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Developmental Study of Repeated-Dose Liver Micronucleus Assay Using Adult Rats

(Kazunori Narumi)

2013
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Kazunori Narumi
## List of Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole dihydrochloride</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>BrdU</td>
<td>bromo-deoxyuridine</td>
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<tr>
<td>CSGMT</td>
<td>Collaborative Study Group for the Micronucleus Test</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>2, 4-DAT</td>
<td>2, 4-diaminotoluene</td>
</tr>
<tr>
<td>2, 6-DAT</td>
<td>2, 6-diaminotoluene</td>
</tr>
<tr>
<td>DEN</td>
<td>diethyl nitrosamine</td>
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<tr>
<td>DMH</td>
<td>1, 2-dimethylhydrazine</td>
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<tr>
<td>ECA</td>
<td>European Chemicals Agency</td>
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<tr>
<td>ECVAM</td>
<td>European Centre for the Validation of Alternative Methods</td>
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<tr>
<td>EdU</td>
<td>5-ethynyl-2’-deoxyuridine</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol-bis (beta-aminoethyl ether) -N, N, N', N'-tetraacetic acid</td>
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<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
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<tr>
<td>GST-P</td>
<td>Glutathione S-transferase placental form</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
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<tr>
<td>HEP(s)</td>
<td>parenchymal hepatocyte(s)</td>
</tr>
<tr>
<td>IARC</td>
<td>The International Agency for Research on Cancer</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
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<tr>
<td>IκB</td>
<td>inhibitor of kappa B</td>
</tr>
<tr>
<td>JEMS</td>
<td>Japanese Environmental Mutagen Society</td>
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<tr>
<td>MFD</td>
<td>maximum feasible dose</td>
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<td>MI</td>
<td>mitotic index</td>
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<td>MMC</td>
<td>Mitomycin</td>
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<td>MMS</td>
<td>Mammalian Mutagenesis Study subgroup</td>
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MN : micronucleus
MNHEP(s) : micronucleated hepatocyte(s)
MTD : maximal tolerated dose
M-phase : metaphase
NF-κB : nuclear factor kappa B
NTP : National Toxicology Program
OECD : Organisation for Economic Co-operation and Development
Ogg1 : 8-oxo-G-DNA glycosylase 1
8-oxo-G : 8-oxoguanine
PB : peripheral blood
PH : partial hepatectomy
RDLMN : repeated-dose liver micronucleus
RDT : repeated-dose toxicity
REACH : Registration, Evaluation, Authorization and Restriction of Chemicals
TUNEL : terminal deoxynucleotidyl transferase-mediated nick end-labeling
UDS : unscheduled DNA synthesis
UKCOM : United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
WOE : weight of evidence
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Chapter 1

General Introduction
1.1. Genotoxicity study

Industrial processes have resulted in the production of various beneficial substances. However, problems such as side effects of pharmaceuticals, food contamination, or environmental pollution by pesticides or industrial discharges have also increased. In developed countries, people encounter thousands of chemicals every day, but few of these chemicals have been subjected to significant toxicological evaluation. Thus, safety or toxicity tests are mandatory and very important before placing the products on the market.

Regulations governing the use of chemicals exist to protect humans against the probable dangers of drugs and medicine, ensure the safety of foods and beverages and the occupational safety of workers during chemical production, and protect the people and the environment against damages from chemical residues such as pesticides. Registration or production of various types of materials and products requires comprehensive assessment of their genotoxicity (Blakey et al., 2008). “Genotoxicity” refers to the ability of substances to damage DNA and cell components that regulate the fidelity of the genome, such as the spindle apparatus, topoisomerases, DNA repair systems, and DNA polymerases, and includes all adverse effects on genetic information (United Kingdom Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment [UKCOM], 2000). Safety and genotoxicity evaluations of substances are generally based on a combination of tests that assess the effects of the 3 major types of genetic damage associated with human disease: gene mutation (i.e., point mutations or deletions/insertions affecting single or blocks of genes),
clastogenicity (i.e., structural chromosome changes), and aneuploidy (i.e., numerical chromosome aberrations). Extensive studies have shown that no single test is capable of detecting all the genotoxic mechanisms or genotoxic substances (Kramer, 1998). While some genotoxicants preferentially induce gene mutations through base pair substitutions or frameshift mechanisms, others may induce chromosome mutations. The existence of different mechanisms of genotoxicity has necessitated regulatory institutions to define standard test batteries to include complementary tests. For instance, the standard batteries for genotoxicity testing of human pharmaceuticals defined by the International Conference on Harmonisation (ICH) guidance on genotoxicity testing [S2(R1) guideline, ICH, 2011] includes 2 options.

**Option 1**

i. Test for gene mutation in bacteria.

ii. Cytogenetic test for chromosomal damage (*in vitro* metaphase chromosome aberration test or *in vitro* micronucleus test), or *in vitro* mouse lymphoma *Tk* gene mutation assay.

iii. *In vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

**Option 2**

i. Test for gene mutation in bacteria.

ii. *In vivo* assessment of genotoxicity by using 2 different tissues, typically, an assay for micronuclei by using rodent hematopoietic cells and a second *in vivo* assay.
Guidelines for the different types of products follow almost the same battery as for the Option 1 of the ICH S2(R1) guideline.

Compounds showing positive results in genotoxicity tests that detect such damage have the potential to be human carcinogens or mutagens (Müller, 1998; UKCOM, 2000). While the relationship between exposure to particular chemicals and carcinogenesis has been established in humans, these relationships are difficult to prove for heritable diseases; therefore, genotoxicity tests are primarily used to predict carcinogenicity (Brambilla and Martelli, 2003; Kirkland et al., 2005, 2006; Matthews et al., 2006). Results of comparative studies have shown that qualitatively, most germ cell mutagens are likely to be detected as genotoxic in somatic cell tests, and negative results of in vivo somatic cell genotoxicity tests generally indicate the absence of germ cell effects (Dearfield et al., 2002). Nevertheless, because germline mutations are associated with human disease, the suspicion that a compound induces heritable effects is considered as serious as the suspicion that a compound induces cancer. Additionally, the outcomes of genotoxicity tests are valuable for interpreting carcinogenicity studies.

1.2. Liver micronucleus assay

In vitro and in vivo tests measuring chromosomal aberrations in metaphase cells can be used to detect a wide spectrum of changes in chromosomal integrity. Breakage of chromatids or chromosomes can result in micronucleus (MN) formation if an acentric fragment is produced; therefore, assays detecting either chromosomal aberrations or
micronuclei can be used to detect clastogens. Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase; thus, MN tests have the potential to detect some aneuploidy inducers.

In regulatory testing, the rodent erythrocyte MN assay is the most common in vivo assay (UKCOM, 2000; ICH, 2011). The MN assay is a simple method that uses either bone marrow (BM) or peripheral blood (PB) to assess cytogenetic damage in vivo. The hematopoietic system has 2 important characteristics: (i) the ease of scoring MN in the newly formed nucleate reticulocytes derived from BM and (ii) the ability to identify the newly formed erythrocytes that completed their last division during or shortly after exposure to the test compound as those staining positive for RNA content (Heddle, 1973; Schmid, 1975). These important advantages led researchers to focus on the hematopoietic cells as targets.

Positive responses in the rodent erythrocyte MN assay are well correlated with carcinogenicity in multiple organs (Morita et al., 1997). However, it is well known that some compounds require metabolic activation in the liver, and it has been shown that some pro-mutagens elicit a negative response in the BM MN assay. Some active metabolites may have a very short lifespan and do not reach BM at sufficient concentrations to induce micronuclei formation. In fact, some rodent liver carcinogens, including di-alkyl-nitrosamines, nitro aromatic compounds, and azo derivatives, showed negative results in the BM assay (Angelosanto, 1995). This led Mirsalis et al. to develop an in vivo rat-liver assay for unscheduled DNA synthesis (UDS) (Mirsalis and Butterworth, 1980). Until recently, the UDS test was the assay of choice for investigating genotoxicity in the liver (Organisation for
Economic Co-operation and Development [OECD], 1997). However, the liver UDS test is now viewed as not sufficiently sensitive for detecting a range of rodent carcinogens that cannot be detected in the in vivo erythrocyte MN test (Kirkland and Speit, 2008), and recently, the alkaline single-cell gel electrophoresis assay (also known as the Comet assay) has been increasingly used to evaluate liver genotoxicity of different product classes (Eastmond et al., 2009; ICH, 2011; UKCOM, 2011). However, both the UDS assay and comet assay are used to detect DNA damage; therefore, it is important to evaluate MN inducibility in tissues that are relevant sites for distribution and metabolism for follow-up assays when the in vitro cytogenetic assay(s) results are positive.

Recently, various investigators have attempted to develop a liver MN assay. Since the liver of mature animals is not a highly mitotic tissue (MacDonald, 1961), detecting induced micronuclei in short-term exposure experiments requires artificial mitotic activation. Tates et al. reported a method involving partial hepatectomy (PH) before or after chemical treatment for the liver micronucleus assay (Tates et al., 1980, 1983, 1986). Division of hepatocytes is stimulated by PH, and positive responses were obtained following the induction of micronuclei in liver cells after treatment with hepatocarcinogens that showed negative results in the erythrocyte assay. Other investigators also evaluated chemical clastogenicity potential in the liver by using the PH method (Cliet et al., 1989). There are, however, 2 shortcomings of the PH method: (1) technically, it is not easy to perform successful hepatectomy on all animals used in the assay and (2) it has been reported that cytochrome P450 (CYP), styrene mono-oxygenase, epoxide hydrolase, and
glutathione-S-epoxide transferase activities decreased by 50% or more 12 h after PH (Mereto et al., 1994; Rossi et al., 1987; Roy and Das, 1990; Zhurkov et al., 1996). In another method, 4-acetyl aminofluorene, a mitogen for liver cells, was used to activate cell proliferation (Braithwaite and Ashby, 1988). However, the possibility of interaction with the test chemical should be considered. An *in vivo*/*in vitro* assay system has also been reported, in which after treatment of the animals by using test chemicals *in vivo*, the hepatocytes were collected and primary cell cultures were established using growth factors before harvesting cells for slide preparation (Sawada et al., 1991). However, this *in vivo*/*in vitro* method is labor-intensive, costly, and time consuming; thus, it is not widely used. Suzuki et al. developed a liver MN assay by using 4-week-old rats (Suzuki et al., 2004, 2005, 2009; Udroiu et al., 2006; Takasawa et al., 2010a, b). This method does not require physical injury or chemical treatment, and utilizes the developmental proliferation of hepatocytes in young rats for up to approximately 5 weeks of age. The metabolic capacity of young rats in this age is comparable to that of adult rats (Furner et al., 1969; Imaoka et al., 1991). However, the expression of some enzymes, such as those of the CYP species, including CYP 2C7, 2C11, 2C12, and 2C22, have not been completely confirmed to exert activity in young rats (Kato and Yamazoe, 1992); thus, this assay must be performed in adult rats.

