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Inhibitory Effects of Inhibitors of Arachidonic Acid Metabolism on the Evolution of Rat Liver Preneoplastic Foci into Nodules and Hepatocellular Carcinomas with or without Phenobarbital Exposure

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Effects of inhibitors of arachidonic acid (AA) metabolism on the evolution of preneoplastic foci into nodules and of nodules into hepatocellular carcinomas were examined in F344 male rat livers with or without phenobarbital (PB) exposure. *p*-Bromophenacyl bromide (BPB), acetylsalicylic acid (ASA), and quercetin (QU) were used as inhibitors of phospholipase A_2, cyclooxygenase and lipoxygenase, respectively. Preneoplastic liver foci were induced by initiation with N-nitrosodiethylamine (200 mg/kg, i.p.) followed by selection using the procedure of Cayama *et al.* For the nodule experiment, starting 1 week after completion of the selection procedure, animals bearing foci were given diets containing 0.05% PB plus 0.75, 1, or 1.5% of one of the inhibitors, 0.05% PB alone, or 0.75, 1 or 1.5% of inhibitor alone, or basal diet for 9 weeks. For the carcinoma experiment, 3 weeks after completion of the selection procedure, animals bearing nodules were given the same diets mentioned above for 29 weeks. BPB, ASA and QU either with or without PB accelerated the remodeling of preneoplastic foci, significantly decreasing the numbers of persistent nodules and hyperplastic nodules. ASA either with or without PB significantly decreased the number of hepatocellular carcinomas per rat. BPB and QU, however, significantly decreased the numbers of hepatocellular carcinomas with but not without PB. The results suggested an involvement of AA metabolism in the process of evolution of preneoplastic foci into nodules and hepatocellular carcinomas in rat liver with or without PB exposure.

Key words: Arachidonic acid metabolism — Inhibitor — Hepatocarcinogenesis — Phenobarbital — F344 rat

AA[2] metabolites are known to act as mediators of intercellular communication in many important biological systems including immune regulation[1–4] and inflammation.[5] Recent studies have also suggested that they act as intracellular second messengers in signal transduction.[6,7] AA metabolism has long been postulated to be involved in all aspects of carcinogenesis, including initiation, promotion, metastasis, cell differentiation and cell proliferation.[8–10] Recently, much attention has been paid to inhibitors of AA metabolism, particularly indomethacin and aspirin, as cancer therapeutic aids.[11,12] Nevertheless, probably because of the diverse functions of AA metabolites in different cells and tissues, their roles in carcinogenesis are to a large extent still controversial and remain to be clarified.[10] Basic and systematic studies using experimental animal models capable of distinguishing between the postulated initiation, promotion and progression steps would be of particular advantage for elucidating this complex problem.

The involvement of AA metabolism in the mechanisms of tumor promotion by phorbol esters in mouse skin carcinogenesis has been extensively studied,[13–15] with elucidation of a possible relation between PGE_2 and the induction of hyperplasia,[16] a role for PGF_2 in both stage I and II promotion steps[16] and a role of the lipoxygenase pathway in the induction of ODC.[17] Rat urinary bladder tumor promotion by sodium saccharin has also been reported to be inhibited by aspirin.[18] Furthermore, inhibitory effects of indomethacin and aspirin on mammary, colon, and tongue carcinogenesis in rats and pancreas carcinogenesis in hamsters without promoter exposure have been documented.[19–22] Nevertheless, causal relationships of AA metabolism to carcinogenesis remain unclear. Moreover, to the authors' knowledge, no detailed report has appeared on the effects of inhibitors of AA metabolism on hepatocarcinogenesis in rats.

