and apoptosis induction of α-eleostearic and α-calendic acids were completely suppressed by the addition of α-tocopherol to the culture medium, as previously reported (12, 14, 15). It is noteworthy that the cytotoxic effect of β-eleostearic acid and β-calendic acid, which have all-*trans*-conjugated double bonds, were not counteracted completely in the presence of α-tocopherol (Figures 5, 6). These results suggest that β-eleostearic acid and β-calendic acid reduce the cell viability of Caco-2 *via* another pathway in addition to the pathway of lipid peroxidation. On the other hand, the cytotoxic effect of β-eleostearic acid on DLD-1, another colon cancer cell line, was lost by the addition by α-tocopherol (data not shown). Thus, the metabolic and signal transduction systems in Caco-2 cells may be important for different anticancer effects among CLN isomers. Further investigations are required for a better understanding of the specific mechanisms underlying the cytotoxic effects and apoptosis induction by β-eleostearic and β-calendic acids.

In summary, two all-*trans*-CLN isomers, β-eleostearic and β-calendic acids, markedly reduced the cell viability and induced apoptosis in Caco-2 cells. Their effects were stronger than those of α-eleostearic and α-calendic acids, which have the *cis* configuration. Furthermore, since the cytotoxic effects of β-eleostearic and β-calendic acids on Caco-2 cells were not suppressed completely by α-tocopherol, CLN isomers with the all-*trans* configuration are suggested to exert anticancer effects through signaling pathways different from those of the CLN isomers with the *cis* configuration.

Acknowledgements

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Modification by curcumin of mutagenic activation of carcinogenic *N*-nitrosamines by extrahepatic cytochromes P-450 2B1 and 2E1 in rats

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To elucidate the mechanism underlying suppression by curcumin of esophageal carcinogenesis induced by NMBA, we evaluated the CYP level and mutagenic activation of environmental carcinogens, by immunoblot analyses and Ames preincubation test, respectively, and bilirubin, 4-nitrophenol and testosterone UDPGT activities in F344 rats treated with curcumin and/or NMBA. No significant alterations in the hepatic levels of constitutive CYP proteins, mutagenic activation by liver S9 or hepatic UDPGT activities were produced by subcutaneous treatment with 0.5 mg/kg NMBA for 5 weeks and/or feeding of 0.05% and 0.2% curcumin for 6 weeks. In contrast, gavage of 0.2% curcumin decreased esophageal CYP2B1 and 2E1 by up to 60%, compared with vehicle control. Similarly, intragastric treatment with 270 mg/kg curcumin decreased esophageal and gastric CYP2B1 and CYP2E1, but not in lung, kidney or intestine. Conversely, large intestinal CYP2B1 was 2.8-fold higher in the treated rats than in control rats. Mutagenic activities of NOC, including NMBA, in the presence of esophagus and stomach S9 were markedly decreased in the treated rats, whereas those in the presence of large intestine S9 were 2.2–3.0-fold above control. These results show that modifying effects of curcumin on esophageal carcinogenesis can be attributed to a decrease in metabolic activation of NMBA by esophageal CYP2B1 during the initiation phase, without the contribution of metabolic activation and inactivation by liver. Further, the present findings suggest the potential of curcumin for modification of gastric and intestinal carcinogenesis initiated with NOC. (*Cancer Sci* 2006; 97: 896–904)

Human esophageal cancer has been closely associated with exposure to nitrate and nitrite, which are precursors of NOC, and exposure to NMBA is also associated with an increased risk of human cancer in the esophagus in China.[1–4] In fact, NMBA is known to be the most potent carcinogen for rat esophagus, irrespective of its mode of administration.[5,6] It has been shown that esophageal mucosa microsomes from male Sprague–Dawley rats can form benzaldehyde and formaldehyde from NMBA at rates 1/5 and 1/60 of hepatic levels of both metabolites, respectively, and the metabolisms are inhibited more than 95% by CO and 70% by SKF525A, typical CYP inhibitors.[7] O^6-methylated guanine level in rats given NMBA is six times higher in esophagus DNA than in liver DNA,[8] and isothiocyanates markedly decrease the incidence and multiplicity of NMBA-induced esophageal tumors, with inhibition of esophageal DNA methylation in F344 rats.[9] Further, it has been shown that ethanol has an enhancing effect on DEN-induced esophageal tumorigenesis in F344 rats,[10] with enhancement of metabolic activation of DEN by hepatic CYP2E1.[11] These findings indicate the importance of metabolic activation of NMBA by the target organ and/or liver during the initiation phase, and some NOC are known to be metabolized in the esophagus at a relatively high rate, often leading to high levels of esophageal DNA alkylation.[8,12,13] The total CYP content of the esophagus microsomes is only 7% of that of liver microsomes,[14] but little is known about the expression of CYP species in rat esophagus; CYP1A1 and 2A3 proteins are constitutively expressed in rat esophagus microsomes, but CYP2B1/2, 2E1 and 3A2 proteins are not.[14–16] We have recently shown that NMBA is mutagenetically activated by hepatic CYP2B1 and 2B2, but not CYP2E1, in rats.[11]

Curcumin is the major yellow pigment in turmeric (the ground rhizome of *Curcuma longa* Linn), widely used as a spice and coloring agent in several foods, such as curry, mustard and potato chips as well as cosmetics and drugs. This natural dye has been reported to inhibit tumors in various organs, such as the tongue, skin, mammary gland, forestomach, liver, duodenum and colon from laboratory animals, during the initiation and/or promotion phases.[17] In addition, Ushida *et al.*[18] reported that esophageal tumorigenesis induced by NMBA is markedly suppressed by treatment with curcumin during the initiation and promotion phases in F344 rats. Curcumin is not toxic to humans up to 8 g/day when taken by mouth for 3 months,[19] being suggested as an

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Abbreviations: 4-NP, 4-nitrophenol; AFB_1, aflatoxin B_1; BHP, *N*-nitrosobis(2-hydroxypropyl)amine; BP, benzo[a]pyrene; CYP, cytochrome P-450; DEN, *N*-nitrosodiethylamine; DMN, *N*-nitrosodimethylamine; DMSO, dimethyl sulfoxide; EROD, ethoxyresorufin O-deethylase; G6P, glucose 6-phosphate; G6PDH, G6P dehydrogenase; Glu-P-1, 2-amino-6-methyldipyrido[1,2-a:3',2'-*d*]imidazole; GST, glutathione *S*-transferase; HCA, heterocyclic amine; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MC, 3-methylcholanthrene; MeAαC, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; MROD, methoxyresorufin *O*-demethylase; NDMM, *N*-nitroso-2,6-dimethylmorpholine; NMBA, *N*-nitrosomethylbenzylamine; NOC, *N*-nitroso compound; NPYR, *N*-nitrosopyrrolidine; PB, phenobarbital; PROD, pentoxyresorufin O-dealkylase; SD, standard deviation; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; UDP, uridine diphosphate; UDPGT, UDP-glucuronyltransferase.

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effective chemopreventive agent. Turmeric and curcumin are known to reduce BP- or 7,12-dimethylbenz[*a*]anthracene-derived DNA adducts in rat liver[20] and in hamster buccal pouch.[21] Turmeric and curcumin have been reported to be a competitive inhibitor for EROD and PROD,[22] and to decrease total CYP content[23] and EROD, MROD and PROD activities in liver, lung and stomach microsomes from rats.[24] Curcumin also induces 4-NP UDPGT[25,26] and 1-chloro-2,4-dinitrobenzene GST[23,25,27–29] in rat liver. However, to our knowledge, no data have shown the effect of curcumin on hepatic levels of CYP enzymes, metabolic activation by CYP and UDPGT activities towards bilirubin and testosterone in rats, or on extrahepatic levels of CYP isoforms in any animal species.

In order to elucidate the mechanism(s) underlying suppression of NMBA-induced esophageal tumorigenesis by curcumin, hepatic and extrahepatic levels of microsomal CYP enzymes known to activate typical environmental carcinogens, mutagenic activation of these carcinogens by tissue S9 and three kinds of hepatic UDPGT activities were assayed in male F344 rats treated with curcumin and/or NMBA.

Materials and Methods

Chemicals

BP, DMN, DEN, 4-NP, testosterone, bilirubin, UDP-glucuronic acid, Trp-P-2 and MeAαC acetates, Glu-P-1 hydrochloride and IQ were purchased from Wako Pure Chemicals (Osaka, Japan) and NMBA was from Sakai Laboratory (Fukui, Japan). Curcumin (>98% pure) was obtained from Nacalai Tesque (Kyoto, Japan), NPYR was from Aldrich Chemical (Milwaukee, WI, USA) and AFB_1 was from Makor Chemicals (Jerusalem, Israel). UDP-[^{14}C(U)]glucuronic acid was purchased from American Radiolabeled Chemicals (St Louis, MO, USA) and G6P, G6PDH, $NADP^+$, NADPH, NADH and ATP were from Oriental Yeast (Tokyo, Japan). All other commercial products were of the purest grade available. BHP and NDMM were synthesized in our laboratory as described previously.[30]

Animal treatment and tissue preparation

All animal experiments were undertaken following guidelines for the care and use of experimental animals set by Gifu Pharmaceutical University and Gifu University. Male 4- or 5-week-old F344 rats purchased from Japan SLC (Hamamatsu, Japan) were housed in wire cages (two or three rats/cage) and maintained under standard laboratory conditions. Thirty male rats, 5 weeks old, were divided into six groups consisting of five animals. Rats in Groups 3 and 5 were given 0.05% curcumin in the basal diet CE-2 (CLEA Japan, Tokyo, Japan) and those in Groups 4 and 6 were given 0.2% curcumin throughout the experiment. One week after initiation of the respective diets, rats in Groups 1, 3 and 4 were subcutaneously treated with 20% DMSO three times per week for 5 weeks, and rats in Groups 2, 5 and 6 received 0.5 mg/kg NMBA dissolved in 20% DMSO.[18] All the animals were decapitated 24 h after the last dose of the vehicle or NMBA. Alternatively, 40 male rats, 6 weeks old, were divided into four groups. Ten rats were given 20% DMSO, serving as a control, 20 rats were treated with 70 or 270 mg/kg curcumin through an intragastric tube, and 10 rats were subcutaneously treated with 0.5 mg/kg NMBA as a single dose. All animals were killed 6 h (NMBA-treated rats) or 24 h after treatment. In addition, 32 rats, 6 weeks old, were divided into two groups and were given DMSO or 270 mg/kg curcumin as a single dose, then killed 24 h after injection. Livers, kidneys and lungs were perfused *in situ* with ice-cold sterile 1.15% KCl and 25% homogenates in 1.15% KCl were prepared. Esophagus, stomach, small intestine and large intestine were rinsed in ice-cold 1.15% KCl after harvesting, and these mucosae were then stripped off the submucosae and any muscular tissues. S9 and microsomal fractions from these tissues were prepared using established procedures.[30]

Assay of total CYP content

Total CYP content in liver and esophagus microsomes was spectroscopically determined by the method of Omura and Sato.[31]

Western blot analysis

Gel electrophoresis and blot analyses were carried out as described in detail previously,[32] according to the established methods of Laemmli[33] and Towbin *et al.*[34], respectively. Separated proteins were transferred by semi-dry electroblotting from the sodium dodecylsulfate–polyacrylamide gel to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) in 25 mM Tris buffer (pH = 8.3) containing 0.19 M glycine and 20% (v/v) methanol. The membranes were blocked by incubation with phosphate-buffered saline containing 5% skim milk for 1 h. The blocked membranes were incubated with goat antirat polyclonal antibodies for CYP1A1/2, CYP2B1/2, CYP2E1 and CYP3A2 (Daiichi Pure Chemicals, Tokyo, Japan) then stained using biotinylated antigoat immunoglobulin G (Vector Laboratories, Burlingame, CA, USA) and a Wako ABC-POD kit (Wako Pure Chemicals).

