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A study of micronucleated hepatocytes detection in the liver micronucleus assay using young adult rats

（成熟ラットを用いた肝臓小核試験における小核を有する肝細胞の検出に関する研究）

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Abbreviations

AAALAC International	Association for Assessment and Accreditation of Laboratory Animal Care International
AO	acridine orange
Ass. Prof.	associate professor
BrdU	5-bromo-2'-deoxyuridine
CAS No.	Chemical Abstracts Service Registry Number
DAB	3.3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DEN	diethylnitrosamine
DMN	dimethylnitrosamine
DNA	deoxyribonucleic acid
EdU	5-ethnyl-2’-deoxyuridine
G1 phase	gap 1 phase
G2 phase	gap 2 phase
h	hour/hours
HBSS	Hank’s balanced salt solution
HE	hematoxylin and eosin
HEPs	hepatocytes
HRP	horseradish peroxidase
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

IMEs	immature erythrocytes
ip	intraperitoneal
IWGT	International Workshop on Genotoxicity Testing
JEMS	Japanese Environmental Mutagen Society
LD_{50}	lethal dose 50%
Lot No.	lot number
MI	mitotic index
min	minute/minutes
MMC	mitomycin C
MMS	Mammalian Mutagenicity Study
M-phase	mitosis phase
MTD	maximum tolerated dose
MNHEPs	micronucleated hepatocytes
MNIMEs	micronucleated immature erythrocytes
No.	number
pH	potential hydrogen
Prof.	professor
RDLMN	repeated-dose liver micronucleus
rpm	revolutions per minute
SD	standard deviation
S phase	synthesis phase
TBS	Tris-buffered saline
UV	ultraviolet

Preface

For drug development, drug candidate compounds are needed to satisfy the regulatory requirements of drug licensing authorities. Revealing the toxicity of a novel compound prior to human clinical trials is one of the most important things in preclinical phase. Therefore, pharmaceutical company should conduct many types of toxicity tests in this phase. Genotoxicity test is one of the safety tests. Genotoxicity describes the property of chemical agents that damages the genetic information within a cell. The genotoxic chemicals cause DNA repair, apoptosis or mutation, which may lead to cancer. Basically, compounds that have carcinogenicity cannot develop as a drug for human use. Therefore, the information of carcinogenicity is important for drug development. However, pharmaceutical companies have to conduct carcinogenicity tests in later clinical phase because carcinogenicity tests require a certain amount of cost and time. Therefore, genotoxicity tests that can accurately predict genotoxic carcinogenicity are expected.

Genotoxicity is induced by different mechanisms. Therefore, the development of tests to detect different mechanisms of genotoxicity is needed to assess the safety of pharmaceuticals. Because no single test is capable of detecting all of the relevant genotoxic mechanisms, pharmaceutical development requires the use of multiple genotoxicity tests. Before the initiation of the first clinical trial of the new drug candidates, regulatory agencies require a battery of tests to ensure the safety of clinical trial participants. Previous ICH guideline calls for a battery of genotoxicity tests consisting of the following three types of assays: a bacterial mutation assay (Ames test), an in vitro mammalian cell assay (metaphase analysis for chromosome breakage or a

mouse lymphoma thymidine kinase gene mutation assay) and an in vivo rodent bone marrow assay for chromosomal damage (rodent micronucleus assay) [1]. This guideline also described positive results in vitro is followed up by a second in vivo study using tissue other than bone marrow [1]. Therefore, it became a duplication of effort in some cases. In 2011, the genotoxicity guideline set by the ICH were revised and the second battery of tests for assessing genotoxicity is offered in addition to the previous battery [2]. The second option includes a bacterial mutation assay and an in vivo test with two endpoints [2]. In addition, the ICH recommended the integration of in vivo genotoxicity studies into multiple administration studies at the pre-clinical stage of pharmaceutical development [2].

The in vivo micronucleus assay of rodent hematopoietic cells is widely used to assess the genotoxicity of chemicals. However, this assay is less sensitive for carcinogens requiring metabolic activation, such as hepatocarcinogens, than direct acting carcinogens [3]. An micronucleus assay utilizing hepatocytes in the liver would therefore be a useful method of evaluating the genotoxicity of hepatocarcinogens [4]. In addition, the combination of results from micronucleus assays of hepatocytes and hematopoietic cells might improve sensitivity in the evaluation of carcinogen genotoxicity. Given that micronuclei form during cell division [5, 6], adult rats are considered unsuitable for the liver micronucleus assay, as hepatocytes have low mitotic activity [7]. As intact adult rats could not be used, researchers instead used young rats [8-11] or adult rats with partial hepatectomy [7, 12, 13] for the liver micronucleus assay. However, these treatments make difficult to integrate the liver micronucleus assay into a general toxicity study. In addition, the entire liver is perfused with collagenase for hepatocyte isolation in most liver micronucleus assays. This procedure also prevents the

evaluation of multiple endpoints, which is required in a histopathological examination. To resolve these problems, the RDLMN assay using young adult rats was developed [14, 15]. This new assay can integrate into general toxicity test, because it uses young adult rats without partial hepatectomy and is not required a collagenase perfusion. For assessing the effectiveness of this assay, the RDLMN assay using MMC was conducted in Chapter 1.

It has been considered that liver micronucleus assay using adult rats cannot detect the genotoxicity due to low mitotic activity. However, it could be detected in the RDLMN assay that used non-hepatectomized young adult rats [14-16]. The accumulation of MNHEPs following repeated dosing and the persistence of MNHEPs were thought to be key factors in the successful detection of MNHEPs using this method [17]. However, I have hypothesized that the period following chemical exposure enabled the detection of MNHEP induction in young adult rats, namely that MNHEPs could be generated from chromosomally damaged cells and accumulated following initiation of chemical exposure until sampling. To confirm this hypothesis, MNHEPs of young adult rats were measured at 2 and 4 weeks after a single dose of both DEN and MMC in Chapter 2.

Contents of this research were published in following articles.

1. K. Shimada, M. Yamamoto, M. Takashima, Y. Wako, K. Kawasako, Y. Aoki, J. Seki, Y. Miyamae, A. Wakata, Repeated-dose liver micronucleus assay of mitomycin C in young adult rats, *Mutat. Res.*, **780–781** (2015) 85-89.
2. K. Shimada, M. Yamamoto, M. Takashima, J. Seki, Y. Miyamae, A. Wakata, Prolonged rest period enables the detection of micronucleated hepatocytes in the liver of young adult rats after a single dose of diethylnitrosamine or mitomycin C, *Mutat. Res.*, **791** (2015) 38-41.

Chapter 1

Repeated-dose liver micronucleus assay of mitomycin C in young adult rats

1. Introduction

The development of tests to detect different mechanisms of genotoxicity is needed to assess the safety of chemicals, including pharmaceuticals. Because no single test is capable of detecting all of the relevant genotoxic mechanisms, pharmaceutical development requires the use of multiple genotoxicity tests. Recently, the genotoxicity guidelines set by the ICH were revised, and the ICH recommended the integration of in vivo genotoxicity studies into multiple administration studies at the pre-clinical stage of pharmaceutical development [2]. This integration of genotoxicity studies into a general toxicity study would reduce the number of experimental animals used throughout the process.

