidized in that order[43]. In this study, we used 1% LA, a concentration that is 7 times greater than that used in their study[43]. To make certain our findings are in line with the review by Lu *et al.*[52] further studies using different experimental models and conditions (different doses of LA) are warranted.

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References

1. Parkin DM, Bray F, Ferlay J, and Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. **55**: 74–108. 2005.
2. Norat T and Riboli E. Meat consumption and colorectal cancer: a review of epidemiologic evidence. Nutr Rev. **59**: 37–47. 2001.
3. Glade MJ. Food, nutrition, and the prevention of cancer: a global perspective. American Institute for Cancer Research/ World Cancer Research Fund, American Institute for Cancer Research, 1997. Nutrition. **15**: 523–526. 1999.
4. Kushi LH, Lenart EB, and Willett WC. Health implications of Mediterranean diets in light of contemporary knowledge. 2. Meat, wine, fats, and oils. Am J Clin Nutr. **61**: 1416S–1427S. 1995.
5. Wynder EL, Kajitani T, Ishikawa S, Dodo H, and Takano A. Environmental factors of cancer of the colon and rectum. II. Japanese epidemiological data. Cancer. **23**: 1210–1220. 1969.
6. Howe GR, Aronson KJ, Benito E, Castelleto R, Cornee J, Duffy S, Gallagher RP, Iscovich JM, Deng-ao J, Kaaks R, Kune GA, Kune S, Lee HP, Lee M, Miller AB, Peters RK, Potter JD, Riboli E, Slattery ML, Trichopoulos D, Tuyns A, Tzonou A, Watson LF, Whittemore AS, and Shu Z. The relationship between dietary fat intake and risk of colorectal cancer: evidence from the combined analysis of 13 case-control studies. Cancer Causes Control. **8**: 215–228. 1997.
7. Tsai WS, Nagawa H, Kaizaki S, Tsuruo T, and Muto T. Inhibitory effects of n-3 polyunsaturated fatty acids on sigmoid colon cancer transformants. J Gastroenterol. **33**: 206–212. 1998.
8. Willett WC, Stampfer MJ, Colditz GA, Rosner BA, and Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. N Engl J Med. **323**: 1664–1672. 1990.
9. Nkondjock A, Shatenstein B, Maisonneuve P, and Ghadirian P. Specific fatty acids and human colorectal cancer: an overview. Cancer Detect Prev. **27**: 55–66. 2003.
10. Clausen MR, Bonnen H, and Mortensen PB. Colonic fermentation of dietary fibre to short chain fatty acids in patients with adenomatous polyps and colonic cancer. Gut. **32**: 923–928. 1991.
11. Slattery ML, Potter JD, Duncan DM, and Berry TD. Dietary fats and colon cancer: assessment of risk associated with specific fatty acids. Int J Cancer. **73**: 670–677. 1997.
12. Schloss I, Kidd MS, Tichelaar HY, Young GO, and O'Keefe SJ. Dietary factors associated with a low risk of colon cancer in coloured west coast fishermen. S Afr Med J. **87**: 152–158. 1997.
13. Ruemmele FM, Schwartz S, Seidman EG, Dionne S, Levy E, and Lentze MJ. Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway. Gut. **52**: 94–100. 2003.
14. Latham P, Lund EK, and Johnson IT. Dietary n-3 PUFA increases the apoptotic response to 1,2-dimethylhydrazine, reduces mitosis and suppresses the induction of carcinogenesis in the rat colon. Carcinogenesis. **20**: 645–650. 1999.
15. Reif S, Klein I, Lubin F, Farbstein M, Hallak A, and Gilat T. Pre-illness dietary factors in inflammatory bowel disease. Gut. **40**: 754–760. 1997.
16. Sakamoto N, Kono S, Wakai K, Fukuda Y, Satomi M, Shimoyama T, Inaba Y, Miyake Y, Sasaki S, Okamoto K, Kobashi G, Washio M, Yokoyama T, Date C, and Tanaka H. Dietary risk factors for inflammatory bowel disease: a multicenter case-control study in Japan. Inflamm Bowel Dis. **11**: 154–163. 2005.
17. Vagefi PA and Longo WE. Colorectal cancer in patients with inflammatory bowel disease. Clin Colorectal Cancer. **4**: 313–319. 2005.
18. Hlongwane C, Delves IG, Wan LW, and Ayorinde FO. Comparative quantitative fatty acid analysis of triacylglycerols using matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry and gas chromatography. Rapid Commun. Mass Spectrom. **15**: 2027–2034. 2001.
19. Hornung B, Amtmann E, and Sauer G. Lauric acid inhibits the maturation of vesicular stomatitis virus. J Gen Virol. **75**: 353–361. 1994.
20. Petschow BW, Batema RP, and Ford LL. Susceptibility of Helicobacter pylori to bactericidal properties of medium-chain monoglycerides and free fatty acids. Antimicrob. Agents Chemother. **40**: 302–306. 1996.
21. Sun CQ, O'Connor CJ, and Roberton AM. The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. Chem Biol Interact. **140**: 185–198. 2002.
22. Lala PK and Chakraborty C. Role of nitric oxide in carcinogenesis and tumour progression. Lancet Oncol. **2**: 149–156. 2001.
23. Rao CV. Nitric oxide signaling in colon cancer