Mutation assay

All tests were carried out using the Ames preincubation assay.[35] Six NOC were dissolved in 100 μL of water and all the other carcinogens in 50 μL of DMSO. The mutagenicities of IQ (dose, 0.03 μg/plate), Trp-P-2 (0.03 or 0.3 μg/plate), Glu-P-1 (0.3 μg/plate), MeAαC (10 μg/plate), BP (5 μg/plate), AFB_1 (1 μg/plate), NPYR (0.25 mg/plate), NMBA (1 mg/plate), DMN and DEN (1 or 10 mg/plate), and BHP and NDMM (10 mg/plate) were checked in the presence of liver, esophagus, stomach or intestine S9 mix, using established procedures.[11,36–39] The amount of tissue S9 fraction was 10 μL/plate for the HCAs, 50 μL for BP and 150 μL for the NOC and AFB_1. *Salmonella typhimurium* tester strains TA100 and TA98 were used for the six NOC and the other carcinogens, respectively. The S9 mix contained 4 mM NADPH, 4 mM NADH, 0.5 U G6PDH, 5 mM G6P and 5 mM ATP, except for the NOC, for which 4 mM $NADP^+$ and 5 mM G6P were used.[32]

Assay of UDPGT activity

UDPGT activities towards bilirubin and 4-NP in liver microsomes were assayed according to the methods of Heirwegh *et al.*[40] and Isselbacher *et al.*,[41] respectively, and that towards testosterone was determined using UDP-[^{14}C(U)]glucuronic acid as described by Matern *et al.*[42]

Statistical analysis

Statistical analyses by Student's *t*-test were carried out to determine the significance of the differences between groups. All statements of significance are either $P < 0.05$ or $P < 0.01$.

Results

Determination of total CYP content and CYP isoform in several tissues

Figure 1 shows immunoblots and levels of microsomal CYP proteins in male F344 rats treated with NMBA and curcumin for up to 6 weeks. Esophageal CYP2B1 and CYP2E1 were constitutively detected with an antibody against hepatic CYP2B1/2 and CYP2E1 in the vehicle group (Group 1). In Group 2 rats subcutaneously treated with 0.5 mg/kg NMBA for 5 weeks the CYP2B1 level was not changed, but that in Group 3 and 4 rats fed 0.05% and 0.2% curcumin, respectively, for 6 weeks was decreased by 30%–40% relative to Group 1 rats. Similarly, the constitutive CYP2B1 level was 40% and 60% lower in Group 5 and 6 rats, respectively, than in Group 1 rats. Treatment with NMBA and/or 0.05% curcumin caused no decrease in the constitutive CYP2E1 level, whereas that in Group 4 and 6 rats was decreased by 30% relative to Group 1 rats. However, there were no significant differences in hepatic levels of constitutively detected CYP2B2, 2E1, 1A2 or 3A2 among the six groups, and neither CYP2B1 nor 1A1 were expressed in any group of rats. In addition, no significant alterations in hepatic and esophageal levels of total CYP content spectroscopically determined were observed in the six groups (liver, 1.35–1.60 nmol/mg; esophagus, 0.30–0.39 nmol/mg).

In order to confirm the new evidence for CYP expression and suppression, the potency of curcumin was further checked in hepatic and extrahepatic microsomes from rats orally treated with 70 and 270 mg/kg curcumin, corresponding to the daily intake in the diet containing 0.05% and 0.2% curcumin, respectively, as a single dose. As shown in Figure 2, esophageal CYP1A1, 1A2 and 3A2, in addition to CYP2B1 and 2E1, were detected with antibodies against hepatic CYP in the vehicle group. The constitutive levels of CYP2B1 and 2E1 in rats given 270 mg/kg curcumin were significantly

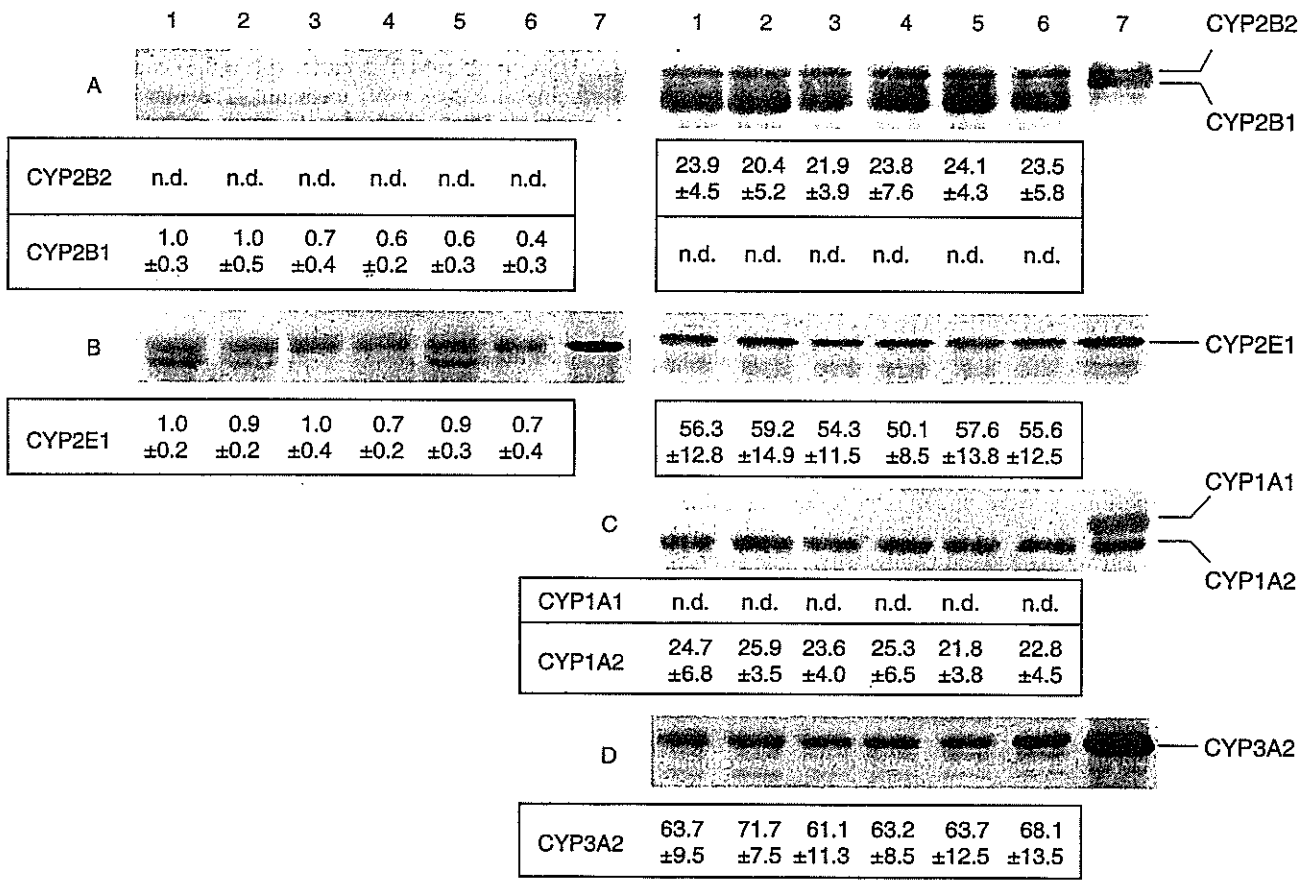

Fig. 1. Immunoblots and densitometric determination of expression of CYP protein in esophagus (left) and liver (right) microsomes from rats treated with NMBA, curcumin or both. Both microsomes were pooled from five rats treated with vehicle (lane 1) or 0.5 mg/kg NMBA (lane 2) three times per week for 5 weeks, 0.05% (lane 3) or 0.2% (lane 4) curcumin for 6 weeks, and NMBA + 0.05% (lane 5) or + 0.2% (lane 6) curcumin. Lane 7 contains CYP standards from male Sprague–Dawley rats treated with PB (A and D), acetone (B) or MC (C). Liver microsomes contain 0.4 μg (A–C) and 1.0 μg (D) microsomal protein, and the values represent means ± SD of pmol/mg microsomal protein obtained from four experiments. Esophagus microsomes (A, B) contain 30 μg microsomal protein, and the values represent means ± SD of the ratio to arbitrary units obtained with the vehicle group. n.d., not detected.

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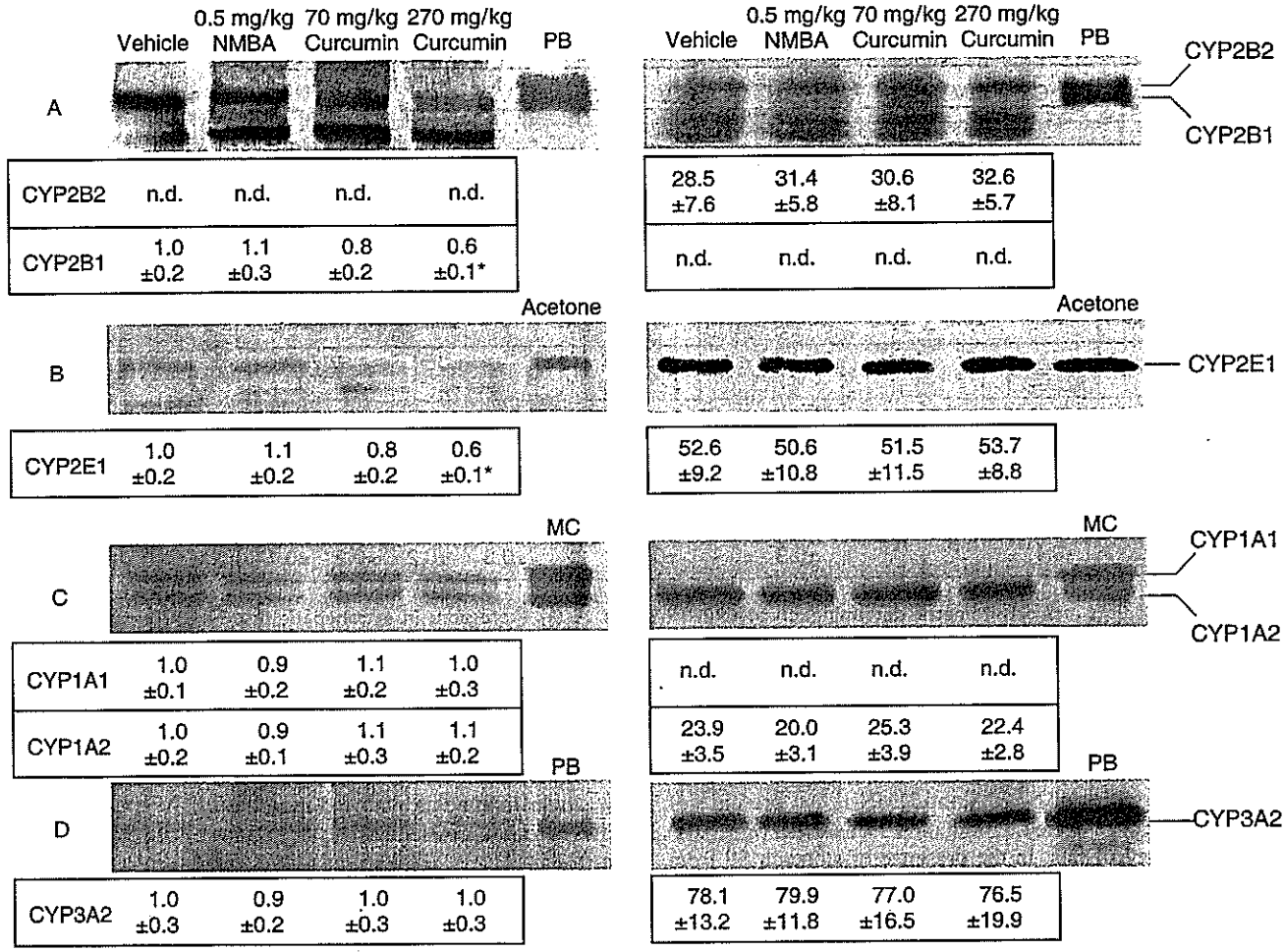