The methods described above have been shown to be very useful for predicting genotoxic hepatocarcinogenicity and are used on a case-by-case basis, but they have not been used routinely to evaluate genotoxicity of chemicals due to their experimental difficulty, labor-intensiveness, and abnormal metabolic conditions required in the
liver.

1.3. Integration of liver micronucleus assay into general toxicity studies

In vivo studies will continue to be important for risk characterization because conditions occurring in vivo, such as exposure and metabolism, are more applicable to humans than conditions in cultured cells. Depending on the regulatory requirements in place for different product classes, such as pharmaceuticals, industrial chemicals, agrochemicals, and consumer products, in vivo genetic toxicity tests are conducted as an integral part of a test battery and to follow-up on positive results from in vitro genotoxicity testing. Historically, in vivo genetic toxicity studies have been conducted as stand-alone studies involving 1–3 acute administrations up to the maximal tolerated dose (MTD).

However, there is increasing interest in implementing the “3 Rs,” the concepts of reduction, replacement, and refinement of animal use in regulatory toxicity testing, including in the genetic toxicology field. In some areas of product development, such as cosmetics, the European Union (EU) and national animal protection laws have already enforced complete replacement of acute in vivo genotoxicity tests (EU, 2003). Moreover, refinements to in vivo genotoxicity tests have been proposed in several recent guidelines for pharmaceuticals and chemicals, and expert recommendations for genotoxicity testing:
Within the EU Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) legislation, refinements of in vivo genotoxicity testing are outlined in the Integrated Testing Strategy guidance and possible integration of genotoxicity endpoints into repeated-dose toxicity (RDT) studies, if scientifically justified (European Chemicals Agency [ECA], 2012).

The ICH S2(R1) guideline encourages integration of in vivo genotoxicity endpoints into RDT studies, if scientifically justified, although acute regimens are also acceptable. When more than one endpoint is evaluated in vivo, it is preferable that they are incorporated into a single study, when possible (ICH, 2011).

A recent workshop attended by genotoxicity experts from academia, regulatory agencies, and industry, and hosted by the European Centre for the Validation of Alternative Methods (ECVAM), identified several options for reducing animal use in genotoxicity testing (Pfuhler et al., 2009). These options include the combining the acute in vivo MN and Comet assays into a single study as well as integrating the MN endpoint into RDT studies.

Integrating genetic toxicology assays into RDT studies has considerable potential benefits, such as providing concurrent information on toxicokinetics, histopathology, and clinical pathology, which may aid in the interpretation of genotoxicity study results. The RDT study is designed for use with rats (OECD, 1995). Rat splenic function preferentially eliminates micronucleated erythrocytes from the
circulating peripheral blood (Schlegel and MacGregor, 1984), unlike the mouse in which micronucleated erythrocytes accumulate and reach a steady state. Thus, the rat erythrocyte micronucleus assay, in which the sensitivity depends on the maximum plasma concentration of the test compound, is not always appropriate for integration into RDT studies. Since RDT studies often employ lower top doses than those used in acute studies, a concern that has been raised regarding integrating genotoxicity endpoints into RDT studies, such as whether sensitivity is reduced. For compounds that are very toxic after repeated dosing, but for which a single dose is well tolerated, there is a concern that sufficiently high doses cannot be achieved in an RDT study. In a study by Hamada et al., 4 of 15 genotoxins: benzo[a]pyren, 1, 2-dimethylhydrazine (DMH), Mitomycin, and monocrotaline were positive in the rat erythrocyte MN test after acute but not after 4-week treatment, presumably because MTD after 28 days was significantly lower than that for the acute dosing (Hamada et al., 2001). If in vitro genotoxicity results are unavailable or positive, the requirements for designing in vivo studies become more stringent in order to assure the sensitivity of the study.

Based on the characteristics of the rat erythrocyte MN test, the ICH S2(R1) guideline defined the top dose in RDT studies for integrating genotoxicity endpoints when in vitro mammalian cell genotoxicity data are positive or not available. Specifically, criteria rendering the RDT study sufficient for assessment of genotoxicity included the following: (1) maximum feasible dose, based on the physical properties of the compound, or (2) plasma exposure plateau with increasing dose, or an accumulation of the compound with repeated
dosing, or (3) a limit dose of 1000 mg/kg/day, in accordance with general toxicology guidance (for 14 days or longer), or (4) a dose that is at least half of the top dose to be used in an acute-regimen assay, if acute toxicity data are available. However, MTD, defined by the typical criteria used in toxicology, is not considered a sufficient criterion for allowing pharmaceutical integration.

The liver is a key site of xenobiotic metabolism and accumulation in vivo, and thus, the repeat-dose MN method, which appears to be applicable in adult rats, is an important assay that should be incorporated into general toxicological studies. However, previously reported liver MN assays are typically performed using in situ liver perfusion via the portal vein or vena cava to isolate hepatocytes. Liver perfusion precludes conducting other tests that are commonly performed as part of general toxicology studies, such as histopathological examination. Importantly, previously described liver assays cannot be conducted using intact adult rat liver; therefore, integration into general RDT studies not possible.

In the present study, I focused on the accumulation effects on micronucleated hepatocytes (MNHEPs) since hepatocytes in the adult rat liver have a long life span (at least 200 days) and slow turnover. In Chapter 2, the rodent hepatocarcinogens diethylnitrosamine (DEN) and 2, 4-diaminotoluene (2, 4-DAT) were investigated using the repeated administration protocol, as well as a new method without the use of in situ perfusion to overcome the limitations of in situ liver perfusion. In Chapter 3, I directly observed the long-term persistence of MNHEPs in the liver by pulse-labeling rat hepatocytes with the thymidine analog 5-ethynyl-2’-deoxyuridine (EdU), followed by chasing during a period of

11
repeated administration of DEN. Additionally, to confirm the cumulative effect of MNHEPs, a theoretical model of MNHEP frequencies was constructed and verified against the actual values measured in the micronucleus test.
Chapter 2

Development of a Repeated-Dose Liver MN Assay Using Adult Rats: An Investigation of Diethylnitrosamine and 2, 4-Diaminotoluene
2.1. INTRODUCTION

Liver MN assays are highly sensitive in predicting hepatocarcinogenicity and useful in evaluating genotoxic substances that target the liver (Angelosanto, 1995; Braithwaite and Ashby, 1988; Clet et al., 1989; Suzuki et al., 2005, 2009; Takasawa et al., 2010a, b; Zhurkov et al., 1996). Because adult rat hepatocytes proliferate slowly, the detection of induced micronuclei in short-term exposure experiments requires mitotic activation by PH (Tates et al., 1980, 1983, 1986) or mitogen treatment (Braithwaite and Ashby, 1988). These procedures have the disadvantages of reducing drug metabolism activity (Rossi et al., 1987), requiring intensive labor such as the implementation of a surgical operation, and a possible interaction between the mitogen and the substance (Parton and Garriott, 1997). To resolve these challenging issues, an assay utilizing autonomous proliferation of young (4 weeks old) rat hepatocytes has been developed (Suzuki et al., 2004). This assay does not require any physical injury or chemical treatment, and young rats have been reported to be similar to adult animals with respect to metabolic enzyme profiles (Furner et al., 1969; Imaoka et al., 1991). However, some enzymes, such as those of the P450 2C family, do not completely exert their activity in juvenile animals (Kato and Yamazoe, 1992), and the need to perform assays in adult animals still exists.

Recent international guidelines, such as the ICH S2(R1) guideline recommends the integration of in vivo genotoxicity endpoints into routine general toxicity studies with the aim of reducing the number of experimental animals used (ICH, 2011). If the evaluation of the induction of micronuclei in hematopoietic cells or hepatocytes is
possible in rat RDT test, it will be extremely useful not only for animal number reduction but also for overall evaluation for toxicity. In most liver MN assays that have been established, the entire liver is perfused with collagenase for hepatocyte isolation, which prevents the evaluation of multiple endpoints in the same organ.

In this study, the focus was on the fact that hepatocytes in the adult rat liver have a long life span (at least 200 days) and show a slow turnover (MacDonald, 1961), and it was examined whether repeated administration of hepatocarcinogens at relatively low doses causes an accumulation of MNHEPs. At the same time, it was attempted to establish a method of hepatocyte isolation from a portion of the liver without *in situ* perfusion to be able to integrate liver MN assays into general toxicity studies.
2.2. MATERIALS AND METHODS

2.2.1. Animals

Male Crl:CD (SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan) and were aged six weeks and weighed approximately 200–250 g at the beginning of the experiments. They were housed two to three per cage in an air-conditioned room with a 12-h light/dark cycle and free access to food and drinking water. The experimental protocol was approved by the Institutional Animal Care and Use Committee prior to its implementation.

2.2.2. Chemicals

DEN (CAS No. 55-18-5, >99.0% purity) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 2, 4-DAT (CAS No. 95-80-7, >95.0% purity) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Both substances were dissolved in physiological saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) before use.

2.2.3. Dose levels and treatment

The high doses for DEN (12.5 mg/kg/day) and 2, 4-DAT (50 mg/kg/day) were set at the lowest and slightly lower dose levels, respectively, at which the test results for these chemicals were positive in young rat liver MN assays (Takasawa et al., 2010b). The low doses for
DEN (6.25 mg/kg/day) and 2, 4-DAT (25 mg/kg/day) were set at a half of the high doses. Animals were divided into groups of 5 and received the test chemicals or the vehicle alone by oral gavage (10 mL/kg of body weight volumes) once a day for 5, 14, or 28 consecutive days.

2.2.4. Liver MN assay

Twenty-four hours after the last administration for each time point, rats were euthanized under thiopental anesthesia. Various conditions for hepatocyte isolation were investigated, and it was decided to employ the procedures described as follows, without using the perfusion method. First, about one-third of the left lateral liver lobe was excised and minced into 2–3 mm$^3$ pieces using a razor blade. These pieces were rinsed with Hanks’ balanced salt solution (HBSS; GIBCO-Invitrogen, Carlsbad, CA, USA) and treated with HBSS containing 0.05% collagenase (Wako Pure Chemical Industries, Ltd.) in a screw-capped tube with shaking at 37°C for 1 h, and then they were strongly shaken by hand approximately 50 times every 30 min. Next, the resulting material was repeatedly pipetted approximately 50 times vigorously and forced through a cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ, USA). The obtained cell suspension was then rinsed with 10% neutral buffered formalin and centrifuged at 50 g for 1 min. Finally, the hepatocyte pellet was resuspended in 10% neutral buffered formalin and kept in a refrigerator until analysis. Immediately before observation, 10 µL of the hepatocyte suspension was mixed with an equal volume of an acridine orange (AO: 0.5 mg/mL) and 4’, 6-diamidino-2-phenylindole dihydrochloride (DAPI: 10 µg/mL)
staining solution, dropped onto a glass slide, and covered with a coverslip (24 × 40 mm) (Suzuki et al., 2004, 2005, 2009; Takasawa et al., 2010a, b).

