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## Inhibition of the development of hepatocellular carcinomas by phenyl *N-tert*-butyl nitron in rats fed with a choline-deficient, L-amino acid-defined diet

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### Abstract

Effects of phenyl *N-tert*-butyl nitron (PBN), a spin-trapping agent, on the development of frank cancers were examined in male Wistar rats fed with a choline-deficient, L-amino acid-defined (CDAA) diet for 70 weeks. PBN (0.065% in the drinking water) reduced incidences, multiplicities and possibly sizes of both hepatocellular adenomas and carcinomas when administered for all 70 weeks or only for the first 26 weeks, and those of carcinomas but not adenomas, when administered only for the last 44 weeks. These results indicate that PBN can prevent the development of frank HCCs in the CDAA diet model. The anti-carcinogenic effect of PBN may be ascribed to the prevention of both the development of HCAs and their malignant conversion to HCCs. If such findings can be generalized, PBN may be able to serve as a good tool to investigate molecular mechanisms underlying carcinogenic processes.

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**Keywords:** Phenyl *N-tert*-butyl nitron; A choline-deficient, L-amino acid-defined diet; Hepatocellular carcinoma; Hepatocellular adenoma; Rat

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## 1. Introduction

Cancer is one of the most serious problems for the human health. Large efforts have been accumulating substantial knowledge regarding methods for improved diagnosis and treatment, and for molecular mechanisms underlying multi-step carcinogenic processes, which are well contributing to the progress to control various types of cancers. However, several types of cancers still lack sufficient amount of such knowledge and remain with poor prognoses. As one of these cancers, hepatocellular carcinoma (HCC) is common in Asia and Africa, and increasing in Europe and North America [1–4]. Its prognosis is extremely poor because of its malignant biological nature due to the frequent recurrence after surgical treatments and intra-hepatic metastases [5–7]. Establishment of an efficient strategy to control HCC is thus demanded, but molecular mechanisms underlying hepatocarcinogenesis have not been as yet fully understood and should be urgently elucidated. In this context, we have been investigating hepatocarcinogenic mechanisms using various animal models. As a part of such studies, we developed a unique hepatocarcinogenesis model in rats chronically fed with a choline-deficient, L-amino acid-defined (CDAA) diet in 1990 [8]. In this model, no carcinogen is administered, yet HCC eventually forms with a high incidence through the induction and growth of preneoplastic hepatocellular lesions, their progression to hepatocellular adenomas (HCAs) and finally the subsequent malignant conversion from HCA to HCC [9–11]. Continuous hepatic damage, represented by death and proliferation of hepatocytes, and fibrosis resulting in cirrhosis, is induced in the background of hepatocarcinogenesis in association with the sequential induction and accumulation of a variety of oxidative sub-hepatocellular injuries, signaling alterations and genetic as well as epigenetic changes on several specific genes and their products [9–15]. These hepatocarcinogenic processes of the CDAA diet model are at least partly similar to those of the human counterparts progressed under chronic viral hepatitis, hemochromatosis, Wilson's disease, etc.; in terms of a sequence of morphological changes, a profile of involved signaling alterations and a possible role of reactive oxygen and/or nitrogen oxide species-induced stress [16–21].

Phenyl *N*-*tert*-butyl nitron (PBN) is a nitron-based compound that traps or scavenges a variety of free radical species, including biologically relevant hydroxyl radical and superoxide anion, and has been used for over 30 years as an analytical reagent to trap and characterize free radicals [22,23]. In addition, PBN has recently been shown to inhibit the induction of inducible nitric oxide synthase and of various cellular signaling alterations mediated by factors including nuclear factor (NF)- $\kappa$ B, pro-inflammatory cytokines, inducible cyclo-oxygenase (COX2) and pro-apoptotic gene products in several *in vitro* and *in vivo* conditions [23]. In turn, PBN is preventive for a variety of animal disorders such as endotoxin shock, ischemia-reperfusion injuries, neurodegenerative diseases and diabetes, *id est*, where reactive oxygen and/or nitrogen oxide species-induced stress is involved in association with concomitant alterations on various signal transduction activities [23]. Because reactive oxygen and/or nitrogen oxide species-induced stress and concomitant signaling alterations are known to be involved also in carcinogenesis [24–31], the aforementioned properties of PBN led to an idea that this compound may serve as a good tool to investigate molecular mechanisms underlying carcinogenic processes.

With these considerations in mind, we set our aim to assess effects of PBN on hepatocarcinogenesis in rats fed with the CDAA diet. In our previous studies, it was clearly demonstrated that PBN inhibits the induction and, more prominently, growth of putatively preneoplastic, glutathione *S*-transferase placental form (GST-P)-positive foci of cellular alteration in the early stage of hepatocarcinogenesis in rats fed with the CDAA diet for 12–16 weeks [32,33]. Such an inhibitory effect of PBN is attributed to the selective induction of apoptosis among preneoplastic hepatocytes and to the prevention of the generation of oxidative sub-hepatocellular injuries and of the induction of COX2 [32,33]. To our knowledge, these were the first and only reports to indicate the potential anti-carcinogenic effects of PBN, but we thought it necessary to ascertain that this compound can indeed prevent the development of frank cancers, before using it for further investigations to elucidate detailed mechanisms underlying (hepato)carcinogenesis. The present study was thus conducted in order to assess effects

of PBN on the entire hepatocarcinogenic processes in rats fed with the CDAA diet, using HCA and HCC as endpoint markers.