The in vivo micronucleus assay using rodent hematopoietic cells is widely used for the assessment of genotoxicity. However, chemicals that exhibit genotoxic potential following metabolic activation, such as hepatocarcinogens, tend to test negative in the bone marrow micronucleus assay [18]. Based on this observation, liver micronucleus assays have been developed. However, it is difficult to integrate the liver micronucleus assay into a general toxicity study because this assay requires the use of young rats [8-11] or the performance of a partial hepatectomy [7, 12, 13]. Additionally, in most liver micronucleus assays, the entire liver is perfused with collagenase for hepatocyte isolation, which prevents the evaluation of multiple endpoints, which is required in a histopathological examination. To resolve these problems, liver micronucleus assay using young adult rats without performing partial hepatectomy and perfusion was developed [14, 15]. These studies examined this new method through the administration of repeated doses and demonstrate that this method was effective for the detection of genotoxicity in the liver.

A collaborative study performed by the MMS group, which is a subgroup of the JEMS, was conducted to evaluate the suitability of the RDLMN assay using young adult rats. I performed the RDLMN assay using MMC as part of the referenced collaborative study.

MMC is a well-known anticancer agent [19] that forms interstrand DNA cross-links [20], resulting in cytotoxicity and mutagenicity. Additionally, MMC is carcinogenic in rats. The ip administration of MMC induces peritoneal sarcomas [21]. MMC has been previously evaluated in a liver micronucleus assay, and MMC has tested positive in all previous studies [9, 11, 22, 23].

In this chapter, I investigated the effectiveness of the novel RDLMN assay for the detection of MMC-induced genotoxicity in the liver. MMC was repeatedly administered to young male adult rats for 14- and 28-day period, and the frequencies of MNHEPs and MNIMEs were determined concurrently.

2. Materials and methods

2.1. Animals

Five-week-old male rats [Crl: CD (SD)] were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The animals were housed in an air-conditioned room, which was maintained at 23 ± 3 °C with 55 ± 15% relative humidity under a 12-h/12-h light/dark cycle, and were allowed free access to a commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. The rats were quarantined and acclimated for a period of one week prior to the start of the experiments. All of the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Furthermore, Astellas Pharma Inc. Kashima Facility was awarded accreditation by the AAALAC International (Frederick, MD, USA).

2.2. Chemicals

MMC (CAS No. 50-07-7, Lot No. 562AAH, 2 mg potency) was purchased from Kyowa Hakko Kirin Co., Ltd (Tokyo, Japan). MMC was dissolved in distilled water and diluted with physiological saline (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan).

2.3. Treatment and dose levels

Five rats per group received ip injections (5 mL/kg of body weight volume) of MMC at 0, 0.25, 0.5 and 1.0 mg/kg/day for 14 and 28 days. The ip injection route was selected as a substitute for intravenous injection, which is the route used in humans. The

highest dose level was set to correspond to the expected MTD in a 28-day study period based on a previous report [24].

2.4. Liver micronucleus assay

The preparation and observation of hepatocyte slides were performed according to the common method described in the summary paper of this collaborative study [16]. Twenty-four h after the last administration for each time point (14 and 28 days), the rats were euthanised under isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan) anesthesia after fasting overnight. However, the all surviving animals treated with 1.0 mg/kg/day of MMC in the 28-day study were humanely sacrificed after 23 days of treatment without overnight fasting because of moribund conditions. The hepatocytes were isolated using collagenase without the perfusion method and stained with AO and DAPI. The number of MNHEPs per 2,000 parenchymal hepatocytes was counted for each animal, and the number of M-phase cells among the 2,000 hepatocytes was also counted to determine the mitotic MI.

2.5. Bone marrow micronucleus assay

The micronucleus assays using bone marrow were performed according to the protocol described in the summary report of this collaborative study [16]. The bone marrow cells were collected from the femur 24 h after the last administration, fixed with methanol and stained with either AO or Giemsa solution. After staining, 2,000 immature erythrocytes were scored for each animal to determine the frequency of MNIMEs. In addition, the number of IMEs out of 1,000 erythrocytes was counted for each animal to evaluate the cytotoxicity.

2.6. Histopathological examination

A part of the left lateral lobe of the liver was fixed with 10% phosphate-buffered formalin, embedded in paraffin, and stained with HE according to the standard method.

Additionally, Ki-67 (clone MIB-5) staining was conducted for quantitative evaluation of the hepatocyte proliferation rate. Two-micrometer sections were mounted on glass slides. The sections were treated with xylene to remove the paraffin and then washed with ethanol to remove the xylene. Antigen retrieval was performed in citrate buffer (Code No., S2031, Dako, Carpinteria, CA, USA), which had been autoclaved for 10 min at 121 °C. Endogenous peroxidase was blocked by incubating the slides in 3% H_2O_2 for 5 min. Subsequently, the slides were rinsed with TBS at pH 7.6 and incubated for 20 min with 1% bovine serum albumin. The sections were then incubated with monoclonal mouse anti-rat Ki-67 antibody (dilution 1:50, Dako) overnight at 4 °C. After rinsing with TBS, the sections were incubated with secondary antibody conjugated with biotin and streptavidin-HRP (Dako-LSAB 2 System-HRP for use on Rat Specimens, Code No. K0609, Dako), and visualized with DAB. The sections were rinsed with water, and incubated in Mayer's hematoxylin as a nuclear counterstain. The slides were scanned using the Aperio ScanScope XT (Aperio, Vista, CA, USA) at 20× magnification. The number of Ki-67 positive nuclei out of at least 10,000 nuclei was counted using an Aperio nuclear v9 algorithm (Aperio) to determine the frequency of Ki-67-positive hepatocytes.

2.7. Statistical analysis

The statistical analysis was performed using the same method described in the summary paper [16]. Differences in the incidences of MNHEPs and MNIMEs between the test chemical and the vehicle control groups were analyzed statistically using Kastenbaum and Bowman's table [25]. The other quantitative data were analyzed for their statistical significance by multiple comparison tests.

3. Results

3.1. Body weight change and lethality

The MMC-treated rats exhibited a statistically significant reduction in body weight gain compared with the vehicle-treated controls in all of the treatment groups (Fig. 1). Because one of the animals that received the 1.0 mg/kg/day dose of MMC died on day 22 prior to administration and the other animals looked abnormally thin, all of the remaining animals in the group were humanely sacrificed on day 23 after administration. Therefore, the samples of the group treated with 1.0 mg/kg/day of MMC in the 28-day study were prepared on day 23 and analyzed. The rats of this group were evaluated only histopathological results but not evaluated other items, because the number of dosing was different from other groups and the rats were in a fatal condition.

3.2. Liver micronucleus assay

The results obtained from the liver micronucleus assays of rats treated with MMC for 14 and 28 days are shown Table 1 and 2. The MMC treatment significantly increased the frequency of MNHEPs in both the 14- and the 28-day studies. The results revealed 11.3-fold and 3.8-fold higher amounts of MNHEPs induced by the 0.25 mg/kg/day dose in the 14-day and the 28-day studies, respectively, compared with the vehicle-treated controls. The maximum occurrence of MNHEPs was found in the group treated with the 0.25 mg/kg/day dose of MMC, and the frequency of MNHEPs was found to be dose-independent in both of the dosing periods. The frequency of M-phase HEPs was significantly decreased in the groups treated with 0.25 and 1.0 mg/kg/day of MMC during the 14-day study, but the values of the control group were slightly higher compared with the facility's background data (0.08 ± 0.08%, n=28).

