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Differential Effects of Partial Hepatectomy and Carbon Tetrachloride Administration on Induction of Liver Cell Foci in a Model for Detection of Initiation Activity

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Differential effects of partial hepatectomy (PH) and carbon tetrachloride (CCl_4) administration on induction of glutathione S-transferase placental form (GST-P)-positive foci were investigated in a model for detection of initiation activity. Firstly, we surveyed cell proliferation kinetics and fluctuation in cytochrome P450 (CYP) mRNA levels by means of relative-quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and CYP 2E1 apoprotein amount by immunoblotting (experiment I) after PH or CCl_4 administration. Next, to assess the interrelationships among cell proliferation, fluctuation of CYPs after PH or CCl_4 administration and induction of liver cell foci, the non-hepatocarcinogen, 1,2-dimethylhydrazine (DMH) was administered to 7-week-old male F344 rats and initiated populations were selected using the resistant hepatocyte model (experiment II). In experiment I, the values of all CYP isozyme mRNAs after PH or CCl_4 administration were drastically decreased at the 12-h time point. From 72 h, mRNAs for all CYP isozymes began increasing, with complete recovery after 7 days. The CYP 2E1 apoprotein content in the PH group fluctuated weakly, whereas in the CCl_4 group it had decreased rapidly after 12 h and was still low at the 48 h point. In experiment II, induction of GST-P-positive foci was related to cell kinetics in the PH group, with about a 6-h time lag between time for carcinogen administration giving greatest induction of GST-P-positive foci and peaks in bromodeoxyuridine (BrdU) labeling, presumably due to the necessity for bioactivation of DMH. With CCl_4 administration, induction of foci appeared dependent on the recovery of CYP 2E1. In conclusion, PH was able to induce cell proliferation with maintenance of CYP 2E1, therefore being advantageous for induction of liver cell foci in models to detect initiation activity.

Key words: Medium-term bioassay — Partial hepatectomy — Carbon tetrachloride — GST-P-positive foci — Initiation

Medium-term liver bioassay systems based on the two-stage hypothesis of carcinogenesis have found application as bridges to fill the gap between long-term carcinogenicity tests and short-term *in vitro* screening assays such as the Ames test.[1–3] The importance of cell proliferation in the medium-term liver bioassay for detection of initiation activities is now well recognized.[4] Partial hepatectomy (PH) strongly induces cell proliferation in rat liver, and therefore has been widely utilized to stimulate DNA synthesis and cell division of hepatocytes in medium-term liver bioassay systems.[1,2,4–6] Similarly, chemical hepatectomy with administration of a necrogenic dose of carbon tetrachloride (CCl_4) is able to induce cell proliferation.[5,7] Another important factor for initiation is metabolic activation of carcinogens, the majority of which need conversion to ultimate carcinogenic species within the body. Most often, the activation steps include oxidation by the cytochrome P450 (CYP)-dependent mixed function oxidase system.[8] CYP consists of a large superfamily of proteins, and families 1, 2, 3 are predominantly involved in the metabolism of carcinogens,[9] mainly in the liver. Therefore, knowledge of the regulation of members of the CYP superfamily during liver regeneration may also have important implications for chemical carcinogenesis.

For detection of initiation activity, we must consider proliferative and metabolic aspects at the time of chemical administration. We have reported initiation activity of a 'non-hepatocarcinogen' in rat liver after administration of necrogenic doses of CCl_4.[7] However, comparative effects of different stimuli to regeneration, and the underlying mechanisms, remain to be clarified. In the present study, we therefore surveyed cell proliferation kinetics, monitored fluctuation of CYP mRNAs by using the relative-quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR),[10] and quantitated amounts of CYP 2E1 apoprotein, which plays an important role in carcinogenic activation after PH or CCl_4 administration.

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Further, we analyzed the relations of the number and area of glutathione S-transferase placental form (GST-P)-positive foci[2, 6, 11] with cell proliferation and CYP levels.