chemoprevention. Mutat Res. **555**: 107–119. 2004.
24. Reddy BS. Studies with the azoxymethane-rat preclinical model for assessing colon tumor development and chemoprevention. Environ Mol Mutagen. **44**: 26–35. 2004.
25. Takahashi M and Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci. **95**: 475–480. 2004.
26. Lee JY, Sohn KH, Rhee SH, and Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. J Biol Chem. **276**: 16683–16689. 2001.
27. Lee JY, Ye J, Gao Z, Youn HS, Lee WH, Zhao L, Sizemore N, and Hwang DH. Reciprocal modulation of Toll-like receptor-4 signaling pathways involving MyD88 and phosphatidylinositol 3-kinase/AKT by saturated and polyunsaturated fatty acids. J Biol Chem. **278**: 37041–37051. 2003.
28. Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, and Wakabayashi K. 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**: 229–238. 2005.
29. Reddy BS, Tokumo K, Kulkarni N, Aligia C, and Kelloff G. Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related compounds. Carcinogenesis. **13**: 1019–1023. 1992.
30. Rao CV and Reddy BS. NSAIDs and chemoprevention. Curr Cancer Drug Targets. **4**: 29–42. 2004.
31. Reddy BS, Hirose Y, Cohen LA, Simi B, Cooma I, and Rao CV. Preventive potential of wheat bran fractions against experimental colon carcinogenesis: implications for human colon cancer prevention. Cancer Res. **60**: 4792–4797. 2000.
32. Reddy BS and Rao CV. Novel approaches for colon cancer prevention by cyclooxygenase-2 inhibitors. J Environ Pathol Toxicol Oncol. **21**: 156–164. 2002.
33. Giardiello FM, Hamilton SR, Krush AJ, Piantadosi S, Hylind LM, Celano P, Booker SV, Robinson CR, and Offerhaus GJ. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. N Engl J Med. **328**: 1313–1316. 1993.
34. Pretlow TP, O'Riordan MA, Somich GA, Amini SB, and Pretlow TG. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. Carcinogenesis. **13**: 1509–1512. 1992.
35. Bird R. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett. **93**: 55–71. 1995.
36. Alrawi SJ, Schiff M, Carroll RE, Dayton M, Gibbs JF, Kulavlat M, Tan D, Berman K, Stoler DL, and Anderson GR. Aberrant crypt foci. Anticancer Res. **26**: 107–119. 2006.
37. Takahashi M, Fukutake M, Yokota S, Ishida K, Wakabayashi K, and Sugimura T. Suppression of azoxymethane-induced aberrant crypt foci in rat colon by nimesulide, a selective inhibitor of cyclooxygenase 2. J Cancer Res Clin Oncol. **122**: 219–222. 1996.
38. Kohno H, Suzuki R, Sugie S, and Tanaka T. Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands. BMC Cancer. **5**: 46. 2005.
39. Barrachina MD, Panes J, and Esplugues JV. Role of nitric oxide in gastrointestinal inflammatory and ulcerative diseases: perspective for drugs development. Curr Pharm Des. **7**: 31–48. 2001.
40. Watanabe K, Kawamori T, Nakatsugi S, and Wakabayashi K. COX-2 and iNOS, good targets for chemoprevention of colon cancer. Biofactors. **12**: 129–133. 2000.
41. Tanaka T, Kohno H, Suzuki R, Yamada Y, Sugie S, and Mori H. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. Cancer Sci. **94**: 965–973. 2003.
42. Murakami A, Shigemori T, and 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. **135** (12 Suppl.): 2987S–2992S. 2005.
43. DeLany JP, Windhauser MM, Champagne CM, and Bray GA. Differential oxidation of individual dietary fatty acids in humans. Am J Clin Nutr. **72**: 905–911. 2000.
44. Tanaka T, Kawabata K, Kakumoto M, Makita H, Hara A, Mori H, Satoh K, Hara A, Murakami A, Kuki W, Takahashi Y, Yonei H, Koshimizu K, and Ohigashi H. Citrus auraptene inhibits chemically induced colonic aberrant crypt foci in male F344 rats. Carcinogenesis. **18**: 2155–2161. 1997.
45. Suzuki R, Kohno H, Sugie S, and Tanaka T. Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. Histol Histopathol. **20**: 483–492. 2005.
46. Rogler G, Brand K, Vogl D, Page S, Hofmeister R, Andus T, Knuechel R, Baeuerle PA, Scholmerich J, and Gross V. Nuclear factor kappaB is activated in macrophages and epithelial cells of inflamed intestinal mucosa. Gastroenterology. **115**: 357–369. 1998.
47. Yamamoto Y and Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest. **107**: 135–142. 2001.
48. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, and DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology. **107**: 1183–1188. 1994.
49. Elder DJ, Baker JA, Banu NA, Moorghen M, and Paraskeva C. Human colorectal adenomas demonstrate a size-dependent increase in epithelial cyclooxygenase-2 expression. J Pathol. **198**: 428–434. 2002.
50. Jacoby RF, Seibert K, Cole CE, Kelloff G, and Lubet RA. The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the min mouse model of adenomatous polyposis. Cancer Res. **60**: 5040–5044. 2000.
51. Oshima M, Murai N, Kargman S, Arguello M, Luk P, Kwong E, Taketo MM, and Evans JF. Chemoprevention of intestinal polyposis in the Apcdelta716 mouse by rofecoxib, a specific cyclooxygenase-2 inhibitor. Cancer Res. **61**: 1733–1740. 2001.
52. Lu H, Ouyang W, and Huang C. Inflammation, a key event in cancer development. Mol Cancer Res. **4**: 221–233. 2006.