## 2. Materials and methods

### 2.1. Ethical considerations

The experimental protocols have been approved by the Animal Experimentation Committee of Sasaki Institute prior to the execution. The experiment was conducted under the monitoring by the committee in accordance with the National Institutes of Health Guideline for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105 and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

### 2.2. Animals, diets, and chemicals

A total of 60 male Wistar rats, 6-week-old, were purchased from Japan SLC, Inc., Hamamatsu, Shizuoka, Japan. Rats were housed five each to a plastic cage with white flake bedding in an air-conditioned room ( $25 \pm 3^\circ\text{C}$  temperature,  $55 \pm 8\%$  relative humidity, 10–12/h ventilation and 12-h dark/light cycle). Rats were used for the experimentation after a 1-week acclimation on a basal diet (CRF-1, Oriental Yeast Corporation, Limited, Itabashi, Tokyo, Japan) and allowed free access to food and tap water throughout the acclimation and experimental periods. Body weight, food consumption, and water intake were monitored weekly. The CDAA diet and a control, choline-supplemented, L-amino acid-defined (CSAA) diet were obtained from Dyets, Incorporated, Bethlehem, PA, USA. PBN was synthesized to achieve a purity of 99.997% in our laboratories according to the method of Janzen and Haire [22].

### 2.3. Animal treatment

Rats were equally divided into six groups. Group 1 received the CDAA diet for 70 weeks. Group 2 received the CDAA diet with PBN at a concentration of 0.065% in the drinking water for 70 weeks. Group 3 received the CDAA diet with PBN for 26 weeks and

then the CDAA diet alone for 44 weeks. Group 4 received the CDAA diet alone for 26 weeks and then the CDAA diet with PBN for 44 weeks. Groups 5 and 6 received the CSAA diet alone and with PBN, respectively, for 70 weeks. The PBN dose was equivalent to the median level used in our previous study [32], and this decision for the PBN dose was made on the basis of the fact that PBN was sufficient to inhibit the induction and growth of GST-P-positive foci of cellular alteration similarly at the middle and high level doses in such a study [32]. In addition, we wished to avoid a possible long-term toxicity caused by PBN (unpredictable at the time of the experimental designing) as much as we could. The time-point of the change of the treatments in groups 3 and 4 was decided based on the preliminary findings that HCAs were induced by a 26-week feeding of the CDAA diet, while HCCs were induced by a 26-week feeding of the CDAA diet and then a 44-week feeding of the CSAA diet, both at an incidence of 40% (data not shown). A period of 26 weeks was thus enough to develop HCAs but not HCCs, and the developed HCAs could be further progressed and converted to HCCs within an additional period of 44 weeks in the absence of any carcinogenic stimuli. All surviving rats were sacrificed 70 weeks after the commencement, when the macroscopic examination was done throughout the animal bodies. The livers were then taken and macroscopically observed in a careful manner. One to three slices each from all liver lobes (numbers of slices from a particular lobe being fixed for all animals) were taken at a thickness of 5 mm, placed in 10%-neutrally buffered formalin, fixed for 24 h and then paraffin-embedded. From each of these slices, two serial 4- $\mu\text{m}$ -thick specimens were prepared and stained routinely by hematoxylin-and-eosin and Masson's trichrome procedures for the histological assessments as specified below.

### 2.4. Histological assessments

The histological examination for the livers was conducted as carefully as possible. HCAs and HCCs were diagnosed strictly according to the well-established criteria described in the literature [34, 35]. The development of HCAs and HCCs was evaluated by their incidences, multiplicities and sizes. Regarding the multiplicities, the number of

hepatocellular neoplasms per rat was defined as their total number detected in all assessed liver specimens of each animal. To evaluate the sizes of hepatocellular neoplasms, the area of each neoplasm was obtained using an Image Processor for Analytical Pathology (IPAP) system (Sumika Technoservice Corporation, City of Osaka, Osaka, Japan). The numbers of apoptotic bodies and mitoses were counted among up to 5000 neoplastic hepatocytes in every HCA and HCC (the numbers of the counted cells being varied because of the varied tumor sizes) and among 20,000 hepatocytes in non-tumoral liver tissues, and their percentages were calculated as apoptotic and mitotic indices, respectively. The grade of fibrosis was evaluated by analyzing percent area occupied by collagen fiber in the Masson's trichrome-stained liver specimens using IPAP.

### 2.5. Statistics

Statistical significance of inter-group differences of the data was assessed as follows. Fisher's exact test was used for the incidences of hepatocellular neoplasms. One-way analysis of variance (ANOVA) was chosen for the sizes of hepatocellular neoplasms, apoptotic indices and mitotic indices among groups 1–4, because there were some groups with only 1 or 2 data. Student–Newman–Keuls multiple comparison test was conducted after ANOVA for the other category data. The inter-group difference was considered statistically significant, when the *P*-value was at least less than 0.05.

### 3. Results

Rat mortalities in groups 1–4 were 3, 2, 3 and 2, respectively, with no specific causes of deaths, while all rats survived in groups 5 and 6 (Table 1). There were no differences among groups in terms of food consumption or water intake (data not shown). The final body and relative liver weights were higher and lower in group 5 than in group 1, respectively (Table 1). The administration of PBN did not affect the final body or relative liver weights in groups 2, 3, 4 and 6 (Table 1).