MATERIALS AND METHODS

Animals Male F344 rats (Charles River Japan Inc., Atsugi), housed five per plastic cage on wood chips for bedding, were maintained under constant conditions (12 h light/dark cycle, 60% humidity at 22±2°C) on Oriental NMF diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. They were allowed to acclimatize for 1 week before the start of experiments, at which time they were 7 weeks old and weighed 140–150 g.

Chemicals and reagents 1,2-Dimethylhydrazine (DMH) was purchased from Tokyo Kasei Co. (Tokyo), CCl_4 from Wako Pure Chemical Industries, Ltd. (Osaka) and bromodeoxyuridine (BrdU) from Sigma Chemical Co. (St. Louis, MO). Diet containing 0.015% 2-acetylaminofluorene (2-AAF) was purchased from Japan Clea Co. (Tokyo). The polyclonal antibody against CYP 2E1 apoprotein was obtained from Chemicon International, Inc. (Temecula, CA).

Experimental protocols In experiment I (Fig. 1A), groups of 3 or 4 rats underwent PH or CCl_4 administration at day 0 and were given an i.p. injection of BrdU (100 mg/kg) 1 h before sacrifice at 0, 12, 18, 24, 30, 36, 48, 60, 72 h and 7 days thereafter. Liver slices were fixed in 10% neutral buffered formalin for immunostaining. In addition to fixed samples, pieces of fresh liver tissue after 0, 12, 24, 36, 48, 72 h and 7 days were immediately frozen in liquid nitrogen and stored at −80°C for quantification of CYP mRNAs by RT-PCR and western blotting.

In experiment II (Fig. 1B), in order to clarify any correlation among cell proliferation, CYP expression and induction of GST-P-positive foci, rats were divided into 14 groups. Six groups (G1–G6) were administered a single dose of DMH (1 mg/kg b.w., i.g.) at 12, 18, 24, 30, 36 or 60 h after PH, and a control group (G7) received the saline vehicle alone. Similarly, after CCl_4 administration, 6 groups (G8–G13) were given a single dose of DMH (10 mg/kg b.w., i.g.) 12, 18, 24, 30, 36 or 60 h after CCl_4 administration and a control group (G14) received the saline vehicle alone after CCl_4 administration. Subsequently, the rats were fed on basal diet for 2 weeks, and then diet containing 0.015% of 2-AAF for the following 2 weeks. Three weeks after DMH administration, all animals received a single dose of CCl_4 (0.8 ml/kg b.w., i.g.). At the end of week 5 the survivors were killed and slices of right liver lobes were fixed in 10% neutral buffered formalin for immunohistochemical examination of GST-P-positive foci.

Relative-quantification of CYP mRNA by real-time RT-PCR using a LightCycler Total RNA was isolated from frozen liver samples using TRIzol Reagent (Gibco BRL, Grand Island, NY). For conversion of total RNA to cDNA, 1 μg of total RNA was treated with 1 U deoxyribonuclease (DNase) I (Amplification grade, Gibco BRL) at room temperature for 15 min. Then, cDNA was synthesized from DNase I-treated total RNA with the Thermoscript RT-PCR System (Gibco BRL) according to the manufacturer's instructions.

Relative-quantificative PCR of CYPs 1A2, 2B1/2, 2C11, 2E1 and 3A1 was performed with the LightCycler system (Roche Diagnostics, Mannheim, Germany), with β-actin as a reference.[10] PCR was performed basically as described earlier[10] using a SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems, Foster

