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Positive Foci of Glutathione *S*-Transferase Placental Form in the Liver of Rats Given Furfural by Oral Administration

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We observed GST-P-positive liver foci in rats during the course of developing liver cirrhosis by oral administration of furfural, an organic solvent. Male Wistar rats were given furfural-containing diet (20–30 mg/kg diet) for 15–150 days, and killed 14 days after terminating furfural feeding. Immunohistochemical investigation of GST-P-positive liver foci which appeared in rats fed furfural for more than 30 days revealed an increase in number and size of the foci in proportion to the duration of furfural administration. Since furfural is known not to be carcinogenic in rats, this finding will be helpful to understand the enhancing effect of furfural-induced cirrhosis on chemical hepatocarcinogenesis.

Key words: Glutathione *S*-transferase placental form — Liver — Rat — Furfural

Furfural, (C_4H_3OCHO), is one of the furan derivatives that can be readily synthesized by oxidation of pentose[1]; it is found in fermented products such as wine, brandy, Japanese sake, etc.[2–6] Based upon the first report of Nakahara and Mori,[7] we showed that long-term oral administration of furfural to rats induces liver cirrhosis which resembles human B type cirrhosis.[8] We also pointed out that the rat liver was more sensitive to carcinogenesis by a chemical such as acetylaminofluorene (2-AAF), when cirrhosis was established in the liver.[9] During the course of furfural-induced cirrhosis, there were no morphological changes suggesting a carcinogenic effect of this chemical.[10]

Since there are only a few appropriate experimental models to study the relationship between liver cirrhosis and hepatocarcinogenesis, we thought that furfural might provide a useful model system. It is important to characterize furfural-induced liver cirrhosis in detail to study the relationship between liver cirrhosis and hepatocarcinogenesis. In the present study, we observed by immunohistochemical methods positive foci of glutathione *S*-transferase placental form (GST-P) in the cirrhosis-developing liver of rats given furfural.

Sixty, specific-pathogen-free, male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu), 5 weeks old, were divided into 6 groups. Each group consisted of 6 experimental animals and 4 controls. All the animals were housed in autoclaved plastic cages set in a room provided with HEPA filtered air. The room temperature was maintained at 22±2°C, and the humidity at 50±20%. The animals were given commercial diet, CRF-1 (Oriental Yeast Co. Ltd., Tokyo) or experimental diet described below, supplied freshly each day; tap water with 10 ppm chlorine added was available *ad libitum*. To prepare experimental diet, a prescribed amount of furfural (Wako Pure Chemical Industries, Osaka) was sprinkled evenly on the CRF-1 pellets, and the mixture was stored in a dark and air-tight container for 1–2 days to allow the furfural to permeate evenly through the pellets. The experimental animals were given the diet containing 20 ml of furfural per 1 kg of CRF-1 for the first 30 days of the experiment; thereafter they were fed the diet containing 30 ml of furfural per 1 kg of CRF-1. For groups 1 to 6, supply of the experimental diet was terminated by the 15th, 30th, 60th, 90th, 120th, and 150th, experimental day respectively. Animals in each group were killed 14 days after termination of furfural administration by cutting the abdominal aorta under ether anesthesia. The liver was removed, observed macroscopically, and fixed with 10% phosphate-buffered formaline. One tissue slice from the maximum cut surface of each lobe was embedded in paraffin and cut into 4 μm sections. Sections were stained with hematoxylin and eosin (HE), with Masson-trichrome and by the avidin-biotin-peroxidase complex method[11] with anti GST-P antibody[12] as the first antibody. The numbers of GST-P-positive foci were counted, divided by the area of the liver sections, and expressed as number per square centimeter. The numbers of liver cells which constituted GST-P-positive foci were also counted as an indicator of the size of each

focus. Since the distribution of the number of constituent cells followed a logarithmic normal distribution, statistical analysis was carried out after logarithmic transformation.

