Variation in the Frequency of Micronucleated Hepatocytes Following Time Course Treatment in the Mouse: Liver Micronucleus Assay of Partially Hepatectomized Tissues

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The time-course pattern of the frequency of micronucleated hepatocytes *in vivo* after partial hepatectomy (PH) was studied in mice using *N*-nitrosodimethylamine (DMN), *N*-nitrosodiethylamine (DEN), and 1,2-dimethylhydrazine (DMH), which are rodent liver carcinogens with potent clastogenic activity in the liver. With all chemicals, production of micronucleated hepatocytes was not clearly observed at 3 d after PH, but was clear 4 or 5 d after PH. We propose that it is preferable to perform a preliminary assay prior to the main assay when estimating the clastogenic potential of certain chemicals towards hepatocytes *in vivo*.

Key words rodent liver carcinogen; *in vivo* mouse liver micronucleus assay; partial hepatectomy

Mouse bone marrow or peripheral blood micronucleus assay has been widely used as a simple and economic method for assessing chromosomal breakage *in vivo*. It was reported that a good correlation exists between micronucleus induction and human carcinogens, as well as possible carcinogens identified by the IARC (International Agency for Research on Cancer).¹⁾ The positive results in the micronucleus assay may be due to carcinogens that target multiple organs.¹⁾ Another report suggests that a positive response in this assay may relate to human carcinogens.²⁾ However, rodent liver carcinogens such as nitrosoamines, nitroaromatic compounds and azo derivatives are rarely sensitive in mouse bone marrow micronucleus assay.^{3,4)} Thus, micronucleus assays using target organ cells, such as liver, stomach, small intestine and colon, have been developed to clarify the relationship between micronucleus induction and carcinogenicity in target organs.^{3,5–12)} The liver micronucleus assay is considered to be an important method to complement the bone marrow assay, especially since many mutagens acquire genotoxic potential through metabolic activation. This assay can confirm the generation of substances with clastogenic activity on hepatocytes. The *in vivo* liver micronucleus assay is recommended as one choice for *in vivo* studies when a chemical has no gene mutagenic potential, especially when clastogenic activity is achieved through metabolic activation *in vitro*.¹³⁾ There are a number of reports concerning this assay,^{7–11)} but a standard method has not been established. Tate *et al.*⁶⁾ reported that micronuclei in hepatocytes induced by mutagens were observed for several weeks. Although this finding is important for identifying micronuclei inducibility in hepatocytes, the screening assay has to be both rapid and accurate. However, there are few studies of the sampling time after PH in the liver micronucleus assay. For these reasons, we investigated the time-course frequencies of micronucleated hepatocytes (MNHEPs). Namely, the genotoxic chemical was administered 24 h before PH and mice were treated for specimen preparation at 3, 4 and 5 d after PH. The rodent liver carcinogens^{1,14)} used in this study were *N*-nitrosodimethylamine (DMN), *N*-nitrosodiethylamine (DEN) and 1,2-dimethylhydrazine (DMH). In this assay, PH was used to stimulate mitosis and proliferation of hepatocytes. It was reported that 4-acetylaminofluorene (4AAF) has effects similar to

those of PH.^{15–17)} Therefore, we wondered if 4AAF would be useful for detecting a chemical with clastogenic activity in hepatocytes.

MATERIALS AND METHODS

Chemicals DMN (CAS No.62-75-9), DEN (CAS No. 55-18-5) and collagenase type I were purchased from Wako Pure Chemical Co. (Japan). DMH (CAS No.306-37-6) was obtained from Tokyo Kasei Co. (Japan); 4AAF (CAS No.28322-02-3) was obtained from Lancaster (U.K.); and S9 (9000×*g* supernatant prepared from rat liver homogenates pretreated with phenobarbital and 5,6-benzoflavone)¹⁸⁾ was obtained from the Oriental Yeast Co., Ltd.

Mouse Liver Micronucleus Assay Male ddY mice, 10–14 weeks of age, were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The test chemical was dissolved in saline. We performed an acute toxicity assay to calculate the 50% lethal dose (LD₅₀) of DMN, DEN and DMH, following oral gavage.¹⁹⁾ Test chemicals at 1/2, 1/4 and 1/8 LD₅₀ were administered as a single oral gavage. After 24 h exposure, mice underwent partial hepatectomy (PH) following ether administration.