3.3. Bone marrow micronucleus assay

The results obtained from the bone marrow micronucleus assays of rats treated with MMC for 14 and 28 days are also shown Table 1 and 2. In both the 14- and the 28-day studies, the results showed that the frequency of MNIMEs significantly increased in the MMC-treated groups in a dose-dependent manner. The frequency of IMEs was decreased in the rats treated with 0.5 and 1.0 mg/kg/day of MMC in the 14-day study and 0.5 mg/kg/day of MMC in the 28-day study indicating bone marrow toxicity.

3.4. Histopathology

Hypertrophy of the bile duct epithelium was observed in the rats treated with 1.0 mg/kg/day of MMC in the 14-day study. Hypertrophy of bile duct epithelium and prominence of hepatocytic nucleolus were observed in the rats treated with 1.0 mg/kg/day of MMC in the 28-day study. However, no change was observed as a result of the 0.25 and 0.5 mg/kg/day doses of MMC. There were no histopathological findings that suggest obvious hepatocyte cytotoxicity in both study periods (Table 3).

The results of Ki-67 staining of cells from the rats treated with MMC for 14 and 28 days are also shown in Table 1 and 2. The frequency of Ki-67 positive hepatocytes was significantly increased in the rats treated with 0.25 mg/kg/day of MMC in the 28-day study. The frequency was lowest at the 1.0 mg/kg/day dose of MMC in the chemical treatment groups in both the 14- and the 28-day studies.

4. Discussion

The present study revealed that repeated administration of MMC induced micronuclei in rat hepatocytes (Table 1 and 2). However, the highest and the second highest occurrences of MNHEPs obtained in both the 14- and 28-day studies were observed in the rats treated with 0.25 and 0.5 mg/kg/day of MMC, respectively. It has been reported that a single ip injection of MMC (0, 1.0 and 2.0 mg/kg) induces MNHEPs in young rats in a dose-dependent manner [9]. However, in young rats given an ip injection of MMC (0, 0.5, 1.0 and 2.0 mg/kg) twice, the formation of MNHEPs increased significantly, although the frequency of MNHEPs decreased at the highest doses of MMC [11, 23].

The animals treated with MMC at 1.0 mg/kg/day exhibited an inhibition of body weight gain and deteriorated condition. The rats treated with MMC at 0.25 and 0.5 mg/kg/day also showed an inhibition of body weight gain compared with the control group (Fig. 1). However, obvious hepatotoxicity was not observed in the histopathological examination in all dose groups (Table 3). These results indicate that 1.0 mg/kg/day of MMC in the 28-day study leads to a fatal condition without hepatotoxicity.

Micronuclei are formed during cell division [5, 6]. Therefore, a micronucleus assay using hematopoietic cells is able to distinguish newly formed erythrocytes that completed their last division during or shortly after exposure to the test agent [3]. This feature makes the assay sensitive and efficient for the detection of genotoxic agents. Because hepatocytes in all cell cycle stages are scored in a liver micronucleus assay, only a small number of divided hepatocytes can be measured. First, I used the frequency of M-phase HEPs as an indicator of cell division in this study and did not observe an

obvious decrease in the frequency of M-phase HEPs. Narumi et al. mentioned that the MI should not be used as a mitotic parameter, because the M-phase is only a small fraction of the cell cycle [14]. These researchers found BrdU incorporation in approximately 10%, 5% and 2.5% of the hepatocytes in 6-, 8- and 11-week-old rats, respectively. Therefore, hepatocytes undergo slow but sufficient proliferation to allow the detection of MNHEPs even in 6- to 10-week-old rats. Because M-phase HEPs are thought to be an inappropriate measure for the estimation of hepatocyte proliferation, an alternative measurement should be performed in the RDLMN assay. For this reason, I performed Ki-67 staining of hepatocytes. Because the Ki-67 protein is expressed during all active parts of the cell division cycle (G1, S, G2 and M phase) [26], this method is thought to be sensitive to measure hepatocyte proliferation. And also, researchers can use paraffin blocks for this histopathological examination.

The occurrence of Ki-67 positive hepatocytes was increased in the rats treated with 0.25 mg/kg/day of MMC compared with the vehicle-treated controls in the 28-day study and decreased in the rats treated with 0.5 mg/kg/day of MMC compared with the 0.25 mg/kg/day of MMC (Table 2). In the 14-day study, the occurrence of Ki-67-positive hepatocytes was increased, but not significantly, in the 0.25 and 0.5 mg/kg/day groups of MMC compared with vehicle treated controls and was decreased with 1.0 mg/kg/day of MMC compared with 0.25 and 0.5 mg/kg/day of MMC (Table 1). Based on these results, the lowered induction of MNHEPs in the rats treated with 1.0 mg/kg/day of MMC might be explained by suppression of hepatocyte cell division. While the treatment period was short, the 14-day study showed a higher frequency of MNHEPs compared with the 28-day study. However, the reason for this was not clear.

Although the highest doses examined in the present study might be too high for a general toxicity study, I could detect micronuclei induction of MMC by repeated dosing for 14 and 28 days even at the lowest dose examined, which was adequate for a general toxicity study. Therefore, the RDLMN assay could be integrated into a general toxicity study to detect liver genotoxicity of MMC.

I also showed that the frequency of MNIMEs was increased in the rats treated with any of the tested doses of MMC for 14 and 28 days (Table 1 and 2). This result differs from that reported in a previous study [24], which found that the ip administration of up to 0.5 mg/kg/day of MMC for 28 days did not induce MNIMEs in rats, although positive results were observed in mice. It is unclear why different results were obtained.

In conclusion, these data revealed that the new RDLMN assay is useful for the detection of MMC-induced genotoxicity in the liver. In addition, MI is thought to be inappropriate for detecting hepatotoxicity, and monitoring proliferating hepatocytes is useful for evaluation of the RDLMN assay. Therefore, further analysis of proliferating hepatocytes is needed to develop this assay into a routine genotoxicity test and to integrate it into a general toxicity study.

5. Summary

The RDLMN assay has been previously reported to be effective for the detection of hepatocarcinogens and suitable for integrating into general toxicology studies. A collaborative study was conducted to evaluate whether this RDLMN assay using young adult rats without collagenase perfusion of the liver can be used to detect genotoxic carcinogens. In this chapter, I performed the RDLMN assay in young adult rats that received ip injections of 0.25, 0.5 and 1.0 mg/kg/day of MMC for 14 and 28 days. The micronucleus induction in the bone marrow was concurrently measured, and a histopathological examination of the liver was conducted. The results revealed that the frequency of MNHEPs was significantly increased in all of the treatment groups. However, the highest occurrence of MNHEPs was observed in the low-dose treatment group in both the 14- and the 28-day study periods. In addition, histopathological changes indicating hepatotoxicity were not observed even in the group that received the highest dose of MMC. There was no change in the frequency of M-phase HEPs in any of the treatment groups compared with facility's background data. However, the frequency of proliferating hepatocytes, as assessed by Ki-67 positivity, was decreased at the highest dose, as was the frequency of MNHEPs. Therefore, the decreased induction of MNHEPs in the high-dose groups might be explained by suppression of hepatocyte cell division. In contrast, the frequency of MNIMEs in the bone marrow significantly increased in a dose-dependent manner in all of the treatment groups in both study periods. These results suggest that the novel RDLMN assay can be used to detect MMC genotoxicity in the liver.