Histopathological findings from sections stained with HE and Masson-trichrome were similar to those that we reported earlier.[10] Briefly, slight fibrous widening of the portal area and pericellular fibrosis were demonstrated after 30 days of furfural administration, followed by porto-portal and porto-central bridging fibrosis after 90 days of furfural ingestion. Partial pseudolobule formation was observed in the animals given furfural for 150 days. Massive or zonal hepatic cell necrosis was not observed throughout the experimental period.

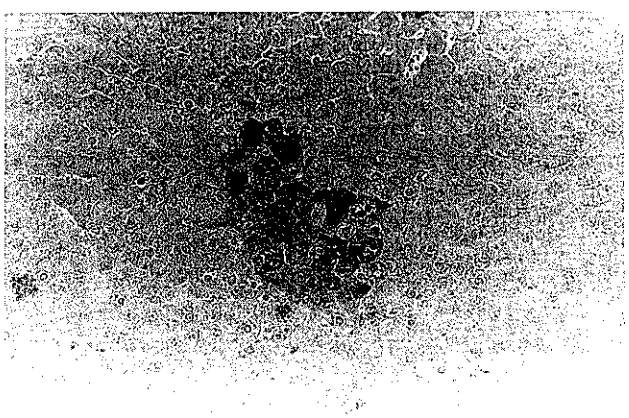

Fig. 1. A representative microphotograph of a GST-P-positive focus seen in the liver of a rat given furfural for 30 days. GST-P immunostain, ×160.

Table I. Number of GST-P-positive Foci-bearing Animals

	Duration of furfural administration (days)					
	15	30	60	90	120	150
Experimental animals	0/6	4/6	4/6	5/6	5/6	6/6
Control animals	0/4	0/4	0/4	0/4	0/4	0/4

GST-P-positive foci were found in the animals treated with furfural for more than 30 days (Fig. 1). Table I shows the number of foci-bearing animals in each group: Table II shows the mean number of foci per square centimeter of the liver sections and the mean number of cells per single focus, which was restored to the raw value by anti-logarithmic transformtion. The range of SD of the number of cells per single focus is also expressed in Table II as the raw value obtained similarly. In the animals given furfural, the incidence of foci-bearing animals, as well as the mean number of foci per square centimeter of the liver sections, increased as the period of furfural feeding was extended. In untreated control animals without administration of furfural, no GST-P-positive focus was observed. Within each group, there was a statistically significant difference between the experimental and control rats ($P<0.01$). The relationship between duration of furfural administration and the number of foci-bearing animals or the mean number of the foci per square centimeter of the liver was significant by one-way analysis of variance ($P<0.05$). The mean number of cells per single focus of each group also tended to increase in proportion to duration of furfural feeding, and the relationship is statistically significant by one-way analysis of variance after normalization by logarithmic transformation ($P<0.01$).

Previously, we reported that the furfural-induced cirrhotic liver of the rat was more sensitive to carcinogenic stimulation by 2-AAF, though long-term furfural administration alone did not cause hepatoma or neoplastic nodules.[9] We also reported that cirrhotic changes produced by furfural administration did not induce focal hepatocytic proliferation suggestive of tumorigenic changes.[10] In the first study by Nakahara and Mori on the effect of long-term furfural administration to rats, they found no hepatoma up to 548 days of furfural administration.[7]

On the contrary, Reynolds *et al.* reported that unique oncogene was defined from hepatic tumors in B6C3/F1 mice induced by furfural administration.[13] According to the two-year study of the National Toxicological Program of the National Institute of Environmental Health Science, USA, carcinogenicity of furfural was only

Table II. Number of GST-P-positive Foci per Unit Area of the Liver and the Number of Constituent Cells per Focus

	Duration of furfural administration (days)					
	15	30	60	90	120	150
No. of foci (/cm^2)	0	0.168±0.144	0.206±0.183	0.496±0.370	0.740±0.474	1.350±0.803
Mean number of constituent cells (Range of SD)	0 —	2.72 (0–8.79)	18.03 (6.75–48.18)	11.27 (1.64–77.25)	29.22 (8.65–98.79)	49.90 (16.53–150.66)

demonstrated in B6C3/F1 mice, and was not found in Fischer 344 rats (S. H. Reynolds, personal communication). Therefore, the carcinogenicity and also the enhancing effect of furfural on chemical carcinogenesis can not be regarded as fully established though our and other investigators' observations suggest that furfural is not carcinogenic in rats.

