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Suppression of Diethylnitrosamine-initiated Preneoplastic Foci Development in the Rat Liver by Combined Administration of Four Antioxidants at Low Doses

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Potential synergism between 4 antioxidants acting at low doses on development of glutathione *S*-transferase placental form (GST-P)-positive liver cell foci was examined in male rats initially given diethylnitrosamine (200 mg/kg, i.p.). Beginning 2 weeks after the initiation, rats received the antioxidants, individually or in combination, in the diet for 6 weeks. All rats were subjected to two-thirds partial hepatectomy at week 3 and killed at week 8. The numbers and areas of GST-P-positive foci were significantly decreased by single treatment with butylated hydroxyanisole (BHA, 1%), *tert*-butylhydroquinone (TBHQ, 1%) and catechol (0.8%), but not with sesamol (0.5%). Combined treatments (BHA+TBHQ, catechol+sesamol, or all 4 chemicals) at a quarter of the above dose levels resulted in decrease in numbers and areas of foci to levels less than the sums of individual inhibition data obtained with the one-quarter levels. Although these combined effects were not statistically significant in the additive model, the results indicate possible synergistic suppression of carcinogenesis by low-dose combined treatment with anti-cancer agents and the usefulness of the present protocol for this type of analysis.

Key words: Hepatocarcinogenesis — Inhibition — GST-P — Antioxidant — Low-dose combination

Since most human cancers are considered to be caused by exposure of individuals to environmental carcinogenic agents,[1-3] primary prevention of cancer development in human populations may be a practical possibility. For rapid detection of carcinogenic agents, we have established a medium-term bioassay method using preneoplastic glutathione *S*-transferase (GST-P)[3]-positive liver cell foci as the endpoint marker lesions.[4, 5] We have reported possible detection of not only carcinogenic or tumor-enhancing agents but also tumor-inhibitory ones with this system.

A number of chemicals have inhibitory potential in animal carcinogenesis models.[6-8] One group of such antitumorigenic agents, the antioxidants, has been extensively examined.[8-10] It is thus of interest that many of the chemicals that we found to exert inhibitory effects among more than 190 chemicals examined in our medium-term liver system are synthetic or naturally occurring antioxidative agents.[5, 11] Inhibitory effects of antioxidants have similarly been demonstrated in other organs.[8, 9, 12-14] Although unexpected carcinogenic action or promoting potency has also been found,[9, 12, 13, 15-22] chemicals in this category are promising candidates for practical use as chemopreventive agents.

In the present study, the influence of combined treatment of rats with four synthetic or naturally occurring antioxidants at low dose levels was investigated using our medium-term carcinogenicity bioassay method to investigate a possible synergism of inhibition among antioxidants, since it has been demonstrated that synergistic effects of various chemicals in liver carcinogenesis can be evaluated with this rapid method.[23-25] The four antioxidants chosen were butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ) as synthetic antioxidants and catechol and sesamol as naturally occurring ones. All four chemicals were earlier found to exert inhibitory effects on diethylnitrosamine (DEN)-initiated preneoplastic liver cell foci development in our system.[5, 11]

MATERIALS AND METHODS

Animals and chemicals A total of 180 male F344 rats, 5 weeks old, were obtained from Charles River Japan Inc., Atsugi. They were housed, 5 rats per cage, with woodchip bedding in an air-conditioned animal room at 23±2°C and 55±5% humidity. Food (Oriental MF, Oriental Yeast Co., Tokyo) and water were available *ad libitum*

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[3] Abbreviations: GST-P; placental form of glutathione *S*-transferase, BHA; butylated hydroxyanisole, TBHQ; *tert*-butylhydroquinone, DEN; diethylnitrosamine, PH; two-thirds partial hepatectomy, BUdR; 5-bromo-2′-deoxyuridine.