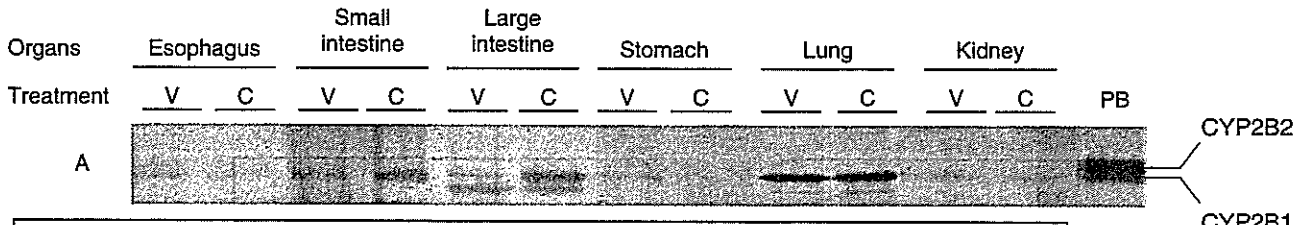
Fig. 2. Immunoblots and densitometric determination of expression of CYP protein in esophagus (left) and liver (right) microsomes from rats treated orally with curcumin or subcutaneously with NMBA as a single dose. Both microsomes were pooled from 10 rats 24 h after each treatment. Lanes PB, acetone and MC contain CYP standards. The amounts of each microsomal protein, including (C) and (D) in the esophagus, and the means ± SD are identical to those described in Figure 1 legend. *$P < 0.01$, compared with the vehicle group (Student's *t*-test). n.d., not detected.

decreased by 40% ($P < 0.01$), relative to control rats, whereas levels of the other CYP isoforms were not changed, and NMBA produced no significant changes in the five CYP isoforms. There were also no significant differences in hepatic levels of constitutive CYP2B2, 2E1, 1A2 and 3A2 among the four groups, and neither CYP2B1 nor 1A1 were detected in any group of rats. Figure 3 shows immunoblots of CYP2B1 and 2E1 in six extrahepatic tissues, including the esophagus, from rats treated with 270 mg/kg curcumin as a single dose. CYP2B1 and 2E1 proteins, but not CYP2B2, were constitutively detected in all the extrahepatic tissues, except for CYP2E1 in the small intestine. The treatment with curcumin significantly decreased gastric and esophageal CYP2B1 by 30% and 40% ($P < 0.05$), respectively, relative to the vehicle group. It also decreased esophageal CYP2E1 by 60% ($P < 0.01$) and gastric CYP2E1 to an undetectable level. In contrast, the CYP2B1 level in the large intestine was 2.8-fold higher ($P < 0.01$) in the curcumin-treated rats than in control rats; there were no significant alterations in the levels of CYP2B1 or 2E1 observed in the small intestine, lung or kidney.

Mutagenic activation of environmental carcinogens by tissue S9

To clarify the potential of liver S9 to mediate mutagenic activation of carcinogens, the mutagenicities of NMBA and 11 other carcinogens known to be metabolically activated by CYP2B1/2, 2E1, 1A1/2 and 3A2, were tested in *Salmonella* strains TA98 and TA100. Figure 4 shows the mutagenic activities of six NOC including NMBA, four HCA, BP and AFB_1 in the presence of liver S9 mix from rats treated with NMBA and/or curcumin for up to 6 weeks (five groups excluding Group 5). There were no significant differences in the mutagenic activities of any NOC in the strain TA100, nor in HCAs, BP or AFB_1 in the strain TA98, among the five groups. Similarly, the single treatment with 0.5 mg/kg NMBA or 270 mg/kg curcumin produced no significant alteration in mutagenicity of five NOC excluding BHP, Glu-P-1 and Trp-P-2 (data not shown).

Figure 5 shows the mutagenic activities of NMBA, DMN and DEN in the presence of esophagus, stomach or large intestine S9 from rats orally treated with the vehicle or 270 mg/kg curcumin as a single dose. In the curucumin-treated rats the



CYP2B2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
CYP2B1	1.0 ±0.1	0.6 ±0.1*	1.0 ±0.3	1.0 ±0.3	1.0 ±0.3	2.8 ±0.7**	1.0 ±0.1	0.7 ±0.1*	1.0 ±0.1	1.2 ±0.2	1.0 ±0.1	1.0 ±0.2

CYP2E1	1.0 ±0.2	0.4 ±0.1**	n.d.	n.d.	1.0 ±0.3	0.9 ±0.4	1.0 ±0.2	n.d.	1.0 ±0.3	1.1 ±0.2	1.0 ±0.2	1.0 ±0.1

Fig. 3. Immunoblots and densitometric determination of CYP2B (A) and 2E (B) expressions in extrahepatic tissue microsomes from rats orally treated with 270 mg/kg curcumin as a single dose. These microsomes were pooled from 16 rats 24 h after treatment with vehicle (V) or curcumin (C). Lanes PB and acetone contain CYP standards and the values represent means ± SD of the ratio to arbitrary units obtained with the vehicle group from between four and eight experiments. The assays of electrophoresis were carried out with 30 μg microsomal protein, except for lung CYP2B1/2 and kidney CYP2E1, for which 5 μg microsomal protein was loaded. $*P < 0.05$ and $**P < 0.01$, compared with the vehicle group (Student's *t*-test). n.d., not detected.

mutagenic activities of NMBA and DMN in the presence of esophagus S9 were decreased to almost half or less ($P < 0.01$) of those in control rats. In the presence of stomach S9 from the treated rats, the mutagenic activities of DMN and DEN were decreased by 82%–85% ($P < 0.01$) relative to control rats and that of NMBA to the spontaneous revertants. However, the mutagenic activities of NMBA and DMN in the presence of large intestine S9 were increased by curcumin treatment to 2.2- ($P < 0.05$) and 3.0-fold ($P < 0.01$), respectively, relative to those in control rats.

Three kinds of hepatic UDPGT activities

Table 1 summarizes the effects of NMBA and curcumin treatment for up to 6 weeks on hepatic UDPGT activities towards bilirubin, 4-NP and testosterone in liver microsomes (Groups 1–6). There were no significant differences in three kinds of UDPGT activities among the six groups. Similarly, no significant alteration in these activities was observed in the single treatments with 0.5 mg/kg NMBA or 270 mg/kg curcumin (data not shown).

Discussion

It has been reported that CYP1A1, but not CYP2B1, 2E1 or 3A1/2, is detectable in esophageal microsomes from male F344[15] and Wistar[14] rats and CYP2E1 in those from ethanol-treated rats.[43] However, the present results indicate that CYP2B1, 2E1, 1A1, 1A2 and 3A2 are constitutively detected in esophageal microsomes from male F344 rats. The reasons for the discrepancies are currently unknown, but it is suggested that the differences might be due to experimental conditions, such as the transfer conditions, the antibodies used against rat or human CYP species or the detection method. In any case, this is, to our knowledge, the first demonstration of the presence of CYP1A2, 2B1, 2E1 and 3A2 in the esophagus from uninduced rats; our results

Table 1. UDPGT activities in liver microsomes from male F344 rats repeatedly treated with NMBA, curcumin or both for up to 6 weeks

Group	Treatment	UDPGT activity (nmol/min/mg protein)		
		Bilirubin	4-Nitrophenol	Testosterone
1	Vehicle	1.10 ± 0.07	26.8 ± 7.8	0.10 ± 0.03
2	NMBA	1.05 ± 0.14	32.3 ± 4.5	0.12 ± 0.01
3	0.05% curcumin	1.07 ± 0.05	27.7 ± 5.8	0.12 ± 0.02
4	0.2% curcumin	1.08 ± 0.12	30.4 ± 5.7	0.10 ± 0.02
5	NMBA + 0.05% curcumin	1.08 ± 0.08	31.2 ± 5.4	0.13 ± 0.03
6	NMBA + 0.2% curcumin	1.04 ± 0.11	29.8 ± 3.2	0.10 ± 0.01

Each test was carried out with liver microsomes pooled from five rats. The results are expressed as means ± SD of between four and eight experiments.