In group 1, the livers were macroscopically yellowish-white and appeared cirrhotic in all rats, and one or two large tumoral nodules with turbid and dark color were observed in four out of seven animals (57.1% incidence) (Fig. 1A and Table 2). No apparent tumoral nodules were macroscopically found in the livers of any other groups, and their incidence of group 1 was thus significantly higher than those of groups 2–5 (Table 2). Besides, group 2 livers were macroscopically yellowish-white with relatively smooth surface (Fig. 1B). While the livers of group 3 macroscopically resembled those of group 2, group 4 livers looked similar to those of group 1 with the exception of a lack of tumoral nodules (data not shown). No remarkable macroscopic changes were noted for the livers of groups 5 and 6 (data not shown).

Upon the histological examination, HCAs showed irregular and intricate architecture consisting of neoplastic hepatocyte trabeculae with a few cell-thick layers (Fig. 2A), and were detected in seven out

Table 1  
Final body weights, relative liver weights and histological grade of liver fibrosis

Group	Treatments		Initial number of rats	Effective number of rats	Final body weight (g)	Relative liver weight (g/100 g body weight)	Histological grade of liver fibrosis (percent area occupied by collagen fiber in the specimen)
	First 26 weeks	Last 44 weeks					
1	CDAA	CDAA	10	7	415 ± 27 <sup>a</sup>	4.45 ± 0.26 <sup>a</sup>	13.32 ± 1.57 <sup>a</sup>
2	CDAA + PBN	CDAA + PBN	10	8	408 ± 34	4.52 ± 0.07	9.95 ± 1.17 <sup>b</sup>
3	CDAA + PBN	CDAA	10	7	406 ± 58	4.40 ± 0.24	8.71 ± 1.12 <sup>b</sup>
4	CDAA	CDAA + PBN	10	8	399 ± 39	4.23 ± 0.63	12.86 ± 1.65
5	CSAA	CSAA	10	10	475 ± 31 <sup>b</sup>	2.90 ± 0.10 <sup>b</sup>	1.61 ± 0.22 <sup>b</sup>
6	CSAA + PBN	CSAA + PBN	10	10	446 ± 15	3.26 ± 0.26	1.58 ± 0.13

<sup>a</sup> Values are presented as means ± SDs in the column.

<sup>b</sup> Significantly different from the group 1 value.

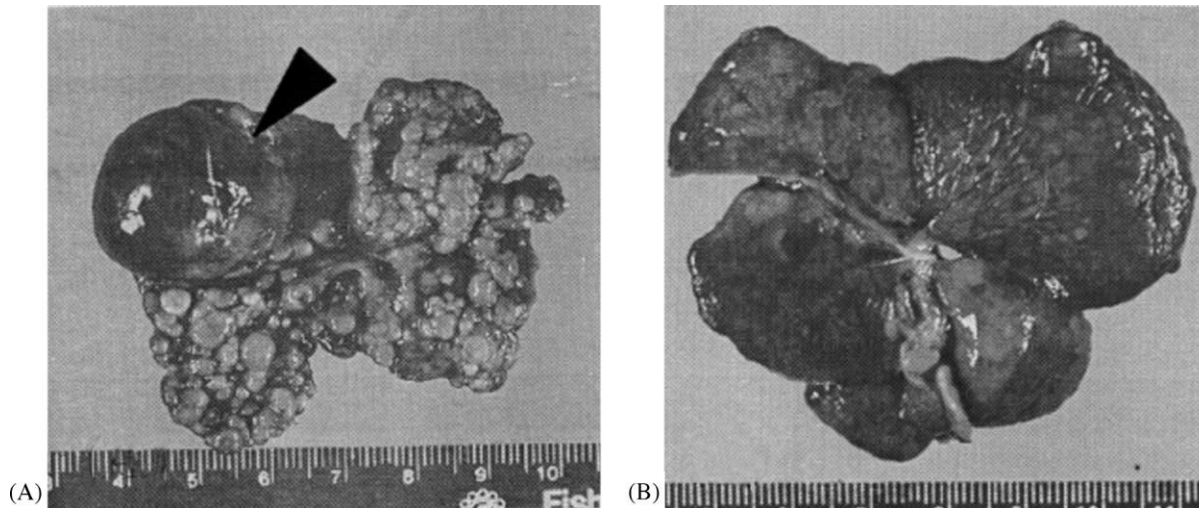


Fig. 1. Representative macroscopic appearances of the livers. (A) A liver of a rat fed with the CDAA diet for 70 weeks alone (group 1), demonstrating a cirrhotic surface with a tumoral nodule (arrowhead). (B) A liver of a rat fed with the CDAA diet and administered PBN for 70 weeks (group 2), showing a smooth surface without a tumoral nodule.

of seven (100%), three out of eight (37.5%), two out of seven (28.6%) and six out of eight (75.0%) rats of groups 1–4, respectively, while being absent (0%) in groups 5 and 6 (Table 2). The HCA incidence of group 1 was significantly higher than those of groups 2, 3 and 5 but not 4 (Table 2). As for the multiplicities, mean HCA numbers per rat of groups 1–5 were 4.14, 1.00, 0.57, 3.00 and 0, respectively, and the group 1 value was significantly higher than those of groups 2, 3 and 5 but not 4 (Table 2). The inter-group differences of the sizes of HCAs were statistically insignificant due to the extreme dispersion of the values in each group. From the biological point of view, however, it may be better to consider that the HCA size of group 1 was larger than those of groups 2 and 3 but not 4, when evaluating individual data in detail. For instance, HCAs larger than  $1 \text{ mm}^2$  were frequent in groups 1 (12 out of 29 HCAs, 41.4%) and 4 (11 out of 25, 44.0%) but rare in group 2 (only one out of eight, 12.5%) and absent (0%) in group 3 (Fig. 3). HCCs were all relatively well-differentiated mostly showing a trabecular architecture (Fig. 2B) and detected in six out of seven (85.7%), two out of eight (25.0%), one out of seven (14.3%) and two out of eight (25.0%) rats of groups 1–4, respectively, while being absent (0%) in groups 5 and 6 (Table 2). The HCC incidence of group 1 was significantly

