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Strong carcinogenic stress response induction of preneoplastic cells positive for GST-P in the rat liver: Physiological mechanism for initiation

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2-acetylaminofluorene (PubChem CID: 5897, 4737)
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ABSTRACT

Aims: To identify experimental conditions that induce preneoplastic cells positive for glutathione S-transferase P-form (GST-P) in the rat liver by new approaches, and analysis of the mechanism of cancer initiation based on the findings.

Main methods: The experimental protocols employed to induce GST-P$^+$ preneoplastic cells in rat liver were as follows. Protocol 1: adult rats were fed basal diet containing 2-acetylaminofluorene (AAF, 0.02% by wt) and high concentrations of *N*-acetyl-L-cysteine (0.5%) over 10 weeks. Protocol 2: rats were subjected to partial hepatectomy (2/3PH), followed by an AAF (0.04%) diet for two more weeks. Vibratome-prepared liver sections were then immunostained for GST-P.

Key findings: GST-P was inducible in the rat liver in response to the strong carcinogenic stress by AAF in the two experimental protocols. When examined immunocytochemically with vibratome sections, the biliary tracts of hepatocytes, GST-P$^+$ single hepatocytes and foci were heavily positive for the marker enzyme in addition to ordinary cytosolic staining of preneoplastic cell populations. The biliary tracts of hepatocytes were severely injured, and the excretory portions of GST-P$^+$ single hepatocytes were significantly injured.

Significance: The cytotoxic action of AAF that give rise to the GST-P$^+$ single hepatocytes was suggested to be an injury to the excretory pump(s) and the duct of hepatocytes. A new physiological mechanism was hypothesized for the induction of preneoplastic cell populations in the rat liver instead of a genetic mechanism.

1. Introduction

The molecular and cellular mechanisms of cancer initiation, *i.e.*, cause of cancer, require elucidation in basic cancer research as well as clinical oncology. However, Pitot pointed earlier that the initial carcinogenic changes might be minor and latent ones that are 'unknownable' in principle [1], and thus, experimental approaches have been very limited. Glutathione S-transferase P-form (GST-P, EC 2.5.1.18) is an isoenzyme of GST that catalyses the conjugation of GSH with a number of endogenous as well as exogenous compounds including drugs and carcinogens [2]. In rat chemical hepatocarcinogenesis, GST-P has been employed as a specific marker enzyme for preneoplastic foci and nodules [3–5]. In 1987, our group reported the presence of single cells/hepatocytes and minifoci that were strongly positive for the marker enzyme in the rat liver [6]. Based on the phenotypic identity of the cells and the fact that the positive cells were inducible prior to the formation of foci and nodules in animal livers, the former cell populations were considered the precursors of the latter cell populations [6–8]. Accordingly, this positive cell induction could be an excellent model system to experimentally obtain insight into the mechanism of cancer initiation *in vivo*. In this model, one of the main problems is whether these positive cells are formed genetically according to the initiated cell theory proposed by Farber et al. [9,10]. However, when the rats were fed a basal diet containing high concentrations of 2-acetylaminofluorene (AAF), the frequency of induction of positive single cells per hepatocyte was more than three orders of magnitude greater than the value of the mutational changes estimated by Farber et al. Accordingly, the transformation was considered to be non-genetic and physiological [11]. In addition, the foci and nodules were suggested to grow mainly non-clonally without cell division following the entry of carcinogens into the cells because their growth was far beyond the rate of cell division under appropriate conditions.

In the present study, two experimental conditions were identified to effectively induce GST-P and the positive cell populations in animal livers. Based on the findings and together with those obtained thus far, preneoplastic cell populations were suggested to be inducible through nongenetic and physiological reactions consecutively rather than through genetic reactions.