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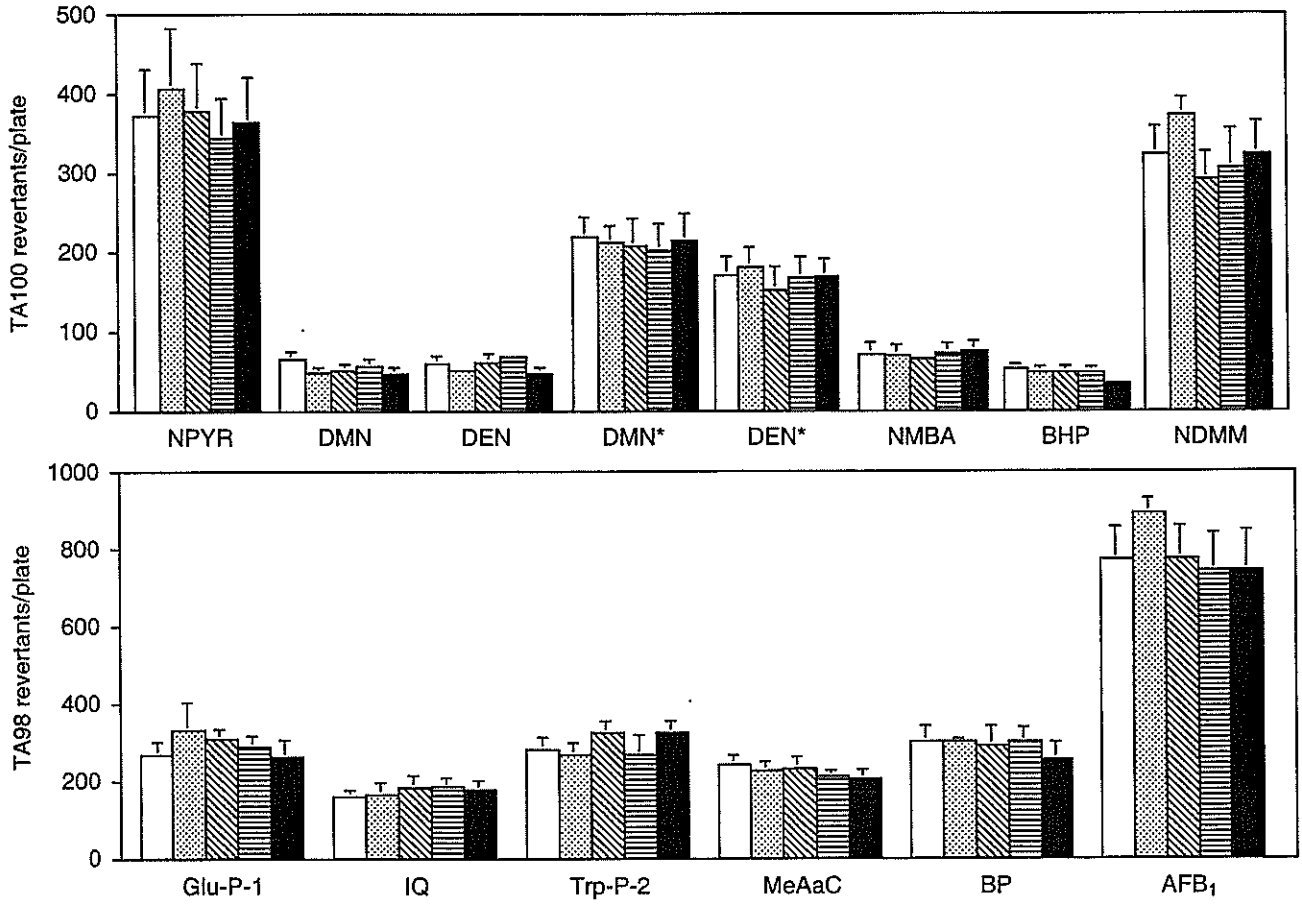

Fig. 4. Mutagenic activities of various carcinogens in the *Salmonella typhimurium* tester strains TA100 (upper, six NOC) and TA98 (lower, four HCA, BP and AFB_1) in the presence of liver S9 from rats treated with NMBA, curcumin or both. Each test was carried out in duplicate (four to eight plates) with liver S9 pooled from five rats each treated with vehicle (Group 1; clear), NMBA (Group 2; light stippled), 0.05% curcumin (Group 3; hatched), 0.2% curcumin (Group 4; horizontal) or NMBA + 0.2% curcumin (Group 6; solid) for up to 6 weeks. Each bar represents the means ± SD after subtraction of spontaneous revertants (TA100, 108–136; TA98, 9–14). DMN and DEN were assayed in both doses of 1 and *10 mg/plate, and Trp-P-2 was in 0.3 μg/plate.

support the previous finding that CYP1A1, 1A2, 2B6, 2E1 and 3A4 and CYP1A2 mRNA are detected in esophageal mucosa from human and rat, respectively.[44–47] In this study it has also been demonstrated that CYP2B1 and 2E1 are constitutively detected in five other extrahepatic microsomes, except for CYP2E1 in the small intestine. Both CYPs are known to be present in the lung, small intestine, kidney[48–50] and large intestine,[51] except for CYP2E1 in the small intestine and CYP2B1 in the large intestine, in rats, and CYP2E1 in the stomach from ethanol-induced rats.[43] Accordingly, this is the first report of the presence of CYP2B1 protein in the stomach and large intestine and CYP2E1 protein in the stomach from uninduced rats, in agreement with the finding of CYP2B1 mRNA expression in both tissues of rats.[52,53]

CYP2A3 expressed in a baculovirus system metabolizes NMBA, predominantly by methylene hydroxylation, and CYP2A3 and its mRNA are constitutively expressed in rat esophagus, but their expressions are 15- and 1600-fold less than in the nasal mucosa, respectively.[16,54] Therefore, Gopalkrishnan *et al.* have suggested that there must be an enzyme other than CYP2A3 responsible for activating NMBA in the esophagus,[54] taking account of the finding that rat esophageal explants efficiently metabolize NMBA.[55] We have shown that CYP2B1 and 2B2 are equally involved in the mutagenic activation of NMBA by PB-induced liver, and CYP2B2 in uninduced liver, in rats.[11] In the rat, hepatic CYP1A1/2, CYP2B1/2 and CYP3A2 have been reported to contribute to the mutagenic activation of HCAs, BP and AFB_1.[36,56] It has been shown that rat CYP2E1 and CYP2B1/2 contribute differently to the mutagenic activation of various *N*-nitrosodialkylamines, depending on the length of the alkyl chain and the dose of substrate.[57–60] CYP2E1 activates NPYR and 1 mg dose of DMN and DEN to mutagens and CYP2B1/2 do BHP, NDMM and 10 mg dose of DMN and DEN. Thus, it seems reasonable that feeding a 0.05% or 0.2% curcumin-containing diet for 6 weeks and intragastric treatment produce no effect on the mutagenic activation of these carcinogens by liver S9, reflecting no alteration of hepatic CYP2B1/2, 2E1, 3A2 and 1A1/2 in rats.

Differing results have been reported for the effect of curcumin or turmeric on metabolic activities specific to each CYP species in rodent liver, lung and stomach. Feeding of 2% curcumin for 2 weeks causes a modest reduction of hepatic EROD activity in female A/J mice,[28] whereas 10%

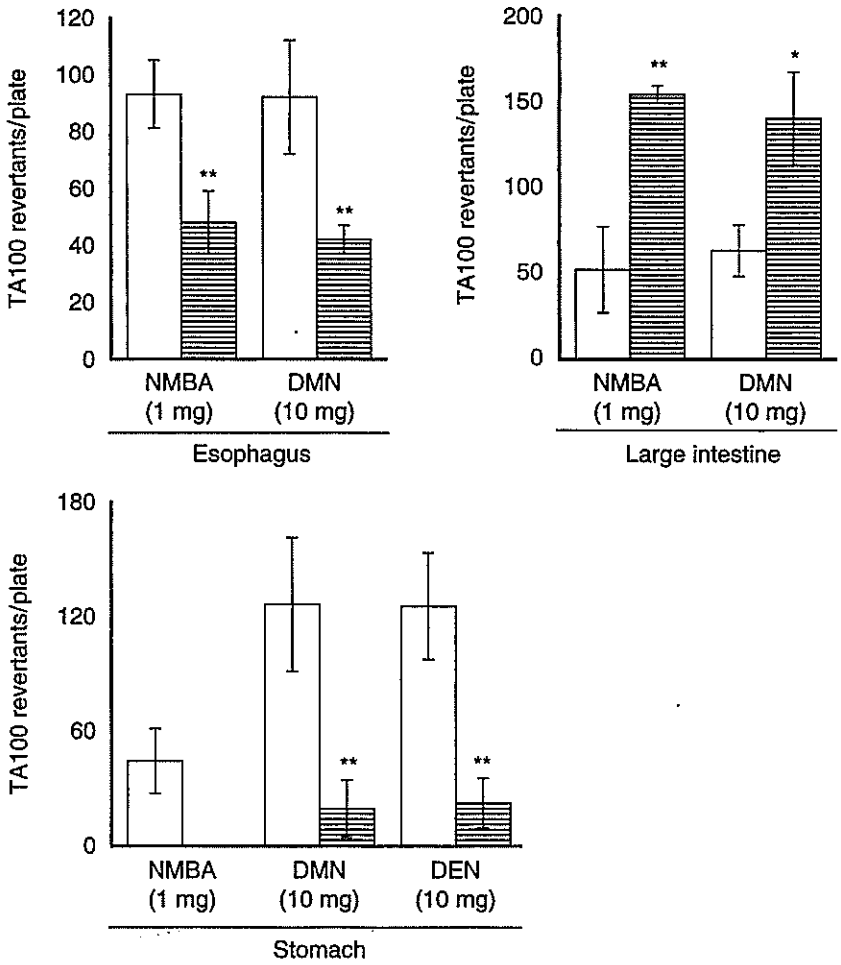

Fig. 5. Effect of a single treatment with 270 mg/kg curcumin on the mutagenic activities of NMBA, DEN and DMN in the *Salmonella typhimurium* strain TA100 in the presence of esophagus, stomach or large intestine S9. Each test was carried out in duplicate (four to six plates) with tissue S9 pooled from 16 rats each treated with vehicle (clear) or 270 mg/kg curcumin (horizontal). Each bar represents the means ± SD after subtraction of spontaneous revertants (111–124). $*P < 0.05$ and $**P < 0.01$, compared with the vehicle group (Student's *t*-test).

turmeric (equivalent to 0.5% curcumin) for 4 weeks shows no alteration of hepatic aryl hydrocarbon hydroxylase activity in male Wistar rats.[25] Feeding of 1% turmeric for 15 days produces no alteration of EROD or PROD activities, but exerts a suppressive effect on MROD activity, BP-induced EROD and MROD activity and PB-induced PROD activity in rat liver.[24] In conjunction with the present findings, with the mutagenic activation of seven carcinogens by hepatic CYP in curcumin-treated rats, it is concluded that curcumin and turmeric exert no suppression of metabolic activities specific to each CYP species in uninduced rodent liver, with the exception of MROD activity, but cause clear suppression in rats induced by PB and MC.

In rat liver, UDPGT1A1, 1A6 and 2B1 are major enzymes inducible by clofibrate, MC and PB, respectively.[61] Carcinogenic NOC, including NMBA, are known to be substrates for UDPGT,[62–64] and UDPGT2B1 is suggested to be the probable enzyme responsible for the glucuronidation of DEN and *N*-nitrosomethyl-*n*-pentylamine.[65] Feeding of 10% turmeric for 4 weeks clearly elevates 4-NP UDPGT activity,[25] but feeding of 5% turmeric (equivalent to 0.25% curcumin) does not. Therefore, it is reasonable that no alterations in three kinds of UDPGT activity were observed in rats fed 0.05% or 0.2% curcumin for 6 weeks. Together with the results of UDPGT activities in rats treated with 70 or 270 mg/kg curcumin as a single dose, treatment with a higher dose of curcumin might be needed for induction of UDPGT activities. The present finding on hepatic UDPGT activities suggests that neither 0.05% nor 0.2% curcumin modify NMBA-induced esophageal carcinogenesis through detoxification by the enzymes under the experimental conditions used. However, curcumin caused a decrease in esophageal and gastric levels of CYP2B1 and 2E1 and the mutagenic activation of NMBA, DEN and DMN by the tissue S9, in agreement with the previous finding that curcumin suppresses the metabolic activity specific to CYP2B1 and 2B2 more strongly in stomach microsomes than in liver microsomes in rats.[24] In contrast, curcumin increased large intestinal CYP2B1 and the mutagenic activation of NMBA and DMN. Together with the findings that high levels of esophageal DNA alkylation are induced by NOC,[8,12,13] and DNA methylation by NMBA and *N*-nitrosomethyl-*n*-butylamine in rats occurs to a higher extent in esophagus than in liver,[66] it suggests that modification of metabolic activation of NOC by the target organ plays a critical role in chemoprevention of NOC-induced carcinogenesis in rats. It has been reported that curcumin inhibits the expression of c-Jun and c-Fos/AP-1,[67] a transcriptional factor that plays an important role in the expressions of CYP2B1/2[68] and CYP2E1,[69] but not CYP1A,[70] and 3 A, suggesting that the

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suppression of AP-1-induced transcription by curcumin might produce a decrease in CYP2B1 and 2E1 expressions in the esophagus and stomach. However, this is not consistent with the present findings of enhancement in mutagenic activation by large intestinal CYP and no suppression in that by liver CYP. As there are no reports on enhancement by curcumin of CYP2B1 or 2E1 expression in any tissue, the mechanisms underlying modification of tissue-specific actions by curcumin remain to be elucidated, and further investigations are now required.