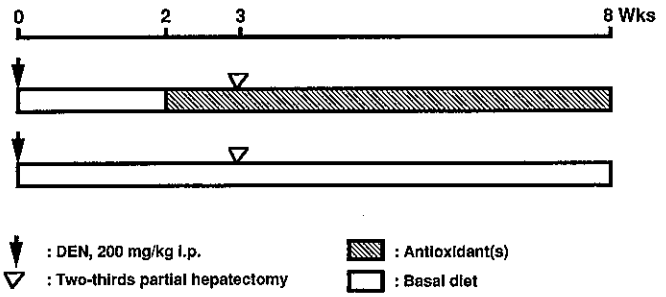

Fig. 1. Experimental design. As test antioxidants, 1% BHA, 1% TBHQ, 0.8% catechol or 0.5% sesamol were given in the diet. Further groups were treated with one-quarter doses of the above chemicals or in combinations of 2 or 4.

throughout the experiment. DEN was obtained from Tokyo Chemical Industry Co., Tokyo. BHA (purity >98%), TBHQ (purity >98%), and catechol (purity >99%) were purchased from Wako Pure Chemical Industries, Osaka, and sesamol (purity >98%) was from Fluka Chemie, AG, Switzerland. The chemicals were incorporated into powdered basal diet (Oriental MF) using a mixer without adding oil and the prepared diets were stored in a cold room until use.

Treatment of animals The experimental protocol is shown in Figure 1. After a 1-week initial observation period, the rats (15 animals for each chemical treatment or control group) were intraperitoneally injected with DEN dissolved in 0.9% NaCl at a dose of 200 mg/kg body weight. Starting 2 weeks later, the animals were maintained on powdered diet supplemented with antioxidant(s) or the basal diet. Two dose levels were used for each antioxidant. The high dose was determined based on the results of previous experiments in our laboratory as follows: 1% BHA, 1% TBHQ, 0.8% catechol, and 0.5% sesamol. The low dose in each case was a quarter of these. Sub-groups in group 1 were administered the 2 synthetic chemicals (BHA and TBHQ), the 2 naturally occurring chemicals (catechol and sesamol) or all 4 chemicals in combination at the one-quarter levels.

All rats were subjected to two-thirds partial hepatectomy (PH) at the end of week 3 and killed at the end of week 8. One hour before death, all rats were intraperitoneally injected with 5-bromo-2′-deoxyuridine (BUdR, Sigma Chemical Corp., St. Louis, MO) at a dose of 100 mg/kg body weight for examination of DNA synthesis levels in the liver.

Immediately upon killing, the livers were excised and cut into 2–3 mm thick slices, three of which, one from the caudate lobe and two from the right anterior lobe, were fixed in ice-cold acetone for subsequent immunohistochemical examination of GST-P expression. GST-P and BUdR immunohistochemistry was separately performed as previously described.[26] The numbers and areas of GST-P-positive foci of more than 0.2 mm in diameter and the total areas of the liver sections examined were measured using a video image processor (VIP-21C, Olympus Co., Tokyo). For examination of BUdR labeling indices, 1000 parenchymal cells were counted in randomly selected fields for each animal.

Statistical analysis Statistical analysis of differences between means was carried out by using Student's t test and Welch's t test in combination with the F test. To determine whether the combined treatments acted additively or synergistically, a t test using test statistic T and the degrees of freedom df defined below was carried out.[27] In the case of simultaneous treatment with all 4 chemicals;

$$T=\frac{Y_{comb}+3\times Y_0-Y_1-Y_2-Y_3-Y_4}{\sqrt{Ve\times(1/n_{comb}+9/n_0+1/n_1+1/n_2+1/n_3+1/n_4)}}$$

$$df=(n_{comb}-1)+(n_0-1)+(n_1-1)+(n_2-1)+(n_3-1)+(n_4-1)$$

where Y is the mean value of foci and n is the number of rats in each combined treatment (comb), control (0) and single chemical treatment (1–4) group, and Ve is the mean square error term. The criterion of $P<0.05$ for significance was chosen for all operations.