The advantage of the rat liver is that the neoplastic processes have been extensively studied and lesions are well characterized.[23,24] Further, recent evidence has indicated AA metabolites to act as mediators of the inter- and intracellular communication which is important for

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[2] Abbreviations used are: AA, arachidonic acid; DEN, N-nitrosodiethylamine; 2-AAF, 2-acetylaminofluorene; BPB, *p*-bromophenacyl bromide; ASA, acetylsalicylic acid; QU, quercetin; GGT, γ-glutamyltranspeptidase; PG, prostaglandin; TX, thromboxane; ODC, ornithine decarboxylase; PB, phenobarbital; HE, hematoxylin and eosin.

various biological functions, including hepatocyte proliferation[25–27] and glycogenolysis.[28] In the liver, Kupffer cells reportedly are the main producers of PGs D_2, E_2, F_2, and I_2, and TXA_2, with hepatocytes having receptors and pronounced catabolic capacity.[29, 30] Thus, rat hepatocarcinogenesis should be a good experimental model for elucidating the involvement of AA metabolism in the carcinogenic processes.

Previously, we reported an inhibitory effect of inhibitors of AA metabolism on preneoplastic liver foci development induced in rats by a well-known liver tumor promoter, PB.[31] In the present study, in order to examine further the role of AA metabolism in rat hepatocarcinogenesis with PB or without PB, the effects of three inhibitors of AA metabolism on the evolution of preneoplastic foci into nodules and that of nodules into hepatocellular carcinomas were examined in rats with or without PB exposure. The inhibitors selected were BPB, an inhibitor of phospholipase A_2, ASA, an inhibitor of cyclooxygenase, and QU, an inhibitor of lipoxygenase.

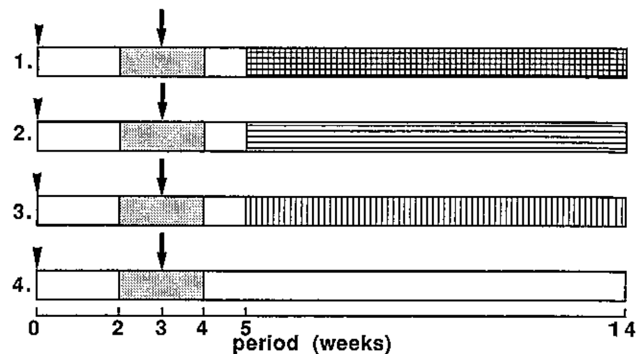

Fig. 1. Experimental protocol. ▼: DEN (200 mg/kg body wt.) i.p. injection, ↓: CCl_4 (1 ml/kg body wt.) i.g., ▒: diet containing 0.02% 2-AAF, ▦: diet containing 0.05% PB plus either 0.75, 1.0 or 1.5% inhibitor, ▤: diet containing 0.05% PB, ▥: diet containing either 0.75, 1.0 or 1.5% inhibitor, □: basal diet.

MATERIALS AND METHODS

Chemicals ASA, QU, 2-AAF and CCl_4 were purchased from Nacalai Tesque Inc., Kyoto, and BPB and DEN from Wako Pure Chemical Industries Ltd., Osaka. PB was obtained from Maruishi Pharmaceutical Co., Inc., Osaka.

Animals and diets Male Fischer 344 rats were obtained from Shizuoka Laboratory Animal Center, Shizuoka and were 6–7 weeks old, weighing about 170–200 g at the commencement of the experiments. The rats were housed in wire cages in an air-conditioned room at 23±2°C and 60% humidity with a 12 h light-dark cycle and given a commercial stock diet, Oriental MF (Oriental Yeast Co., Tokyo) and water *ad libitum*. Diets containing PB and inhibitors were made by mixing the chemicals into powder basal diet, Oriental MF.

Experimental protocol for Experiment I Effects of inhibitors on the evolution of foci into nodules were examined in Experiment I using the protocol shown in Fig. 1. The inhibitor doses adopted in the present experiment were the same as in our previous study.[31] Preneoplastic foci were induced in all the animals by initiation with a single intraperitoneal injection of DEN (200 mg/kg body weight) followed by selection using the procedure of Cayama *et al.*,[32] comprising feeding of 0.02% 2-AAF diet for 2 weeks with intragastric intubation of CCl_4 (1 ml/kg body weight) midway. One week after completion of the selection procedure, animals bearing foci were divided into 4 groups: group 1 was given diet containing 0.05% PB plus either 0.75, 1.0 or 1.5% inhibitor; group 2 received 0.05% PB alone; group 3 was given diet containing 0.75, 1.0 or 1.5% inhibitor alone; group 4 received basal diet for 9 weeks. All animals were sacrificed 14 weeks after the beginning of the experiment, after being starved for 17 h. Livers were excised and a single slice was taken from each liver lobe. Numbers and sizes of persistent and remodelling nodules were examined by GGT staining, and numbers and incidences of histologically diagnosed hyperplastic nodules by HE stainings.