In conclusion, it has been demonstrated that dietary feeding or intragastric treatment with curcumin shows a suppressive effect on the mutagenic activation of three NOC, including NMBA, by either esophageal or gastric CYP2B1 and 2E1, but shows a promotive effect on the mutagenic activation of two NOC by large intestinal CYP2B1. Consequently, it suggests that suppression by curcumin of NMBA-induced esophagus carcinogenesis in F344 rats can be attributed to a decrease in the metabolic activation of NMBA by esophageal CYP2B1 during the initiation phase, without the contribution of metabolic activation or inactivation by glucuronidation in rat liver. The present data also suggest that dietary exposure to curcumin might suppress esophageal carcinogenesis initiated by DEN, *N*-nitrosopiperidine and methyl-*n*-amylnitrosamine,[71–73] or gastric carcinogenesis initiated by other carcinogens activated by CYP2B1/2 and 2E1, but that curcumin might enhance large intestinal carcinogenesis induced by these carcinogens, although curcumin is known to suppress colon carcinogenesis initiated with azoxymethane, a direct carcinogen.[74] Together with findings that anti-inflammatory[75] and antioxidant actions[76] by curcumin are possible inhibitory mechanisms, it is reasonable to assume that curcumin could affect chemically-induced carcinogenesis through multiple mechanisms.

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Troglitazone and 9*cis*,11*trans*,13*trans*-Conjugated Linolenic Acid: Comparison of Their Antiproliferative and Apoptosis-Inducing Effects on Different Colon Cancer Cell Lines

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Key Words
Troglitazone · 9*cis*,11*trans*,13*trans*-conjugated linolenic acid · Apoptosis · HT-29 · Caco-2 · Colon cancer cells · PPARγ

Abstract
Background: We have previously reported that troglitazone, a synthetic ligand for peroxisome proliferator-activated receptor γ (PPARγ), and bitter gourd seed oil rich in 9*cis*,11*trans*,13*trans*-conjugated linolenic acid (9*c*,11*t*,13*t*-CLN) prevent colon carcinogenesis. To evaluate the chemotherapeutic effect and potency of these compounds on colon cancer cells, we investigated their antiproliferative and apoptosis-inducing effects using different human colon cancer cell lines. ***Methods:*** The antiproliferative and apoptosis-inducing effects of troglitazone and 9*c*,11*t*,13*t*-CLN were evaluated and compared using HT-29, DLD-1 and Caco-2 cells at different stages of enterocytic differentiation. ***Results:*** Troglitazone and 9*c*,11*t*,13*t*-CLN decreased cell viability and induced apoptosis in three colon cancer cell lines. The susceptibility of HT-29, which expresses PPARγ at high levels, to troglitazone and 9*c*,11*t*,13*t*-CLN was higher than that of Caco-2 cells with low levels of PPARγ. ***Conclusion:*** Troglitazone and 9*c*,11*t*,13*t*-CLN exhibited more effective chemotherapeutic effects on HT-29 cells than on Caco-2 cells.

Introduction

Colon cancer is one of the most malignant neoplasias in the world [1]. In Japan, the incidence of this malignancy has been increasing. Cancer chemoprevention utilizing natural or synthetic compounds is one of the attractive approaches for fighting against this malignancy.

Peroxisome proliferator-activated receptor γ (PPARγ) has attracted attention as one of the target molecules to prevent cancer [2–4]. PPARγ is predominantly found in adipose tissue [5] and is also expressed in colon [6, 7], breast [8] and prostate cancer cells [9]. Recent research has shown that PPARγ activation suppresses colon carcinogenesis. We have reported that troglitazone, a specific PPARγ ligand, effectively suppressed the development of aberrant crypt foci (ACF), which are putative precursor lesions of colonic adenocarcinoma, induced by treatment with azoxymethane (AOM) and dextran sodium sulfate in rats [10, 11]. Another specific PPARγ li-

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gand, pioglitazone, also suppressed intestinal polyp formation in *APC*^{1309} mice [12].

On the other hand, we have demonstrated that dietary bitter gourd *(Momordica charantia)* seed oil (BGO) and pomegranate seed oil (PGO), which contain more than 60% 9*cis*,11*trans*,13*trans*-conjugated linolenic acid (9*c*,11*t*,13*t*-CLN), remarkably inhibited AOM-induced colonic ACF and adenocarcinoma in rats [13–15]. In those studies, BGO administration enhanced apoptotic cells in ACF, without affecting the surrounding crypts. Furthermore, we have reported that free fatty acid prepared from BGO induced apoptosis in colon cancer cells [16]. These findings suggest that 9*c*,11*t*,13*t*-CLN has a high potential as a chemotherapeutic compound on colon cancer cells.

In the current study, we compared the antiproliferative and apoptosis-inducing effects of troglitazone and 9*c*,11*t*,13*t*-CLN on three human colon cancer cell lines at different stages of enterocytic differentiation in order to characterize their anticancer effects and extend our investigations.

Materials and Methods

Materials

Colon cancer cells lines HT-29 (HTB-38), Caco-2 (HTB-37) and DLD-1 (CCL-221) were obtained from the American Type Culture Collection (Rockville, Md., USA). Troglitazone was a kind gift from Sankyo Co., Tokyo, Japan. Bezafibrate was purchased from Sigma Chemical Co. Ltd. (St. Louis, Mo., USA). 9*c*,11*t*,13*t*-CLN was purchased from Matreya Inc. (Pleasant Gap, Pa., USA).

Cell Lines and Cell Culture

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin. HT-29 and DLD-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell Viability Assay

Human colon cancer cells (2×10^3 cells/well) were cultured in 96-well microplates with 100 μl medium per well for 24 h. Troglitazone, bezafibrate and 9*c*,11*t*,13*t*-CLN were dissolved in 1% dimethyl sulfoxide solution and 10 μl of sample solution was then added to the culture. After incubation, 10 μl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1, Wako Chemical Co., Tokyo, Japan) solution was added to each well, and then the plate was incubated for an additional 3 h. The number of viable cells was measured at 450 nm. Viability was expressed as the percentage of viable cells in a control culture.

Extraction of mRNA and RT-PCR

Poly(A)+ RNA in colon cancer cells was extracted from cell lysate using an mRNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) with a biotinylated oligo(dT)$_{20}$ probe. RT-PCR was performed using a Titan One-Tube RT-PCR kit (Roche Diagnostics GmbH). The primer pair used to amplify PPARγ was: sense 5′-GAGTCCATGCTTGCGAAGGATGC-3′, antisense 5′-CGATATCACTGGAGATCTCCGCC-3′; and β-actin was: sense 5′-CCAAGGCCAACCGCGAGAAGATGAC-3′, antisense 5′-AGGGTACATGGTGGTGCCGCCAGAC-3′. Cycling conditions were as follows: 1× (55°C for 30 min, 94°C for 2 min), 10× (94°C for 30 s, 55–61°C for 30 s, 68°C for 45 s), 18× (94°C for 30 s, 55–61°C for 30 s, 68°C for 45 s cycle elongation of 5 s for each cycle), 68°C for 7 min. Each product was electrophoresed in 2% agarose gels, stained with ethidium bromide and visualized in UV light.

Western Blot Analysis

Colon cancer cells were lyzed with a cold RIPA buffer (pH 7.4) containing 20 m*M* Tris-HCl, 150 m*M* NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin and 1 m*M* Na_3VO_4. The supernatant (40 μg protein/lane) of cell lysate was separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane. The membrane was incubated with anti-human PPARγ antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) for 1 h. Thereafter, the membranes were incubated with a secondary antibody, anti-rabbit IgG-conjugated horseradish peroxidase (Santa Cruz Biotechnology) for 1 h at room temperature and treated with the reagents in a chemiluminescent detection kit (ECL system, Amersham Pharmacia Biotech, Piscataway, N.J., USA) according to the manufacturer's instructions. Actin was detected as a control with human Actin antibody (Santa Cruz Biotechnology).

Measurement of DNA Fragmentation

Quantitative measurement of apoptotic cells was performed using a commercial kit (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH) according to the manufacturer's instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. Cell culture conditions were the same as in cell viability assays.

Statistical Analysis

Data are expressed as means ± SD. Statistical comparisons were made by Scheffé's F test. Differences with $p < 0.05$ were considered significant.

Results

PPARγ Expression in Colon Cancer Cell Lines

We first investigated the expression level of PPARγ in three colon cancer cell lines, HT-29, DLD-1 and Caco-2 cells. PPARγ mRNA was detected in all of the cell lines. The expression level of PPARγ mRNA in HT-29 cells was the highest in the three cell lines as shown in figure 1.

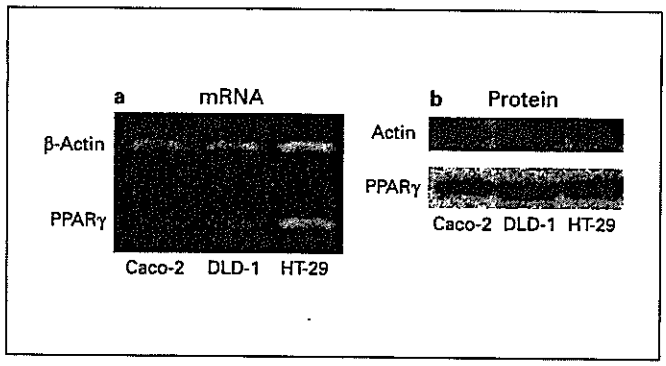

Fig. 1. Expression of PPARγ in human colon cancer cell lines. **a** PPARγ mRNA was detected by RT-PCR as described in Materials and Methods. **b** PPARγ protein was detected by Western blot analysis using PPARγ antibody.