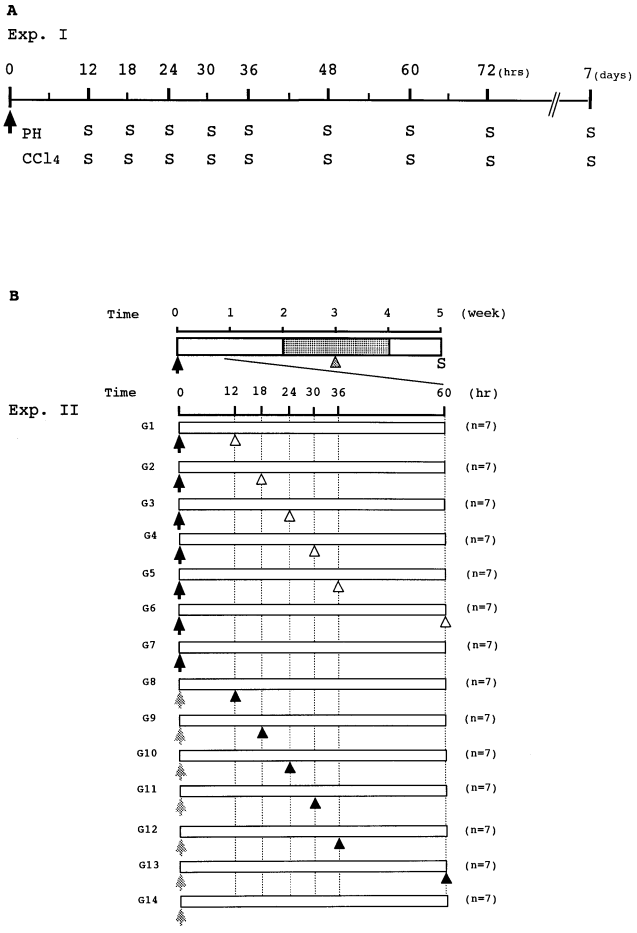

Fig. 1. Schematic representation of the protocols in (A) experiment I, and (B) experiment II. See "Materials and Methods" for further details. (A) ↑, PH or CCl_4 1 ml/kg b.w., i.g.; S, sacrifice. BrdU 100 mg/kg i.p. at 1 h before sacrifice. (B) △, DMH 1 mg/kg b.w., i.g.; ↑, CCl_4 1 ml/kg b.w., i.g.; ▲, CCl_4 0.8 ml/kg b.w., i.g.; ▲, DMH 10 mg/kg b.w., i.g.; ■, 0.015 % 2-AAF in the diet; ↑, PH; S, sacrifice.

City, CA). Briefly, 20 μl of reaction mixture was prepared containing 1 μl of cDNA, 200 μM each dATP, dCTP and dGTP, 400 μM dUTP, 200 nM of 5′ and 3′ specific PCR primers, 0.025 U/μl AmpliTaq Gold polymerase and the provided buffer. The $MgCl_2$ concentration was 1.5 mM for 2B1/2 and 2C11, and 3 mM for 1A2, 2E1 and 3A1. Primer sequences for CYP isozymes and β-actin were as described by Morris *et al.*[12] and Nudel *et al.*,[13] respectively. Primers for 2B1/2 were not able to distinguish between the two 2B isoenzymes because of their high sequence homology (97%).[12]

PCR reaction mixtures were incubated at 95°C for 10 min in order to activate enzymes, and then melting, annealing and extension cycles were carried out at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min.[12] Amplification of β-actin cDNA was accomplished with cycle conditions of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min.[10] All amplifications were carried out for 35 cycles. SYBR Green fluorescence was measured at the end of each extension. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase using the LightCycler system by calculating the ratio of the cycle number of each enzyme relative to that of β-actin cDNA.[10]

Immunohistochemical staining of sections Immunohistochemical staining of sections for GST-P and BrdU labeling, measurement of number and area of GST-P-positive foci and generation of BrdU labeling indices were performed as described previously.[7] Immunohistochemical staining of CYP 2E1 apoprotein in paraffin-embedded liver sections was as detailed earlier.[14] The specificity of this polyclonal antibody was reported by Edwards *et al.*[15]