Haploinsufficiency and acquired loss of *Bcl11b* and *H2AX* induces blast crisis of chronic myelogenous leukemia in a transgenic mouse model

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Chronic myelogenous leukemia (CML) is a hematological malignancy that begins as indolent chronic phase (CP) but inevitably progresses to fatal blast crisis (BC). p210BCR/ABL, a chimeric protein with enhanced kinase activity, initiates CML CP, and additional genetic alterations account for progression to BC, but the precise mechanisms underlying disease evolution are not fully understood. In the present study, we investigated the possible contribution of dysfunction of Bcl11b, a zinc-finger protein required for thymocyte differentiation, and of H2AX, a histone protein involved in DNA repair, to the transition from CML CP to BC. For this purpose, we crossed CML CP-exhibiting *p210BCR/ABL* transgenic (*BA*$^{tg/-}$) mice with *Bcl11b* heterozygous (*Bcl11b*$^{+/-}$) mice and *H2AX* heterozygous (*H2AX*$^{+/-}$) mice. Interestingly, *p210BCR/ABL* transgenic, *Bcl11b* heterozygous (*BA*$^{tg/-}$*Bcl11b*$^{+/-}$) mice and *p210BCR/ABL* transgenic, *H2AX* heterozygous (*BA*$^{tg/-}$*H2AX*$^{+/-}$) mice frequently developed CML BC with T-cell phenotype and died in a short period. In addition, whereas p210BCR/ABL was expressed in all of the leukemic tissues, the expression of Bcl11b and H2AX was undetectable in several tumors, which was attributed to the loss of the residual normal allele or the lack of mRNA expression. These results indicate that Bcl11b and H2AX function as tumor suppressor and that haploinsufficiency and acquired loss of these gene products cooperate with p210BCR/ABL to develop CML BC. (*Cancer Sci* 2009; 100: 1219–1226)