6. Figures and tables

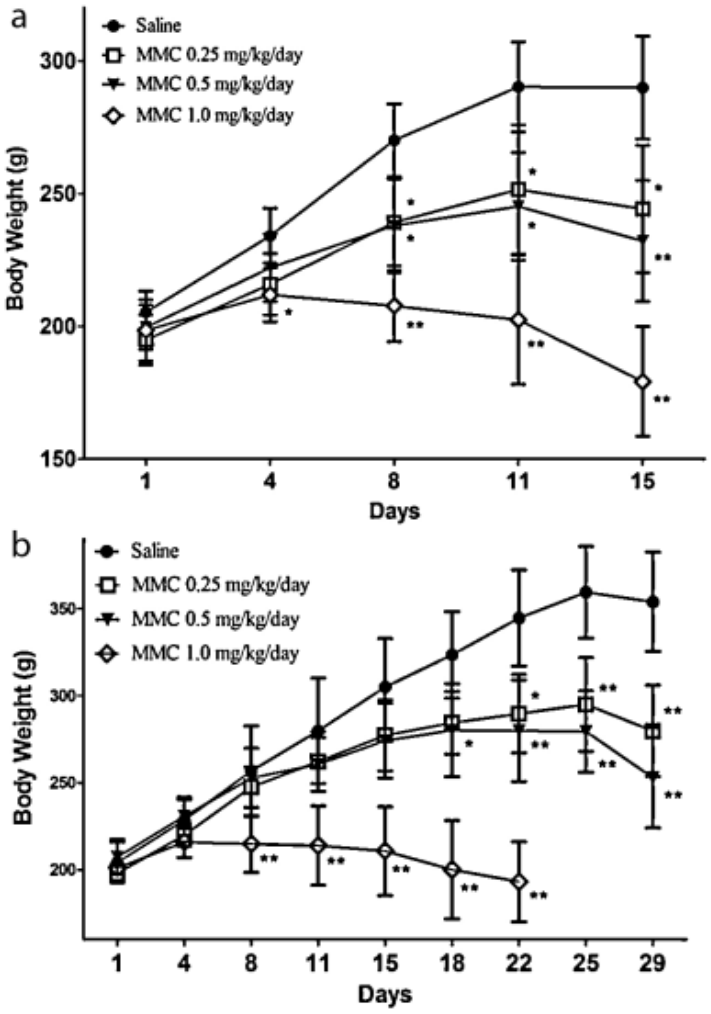

Fig. 1. Changes in body weight of rats treated with MMC for both 14- (a) and 28- (b) day periods. The body weight data represent the means ± SD. **P* < 0.05 and ***P* < 0.01 compared with the vehicle-treated control group; statistical significance was assessed by Dunnett's multiple comparison test.

Table 1. Results obtained from the liver and the bone marrow micronucleus assays of rats treated with MMC for 14 days.

Test substances	No. of animals	Dose (mg/kg/day)	Liver micronucleus assay			Bone marrow micronucleus assay	
			MNHEPs (%) (mean±SD)	M-phase HEPs (%) (mean±SD)	Ki-67 positive HEPs (%) (mean±SD)	MNIMEs (%) (mean±SD)	IMEs (%) (mean±SD)
Saline	5	-	0.03 ± 0.03	0.17 ± 0.08	0.13 ± 0.07	0.23 ± 0.04	49.5 ± 6.6
MMC	5	0.25	0.34 ± 0.20 **	0.05 ± 0.05 *	0.43 ± 0.41	0.55 ± 0.26 **	38.8 ± 4.7
	5	0.5	0.28 ± 0.14 **	0.14 ± 0.04	0.57 ± 0.37	0.69 ± 0.27 **	34.2 ± 9.0 *
	5	1.0	0.17 ± 0.15 **	0.05 ± 0.06 *	0.21 ± 0.17	2.98 ± 1.32 **	18.0 ± 10.8 **

*P < 0.05 and **P < 0.01 compared with the vehicle-treated control group; the statistical significance of the differences was assessed by the Kastenbaum and Bowman test (MNHEPs and MNIMEs) or Dunnett's multiple comparison test (M-phase HEPs, Ki-67 positive HEPs and IMEs).

Table 2. Results obtained from the liver and the bone marrow micronucleus assays of rats treated with MMC for 28 days.

Test substances	No. of animals	Dose (mg/kg/day)	Liver micronucleus assay			Bone marrow micronucleus assay	
			MNHEPs (%) (mean±SD)	M-phase HEPs (%) (mean±SD)	Ki-67 positive HEPs (%) (mean±SD)	MNIMEs (%) (mean±SD)	IMEs (%) (mean±SD)
Saline	5	-	0.05 ± 0.04	0.06 ± 0.04	0.21 ± 0.08	0.28 ± 0.13	42.7 ± 5.4
	5	0.25	0.19 ± 0.22 **	0.08 ± 0.06	1.93 ± 1.01 **	0.52 ± 0.08 **	36.0 ± 6.3
MMC	5	0.5	0.14 ± 0.11 *	0.07 ± 0.08	0.27 ± 0.22	0.59 ± 0.23 **	25.0 ± 7.3 **
	4	1.0	0.12 ± 0.03 #	0.08 ± 0.08 #	0.26 ± 0.44 #	5.13 ± 4.60 #	27.7 ± 8.7 #

*P < 0.05 and **P < 0.01 compared with the vehicle-treated control group; the statistical significance of the differences was assessed by the Kastenbaum and Bowman test (MNHEPs and MNIMEs) or Dunnett's multiple comparison test (M-phase HEPs, Ki-67 positive HEPs and IMEs).

#Moribund and sacrificed on day 23 (excluded from the statistical analysis because of the number of dosing was different from other groups and the rats were a fatal condition).

Table 3. Histopathological findings in the livers of rats treated with MMC.

Organ	Test substances	Saline	MMC		
Findings	Dose (mg/kg/day)	-	0.25	0.5	1.0
14-day study	No. of animals examined	5	5	5	5
Liver	Grade				
Hypertrophy, bile duct epithelium	-	5	5	5	1
	1+	0	0	0	4
28-day study	No. of animals examined	5	5	5	4[#]
Liver	Grade				
Hypertrophy, bile duct epithelium	-	5	5	5	3
	1+	0	0	0	1
Prominent, hepatocytic nucleolus	-	5	5	5	3
	1+	0	0	0	1

-: none, 1+: minimal.

#Moribund and sacrificed on day 23.