higher than those of groups 2–5 (Table 2). As for the multiplicities, mean HCC numbers per rat of groups 1–5 were 1.71, 0.25, 0.14, 0.38 and 0, respectively, and the group 1 value was significantly higher than those of groups 2–5 (Table 2). The inter-group differences of the sizes of HCCs were statistically insignificant due to the extreme dispersion of the values in each group. From the biological point of view, however, it may be better to consider that the HCC size of group 1 was larger than those of groups 2–4, when evaluating individual data in detail. For instance, HCCs larger than  $10 \text{ mm}^2$  were frequent in group 1 (five out of 12 HCCs, 41.7%) but absent (0%) in groups 2–4 (Fig. 3). In each of groups 1–4, apoptotic bodies and mitoses were observed prominently in HCCs (Fig. 2B), while they were seen in HCAs with the significantly lesser degree and in non-tumoral liver tissues with further significantly lesser degree (Figs. 4 and 5). The apoptotic (Fig. 4) and mitotic (Fig. 5) indices were significantly lower and higher, respectively, in HCCs and also in HCAs of group 1 than in those of groups 2–4. In contrast, the apoptotic and mitotic indices in non-tumoral liver tissues of group 1 ( $0.73 \pm 0.08$  and  $0.63 \pm 0.07\%$ , respectively) were both significantly higher than those of groups 2 ( $0.41 \pm 0.02$  and  $0.37 \pm 0.09$ ), 3 ( $0.40 \pm 0.05$  and  $0.41 \pm 0.07$ ) and 4 ( $0.28 \pm 0.03$



Table 2  
Incidences of macroscopically observed liver tumoral nodules and incidences and multiplicities of histologically detected hepatocellular neoplasms

Group	Treatments		Effective number of rats	Incidences of macroscopically observed liver tumoral nodules		Incidence and multiplicities of histologically detected hepatocellular neoplasms					
	First 26 weeks	Last 44 weeks		Number of bearing rats	Incidence (%)	HCA			HCC		
1	CDA	CDA	7	4	57.1	7	100	4.14 ± 0.9 <sup>0a</sup>	6	85.7	1.71 ± 1.11 <sup>a</sup>
2	CDA + PBN	CDA + PBN	8	0	0 <sup>b</sup>	3	37.5 <sup>b</sup>	1.00 ± 1.51 <sup>b</sup>	2	25.0 <sup>b</sup>	0.25 ± 0.46 <sup>b</sup>
3	CDA + PBN	CDA	7	0	0 <sup>b</sup>	2	28.6 <sup>b</sup>	0.57 ± 0.98 <sup>b</sup>	1	14.3 <sup>b</sup>	0.14 ± 0.38 <sup>b</sup>
4	CDA	CDA + PBN	8	0	0 <sup>b</sup>	6	75.0	3.00 ± 2.00	2	25.0 <sup>b</sup>	0.38 ± 0.74 <sup>b</sup>
5	CSA	CSA	10	0	0 <sup>b</sup>	0	0 <sup>b</sup>	0 <sup>b</sup>	0	0 <sup>b</sup>	0 <sup>b</sup>
6	CSA + PBN	CSA + PBN	10	0	0	0	0	0	0	0	0

<sup>a</sup> Values are presented as means ± SDs in the column.

<sup>b</sup> Significantly different from the group 1 value.

and  $0.31 \pm 0.07$ ) (Figs. 4 and 5) as well as group 5 ( $0.13 \pm 0.03$  and  $0.11 \pm 0.04$ ). On the other hand, in the groups given the control CSA diet, the administration of PBN did not exert any effects regarding the development of hepatocellular neoplasms (compare the data of group 5 with those of group 6 in Table 2), or the apoptotic ( $0.13 \pm 0.04\%$  in group 6) or mitotic ( $0.12 \pm 0.04\%$  in group 6) indices. It should be noted that data for total hepatocellular neoplasms (combining HCA and HCC) are not presented, because all rats bearing HCC bore HCA so that the data for total hepatocellular neoplasms, if presented, become fundamentally identical to those for HCA, and the histological examination was conducted in a very critical manner.

As histological findings other than hepatocellular neoplasms, fatty liver with cirrhosis was observed in all group 1 rats (data not shown). In group 2, fatty liver was still evident, but fibrosis was scarce (data not shown). The histological appearances of the livers of groups 3 and 4 closely resembled those of groups 2 and 1, respectively (data not shown); no remarkable lesions were detected in groups 5 or 6 (data not shown). With regard to cirrhotic liver change, the grade of fibrosis of group 1 (13.32%) was significantly greater than those of groups 2 (9.95%), 3 (8.71%) and 5 (1.61%) but not 4 (12.86%) (Table 1). In the meantime, various types of foci of cellular alteration were observed in the livers of all rats of all groups with varied numbers and sizes (data not shown), but their development or nature was not evaluated in detail to avoid unnecessary confusion.

#### 4. Discussion

The present results indicate a clear phenomenon that PBN inhibits the development of HCCs in rats fed with the CDA diet for 70 weeks, while PBN is not hepatotoxic or hepatocarcinogenic in rats at least during its administration with a control CSA diet for such a period. PBN reduced the development of HCA and HCC when administered only for the first 26 weeks, whereas reducing the development of HCC without alteration on the development of HCA when administered only for the last 44 weeks.