Chronic myelogenous leukemia (CML) is a disorder of hematopoietic stem cells, characterized by excessive and uncontrolled proliferation of differentiated myeloid cells.[1–3] Clinically, CML undergoes two different stages.[1–3] In the initial stage, chronic phase (CP), the leukemic cells retain the ability to differentiate into mature granulocytes and are sensitive to conventional therapies. However, after several years' duration of CP, the disease inevitably accelerates and ultimately progresses to the terminal stage, blast crisis (BC), which exhibits aggressive proliferation of immature blast cells and is resistant to intensive therapies.[1–3]

The cytogenetic hallmark of CML CP is t(9;22)(q34;q11) (known as Philadelphia chromosome, Ph), which generates a *BCR–ABL* fusion gene encoding a 210-kDa chimeric protein (p210BCR/ABL).[1–3] p210BCR/ABL possesses a constitutively active tyrosine kinase activity, which plays an essential role in the initiation of the disease.[1–3] Although Ph is the unique and sole chromosomal abnormality in CP, additional and non-random chromosomal abnormalities are frequently observed in BC, indicating that secondary genetic events account for the disease progression.[1–3]

To understand the pathogenesis of the disease, it is necessary to establish animal models that express *p210BCR/ABL* and recapitulate the clinical course of CML. For this purpose, we generated transgenic mice expressing *p210BCR/ABL* under the control of the mouse *TEC* promoter.[4] The *p210BCR/ABL* transgenic (hereafter, designated as *BA*$^{tg/-}$) mice reproducibly exhibited a myeloproliferative disorder closely resembling human CML CP.[4] In addition, by crossing *BA*$^{tg/-}$ mice with *p53* heterozygous mice and *Dok-1/Dok-2* knockout mice, we showed that the loss of p53 and absence of Dok-1/Dok-2 accelerated the disease and caused CML BC.[5,6] Furthermore, by applying retroviral insertional mutagenesis to *BA*$^{tg/-}$ mice, we demonstrated that overexpression and enhanced kinase activity of p210BCR/ABL and altered expression of Notch1 contribute to CML BC.[7] These results demonstrated that the *BA*$^{tg/-}$ mouse is not only regarded as a model for CML CP, but is also useful for investigating the molecular mechanisms underlying the progression from CP to BC.

Chromosomal and molecular analyses have revealed that several mechanisms are implicated in this process, such as: (i) loss of tumor suppressor; (ii) differentiation arrest; and (iii) chromosomal instability.[3] Indeed, as an example of (i), we demonstrated that loss of p53 cooperates with p210BCR/ABL and induces CML BC.[5,6] In the present report, as candidate genes for (ii) and (iii), we chose *Bcl11b* (also known as *Rit1* and *Ctip2*), encoding a transcription factor required for thymocyte differentiation,[8] and *H2AX*, encoding a histone protein involved in DNA repair,[9] and examined the possible contribution that dysfunction of these gene produces for the disease progression of CML. For this purpose, we crossed *BA*$^{tg/-}$ mice with mice heterozygous for *Bcl11b* (*Bcl11b*$^{+/-}$) or *H2AX* (*H2AX*$^{+/-}$) and generated *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ mice and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice. Interestingly, both types of double transgenic mouse frequently developed CML BC and died in a short period. The pathological, flow cytometric, molecular, and chromosomal analyses of the diseased mice are described.

Materials and Methods

Mice. *p210BCR/ABL* transgenic, *Bcl11b* heterozygous, and *H2AX* heterozygous mice were generated as described previously.[4,8,10] Crossing and genotyping of the mice were carried out as described previously.[5] All of the mice were kept according to the guidelines of the Institute of Laboratory Animal Science, Hiroshima University.