The slide preparation was observed by fluorescence microscopy (at 400× magnification with UV excitation), and the number of MNHEPs per 2000 parenchymal hepatocytes (HEPs), including mono-, bi-, and multi-nucleate cells, was counted for each animal. MNHEPs were identified as HEPs with round or distinct micronuclei stained with the same color as the nuclei and with diameters of 1/4 or less than those of the main nuclei (Braithwaite and Ashby, 1988; Cliet et al., 1989). MNHEPs were confirmed by focusing up and down while taking into account the thickness of the HEPs. The number of metaphase (M-phase) cells among the 2000 HEPs was also counted to determine the mitotic index (MI). M-phase cells were defined as those in a division stage from prophase to telophase, with a poorly defined nuclear envelope, identifiable chromosomes and two unevenly shaped nuclei that strongly fluoresced (Suzuki et al., 2004, 2005, 2009; Takasawa et al., 2010a, b).

2.2.5. Statistical analyses

Differences in the incidence of MNHEPs between the test groups and the vehicle control were analyzed statistically using Kastenbaum and Bowman’s tables (Kastenbaum and Bowman, 1970).
2.3. **RESULTS**

2.3.1. **Perfusion-independent hepatocyte isolation method**

First, the conditions under which hepatocytes could be isolated without using the *in situ* perfusion method were investigated, and cell samples suitable for MN observation were obtained (Figures 1a–d and 2). When the specimens were homogenized with a Dounce homogenizer (loose pestle) or minced into very small pieces (<1 mm$^3$), the cytoplasms were damaged and the cells aggregated (Figure 1a and b). These cells were therefore not appropriate for observation because the border of individual cells was unclear. The optimum size was considered to be 2–3 mm$^3$. In this condition, individual cells were clear in morphology, and their nuclei were clearly identifiable (Figure 1c and d). Pretreatment with a chelating buffer [HBSS containing 0.5 mM ethylene glycol-bis (beta-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) (GIBCO-Invitrogen)] used in the *in situ* perfusion method did not affect the status of cell isolation; therefore, we concluded that chelating is not necessary (Figure 1c and d).
Figure 1. Fluorescence microscopy images of hepatocytes isolated under various conditions of liver tissue treatment [tissue size: homogenized (a), minced into <1 mm$^3$ (b), minced into 2–3 mm$^3$ (c, d); pretreatment with chelating buffer including EGTA: chelated (a, b, c) and non-chelated (d)], stained with AO and DAPI.
Figure 2. Fluorescence microscopy image of hepatocytes isolated using the non-perfusion method (stained with AO and DAPI). The hepatocytes are from rats treated with 12.5 mg/kg DEN daily for 28 days. The arrow indicates a hepatocyte MN.
2.3.2. Liver MN assay

The results of the liver MN assays are given in detail in Table 1 and are graphically summarized in Figures 3 and 4a and b.

The mean MI values for hepatocytes in the vehicle-treated control groups were 0.1% or less at each time point (age 6.5, 8 and 10 weeks; Figure 3). The MI values for the groups treated with the test chemical were similar to those of the vehicle-treated control groups.

The mean MNHEP frequencies in the vehicle-treated control groups at each time point ranged from 0.05% to 0.07%. The MNHEP frequencies significantly increased after repeated administration of DEN at 12.5 mg/kg/day for 5 days or longer and 2, 4-DAT at 50 mg/kg/day for 14 days or longer. The frequency exhibited an increasing trend with dependence on the number of administrations. For both DEN and 2, 4-DAT, repeated administration even at half the high dosage level (6.25 mg/kg/day for DEN and 25 mg/kg/day for 2, 4-DAT) for 28 days resulted in significant induction of MNHEPs and showed a dose-dependent increasing trend.

In the group treated with 2, 4-DAT at 50 mg/kg for 28 days, 2 of the 5 rats died on days 13 and 24. Clinical symptoms were observed in the surviving animals after receiving 2, 4-DAT. All surviving animals showed signs of ptosis during the first few days of treatment, and a few animals presented with salivation during the last few days of the 28-day treatment period. No mortality or clinical symptoms were observed in any animals treated with DEN. Similarly, in the 2, 4-DAT and DEN treatment groups, the mean body weight increased during the treatment period, but the rate of weight gain was suppressed in a dose-dependent
manner. As a result, the mean body weights at the termination of the 14- or 28-day treatment were approximately 95% and 90% or 90% and 80%, respectively, of the vehicle control group (which was set to 100%) in the low- and high-dose groups (data not shown). These results suggest that the dose levels used in this study were around or lower than the MTD of the test chemicals for a 14- or 28-day treatment period.
Table 1
Results of the liver MN assay.

<table>
<thead>
<tr>
<th>Chemical (mg/kg/day)</th>
<th>No. of dosings</th>
<th>No. of animals</th>
<th>MNHEPs (%) Individual data (Mean ± SD)</th>
<th>MI (%) Individual data (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0.05, 0.15, 0.05, 0.00, 0.00 (0.05 ± 0.07)</td>
<td>0.00, 0.05, 0.10, 0.15, 0.25 (0.11 ± 0.10)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>14</td>
<td>0.10, 0.10, 0.05, 0.05, 0.05 (0.07 ± 0.03)</td>
<td>0.00, 0.05, 0.05, 0.00, 0.00 (0.02 ± 0.03)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>28</td>
<td>0.05, 0.05, 0.05, 0.05, 0.10 (0.06 ± 0.02)</td>
<td>0.05, 0.00, 0.05, 0.00, 0.10 (0.04 ± 0.04)</td>
</tr>
<tr>
<td><strong>2, 4-DAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>5</td>
<td>0.05, 0.05, 0.05, 0.00, 0.40 (0.11 ± 0.15)</td>
<td>0.10, 0.05, 0.00, 0.00, 0.10 (0.05 ± 0.05)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>14</td>
<td>0.40, 0.35, 0.55, 0.10, 0.60 (0.40 ± 0.20)**</td>
<td>0.15, 0.00, 0.10, 0.10, 0.05 (0.08 ± 0.06)</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>28</td>
<td>0.60, 1.35, 0.40, 0.85, 0.60 (0.76 ± 0.37**)</td>
<td>0.05, 0.05, 0.10, 0.00, 0.00 (0.04 ± 0.04)</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>3a</td>
<td>1.55, 0.95, 1.25 (1.25 ± 0.30)**</td>
<td>0.00, 0.00, 0.05 (0.02 ± 0.03)</td>
</tr>
<tr>
<td><strong>DEN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>5</td>
<td>5</td>
<td>0.20, 0.25, 0.20, 0.10, 0.25 (0.20 ± 0.06**)</td>
<td>0.15, 0.20, 0.15, 0.05, 0.25 (0.16 ± 0.07)</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>14</td>
<td>2.15, 1.95, 0.95, 0.55, 1.60 (1.44 ± 0.68**)</td>
<td>0.15, 0.10, 0.05, 0.15, 0.10 (0.11 ± 0.04)</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>5</td>
<td>0.90, 1.25, 1.05, 1.20, 0.95 (1.07 ± 0.15***)</td>
<td>0.00, 0.15, 0.05, 0.05, 0.05 (0.06 ± 0.06)</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>28</td>
<td>2.80, 2.60, 2.15, 2.25, 1.90 (2.34 ± 0.36***)</td>
<td>0.20, 0.10, 0.20, 0.25, 0.25 (0.20 ± 0.06)</td>
</tr>
</tbody>
</table>

*aTwo rats died because of toxicity during the treatment period.
** p≤0.01, Kastenbaum & Bowman's statistical method.
Figure 3. Age-related changes in the frequency of mitotic index (MI) of rat hepatocytes and effects of repeated administration of DEN and 2, 4-DAT. The data for 4.5-week-old rats were prepared by plotting mean vehicle control value of our historical data from young rat studies. The data for 6.5-, 8- and 10-week-old rats represent the MI (%) evaluated 24 h after the end of repeated administration to 6-week-old rats for 5, 14, and 28 days, respectively.
Figure 4. Frequencies of MNHEPs evaluated 24 h after the end of repeated administration of DEN (a) and 2, 4-DAT (b) to 6-week-old rats for 5, 14, and 28 days (**: p≤0.01, Kastenbaum & Bowman's method).
2.4. DISCUSSION

In the in vivo liver MN assay methods previously developed, MN inducibility is detectable by a short-term treatment of a test chemical at relatively high doses. These previous methods require artificial mitotic stimulation by PH (Tates et al., 1980) or mitogen treatment (Braithwaite and Ashby, 1988) or the use of young rat liver, which undergoes a higher rate of cell proliferation in comparison with adult rat liver (Suzuki et al., 2004). These assay methods cannot use normal adult liver, and hepatocytes are isolated with the in situ perfusion of the entire liver. For both of these reasons, the liver MN assay methods reported previously cannot be integrated into general toxicity studies.

As an alternative to the in situ perfusion method, I investigated a hepatocyte isolation technique in which a portion of the liver tissue was incubated and digested with collagenase in a test tube. Efficient collagenase digestion was achieved by mincing the liver tissue into 2–3 mm$^3$ pieces, and pretreatment with a chelating buffer was unnecessary. This method does not require a large-scale perfusion procedure and thus saves labor in the experiment. More importantly, hepatocyte samples are prepared from only a portion of the liver tissue, enabling toxicity evaluations of multiple endpoints, including other genotoxic studies (e.g., comet assay) in addition to typical histopathological tests; this is of substantial significance.

In a liver MN assay in which 4-week-old rats were used and in which the specimens were prepared at the age of approximately 4.5 weeks, the historical values of MI for the vehicle-treated control group at the testing institution ranged from approximately 0.5% to 1%. The MI
for adult rats was 0.2% or less when the specimens were prepared at approximately 6.5–10 weeks of age. The growth of S phase cells in adult rats is 40 times less than that in 4-week-old rats (Parton and Garriott, 1997). Because adult rat hepatocytes proliferate slowly and because the M-phase occupies only a small fraction of the cell cycle, the use of the MI as a mitotic parameter seems to be inappropriate.

According to unpublished data, an evaluation of 3-day division/proliferation demonstrated that at the age of 6, 8, and 11 weeks, bromo-deoxyuridine (BrdU) intake was found in approximately 10%, 5%, and 2.5% of hepatocytes, respectively (data provided kindly by Dr. Muto of Mitsubishi Tanabe Pharma Corporation). Thus, it is likely that the hepatocytes of even 6- to 10-week-old rats undergo slow but steady proliferation and that we need not seek highly proliferating conditions of hepatocytes in a long-term repeat-dose protocol using adult rats.