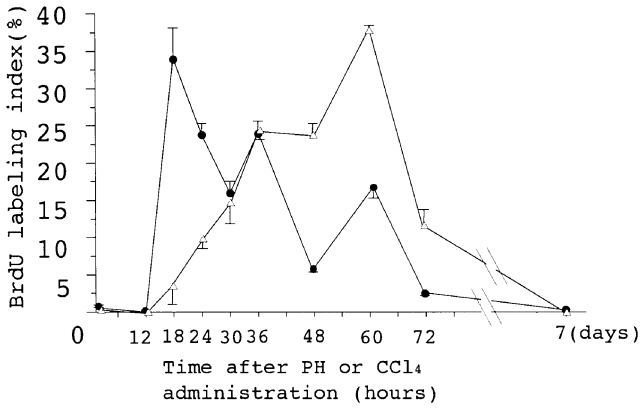

Fig. 2. BrdU labeling indices in the livers of rats at various times after PH (●) or CCl_4 administration (△). Data are mean±SEM values for 3–4 animals in each group.

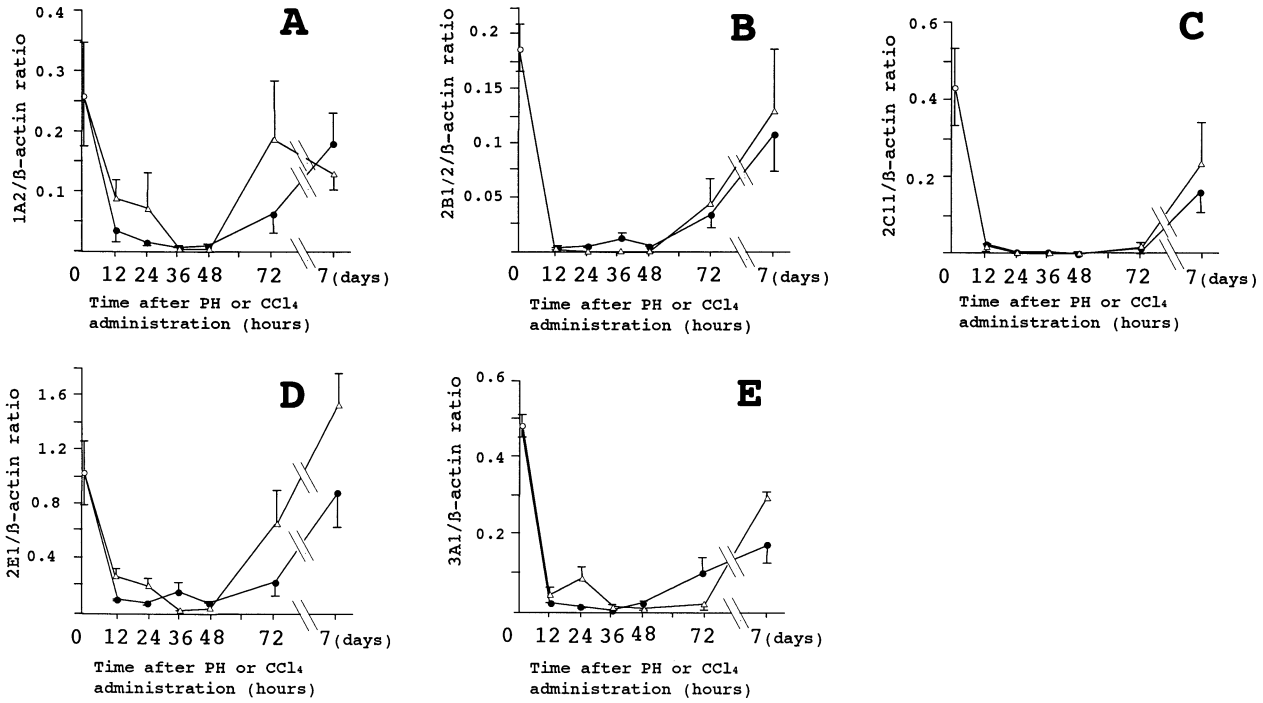

Fig. 3. Relative quantitation of CYPs mRNA normalized with respect to β-actin (A–E) in the livers of rats at various times after PH (●) or CCl_4 administration (△). Data are mean±SEM values for 3–4 animals in each group.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting analysis of CYP 2E1 Liver microsomes were prepared essentially as described by Burke and Meyer.[16] Briefly, livers were transferred into 0.25 *M* glycerol/50 m*M* Tris pH 7.4, homogenized with a glass Potter-Elvehjem type homogenizer with a Teflon pestle and centrifuged at 13 500*g* for 20 min. The supernatant was then recentrifuged at 105 000*g* for 60 min and the resultant pellet of the microsomal fraction was washed once in 0.15 *M* KCl/10 m*M* Tris pH 7.6.[17] After centrifugation, the protein concentration was determined by means of the BioRad DC protein assay (BioRad, Hercules, CA), based on Lowry's method.[18] Microsomal preparations were snap-frozen in liquid nitrogen and stored at −80°C until used.

Liver microsomes were separated on a 10% SDS-polyacrylamide gel[19] and blotted onto Hybribond P (Amersham, Buckinghamshire, England) in 20% methanol, 400 m*M* glycine, 50 m*M* Tris-HCl (pH 8.3) at 4°C for 60 V for 4 h. CYP 2E1 was detected with an antibody kit and the enhanced chemiluminescence (ECL) detection system as recommended, with a positive control supplied by the manufacturer (Amersham). Samples at each time point were analyzed in triplicate or quadruplicate. The blots were scanned and quantitated as described by Trautwein *et al*.[17]

Statistical analysis The significance of differences in the quantitative data from experiment II were statistically evaluated using ANOVA.

RESULTS

