Detection of Initiation Activity of 1,2-Dimethylhydrazine in *in vivo* Medium-Term Liver Initiation Assay System using 4-Week-Old Rats without Hepatocellular Proliferative Stimuli during the Test Chemical Treatment Period

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ABSTRACT. We have developed an *in vivo* medium-term liver initiation assay system to detect initiation activities of chemicals on multi-organ carcinogenesis. However, cell proliferation stimuli during the test chemical treatment period, required in the previously used assay models using adult rats, are laborious; moreover, those cause decrease of hepatic metabolic enzymes and psychological and physical discomfort to animals resulting in inaccurate interpretation. Therefore, we investigated the utility of another *in vivo* medium-term liver initiation assay model using 4-week-old rats without the cell proliferation stimuli. In this study, we confirmed that 4-week-old and 4.5-week-old male rats have high hepatocyte proliferation activity and similar enzyme activities of hepatic Cytochrome P450 subtypes as compared with 8-week-old male rats. Next, the *in vivo* medium-term liver initiation assay model using 4-week-old rats without cell proliferation stimuli was evaluated for the detection of the initiation activity of 1,2-dimethylhydrazine (DMH), which is a well-known genotoxic carcinogen. Four-week-old rats were orally administered DMH (single dose, 4 or 16 mg/kg; or 4-day repeat, 1 or 4 mg/kg); subsequently, these rats were treated promotion treatment consisted of administration of 2-acetylaminofluorene and carbon tetrachloride. Four weeks after the first DMH administration, the glutathione S-transferase placental form (GST-P)-positive foci induced by DMH in the liver was measured immunohistochemically. The inductions of GST-P-positive foci in all DMH-treated groups were dose-dependent, duration-dependent and significantly higher than that in non-DMH-treated group. From these results, our assay model was detected the initiation activity of DMH simply, and would be useful to evaluate the carcinogenicity of chemicals.

KEY WORDS: 1,2-dimethylhydrazine (DMH) Introduction, carcinogenesis, GST-P-positive cell foci, liver, rat.

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Several models are available today for studying the mechanism and activity of carcinogenesis *in vivo*, most comprising multistage studies involving initiation, promotion, and progression [15]. *In vivo* medium-term carcinogenic assays based on the multi-stage carcinogenesis hypothesis have been developed in order to bridge the gap between long-term *in vivo* carcinogenicity studies and short-term *in vitro* screening tests [16, 22, 47, 53]. The Ito model for the detection of the promotion activity in the liver has already been validated using a long list of chemicals and is widely used to detect the promotion activities of chemicals [23, 24]. Initiation is also a key event in multi-stage carcinogenesis, resulting from complicated processes *in vivo* [17]. Therefore, an *in vivo* medium-term liver initiation assay model has also been developed to detect initiation activity of chemicals, and 26 well-known chemicals have been evaluated [38].

The medium-term liver initiation assay model could detect genotoxic carcinogens, regardless of the target organ, with reference to the induction of glutathione S-transferase

placental form (GST-P) positive foci in rat livers [38]. GST-P-positive cells were greatly increased in putative neoplastic hepatocellular foci, nodules, and carcinomas [48]. GST-P-positive cells were detectable within days of a single administration of various initiating hepatocarcinogens including diethylnitrosamine (DEN) and aflatoxin B1, but not with promoters and non-carcinogenic agents [30, 31]. Hence, the initiation activity of chemicals can be detected by the measurement of GST-P-positive foci as an end-point marker within a relatively short period because GST-P-positive cells can be considered to be initiated and to be capable of evolving into hepatic preneoplastic lesions [29, 32].

Cell proliferation and metabolism are an essential part of the carcinogenic process. Cell proliferation participates in inducing the mutation of DNA during the initiation of cancer [5, 6]. Most chemical carcinogens are procarcinogens that need to be converted to true carcinogens by metabolic activation [18]. The metabolic enzymes in the liver, e.g., cytochrome P450 (CYP), are responsible for such bioactivation and play key roles. For this reason, high cell proliferation activity and adequate metabolic enzyme activity in the liver during administration of test chemical are essential to detect initiation activity.