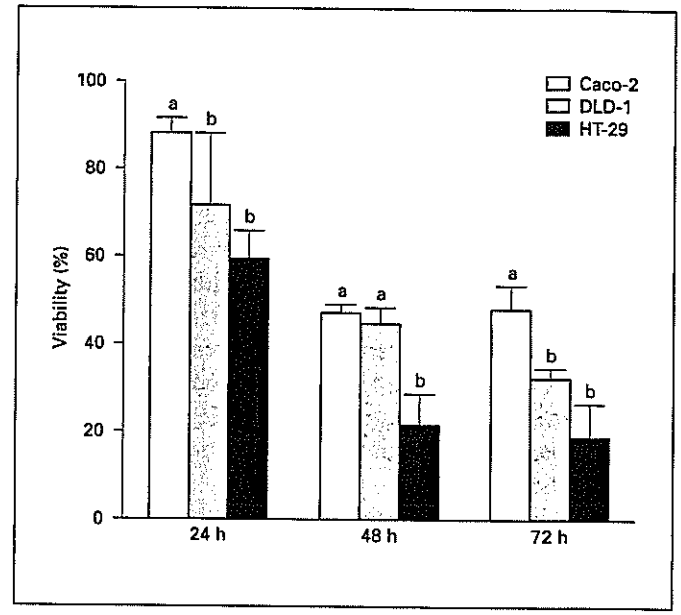

Fig. 2. Effect of troglitazone on the viability of human colon cancer cells. Colon cancer cells were preincubated for 24 h and then 100 μ*M* troglitazone was added to the culture medium. Cells were incubated for an additional 24–72 h and cell viability was measured by the WST-1 assay. Presented data were shown as relative cell number to control. All data are expressed as means ± SD of three experiments. At each incubation time, the values with different letters were significantly different from each other. $p < 0.05$ (Scheffé's F test).

The PPARγ protein level in HT-29 and DLD-1 cells was higher than in Caco-2 cells.

Effect of Troglitazone and 9c,11t,13t-CLN on the Viability of Colon Cancer Cells

The effects of troglitazone, bezafibrate and 9*c*,11*t*,13*t*-CLN on cell viability were examined in three human colon cancer cell lines. A specific PPARγ ligand, troglitazone reduced the viability of HT-29, Caco-2 and DLD-1 cells in a time-dependent manner (fig. 2). Susceptibility to 100 μ*M* of troglitazone was in the following order: HT-29 > DLD-1 > Caco-2 cells after 72 h of incubation. The viability of HT-29 cells was reduced to less than 20% of control. In contrast, a PPARα ligand, bezafibrate, did not affect the viability of three colon cancer cell lines after 72 h of incubation (data not shown).

On the other hand, 9*c*,11*t*,13*t*-CLN also reduced the viability of three colon cancer cell lines in a dose-dependent manner (fig. 3a, b). The viability of HT-29 and DLD-1 cells was drastically decreased by 9*c*,11*t*,13*t*-CLN compared with Caco-2 cells. When HT-29 and DLD-1 cells were treated with 12.5 μ*M* of 9*c*,11*t*,13*t*-CLN, their viability was reduced to approximately 10% after 24 h of incubation.

Induction of Apoptosis of Colon Cancer Cells by Troglitazone and 9c,11t,13t-CLN

To examine the induction of apoptosis by troglitazone and 9*c*,11*t*,13*t*-CLN in HT-29 and Caco-2 cells, cytoplasmic-histone-associated DNA fragments as an indicator of apoptosis was measured by a cell death detection ELISA kit. As shown in figure 4, troglitazone induced DNA fragmentation in HT-29 cells with high expression of PPRAγ (fig. 4). Treatment with 100 μ*M* troglitazone for 72 h increased DNA fragmentation to more than 3-fold compared to control cells. However, in Caco2 cells with low expression of PPARγ, DNA fragmentation by troglitazone was not observed at 100 μ*M*.

9*c*,11*t*,13*t*-CLN also markedly induced DNA fragmentation in HT-29 cells (fig. 5a). The fragmentation level by incubation with 6.25 μ*M* 9*c*,11*t*,13*t*-CLN reached up to more than 10-fold of control after 48 h of incubation. However, in Caco-2 cells, 9*c*,11*t*,13*t*-CLN did not induce DNA fragmentation during 24 h of incubation (fig. 5b). By 48 h of incubation with 12.5 μ*M* 9*c*,11*t*,13*t*-CLN, DNA fragmentation was induced in Caco-2 cells to 5-fold of control. The apoptosis-inducing effect of 9*c*,11*t*,13*t*-CLN on HT-29 and Caco-2 cells, at least partly, corresponded to the reduction in viability by 9*c*,11*t*,13*t*-CLN in HT-29 and Caco-2 cells.

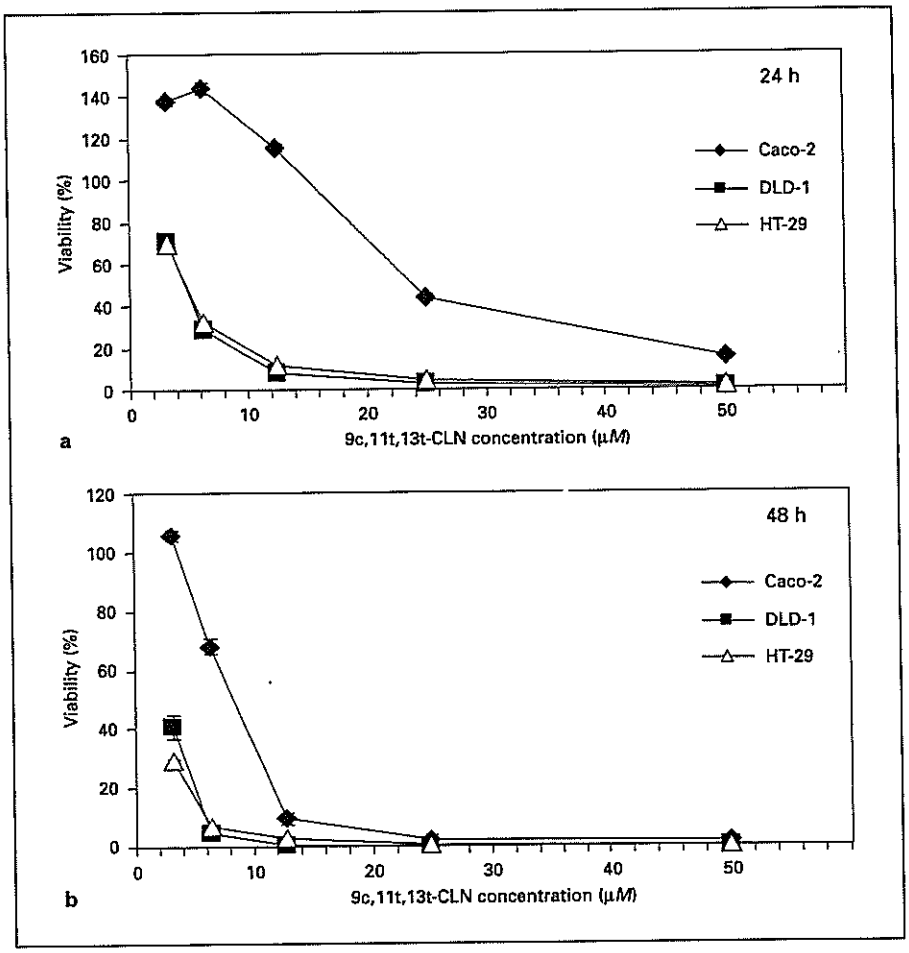

Fig. 3. Effect of 9*c*,11*t*,13*t*-conjugated linolenic acids on the viability of human colon cancer cells. Colon cancer cells were preincubated for 24 h and then 9*c*,11*t*,13*t*-CLN was added to the culture medium. Cells were incubated for an additional 24 h (**a**) and 48 h (**b**). Viability was measured by the WST-1 assay. Presented data are shown as relative cell number versus control. All data are expressed as means ± SD of three experiments.

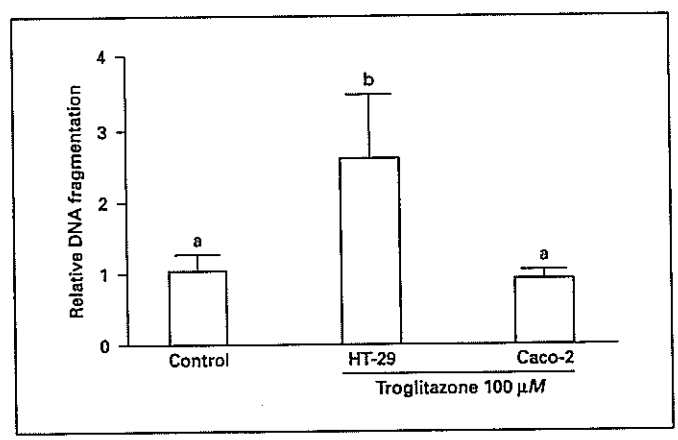

Fig. 4. DNA fragmentation in HT-29 and Caco-2 cells treated with troglitazone. Cells were preincubated for 24 h and then 100 μ*M* troglitazone was added to the culture medium. DNA fragmentation was measured by a sandwich enzyme immunoassay using anti-histone antibody and anti-DNA antibody after 72 h of incubation. Relative DNA fragmentation; the control was assigned a value of 1.0. Values are means ± SD of three experiments. The values with different letters were significantly different from each other. $p < 0.05$ (Scheffé's F test).

Discussion

PPARγ ligands such as troglitazone and 15d-prostaglandin (PG) J_2 were reported to cause growth inhibition and induce apoptosis in cancer cells [17–19]. PPARγ activation is suggested to be associated with suppression of cell growth and apoptosis induction on colon cancer cells [17, 20]. In the present study, expression of PPARγ protein in HT-29 and DLD-1 cells was higher than that of Caco-2 cells. Our results are in accordance with those in previous reports [6, 7]. Decrease in cell viability and apoptosis induction by troglitazone were remarkable in

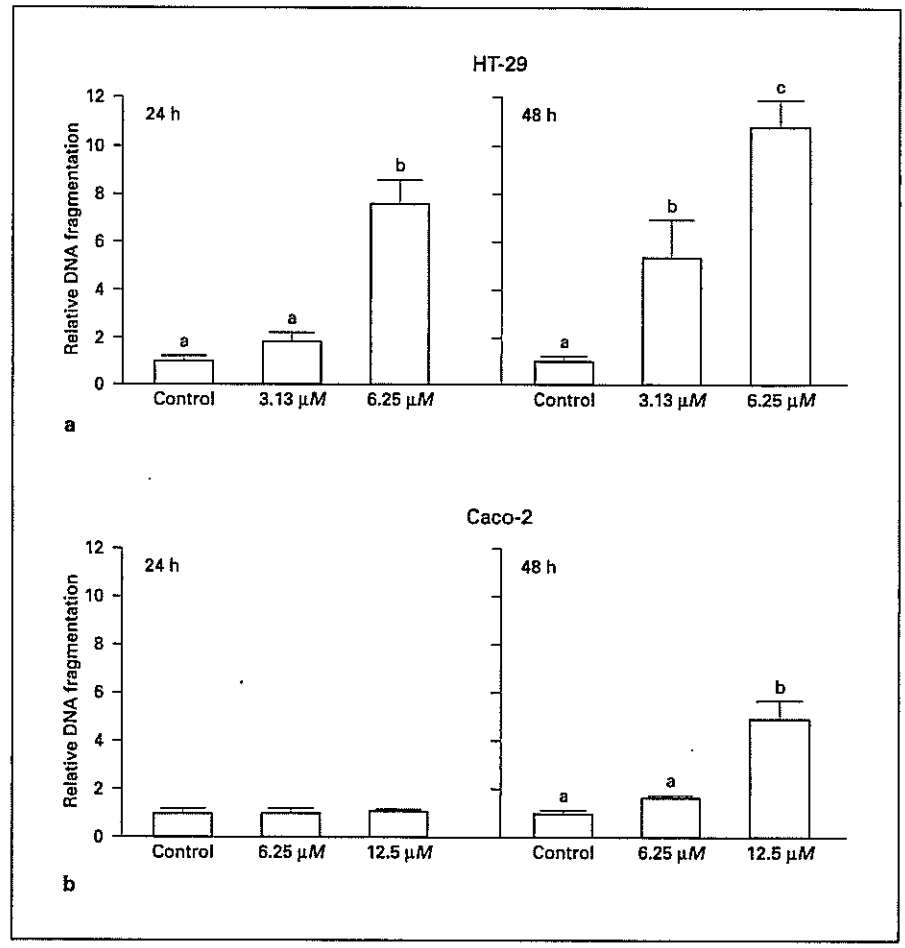

Fig. 5. DNA fragmentation in HT-29 and Caco-2 cells treated with 9*c*,11*t*,13*t*-CLN. Cells were preincubated for 24 h and then 9*c*,11*t*,13*t*-CLN was added to the culture medium. DNA fragmentation was measured by a sandwich enzyme immunoassay using anti-histone antibody and anti-DNA antibody after 24 h (**a**) and 48 h (**b**) of incubation. Relative DNA fragmentation; the control was assigned a value of 1.0. Values are means ± SD of three experiments. The values with different letters were significantly different from each other. $p < 0.05$ (Scheffé's F test).