Experimental protocol for Experiment II Effects of inhibitors on the evolution of nodules into hepatocellular carcinomas were examined in Experiment II. The doses of the inhibitors adopted in the present experiment were those appearing to be the maximum well-tolerated doses during the experimental period of Experiment II. Preneoplastic foci were induced in all animals by initiation with DEN and the selection procedure described above. Three weeks after completion of the selection procedure, all the animals bearing persistent nodules were divided into the same 4 groups as in Experiment I, and given the diets for 29 weeks. All surviving animals were killed 36 weeks after the commencement of the experiment, after being starved for 17 h and the livers were immediately excised. All liver lobes were sliced at 3 mm thickness, with every other slice being taken and processed for HE staining. Incidences and numbers per rat of hepatocellular carcinomas were assessed.

Intake of food and chemicals Food intake and body weight were measured weekly during the experimental periods. The average intake values for food, PB and inhibitors were calculated in terms of either the intake per rat per day or per kg body weight per day over the entire period.

Histochemical and histological studies The liver slices were fixed in ice-cold 95% ethanol containing 1% acetic

acid for 2–3 h, followed by 99.5% ethanol at 4°C overnight, routinely processed for paraffin sectioning and stained for GGT and HE. GGT activity was histochemically demonstrated by the method of Rutenberg *et al.*[33] Nodules homogeneously stained with GGT were designated as persistent nodules and those only patchily stained with GGT as remodeling nodules.[34] The numbers and areas of persistent and remodeling nodules were analyzed with the aid of an image analyzer, model HTB-C995 (Hamamatsu Television Co. Ltd., Shizuoka) connected to a Desktop Computer System-45 (Hewlett-Packard Co., USA). The numbers of nodules per cm^3 of liver were calculated using the procedure of Campbell *et al.*[35] Hyperplastic nodules and hepatocellular carcinomas were histopathologically diagnosed according to the criteria of Squire and Levitt.[36] Both persistent and hyperplastic nodules have been postulated to be preneoplastic nodules that evolve into hepatocellular carcinomas. Persistent nodules are based upon a biochemical phenotypic marker and hyperplastic nodules upon a morphological one. Not all of the persistent nodules exhibit the phenotype of hyperplastic nodules, and *vice versa*. In the present study, the two phenotypic markers were adopted for diagnosing preneoplastic nodules.

Statistical analysis Quantitative differences between groups were statistically analyzed by Student's t test and the chi-squared test.

RESULTS

Inhibitory effects of the inhibitors on the evolution of foci into nodules Body and liver weights and food and chemical intake data for Experiment I are summarized in Table I. The inhibitors BPB and ASA with or without PB did not significantly affect the final body and liver weights as compared with values for either basal diet or PB-alone groups. The QU-alone groups at both 0.75 and 1.5% dose levels had slightly but significantly increased final body weights and significantly decreased liver weights in terms of ratio to body weight as compared with the basal diet group. No influence of inhibitors on food intake was observed.

Data for numbers and size of persistent and remodeling nodules and incidences and numbers of hyperplastic nodules are summarized in Table II. All the inhibitors BPB, ASA and QU at both doses tested, with or without PB, significantly decreased the numbers of both persistent and hyperplastic nodules per cm^2 of liver and simultaneously increased the numbers of remodeling nodules compared with either basal diet or PB-alone groups. Similar results were obtained in terms of the numbers of persistent and remodeling nodules per cm^3 (data not shown). Nevertheless, all the inhibitors appeared to increase the sizes of persistent nodules, although this was not always statistically significant or dose-dependent.