BrdU labeling indices after PH or CCl_4 administration The data for cell proliferation kinetics are summarized in Fig. 2. Liver cell proliferation after PH was at a maximum after 18 h, approximately 35% of the cells being positive for BrdU. Labeling indices increased again at 36 and 60 h after PH. After CCl_4 administration, centro- to mid-zonal necrosis of hepatic lobules was seen from 24 to 36 h. Subsequently, liver cell proliferation in the periportal area gradually increased and the mean BrdU index reached approximately 40% at 60 h. Seven days after PH or CCl_4 administration, the labeling indices showed no differences from control values, suggesting cessation of liver cell regeneration.

Amounts of CYP mRNAs after PH or CCl_4 administration The data for relative-quantification of CYP mRNAs in livers of hepatectomized or CCl_4-administered rats using β-actin as an internal control are summarized in Fig. 3, A–E. At 12 h after PH or CCl_4, the values for CYP mRNAs decreased drastically. From 72 h after PH or CCl_4, all the CYP isozyme mRNAs began increasing and recovered to nearly control levels at 7 days after PH.

After the LightCycler reaction, the samples were electrophoresed in 2.5% agarose gels, and visualized with ethidium bromide to confirm that there was no obvious primer dimer formation or amplification of any extra bands. Total RNA samples without RT provided no apparent PCR amplification (data not shown).

Quantitation of CYP 2E1 apoprotein After PH, the CYP 2E1 apoprotein contents in the microsomal proteins decreased at 24 h, but were not less than 60% of the controls (Fig. 4, A and B). On the other hand, the CYP 2E1 level decreased rapidly to approximately 10% of the control value at 12 h after CCl_4 administration, remaining low until 48 h, then recovering by 72 h. Immunohistochemistry supported these results (data not shown). Briefly, the centrilobular pattern of CYP 2E1-positive cells did not change after PH. On the other hand, most hepatocytes were negative 12 to 48 h after CCl_4 administration. Staining recovered after 7 days.