The mean frequency of MNHEPs in the negative control group was low (0.05–0.07%) and was similar to the result obtained by the liver MN assay using young rats (range of historical values at the testing institution: approximately 0.06–0.12%). It was therefore suggested that even in the liver of adult rats (approximately 6.5–10 weeks old), the frequency of naturally occurring micronuclei is quite low.

A recent review of non-bone marrow MN assays in rodents revealed the existence of one study examining the liver MN assay with repeated-dose for 21 days (Morita et al., 2011). The frequencies of MNHEPs were 0.47%, 0.43%, and 0.40% after dosing for 7, 14, and 21 days, respectively, in a study by Singh et al. (Singh et al., 2008). These values are higher than our values. The liver cell suspensions were prepared by homogenization, and the cells were stained with
DNA-nonspecific stains (May-Grunwald and Giemsa) by Singh *et al.* (Singh *et al.*, 2008); these differences might affect the MNHEP frequency.

DEN is a well-known rodent hepatocarcinogen. DEN gave positive results under metabolism-activated conditions in *in vitro* genotoxic studies, but negative or weak positive results in bone marrow MN assays (Morita *et al.*, 1997). Previously performed liver MN assays have demonstrated that DEN gives clearly positive test results (Suzuki *et al.*, 2004, 2005, 2009; Takasawa *et al.*, 2010a, b). The present study confirmed that repeated administration of DEN for 5 days or longer resulted in significant induction of MNHEPs, even in adult rat liver without additional treatment to stimulate cell division. The MNHEP frequency after a 5-day repeated administration of DEN in the present study was half the frequency (0.47±0.20%) obtained in juvenile rats at an identical dose (Takasawa *et al.*, 2010b). This result was considered to be attributable to an age-related reduction in proliferation. However, continued administration following the 5-day treatment period in the present study increased the MNHEP frequencies. This finding could be explained by the idea that hepatocytes with micronuclei have remained over a long period of time and have accumulated additively.

2, 4-DAT is known to be carcinogenic in the rodent liver (The International Agency for Research on Cancer [IARC], 1978). In *in vitro* genotoxic studies, it gave positive test results primarily under metabolism-activated conditions (George and Westmoreland, 1991). However, 2, 4-DAT was equivocally positive in rat bone marrow MN assays (George and Westmoreland, 1991), and it gave weakly positive responses in rat hepatic UDS tests (George and Westmoreland, 1991;
Mirsalis et al., 1982), which are frequently used as the 2nd in vivo genotoxicity study. In contrast, 2, 4-DAT was positive in the liver MN assay using juvenile rats: the liver MN assay is considered to be highly sensitive in detecting a genotoxic substance that exerts its effects via metabolic activation (Suzuki et al., 2009; Takasawa et al., 2010b). In the present study, repeated administration of 2, 4-DAT to adult rats for 14 days or longer significantly induced micronuclei at a frequency similar to that obtained in the test using young rats (the maximum frequency of MNHEPs: 0.37±0.12%) (Takasawa et al., 2010b).

Although the total dose amount of the test chemical was the same for the 14-day administration at the prescribed dose and the 28-day administration at half the prescribed dose, the frequency of MNHEPs showed a different pattern between DEN and 2, 4-DAT. The MNHEP frequency was higher when DEN was administered at the higher dose for 14 days (12.5 mg/kg/day × 14 days > 6.25 mg/kg/day × 28 days), whereas the frequency was higher when 2, 4-DAT was administered at the lower dose for 28 days (50 mg/kg/day × 14 days < 25 mg/kg/day × 28 days). Considering age-related reductions in hepatocyte proliferation rates, the chemically induced frequencies of micronuclei are expected to be higher when administered at higher doses to younger animals, as in the case of DEN. In the study using young rats, DEN administered at the dose inducing micronuclei did not clearly inhibit hepatocyte proliferation (Takasawa et al., 2010b). However, 2, 4-DAT induced micronuclei in the dose range in which this chemical inhibited cell division to some extent, and 2, 4-DAT did not induce micronuclei strongly in the higher dose range in which it markedly inhibited division (Takasawa et al., 2010b). Accordingly, if a test chemical exerts its
genotoxicity only at near-cytotoxic levels in a short-term treatment, then it is expected that a long-term repeated administration of the chemical at doses at which it exerts lower toxicity would result in an effective accumulation of MNHEPs, contributing to an increased sensitivity in detecting positive reactions. It will be difficult to evaluate hepatocyte toxicity directly in the repeated-dose liver MN assay (RDLMN). However, the examination of clinical chemistry or histopathology of the liver may be an alternative in repeated-dose general toxicity tests.

The present results indicate that even in adult rat liver tissues with low mitotic activity, MN induction is detectable with satisfactorily high sensitivity by repeated-dose of the test chemical for 14 days or longer, as the adult rat liver is characterized by a long life span and relatively slow turnover of hepatocytes. The establishment of the hepatocyte isolation technique without using the \textit{in situ} perfusion method enables integration of liver MN assays into general toxicity studies, contributing to a reduction of the number of animals required, and it enables comprehensive evaluation of toxicological effects of a test chemical.
2.5. SUMMARY

Various liver MN assay methods, such as those involving PH, treatment with mitogens, and the use of young animals, have been developed. These assays have been proven to be of high sensitivity and specificity to predict hepatocarcinogenicity of compounds that cannot be detected by BM MN assays. On the contrary, the existing assays have only been evaluated for their use in detecting MN induction in the settings of relatively short-term cell proliferation. However, the integration of in vivo genotoxicity endpoints into routine toxicity studies is increasingly desired from the viewpoint of animal welfare to reduce the number of animals used. In this study, the rodent hepatocarcinogens DEN and 2, 4-DAT were repeatedly administered orally to male Crl:CD (SD) rats (6 weeks old at the beginning of administration) for 5, 14, and 28 days, and changes in the frequency of MNHEPs in liver tissues that had undergone no artificial treatment to accelerate cell proliferation were evaluated. At the same time, a new method of hepatocyte isolation involving the treatment of a portion of the liver with collagenase in a centrifuge tube, without the use of in situ perfusion, was established. The induction of MNHEPs was achieved after the repeated administration of DEN for 5 days or longer and of 2, 4-DAT for 14 days or longer. MN frequencies were increased depending on the number of administrations, indicating that MNHEPs had possibly remained for a long period of time and accumulated additively. It therefore appears that even in adult rat liver with low mitotic activity, a repeated-dose of a chemical substance for 14 days or longer enables the detection of MN induction. In addition, the
establishment of a method to isolate hepatocytes without perfusion using only a part of the liver enables the integration of liver MN assays into general toxicity studies.
Chapter 3

Persistence and Accumulation of Micronucleated Hepatocytes in Liver of Rats after Repeated Administration of Diethylnitrosamine
3.1. INTRODUCTION

The bone marrow is not sufficiently exposed to the active metabolites of some pro-mutagens, such as the well-known rodent carcinogen DEN, due to their short life span, resulting in poor detectability of MN induction (Morita et al., 1997; Tates et al., 1980). Therefore, the liver, the main organ of metabolism, is a prime target for genotoxicity evaluations in vivo that can supplement the bone marrow MN assay. A number of different liver MN assay methods have been developed to date, and these methods have been reported to be highly sensitive to hepatocarcinogens that have not been shown to induce micronuclei in hematopoietic cells (Braithwaite and Ashby, 1988; Suzuki et al., 2004; Tates et al., 1980).

From the perspective of animal welfare, some regulatory guidelines for genotoxicity testing recommend to reduce the number of experimental animals by incorporating in vivo genotoxicity tests into general toxicity studies (ECA, 2012; ICH, 2011). A variety of measures have been used in previous liver MN assays to compensate for the low mitotic activity of adult rat hepatocytes, including artificial enhancement of hepatocyte proliferation by PH (Tates et al., 1980) or mitogen treatment (Braithwaite and Ashby, 1988) or by use of livers from young (4 weeks old) rats, which have higher mitotic activity (Suzuki et al., 2004). This means that these existing liver MN tests cannot be integrated into general toxicity studies, which utilize intact adult animals. Moreover, there is an assumption that the dose levels applied for the repeated administration protocol of general toxicity tests are not high enough to evaluate the genotoxic potential of test
compounds. Organs with high mitotic activity, such as those composed of erythrocytes, spermatids, or epithelial cells of the skin or gastrointestinal tracts, are used for efficient MN induction (Morita et al., 2011). However, because of the high turnover rate in these target organs, micronucleated cells disappear after a short time. For this reason, lower doses could be directly linked to attenuation of detection sensitivity (Hamada et al., 2001).

In Chapter 2, a RDLMN assay using adult rats was developed, taking advantage of the characteristically long turnover period of adult rat livers, although the belief that the low mitotic ability of these cells was disadvantageous for MN detection (Narumi et al., 2012). At the same time, a new method of hepatocyte isolation from a part of the liver was also established instead of the whole-liver perfusion method used in other liver MN assays (Narumi et al., 2012). This new method enabled the liver of a single animal to be shared and evaluated at several different endpoints, including histopathological tests. On the basis of the results of studies that used several carcinogenic substances, it has been reported that the frequency of MNHEPs increases in accordance with the number of days of repeated administration, providing an indicator that detects genotoxicity with high sensitivity even at comparatively low doses (Narumi et al., 2012; Takasawa et al., 2013).

Because adult rat hepatocytes are known to have a long lifespan of approximately 200 days (MacDonald, 1961), the detectability of micronucleus formation in these cells is thought to be due to the long-term persistence of hepatocytes with induced micronuclei in the liver and the resulting increase in number following repeated administration. Thus, these properties of hepatocytes make them
suitable for risk evaluation-type protocols that involve long-term administration of low doses, enabling their incorporation into general toxicity testing. To date, however, no data that directly indicate the persistence of MNHEPs and their capacity to accumulate have been reported.

In this study, rat hepatocytes were pulse-labeled with the thymidine analog EdU and subsequently chased during the period of repeated administration of DEN to verify the persistence of MNHEPs. I also calculated the theoretical frequency of MNHEPs induced on each day of administration from the results of the EdU-labeling analysis, derived the cumulative values over time, and compared these with the actual values measured in the MN test to verify the presence of a cumulative effect. I further interpreted shifts over time in the proliferation rates or MNHEP frequency as early changes during the process of DEN-induced hepatocarcinogenesis and performed a simultaneous histological analysis to investigate these changes in more detail.
3.2. MATERIALS AND METHODS

3.2.1. Animals

Male Crl:CD (SD) rats were purchased from Charles River Japan Inc. (Yokohama, Japan) and were aged six weeks and weighed approximately 170–210 g at the beginning of the experiments. They were housed two to three per cage in an air-conditioned room with a 12-h light/dark cycle and free access to food and drinking water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the test facility prior to its implementation.

3.2.2. Chemicals

DEN (CAS No. 55-18-5, >99.0% purity) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and EdU was purchased from Invitrogen/Life Technologies, Inc. (OR, USA). Both substances were dissolved in distilled water for injection (Fuso Pharmaceutical Factory, Inc., Tokushima, Japan) before use.