Table. I Body and Liver Weights and Food and Chemical Intake Data for Rats Initiated by DEN, Selected and Fed PB and/or BPB, ASA, QU during the Evolution of Foci into Nodules[a]

Group No.	Treatments			No. of rats	Body weight	Liver weight		Average food intake		Average chemical intake mg/kg body wt./day	
	DEN	PB	BPB/ASA/QU(dose%)		Final	g	Ratio to body wt.×10^2	g/rat/day	g/kg, body wt./day	PB	BPB/ASA/QU
Experiment I-A											
1-a	+	+	BPB(0.75)	7	293.6±15.0	15.8±3.5	5.4±1.0	14.4±1.7	48.1±6.4	24.1±3.8	361.4±47.6
-b	+	+	BPB(1.5)	9	278.8±29.3	16.4±1.5	6.0±1.1	12.5±1.8	46.2±6.1	23.1±3.1	692.2±91.4
-c	+	+	ASA(0.75)	7	291.0±35.2	17.9±3.0	6.1±1.7	15.0±1.6	52.0±6.4	26.0±3.2	390.0±47.8
-d	+	+	ASA(1)	9	297.3±23.0	17.2±2.4	6.1±1.2	14.7±2.6	48.5±5.6	24.2±2.7	483.0±54.7
2	+	+	—	7	314.4±26.1	17.2±4.1	5.5±1.3	15.9±2.3	53.8±8.9	26.9±4.5	0
3-a	+	−	BPB(0.75)	8	298.5±36.6	14.3±3.6	4.9±1.5	15.2±2.0	51.4±8.4	0	383.6±63.3
-b	+	−	BPB(1.5)	8	286.3±70.6	12.2±1.2	5.2±2.8	13.2±2.0	46.4±7.5	0	692.3±112.8
-c	+	−	ASA(0.75)	8	305.9±22.6	12.0±2.0	3.9±0.7	14.8±1.5	51.5±4.9	0	386.2±36.8
-d	+	−	ASA(1)	8	292.6±16.5	12.3±1.4	4.2±6.5	14.2±4.3	51.8±6.9	0	581.1±68.9
4	+	−	—	8	311.5±26.9	13.3±1.5	4.3±0.1	16.2±3.0	56.7±10.5	0	0
Experiment I-B											
1-a	+	+	QU(0.75)	10	278.0±14.9	19.6±5.5	7.0±1.8	12.5±2.2	48.6±9.6	24.3±4.5	364.4±68.1
-b	+	+	QU(1.5)	10	277.8±12.9	22.1±2.7	8.1±1.1	12.5±1.9	50.8±7.1	25.4±3.6	761.8±107.0
2	+	+	—	10	286.9±22.2	22.5±3.0	7.8±0.9	12.7±3.4	50.0±12.6	25.0±6.3	0
3-a	+	−	QU(0.75)	10	308.0±19.8[b]	16.8±2.3	5.5±0.7[b]	13.1±2.0	49.9±7.3	0	374.0±55.0
-b	+	−	QU(1.5)	10	305.3±23.2[b]	16.1±1.7	5.3±0.8[b]	13.0±2.1	49.6±7.3	0	743.6±109.4
4	+	−	—	10	278.8±27.8	17.5±2.9	6.3±0.9	14.9±3.7	59.5±15.2	0	0

a) Values are mean±SD.
b) Significantly different from group 4 in Exp. I-B ($P<0.05$).

Table II. Numbers and Areas of Persistent and Remodeling Nodules and Incidence and Numbers of Hyperplastic Nodules in Rats Initiated, Selected and Fed PB and/or BPB, ASA, QU during the Evolution of Foci into Nodules$^{a)}$