HT-29, which highly expresses PPARγ protein. In contrast, bezafibrate, a PPARα ligand, did not show an antiproliferative effect on colon cancer cells. In previous studies, it was reported that PPARγ ligand induced apoptosis in colon cancer cells [17, 19]. Moreover, troglitazone has been reported to induce PPARγ in colon cancer cells [21, 22]. We also reported that increased expression of PPARγ was observed in the colon of rats fed the diets containing BGO rich in 9*c*,11*t*,13*t*-CLN [14]. Thus, activation and regulation of PPARγ are suggested to be important in the prevention of colon carcinogenesis. In the current study, it was demonstrated that the antiproliferative and apoptosis-inducing effects of troglitazone are effective on HT-29 cells with a high expression level of PPARγ.

On the other hand, we recently reported that dietary BGO rich in 9*c*,11*t*,13*t*-CLN effectively suppressed the AOM-induced ACF and adenocarcinoma in F344 rats [13, 14]. Dietary BGO enhanced apoptotic cells in ACF, without affecting the surrounding crypts. In addition, we and others reported that purified 9*c*,11*t*,13*t*-CLN induced apoptosis in colon cancer cells [16, 23]. These results indicate that 9*c*,11*t*,13*t*-CLN is an advantageous compound in chemotherapy and chemoprevention of colon cancer. In the current study, it is noteworthy that 9*c*,11*t*,13*t*-CLN reduced cell viability and induced apoptosis in HT-29 cells much more than in Caco-2 cells. Similar findings were observed in troglitazone-treated colon cancer cell lines. These results indicate that 9*c*,11*t*,13*t*-CLN as well as troglitazone are more effective in inhibiting growth and inducing apoptosis of colon cancer cells expressing PPARγ protein at a high level. HT-29 cells are known to display poor enterocytic features under standard culture conditions, while Caco-2 cells exhibit a differentiated phenotype and DLD-1 cells exhibit an intermediate aspect [24]. In addition, the anticancer effects of the irinotecan/5-fluorouracil combination varied with

colon cancer cell lines [25]. The differential response of HT-29, Caco-2 and DLD-1 cell lines to troglitazone and 9*c*,11*t*,13*t*-CLN is suggested to be depend on cell features, including the expression level of PPARγ protein, an expected molecular target for cancer chemoprevention. Further investigation is required for a better understanding of the mechanisms involved in growth inhibition and apoptosis induction by troglitazone and 9*c*,11*t*,13*t*-CLN.

In summary, we demonstrated that troglitazone and 9*c*,11*t*,13*t*-CLN reduced cell viability and induced apoptosis in human colon cancer cell lines. The effects of troglitazone and 9*c*,11*t*,13*t*-CLN differed among human colon cancer cell lines at different stages of enterocytic differentiation. Especially, the antiproliferative and apoptosis-inducing effects of troglitazone and 9*c*,11*t*,13*t*-CLN were stronger on HT-29 cells than on Caco-2 cells.

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Regular article

Chemopreventive Effects of Nobiletin against Genotoxicity Induced by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the Lung of *gpt* delta Transgenic Mice

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Nobiletin, a major component of citrus polymethoxyflavones, possesses anticancer, antiviral and anti-inflammatory activities. To evaluate the chemopreventive potential against lung cancer induced by cigarette smoke, we examined suppressive effects of nobiletin against genotoxicity induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most carcinogenic tobacco-specific nitrosamine, in the lung of *gpt* delta transgenic mice. Male and female *gpt* delta mice were fed nobiletin at a dose of 100 or 500 ppm in diet for seven days and treated with NNK at a dose of 2 mg/mouse/day, i.p. for four consecutive days. Dietary administration of nobiletin continued at the doses during the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced the *gpt* mutant frequency (MF) in the lung 19- and 9-fold, respectively, over the values of untreated female and male mice. Interestingly, nobiletin reduced the NNK-induced MFs by 25–45% in both sexes and the reduction at a dose of 100 ppm in females and 500 ppm in males was statistically significant ($P<0.05$). To further characterize the suppressive effects, we conducted bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits S9-mediated genotoxicity of NNK. Nobiletin as well as 8-methoxypsoralen, an inhibitor of CYP2A, reduced the genotoxicity of NNK by more than 50%. These results suggest that nobiletin may be chemopreventive against NNK-induced lung cancer and also that the chemopreventive efficacy may be due to inhibition of certain CYP enzymes involved in the metabolic activation of NNK.

Key words: nobiletin, NNK, chemoprevention, cigarette smoking, *gpt* delta transgenic mice

Introduction

Humans are exposed to a variety of exogenous and endogenous genotoxic agents. Of various hazardous environmental factors, cigarette smoke may be the most causative factor associated with the incidence of human cancer (1). Although cigarette smoke contains more than 4,000 compounds including 40 known human carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nicotine-derived nitrosamino ketone, NNK) is the most carcinogenic tobacco-specific nitrosamine (2,3). NNK is estimated to be present at levels of 17–430 and 390–1,440 ng, respectively, per cigarette in mainstream and sidestream of cigarette smoke (3). NNK induces lung tumors in rats, mice and hamsters and is classified into Class 2B carcinogen (possibly carcinogenic to humans) by International Agency for Research on Cancer (4). NNK is metabolically activated by CYP (P-450) enzymes, and the metabolites generate methylated and pyridyloxobutylated DNA, which can induce G:C-to-A:T and G:C-to-T:A mutations, respectively. O^6-Methylguanine in the lung may be a causative lesion of NNK leading to activation of Ki-*ras* protooncogene, an initiation of tumor development (5,6).

With smoking the major etiological factor for lung cancer, a number of naturally occurring and synthetic chemicals have been proposed as candidates of chemopreventive agents to protect smokers who are unwilling or unable to quit smoking. Examples of the candidates include inhibitors of metabolic activation of NNK, e.g., phenethyl isothiocyanate and curcumins (7–10), enhancers of detoxication enzymes, e.g., prodrugs of L-selenocystein (11), antioxidants, e.g., vitamine E and carotenoids (12,13) and inhibitors of signal transduction downstream from the activated oncogenes, e.g., perillyl alcohol and deguelin (14,15).

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) is a

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polymethoxyflavone found in *Citrus depressa* Rutaceae, a popular citrus fruit in Okinawa, Japan (16). Interestingly, nobiletin seems to possess anticancer activities by inhibiting critical steps of carcinogenesis, i.e., initiation (13,17), promotion (18,19) and metastasis (16,20,21). In addition, nobiletin inhibits the P-glycoprotein drug efflux transporter, suggesting the ability to reverse multi-drug resistance of tumor cells (22).

To evaluate the chemopreventive efficacy against lung cancer induced by cigarette smoke, we examined suppressive effects of dietary administration of nobiletin in the lung of *gpt* delta mice treated with NNK. In this mouse model, base substitutions such as G:C-to-A:T or G:C-to-T:A can be detected by *gpt* selection. In fact, Miyazaki *et al.* (23) have employed the mice to demonstrate the chemopreventive effects of 8-methoxypsoralen against NNK-induced mouse lung adenoma. Besides *in vivo* genotoxicity assays, we conducted a bacterial mutation assay with *Salmonella typhimurium* YG7108 to examine whether nobiletin inhibits the genotoxicity of NNK in the presence of S9 metabolic activation system. The bacterial strain lacks O^6-methylguanine methyltransferase activity, so that it is highly sensitive to base substitution mutations by NNK and other alkylating agents (24,25). The results suggest that nobiletin clearly suppresses the genotoxicity of NNK *in vivo* and *in vitro*. We discuss the mechanisms underlying the suppressive effects and the possible usage of nobiletin as a chemopreventive agent against lung cancer induced by cigarette smoke.

Material and Methods

Materials: Nobiletin (>99.9% purity) was chemically synthesized according to the method described by Tsukayama *et al.* (26) with slight modifications. Sources of other chemicals used in this study are as follows: NNK, Toronto Research Chemicals (Toronto, Canada); benzo[a]pyrene (BP), Wako Pure Chemicals (Osaka, Japan); 8-methoxypsoralen and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), Sigma-Aldrich Japan K. K. (Tokyo, Japan). S9 prepared from male Sprague-Dawley rats pretreated with phenobarbital and 5,6-benzoflavone was purchased from Kikkoman Cooperation, Chiba, Japan.

Treatment of *gpt* delta mice: Male and female *gpt* delta C57BL/6J transgenic mice, obtained from Japan SLC, Inc. (Shizuoka, Japan), were maintained in Animal Facility of Kanazawa Medical University, according to the institutional animal care guidelines. The animals were housed in plastic cages with free access to tap water and powdered basal diet CRF-1 (Oriental Yeast, Tokyo, Japan) under controlled conditions of temperature at 23 ± 2°C, humidity of 10% and lighting (12 h light-dark cycle). Twenty female and 25 male *gpt* delta mice were each divided into four experimental and one control groups (Fig. 1). When the mice were 8 weeks of age, they were fed diet supplemented with nobiletin at a concentration of 100 ppm (Group 2) or 500 ppm (Groups 3 and 4) for 38 days. Groups 1 through 3 were treated with a single i.p. injection of NNK dissolved in saline at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 4 and 5 were treated with saline as vehicle. Mice were sacrificed under ether anesthesia at day 38. The lung was removed, placed immediately in liquid nitrogen, and stored at −80°C until analysis.

DNA Isolation, *in vitro* packaging and *gpt* mutation assay: High-molecular-weight genomic DNA was extracted from the lung using the RecoverEase DNA Isolation Kit (Stratagene, La Jolla, CA). λEG10 phages were rescued using Transpack Packaging Extract (Stratagene, La Jolla, CA). The *gpt* mutation assay was performed according to previously described methods (27,28). *gpt* MFs were calculated by dividing the number of colonies growing on agar plates containing chloramphenicol and 6-thioguanine by the product of the number of colonies growing on plates containing chloramphenicol and the dilution factor.