RESULTS

Final body and liver weights and data for BUdR labeling indices in whole liver are summarized in Table I. Body weights as compared to DEN-alone group values were significantly decreased in groups treated with TBHQ and catechol at the high dose, and in the case of combined treatment with all 4 chemicals at the one-quarter doses. Liver weights were increased in all groups treated with BHA and/or TBHQ irrespective of any other treatment, and with sesamol at high dose. Relative liver weight to body weight values were significantly higher with all treatments except for catechol and sesamol at the one-quarter dose levels. BUdR labeling index was increased by all antioxidants. The effect was highest with catechol and then with TBHQ, followed by sesamol and BHA. BHA slightly increased te labeling index.

Numbers and areas of GST-P-positive liver cell foci are summarized in Table II. With all antioxidants except for sesamol, the foci induction was significantly suppressed in the high-dose groups. TBHQ exerted the most effective inhibition, but not at the low dose level, under the present experimental conditions. Sesamol slightly, but not significantly, decreased the number of foci. At the one-quarter dose level, only BHA exerted a significant inhibitory effect.

Table I. Final Body and Liver Weights and Hepatocyte BUdR Labeling Indices in Rats Initiated with DEN

Group/Treatment	Number of rats	Body weight (g)	Liver weight		BUdR labeling index	
			Absolute (g)	Relative to body weight (%)	No. of rats	No. per 1000 cells
1. BHA	12	270±12	11.3±0.6***	4.2±0.1***	4	3.7±0.9*
2. TBHQ	15	247±10***	11.2±0.8***	4.5±0.2***	4	4.2±0.8**
3. Catechol	15	223±12***	8.8±0.7	3.9±0.2***	4	4.6±0.6***
4. Sesamol	12	286±12	9.4±0.8**	3.3±0.2*	5	4.0±0.9**
5. 1/4 BHA	14	277±14	10.3±0.8***	3.7±0.1***	4	1.7±0.6
6. 1/4 TBHQ	13	273±8	9.9±0.4***	3.6±0.2***	5	3.2±1.9
7. 1/4 Catechol	15	279±10	9.0±0.4	3.2±0.1	5	5.1±1.0***
8. 1/4 Sesamol	14	273±16	8.7±0.8	3.2±0.2	4	2.2±0.6
9. 1/4 (BHA+TBHQ)	14	269±16	10.8±0.7***	4.0±0.3***	5	2.7±0.5
10. 1/4 (Catechol+Sesamol)	14	274±16	9.0±0.8	3.3±0.1**	5	2.6±1.3
11. 1/4 (BHA+TBHQ+Catechol+Sesamol)	15	256±21***	10.6±0.8***	4.2±0.4***	3	2.8±0.4
12. None	15	278±17	8.6±0.6	3.1±0.1	5	2.4±0.6

Data are mean±SD values.
Significantly different from group 12 at; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Table II. Numbers and Areas of GST-P-positive Liver Cell Foci in Rats Initiated with DEN

Group/Treatment	Number of rats	Number (No./cm^2)		Area (mm^2/cm^2)	
		Observed[a]	Net[b]	Observed[a]	Net[b]
1. BHA	12	4.05±1.63***	−2.60	0.26±0.13**	−0.24
2. TBHQ	15	3.57±1.13***	−3.08	0.21±0.10**	−0.29
3. Catechol	15	4.45±1.58***	−2.20	0.26±0.11**	−0.24
4. Sesamol	12	5.79±2.37	−0.86	0.49±0.26	−0.01
5. 1/4 BHA	14	5.31±1.38*	−1.34	0.33±0.13*	−0.17
6. 1/4 TBHQ	13	8.11±1.99	1.46	0.55±0.17	0.05
7. 1/4 Catechol	15	7.03±2.20	0.38	0.49±0.17	−0.01
8. 1/4 Sesamol	14	6.10±1.89	−0.55	0.46±0.19	−0.04
9. 1/4 (BHA+TBHQ)	14	4.47±1.65**	−2.18	0.27±0.12**	−0.23
10. 1/4 (Catechol+Sesamol)	14	5.92±2.01	−0.73	0.40±0.16	−0.10
11. 1/4 (BHA+TBHQ+Catechol+Sesamol)	15	4.42±1.27***	−2.23	0.24±0.09**	−0.26
12. None	15	6.65±1.77		0.50±0.26	

a) Data are mean±SD values.
b) Values obtained by subtracting the background level (group 12).
Significantly different from group 12 at; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