Partial hepatectomy (PH) strongly induces hepatocyte

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proliferation and is widely utilized as a cell proliferation stimulus in the medium-term liver initiation assay model [19, 25, 38, 46]. However, performing PH involves certain problems. First, surgical hepatectomy requires technical expertise and is time consuming, especially when large numbers of experimental animals are to be used. Second, PH causes pain to the animals; moreover, a high mortality is observed after the administration of toxic chemicals because of the high hepatotoxicity induced by the synergistic effect of PH and the toxic chemical. Third, hepatocyte proliferation after PH is sharply variable; it is therefore easy to miss the time points of high hepatocyte proliferation and high initiation activity [37].

Chemical hepatectomy (CH) and the fasting/refeeding method are very simple as compared with PH, but these methods also entail certain problems. Hepatotoxicants such as carbon tetrachloride (CCl_4), allyl alcohol, and d-galactosamine have been used for CH; however, although they induced hepatocyte proliferation, the induction of GST-P-positive foci in the medium-term liver initiation assay model with these cell proliferation stimuli was lower than that with PH in previous studies [27, 33, 39–41]. The reason is possibly that the induction of hepatocyte proliferation after the administration of these hepatotoxicants was lower than that after PH; moreover, when hepatocyte proliferation activity was increased, the hepatic metabolic enzymes causing metabolic activation of procarcinogen were markedly decreased by hepatotoxicity of these hepatotoxicants. The fasting/refeeding method consists of fasting for 4 days and refeeding for 1 day before administering the test chemical. The induction of GST-P-positive foci in the medium-term liver initiation assay model with the fasting/refeeding method was similar to that observed with PH [9]. However, the fasting/refeeding method has only been tested using diethylnitrosamine (DEN)-a well-known liver carcinogen; moreover, 4-day fasting causes physical and psychological discomfort to the animals, including a decrease in body weight.

The use of proliferating tissue is a prerequisite for the medium-term liver initiation assay model, and another method that can induce high hepatic proliferation activity without proliferation stimuli such as PH is desirable. Espaniari *et al.* [9] reported that the medium-term liver initiation assay model using neonatal rats during the initiation phase achieved satisfactory results without cell proliferation stimuli identical to the model with PH in 8-week-old rats. Nevertheless, the model with neonatal rats has only been tested using DEN, and requires a long study period of 14 weeks. Moreover, it is considered that neonatal rats have inadequate metabolic enzyme activities in the liver for the metabolic activation of test chemical.

On the other hand, the livers of 4-week-old rats are still growing, and have high hepatocyte proliferation activity and similar enzyme activities of hepatic CYP as compared with that in adult rats [12, 26, 35]. Hence, our study aimed to investigate the utility of 4-week-old rats in the medium-term liver initiation assay model without a cell proliferation stimulus, and current study was consisted of 2 experiments. In

experiment I, the hepatocyte kinetics and the enzyme activities of several hepatic CYP isozymes in 4-week-old, 4.5-week-old, and 8-week-old Fischer-344 male rats were evaluated to confirm superiority and continuousness of hepatocyte proliferation activity and similarity of hepatic metabolism activity at 4 weeks of age as compared with those at 8 weeks of age. In experiment II, the *in vivo* medium-term liver initiation assay model using 4-week-old rats without a proliferation stimulus was evaluated for the detection of the initiation activity of 1,2-dimethylhydrazine (DMH), which is a well-known genotoxic carcinogen targeting the colon and a non-hepatocarcinogen in rodents [52, 55]; the utility of this model using 4-week-old rats was evaluated.