Pathological analysis. Autopsies were carried out on dead or moribund animals. Peripheral blood smears were stained with Wight-Giemsa. After gross examination, tissues were fixed in

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10% neutral buffered formaldehyde and representative slices were stained with hematoxylin–eosin (HE).

Western blot analysis. Proteins were extracted from tissues, separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with appropriate antibodies as described previously.[4,8] The antibodies used in this study were: anti-ABL monoclonal antibody, Ab3 (Oncogene Science, Cambridge, MA, USA); an anti-Bcl11b polyclonal antibody;[8] and an antihistone H2AX antibody (Millipore, Bedford, MA, USA). Positive signals were detected with the enhanced chemiluminescence system.

Southern blot analysis and genomic PCR. For Southern blotting, DNA was digested with restriction enzymes, separated in an agarose gel, blotted to a nylon membrane, and hybridized with a ^{32}P-dCTP-labeled *TCRβ* probe. Genomic PCR was carried out using the following primers as described previously:[11] P1 (5′-TGCAGCTTTCCGGGCGATGCCA-3′), P2 (5′-ACTTTCCCAG-AACCCCACGC-3′), and P3 (5′-CCTGCTTGCCGAATATCAT-GGTGG-3′) for *Bcl11b*; and P1 (5′-TCACATTGTTTCCTTCG-GTGTCAC-3′), P2 (5′-AAGTGTTGTGATTGGGAAGCGTAG-3′), P3 (5′-AGATCCCGTTGACTGAACACAGG-3′), P4 (5′-TTCAG-GTTTTGTTGTCGCGCCGTAG-3′), and P5 (5′-TCAGCTCTTT-CTGTGAGGGAGGTGG-3′) for *H2AX*.

Northern blot analysis and RT-PCR. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), separated in 1.2% formaldehyde gel, blotted to a nylon membrane, and hybridized with a ^{32}P-dCTP-labeled *H2AX* probe. RT-PCR was carried out using the following primers as described previously:[11] 5′-CGAGCTCA-GGAAAGTGTCCGAG-3′ and 5′-GGAAATTCATGAGCGGG-GACTG-3′ for *Bcl11b*; 5′-CCTTCTGGAAGACTTGGCCTTC-3′ and 5′-GAGGAAGATGTGCCTGTTACC-3′ for *H2AX*; and 5′-TTCAACACCCCAGCCATGTA-3′ and 5′-CTCAGGAGGAG-CAATGATCT-3′ for *β-actin*.

Flow cytometric analysis. Cells were stained with FITC- or phycoerythrin (PE)-conjugated anti-Thy-1.2, anti-B220, anti-Mac1, and anti-Gr1 monoclonal antibodies (Pharmingen, San Diego, CA, USA), as described previously.[5]

Chromosomal analysis. Chromosomes were prepared by means of standard culture procedures for tumor cells and treated with trypsin-Giemsa as described previously.[12]

Patient samples and normal bone marrow cells. Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University.[13] Diagnosis of CML CP or CML BC (myeloid or B-lymphoid lineage) was carried out based on morphological, cytogenetic, and immunophenotypic analyses. Normal bone marrow cells were obtained from a healthy volunteer.