Combined treatment with 2 or 4 antioxidants at the one-quarter dose levels resulted in significant suppressive effects in groups including BHA and TBHQ. The combination of catechol and sesamol reduced the foci levels, but less than in the BHA+TBHQ group. Comparison using net values revealed strongest inhibition in BHA+TBHQ combined groups irrespective of supplementation with catechol and sesamol. Number of foci in the BHA and TBHQ combined group was lower than in the control, the difference being −2.18, as compared to 0.12 for the sum of individual data (−1.34 +1.46), the respective figures for area being −0.23 against −0.12 (−0.17 +0.05). Combined treatment with catechol and sesamol showed a smaller supraadditive effect, the values

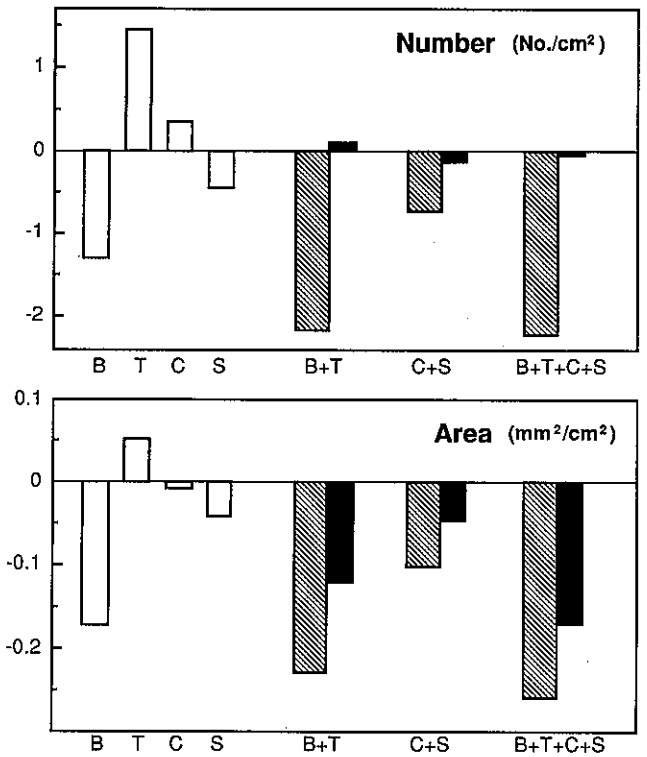

Fig. 2. Effects of combined treatment of antioxidants at the one-quarter dose levels. Net values obtained by subtracting the control level were used. B; BHA, T; TBHQ, C; catechol, S; sesamol. ▭; Single chemical. ▨; Combined treatment. ■; Sum of individual effects. Suppressive effects of combined administration of 2 or 4 antioxidants were higher than the sums of individual data.

for number being −0.73 against −0.17 (0.38 −0.55) and area being −0.10 against −0.05 (−0.01−0.04). Combined treatment with all 4 antioxidants gave the number of −2.23 against −0.05 and the area of −0.26 against −0.17. The effects of combined treatment are illustrated graphically in Figure 2. However, when the effects of the combined treatments were statistically analyzed using the additive model,[27] no significant synergism was found for any of the combinations (BHA+TBHQ, catechol+ sesamol, and all four chemicals).