MATERIALS AND METHODS

Chemicals: DMH dihydrochloride and 2-acetylaminofluorene (2-AAF) were purchased from Tokyo Chemical Industry Ltd. (Tokyo, Japan). The animals' feed (CA-1) containing 0.015% of 2-AAF and 10% corn oil was obtained from Clea Japan Inc. (Tokyo, Japan). CCl_4 was purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan). 5-Bromo-2'-deoxyuridine (BrdU), chlorzoxazone (CZX), 6-hydroxychlorzoxazone (6-OH CZX), resorufin, ethoxyresorufin, 6 β -hydroxytestosterone (6 β -OH TS), and β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). 7 α -hydroxytestosterone (7 α -OH TS) and 16 β -hydroxytestosterone (16 β -OH TS) were obtained from UFC Ltd. (Manchester, Grate Manchester, UK). Testosterone was purchased from Biogenesis Ltd. (Poole, Dorset, UK). The Bio-Rad Protein Assay kit used for the determination of microsomal protein content was obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Animals: Male Fischer-344 rats were obtained from Charles River Japan (Atsugi, Japan), and 3–4 animals were housed per plastic cage on softwood chip bedding. These animals were maintained under constant conditions (12 hr light/dark cycle, 60% humidity at $22^\circ\text{C} \pm 2^\circ\text{C}$), and fed on an CA-1 diet (Clea Japan Inc.) and tap water *ad libitum*.

Experimental protocols: In experiment I, hepatocyte proliferation kinetics were evaluated in 4-week-old, 4–5-week-old, and 8-week-old rats. The rats were intraperitoneally administered BrdU (100 mg/kg) dissolved in saline, 1 hr before autopsy. After euthanasia by exsanguination under diethyl ether anesthesia, the liver slices were fixed in 10% neutral buffered formalin for approximately 24 hr and processed for embedding in paraffin for hematoxylin and eosin (H.E.) staining as well as immunohistochemical examination of BrdU-labeled cells. Pieces of fresh liver tissue were rinsed in ice-cold saline and immediately frozen in liquid nitrogen and stored at approximately -80°C until use for the measurement of enzyme activities of several hepatic CYP isozymes.

In experiment II (Fig. 1), the *in vivo* medium-term liver

Fig. 1. Schematic representation of the protocol in experiment II: See Materials and Methods for further details.

initiation assay model using 4-week-old rats was evaluated to detect the initiation activity of DMH. In accordance with the dose and administration period of DMH, 4-week-old rats after 1-week acclimatization were divided into 5 groups as follows: 1 mg/kg/day single administration, 1 mg/kg/day 4-day administration, 4 mg/kg/day single administration, 4 mg/kg/day 4-day administration, and 16 mg/kg/day single administration. DMH was dissolved in saline and administered orally once daily. The weights of the 4-week-old rats ranged from 52.1 to 73.3 g at the first administration of DMH. The control group received no DMH. All rats were fed basal diet (CA-1) for 1 week after the first DMH administration, and then the diet containing 0.015% 2-AAF for the following 2 weeks. At 2 weeks after the first DMH administration, all animals were orally administered a single dose of CCl₄ (0.8 mL/kg) dissolved in corn oil. Additionally, the non-promotion group was only administered 4 mg/kg/day DMH for 8 days without providing the diet containing 2-AAF and orally administration of CCl₄. At the end of week 4 after the first DMH administration, the survivors were euthanized, and their liver slices were fixed in 10% neutral buffered formalin for immunohistochemical examination of GST-P-positive foci.

All procedures described in this study were approved by the Gifu University Committee on Animal Care and Supply.

Preparation of hepatic microsomes: Hepatic microsomes were prepared as described previously [36, 54]. Approximately 1–2 g of liver tissue was homogenized (Polytron Homogenizer; GTR-1000, Tokyo Rikakiki Co., Ltd., Tokyo, Japan) in a 3-volume homogenization buffer (0.1-M Tris acetate, 0.1-M KCl, 1-mM EDTA, 5 μ g/mL butylated hydroxytoluene, and 40 μ g/mL phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 700 $\times$ g for 10 min at 4°C, and the supernatant was centrifuged at 12,000 $\times$

g for 20 min at 4°C. The supernatant was ultracentrifuged at 105,000 $\times$ g for 60 min at 4°C; subsequently, the pellet was suspended in sucrose phosphate buffer (0.1-M phosphate buffer, 0.25-M sucrose, and 0.1-mM EDTA). These aliquots were stored at –80°C until further use.

Microsomal protein concentrations were determined by the Bradford's protein assay method using the Bio-Rad Protein Assay kit (Coomassie Brilliant Blue G-250) and bovine serum albumin as the standard, according to the manufacturer's protocol.