3.2.3. Dose levels and treatment

A single dose of DEN (12.5 mg/kg/day) at which significant MN induction in adult rat livers had been shown in Chapter 2 (Narumi et al., 2012) was used. Treatment with DEN, EdU labeling, and sampling were performed according to the six-fold test schedule shown in Figure 5, with each schedule being applied to three animals. In schedules 1–3,
DEN was repeatedly administered by oral gavage (10 mL/kg of body weight) daily for 7 or 14 days, with a single intraperitoneal injection of EdU (30 mg/kg) at the time of the initial administration, or on day 7 (1 or 2 weeks before sacrifice), in order to confirm the persistence of MNHEPs. In schedules 4–6, DEN or vehicle were administered for 1, 7, or 14 days, with a single injection of EdU at the time of final administration (24 h before sacrifice), in order to observe changes in the proliferative activity of hepatocytes and the rate of induction of MNHEPs during the period of repeated administration.
Figure 5. Experimental design for EdU-labeling analysis.
3.2.4. Liver MN assay

Animals were euthanized by exsanguination under thiopental anesthesia. The hepatocyte preparations were obtained according to the methods established in Chapter 2 (Narumi et al., 2012), with slight modifications. The livers were excised, and an approximately 1 g portion of the left lateral lobe was cut into slices of approximately 0.5- to 1-mm thickness with a razor blade. These strips were rinsed in HBSS (GIBCO-Invitrogen, Carlsbad, CA, USA) and incubated with HBSS containing 100 units/mL of collagenase (Collagenase Yakult-S, Yakult Pharmaceutical Industry, Co., Ltd., Tokyo, Japan) in a screw-capped flask at 37°C for 1 h with shaking at approximately 90 rotations per minute (rpm). The strips were then shaken at the maximum speed (approximately 180 rpm) for 1 min, every half hour. The resulting material was repeatedly pipetted vigorously to break apart cell clumps and then forced through a cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ, USA). The cell suspension was then rinsed with 10% neutral buffered formalin and centrifuged at 50 × g for 2 min. Finally, the hepatocyte pellet was resuspended in 10% neutral buffered formalin and kept in a refrigerator until analysis. Immediately before observation, 10 μL of the hepatocyte suspension was mixed with an equal volume of an AO (500 μg/mL) and DAPI (2.5 μg/mL) staining solution, dropped onto a glass slide, and covered with a coverslip (24 mm × 60 mm). The slide preparation was observed by fluorescence microscopy (at 400 × magnification with UV excitation), and the number of MNHEPs per 2000 HEPs was counted for each animal according to the previous reports (Narumi et al., 2012; Suzuki et al., 2004).
3.2.5. Determination of EdU incorporation in hepatocyte suspensions

Detection of EdU incorporation into the DNA was done with Click-iT™ EdU Alexa Fluor® Imaging Kits (Invitrogen/Life Technologies, Inc., OR, USA) basically according to the manufacturer’s instructions. In brief, approximately 0.1 mL of the hepatocyte suspension that was prepared for the liver MN assay was washed twice with HBSS and permeabilized in HBSS containing 0.5 % Triton X-100 for 0.5 h, and washed again. Click-iT™ EdU reaction cocktail (1×) was prepared according to the manufacturer’s instructions and immediately added to the cell pellet. The samples were incubated for 30 min at room temperature in the dark and washed twice with HBSS. Finally, the sample pellet was resuspended in antifade mounting medium (VECTASHIELD®, Vector Laboratories, Inc., CA, USA) and kept in a cool, dark place until analysis.

Immediately prior to observation, the samples were mixed with DAPI (25 µg/mL) at a ratio of 1:4, dropped onto a glass slide, and covered with a coverslip (24 mm x 60 mm). The slide preparations were observed by fluorescence microscopy (at 600 × magnification). The number of EdU-labeled HEPs [EdU(+ )HEPs] with nuclei exhibiting green fluorescence under blue excitation per 2000 HEPs with nuclei exhibiting blue fluorescence under UV excitation was counted for each animal, and their proportion was calculated. The number of EdU-labeled MNHEPs [EdU(+ )MNHEPs] per 1000 EdU(+ )HEPs was also counted, and their frequency was calculated.
3.2.6. Theoretical model of the cumulative effects of MNHEP frequency

As a preliminary experiment, EdU was injected into rats according to the same methods used in this study and sequentially the incorporation of EdU into hepatocytes was monitored for up to 48 h. The proportion of EdU(+)HEPs peaked at approximately 12 h and remained at that level thereafter. It was therefore assumed that i.p.-injected EdU would be rapidly consumed during hepatocyte growth, contributing to hepatocyte labeling up to approximately 12 h.

The theoretical frequency of MNHEPs induced in rat livers on each day \( (n \text{th day, 24-h period}) \) of DEN administration \( [f(n)] \) was calculated by multiplying the proportion of EdU(+)HEP by the frequency of EdU(+)MNHEP, and by the constant 2 \((24 \text{ h} / 12 \text{ h})\), and then dividing by 100. Values of \( f(n) \) at 1, 7, and 14 days were calculated from the results of schedules 4–6. Proportions of EdU(+)HEP and frequencies of EdU(+)MNHEP for days 2–6 were calculated temporarily according to a linear formula from those on days 1 and 7, and those for days 8–13 were calculated in a similar manner from those on days 7 and 14. Values of \( f(n) \) for days 2–6 and 8–13 were calculated in the same manner as those for days 1, 7, and 14. To reflect the persistence/loss of MNHEPs under conditions of repeated DEN administration (days 2–7 or 8–14), the difference between the \( f(n) \) values of schedule 4 or 5 \((24 \text{ h after EdU-labeling at the first or seventh administration})\) and the values similarly calculated from the results of schedule 1 or 3 \((6 \text{ or 7 days later})\) was divided by the number of intervening days to calculate the rate of loss per day \( (r) \). Based on the
theoretical frequencies of MNHEPs \([f(n)]\) for each day of administration and the rate of loss \((r)\) calculated, a cumulative model of MNHEP frequency over time was constructed [cumulative value, \(Cf(n) = Cf(n – 1) \times (1 – r) + f(n)\)]. For example, the cumulative value on day 2 of administration \([Cf(2)]\) was calculated by subtracting the portion lost \([f(I) \times (1 – r)]\) from the frequency of micronucleated cells induced on day 1 \([f(I)]\) and adding the frequency of micronucleated cells newly induced on day 2 \([f(2)]\), as \([Cf(2) = Cf(I) \times (1 – r) + f(2)]\). For this model, it was assumed that the total number of whole liver hepatocytes remained almost constant during the period of DEN administration.

3.2.7. Histological analyses

For histopathological/immunohistochemical examinations, the remainder of the left lateral lobe of the liver was fixed with 10% phosphate buffered formalin for routine embedding in paraffin. Sections were stained with hematoxylin and eosin for histopathological examination.

For staining of Ki-67, proliferation-associated protein, the sections were microwaved in Target Retrieval Solution (DAKO/Agilent Technologies Inc., CA, USA) for 30 min after deparaffinizing and rehydrating, and then blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol for 5 min. The sections were incubated with a mouse anti-Ki-67 antibody (clone MIB-5, catalog No. M7428, DAKO/Agilent Technologies Inc., 1:500 dilution) for 1 h, followed by incubation with a biotinylated rabbit anti-mouse immunoglobulin (catalog No. E0464, DAKO/Agilent Technologies Inc.,
1:200 dilution) for 30 min, and with streptavidin–horseradish peroxidase (catalog No. P0397, DAKO/Agilent Technologies Inc., 1:300 dilution) for 30 min. Staining was developed with diaminobenzidine, and the sections were counterstained with hematoxylin, dehydrated, and mounted. The numbers of Ki-67 positive cells were counted in 5 random fields per section at 200 × magnification directly under a microscope for each animal.

Glutathione S-transferase placental form (GST-P) staining was performed according to the same procedure, but without antigen retrieval. A rabbit anti-rat GST-P antibody (catalog No. 311-H, Medical and Biological Laboratories Co., Ltd., Nagoya, Japan, 1:2000 dilution) and a horseradish peroxidase-labeled dextran polymer conjugated goat anti-rabbit antibody (Envision, catalog No. K4003, DAKO/Agilent Technologies Inc.) were used as the primary and secondary antibodies, respectively. The numbers of GST-P positive single cells and mini-foci (comprising 2–10 cells) were counted in 50 random fields per section at 400 × magnification directly under a microscope for each animal.

Terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) staining was performed by means of an apoptosis detection kit (DeadEnd™ Colorimetric TUNEL System, Promega Corp., WI, USA) according to the manufacturer's recommended instructions. The numbers of TUNEL positive cells were counted in 50 random fields per section at 400 × magnification directly under a microscope for each animal.
3.3. RESULTS

3.3.1. Liver MN assay

The MNHEP frequency among all hepatocytes increased in the liver MN assay as the number of days of DEN-administration increased (Figure 6). Body- and liver-weight gain were reduced in the DEN-treated groups (Figures 7a and b).

![MNHEPs Frequencies](image_url)

**Figure 6.** Frequencies of MNHEPs in rats that were repeatedly given the vehicle or DEN. Bars represent mean values + SD of all animals for each day (n = 6 and 9 for DEN, n = 3 for vehicle).
Figure 7. Body (a) and liver (b) weight changes during the experiment. Symbols represent mean values of all animals for each day (n = 3–9 for DEN, n = 3 for vehicle).
3.3.2. EdU-labeling analyses

As shown in Figures 8a and b, EdU-labeled main nuclei and micronuclei of hepatocytes were clearly detectable in the suspension specimens.

The results of EdU-labeling experiments conducted under the conditions of schedules 1–6 are presented in Table 2. To show time-dependent changes (1 or 2 weeks after EdU labeling), the results of schedules 1–5 are graphically summarized in Figures 9a and b. The EdU(+)HEPs and EdU(+)MNHEPs ratios remained at a certain value for 7 or 14 days. The initial frequencies of EdU(+)MNHEPs were decreased by approximately 35% over the next week. The results of schedules 4–6 are graphically summarized in Figures 10a and b as treatment-dependent changes (24 h after EdU labeling). The proportion of EdU(+)HEPs and the frequency of EdU(+)MNHEPs increased as the number of days of DEN-administration increased.
Figure 8. Fluorescence-microscope images of isolated hepatocytes stained with fluorescein azide and counterstained with DAPI. All hepatocyte nuclei fluoresced blue under UV excitation (a); EdU-labeled main nuclei and micronuclei fluoresced green under blue excitation (b). Arrows indicate hepatocyte micronuclei.
Table 2

Results of EdU-labeling analysis.

