PBO did not induce oxidative DNA damage irrespective of ROS generation in mice, we must perform further analyses as a future work to clarify the difference in the formation of 8-OHdG between the rat and mouse.

Excessive ROS production and oxidative stress produces a rapid and transient increase in mRNA levels of the early response genes, such as *c-Fos*, *c-Jun*, and *c-Myc* (Iemitsu et al. 2006; Luna et al. 1994; Mattie et al. 2008). Early response genes encode transcription factors and therefore play a role in the regulation of cellular responses following endogenous or exogenous stress (Luna et al. 1994). C-myc induced by ROS generation then regulates the expression of E2F1 (Matsumura et al. 2003; Mann and Jones 1996). E2F1 can suppress NF-kB activity to increase the susceptibility to apoptosis in in vitro transfection studies (Matsumura et al. 2003). c-Myc and E2F1 are higher in lymphoma cells during middle tumor development and, like MAPK, regulate cell proliferation without changing cell size (Panchuk et al. 2008). Sustained overexpression of E2F and c-Myc during the terminal stage of tumor development can increase cell size due to the blockage of G_1/S transition or facilitation of apoptosis. This finding suggests that increased expression of *c-Myc* and *E2f1* during carcinogenesis involves complex phenomena consisting of cell proliferation, cell quiescence, and apoptosis, depending on the stage of tumor development. In addition, the PCNA-positive ratio significantly increased in intact hepatocytes, CK8/18-positive foci, and adenomas in the DEN + PBO group compared with the DEN-alone group. Considering the increased PCNA-positive ratio and *Ccnd1*, and unchanged expression of *p16*, *p21*, *p27*, *p53*, and *p57*, upregulation of *c-Myc* resulting from the excessive ROS production and *E2f1* may reflect for a role on cellular proliferation in hepatocellular proliferative lesions induced by PBO in mice.

Here, PBO decreased *Egfr* levels. EGFR belongs to the family of c-erbB proteins that can heterodimerize for ligand-dependent activation of downstream signaling. Downregulation of EGFR occurs in preneoplastic liver cell foci and neoplastic lesions positive for GST-P after treatment with non-genotoxic hepatocarcinogens early in a rat two-stage hepatocarcinogenesis model (Taniai et al. 2009). We found similar EGFR downregulation in GST-P-positive foci here. EGFR inactivation can be induced through transcriptional silencing by CpG island hypermethylation in tumor cells (Montero et al. 2006) and promote the formation of proliferative lesions in mice and rats. Taking into account the above references, the downregulation of EGFR is considered to play a role for the formation of liver proliferative lesions in mice as well as in rats.

In conclusion, PBO has the potential to generate ROS via the metabolic pathway in the liver of mice but does not induce oxidative DNA damage. Such an excessive ROS production probably activates cell growth involved in c-Myc- and E2F1-related pathways and acts as a liver tumor promoter of DEN-initiated hepatocarcinogenesis in mice.

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Title: Cytokeratin 8/18 is a useful immunohistochemical marker for hepatocellular proliferative lesions in mice

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Running head: CK8/18 AS LIVER TUMOR MARKERS IN MICE

Abstract

In order to clarify whether cytokeratin (CK) 8/18 is a useful immunohistochemical marker for hepatocellular proliferative lesions in mice, partially hepatectomized male ICR mice were given 0.6 % piperonyl butoxide (PBO) for 8 (Experiment I) or 25 weeks (Experiment II) after *N*-diethylnitrosamine (DEN) initiation treatment, and the livers were subjected to histological examinations on hematoxylin and eosin (HE) stained sections, CK8/18 immunohistochemistry and gamma-glutamyl transpeptidase (GGT) histochemistry. In Experiment I, the multiplicity of hepatocellular foci in paraffin-embedded sections which were observed in HE-stained sections and positive for CK8/18 was 10.17 and 18.50, respectively, while that of hepatocellular foci in frozen sections which were observed in HE-stained sections and positive/negative for GGT was 6.17 and 8.17, respectively. In Experiment II, the total multiplicity of hepatocellular foci in paraffin-embedded sections which were observed in HE-stained sections and positive/negative for CK8/18 was 4.47 and 23.17, respectively, while that of hepatocellular foci in frozen sections which were observed in HE-stained sections and positive/negative for GGT was 2.50 and 3.50, respectively. Most of the hepatocellular adenomas and carcinomas observed in HE-stained sections were positive for CK8/18, but some of the adenomas were negative for CK8/18. These findings indicate that more hepatocellular proliferative lesions can be detected in CK8/18 immunohistochemistry in

addition to those observed in HE-stained sections, and suggest that CK8/18 may become a useful immunohistochemical marker for detecting hepatocellular proliferative lesions in mice.