Quantitation of GST-P-positive foci The data for numbers and areas of GST-P-positive foci per unit area of liver

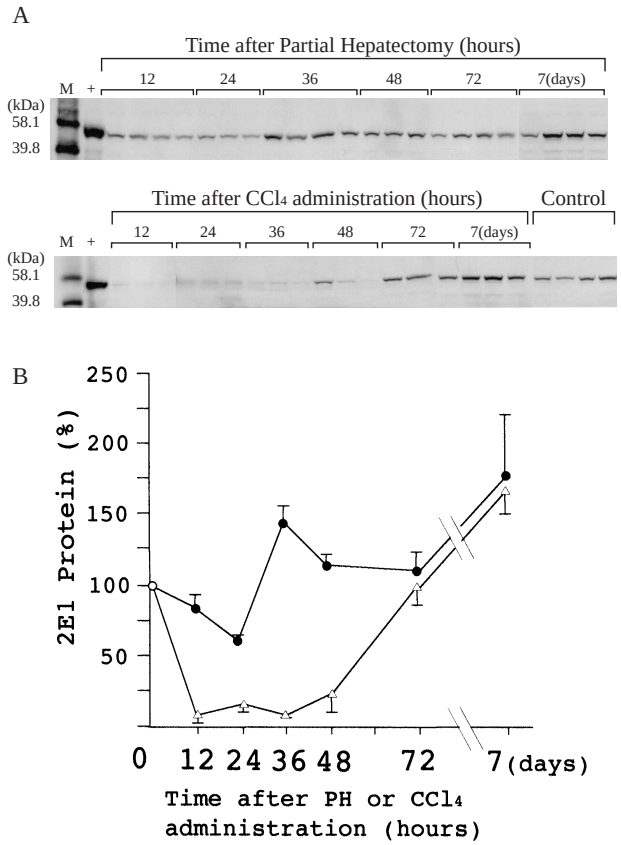

Fig. 4. (A) Western blot analysis of CYP 2E1 in hepatic microsomes from rat livers at various times after PH or CCl_4 administration. M, molecular weight marker; +, isoniazid-treated rat liver microsomes provided as a positive control in the kit. (B) Relative amounts of CYP 2E1 apoprotein. PH (●) or CCl_4 administration (△).

section in experiment II are summarized in Fig. 5. In experiment II, control groups (G7 and G14) without DMH administration had no significant numbers or areas of GST-P-positive foci. However, GST-P-positive foci were highly induced by application of the carcinogen at 12 and 30 h after PH, each of these time points being 6 h prior to a peak of DNA synthesis (18 and 36 h after PH), as shown in Fig. 2. In contrast, administration of DMH at 18 and 36 h after PH, when the highest BrdU indices were evident, was not effective for induction of GST-P-positive foci. There was thus an approximately 6-h time lag between the most effective time for induction of GST-P-positive foci and the highest BrdU labeling. The numbers and areas of GST-P-positive foci in G8–10 increased in proportion to the cell proliferation activity at the time of carcinogen administration.

DISCUSSION

Most carcinogens are indirect-acting, requiring metabolic activation mainly catalyzed by CYP mixed functional oxidases. Therefore, fluctuations in the amounts of