<table>
<thead>
<tr>
<th>Chemical Schedule</th>
<th>No. of days of administration</th>
<th>EdU-labeling</th>
<th>Rate of EdU-labeled HEPs (%) (mean ± SD)</th>
<th>Frequency of MNed cells in EdU-labeled HEPs (%) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day of injection</td>
<td>Days after injection</td>
<td></td>
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<tr>
<td>DEN</td>
<td>1</td>
<td>7</td>
<td>1</td>
<td>7</td>
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<td></td>
<td>2</td>
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<td>14</td>
<td>14</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 9. Proportions of EdU(+)HEPs and frequencies of MNHEPs among EdU(+)HEPs on days after a single injection of EdU in rats that were repeatedly given DEN. Circles and bars represent mean values for schedules 4, 1, and 2 (a), and schedules 5 and 3 (b).
Figure 10. Proportions of EdU(+)HEPs and frequencies of MNHEPs among EdU(+)HEPs 24 h after single EdU injection in rats that were repeatedly administered vehicle (a) or DEN (b). Circles and bars represent mean values for schedules 4–6.
3.3.3. Theoretical model of the cumulative effects of MNHEP frequency

The theoretical model of the cumulative effects of MNHEP frequency, based on the results of the EdU-labeling analyses, is illustrated in Figure 11. The theoretical frequencies of MNHEPs that were newly induced on the 7th and 14th day of DEN administration were 0.14% and 0.37%, respectively. The cumulative values for MNHEP frequency were close to the actual values measured in the liver MN test.
Figure 11. Theoretical model of the cumulative effect on MNHEP frequency. Solid bars indicate the theoretical frequencies of MNHEPs that were newly induced on each day ($n$th day) of DEN administration 

\[ f(n) = \text{proportion of EdU}(+)\text{HEP} \times \text{frequency of EdU}(+)\text{MNHEP} \times 2 (24 \text{ h} / 12 \text{ h})\].

Open bars with dotted lines indicate cumulative values of MNHEP frequency from the previous day ($Cf(n - 1)$), corrected for the time-dependent loss rate ($r$: 0.080, 2nd–7th day; 0.067, 8th–14th days), as 

\[ Cf(n - 1) \times (1 - r) \].

Therefore, the cumulative model of MNHEP frequency over time was constructed by adding both frequencies, as 

\[ (Cf(n)) = Cf(n - 1) \times (1 - r) + f(n) \].

The actual measured values in the liver MN test (circles with SD bars), as shown in Figure 6, are plotted against this cumulative model to compare the time-course of changes in MNHEP frequency.
3.3.4. Histological analyses

Histopathologically, acidophilic changes and single cell necrosis of hepatocytes and inflammatory cell infiltration were observed in the centrilobular region of livers of rats treated with DEN for 7 or 14 days. Increased mitotic figures were also observed diffusely in the livers of rats treated with DEN for 1, 7 or 14 days.

Immunohistochemically, the number of Ki-67-positive hepatocytes increased gradually after DEN exposure in a frequency of administration-dependent manner. In contrast, the number of Ki-67-positive hepatocytes decreased in a time-dependent manner in the vehicle-treated control group, probably due to aging of the rats (Figure 12a).

TUNEL-positive hepatocytes appeared after the initial DEN treatment and their numbers increased greatly after 7 or more days of exposure (Figure 12b). Occasionally, some of the TUNEL-positive cells appeared to be MNHEPs (Figure 14).

GST-P-positive single hepatocytes appeared after the initial DEN treatment and their numbers increased as the number of days of DEN-administration increased (Figure 13a). GST-P-positive mini-foci appeared on the 7th day of DEN treatment, although the total number of mini-foci was low and increased markedly on the 14th day of DEN treatment (Figures 13b and 15).
Figure 12. Time course changes of Ki-67 (a) and TUNEL (b) positive hepatocytes in the livers of rats that were repeatedly administered DEN. Symbols represent mean values of all animals for each day (n = 2, 4, and 6 for days 1, 7, and 14, respectively).
Figure 13. Time course changes of GST-P positive single cells (a) and mini-foci (b) in the livers of rats that were repeatedly administered DEN. Symbols represent mean values of all animals for each day (n = 2, 4, and 6 for days 1, 7, and 14, respectively).
Figure 14. TUNEL-positive hepatocytes (brown) in the livers of rats that were given DEN for 14 days. The livers were counterstained with hematoxylin. Arrows indicate micronuclei.
Figure 15. GST-P positive hepatocytes (brown) in the livers of rats that were administered DEN for 14 days. The livers were counterstained with hematoxylin.
3.4. DISCUSSION

Similar to the results in Chapter 2 (Narumi et al., 2012), the frequency of MNHEPs increased as the number of days of DEN administration increased. This responsiveness in the liver MN assay could be due to the long-term persistence of MNHEPs in the liver and the resulting increase in their number following repeated administration. These mechanisms are important factors that would support the suitability of the liver MN assays for repeated administration protocols. However, to date, no data have been published that provide evidence for the existence of these mechanisms. In this study, I attempted to verify these mechanisms by use of EdU uptake to label cells that were undergoing mitotic proliferation.

\[^3\text{H}\]\ thymidine- and BrdU-labeling methods have been widely used to study DNA replication and sister chromatid exchange as well as to assess cell proliferation. These methods use autoradiography or anti-BrdU immunostaining to detect the incorporated label and have several limitations, including low resolution, high background, and nuclear counterstaining due to a harsh DNA denaturation step that is required to expose the BrdU epitope (Takagi et al., 1993). Recently, EdU, which is structurally similar to the natural nucleoside, has been used as an alternative for the labeling of DNA in proliferating cells (Buck et al., 2008; Diermeier-Daucher et al., 2009; Salic and Mitchison, 2008). EdU detection is based on a copper-catalyzed covalent reaction between a fluorescent azide and an alkyne group of EdU (Rostovtsev, 2002). This method is highly sensitive and much faster than detection with \[^3\text{H}\]\ thymidine or BrdU detection. In addition, because the size of
fluorescent azide is much smaller in size than the anti-BrdU antibody, it can easily diffuse through tissues and approach the incorporated EdU without denaturing the DNA. In this study, the labeled nuclei (including micronuclei) of hepatocytes were easily and clearly detectable in the isolated suspensions with the EdU-labeling method. In addition, this method allowed for perfect counterstaining with DAPI. Therefore, it was considered to be very useful for the analysis of a large number of hepatocyte samples.

The presence of EdU(+)HEPs in isolated liver cells after 1 and 2 weeks suggested that newly proliferated hepatocytes may persist for long periods, even under conditions of repeated DEN administration. Similarly, the detection of EdU(+)MNHEPs after 2 weeks demonstrated that hepatocytes persist for long periods, even after the induction of micronuclei. The initial frequencies of EdU(+)MNHEPs were not constant; they decreased by approximately 35% over the next week. These changes indicate that micronucleated cells preferentially disappeared among EdU-labeled HEPs. In vitro live cell imaging studies have shown that cells with micronuclei tend to die during the progressing cell cycle with higher frequency than cells without micronuclei (Yasui et al., 2010). In the present study, the simultaneous increase in both MNHEP frequency and the number of TUNEL-positive cells in liver sections as well as the enhancement of hepatocyte proliferation suggested that a mechanism for the selective elimination of hepatocytes with micronuclei may exist. It has been reported that the number of single GST-P-positive cells increased dramatically within the first week in the livers of rats that were given a single large dose of DEN and subsequently declined rapidly, with approximately 70% lost after 3
weeks (Satoh et al., 1989). These authors showed that the increase in aggregate GST-P positive cell counts (2–10 cells, mini-foci; 11–50 cells, larger lesion; >50 cells, larger foci) and their frequencies over time suggested that most of the single GST-P-positive altered cells that transiently increased after treatment underwent apoptosis, but that a proportion remained and may have developed into mini-foci or even large lesions (Satoh et al., 1989). Because DEN was repeatedly administered to the rats in this study, the decline in the number of GST-P-positive single cells over time was not observed. However, it is conceivable that some of the micronucleated cells induced by repeated DEN administration may have avoided apoptosis and been initiated to undergo repeated mitotic proliferation, resulting in an increase in the number of GST-P-positive mini-foci in the liver on day 14.

DEN, which is a simple alkylating agent, is mainly activated metabolically by CYP 2E1 to generate ethylating electrophiles that form DNA adducts (Belinsky et al., 1982; Eadle et al., 1984). It has been reported that although O\(^6\)-ethylguanine, the major promutagenic adduct, is formed transiently in rat liver after a single administration of DEN, it is subsequently rapidly repaired and decreases to approximately 4% of its initial levels after 24 h (Williams et al., 1996). Therefore, it was envisaged that the frequency of MNHEPs induced in a 1-day (24-h) period might be constant among EdU-labeled newly divided cells, irrespective of the number of DEN administration. The frequency of EdU(+)MNHEPs, however, gradually increased dependent on the number of doses, and it has been suggested that repeated administration of DEN over several days may increase the risk of inducing chromosomal aberrations in hepatocytes. In a previous paper, analysis
of DNA-adduct kinetics indicated that hepatocytes in the S-phase of the cell cycle may be more vulnerable to carcinogen-induced DNA damage than hepatocytes in other phases (Kaufmann et al., 1991). These findings are consistent with the increases in the frequency of MNHEP induction, along with the increased proportion of EdU-labeled hepatocytes, as observed in the present study. The levels of the DNA oxidative damage marker 8-oxoguanine (8-oxo-G) in the liver have also been reported to rise 6 h after DEN administration and to be subsequently maintained at a high level, up to 72 h, throughout the experimental period (Nakae et al., 2008). Similar effects are also evident after administration of heterocyclic amine or dimethylarsinic acid. Expression of mRNA of 8-oxo-G-DNA glycosylase 1 (Ogg1) gene, encoding a specific repair enzyme for 8-oxo-G, is down-regulated in the livers of these animals, in what is regarded as a characteristic mechanism of initiation by hepatocarcinogens (Nakae et al., 2008). This mechanism may also have resulted in the increase in MNHEPs over time.