Key word: cytokeratin 8/18, liver, mouse, piperonyl butoxide.

INTRODUCTION

The rat liver medium-term bioassay system first established by Ito *et al.* [14] has been repeatedly used for the detection of carcinogenic or tumor promoting potentials of chemical substances and has great advantages due to reproducibility and reliability for generation of data within 8 weeks [8]. For assessment of promoting effects of the test chemicals, glutathione S-transferase placental form (GST-P)-positive foci are used as the primary endpoint. Moreover, since production of GST-P positive foci has been closely correlated with the actual tumor yields [11, 21], they are regarded as reliable preneoplastic lesions in rats. However, it is generally recognized that this immunohistochemical marker is not reactive for liver preneoplastic and neoplastic lesions of mice. It has been shown that gamma-glutamyl transpeptidase (GGT) play a role in multistage hepatocarcinogenesis and the enhanced expression of this enzyme is observed in preneoplastic altered hepatocellular foci, hepatocellular adenomas, and hepatocellular carcinomas in rats and mice [13]. Therefore, GGT is used as a histochemical marker for these proliferative lesions in mice. However, this histochemical staining of GGT is not suitable for routine histopathological examinations, because frozen sections are necessary for this staining. In addition, there is a disadvantage that almost all the proliferative lesions are not always stained with GGT [7]. Therefore, more reliable markers for liver preneoplastic and neoplastic lesions are absolutely necessary in mice.

It has been shown that cytokeratin (CK) 8/18 overexpression may drive neoplastic transformation of preneoplastic cells in GST-P-positive foci during rat hepatocarcinogenesis [16]. Moreover, we previously reported that hepatocellular altered foci induced in rasH2 mice given fenofibrate for 8 weeks after *N*-diethylnitrosamine (DEN) initiation were immunohistochemically stained with CK8/18 [18].

Piperonyl butoxide (PBO) is a pesticide synergist that is widely used with pyrethroids for grain protection and as a domestic insecticide. PBO is a hepatocarcinogen in F344 rats fed a diet containing 1.2 % PBO for 2 years [29] and CD-1 mice fed a diet containing 300 mg/kg/day PBO for 79 weeks [4]. In our previous study in which male mice were subjected to a two-thirds partial hepatectomy, followed by DEN initiation, and given a diet containing 0.6 % PBO for eight weeks to clarify the mechanism of PBO-induced hepatocarcinogenesis in mice, the incidence of GGT-positive foci were significantly increased in the DEN + PBO group compared with the DEN-alone group [19].

In the present study, we performed two different experiments using the mouse two-stage hepatocarcinogenesis model subjected to 8- or 25-weeks tumor promotion treatment of PBO to clarify whether CK8/18 is a useful immunohistochemical marker for hepatocellular altered foci, adenomas, and carcinomas in mice.

MATERIALS AND METHODS

Chemicals

Piperonyl butoxide (PBO), [2-(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene (CAS register number 51-03-6, EU Number piperonyl butoxide, purity 90 %), was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). DEN was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Animals and experimental design

Six-week-old male ICR mice were obtained from Japan SLC Inc. (Shizuoka, Japan). They were housed in plastic cages (five animals/cage) with absorbent paper chip bedding in an animal room maintained under standard conditions (room temperature, 22 ± 2 °C; relative humidity, 55 % ± 5 %; and light/dark cycle, 12 h) and given free access to a powdered diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. The animals were acclimatized for one week prior to beginning the experiment. The experiment was performed in accordance with the guidelines for animal experimentation of the Tokyo University of Agriculture and Technology.

We performed two different experiments using a short-term two-stage liver carcinogenesis model [23] in mice. In Experiment I, to enhance hepatocellular proliferation, mice were subjected to a two-thirds partial hepatectomy. Twenty-four hours after the

hepatectomy, mice were given a single i.p. injection of DEN (20 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. One week after the DEN injection, 10 animals were given a powdered diet containing 0.6 % PBO for 8 weeks. Nine mice survived in this group, and 6 of 9 mice were subjected to histological examinations including histochemical and immunohistochemical examinations. In Experiment II, mice were similarly subjected to a two-thirds partial hepatectomy. Twenty-four hours after the hepatectomy, mice were given a single i.p. injection of DEN (20 mg/kg body weight). One week after the injection, 16 animals were given a powdered diet containing 0.6 % PBO for 25 weeks. Fifteen mice survived, and 6 of 15 mice were subjected to the same morphological examinations as the Experiment I.