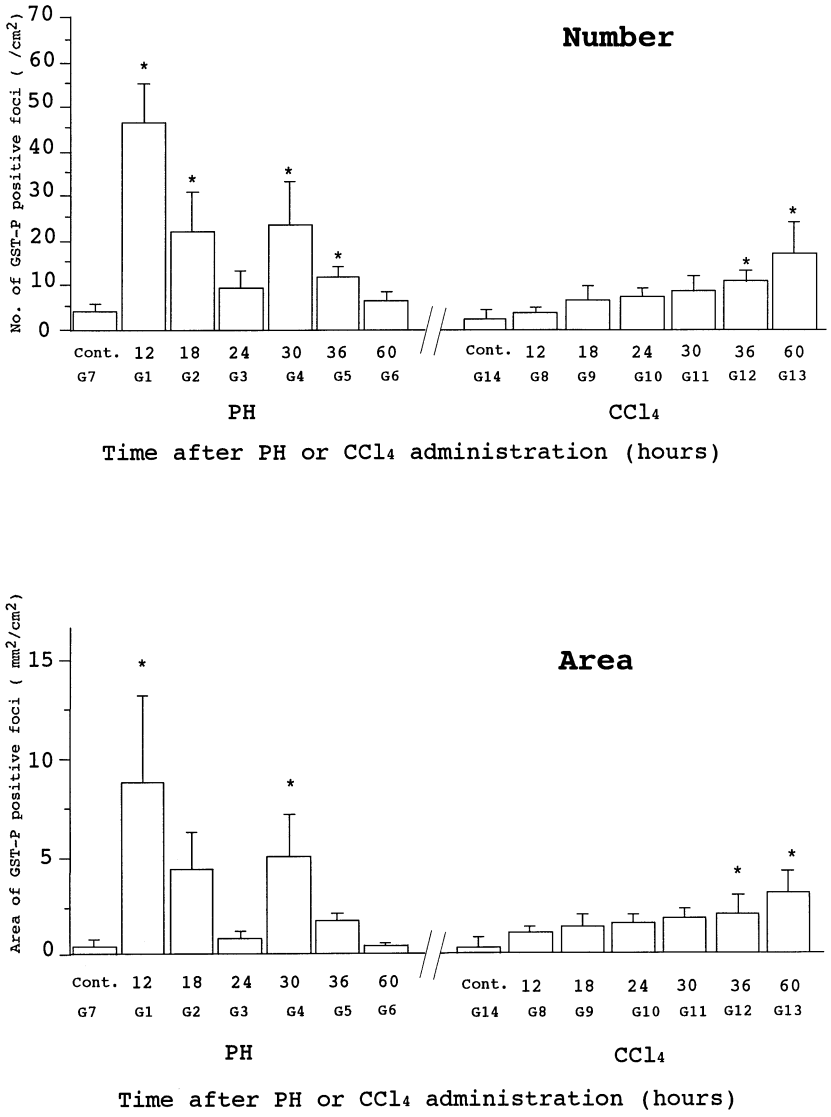

Fig. 5. Numbers and areas of GST-P-positive liver cell foci >0.1 mm diameter in experiment II. Data are mean±SD values for 7–15 animals in each group. *, $P<0.05$ as compared with animals without DMH administration (Cont., G7 and G14) in both PH and CCl_4 administration groups.

such metabolic enzymes are thought to influence initiation activity. In the first period of high BrdU labeling (~24 h after PH) after PH in the present experiment I, CYP mRNAs and 2E1 apoprotein contents decreased. The CYP mRNAs of most isozymes, such as 1A2, 2B1/2, 2C11, 2E1 and 3A1, are also known to be reduced during rapid proliferation of isolated hepatocytes in culture.[20] The time course of change in total CYP content in culture after isolation was similar to that of regenerating rat liver after PH, so that there seems to be a similarity in the mode of de-differentiation between *in vitro* and *in vivo* regeneration cases.[20] Ishizuka and coworkers[21] reported isozyme-selective alterations in the expression of CYP apoproteins. Changes of 2B1 and 2E1 were not significant for 2 weeks after PH. In contrast, the content of 3A2 was remarkably suppressed in regenerating liver.[21] In our study, decrease of CYP 2E1 apoprotein at 24 h was to approximately 60% of the control level at the most, followed by rapid recovery. In addition, the most intense immunohistochemical staining with the CYP 2E1 apoprotein antibody was obtained in the hepatocyte layers nearest the central vein at all times after PH,[14] as in the control group. Although the content of 2E1 apoprotein at 24 h after PH was approximately a half that after 36 h, no significant difference was observed regarding induction of GST-P-positive foci between the two time points in experiment II. Therefore, the content of 2E1 apoprotein at 24 h after PH appeared to be sufficient to activate 1 mg/kg DMH.