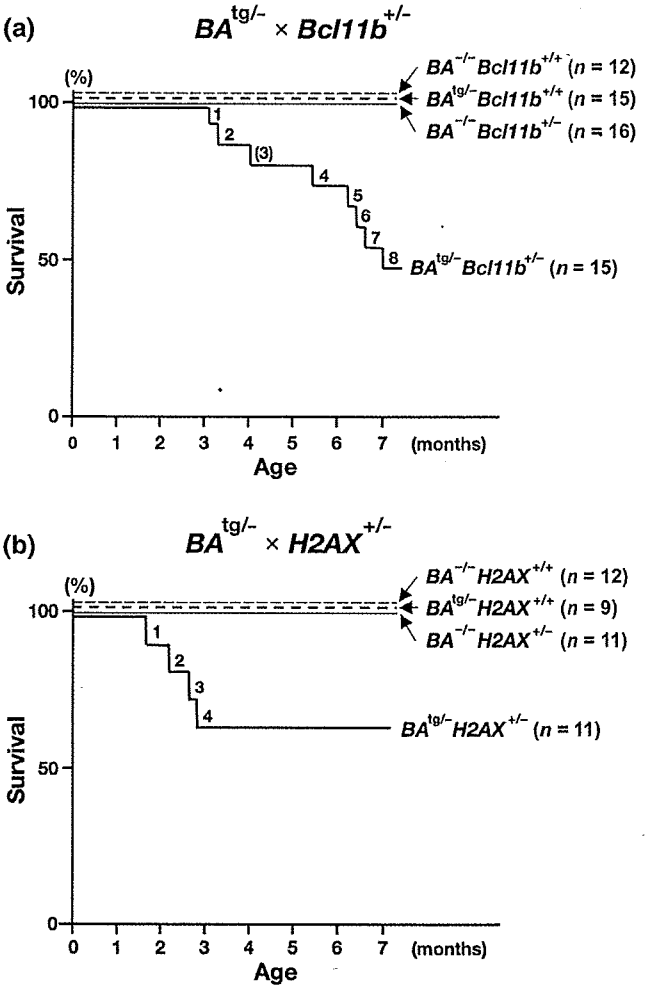

Fig. 1. Survival curves of mice generated by (a) $BA^{tg/-} \times Bcl11b^{+/-}$ and (b) $BA^{tg/-} \times H2AX^{+/-}$. The survival curves of $BA^{-/-}Bcl11b^{+/+}$ and $BA^{-/-}HX2A^{+/+}$, $BA^{tg/-}Bcl11b^{+/+}$ and $BA^{tg/-}H2AX^{+/+}$, $BA^{-/-}Bcl11b^{+/-}$ and $BA^{-/-}H2AX^{+/-}$, and $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ mice are shown as thin dotted, thick dotted, thin continuous, and thick continuous lines respectively. In the $BA^{tg/-} \times Bcl11b^{+/-}$ group, 8 of 15 $BA^{tg/-}Bcl11b^{+/-}$ mice died within 7 months of age and in the $BA^{tg/-} \times H2AX^{+/-}$ group, 4 of 11 $BA^{tg/-}H2AXb^{+/-}$ died within 3 months of age. The number of an unanalyzable $BA^{tg/-}Bcl11b^{+/-}$ mouse due to death (no. 3) is shown in parentheses.

Results

***$BA^{tg/-}Bcl11b^{+/-}$* and *$BA^{tg/-}H2AX^{+/-}$* mice developed acute leukemia and died in a short period.** To investigate the contribution of haploinsufficiency of *Bcl11b* and *H2AX* to the disease progression of CML, we crossed CML-exhibiting $BA^{tg/-}$ mice with $Bcl11b^{+/-}$ mice and $H2AX^{+/-}$ mice. Mice with four different genotypes were generated by each crossing: $BA^{tg/-} \times Bcl11b^{+/-}$ created $BA^{-/-}Bcl11b^{+/+}$ (wild type), $BA^{tg/-}Bcl11b^{+/+}$ (*p210BCR/ABL* transgenic), $BA^{-/-}Bcl11b^{+/-}$ (*Bcl11b* heterozygous), and $BA^{tg/-}Bcl11b^{+/-}$ (*p210BCR/ABL* transgenic, *Bcl11b* heterozygous); and $BA^{tg/-} \times H2AX^{+/-}$ produced $BA^{-/-}H2AX^{+/+}$ (wild type), $BA^{tg/-}H2AX^{+/+}$ (*p210BCR/ABL* transgenic), $BA^{-/-}H2AX^{+/-}$ (*H2AX* heterozygous), and $BA^{tg/-}H2AX^{+/-}$ (*p210BCR/ABL* transgenic, *H2AX* heterozygous). Mice with these genotypes were normally born approximately at the expected Mendelian ratio (see the mouse number shown in parentheses in Fig. 1), indicating that the crossing did not affect the embryonic development of the mice.