Abbreviations: AAF, 2-acetylaminofluorene; BD, interlobular bile duct; GST-P, pi class glutathione S-transferase; NAC, *N*-acetyl-L-cysteine; LPO, lipid peroxidation

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2. Materials and methods

2.1. Chemical and diet preparation

The affinity-purified rabbit anti-GST-P antibody was obtained from the Medical and Biologic Laboratories (Nagoya, Japan). Labelled polymer-HRP anti-rabbit IgG was purchased from DakoCytomation (Tokyo, Japan). NAC and the AAF carcinogen were purchased from Nacalai Chemical Co. (Osaka, Japan). The basal powdered diet (MF diet) was obtained from Oriental Yeast Co. (Tokyo, Japan).

The basal diet containing AAF (0.02%) and NAC (0.5%) was prepared as follows. First, crystalline AAF (400 mg) was mixed with approximately 5 ml of canola oil (Nissin Oil Mills, Tokyo) in a ceramic mortar and ground well manually with a pestle until a homogeneous suspension was formed. An additional amount of canola oil (95 ml) together with NAC (10 g) was then added to the suspension and was mixed well. The oil suspension (approximately 100 ml) was then poured slowly onto 1.9 kg of the basal diet in a large mixer, and both were mixed well for 10 to 15 min. The low-AAF (0.01%) diet and high-AAF (0.04%) diet were also prepared in 2 kg lots.

2.2. Animals and treatments

All animal experiments were approved in advance by the Ethics Committee and were conducted according to the Hirosaki University Guidelines for Animal Experimentation. Male Sprague-Dawley rats (5 week-old) were purchased from CLEA Japan, Inc. (Tokyo) and acclimated in the animal facility for one week before the start of the experiments. GST-P$^+$ cells were induced in the rat liver according to the two experimental protocols, as shown in Fig. 1. In protocol 1, a total of 25 rats were divided into groups of 3 to 4 animals and were fed a basal diet containing AAF (0.02%) and NAC (0.5%) *ad libitum* for a period of 10 weeks. Control animals were fed either the basal diet containing AAF (0.02%) for the appropriate time intervals [11]. In protocol 2, a total of 7 animals were fed the AAF (0.01%) diet for one week, were subjected to partial hepatectomy (2/3PH, arrowhead), and were subsequently fed the AAF (0.04%) diet for one or two additional weeks. All animals were anaesthetized intramuscularly with pentobarbital (50 mg/kg) and were then euthanized. The livers obtained were excised and cut into 3- to 4-mm-thick slices for fixation in 10% phosphate-buffered formalin at 4°C. The fixative was replaced the next day as reported previously [11,12].

2.3. Immunocytochemical staining of vibratome-prepared liver sections

Liver specimens were sectioned automatically into 25 μm-thick sections in phosphate-buffered saline (PBS) using a microslicer (Vibratome 1500 Sectioning System, Vibratome Products, NY). The sections were stained immunocytochemically for GST-P and then examined by digital light microscopy after being mounting on slides in 50% glycerol (Coolscope, Nikon, Tokyo) as described previously [11,12].

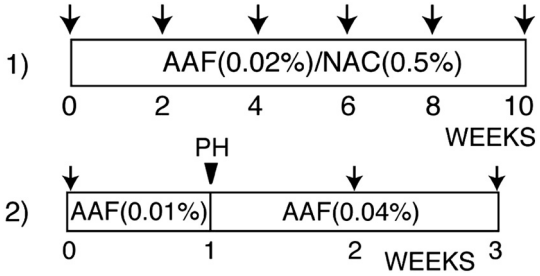

Fig. 1. Experimental protocols for the induction of GST-P$^+$ cell populations in the rat liver. The values in parentheses denote the concentrations of AAF and NAC contained in the basal diet. The arrows indicate the time of euthanasia.