Chapter 2

Prolonged rest period enables the detection of micronucleated hepatocytes in the liver of young adult rats after a single dose of diethylnitrosamine or mitomycin C

1. Introduction

The in vivo micronucleus assay of rodent hematopoietic cells is widely used to assess the genotoxicity of chemicals. However, this assay is less sensitive for carcinogens requiring metabolic activation, such as hepatocarcinogens, than direct acting carcinogens [3]. An micronucleus assay utilizing hepatocytes in the liver would therefore be a useful method of evaluating the genotoxicity of hepatocarcinogens [4]. In addition, the combination of results from micronucleus assays of hepatocytes and hematopoietic cells might improve sensitivity in the evaluation of carcinogen genotoxicity. Based on this concept, liver micronucleus assays have been developed along with liver unscheduled DNA synthesis assays and comet assays. Given that micronuclei form during cell division [5, 6], adult rats are considered unsuitable for the liver micronucleus assay, as hepatocytes have low mitotic activity [7]. As intact adult rats could not be used, researchers instead used young rats (aged 4 weeks) [8-11] or adult rats with partial hepatectomy [7, 12, 13] for the liver micronucleus assay.

An RDLMN assay using young adult rats was recently developed [14, 15] and evaluated in a collaborative study by the MMS group which is a subgroup of the JEMS [16]. This study confirmed the high sensitivity of the RDLMN assay using young adult rats to genotoxic hepatocarcinogens [16]. The accumulation of MNHEPs following repeated dosing and the persistence of MNHEPs for a relatively long period are considered key factors in the successful detection of MNHEPs using this method [17], despite the low mitotic activity of young adult rat hepatocytes.

I hypothesized that not only the accumulation of MNHEPs following repeated dosing but also an adequate period for cell proliferation of chromosomally damaged hepatocytes were key factors in the detection of MNHEPs in an RDLMN assay using

young adult rats. Following a single dose of a genotoxic compound and an adequate period for cell proliferation, MNHEP induction might therefore be detected in young adult rats. In fact, Tates et al. has shown that MNHEPs can be generated several weeks after a single dosing of DEN in adult rats that were partially hepatectomized 3 days before sampling [12]. To confirm this hypothesis, MNHEPs of young adult rats were measured at 14 and 28 days after a single dose of DEN and at 30 days after a single dose of MMC.

2. Materials and methods

2.1. Animals

Five-week-old male Crl:CD (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Rats were housed in an air-conditioned room maintained at 23 ± 3 °C with 55 ± 15% relative humidity under a 12-h/12-h light/dark cycle and allowed free access to a commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Rats were quarantined and acclimated for one week before experiments. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Further, the Kashima facility of Astellas Pharma Inc. was awarded accreditation by the AAALAC International (Frederick, MD, USA).

2.2. Chemicals

DEN (CAS No. 55-18-5, Lot No. PEI3F, ≥99.0% purity) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and MMC (CAS No. 50-07-7, Lot No. 562AAH, 2 mg potency) from Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan). DEN was dissolved in distilled water (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan), and MMC was dissolved in distilled water and diluted with physiological saline (Otsuka Pharmaceutical Factory, Inc.).

2.3. Treatment and dose levels

Five rats per group received a single dose of DEN, MMC and appropriate vehicle. In the experiments using DEN, the rats received a single oral administration of 12.5, 50, and 100 mg/kg of DEN (10 mL/kg of body weight). The highest dose of DEN was 100

mg/kg, which is approximately half of LD_{50} [27]. The lowest dose of DEN was 12.5 mg/kg, at which significant MNHEP induction in a repeated-dosing assay has been reported [14, 17]. In the MMC experiment, rats received a single ip administration of 0.5, 1.0, and 2.0 mg/kg of MMC (5 mL/kg of body weight). The highest dose for MMC was 2.0 mg/kg, which is approximately half of LD_{50} [28]. The lowest dose of MMC was 0.5 mg/kg, at which significant MNHEP induction in the RDLMN assay for 14 and 28 days has been described (Chapter 1).

2.4. Liver micronucleus assay

Hepatocytes were sampled 14 and 28 days after DEN administration, and 30 days after MMC administration (Fig. 2). After fasting overnight, rats were euthanised by exsanguination under anesthesia induced by isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan). Hepatocyte specimens were prepared as previously described [16, 17], with slight modifications. Briefly, approximately 1.0 g of the left lateral lobe of the liver was excised and finely sliced with a razor blade. These slices were rinsed with HBSS (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), transferred to a 50-mL tube, and incubated with HBSS containing 100 units/mL of collagenase (Collagenase Yakult-S; Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) for 1 h at 37 °C with shaking at approximately 50 rpm. Tubes were manually shaken for 1 min at every 30 min. The resulting material was repeatedly pipetted, and tissue fragments were removed via filtration through cotton gauze. Cell suspensions were collected by filtration through a 70 μm-diameter cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and then centrifuged at 500 rpm for 2 min. After the supernatant was discarded, cells were rinsed with 10% neutral-buffered formalin (Sigma-Aldrich Co., St. Louis, MO, USA) and

centrifuged at 500 rpm for 2 min. Supernatant was then discarded, and the hepatocyte pellet was resuspended in 10% neutral-buffered formalin and kept at room temperature until analysis. The hepatocyte suspension (10 μL) was mixed with an equal volume of AO (500 μg/mL; Nacalai Tesque, Inc., Kyoto, Japan) and DAPI (10 μg/mL; Dojindo Laboratories, Kumamoto, Japan) staining solution, dropped onto a glass slide, and covered with a coverslip. The slide was observed using fluorescence microscopy (at 600× magnification with UV excitation), and the number of MNHEPs per 2,000 parenchymal hepatocytes was counted for each rat. MNHEPs were defined as hepatocytes with round or distinct micronuclei stained the same color as the main nuclei and with diameters of one quarter or less than that of the main nuclei [29]. The number of M-phase cells per 2,000 hepatocytes was also counted to determine the MI.

2.5. Statistical analysis

Differences in the incidence of MNHEPs between the test chemical and vehicle control groups were analyzed using Kastenbaum-Bowman tables [25]. Other quantitative data were analyzed using multiple comparison tests.

3. Results

3.1. Diethylnitrosamine

Fig. 3 shows the frequency of MNHEPs and M-phase HEPs 14 and 28 days after a single dose of DEN. The frequencies of MNHEPs showed a statistically significant increase in a dose-dependent manner at 14 and 28 days, with a higher frequency on day 14 than on day 28 for all dosage groups. The frequency of M-phase HEPs was unchanged on both day 14 and 28.

3.2. Mitomycin C

Fig. 4 shows the frequency of MNHEPs and M-phase HEPs 30 days after a single dose of MMC. The frequency of MNHEPs showed statistically significant increase in a dose-dependent manner. The frequency of M-phase HEPs was unchanged.