In this study, after the initial administration of DEN, results suggesting enhanced proliferation of liver cells were obtained for mitotic figures in liver tissue and for the rate of EdU incorporation in isolated hepatocytes. The Ki-67 index, a nuclear antigen expressed during all phases of the cell cycle except in the G0 phase (Gerdes et al., 1984), the early proliferative response was not clear probably because the 6-week-old rat livers partially maintain their autonomous growth state. On day 1, no necrotic lesions were evident in the liver tissues. This cannot be accounted for by compensatory enhancements in proliferation. Overproduction of reactive oxygen not only induces mutations as a result
of oxidative damage to DNA, but is also known to activate signal-transduction pathways, including those involving activator protein-1 (AP-1) and nuclear factor kappa B (NFκB), inducing the transcription of genes related to pathways that regulate cell proliferation (Klaunig and Kamendulis, 2004). It is possible that the early actions of DEN enhancing proliferation may evoke a similar mechanism. Each of the cell proliferation parameters examined in the present study subsequently increased with continued DEN administration, with a particularly pronounced increase in the number of EdU(+)HEPs between days 7 and 14. This change resembled the changes in GST-P-positive cell counts. The most pronounced increase occurred in the number of GST-P-positive mini-foci on day 14, suggesting that initiated cells may have begun to progress to clonal proliferation with a concomitant increase in the rate of proliferation. Nevertheless, in this enhanced proliferative states of hepatocytes, a theoretical calculation of the frequency of transiently induced MNHEPs based on the results of EdU-labeling analysis yielded results of 0.14% and 0.37% on days 7 and 14 of DEN administration, respectively, which were only approximately 20–30% of the MNHEP frequencies that were actually obtained in the liver MN test (mean values: 0.43 % and 1.87 %, respectively). However, the values calculated after taking into account the persistence of MNHEPs that were initially induced from the start of administration into account were similar to the actual measured values. These results confirmed the hypothesis that micronucleated cells that are induced in rat liver persist for long durations at a constant rate, with repeated administrations resulting in their accumulation and increased frequency.
In this study, the long-term persistence of MNHEPs was able to be observed directly by means of EdU-labeling analysis. The cumulative effect of MNHEPs due to repeated administration was also confirmed by verifying it against a theoretical model. The proliferative state of hepatocytes may vary according to the dose level or duration of treatment (e.g., 14 or 28 days), thereby affecting the balance between accumulation of MNHEPs and their removal by apoptosis. All the same, however, in the case of repeated administration of genotoxic hepatocarcinogens, rat livers may conceivably undergo such a mutation accumulation process, leading to promotion and/or progression in carcinogenesis. Because of these properties of rat hepatocytes, the liver MN assay is an in vivo test method for genotoxicity that is suitable for comparatively long-term repeated administration protocols, and its incorporation into general toxicity testing may contribute to reducing the number of animal experiments, and to providing a method for evaluating the risk of low-dose exposures.
3.5. SUMMARY

In Chapter 2, the RDLMN assay in adult rat livers was developed (Narumi et al., 2012). This assay demonstrated a high detectability of hepatocarcinogens at relatively low doses, as indicated by dose number-dependent MN induction. Because the adult rat liver is known to have a long life-span, this desirable property of the assay was conjectured to be a cumulative effect of MNHEPs that have persisted for long periods in the liver following repeated administration. However, no data directly supporting the underlying mechanisms have been published to date. In this study, I attempted to verify the mechanisms by means of pulse-labeling of micronucleated hepatocytes with the thymidine analog EdU. The rodent hepatocarcinogen DEN was repeatedly administered orally to male Crl:CD (SD) rats (6 weeks old) for up to 2 weeks, and EdU was injected intraperitoneally on days 1, 7, or 14. Hepatocytes were isolated by use of a non-perfusion technique at 24 h, 1 or 2 weeks after EdU injection and analyzed for EdU incorporation and micronucleus formation. The results of this study confirmed that MNHEPs labeled with EdU on the first day of DEN administration persisted until 2 weeks post-administration in the rat livers. However, the frequency of MHNEPs among EdU-labeled hepatocytes decreased over time. In addition, the number of TUNEL-positive cells in the liver tissue increased, suggesting selective removal of micronucleated cells. Theoretical calculation of the cumulative MNHEP frequency on each of the days on which DEN was administered, taking into account the rate of loss, came out closer to the actual value observed in the liver MN test. Taken together, these
results indicate that although micronucleated cells induced in rat livers by the administration of a genotoxic hepatocarcinogen DEN undergo selective removal, they persist for a long time in a certain proportion, and repeated administration results in their accumulation and increased frequency.
Chapter 4

General Discussion
4.1. Introduction

Genotoxicity tests were originally developed to detect genotoxic substances and carcinogens, and these tests have played important roles in understanding the mechanisms of chemical carcinogenesis and assessing the genetic hazards of chemicals in humans. Predicting chemical carcinogenicity is a major goal of genotoxicity assays. Many evaluations of the results of in vitro genotoxicity tests (Kuroki and Matsushima, 1987; Tennant et al., 1987; Zeiger et al., 1990) and the in vivo MN test (Mavournin et al., 1990; Shelby et al., 1993; Morita et al., 1997) indicate that they correlate with chemical carcinogenicity.

In the regulatory standard battery of genotoxicity tests, the sensitivity (ability to produce a positive response in the presence of a carcinogen) of the bacterial gene mutation assay, in vitro chromosome aberration test, and mouse lymphoma assay was approximately 59%, 66%, and 73%, respectively (Kirkland et al., 2005). In contrast, the sensitivity of rodent BM cytogenetic tests, particularly the MN assay, appears to be relatively high (approximately 80%) for human carcinogens (Nersessians, 1992; Shelby, 1988; Shelby and Zeiger, 1990). It was also reported that the mouse erythrocyte MN assay detected IARC human carcinogens according to the degree of evidence for carcinogenicity; 91, 65, and 60% for Group 1 (known human carcinogens), 2A (probable human carcinogens), and 2B (possible human carcinogens), respectively, taking into account the structure-activity relationship (Morita et al., 1997). However, several rodent hepatocarcinogens, including nitro-aromatic amines, alkylnitrosamines, and azo-derived chemicals, show negative results in
the erythrocyte MN assays (Angelosanto, 1995; Morita et al., 1997). These chemicals are pro-mutagens, and their active metabolites are considered to have a very short lifespan and do not reach the BM at sufficient concentrations to induce micronuclei formation. Additionally, one of the major target tissues for many chemical carcinogens is the liver (Gold et al., 1991). Thus, genotoxic hepatocarcinogens may be detected using liver MN assays. Most previous MN assays in the liver have been conducted through PH, mitogen treatment, or using young rats. Studies have shown that the liver MN assay may be useful for predicting chemical hepatocarcinogens, even when these chemicals are insensitive to rodent BM MN assays (Angelosanto, 1995; Braithwaite and Ashby, 1988; Cliet et al., 1989; Suzuki et al., 2005, 2009; Takasawa et al., 2010a, b; Zhurkov et al., 1996).

Recently, the animal welfare movement, which emphasizes the “3 Rs” (reduction, replacement, and refinement of animal use), have generally focused on regulatory toxicity testing, including in the genetic toxicology field. The EU has begun to enforce complete replacement of acute in vivo genotoxicity tests (EU, 2003). Moreover, refinements in conducting in vivo genotoxicity tests have been proposed in several recent guidelines (ECA, 2012; ICH, 2011), as well as expert recommendations for genotoxicity testing (Pfuhler et al., 2009). If the evaluation of micronuclei induction in hepatocytes is possible by using rat RDT study, then, it will be extremely useful to not only reduce the number of animals required but also for overall toxicity evaluation. However, previously developed liver MN assays are typically performed by use of in situ liver perfusion via the portal vein or vena cava to isolate hepatocytes. Liver perfusion precludes the examination of other
endpoints as well as histopathological examination, which is required for general toxicology studies. Above all, previously utilized liver assays cannot be conducted using intact adult liver, and therefore, cannot be integrated into general toxicity studies.

In this study, I focused on the fact that hepatocytes in adult rat liver have a long life span. Thus, I examined whether repeated administration of hepatocarcinogens at relatively low doses causes MNHEP accumulation. Additionally, we attempted to establish a method of hepatocyte isolation from a portion of the liver without using \textit{in situ} perfusion to enable integration of liver micronucleus assays into general toxicity studies. Moreover, to directly observe the persistence of MNHEPs, rat hepatocytes were pulse-labeled using the thymidine analog EdU and subsequently chased during repeated administration of DEN. To confirm the cumulative effect of MNHEPs, a theoretical model of MNHEP frequencies was constructed and verified against the actual values measured in a micronucleus test. To further interpret the results obtained using EdU analysis, simultaneous histological analysis was conducted.

\subsection{Development of new hepatocyte isolation technique}

In the micronucleus assay, it is an indispensable requirement to see each cell border clearly. In that respect, it is easy to prepare cell suspensions for the erythrocyte MN assay. Hepatocytes are commonly separated from the liver through \textit{in situ} collagenase digestion, which is a 2-step process. In the first step, the liver is placed in an isotonic solution, in which calcium is removed to disrupt cell-cell tight junctions.
using a calcium-chelating agent. Next, a solution containing collagenase is added to separate the hepatocytes from the liver stroma. Previously developed liver MN assays as stand-alone acute assays have involved this perfusion technique. Because the in situ technique digests the entire liver, this liver cannot use to examine other toxicological endpoints. Appropriate isolation of hepatocytes for MN observations from a portion of the liver tissue was the primary goal of this study; therefore, various conditions not involving the in situ perfusion technique were investigated in Chapter 2. Conditions used included 2–3 mm³ mincing size, pretreatment with chelating agent was unnecessary, and use of a screw-capped tube with shaking at 37°C for 1 h for collagenase digestion. Moreover, this isolation method was refined to determine more stable conditions for hepatocyte preparation regardless of skill level, such as collagenase brand and cutting shape (cube or slice) of liver tissue, in Chapter 3. The most effective factor was collagenase brand; we found that Yakult-S performs better than the other brands tested (data not shown). Details of the manufacturing process and additives in each brand were not available, but according to the general information provided, Yakult-S has low activity of the other proteases. Therefore, this may have resulted in less damage to hepatocytes during isolation.

This method requires only incubation with collagenase in a flask with shaking but not large-scale perfusion and heavy labor. This is an advantage for widespread use and integration into routine toxicity studies. A joint study by the Collaborative Study Group for the MN Test (CSGMT), a task group belonging to the Mammalian Mutagenesis Study subgroup of the Japanese Environmental Mutagen Society (JEMS-
MMS), confirmed that the hepatocyte isolation method reproduces very low and steady control values in all facilities (in writing for publication).

4.3. **Sensitivity of repeated-dose liver MN assay using adult rats**

In Chapter 2 (Narumi *et al*., 2012), both genotoxic hepatocarcinogens, DEN and 2, 4-DAT induced dose- and duration-dependent increasing of MNHEPs. In contrast, in BM MN assays, short-term DEN and 2, 4-DAT treatments associate with negative or weak responses in rats and mice (George and Westmoreland, 1991; Morita *et al*., 1997). This suggests that genotoxic hepatocarcinogenicity, which is typically undetectable using the standard BM MN assay, can be assessed using the liver MN assay. Particularly, 2, 4-DAT did not induce MNHEPs more than 2-fold that of the negative controls at higher doses, even in juvenile rat livers, which show high mitotic activity (Takasawa *et al*., 2010b). These results indicate that the liver micronucleus assay may be more sensitive using a long-term repeated administration protocol through accumulation of MNHEP, likely because of the long life span of hepatocytes.