In brief, three lobes of the liver were excised and specimens were prepared at 3, 4 and 5 d after PH. Perfusion under ether anesthesia was started with Ca²⁺ and Mg²⁺-free Hanks' balanced salt solution (HBSS) containing 10 mM HEPES and 0.5 mM EDTA for 2 or 3 min, and was maintained at 37 °C. The perfusion was continued for 5 min with HBSS containing 10 mM HEPES, collagenase type I (0.05%), 5 mM CaCl₂ and 0.005% trypsin inhibitor. After treatment, the hepatocytes were dispersed with Ca²⁺ and Mg²⁺ free phosphate buffered saline [PBS(–)] and centrifuged at 500 rpm. The sedimented cells were resuspended in PBS(–) then re-centrifuged. This procedure was repeated three times. Finally, the cell pellets were suspended in a small volume of fetal bovine serum and the hepatocyte sample was smeared on a glass slide. The slides were fixed with 10% formaldehyde solution for 10 min and stained with Schiff reagent for 1 h.²⁰⁾ One thousand hepatocytes with a normal cell morphology were observed at 600× magnification per mouse. The selection and scoring for MNHEPs was done according to the cri-

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Fig. 1. Frequencies of Micronucleated Hepatocytes on DMN, DEN and DMH

Test chemicals were administered by a single oral gavage. After 24 h exposure, mice were subjected to PH. The specimens were prepared at 3 d (PH3), 4 d (PH4) and 5 d (PH5) after PH, respectively. Frequencies of micronuclei represent mean values for hepatocytes sampled at 3–5 d after PH.

teria of Braithwaite and Ashby.¹⁶⁾

Next, we determined if 4AAF would provide an effective replacement for PH.^{16,21)} Namely, DMN (5 mg/kg) was orally administered on day 0 and 4AAF (1000 or 2000 mg/kg) suspended in olive oil was orally given on day 1. Specimens were prepared at 5 days after 4AAF treatment.

Bacterial Reverse Mutation Assay of 4AAF This experiment was performed as described.^{18,22)} *Salmonella typhimurium* strains TA100, TA98 and *Escherichia coli* WP2 *uvrA* were used. Rat liver S9 mix was used as the metabolic activation method for this assay, which was performed using the preincubation method, with or without S9 mix. 4AAF dissolved in dimethylsulfoxide (DMSO, solvent control) was added just prior to the assay. Five doses of 4AAF with duplicate plates per dose were tested for each strain and a positive result was considered when the revertant colonies were above two fold that of the solvent control.²³⁾ The dose-response re-

action was also considered.

RESULTS

The frequencies of MNHEPs for different sampling days after PH are shown in Fig. 1. The frequency of concurrent solvent control in DMN treated mice was below 0.48%. In the PH3 groups, 2.5 mg/kg DMN resulted in a slight increase of MNHEPs, whereas no increases were observed for the middle and high dose groups. In the PH4 groups, no clear increase was seen in any group. In addition, at the high dose of 10 mg/kg DMN, three mice died before specimen preparation. In the PH5 groups, DMN treated mice showed a positive response in the frequencies of MNHEPs compared with the solvent control. However, one mouse died when given 10 mg/kg DMN. The frequencies of MNHEPs for the concurrent solvent control in DEN treated mice were below 0.82%.

Table 1. The Mouse Liver Micronucleus Assay with the DMN and 4AAF Combination

Combination of chemicals	No. of animals	Micronucleated hepatocytes (%)
Solvent+olive oil	5	0.00±0.00
DMN+olive oil	5	0.08±0.13
DMN+4AAF (1000 mg/kg)	5	0.00±0.00
DMN+4AAF (2000 mg/kg)	5	0.00±0.00

Solvent (saline) or DMN (5 mg/kg) was administered on day 0, and 4AAF (1000 or 2000 mg/kg) was given on day 1. The specimens were prepared on day 6. Values represent mean±S.D. for 5 mice.