In this study, we investigated the GST-P-positive foci in livers of rats given furfural to clarify the mechanism whereby furfural enhances the effect of other carcinogens.

A GST-P-positive focus is considered to consist of cells "initiated" by various chemical carcinogens.[14] It is extensively used as one of the most reliable markers of early stages of chemical hepatocarcinogenesis,[15] although some non-genotoxic carcinogens are known not to induce GST-P-positive foci.[16, 17] The actual role of GST-P-positive foci in chemical hepatocarcinogenesis is still unclear, but it is suggested that GST-P is related to detoxification of carcinogens. Recently, Peraino *et al.* investigated enzyme-altered foci, including gamma glutamyl transpeptidase-positive foci; this enzyme is another representative marker for "initiated cells" and the foci are mostly compatible with GST-P-positive foci. They concluded that enzyme-altered foci do not linearly progress to neoplastic nodule and hepatocellular carcinoma, but these foci are indicative of total carcinogenic action.[18]

In furfural-administered rats killed immediately after completion of furfural administration, diffuse GST-P-positive staining was observed in the periportal area (data not shown). Since this finding is thought to be due to the toxicity of furfural, and not to be related to hepatocarcinogenesis, we evaluated GST-P-positive foci in rats killed two weeks after the end of furfural administration. At this time, the diffuse GST-P-positive staining in the periportal area had disappeared, but distinct GST-P-positive foci, which seemed morphologically the same as those seen during chemical hepatocarcinogenesis, remained. This suggests that these foci are long-lasting ones, and not acutely induced. According to our present data, the number of rats with GST-P-positive foci as well as the size and the number of the foci apparently increased as the period of furfural administration was extended, though the size and the number of the foci were small. This finding indicates that furfural significantly induces GST-P-positive foci and that the foci continue growing as long as furfural is given, as cirrhotic changes of the liver become apparent. Concerning our previous report that a diffuse, not localized, S-phase cell increase was observed during the course of furfural-induced cirrhosis,[10] the foci are growing slowly, and single labeling with BrdU cannot detect cell proliferation. The GST-P-positive foci, which increase during the course of developing cirrhosis, might play an important role in the mechanism underlying the fact that cirrhotic liver is more sensitive to carcinogenic stimuli. The fact that a putatively non-hepatocarcinogenic agent, i.e. furfural, induces GST-P-positive foci, which are not distinguishable from those induced by chemical carcinogens, is also important when considering the relationship between GST-P-positive foci and chemical carcinogenesis.

Our finding that furfural significantly induces GST-P-positive foci in rats, but does not induce hepatocellular carcinoma or neoplastic nodules even after a long period, seems to support the suggestion of Peraino *et al.*[18] If GST-P-positive foci do not indicate "precancerous cells" which continuously advance to become cancer cells, but instead indicate a state of reactivity to carcinogenic stimuli, we can understand why hepatocellular carcinoma is much more readily induced by chemical carcinogens in furfural-induced cirrhotic liver. This hypothesis is also compatible with our observation that focal increase of S-phase cells, which is considered to be related to neoplastic changes, was not detected by the BrdU-anti-BrdU technique. In our study of human liver biopsies, a diffuse S phase cell increase was also observed in human B type liver cirrhosis.[19] However, another possibility, that furfural has weak carcinogenicity that is undetectable in our experimental system, or that furfural initiates hepatocarcinogenesis, but does not promote it, like 2-Me-DAB,[20] can not be ruled out.

To answer this question, other experimental systems, such as partial hepatectomy or administration of a known promoter together with furfural feeding, should be used. Further studies are in progress, since we think furfural is an interesting and valuable chemical for a model system not only to investigate the relationship between liver cirrhosis and hepatocarcinogenesis, but also to elucidate the significance of the enzyme-altered "precancerous lesion" of experimental hepatocarcinogenesis.

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