Group No.	Treatments			No. of rats	Persistent nodules			Remodeling nodules			Hyperplastic nodules	
	DEN	PB	BPB/ASA/QU (dose%)		No./cm^2	Size (mm^2)	%area	No./cm^2	Size (mm^2)	%area	Incidence (%)	No./cm^2
Experiment I-A												
1-a	+	+	BPB(0.75)	7	7.0±2.8$^{b)}$	2.2±1.7	15.5±7.3$^{b)}$	14.9±1.9$^{b)}$	1.5±0.9$^{b)}$	22.4±3.1$^{b)}$	7/8 (87.5)	0.21±0.11$^{b)}$
-b	+	+	BPB(1.5)	9	7.2±2.5$^{b)}$	2.7±1.7$^{b)}$	19.5±10.9	15.4±4.2$^{b)}$	1.4±0.9	21.3±5.3$^{b)}$	8/9 (88.9)	0.10±0.06$^{b)}$
-c	+	+	ASA(0.75)	7	8.2±2.1$^{b)}$	2.4±1.8$^{b)}$	19.7±9.0	12.9±2.4$^{b)}$	1.5±1.0$^{b)}$	19.0±5.3$^{b)}$	7/7 (100)	0.28±0.15$^{b)}$
-d	+	+	ASA(1)	9	6.5±2.2$^{b)}$	2.2±2.1	14.4±8.8$^{b)}$	15.2±2.5$^{b)}$	1.4±0.9	21.3±3.7$^{b)}$	8/9 (88.9)	0.13±0.07$^{b)}$
2	+	+	—	7	15.0±5.5	2.0±1.3	29.5±11.6	8.1±2.2	1.3±0.9	10.9±3.0	7/7 (100)	0.61±0.26
3-a	+	−	BPB(0.75)	8	3.3±1.9$^{c)}$	4.0±2.5$^{c)}$	13.1±8.5	20.1±3.2$^{c)}$	1.5±0.9$^{c)}$	28.2±7.0	6/8 (75.0)	0.10±0.04$^{c)}$
-b	+	−	BPB(1.5)	8	2.9±1.0$^{c)}$	2.3±1.5	6.9±2.2$^{c)}$	21.8±4.0$^{c)}$	1.4±0.9$^{c)}$	30.9±6.7	4/6 (66.7)	0.09±0.08$^{c)}$
-c	+	−	ASA(0.75)	8	3.2±2.8$^{c)}$	2.3±1.7	7.6±8.3$^{c)}$	19.7±4.7$^{c)}$	1.2±0.8$^{c)}$	22.7±10.2	7/8 (87.5)	0.10±0.05$^{c)}$
-d	+	−	ASA(1)	8	3.3±2.1$^{c)}$	2.5±1.6	8.2±5.0$^{c)}$	20.7±4.0$^{c)}$	1.2±0.7$^{c)}$	24.9±6.5	6/8 (75.0)	0.08±0.07$^{c)}$
4	+	−	—	8	8.0±3.0	2.2±1.8	17.1±5.8	13.8±2.7	1.7±1.1	22.9±6.4	8/8 (100)	0.38±0.01
Experiment I-B												
1-a	+	+	QU(0.75)	10	9.9±3.4$^{d)}$	2.8±2.0$^{d)}$	27.3±14.7$^{d)}$	18.0±3.7$^{d)}$	2.1±1.2$^{d)}$	37.2±7.9$^{d)}$	9/10 (90.0)	0.20±0.10$^{d)}$
-b	+	+	QU(1.5)	10	10.2±1.9$^{d)}$	2.9±1.8$^{d)}$	28.7±5.1$^{d)}$	18.6±3.0$^{d)}$	2.2±1.2$^{d)}$	40.6±6.3$^{d)}$	9/10 (90.0)	0.15±0.12$^{d)}$
2	+	+	—	10	21.2±3.4	2.4±1.8	50.6±16.1	7.3±1.9	1.8±0.9	13.0±5.2	10/10 (100)	0.74±0.44
3-a	+	−	QU(0.75)	10	5.3±2.8$^{e)}$	2.0±1.5	10.7±7.5$^{e)}$	21.8±4.0$^{e)}$	2.0±1.0$^{e)}$	42.7±8.4$^{e)}$	7/10 (70.0)	0.09±0.07$^{e)}$
-b	+	−	QU(1.5)	10	5.1±1.0$^{e)}$	2.4±2.0	12.4±4.6$^{e)}$	20.9±3.5$^{e)}$	1.9±1.0	39.1±9.8$^{e)}$	9/10 (90.0)	0.08±0.04$^{e)}$
4	+	−	—	10	9.6±3.5	2.2±2.2	21.5±10.1	15.2±1.8	1.8±1.1	27.2±4.7	9/10 (90.0)	0.36±0.19

a) Values are mean±SD.
b) Significantly different from group 2 in Exp. I-A ($P<0.05$–0.001).
c) Significantly different from group 4 in Exp. I-A ($P<0.05$–0.001).
d) Significantly different from group 2 in Exp. I-B ($P<0.01$–0.001).
e) Significantly different from group 4 in Exp. I-B ($P<0.01$–0.001).