4. Discussion

The present study demonstrated that prolonged rest period enables the detection of MNHEP induction in the liver of young adult rats even if a single dose of a genotoxic chemical. Tates et al. have already reported single-dose liver micronucleus assays in rats without hepatectomy [12, 30]. In the first of the two studies, adult rats were intraperitoneally administered a single dose of DEN with or without partial hepatectomy, and hepatocytes were isolated from 3 to 60 days after treatment [12]. As the frequency of MNHEPs without partial hepatectomy was similar to that of control groups with partial hepatectomy, the authors concluded that the frequencies of MNHEPs without partial hepatectomy were at control level. However, the frequencies of MNHEPs without partial hepatectomy from 8 to 60 days after treatment were higher than those in control groups without partial hepatectomy [12]. These authors also examined MNHEPs with a single dose of DMN in rats with or without hepatectomy and obtained similar results [30]. Therefore, when the frequencies of MNHEPs in groups without partial hepatectomy were compared to those without partial hepatectomy control group, MNHEP induction could be detected in rats treated with a single dose of both DEN and DMN in previous studies. In addition, the induction of MNHEPs in adult rats exposed to 400-rad X-rays without hepatectomy was observed 9 days after irradiation, but not 3 days after irradiation [31]. This result also shows that MNHEP induction cannot be detected early after an exposure to mutagen in adult rats without partial hepatectomy treatment. The detection of MNHEPs in young adult rats at 2 and 4 weeks after a single dose was therefore considered reasonable.

MNHEP induction in rats at all doses of DEN was higher on day 14 than on day 28 (Fig. 3). Given that the life expectancy of normal hepatocytes is approximately 200

days [32], MNHEPs might be selectively eliminated between 14 and 28 days after induction. Narumi et al. also reported that hepatocytes with micronuclei disappear more frequently than those without [17]. They administered DEN to young adult rats at single and repeated doses for 7- and 14-day periods with a single injection of EdU at the initial administration and reported that the frequency of EdU-labeled MNHEPs subsequently decreased by approximately 35% of the initial frequency after 1 week [17]. Further, findings from an in vitro live cell imaging study suggested that the rate of micronucleated cell death was approximately 2.5 times that of cells without micronuclei [6].

The dose-dependent induction of MNHEPs in MMC-treated rats after 30 days is shown in Fig. 4. However, the frequency of MNHEPs was decreased at higher doses in my previous RDLMN assay treated with 0.25, 0.5, and 1.0 mg/kg of MMC for both 2 and 4 weeks. The difference between these two results might be due to suppression of hepatocyte cell division observed at high doses in the RDLMN assay (Chapter 1). Repeated dosing might therefore not be appropriate for the detection of MNHEPs induced by compounds that might suppress hepatocyte cell division, such as MMC.

Twenty-two chemicals were recently tested in 14- or 28-day RDLMN assays, with the induction of MNHEPs showing good correlation with hepatocarcinogenicity [16]. The RDLMN assay might therefore be established as a regulatory genotoxicity test in which a concurrent positive control group was required to be included. The RDLMN assay was discussed in the liver micronucleus test working group at the 6th IWGT, and a duration of dosing in a positive control group was listed as a future issue to address [4]. Present study shows that rats receiving a single dose of DEN sampled after both 14- and 28-day of rest period can be used as positive controls in RDLMN assays. Given that frequent dosing of a positive control chemical such as a mutagen is a burden to both

researchers and animals, I recommend a single oral administration of 50 mg/kg of DEN with a 14- and 28-day rest period as a positive control in the RDLMN assay. This method appears more suitable in terms of labor intensity and animal welfare than repeated dosing of DEN.

5. Summary

A repeated-dose micronucleus assay of young adult rat hepatocytes was recently developed to evaluate the genotoxicity. In this assay, accumulation of MNHEPs induced by repeated dosing of genotoxic chemicals is considered a key factor in the detection of micronuclei induction. Then, I hypothesized that the period following chemical exposure enabled the detection of MNHEP induction in young adult rats, namely that MNHEPs could be generated from chromosomally damaged cells and accumulated following initiation of chemical exposure until sampling. I therefore measured MNHEP induction at 2 and 4 weeks after a single oral administration of 12.5, 50, and 100 mg/kg of DEN and an ip administration of 0.5, 1.0, and 2.0 mg/kg of MMC to young adult rats. Results showed a statistically significant and dose-dependent increase in the number of MNHEPs in both DEN- and MMC-treated rats, indicating that prolonged rest period following a single dose of a genotoxic chemical enables the detection of MNHEP induction in the liver of young adult rats. From these results, a single oral administration of 50 mg/kg of DEN with a 2- and 4- week rest period can be used as a positive control in RDLMN assays. This procedure is superior in terms of labor saving and animal welfare to repeated dosing of DEN.

6. Figures and tables

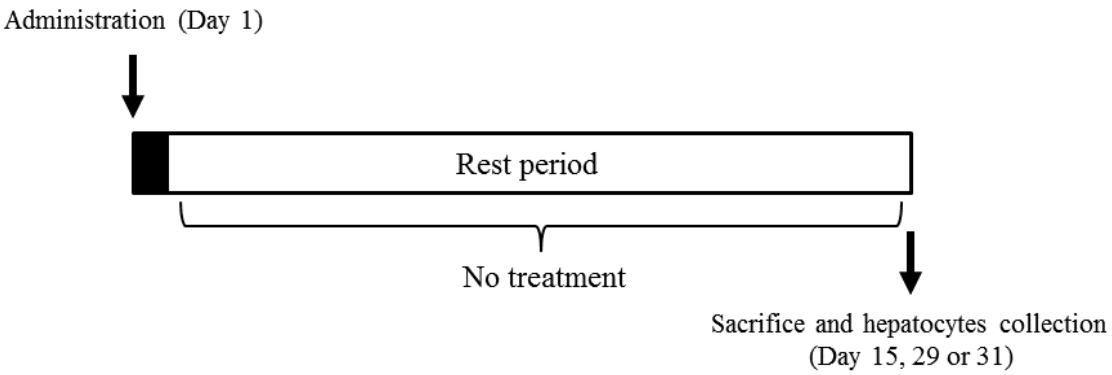

Fig.2. Experimental design.

Each rat received a single dose of either vehicle, DEN or MMC. After dosing, the rats were housed for 2 and 4 weeks (rest period). After a rest period, the rats were sacrificed and hepatocytes were collected to measure the frequency of MNHEP induction.