Total cytochrome P450 measurement: The content of total CYP was measured photometrically using the method described by Omura and Sato [34]. The microsomal sample was further diluted to approximately 1 μ g/mL microsomal protein with phosphate-glycerol buffer (20:1 v/v, 0.1 M phosphate buffer: glycerol). Total cytochrome P450 was determined by the formation of the carbon monoxide difference spectrum between 450 nm and 490 nm absorbance following dithionite reduction.

Enzyme activity determination

Ethoxyresorufin O-deethylase activity: Ethoxyresorufin O-deethylase activity was quantified by the fluorometric measurement of resorufin, which is 7-ethoxyresorufin metabolite [4]. The reaction mixture comprising 15- μ M 7-ethoxyresorufin, 1-mM NADPH, 3-mM magnesium chloride, and phosphate buffer (0.1 M, pH 7.4) was prepared to a total volume of 180 μ L and placed in one well of a microplate. All incubation mixtures were preincubated for 10 min at 37°C. The reactions were initiated by the addition of 20 μ L of 1 μ g/mL microsomal protein and fluorometrically monitored (excitation, 544 nm; emission, 590 nm) at 37°C for 3 min in the microplate reader.

Testosterone hydroxylase activities: CYP subtypes stereospecifically hydroxylate testosterone at certain ring posi-

tions to produce several metabolites [3, 42]. CYP2A, CYP2B, and CYP3A activities were measured by monitoring the 7 α -, 16 β -, and 6 β -hydroxylation of testosterone using high-performance liquid chromatography (HPLC) [56]. The reaction mixture comprising 5 μ l of 20-mM testosterone, 50 μ l of 10-mM NADPH, and phosphate buffer (0.1 M, pH 7.4) was prepared to a total volume of 450 μ l. All incubation mixtures were preincubated for 5 min at 37°C in a shaking water bath. The reactions were initiated by the addition of 50 μ l of 5 mg/ μ l microsomal protein and terminated by the addition of 250 μ l ice-cold methanol after incubation for 10 min at 37°C. Microsomal protein was removed from suspension by centrifugation (14,000 $\times$ g, 10 min), and 50 μ l of the supernatant was injected directly into the HPLC system. A Hypersil BDS C₁₈, 5 μ m (10 cm $\times$ 0.46 cm) analytical column (Phenomenex, Macclesfield, UK) maintained at a temperature of 40°C was used for HPLC. The HPLC analysis used a gradient in which solvent A (water) was varied from 80% (0–10 min) to 72% (10–13 min) to 80% (13–16 min), solvent B (acetonitrile) from 10% (0–10 min) to 14% (10–13 min) to 10% (13–16 min), and solvent C (tetrahydrofuran) from 10% (0–10 min) to 14% (10–13 min) to 14% (13–16 min) of total flow volume. The flow rate was constant at 1.0 μ l/min. Testosterone metabolite formations (7 α -OH TS, 16 β -OH TS, and 6 β -OH TS) were measured by in-line UV detection at 255 nm, and the chromatograms were analyzed.

Chlorzoxazone hydroxylase activity: CYP2E1 activity was monitored by HPLC assay of the 6-hydroxylation of CZX as described by Leclercq *et al.* [28]. The reaction mixture comprising 20 μ g of CZX, 0.6 mg of NADPH, 50 μ l of 0.1-M magnesium chloride, and 0.1-M Tris buffer (pH 7.6) was prepared to a total volume of 450 μ l. The reaction was initiated by the addition of 50 μ l of 3 mg/ μ l microsomal protein and terminated by the addition of 200 μ l ice-cold zinc sulfate (15%, w/v) after incubation for 20 min at 37°C. Microsomal protein was removed from suspension by centrifugation (14,000 $\times$ g, 10 min), and 50 μ l of the supernatant injected directly into the HPLC system. An Aquasil C₁₈, 3 mm (15 cm $\times$ 0.46 cm) analytical column (Thermo Fisher Science Inc., Waltham, MA, U.S.A.) maintained at a temperature of 25°C was used. The mobile phase composition was 10-mM ammonium acetate buffer-acetonitrile (75:25, v/v) at a flow rate of 1.0 μ l/min. Formation of 6-OH CZX was measured by in-line UV detection at 287 nm, following which the chromatograms were analyzed.