Table 2. Mutagenicities of 4AAF Determined by the Metabolic Activation Method

Dose (μg/plate)	Mean revertant colonies/plate		
	TA100	TA98	WP2uvrA
0	134	20	19
78	949 (7.0)	72 (3.6)	29
156	2498 (18.6)	270 (13.5)	33
313	3860 (28.8)	722 (36.1)	58 (3.1)
625	3663 (27.3)	1121 (56.1)	61 (3.2)
1250	3605 (26.9)	991 (49.6)	61 (3.2)

Numbers in parentheses represent relative values compared with solvent control.

In the PH3 groups, no increases in the frequencies of MNHEPs were observed in any DEN treated group. Both PH4 and PH5 groups showed comparatively clear positive responses in the DEN treated mice. The frequencies of MNHEPs in the solvent control DMH treated mice were below 0.35%. In the PH3 groups, increases in the frequencies of MNHEPs were not seen in DMH or DEN treated mice, whereas 7.5 mg/kg DMH in PH4 and 15 mg/kg DMH in PH5 showed comparatively clear positive responses. An evaluation of 15 mg/kg DMH in PH4 could not be made as specimens were defective due to the toxicity of DMH.

Results of the mouse liver micronucleus assay with the combination of 4AAF and DMN are shown in Table 1. The frequencies of MNHEPs using the PH method, shown in Fig. 1, significantly increased with a dose of 5 mg/kg DMN in PH5. However, 4AAF treatment as an alternative to the PH method did not give rise to potent induction of MNHEPs. The results of bacterial reverse mutation assay with 4AAF are shown in Table 2. 4AAF did not show any increase in revertant colonies above two fold without S9 mix with all strains (data not shown). As seen in Table 2, two fold increases in revertant colonies were remarkable, and showed dose dependency in every strain when S9 mix was added. Thus, 4AAF was clearly positive in this assay, as others have reported.²⁴⁾ In the present study, 4AAF was also positive in *Escherichia coli* WP2uvrA.

DISCUSSION

There are several methods used for micronucleus assay using hepatocytes, for example, partial hepatectomy,^{6,7)} infant animals,⁴⁾ primary culture cells^{25,26)} and mitogens.⁸⁾ With the PH method, little work has been done to investigate the appropriate sampling time after PH. This aspect of the PH method should be emphasized in order to confirm if a chemi-

cal has the potential for induction of MNHEPs. Based on this background, we investigated the time-course frequencies of MNHEPs after PH using DMN, DEN and DMH, all rodent liver carcinogens, and which also give positive responses in the liver micronucleus assay.^{7,11,27)} Clearly positive responses were not observed with PH3 treatment but were positive for PH4 and/or PH5. Therefore, it was considered that PH4 and/or PH5 treatment is appropriate for sampling time in the liver micronucleus assay. In the case of DMN, PH5 treatment seems to be favored. Therefore, for the main assay following the preliminary one, dose selection has to be considered. This selection will determine if a chemical has potent clastogenic activity on hepatocytes *in vivo*. The following factors may contribute to the difference in MNHEP frequencies on each PH treatment day; (1) cell death due to over dose; (2) inhibition or delay of hepatocyte regeneration and proliferation after PH.⁶⁾

It was reported that 4AAF has a potent rat and mouse liver mitogenic stimulus effect, similar to that seen with PH treatment.^{15–17)} As a pilot study, we investigated MNHEP inducibility in combination with DMN and 4AAF and induction of MNHEPs was zero in all DMN treated groups. This is presumed to be due to less sensitivity with regards the mitogenic action of 4AAF.¹⁷⁾ Another cause may be the use of an olive oil suspension.²⁸⁾ Although an incorporation study using a labelled compound was not done, the number of binucleated cells per 1000 hepatocytes, (indicating the proliferative and mitotic activity) was similar to the ratio seen with solvent control and without 4AAF administration.^{6,29,30)} 4AAF has no apparent clastogenic activity *in vitro* (data not shown) or *in vivo*.³¹⁾ However, 4AAF generates metabolites which acquire gene mutation potentiality through metabolic activation, and concerns about a possible interaction between a chemical and a mitogen such as 4AAF were discussed by Parton and Garriott.⁴⁾

Our findings indicate that PH treatment after chemical administration and dose selection should be set up based on a preliminary assay prior to doing the main assay.

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