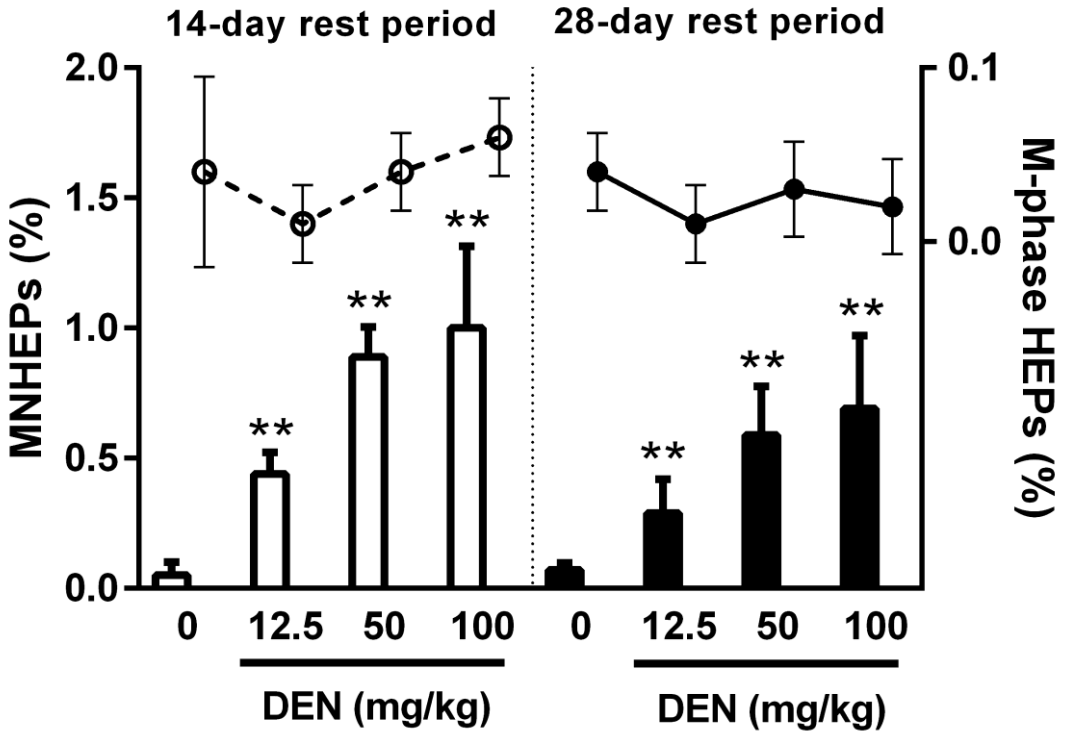

Fig. 3. Frequency of MNHEPs (bars) and M-phase HEPs (lines) in rats treated with a single oral administration of DEN. Hepatocyte specimens were prepared after a 14- (open column and circle) and 28-day (closed column and circle) rest period. Values are presented as mean ± SD (n=5) **P<0.01 compared with the vehicle-treated control group. Statistical significance was assessed by the Kastenbaum and Bowman test.

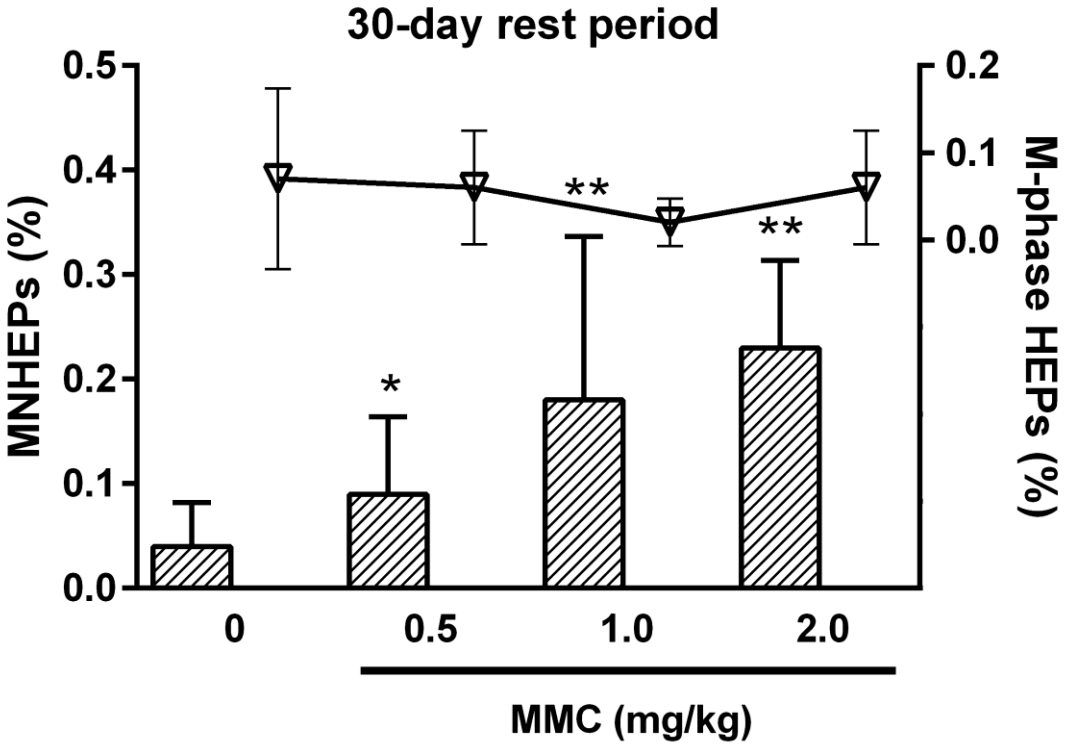

Fig. 4. Frequency of MNHEPs (bars) and M-phase HEPs (lines) in rats treated with a single ip administration of MMC. Hepatocyte specimens were prepared after a 30-day rest period. Values are presented as the mean ± SD (n=5) *P<0.05 and **P<0.01 compared with the vehicle-treated control group. Statistical significance was assessed by the Kastenbaum and Bowman test.

Conclusion

The RDLMN assay was developed as a novel genotoxicity test that can detect carcinogens requires metabolic activation and integrate into general toxicity studies. I participated in the collaborative study for evaluating the RDLMN assay. In this study, MMC was repeatedly administered to young adult male rats for 14 and 28 days, and the frequencies of MNHEPs were determined. This study revealed that repeated administration of MMC induced micronuclei in rat hepatocytes. In addition, this collaborative study showed the high sensitivity of the RDLMN assay to hepatocarcinogens, which were reported negative in the micronucleus assay using hematopoietic cells [16]. This method was discussed at the 6th IWGT in reaction to this result and the working group concluded that liver micronucleus assay including the RDLMN assay could be used as a second in vivo test when a relevant positive result in in vitro mammalian cell genotoxicity tests is noted, and a negative result is observed in the in vivo micronucleus test using hematopoietic cells [4]. Therefore, the formulation of the guideline of the RDLMN assay and further use of the RDLMN assay as a regulatory test are expected in the future.

However, dose-dependent MNHEPs induction was not observed in the RDLMN assay using MMC, despite dose-dependent reduction of body weight and increase of MNIMEs were observed. Because micronuclei are formed during cell division [5, 6], they cannot be detected if the cell division was stopped. MMC is a well-known anticancer agent [19] that forms interstrand DNA cross-links [20], resulting in cytotoxicity, mutagenicity and mitotic inhibition [33]. Then I hypothesized that suppression of cell division caused by MMC was the reason for the reduction of induced MNHEPs observed

at higher doses. The frequency of proliferating hepatocytes, as assessed by Ki-67 positivity, was decreased at the highest dose, as was the frequency of MNHEPs. Therefore, the decreased induction of MNHEPs in the high-dose groups might be caused by suppression of hepatocyte cell division caused by MMC.

It has been considered that liver micronucleus assay using adult rats cannot detect the genotoxicity due to low mitotic activity. However, it could be detected in the RDLMN assay that used non-hepatectomized young adult rats [14-16]. The accumulation of MNHEPs following repeated dosing and the persistence of MNHEPs were thought to be key factors in the successful detection of MNHEPs using this method [17]. However, I have hypothesized that an adequate period for cell proliferation of chromosomally damaged hepatocytes is also a key factor in the detection of MNHEPs in the RDLMN assay using young adult rats. In other words, I have considered that MNHEPs induction can be detected in young adult rats following an adequate period for cell proliferation until sampling even after only a single dose.