Immunohistochemistry: The avidin-biotin complex method has been used to demonstrate BrdU-labeled hepatocytes, in the S-phase of the cell cycle, and GST-P-positive foci [2, 20]. The sections were incubated with peroxidase-blocking solution (DAKO Cytomation Inc., Carpinteria, CA, U.S.A.) for 5 min to block any endogenous peroxidase activity, and incubated with Protein block serum-Free (DAKO Cytomation Inc.) for 10 min for nonspecific binding. For BrdU-immunostaining, the section was treated with 2-N HCl for 30 min and then 0.05% pepsin solution containing 0.01-N HCl for 12 min to reactivate the target

antigen after blocking the endogenous peroxidase activity. All subsequent operations were performed after washing the sections with TBS containing 0.1% Tween 20. The sections were incubated with mouse monoclonal antibody against BrdU (DAKO Cytomation Inc.) diluted at 1:50 or rabbit polyclonal antibody against rat GST-P (MBL, Nagoya, Japan) for 60 min at room temperature. Subsequently, the sections for anti-BrdU and anti-GST-P immunostain were incubated with anti-mouse goat immunoglobulins (DAKO Cytomation Inc.) diluted at 1:400 for 30 min or biotinylated anti-rabbit immunoglobulins in LSAB2 kit (DAKO Cytomation Inc.) for 10 min at room temperature. Next, streptavidin conjugated to horseradish peroxidase was reacted with the sections for the production of the avidin-biotin complex using the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min or the LSAB2 kit (DAKO Cytomation Inc.) for 10 min. The reaction was detected using 3,3'-diaminobenzidine (DAB) as a chromogen in the DAB kit (DAKO Cytomation Inc.).

At least 6 areas per liver lobe were examined at 100 $\times$ magnification, and the percentage of BrdU-labeled hepatocytes from a total of 2000 or more hepatocytes was calculated. On the other hand, the numbers and areas of GST-P-positive foci >0.1 mm in diameter and the total areas of liver sections were quantified using eCognition (Definiens, Munich, Germany), which is an innovative object-based quantitative image analysis system.

Statistical analysis: Descriptive statistics (mean $\pm$ SD) were used to report the liver weight, relative liver weight, BrdU-labeled hepatocyte index, hepatic total CYP content, enzyme activities of hepatic CYP subtypes and numbers and areas of GST-P foci. The statistical significance of differences in quantitative data in experiments I and II was analyzed using *t*-tests and pairwise multiple comparisons using the SAS non-clinical package (SAS Institute Japan, Japan). The level of significance was set at less than 5%.

In the *t*-tests, the homogeneity of the variance was tested using the F-test at a significance level of 5%. Student's *t*-test was applied when homogeneous variance was demonstrated, and Welch's *t*-test was applied when the variance was not homogeneous. In multiple comparisons, the homogeneity of the variance was tested using the Bartlett method at a significance level of 5%. When homogeneous variance was demonstrated, a one-way ANOVA analysis was applied. The mean values were further compared using Dunnett's multiple comparison only when the one-way ANOVA analysis of variance detected significant differences between the control group and the evaluated groups. When the Bartlett test showed that the variance was not homogeneous, the Kruskal-Wallis test was applied. Steel's test of means was performed only when the Kruskal-Wallis test detected significant differences between the control group and the evaluated groups.

RESULTS

Hepatocyte proliferation and hepatic CYP in 4-week-old,

4.5-week-old, and 8-week-old rats: The mean values for liver weight and relative liver weight for the experimental animals are indicated in Fig. 2. The mean liver weights in 4-week-old and 4.5-week-old rats were significantly lower than that in 8-week-old rats, but the mean relative liver weights in 4-week-old and 4.5-week-old rats were significantly higher than that in 8-week-old rats. Histological examination (Fig. 3) showed that the lobular pattern was similar between 4-week-old rats and 8-week-old rats; however, the hepatocytes in the middle zone of the liver lobule in 4-week-old rats seemed to be smaller than those in the 8-week-old rats. Several mitotic figures in hepatocytes were observed similarly in the middle and periportal zones in both 4-week-old rats and 8-week-old rats. However, the BrdU-labeled hepatocytes in 4-week-old rats were observed more frequently in the pan-lobule, but those in 8-week-old rats were few in the middle and periportal zones. The BrdU-labeled hepatocyte index is indicated in Fig. 4. The BrdU-labeled hepatocyte index values in 4-week-old and 4.5-week-old rats were approximately 3 and 2 times higher than that in 8-week-old rats, respectively.