NF- $\kappa$ B is generally known to promote cell survival and cell proliferation by causing an anti-apoptotic and

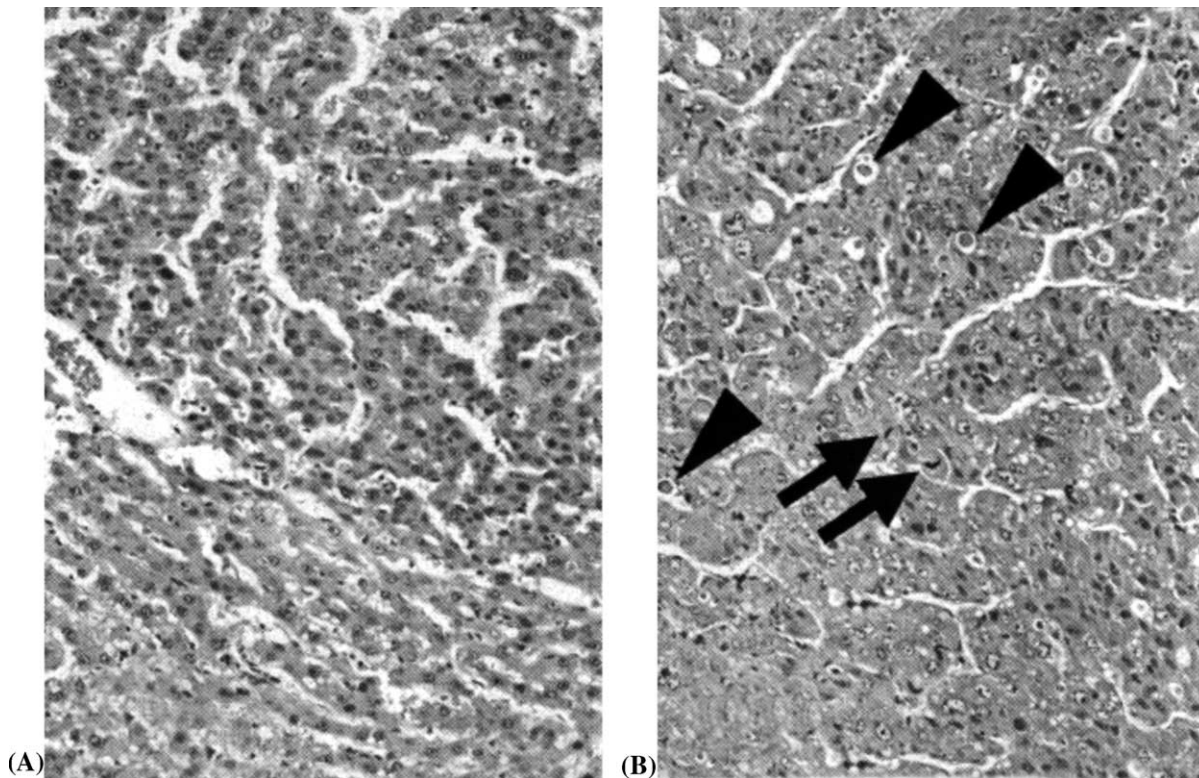


Fig. 2. Representative histological appearances of the hepatocellular neoplasms (hematoxylin-and-eosin staining,  $\times 25$ ). (A) HCA, located in the upper right part of the figure, demonstrated irregular and intricate architecture consisting of neoplastic hepatocyte trabeculae with a few cell-thick layers. The neoplasm was demarcated from the surrounding liver parenchyma in a relatively sharp manner. (B) HCCs were relatively well-differentiated and showed a trabecular pattern with numerous apoptotic bodies (arrowheads) and mitoses (arrows).

pro-proliferative signaling profile [36]. It is activated by various carcinogenic stimuli, and its constitutive expression is demonstrated in various cancer cell lines and cancers in vivo [36]. In the human hepatocarcinogenesis, this transcription factor is suggested to play pivotal roles especially in its early stage [18,19,37–40]. We found that NF- $\kappa$ B was activated in the livers of rats fed with the CDAA diet in a preliminary study, which has been postulated as one of the causes of the fact that preneoplastic hepatocytes are resistant towards the host activity to eliminate them through apoptotic processes and possess high proliferating activity [9,33]. Likewise, it is shown in vitro that an immortalized rat hepatocyte cell line, CWSV1, rapidly dies through apoptotic processes by acute choline deficiency but adapts to survive under the chronic and gradual deficient condition in choline [41,42]. This is caused by virtue of the activation of

NF- $\kappa$ B, and CWSV1 becomes resistant towards apoptosis and in turn transformed [41,42]. It may thus be safe to consider that NF- $\kappa$ B plays a key role in the development of preneoplastic hepatocellular lesions in the early stage of the CDAA diet model, which is phenomenally evidenced by the anticarcinogenic effects of PBN, as well as *N*-(4-hydroxyphenyl)retinamide and 1'-acetoxychavicol acetate, all inhibitors of the activation of NF- $\kappa$ B [23,36,43,44], shown in our previous [32,33,45,46] and present results. PBN induces apoptosis selectively in preneoplastic hepatocytes and thereby reduces the number and size of GST-P-positive lesions in the early stage of hepatocarcinogenesis in rats fed with the CDAA diet for 16 weeks [33]. Although PBN does not alter the proliferating activity of preneoplastic hepatocytes by a 16-week administration with the CDAA diet, its physiological 4-hydroxylated