To confirm this hypothesis, MNHEPs of young adult rats were measured at 2 and 4 weeks after a single dose of both DEN and MMC. As a result, induction of MNHEPs were shown in both DEN- and MMC-treated young adult rats. These results demonstrated that a prolonged rest period enables the detection of MNHEP induction in the liver of young adult rats even if a single dose of a genotoxic chemical. In addition, a dose-dependent induction of MNHEPs in the rats received a single-dose of MMC was observed, despite a dose-independent induction was confirmed in the RDLMN assay. Therefore, repeated dosing might not be appropriate for the detection of MNHEPs induced by compounds that suppress hepatocyte cell division, such as MMC.

If the RDLMN assay was established as a regulatory genotoxicity test, a

concurrent positive control group was required to be included. Present study showed that rats receiving a single dose of DEN sampled after 14- and 28- day of rest period can be used as positive controls in the RDLMN assays. Given that frequent dosing of a positive control chemical such as a mutagen is a burden to both researchers and animals, I recommend a single oral administration of 50 mg/kg of DEN with a 14- and 28-day rest period as a positive control in the RDLMN assay. This method appears more suitable for a positive control in terms of labor intensity and animal welfare than repeated dosing of DEN.

Although further study is needed, single-dose liver micronucleus assay with a 14-day rest period using young adult rats may also be developed as a regulatory genotoxicity test. As this method can integrate into a single dose acute toxicity test, this method is also reasonable in the view of cost and labor saving as well as animal welfare. I hope the liver micronucleus assays are used as a regulatory genotoxicity test and become an excellent assay for detecting genotoxic hepatocarcinogens.

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Summary in Japanese

成熟ラットを用いた肝臓小核試験における小核を有する肝細胞の検出に関する研究

げっ歯目の造血細胞を用いる in vivo 骨髄小核試験は化合物の遺伝毒性を評価するために広く用いられている。しかしこの試験は肝発がん物質のような代謝活性化を受けて発がん性を示す化合物の検出には適していない。肝臓小核試験は代謝活性化体が発がん性を示す化合物の遺伝毒性評価に優れており，さらに骨髄小核試験と肝臓小核試験の結果を組み合わせることは発がん物質の検出力を上げることになると考えられてきた。しかし小核は細胞分裂の際に形成され，細胞分裂頻度が低い成熟ラットの肝細胞を用いて小核を観察することは不適切であると考えられてきたため，細胞分裂頻度が高い幼若ラットや部分肝切除を施した成熟ラットを用いた肝臓小核試験が開発されてきた。しかしこれらの試験は代謝が成熟動物とは異なる幼若動物や，外科手術を必要とする部分肝切除を施した動物を用いるため遺伝毒性試験として広く使用されることはなかった。さらにこれらは一般毒性試験に組込むことができず，またコラゲナーゼ灌流という方法を用いて肝細胞を採取していたため病理学的検査を施すことができなかった。これらの問題点を解決するためコラゲナーゼ灌流をおこなわない，成熟ラットを用いた反復投与肝臓小核試験が開発された。この新しい肝臓小核試験の有用性を確かめるため，MMC 反復投与による肝臓小核試験を実施した。

MMC を 14 及び 28 日間，0.25, 0.5, 1.0 mg/kg/day の用量で雄成熟ラットに反復投与し，肝臓及び骨髄小核試験を実施した。幼若赤血球を用いる骨髄小核試験では用量依存的な小核の誘発が観察されたが，肝臓では陰性対照群と比較して有意な小核の誘発が検出されたものの用量依存性は認められなかった。また用量依存的な体重増加抑制及び体重減少は

認められたものの，病理学的検査では肝毒性を示す所見は観察されなかった。これらの結果から MMC の反復投与により小核の誘発は検出されたが，用量依存性が見られなかったのは MMC による細胞分裂抑制に原因があるのではないかと考え，抗 Ki-67 抗体を用いた免疫組織学的検査をおこなったところ，高用量群で細胞増殖頻度が低下していた。そのため MMC 反復投与肝臓小核試験において，肝小核の誘発に用量依存性が認められなかったのは MMC による細胞分裂の抑制によるものである可能性が考えられた。

成熟動物は細胞分裂頻度が低いため肝臓小核試験には向いていないと考えられていたが，反復投与肝臓小核試験では成熟ラットを用いているにも関わらず肝小核の検出が可能であった。これについて反復投与により生じた小核を有する肝細胞の蓄積と小核を有する肝細胞が肝臓内にとどまり続けることが反復投与肝臓小核試験において肝小核の検出が可能となった理由と考えられてきた。しかし染色体に損傷を受けた肝細胞が細胞分裂をするまでに十分な期間があったことも反復投与肝臓小核試験において肝小核の検出が可能となった原因の 1 つであるとも考えられた。つまり単回投与であっても細胞分裂がおこなわれるまでの十分な期間を設けた後に肝細胞を採取すれば，成熟ラットの肝小核の誘発が検出できるのではないかと考えた。

この仮説を確かめるために DEN 及び MMC を単回投与後，2 及び 4 週間の休薬期間を置いたのちに肝臓小核試験を実施した。その結果，DEN 及び MMC ともに陰性対照群と比較して有意な肝小核の誘発が観察された。この結果から成熟ラットにおいても休薬期間を置くことで単回の遺伝毒性物質の投与により肝小核の誘発が検出できることが明らかになった。さらに反復投与肝臓小核試験では用量依存性が見られなかった MMC においても，単回投与後に休薬期間を置くことで用量依存的な小核の誘発が観察された。従って細胞分裂頻度を抑制する MMC のような遺伝毒性物質の評価には反復投与肝臓小核試験は向いておらず，単回投与後に休薬期間を置く肝臓小核試験の方が適しているのかもしれない。

反復投与肝臓小核試験が遺伝毒性試験として確立されるためには適切な陽性対照

群の設置が必要となってくる。今までは陽性対照群に関する議論はあまりされてこなかったが，今回の研究の結果から DEN 単回投与後に 14 及び 28 日間の休薬期間を置いた群は反復投与肝臓小核試験の陽性対照群として使用できることが明らかになった。陽性対照として遺伝毒性物質を反復投与することは試験実施者にとっても実験動物に対しても負担となるため，50 mg/kg の用量の DEN を単回投与したのち休薬期間を置いた群を反復投与肝臓小核試験の陽性対照群として用いることを推奨したい。

さらなる研究が必要となることではあるが，単回投与後に 14 日間の休薬期間を置いた成熟ラットを用いた肝臓小核試験もまた遺伝毒性試験として開発することが可能であるかもしれない。この方法は通常 14 日間の休薬期間を設ける単回投与急性毒性試験に組込むことが可能であるため，コストと労力さらには動物福祉の点からも合理的であると考えられる。どちらにせよ肝臓小核試験が遺伝毒性試験の 1 つとして広く用いられ，遺伝毒性肝発がん物質の検出のために使われていき，安全な医薬品開発の一助となることを望む。