The hepatic CYP contents and enzyme activities of CYP1A, CYP2A, CYP2B, CYP2E, and CYP3A in each age group are indicated in Fig. 5. The CYP contents in 4-week-old and 4.5-week-old rats were similar to those in 8-week-old rats. The enzyme activities of CYP1A, CYP2B, CYP2E, and CYP3A in the livers of 4-week-old and 4.5-week-old rats were almost equivalent to those in 8-week-old rats. The enzyme activity of hepatic CYP2A in 4-week-old and 4.5-week-old rats was approximately 2 times higher than that in 8-week-old rats.

GST-P-positive Foci induced by DMH administration in the medium-term liver initiation assay model using 4-week-old rats: Data for the numbers and areas of GST-P-positive foci per unit area of liver sections in experiment II are summarized in Fig. 6.

The numbers and areas of GST-P-positive foci in the rats administered DMH were significantly increased as compared with those in the control rats, and they showed a dose-dependency. The values for GST-P-positive foci were similar between the rats treated with DMH 1 mg/kg/day for 4 days and the rats treated with DMH 4 mg/kg/day for 1 day. In the same way, GST-P-positive foci in the rats treated with DMH 4 mg/kg/day for 4 days were similarly induced as compared with those in the group treated with DMH 16 mg/kg/day for 1 day. Histopathologically, large GST-P foci occupying several lobules and causing distinct compression of adjacent parenchyma were observed in 3 rats treated with DMH 4 mg/kg/day for 4 days and 1 rat treated with DMH 16 mg/kg/day for 1 day (Fig. 7). Numbers of the large GST-P foci in the liver of these rats was mainly 1–2, but 7 large GST-P foci were observed in the liver of 1 rat treated with DMH 4 mg/kg/day for 4 days. On the other hand, the numbers and areas of GST-P-positive foci in the rats administered 4 mg/kg/day DMH for 8 days without the promotion treatment consisting of CCl₄ and 2-AAF administration were not increased as compared with that in the control rats.

Fig. 2. Liver weight and relative liver weight as compared with body weights in the 4-week-old, 4.5-week-old, and 8-week-old rats. Data are represented as mean $\pm$ SD values for 4–5 animals in each group. ** $P < 0.01$, significantly different as compared with values for 8-week-old rats.

DISCUSSION

Several *in vivo* carcinogenic study methods, including the Solt-Farber protocol, have been developed to study multistage carcinogenesis in the liver [10]. Initiation is an important step in multistage carcinogenesis, and the initiation activity of test chemicals can be detected in a medium-term liver initiation assay model by cell proliferation stimuli including PH just before the treatment of test chemical, in combination with promotion treatment consisted of administration of a necrogenic dose of CCl₄ and a low dose of 2-AAF after test chemical treatment. High hepatocyte proliferation activity and sufficient metabolic activation by metabolic enzymes for administration of the test chemical are important in detecting the initiation activity of the test chemical. This study investigated the utility of using 4-week-old rats to replace an *in vivo* liver initiation assay model using a cell proliferation stimulus.

As shown in the results of BrdU-labeled cell kinetics (Fig. 4), the hepatocyte proliferation index in the liver of 4-week-old rats is approximately 3 times higher than that in 8-week-old rats, and the GST-P-positive foci in the rats administered a single DMH dose at 4 weeks of age were induced significantly as compared with those in the control rats in experiment II (Fig. 6). From these results, 4-week-old rats is considered to be advantageous in the medium-term liver initiation assay model because it does not require cell proliferation stimuli causing psychological/physical pain and unexpected effect before administration of test chemical. In the previous articles, Furukawa *et al.* [12] reported that BrdU labeling index of hepatocyte in the male Wistar-rats at 4, 5, 6, 7 and 8 weeks of age were approximately 2.5%, 1.5%, 1.2%, 1.0% and 0.3%, respectively. In the liver micronucleous assay, in which high hepatocyte proliferation activity facilitates the detection of mutagenicity, Suzuki *et al.* [45] reported that the incidence of the num-