On completion of treatment, the mice were killed by exsanguination from the posterior *vena cava* under ether anesthesia, and livers were immersed in 4 % paraformaldehyde solution for microscopy. Two sections obtained from different portions of livers/mouse were embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, USA) to obtain frozen sections for histochemical evaluation of GGT-positive foci, a marker of preneoplastic foci, in mouse livers [7].

Histopathology, histochemical, and immunohistochemical evaluations, and quantitative analyses

After sacrifice, two different sections from the livers fixed in 4 % paraformaldehyde were embedded in paraffin, sectioned at 3 μm thickness, and stained with hematoxylin and eosin (HE) for histopathological examinations. The incidence and multiplicity of liver proliferative lesions, such as hepatocellular altered foci, adenomas, and carcinomas, in these HE-stained sections/mouse were counted under a light microscope.

For immunohistochemistry, serial paraffin-embedded liver sections that were continuous to the HE-stained sections were deparaffinized in xylene and rehydrated in ethanol. Anti-CK8/18 polyclonal antibody was purchased from PROGEN Biotechnik GmbH (Heidelberg, Germany). The liver sections for CK8/18 were incubated with citrate buffer [0.1 mol/L citrate (pH 6.0)] and heated in a microwave oven at 98 °C for 30 min before incubation with 0.3 % hydrogen peroxide in PBS. Non-specific binding sites were blocked with blocking normal serum. The specimens were incubated overnight with the CK8/18 antibody at a dilution of 1:100 in 0.5 % casein-PBS at 4 °C. The sections were incubated with a guinea pig peroxidase-conjugated secondary antibody (Fitzgerald Industries International Inc., MA, USA) diluted in PBS supplemented with 0.5 % casein. Subsequently, 3, 3'-diaminobenzidine (DAB, Dojindo laboratories, Kumamoto, Japan) was applied as a chromogen. The sections

were finally counterstained with hematoxylin. The incidence and multiplicity of CK8/18-positive and -negative foci, adenomas and carcinomas were counted under a light microscope. The numbers of CK8/18-positive/negative lesions greater than 0.2 mm in diameter were counted.

Two different sections per mouse obtained from frozen sections were sectioned at 7 μm thickness, and stained with HE for histopathological examinations. The incidence and multiplicity of liver proliferative lesions, such as hepatocellular altered foci, adenomas, and carcinomas, in these HE-stained sections/mouse were counted under a light microscope.

Histochemical staining of GGT was performed by the modifying methods of Rutenberg *et al.* (1969) [27]. Two frozen sections/mouse obtained from the different portions of livers were cryosectioned and fixed using methanol. After air-drying, a freshly prepared solution containing the substrate, *L*-glutamic acid-γ-(4-metoxy-β-naphthylamide) (Sigma–Aldrich, St Louis, MO, USA), and fast blue BBN (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M Tris-buffered saline (pH 7.4) was coated onto the section. Following incubation, the slides were transferred into a 0.1 M cupric sulfate solution. The sections were then stained with hematoxylin and mounted in Apathy's mounting media (Wako Pure Chemical Industries, Osaka, Japan). The incidence and multiplicity of liver proliferative lesions in GGT-stained sections/mouse obtained from two different portions of livers were

counted under a light microscope. Single GGT-positive cells were ignored, while the numbers of GGT-positive/negative lesions greater than 0.2 mm in diameter were counted.

RESULTS

Experiment I

In HE staining of paraffin-embedded sections, all treated mice had hepatocellular altered foci (Table 1). These foci are composed of eosinophilic cell type. In immunohistochemistry, CK8/18-positive foci were observed in all treated mice. In these mice, hepatocellular foci observed in HE-stained sections were positive for CK8/18 (Figs. 1A and B), but foci that could not be detected in HE-stained sections were also positive for CK8/18 (Figs. 1N and 1O). The multiplicity of hepatocellular foci from the treated mice which were observed in HE-stained sections and positive for CK8/18 was 10.17 and 18.5, respectively.

In HE staining of frozen sections, all treated mice had hepatocellular altered foci (Table 1). GGT histochemistry revealed that all treated mice had GGT-positive (Figs. 1C) and -negative foci. The multiplicity of hepatocellular foci from the treated mice that were observed in HE-stained sections, positive for GGT and negative for GGT was 6.17, 3.67 and 4.50, respectively. The total multiplicity of hepatocellular foci from the treated mice which were observed in HE-stained sections and positive/negative for GGT was 6.17 and 8.17,

respectively, and the number of hepatocellular foci observed in HE-stained sections was slightly lower than that of GGT-positive/negative foci.

Neither hepatocellular adenomas nor carcinomas were observed in these treated mice.