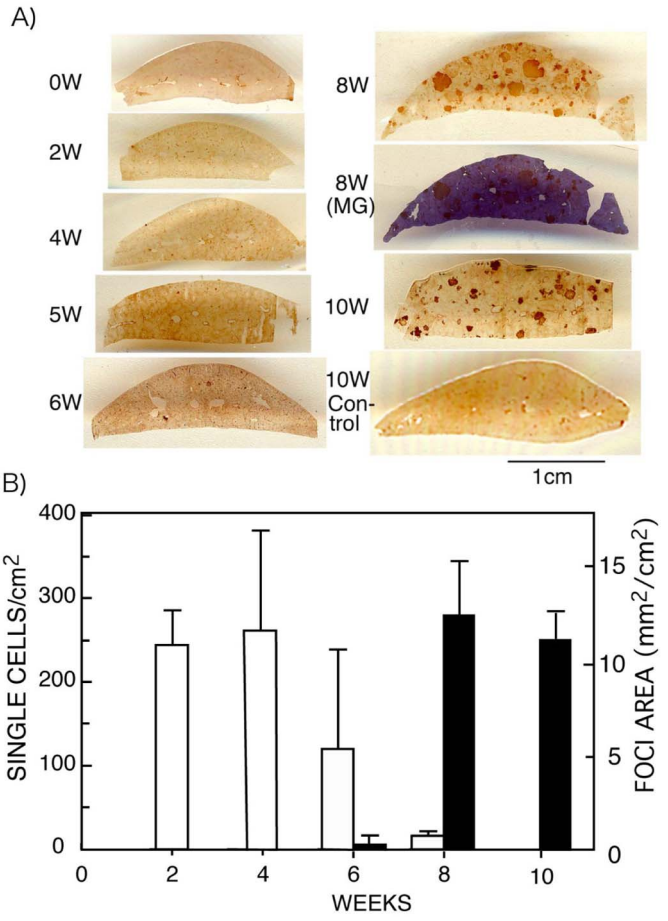

Fig. 2. Induction of GST-P$^+$ cells in the rat liver by AAF and NAC. (A) Macroscopic view of GST-P$^+$ foci and nodules induced in the rat liver. The time point of euthanasia is indicated on the individual liver samples immunostained for GST-P. MG: nuclear staining of the specimen with methyl green solution; 10W Control, liver of a rat fed the basal diet alone for 10 weeks. B) Induction time course of GST-P$^+$ cells. Open bars, GST-P$^+$ single hepatocytes (cells/cm^2) [6]; closed bars, area of foci and nodules (mm^2/cm^2) [5] induced in the liver. The data are expressed as the means ± SD.

3. Results

3.1. Strong carcinogenic stress response in experimental protocol 1

3.1.1. Time course of induction of GST-P$^+$ single cells, foci and nodules

The rats were fed a basal diet containing AAF (0.02%) and high concentrations of NAC (0.5%) over 10 weeks according to the protocol 1 illustrated in Fig. 1. Fig. 2A shows macroscopic immunostaining patterns of vibratome-prepared liver sections using an antibody for GST-P. As seen in Fig. 2B, GST-P$^+$ single cells were induced from 2 to 6 weeks, with the peak number noted after 4 weeks (264 ± 119 cells/cm^2), followed by a gradual decrease. Foci and nodules were induced after 6 weeks. The percentage of the GST-P$^+$ foci area was very small (0.36 ± 0.24 mm^2/cm^2, %) after 6 weeks, but increased rapidly to 12.5 ± 2.8 and 11.3 ± 1.2% after 8 and 10 weeks, respectively. Numerous vacuolated cells, which were negative for nuclear staining with methyl green, were found in nodule-bearing rat livers [Fig. 2A, 8 W (MG)] (see Supplementary Fig. 1.).

3.1.2. Immunostaining of bile canaliculi/canalicular networks of hepatocytes

Numerous GST-P$^+$ single cells were induced and found to be dispersed throughout the liver in rats fed the AAF/NAC-containing diet for 2 to 6 weeks (Fig. 3A). It is noteworthy that all of the canaliculi/canalicular networks were heavily but heterogeneously stained for the