Fig. 3. Histological differences in livers of 4-week-old and 8-week-old rats. HE staining (A: 4-week-old, C: 8-week-old) and BrdU immunostaining (B: 4-week-old, D: 8-week-old) in experiment I. The lobular pattern was similar in 4-week-old rats (A) and 8-week-old rats (C), but the hepatocytes in the middle zone of the liver lobule of 4-week-old rats seemed to be smaller than the hepatocytes of 8-week-old rats. Although several mitotic figures (arrowhead) were observed similarly in the middle and periportal zones in both 4-week-old rats and 8-week-old rats, the BrdU-labeled hepatocytes in 4-week-old rats (B) were observed more frequently in the pan-lobule as compared with those in 8-week-old rats, which are few in the middle and periportal zones (D). CV: central vein, P: portal triad. Bar is 300 micrometers.

Fig. 4. Data plot of BrdU-labeled hepatocyte indices in the 4-week-old, 4.5-week-old, and 8-week-old rats. Middle line indicates mean values, and the upper and lower lines represent standard deviation. ** $P < 0.01$, significantly different as compared with 8-week-old rats.

ber of micronucleated hepatocytes (MNHEP) induced by DEN administration in the livers of 4-week-old rats was significantly higher than that in 7-week-old and 9-week-old rats; moreover, the models using 4-week-old rats without cell proliferation stimulus during administration of test chemical indicated equal or even higher practicability than did 6-week-old rats undergoing PH before test chemical dosing. Hence, using 4-week-old rats is able to replace using cell proliferation stimuli as induction of hepatocyte proliferation activity before administration of test chemical because the hepatocyte proliferation activity in 4-week-old rats is significantly higher than adult rats, and it is suggested that using 4-week-old rats is reasonable for detecting initiation activity.

Other advantages in using 4-week-old rats concern the animal experiment period and the requisite amounts of test chemical. The previous assay models required detecting initiation activity of carcinogens for more than 5 weeks, but

Fig. 5. Hepatic total CYP content (A) and enzyme activities of hepatic CYP 2E (B), CYP 1A (C), CYP3A (D), CYP2A (E) and CYP2B (F) in 4-week-old, 4.5-week-old, and 8-week-old rats. Middle lines represent mean values, and upper and lower lines indicate standard deviation. ** $P < 0.01$, significantly different as compared with 8-week-old rats.

the current study assay model required detecting initiation activity of DMH for only 4 weeks. The body weight in 4-week-old rats ranges from 25% to 33% of that of 7- or 8-week-old rats; accordingly, the requisite amount of test chemical for detecting initiation activity in this model using

4-week-old rats is less than that in previous models using older animals.

Multiple low-dose administrations proved superior to the single high-dose administration when the margin of test chemical between toxic/lethal dose level and genotoxic/car-

Fig. 6. Numbers and areas of GST-P-positive foci > 0.1 mm in diameter in the group administered DMH or saline in experiment II. Data represent mean $\pm$ SD values for 8–17 animals in each group, except 4 mg/kg DMH administration for 8 days without promotion treatment consisting of CCl_4 and 2-AAF administration (3 animals). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, statistically significant difference as compared with animals that were administered saline. # $P < 0.05$, significant difference between the 4-day DMH 1-mg/kg/day and 4-mg/kg/day administration groups.

Fig. 7. GST-P-positive foci induced by DMH administration in the medium-term liver initiation assay model using 4-week-old rats. Large GST-P foci occupying several lobules and causing distinct compression of adjacent parenchyma were observed in the liver treated with DMH 4 mg/kg/day for 4 days. A: HE staining, B: GST-P immunostaining. Bar is 800 micrometers.