On the other hand, in the CCl_4 administration groups, not only CYP mRNA, but also CYP 2E1 apoprotein decreased rapidly and remained low for at least 48 h. This reduction is thought to be caused by degradation of the isoenzyme, namely by suicide metabolism.[22] The metabolic intermediates generated from CCl_4 metabolism by CYP 2E1 are extremely reactive trichloromethyl radicals[23] which attract methylene hydrogen from unsaturated lipids at the site of formation, triggering a chain lipid peroxidation in the endoplasmic reticulum[23, 24] and degrading the enzyme responsible for the bioactivation.[25] This is associated with the 20S proteasome.[26] To our knowledge, fluctuation of CYP mRNA after CCl_4 administration has not hitherto been reported. Rapid decline of CYP mRNAs might be affected by degeneration and necrosis of hepatocytes, and subsequent regeneration causing de-differentiation.

In experiment II, initiation activities after CCl_4 administration correlated with BrdU labeling indices at the time of DMH administration, despite the persisting low levels of CYPs, in line with findings in our previous study.[7] However, induction of GST-P-positive foci after PH was stronger at 12 and 30 h than that at 18 and 36 h when the BrdU indices were the highest, when sufficient amounts of CYPs existed. The difference in initiation activity after PH or CCl_4 administration might be caused by the fluctuation of DMH-metabolizing enzymes. Indirect-acting non-hepatocarcinogens such as DMH and metabolic derivatives, e.g. azoxymethane, are principally colon carcinogens in rodents.[27] As the result of metabolism of these chemicals, the proximate carcinogen methylazoxymethanol (MAM) and the ultimate carcinogen methyldiazonium ion are generated.[28] This carcinogenic activation is catalyzed by CYP 2E1 in rat liver.[29] MAM formation peaks at 4.5 h after DMH administration in male F344 rats, as demonstrated by analysis of urine with high-pressure liquid chromatography.[28] In the present CCl_4 administration groups, the BrdU labeling index began to increase at 24 h after administration. However, the contents of CYP 2E1 apoprotein were less than 25% of a control level until 48 h. Therefore, initiation appeared to depend on the content of CYP 2E1 apoprotein rather than cell proliferation. On the other hand, in the PH groups, the decline of CYP 2E1 apoprotein content was 40% relative to controls at most, levels being apparently sufficient to activate DMH to ultimate forms. Therefore, initiation depended on cell proliferation and the time lag between intiation and cell kinetics could be explained by the period required for bioactivation of DMH.

PH induced-hepatocyte proliferation occurs firstly in periportal cells, with mid-lobular and perivenous cell division occurring later.[30] On the other hand, after CCl_4 treatment, cell proliferation occurs in periportal areas after centro- to mid-zonal necrosis of hepatic lobules. Since cell proliferation is important for initiation of foci,[4] this would be expected to occur in the periportal area. Therefore, decrease of initiated hepatocytes owing to CCl_4 hepatotoxicity might be very low.

Initiation activity of the direct carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) using CCl_4- or PH-stimulated initiation assay was reported by Kobayashi *et al*.[5] In their report, regardless of cell proliferative stimulus, initiation activity of MNNG correlated with cell kinetics. Even if CYPs were reduced by necrosis ascribed to CCl_4 hepatotoxicity, initiation activity of MNNG was not influenced. Therefore, the results of their study using a direct carcinogen support our hypothesis that cell proliferation and the content of CYP after PH or CCl_4 treatment influence initiation.

In conclusion, CYP isozyme mRNAs are reduced rapidly after PH or CCl_4 administration. However, the decline of 2E1 apoprotein content is very different in the PH and CCl_4 cases. Expression of 2E1 apoprotein was retained during liver regeneration after PH and induction of GST-P-positive foci appeared dependent on cell kinetics. In contrast, most CYP 2E1 was degraded after CCl_4 administration, and induction of foci was dependent on its recovery. Since PH is able to induce cell proliferation while maintaining CYP 2E1, it has an advantage for induction of liver cell foci to detect initiation activities.

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