Experiment II

Hepatocellular foci: In HE staining of paraffin-embedded sections, all treated mice had hepatocellular altered foci (Table 2). These foci are composed of eosinophilic, basophilic or clear cell type. In immunohistochemistry, CK8/18-positive foci were observed in all treated mice. In these mice, hepatocellular foci observed in HE-stained sections were positive for CK8/18, but foci that could not be detected in HE-stained sections were also positive for CK8/18. The CK8/18-positive foci which were detected in HE-stained sections were mainly composed of eosinophilic cell type. In addition, hepatocellular foci that are negative for CK8/18 were observed in 5 of 6 mice (83 %). These CK8/18-negative foci were composed of basophilic or clear cell type. The multiplicity of hepatocellular foci from the treated mice that were observed in HE-stained sections, positive for CK8/18 and negative for CK8/18 was 4.47, 19.67 and 3.50, respectively. The total multiplicity of hepatocellular foci from the treated mice that were observed in HE-stained sections and positive/negative for CK8/18 was 4.47 and 23.17, respectively.

In HE staining of frozen sections, all treated mice had hepatocellular altered foci (Table 2). It

was difficult to distinguish cell types (eosinophilic, basophilic or clear cells) of altered foci from the HE staining of frozen sections. GGT histochemistry revealed that all treated mice had GGT-positive foci, but GGT-negative foci were noted in 3 of 6 mice (50 %). The multiplicity of hepatocellular foci from the treated mice which were observed in HE-stained sections, positive for GGT and negative for GGT was 2.50, 2.00 and 1.50, respectively. The total multiplicity of hepatocellular foci from the treated mice which were observed in HE-stained sections and positive/negative for GGT was 2.50 and 3.50, respectively. Hepatocellular adenomas: In HE staining of paraffin-embedded sections, all treated mice had hepatocellular adenomas (Table 2). In immunohistochemistry, CK8/18-positive adenomas were observed in all treated mice. In these mice, hepatocellular adenomas observed in HE-stained sections were positive for CK8/18 (Figs. 1D and E). These CK8/18-positive adenomas were mainly composed of eosinophilic cell type. In addition, hepatocellular adenomas which were negative for CK8/18 were observed in 4 of 6 mice (67 %). These CK8/18-negative adenomas were composed of basophilic or clear cell type (Figs. 1J, K, L and M). The multiplicity of hepatocellular adenomas from the treated mice which were observed in HE-stained sections, positive for CK8/18 and negative for CK8/18 was 11.17, 9.83 and 1.33, respectively. The total multiplicity of hepatocellular adenomas from the treated mice which were observed in HE-stained sections and positive/negative for CK8/18 was 11.17 and 11.17,

respectively.

In HE staining of frozen sections, all treated mice had hepatocellular adenomas (Table 2). It was difficult to distinguish cell types (eosinophilic, basophilic or clear cells) of these adenomas from the HE staining of frozen sections. GGT histochemistry revealed that 5 of 6 treated mice (83 %) had GGT-positive adenomas, but GGT-negative foci were also noted in 5 of 6 mice (83 %). The multiplicity of hepatocellular adenomas from the treated mice which were observed in HE-stained sections, positive for GGT and negative for GGT was 10.50, 3.83 and 6.50, respectively. The total multiplicity of hepatocellular adenomas from the treated mice which were observed in HE-stained sections and positive/negative for GGT was 10.50 and 10.33, respectively. Hepatocellular carcinomas: In HE staining of paraffin-embedded sections, 3 of 6 treated mice (50 %) had hepatocellular carcinomas (Table 2). In immunohistochemistry, these carcinomas were also positive for CK8/18 (Figs. 1G and H). There were no hepatocellular carcinomas that were negative for CK8/18 in these treated mice. The multiplicity of hepatocellular carcinomas from the treated mice which were observed in HE-stained sections, positive for CK8/18 and negative for GGT was 1.50, 1.50 and 0, respectively. The total multiplicity of hepatocellular carcinomas from the treated mice which were observed in HE-stained sections and positive for CK8/18 was 1.50 and 1.50, respectively.

In HE staining of frozen sections, 3 of 6 treated mice (50 %) had hepatocellular carcinomas (Table 2). GGT histochemistry revealed that 1 of 6 treated mice (17 %) had GGT-positive carcinomas (Fig. 1I), but GGT-negative carcinomas were also noted in 3 of 6 mice (50 %). It was difficult to distinguish cell types (eosinophilic, basophilic or clear cells) of these carcinomas from the HE staining of frozen sections. The multiplicity of hepatocellular carcinomas from the treated mice which were observed in HE-stained sections, positive for GGT and negative for GGT was 0.67, 0.17 and 0.50, respectively. The total multiplicity of hepatocellular carcinomas from the treated mice which were observed in HE-stained sections and positive/negative for GGT was 0.67 and 0.67, respectively.