All of the mice were observed continuously and peripheral blood parameters were counted routinely. The genotype-based survival curves of the mice in each crossing are shown in Figure 1. During a 7-month observation period, in the $BA^{tg/-} \times Bcl11b^{+/-}$ group, 8 of 15 $BA^{tg/-}Bcl11b^{+/-}$ died of acute leukemia, in contrast $BA^{-/-}Bcl11b^{+/+}$, $BA^{tg/-}Bcl11b^{+/+}$, and $BA^{-/-}Bcl11b^{+/-}$ littermates did not show any disorders (Fig. 1a). As for the $BA^{tg/-} \times H2AX^{+/-}$ group (lower panel), 4 of 11 $BA^{tg/-}H2AX^{+/-}$ mice exhibited proliferation of blast cells and died within 3 months of birth, whereas no disease was observed in $BA^{-/-}H2AX^{+/+}$, $BA^{tg/-}H2AX^{+/+}$, and $BA^{-/-}H2AX^{+/-}$ littermates (Fig. 1b).

The representative results of pathological analysis of $BA^{tg/-}Bcl11b^{+/-}$ and $BA^{tg/-}H2AX^{+/-}$ leukemic mice are shown in Figure 2. Macroscopically, both leukemic mice exhibited marked thymic enlargement with splenomegaly, which were occasionally associated with lymph node swelling or pleural effusion (data not shown). The peripheral blood smears exhibited proliferation of blast cells morphologically resembling lymphoblasts (upper panels of Fig. 2). Tissue sections showed that the blast cells caused destruction of the basic structure of the thymus (second panels of Fig. 2) and infiltrated in non-hematopoietic tissues, such as liver (third panels of Fig. 2). In contrast, the bone marrow

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Table 1. Characteristics of *p210BCR/ABL*$^{tg/-}$ *Bcl11b*$^{+/-}$ leukemic mice

Mouse no.	Age at disease (months)	PB parameters			Macroscopic tumor sites	*TCRβ* status	p210BCR/ABL expression	Bcl11b expression	*Bcl11b* status
		WBC ($\times 10^3/\mu L$)	Hb (g/dL)	Plt ($\times 10^4/\mu L$)					
1	3.1	35.0	12.5	65.6	Thy, Spl	G/R	+	+	G/T
2	3.3	5.0	10.5	64.8	Thy, Spl	G/loss	+	+	G/T
3	4.0†	ND	ND	ND	Thy	ND	ND	ND	ND
4	5.3	2.3	7.1	44.2	Thy	G/loss	+	+	G/T
5	6.0	12.0	12.3	35.5	Thy, PE	G/loss	+	–	T/loss
6	6.1	6.6	13.9	53.5	Thy	G/R	+	–	T/loss
7	6.4	14.6	15.5	47.1	Thy, Spl	G/R	+	+	G/T
8	6.9	1.5	14.1	74.9	Thy, PE	G/R	+	–	T/loss

†Found dead. G, germline; Hb, hemoglobin; ND, not done; PB, peripheral blood; PE, pleural effusion; Plt, platelet; R, rearranged; Spl, spleen; T, targeted; Thy, thymus; WBC, white blood cell.