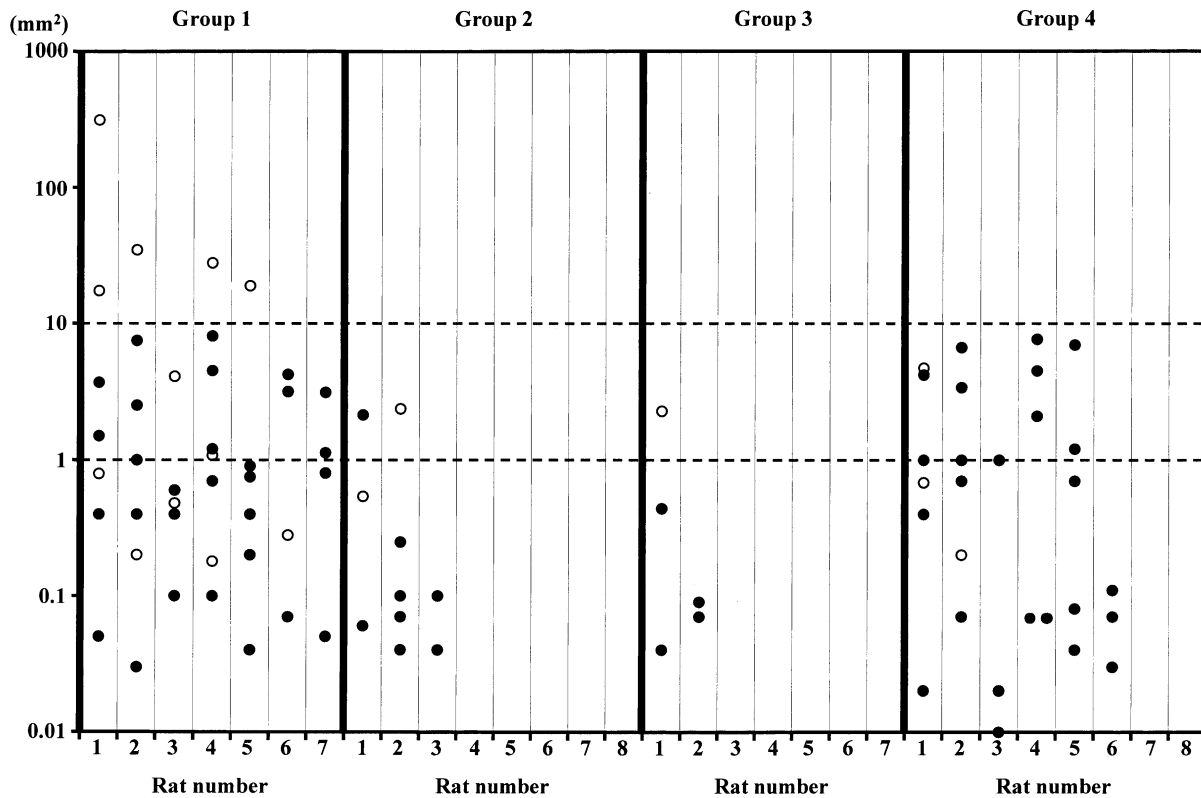


Fig. 3. The sizes of hepatocellular neoplasms in groups 1–4. Closed and open circles represent values of individual HCCs and HCAs, respectively, and they are plotted according to a common logarithm scale as specified in the ordinate. Each column contains the data for a particular animal as specified in the abscissa. It should be noted that the animal numbers in this figure are provisional for the easy understanding and do not have any relationships with those used to specify particular animals in the actual experimentation. Broken lines divide the data into two categories, larger and smaller than 1 and 10 mm<sup>2</sup> for HCCs and HCAs, respectively.

metabolite reduces such an activity in GST-P-positive lesions and reduces the size of such lesions superiorly to its parent compound [33]. PBN might thus be capable of inhibiting the proliferating activity of (pre)neoplastic hepatocytes through its metabolism, when administered for a longer period, for instance 26 weeks. In this context, it is indicated that PBN inhibits the induction, maintenance and progression of HCAs, and suggested that the HCA cells surviving from the anti-carcinogenic effects of PBN may have a reduced capability to progress further. These can explain the present results seen in group 3 (and group 2 also), in which the incidences, multiplicities and possibly sizes of both HCAs and HCCs were lower, while the apoptotic and mitotic indices of HCCs (and HCAs also) being higher and lower, respectively, than in group 1. A signaling profile downstream of NF- $\kappa$ B in

the early stage hepatocarcinogenesis in the CDAA diet model and its relevance to the human situation are still obscure, and investigations on this point are now in progress in our laboratories.

Recent progress of the studies has provided much information regarding molecular mechanisms underlying hepatocarcinogenesis including in its late stage [6,16–20,47], but there remain numerous uncertainties. We have found several genetic and epigenetic changes in the late stage of the CDAA diet model, such as the mRNA overexpression of the *c-myc* gene in HCCs but not HCAs due to the hypomethylation occurring in its 5'-flanking region and the disturbance of the transforming growth factor (TGF)- $\beta$  signaling pathway due to the point mutation of the *Smad2* gene and the down-regulation of the mRNA expression of the *TGF- $\beta$  receptor type II* gene [12,13].