Table III. Body and Liver Weights and Food and Chemical Intake Data for Rats Initiated by DEN, Selected and Fed PB and/or BPB, ASA, QU during the Evolution of Foci into Hepatocellular Carcinomas$^{a)}$

Group No.	Treatment			No. of rats	Body weight	Liver weight		Average food intake		Average chemical intake mg/kg, body wt./day	
	DEN	PB	BPB/ASA/QU (dose%)		Final	g	Ratio to body wt.×10^2	g/rat/day	g/kg, body wt./day	PB	BPB/ASA/QU
1-a	+	+	BPB (0.75)	14	299.0±27.2	26.0±4.3	8.4±2.0	13.3±2.3	43.9±8.4	22.0±4.2	329.5±62.6
-b	+	+	ASA(0.75)	14	308.1±26.6$^{b)}$	25.4±6.4	8.3±2.3	14.7±2.5	47.6±6.3	23.8±3.2	358.2±48.8
-c	+	+	QU (1.5)	14	293.1±14.0	24.7±6.1	8.4±2.0	14.1±2.0	46.8±7.7	23.4±3.8	701.2±115.9
2	+	+	—	12	270.2±41.4	24.2±4.3	9.3±1.7	13.6±1.8	45.0±6.5	22.5±3.3	0
3-a	+	−	BPB (0.75)	11	324.0±24.5	18.3±4.6$^{c)}$	5.8±1.8$^{c)}$	13.7±2.5	41.7±7.4	0	313.0±55.8
-b	+	−	ASA(0.75)	13	309.7±31.5	14.2±3.8$^{c)}$	4.6±1.0$^{c)}$	13.8±2.2	46.4±8.3	0	348.0±62.0
-c	+	−	QU (1.5)	11	313.8±31.8	16.2±5.1$^{c)}$	5.2±1.7$^{c)}$	13.1±1.9	42.4±7.8	0	643.3±108.3
4	+	−	—	8	327.0±21.9	26.1±8.8	8.2±2.9	14.6±3.2	48.9±8.9	0	0

a) Values are mean±SD.
b) Significantly different from group 2 ($P<0.02$).
c) Significantly different from group 4 ($P<0.05$–0.001).

Thus, although all the inhibitors at either dose level decreased the % area occupied by persistent nodules as compared with either the basal diet or PB-alone groups, no significant differences were observed in groups where the sizes of the nodules were significantly increased. None of the inhibitors significantly affected the incidence of hyperplastic nodules.

Inhibitory effects of inhibitors on the evolution of persistent nodules into hepatocellular carcinomas Body and liver weights and food and chemical intake date for Experiment II are summarized in Table III. With the exception of 0.75% ASA in combination with PB, none of the inhibitors with or without PB significantly affected final body weight values. The inhibitors BPB, ASA and

Table IV. Incidences and Numbers of Hepatocellular Carcinomas in Rats Initiated by DEN, Selected and Fed PB and/or BPB, ASA, QU during the Evolution of Foci into Hepatocellular Carcinomas[a]