cinogenic dose level was small. Human exposure to environmental carcinogens is usually limited to low concentrations and human are generally exposed to such carcinogens over a long-term period [1, 7, 49]; therefore, multiple low-dose administration would be expected to be superior to single high-dose administration for extrapolation to carcinogenesis in humans. Generally, previous assay models using a cell proliferation stimulus tested only single or multiple doses over 1-day administration of test chemi-

cals because the induction of hepatocyte proliferation could be maintained only for approximately 1 day [2, 39]. In the current study, hepatocyte proliferation activity in 4-week-old rats was significantly higher than that in 8-week-old rats and continued at high levels until 4.5 weeks of age in experiment I, and the induction of GST-P-positive foci by 4-day divided doses of DMH was approximately equal to that with the same total single dose of DMH in experiment II. From these results, using 4-week-old rats in the medium-term

liver initiation assay models not only makes it unnecessary to use a cell proliferation stimulus but also enables to detect the initiation activity of test chemicals with repeat administration of ones, which could not be detected the initiation activity in previous assay models.

Most carcinogens require metabolic activation, and this is mainly catalyzed by CYP. CYP2E1 metabolizes nitrosamines such as DEN [51] and hydrazine derivatives such as DMH [11, 44]. In previous studies, it has been reported that the enzyme activities of hepatic CYP2A in 3- or 4-week-old SD rats were higher than that in 7- or 8-week-old SD rats, and enzyme activities of hepatic CYP1A, CYP2B, CYP2E, and CYP3A in 3- or 4-week-old rats were similar as compared with that in 7- or 8-week-old rats [8, 43]. Kato and Yamazoe [26] reported that expression of hepatic CYP1A2, CYP2A1, CYP2B1, CYP2B2, CYP2E1, CYP3A1, and CYP3A2 approximately reached maximum levels in 3-week-old rats. On the other hand, expressions of hepatic metabolic enzymes such as CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2B2, and CYP3A2 in neonatal rats aged below 1 week of age were significantly low except for CYP2E1 [8, 21]. Although the utility of the medium-term liver initiation assay model using neonatal rats has been reported [9], it is considered that the metabolic activation of procarcinogens is not adequate in the liver of neonatal rats. In this study, hepatic CYP contents and the enzyme activities of CYP1A, CYP2B, CYP2E, and CYP3A in 4-week-old and 4.5-week-old rats were similar to that in 8-week-old rats, and hepatic CYP2A in 4-week-old and 4.5-week-old rats was approximately 2 times higher than that in 8-week-old rats (Fig. 5). Therefore, the medium-term liver initiation assay model using 4-week-old rats is able to detect the initiation activity of a procarcinogen such as DMH adequately.

GST-P-positive foci can be strongly stimulated by the application of 2-AAF feeding with cell proliferation treatment. In the previous medium-term liver initiation assay models, the initiated cells could be selectively stimulated to produce altered foci by using an appropriate selection pressure consisting of exposure to 2-AAF in combination with a hepatocyte proliferation stimulus by CCl₄ administration [40, 50]. In experiment II, the induction of GST-P foci in the rats treated with only DMH 4 mg/kg/day administration for 8 days without the promotion treatment consisting of diet dosing of 2-AAF and oral administration of CCl₄ was much lower than that in the control group administered saline with the promotion treatment. From these results, the promotion treatment consisting of 2-AAF and CCl₄ administration after formation of initiated cells by test chemical was also necessary for enabling the detection of the initiation activity of test chemicals in medium-term liver initiation assay models using 4-week-old rats. It is likely that juvenile livers may be sensitive to the initiation activity of carcinogens, and carcinogenic risks in children may be higher than those in adults when they are exposed to liver genotoxic carcinogens or liver promotion stimuli such as non-genotoxic carcinogens with mutagens that include various environmental materials. It is considered that further

studies are needed to analyze the carcinogenic risk of environmental materials, such as agricultural chemicals, in children.

In conclusion, the use of 4-week-old rats in medium-term liver initiation assay models enables the utilization of the high and sustained hepatocyte proliferation activity with sufficient levels of hepatic metabolic enzymes such as CYP2E1, activating DMH to the ultimate carcinogenic forms. This *in vivo* assay model without a cell proliferation stimulus during test chemical treatment was capable of evaluating DMH carcinogenicity rapidly and simply with single- or repeat-administration of one, as compared with previous assay models that used cell proliferation stimuli. We suggest that this assay model is better for 3R concept of animal welfare than previous assay models, and expect that the detection sensitivity for initiation activity would improve markedly with this model; especially, with repeat administration of test chemical.

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