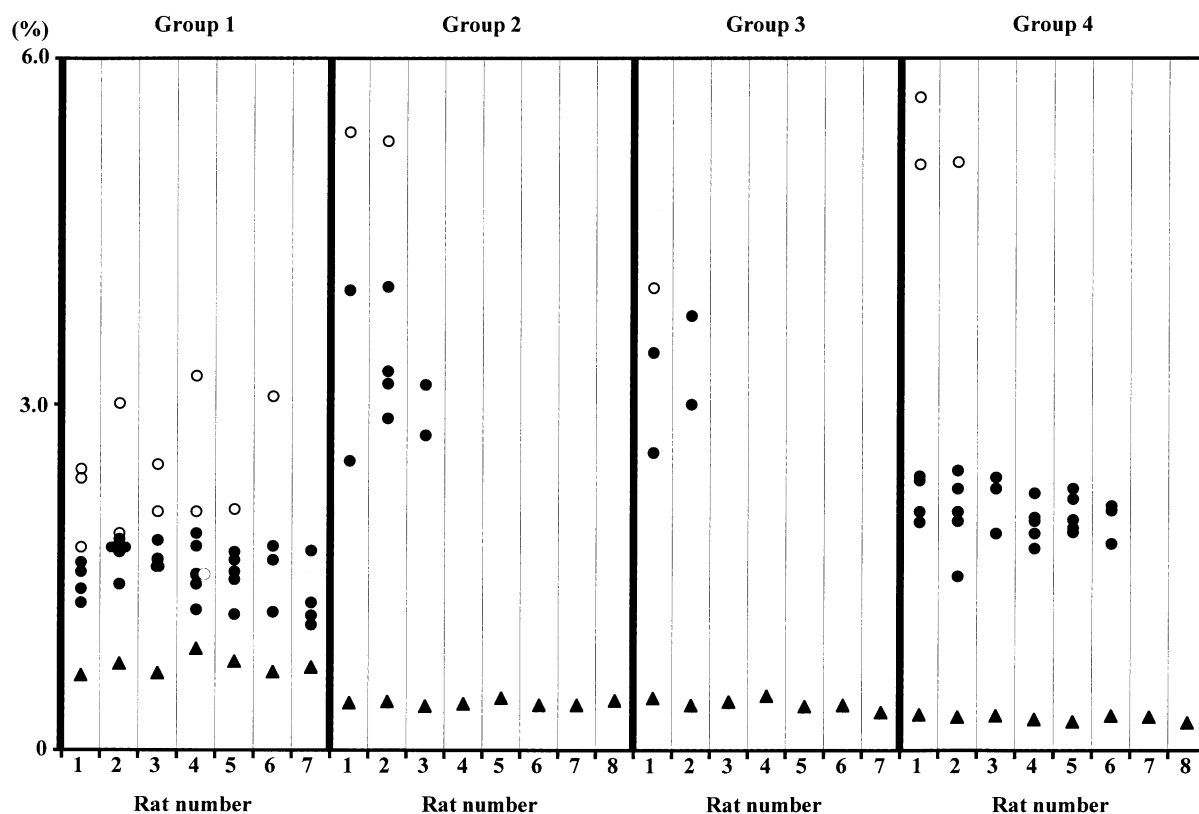


Fig. 4. The apoptotic indices in hepatocellular neoplasms and non-tumoral liver tissues of groups 1–4. Closed and open circles represent values of individual HCAs and HCCs, respectively. Closed triangles represent values of non-tumoral tissues. Each column contains the data for a particular animal as specified in the abscissa. It should be noted that the animal numbers in this figure are provisional for the easy understanding and do not have any relationships with those used to specify particular animals in the actual experimentation.

The expression of Bcl-xL protein is down-regulated in the late stage preneoplastic lesions, HCAs and HCCs [14]. COX2 protein is expressed in some HCAs but not HCCs [15]. The spectrum of these changes show a different pattern from that detected in (pre)neoplastic lesions induced in the livers of rats treated with a genotoxic carcinogen, *N*-nitrosodiethylamine [13,48], and there are both similarities to and differences from that observed in the human counterparts [16–20]. At this moment, the background molecular mechanisms of the late stage hepatocarcinogenesis in the CDAA diet model and their relevance to the human situations are still obscure, and investigations on this point are now in progress in our laboratories. The present results seen in group 4 may give some hints to elucidate such late stage mechanisms. When administered in the period after the appearance of HCAs, PBN

still reduced the incidences, multiplicities and possibly sizes of HCCs but did not affect the maintenance or possibly growth of HCAs. In this case, therefore, PBN is considered to affect the malignant conversion of HCAs to HCCs and the further progression of HCCs. The higher apoptotic and lower mitotic indices of HCCs (and HCAs also) in group 4 than those in group 1 suggest that the PBN exerts pro-apoptotic and anti-proliferative effects towards the neoplastic hepatocytes. Abnormal DNA methylation has been suggested to be one of the critical events in carcinogenesis in both humans and animals [49,50]. As aforementioned, the *c-myc* gene is overexpressed due to a hypomethylating machinery, which is suggested to be involved in the malignant conversion of HCAs to HCCs in the CDAA diet model [12]. Cellular or DNA damages induced by reactive oxygen