Bacterial mutation assay: The mutagenicity assay was carried out with a pre-incubation method with modifications (29). Nobiletin or 8-methoxypsoralen was dissolved in DMSO and the solution (50 μL) was mixed with S9 mix (0.5 mL). They were kept on ice for 5 min and mixed with the solution (50 μL) of chemicals, i.e., NNK, BP or MNNG, dissolved in DMSO. Then, they were mixed with overnight culture (0.1 mL) and incubated for 20 min at 37°C. When the mutagenicity of MNNG was assayed, 1/15M phosphate buffer pH7.4 (0.5 mL) was added instead of S9 mix. The reaction mixture containing bacteria, nobiletin (or 8-methoxypsoralen) and the chemical with or without S9 mix was poured onto agar plates with soft agar and incubated for two days at 37°C. Each chemical was assayed with 6–8 doses on triplicate or duplicate plates. Tester strains for the mutation assays were *S. typhimurium* YG7108 for NNK and MNNG, and *S. typhimurium* YG5161 (30) for BP. Relevant genotypes of the strains are as follows: YG7108 (24,25) as *S. typhimurium* TA1535 but is $\Delta ada_{ST}\,\Delta ogt_{ST}$; YG5161 (30) as *S. typhimurium* TA1538 harboring plasmid pYG768 carrying the *dinB* gene of *Escherichia coli*.

Statistical analysis: All data are expressed as mean ± standard deviations. Differences between groups were tested for statistical significance using a Student's *t*-test. A P value less than 0.05 denoted the presence of a statistically significant difference.

Results

Dietary administration of nobiletin suppresses mutations induced by NNK in the lung of *gpt* delta mice: To examine the suppressive effects of nobiletin against genotoxicity induced by NNK, female and male *gpt* delta mice were fed nobiletin in diet at a dose of 100 or 500 ppm for a week and treated with NNK (Fig. 1). Dietary administration of nobiletin continued during the NNK treatments and in the following period before sacrifice at day 38. NNK treatments enhanced *gpt* MF in the lung 19 times in females and 9 times in males over the control levels (Tables 1 and 2). Since the MFs ($\times 10^{-6}$) of untreated controls were similar between females and males (3.0 ± 1.3 versus 3.1 ± 2.0), NNK-induced MF was higher in females (58.1 ± 16.7) than in males (26.5 ± 11.8). Nobiletin itself was non-genotoxic (Group 4). Nobiletin appeared to reduce the MFs in both sexes. In females, the dietary administration of nobiletin at 100 and 500 ppm (Groups 2 and 3) reduced the NNK-induced MF by 34 and 32%, respectively, and the reduction at 100 ppm was statistically significant ($P<0.04$). In males, nobiletin at 100 and 500 ppm reduced the MF by 25 and 45%, respectively, and the reduction at 500 ppm was statistically significant ($P<0.04$). These results indicate that nobiletin suppresses NNK-induced genotoxicity in the lung of *gpt* delta mice.

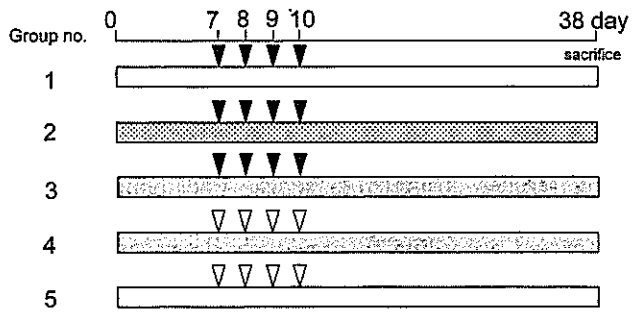

Fig. 1. An experimental design to examine chemopreventive effects of nobiletin against genotoxicity of NNK in the lung of *gpt* delta mice. Twenty female and 25 male eight-week-old *gpt* delta mice were each divided into five groups. Groups 1 through 3 were treated with a single i.p. injection of NNK at a dose of 2 mg/mouse/day for four consecutive days from day 7 through day 10. Groups 2 and 3 were fed diet supplemented with nobiletin at doses of 100 ppm and 500 ppm, respectively, for 38 days. Groups 4 and 5 were treated with saline as vehicle, and Group 4 was fed diet with nobiletin at a dose of 500 ppm for 38 days. Mice were sacrificed at day 38, and the *gpt* MF in the lung were determined. □, basal diet; ▨, nobiletin in diet at a dose of 100 ppm; ■, nobiletin in diet at a dose of 500 ppm; ▼, NNK (2 mg/mouse/day, i.p.); ▽, saline.

Nobiletin inhibits genotoxicity of NNK in the presence of S9 activation in *S. typhimurium* YG7108: To further characterize the suppressive effects of nobiletin against genotoxicity of NNK, we conducted bacterial mutation assays to examine whether nobiletin inhibits genotoxicity of NNK in the presence of S9 activation enzymes (Fig. 2A). NNK at a dose of 500 μg/plate induced mutations in *S. typhimurium* YG7108 and produced about 900 His^+ revertants/plate, which was 40–50 times higher than the value of spontaneous mutations. Nobiletin itself was non-genotoxic either with or without S9 activation (Fig. 2A, C and D).

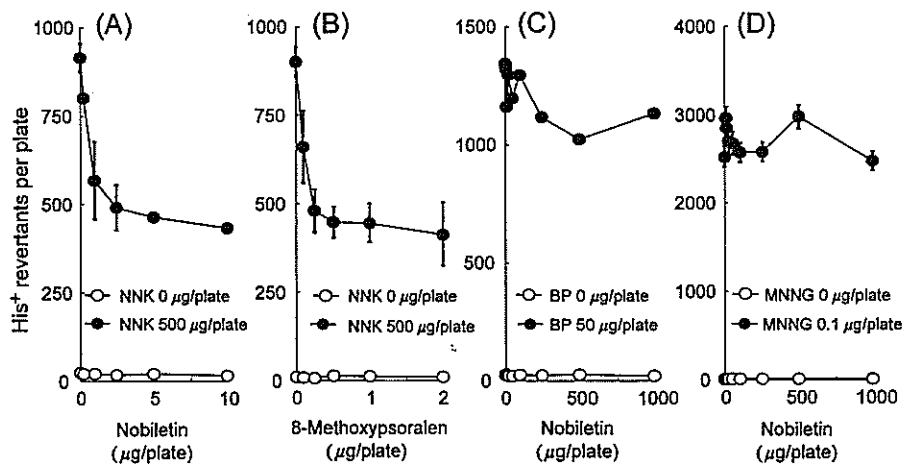

Fig. 2. Suppressive effects of nobiletin against genotoxicity of NNK in the presence of S9 mix in *S. typhimurium* YG7108. Closed circles represent the numbers of His^+ revertants/plate induced by the following compounds: NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (A), NNK (500 μg/plate) in the presence of S9 mix along with the increasing doses of 8-methoxypsoralen (B); BP (50 μg/plate) in the presence of S9 mix along with the increasing doses of nobiletin (C); MNNG (0.1 μg/plate) in the absence of S9 mix along with the increasing doses of nobiletin. Open circles represent the numbers of His^+ revertants/plate when the non-genotoxicity of nobiletin (A, C and D) and 8-methoxypsoralen (B) were confirmed. Strains used are *S. typhimurium* YG7108 (A, B and D) and *S. typhimurium* YG5161 (C). Averages and standard deviations are presented in A, B and D where three plates were used for the assays. Averages are presented in C where two plates were used for the assay.

Table 1. Suppressive effects of nobiletin against genotoxicity of NNK in the lung of female *gpt* delta mice

Group number*	Animal I.D.	Total colonies	No. of mutants	*gpt* MF ($\times 10^{-6}$)	Average ±S.D.†	*P*-value‡
1	F001	898,500	68	75.7		
NNK alone	F002	1,017,000	57	56.1		
	F003	1,464,000	53	36.2		
	F004	1,054,500	68	64.5		
		4,434,000	246	55.5	58.1±16.7	
2	F005	1,134,000	36	31.8		
NNK	F006	1,353,000	48	35.5		
+Nobiletin	F007	1,152,000	54	46.9		
(100 ppm)	F008	916,500	37	40.4		
		4,555,500	175	38.4	38.6±6.6	0.036§
3	F009	1,369,500	33	24.1		
NNK	F010	798,000	36	45.1		
+Nobiletin	F011	1,606,500	66	41.1		
(500 ppm)	F012	1,027,500	48	46.7		
		4,801,500	183	38.1	39.3±10.4	0.052
4	F013	1,059,000	3	2.8		
Nobiletin	F014	1,377,000	4	2.9		
(500 ppm)	F015	1,092,000	6	5.5		
alone	F016	900,000	6	6.7		
		4,428,000	19	4.3	4.5±1.9	<0.001
5	F018	2,856,000	6	2.1		
No treatments	F019	1,560,000	4	2.6		
	F020	1,809,000	9	5.0		
	F021	2,013,000	5	2.5		
		8,238,000	24	2.9	3.0±1.3	<0.001

*Group 1, mice treated with NNK (2 mg/mouse/day × 4 days) alone; Group 2, mice treated with NNK plus nobiletin at a dose of 100 ppm in diet; Group 3, mice treated with NNK plus nobiletin at a dose of 500 ppm in diet; Group 4, mice fed nobiletin at a dose of 500 ppm in diet without NNK treatments; Group 5, mice without treatments with NNK or nobiletin. The Group No. corresponds with group No. in Fig. 1.

†Average ± standard deviation of *gpt* MF of four mice.

‡Differences between *gpt* MF of each group and that of Group 1 were tested for statistical significance using a Student's *t*-test.

§Statistically significant ($P<0.05$) against Group 1. The values in Groups 4 and 5 are also statistically significant. But the mice in Groups 4 and 5 are not treated with NNK so that the values are not marked with §.

An addition of nobiletin in the reaction mixture containing NNK and S9 mix reduced the genotoxicity of NNK in a dose-dependent manner, and the number of His^+ revertants/plate decreased by more than 50% at the highest dose of nobiletin, i.e., 10 μg/plate. There was no obvious reduction of background lawn of bacteria at any dose of nobiletin, suggesting that nobiletin was not very much toxic under the experimental conditions. Similar dose-dependent reduction of the genotoxicity of NNK was observed with 8-methoxypsoralen (Fig. 2B). An addition of 8-methoxypsoralen into the reaction mixture containing NNK and S9 mix reduced the number of His^+ revertants/plate by more than 50%. Despite the similar inhibitory effects, the dose necessary to reduce the genotoxicity of NNK by 50% was 5- to 10-fold higher with nobiletin than with 8-methoxypsoralen (2.5 μg/plate for nobiletin versus 0.25–0.5 μg/plate for 8-methoxypsoralen). In contrast, nobiletin exhibited weak or virtually no inhibitory effects on the genotoxicity of BP or MNNG, respectively (Fig. 2C and D). An addition of nobiletin reduced the genotoxicity of BP in the presence of S9 activation by 20%, while it did not modulate the genotoxicity of MNNG in the absence of S9 enzymes.

Discussion

Lung cancer continues to be the leading cause of cancer death in developed countries. Dietary compounds with potential to inhibit lung cancer may be a promising and practical approach for reducing the risk of lung cancer caused by smoking. In this study, we examined the chemopreventive efficacy of nobiletin