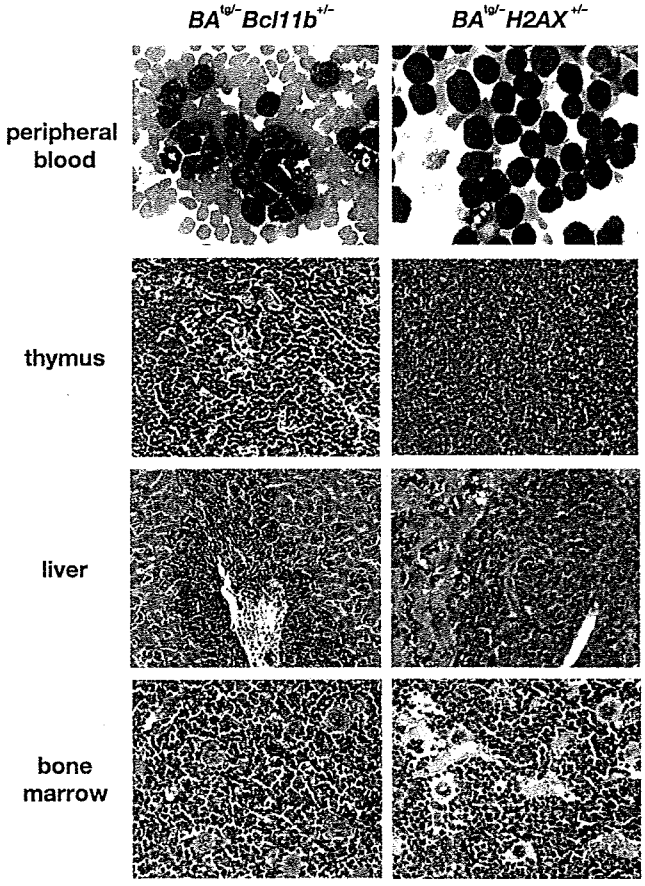

Fig. 2. Representative results of pathological analysis of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ (left panels) and *BA*$^{tg/-}$*H2AX*$^{+/-}$ (right panels) leukemic mice. Wight-Giemsa-stained peripheral blood smears and HE-stained tissue slices are shown. In both leukemic mice, blast cells proliferated in the peripheral blood (upper panels), caused destruction of the basal structure of the thymus (second panels), and infiltrated around the vessel and in the sinusoids in the liver (third panels). In contrast, bone marrow exhibited myeloid cell hyperplasia with differentiation and proliferation of megakaryocytes (bottom panels).

showed a predominance of myeloid cells with differentiation and proliferation of megakaryocytes (bottom panels of Fig. 2). These results demonstrated that haploinsufficiency of *Bcl11b* and *H2AX* cooperated with *p210BCR/ABL*, transformed *p210BCR/ABL*-expressing hematopoietic cells, and caused CML BC. The characteristics of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice are summarized in Table 1 and Table 2, respectively.

Leukemias that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice were of T-cell lineage and were mostly clonal in origin. To determine the cell lineage and clonality of the leukemias that developed in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ mice, blast cells were subjected to flow cytometric and Southern blot analyses.

The representative results of flow cytometric analysis of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic cells are shown in Figure 3(a). In both types of mice, leukemic cells were highly positive for Thy1.2, the antigen specific for T lymphocytes, but were negative for CD19, Gr1, and Mac1, the markers for B lymphocytes, granulocytes, and macrophages respectively.

The clonality of the leukemic cells was examined by gene rearrangement analysis. DNA extracted from a control thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were digested with a restriction enzyme and blotted with the T-cell receptor β (*TCR-β*) gene. As shown in Figure 3(b), more than half of the samples (no. 1 and no. 6–8 in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and no. 1 and 2 in *BA*$^{tg/-}$*H2AX*$^{+/-}$) showed rearranged bands, and in the remaining samples (no. 2, 4, and 5 in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and no. 3 and 4 in *BA*$^{tg/-}$*H2AX*$^{+/-}$), loss of the upper germline band was observed (the positions of germline bands are indicated by arrows and shown as 'G'). These results demonstrated that the blast cells of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were committed to the T-cell lineage and most of the tumors were clonal in origin.

Frequent and acquired loss of Bcl11b and H2AX protein expression in the tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice. We then investigated protein expression in the tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice. Proteins extracted from a control thymus and tumor tissues of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice were blotted with antibodies against c-ABL, Bcl11b, and H2AX.

The results of p210BCR/ABL expression in these tumors are shown in the upper panels of Figure 4(a,b). As shown in both panels, the 210-kDa band was detected in all of the tumor samples, indicating that the blast cells originated from *p210BCR/ABL*-expressing hematopoietic precursors. We next examined the expression of Bcl11b and H2AX proteins in *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic samples respectively. Interestingly, in the anti-Bcl11b western blot, the expression of Bcl11b was found to be lost in three of seven samples (no. 5, 6, and 8, middle panel of Fig. 4a). In addition, in the anti-H2AX blot, the expression of H2AX was undetectable in two of four samples (no. 2 and 3, middle panel of Fig. 4b). These results indicated that the protein expression of Bcl11b and H2AX was lost in several samples of *BA*$^{tg/-}$*Bcl11b*$^{+/-}$ and *BA*$^{tg/-}$*H2AX*$^{+/-}$ leukemic mice.

To investigate the molecular mechanism underlying the loss of Bcl11b and H2AX expression, DNA extracted from tumor