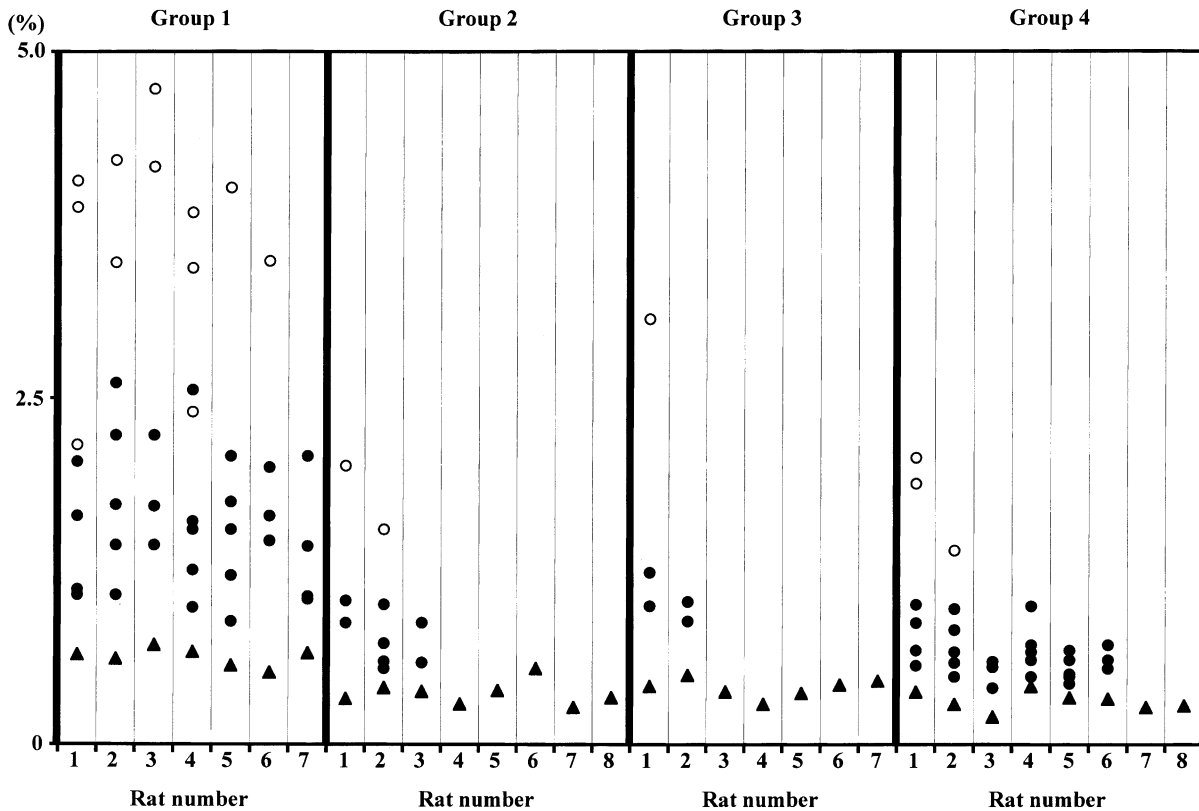


Fig. 5. The mitotic indices in hepatocellular neoplasms and non-tumoral liver tissues of groups 1–4. Closed and open circles represent values of individual HCAs and HCCs, respectively. Closed triangles represent values of non-tumoral tissues. Each column contains the data for a particular animal as specified in the abscissa. It should be noted that the animal numbers in this figure are provisional for the easy understanding and do not have any relationships with those used to specify particular animals in the actual experimentation.

and/or nitrogen-oxide species-induced stress are known to alter the DNA methylation patterns under the intervention of transcription factors [50,51]. PBN is shown to reduce the levels of oxidative injuries on DNA and other components of hepatocytes at least during the early stage hepatocarcinogenesis of the CDAA diet model [32,33]. If PBN also acts similarly in the late stage, the overexpression of the *c-myc* gene might then be suspended or disturbed, resulting in the inhibition of the malignant conversion of HCAs and the introduction of enhanced apoptosis and reduced cell proliferation in the barely induced HCCs by the down-regulation of this anti-apoptotic and pro-proliferative [52] gene.

HCCs are often associated with liver cirrhosis in human cases, and the signaling alterations concerning the fibro/cirrhogenesis in the liver are considered largely involved in the hepatocarcinogenic processes

[7,16–19,47]. Numerous signaling molecules are known to participate in a complex manner, placing the activation, proliferation and functioning of liver stellate cells at the center [53]. Similarly to these human situations, the association of liver cirrhosis with HCC is one of the characters in the CDAA diet model, and the fibro/cirrhogenic processes are considered to be involved in its hepatocarcinogenic processes [9]. In fact, inhibitors of the activation of liver stellate cells inhibit the induction of both fibrosis and GST-P-positive foci of cellular alteration in the livers of rats fed with the CDAA diet [54,55]. While COX2 is one of the most critical signaling factors involved in fibro/cirrhogenesis and carcinogenesis [56,57], this enzyme protein is expressed in the non-parenchymal area by liver stellate cells as well as Kupffer and sinusoid endothelial cells during the early stage of the CDAA diet model and

considered to exert its anti-apoptotic and pro-proliferative [57] effects to (pre)neoplastic hepatocytes in a paracrine manner [15]. Inhibitors of COX2 such as nimesulide and *N*-(4-hydroxyphenyl)retinamide inhibit the induction of fibrosis along with the inhibition of the induction and growth of preneoplastic lesions in the livers of rats fed with the CDAA diet [9,10,15,45]. PBN inhibits COX2 at a catalytic level, which is one of the mechanisms by which this compound inhibits the early stage hepatocarcinogenesis in the CDAA diet model [32]. In the present study, the fibro/cirrhogenic processes are suggested to be involved in the hepatocarcinogenic processes in rats fed with the CDAA diet and can be inhibited by PBN, but the presence of cirrhosis itself may not affect.

In conclusion, the present study demonstrates that PBN can indeed prevent the development of frank HCCs in the CDAA diet model. It is conceivable that the anti-carcinogenic effect of PBN may be ascribed to the prevention of both the development of HCAs and their malignant conversion to HCCs. If such findings are not specific only for this particular model, PBN may be able to serve as a good tool to investigate molecular mechanisms underlying carcinogenic processes. Further studies are apparently warranted.

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