Group No.	Treatment			No. of rats	Hepatocellular carcinomas						
	DEN	PB	BPB/ASA/QU (dose%)		Incidence (%)	No/rat	No. counted (%)				
							Total	Well differ.	Moderately differ.	Poorly differ.	Glandular/papillary
1-a	+	+	BPB (0.75)	14	13/14 (92.9)	2.14±1.46[b]	30	29 (96.7)[b]	1 (3.3)	0	0[b]
-b	+	+	ASA (0.75)	14	12/14 (85.7)	2.21±1.76[b]	31	26 (83.9)	3 (9.7)	0	2 (6.5)
-c	+	+	QU (1.5)	14	13/14 (92.9)	2.36±1.15[b]	33	27 (81.8)	1 (3.0)	0	5 (15.2)
2	+	+	—	12	12/12 (100)	4.08±2.27	50	40 (82.0)	2 (4.0)	0	8 (16.0)
3-a	+	−	BPB (0.75)	11	11/11 (100)	1.45±0.82	16	10 (62.5)	1 (6.3)[c]	0	5 (31.3)[c]
-b	+	−	ASA (0.75)	13	5/13 (38.5)	0.54±0.78[c]	8	3 (37.5)	3 (37.5)	0	2 (25.0)[c]
-c	+	−	QU (1.5)	11	7/11 (63.6)	1.18±1.17	13	7 (53.9)	3 (23.1)	0	3 (23.1)[c]
4	+	−	—	8	7/8 (87.5)	2.00±1.51	17	11 (64.7)	5 (29.4)	1 (5.9)	0

a) Values are mean±SD.
b) Significantly different from group 2 ($P<0.05$–0.01).
c) Significantly different from group 4 ($P<0.05$–0.01).

QU, without PB but not with PB all significantly decreased liver weights in terms of both absolute values and ratios to body weights as compared with data for the basal diet group. None of the inhibitors with or without PB affected food (and therefore chemical) intake.

Data for incidences, numbers and histological types of hepatocellular carcinomas are summarized in Table IV. ASA at a dose of 0.75% irrespective of whether given with or without PB exerted an inhibitory effect on the evolution of persistent nodules into hepatocellular carcinomas. Thus the numbers of hepatocellular carcinomas per rat were significantly decreased as compared with either basal diet or PB-alone group values. ASA without PB but not with PB appeared to decrease the incidence of hepatocellular carcinomas, although this was not statistically significant, probably due to the small number of animals. BPB and QU with PB but not without PB also significantly decreased the numbers of hepatocellular carcinomas. No significant differences in incidence were found. Histologically, well differentiated, moderately differentiated, poorly differentiated and glandular/papillary types of hepatocellular carcinomas were diagnosed. BPB with PB slightly but significantly increased the proportion of well differentiated lesions while decreasing the glandular/papillary type as compared with the PB-alone group. BPB without PB also slightly but significantly decreased the moderately differentiated type compared with the basal diet group. All the inhibitors without PB slightly but significantly increased the glandular/papillary type compared with the basal diet group, probably because no glandular/papillary type developed in the basal diet group in the present experiment. Thus, none of the inhibitors appeared clearly and consistently to affect the histological type of hepatocellular carcinoma developing from DEN-initiated lesions.

DISCUSSION

The present results clearly indicated that evolution of preneoplastic foci into nodules, independent of PB exposure, can be inhibited by all three agents used in the present study, BPB, ASA and QU. In addition, the evolution of persistent nodules into hepatocellular carcinomas under the influence of PB can be inhibited by all the inhibitors, ASA also exerting a similar effect in the absence of PB.

A proportion of the preneoplastic foci induced by the procedure of Cayama *et al.*,[32] which was adopted in the present study, are known to evolve finally into hepatocellular carcinomas, through a well-characterized sequence of changes.[37] Only a minority of the induced preneoplastic foci develop into persistent nodules or hyperplastic nodules, the vast majority of foci and nodules undergoing a process of remodeling.[34, 37] This latter process has been ascribed to a loss of the acquired altered phenotype accompanied by a gradual return to the normal-looking acinar architecture without involvement of total cell death and subsequent regeneration.[34] Nevertheless, it remains unclear what factors are involved in triggering and driving the remodeling process and what determines the fate of individual lesions.