DISCUSSION

It is well known that the expression of GGT and GST-P is used to detect hepatocellular foci and tumors in rats and mice, and histochemical and immunohistochemical methods have been developed to visualize these lesions microscopically [3, 25]). GGT has frequently been used as a marker of biochemical alteration in hepatocellular foci and tumors in rats. This membrane-bound enzyme is found at higher levels in fetal and neonatal livers than in normal adult rat livers and appears in biochemically altered hepatocellular foci soon after initiation with chemical carcinogens [9, 10, 12]. GGT catalyzes the transfer of the gammaglutamyl moiety of glutathione to an amino acid acceptor [12, 31]. It is a Phase II drug-metabolizing enzyme which may be induced by drugs, such as phenobarbital, that modify the carcinogenic process. However, it can also be influenced in normal livers by other factors such as diet, strain, age, and sex of the animal [26, 30]. Therefore, it has been suggested that GST-P may be a superior marker for biochemically altered hepatocellular foci in rats, since GST-P expression appears to be limited to hepatocellular foci and tumors in rats, with very little expression occurring in normal liver parenchyma [26, 28, 30]. On the other hand, it is generally recognized that GST-P is not immunohistochemically reactive for liver preneoplastic and neoplastic lesions of mice and there is a disadvantage that almost all the proliferative lesions are not always stained with GGT [6]. In the present study, all treated mice

had GGT-positive foci, but the number of GGT-positive foci was lower than that of hepatocellular foci observed in HE-stained sections in Experiment I. In addition, some of the hepatocellular foci observed in HE-stained sections were negative for GGT. This finding means that almost all the hepatocellular foci can't be detected in GGT histochemistry. In Experiment II, 6 of 6 treated mice had GGT-positive foci, but the multiplicity of GGT-positive foci was slightly lower than that of hepatocellular foci observed in HE-stained sections, and GGT-negative foci were also noted in 3 of 6 mice (50 %). In addition, 6 of 6 mice (100 %) had hepatocellular adenomas, while 5 of 6 treated mice (83 %) had GGT-positive adenomas. Furthermore, GGT-negative adenomas were noted in 5 of 6 mice (83 %). These findings indicate that hepatocellular adenomas observed in HE-stained sections could not be always detected in GGT histochemistry, as demonstrated by the previous histochemical study in rats [6]. Regarding hepatocellular carcinomas stained with HE, 3 of 6 treated mice (50 %) had hepatocellular carcinomas, while 1 of 6 treated mice (17 %) and 3 of 6 mice (50 %) had GGT-positive carcinomas and GGT-negative carcinomas, respectively. These findings on hepatocellular carcinomas indicate that some of the carcinomas were negative for GGT. As described above, the result of our study also confirmed the disadvantage pointed out by the previous workers that hepatocellular proliferative lesions observed in HE-stained sections could not be always detected in GGT histochemistry [6, 7].

CK8 and CK18 are known to be distributed in cytoplasmic filament networks and as bands associated with the plasma membrane from hepatocytes, epithelia of the intestinal tract, ductal cells of several glands and epithelia of the thymus in mice [1]. The expression of these CKs is also demonstrated in hepatocytes and bile duct epithelia of rats [16] and epithelia of the skin and apocrine glands of dogs [17]. A number of *in vitro* experiments and transgenic mouse model studies have shown that CK8/18 carry out essential functions in protecting hepatocytes from a variety of stresses such as griseofulvin, acetaminophen and cadmium [22, 24]. On the other hand, overexpression of CK8/18 in human hepatocellular carcinomas has been previously demonstrated by immunohistochemistry [2, 15]. In addition, CK8/18 has been reported to be a reliable marker of hepatocellular proliferative lesions during early stage of rat hepatocarcinogenesis [16]. As the possible mechanism of overexpression of CK8/18 in hepatocellular tumors in rats, it has been suggested that CK8 and CK18 complex due to CK8 phosphorylation may drive neoplastic transformation of GST-P-positive foci during rat hepatocarcinogenesis leading to the formation of hepatocellular tumors [16]. Phosphorylation of CK8 and CK18 can be increased in primary cultures of mouse hepatocytes and rat livers by some tumor promoters, such as 12-*O*-tetradecanoylphorbol-13-acetate and phenobarbital [5, 16]. Taking into account the above references, it can be suggested that CK8/18 is a useful immunohistochemical marker for hepatocellular proliferative lesions in mice.