DISCUSSION

All four antioxidants investigated in the present study have been shown to possess inhibitory potential for rat liver carcinogenesis using GST-P or γ-glutamyltranspeptidase-positive foci as endpoint marker lesions.[5, 11-13, 22, 28] In the present experiment, this influence was confirmed, except in the sesamol case, with the high dose levels. Although statistically significant effects in the additive model were not evident, synergistic inhibitory effects of these antioxidants on the development of liver preneoplastic foci were suggested to have occurred. Since final body weights and BUdR labeling indices did not correlate with GST-P-positive foci development, the inhibitory effects were not directly correlated with hepatocellular growth rate. The effects of catechol and sesamol were much less than those of BHA and TBHQ in the present experiment. Inhibitory effect in the catechol and sesamol combined group was less than the potency of each BHA or TBHQ. This may be partly related to the doses used. The dose of sesamol was 2% in a carcinogenicity study in rats.[16]

Increased chemopreventive effects by combined treatment of animals have been reported for retinoids in mouse skin,[29] retinoids and protease inhibitors in mouse skin,[30] retinoids and 2-bromoerocryptine, a suppresser of prolactin release from the pituitary,[31] rat γ-interferon and human tumor necrosis factor α in rat prostatic tumor,[32] selenium, magnesium, ascorbic acid and retinyl acetate in rat mammary carcinogenesis,[33] and piroxicam, α-difluoromethylornithine, 16α-fluoro-5-androsten-17-one and ellagic acid in rat colon carcinogenesis.[34] In many cases, underlying mechanism(s) responsible for the synergistic inhibition of carcinogenesis, as well as individual inhibitory effects, have not been completely elucidated.

Enhancement or inhibition in combined treatment protocols is the result of various biological activities of the chemicals, mainly depending on whether the agents have the same or different sites of primary action (similar or dissimilar) and whether or not the presence of one chemical influences the amount of other agents reaching their site of action (interactive or non-interactive). In the case of independent action (dissimilar and non-interactive), combined treatment effects may be the result of simple addition.[35] In the present case, since the influence was not directly additive the possibility of exerting effects through similar and interactive action must be considered. BHA is known to induce liver microsomal enzymes and to bring about a decrease in oxidizing species.[36] However, the question of whether this is directly relevant to inhibition still requires elucidation, and the effects have not been investigated for the other 3 antioxidants examined in the present study.

The inhibitory influence of antioxidants on carcinogenesis is in general based on the concept that they may exert a scavenging effect on reactive species of carcinogens, thus protecting cell constituents from attack.[8-10] Therefore, most experiments demonstrating chemopreventive effects of antioxidants have been conducted using simultaneous treatment protocols.[8, 10, 13] However, in the rat liver, antioxidants also exert second stage inhibitory

potential after a treatment with DEN, N-ethyl-N-hydroxyethylnitrosamine or aflatoxin B_1, as shown in the present experiment and in previous studies.[11, 22, 28] Similar inhibitory effects were observed for BHA and butylated hydroxytoluene after aflatoxin B_1 treatment.[28] Inhibition of liver carcinogenesis is sometimes associated with severe body weight loss or retardation[37] and commonly observed with simultaneous administration with carcinogen.[13, 38, 39] This was not the case in the present investigation.

BHA[15] and sesamol[16] at high dose have been revealed to be carcinogenic for the rat forestomach and catechol[16] is carcinogenic for the rat glandular stomach. These antioxidants, including TBHQ, also exert tumor-promoting effects in many organs.[18, 20, 21] Combined treatment of rats with these antioxidants has already been demonstrated to act synergistically in the rat forestomach.[40, 41] It is generally agreed that the effects of antioxidants differ in organs.[9] Similarly, retinoids which usually show inhibitory effects on experimental liver carcinogenesis and other organs[29–31, 42, 43] have in addition been reported to possess tumor-enhancing potential.[44, 45] Chemicals which exert only tumor-suppressive influence are apparently rare. To eliminate unwanted effects, protocols with low-dose combination treatment with anti-tumorigenic agents could be a possible practical method for prevention of carcinogenesis.

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