The liver tumor promoter PB has been shown to prevent the remodeling, increasing the number of persistent nodules and hepatocellular carcinomas.[38] Thus, the present finding that all the inhibitors inhibited the promoting activity of PB on nodule persistence, further confirmed an involvement of AA metabolism in the underlying mechanisms of PB action, in line with our previous report.[31] The finding that the three inhibitors similarly prevented foci from becoming persistent nodules, even without PB exposure, suggests that either the

same process is influenced both with and without PB or that AA metabolism plays separate roles in exogenous and endogenous control of remodeling and persistence. In the remodeling mechanisms, various factors, not only cell proliferation and differentiation, but also apoptosis, gap junctional communication and immune regulation, may be assumed to be involved since PB can prevent apoptosis[39] and inhibit gap junctional communication.[40] Further, induction of gap junctional communication by transfection of connexin 32 cDNA in a hepatoma cell line exerts growth retardation effects *in vivo* but not *in vitro.*[41] Although an involvement of AA metabolites, either PGs or TXs, in hepatocyte proliferation has been postulated,[25-27] the present finding of an increase in the size of persistent nodules indicates that simple inhibition of cell proliferation by the inhibitors is not the major influence. Roles for AA metabolism in gap junctional communication[42] and immune regulation[1-4] have also been postulated. No report, however, is available regarding the involvement of AA metabolism in hepatocyte differentiation and apoptosis, although effects on hematologic cell differentiation[43] and roles in either hepatocyte cell death mechanisms[44] or protection against hepatocyte injury,[45] have been indicated. The present results provide a clue for elucidating the mechanisms underlying the destiny of remodeling and persistent nodules.

The present results on inhibition of the evolution of persistent nodules into hepatocellular carcinomas by all the inhibitors under PB exposure might be partly ascribed to inhibition of promotion activity of PB by the inhibitors, further confirming an involvement of AA metabolism in the promotion mechanisms of PB. However, whether the difference observed between BPB and QU on the one hand, and ASA on the other, regarding their effects during hepatocellular carcinoma development with or without PB exposure points to any essential difference in mechanism is unclear. Although factors that may be involved in the progression step have not been completely clarified, those that induce new changes in DNA, including mutation, gene rearrangement or gene amplification, have been postulated as important.[46,47] So far, carcinogens themselves and chemicals that produce activated oxygen species are known to be active progressors.[46,47] Since AA metabolism includes activated oxygen species in its pathway,[48] blockage of activated oxygen production by the inhibitors might partly explain the present results. Blockage by the inhibitors of other factors, such as tumor cell proliferation, differentiation, apoptosis and immune regulation, which may regulate tumor growth capacity and provide an increased probability of DNA alteration, are perhaps also involved. On the other hand, many but not all tumors produce elevated levels of AA metabolites, particularly prostaglandins, and cancer patients are known to excrete abnormal amounts of these.[8-10] Rat hepatoma cell lines also produce elevated levels of AA metabolites, particularly prostaglandins, without any direct correlation with growth rate, and indomethacin inhibits tumor growth *in vitro* and *in vivo.*[49,50] Thus, the present results on inhibition of the development of hepatocellular carcinomas by ASA exposure seems to be partly in line with these reports. Unfortunately, however, there has been no report on the relationship between lipoxygenase pathway metabolites and cancer, so further studies will be required to explain the observed differential effects among ASA, BPB and QU. With regard to the biological significance of the elevated AA metabolites, their involvement in allowing tumor cells to escape immunosurveillance, thereby giving them a growth advantage, has been postulated, but this appears controversial.[1-4,51] Further, suggestive evidence of an abnormal signal transduction pathway for response to PGE_1 in rat hyperplastic nodules and hepatocellular carcinomas has been reported.[52] Nevertheless, the pathophysiological roles of AA metabolism in cancer development remain to be elucidated from the viewpoint of progression mechanisms, and our present results provide another clue.

In conclusion, together with our previous report,[31] the present results clearly indicated an involvement of AA metabolism in the tumor promotion mechanisms of PB, with all the inhibitors inhibiting the promotive developement by PB of all the lesions, foci,[31] nodules and hepatocellular carcinomas. However, it remains to be elucidated which pathway of AA metabolism is involved in the PB promotion mechanisms. Further, differential involvement of AA metabolism in the development of foci, nodules and hepatocellular carcinomas without PB is indicated, with none of the inhibitors exerting any effect on the foci[31] but all inhibiting nodule development, though only ASA exerts a significant effect on carcinoma development. It should, however, be borne in mind that the inhibitors used in the present and previous[31] studies might also have affected other pathways than AA metabolism,[31] and further investigations in this area are clearly warranted.

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