A supplemental study was carried out to further investigate the effectiveness of the RDLMN assay for predicting hepatocarcinogenicity using the genotoxic carcinogen DMH and the non-carcinogen 2, 6-diaminotoluene (2, 6-DAT) (Takasawa *et al*., 2013). The aromatic amines 2, 4-DAT and 2, 6-DAT are structural isomers which are often compared to examine carcinogenic potential. Both 2, 4-DAT and 2, 6-DAT are mutagenic in *Salmonella typhimurium* strains (Toyoda-Hokaiwada *et al*., 2010) and *in vitro* chromosome aberration assays
(Ashby and Tennant, 1991; George and Westmoreland, 1991), and show negative or weakly positive results in the BM MN assay (George and Westmoreland, 1991). Although these chemicals exhibited the same results in genotoxicity studies, only 2, 4-DAT is carcinogenic in rats and mice (National Toxicology Program [NTP], 1979, 1980). Therefore, the carcinogenic potency of these 2 isomers cannot be distinguished using genotoxicity studies commonly required by regulatory paradigms. Whereas 2, 4-DAT showed a clear positive results in this study (Narumi et al., 2012), 2, 6-DAT did not show a significant increase in the supplementary study (Takasawa et al., 2013); thus, the RDLMN assay is thought to show not only higher sensitivity to carcinogens, but also higher specificity to non-carcinogens.

Although it has been reported that the rat BM MN assay can be used in general toxicity studies, a chemical such as DMH, which must be administered at lower levels during repeated dosing than those used for single or double dosing, cannot be detected using the BM MN assay under the repeated dose regimen (Hamada et al., 2001). In the supplementary study, however, DMH significantly induced MNHEPs at lower dose levels than those used in previous reports (Takasawa et al., 2013). Based on these findings and speculation concerning MNHEP accumulation in the liver as described above, the liver MN assay is considered more appropriate for a repeated dose study than that by using the BM MN assay.

Based on these fundamental investigations, a collaborative study, the CSGMT/JEMS-MMS, has conducted joint research to assess the performance of the RDLMN assay using adult rats. In this joint study, 23 chemicals, including liver carcinogens, were investigated by 20
laboratories to confirm the feasibility of the assay, so far. The assay detected 17 of the 19 hepatocarcinogens, while only 4 of these chemicals were positive in the BM assay. These results reinforce the results of this study, and the sensitivity of the RDLMN to hepatocarcinogens is expected.

4.4. Significance of integration of rat liver MN assay into general toxicity studies

This study began with focusing on the long turnover time of adult liver hepatocytes, and it was postulated that hepatocytes remain for a long period, even after MN formation and that MNHEPs accumulate in the liver following repeated treatment with genotoxic carcinogens. In this study (Chapter 2) and the supplemental study, an increased MNHEP frequency was observed in a duration-dependent manner. With integration of the liver MN assay into a routine RDT assay, all toxicological endpoints, including MN inducibility, were evaluated only at the terminal point. Therefore, understanding characteristics of adult liver hepatocytes, such as proliferation and MN inducibility of hepatocytes or persistence and accumulation of MNHEPs, throughout the repeated dosing period is significant. Thus, in Chapter 3, rat hepatocytes were pulse-labeled using the thymidine analog EdU and is subsequently assessed during the chase phase with repeated administration of DEN. The long-term persistence of MNHEPs was directly observed, and the cumulative effect of MNHEPs was confirmed by comparing the constructed theoretical model with the actual values observed in the liver micronucleus test (Narumi et al., 2013). Based on
these observed characteristics of hepatocytes, the liver micronucleus assay is suitable for comparatively long-term repeated administration protocols.

In chapter 3, duration-dependent enhanced proliferation and MN induction of hepatocytes were also obtained by means of EdU-labeling analysis. Additionally, preferential disappearance of MNHEP was observed. These changes in isolated hepatocytes may be closely associated with the immunohistochemical staining scores of Ki-67, TUNEL, and GST-P. A major insight into cancer etiology was revealed by the realization that carcinogenesis occurs through sequential stages known as initiation, promotion, and progression (Berenblum, 1954). These stages were characterized in model systems as well as human neoplasms (Klein and Klein, 1985). Epigenetic (non-genotoxic) factors, such as different substances that trigger cell death, chronic inflammation, or peroxisome proliferation, are likely to act as tumor promoters in the liver (Sarma et al., 1986). In the supplemental study, histopathological examination revealed that the hepatocarcinogens DEN, 2, 4-DAT, and DMH caused cytotoxic injury and likely induced regenerative proliferation, but the non-carcinogen 2, 6-DAT did not (Takasawa et al., 2013). The liver has the unique ability to regulate its growth and mass. Mature hepatocytes proliferate to the extent that homeostasis is maintained after hepatic damage, including chemical injury and PH. Accordingly, most chemical hepatocarcinogens likely exert cytotoxic effects that stimulate liver regeneration, which may be observable through routine histopathological examination. Inflammatory cellular infiltration was observed in livers treated with DEN or 2, 4-DAT (Takasawa et al., 2013). A common
tumor-promoting mechanism may involve inflammation (Philip et al., 2004). A major link between inflammation and carcinogenesis may depend on NF-κB transcription factors, which are critical regulators of innate immune responses and inflammation (Karin et al., 2002). Some hepatocarcinogens, such as DEN, are known induce DNA oxidative damage in liver cells (Nakae et al., 2008); additionally, oxidative stress can activate NF-κB via convergence on the cytoplasmic inhibitor of kappa B (IκB) kinase complex (Hayden and Ghosh, 2004; Wuerzberger-Davis et al., 2007). Once activated, NF-κB dimers stimulate transcription of genes encoding cytokines, growth factors, chemokines, and antiapoptotic factors (Ghosh and Karin, 2002). NF-κB was shown to be instrumental for tumor promotion in inflammation-associated liver cancer (Pikarsky et al., 2004). The death of DEN-exposed hepatocytes activates adjacent myeloid cells (Kupffer cells) to produce hepatomitogens that promote compensatory proliferation of surviving hepatocytes. Production of these mitogens depends on NF-κB in hematopoietic-derived Kupffer cells (Maeda et al., 2005). Therefore, if MNHEP frequencies significantly increase, additional evaluation of NF-κB activation, as well as immunohistochemical staining of Ki-67, TUNEL, or GST-P, may provide important information regarding the ability of test compounds to enhance initiation and to progress to the promotion stage of carcinogenesis.

Until recently, genotoxicity has been regulated primarily based on qualitative outcomes of hazard identification assays; decisions are often based on whether a compound shows positive or negative genotoxic potential. Recently, the field of regulatory genetic
toxicology has moved from overly relying on the outcomes of short-term hazard screening tests to more risk-based assessment and weight of evidence (WOE) approaches (U.S. Food and Drug Administration [FDA], 2006; Thybaud et al., 2007, 2011). In vivo assays are critical components of both WOE analysis and quantitative risk assessments. As described above, the liver micronucleus assay is highly effective when integrated into general RDT studies, and takes advantage of the long-term persistence of hepatocytes to result in the accumulation of micronucleated hepatocytes. Its integration may contribute to a reduction in the number of animal experiments and provide a further risk-based assessment method for predicting carcinogenic potency.

The RDLMN assay using adult rats, developed in this study, was followed by the domestic collaborative study (CSGMT/ JEMS-MMS). The results obtained in this collaborative study have attracted international attention and are scheduled to be published as a special issue in Mutation Research/Genetic Toxicology and Environmental by the end of this year. Additionally, the assay will be presented at the International Workshop on Genotoxicity Testing to be held this autumn. The conference results will subsequently affect the regulatory guidelines for examining various types of compounds worldwide. The final goals of this study are acceptance as international test guidelines by the OECD and/or ICH and contribute to improving world health.
4.5. Conclusions

In previously developed in vivo liver micronucleus assays, micronucleus inducibility is detectable following short-term treatment of a test compound at relatively high doses. These methods require artificial mitotic stimulation through PH (Tates et al., 1980), mitogen treatment (Braithwaite and Ashby, 1988), or use of young rat liver, which undergoes a higher rate of cell proliferation than that by the adult rat liver (Suzuki et al., 2004). These assay methods cannot use normal adult liver, and hepatocytes are isolated using in situ perfusion of the entire liver. Thus, liver micronucleus assay methods reported previously cannot be used for general toxicity studies.

In this study, I found that in adult rat liver tissues that show low mitotic activity, micronucleus induction was detectable to a sufficiently high sensitivity following repeated dosing of the test chemical for 14 days or more, since the adult rat liver is characterized by a long life span and relatively slow turnover of hepatocytes. The long-term persistence of MNHEPs in the liver was directly confirmed by means of EdU-labeling analysis. By constructing a theoretical model of MNHEP frequencies, the cumulative effect on MNHEPs was also verified.

An established method for hepatocyte isolation that does not involve in situ perfusion enables the integration of liver micronucleus assays into general toxicity studies, contributing to a reduction of the number of animals required, and enabling comprehensive evaluation of the toxicological effects of a test compound.
Chapter 5

Summary and Conclusions
In Chapter 2, to enable the liver tissue examined concurrently with other toxicological endpoints, alternatives to the *in situ* whole-liver perfusion method were examined. The established method of hepatocyte isolation involved treatment of a portion of the liver by using collagenase in a centrifuge tube without the use of *in situ* perfusion. This new method enables the integration of liver micronucleus assays into general toxicity studies. To confirm whether repeated administration of hepatocarcinogens at relatively low doses causes accumulation of MNHEPs in the liver, DEN and 2, 4-DAT were repeatedly orally administered to adult male rats for 5, 14, and 28 days. Induction of MNHEPs was achieved after repeated administration of DEN for 5 days or longer and that of 2, 4-DAT for 14 days or longer. Induction levels were increased depending on the number of administrations. These results suggest that MNHEPs remain for a long period and accumulate additively.

In Chapter 3, to directly observe hepatocyte fate after micronucleation, DEN was repeatedly administered orally to adult male rats for up to 2 weeks and the thymidine analog EdU was injected intraperitoneally on day 1, 7, or 14. Hepatocytes were isolated by use of the non-perfusion technique at 24 h, 1 week or 2 weeks after EdU injection. MNHEPs labeled with EdU on the first day of DEN administration persisted until 2 weeks post-administration in the rat livers. However, the frequency of MHNEPs among EdU-labeled hepatocytes decreased over time. Additionally, the number of TUNEL-positive cells in the liver tissue increased, suggesting selective removal of micronucleated cells. Theoretical calculation of the cumulative MNHEP frequency on each of the days on which DEN was
administered, taking into account the rate of loss, came out closer to the actual observed value in the liver MN test. These results indicate that although micronucleated cells induced in rat livers by administration of genotoxic hepatocarcinogens undergo selective removal, they persist for a long period in a certain proportion, and repeated administration results in their accumulation and increased frequency.

In this study, it was revealed that (1) the established method for hepatocyte isolation without using the in situ perfusion technique enables the integration of liver micronucleus assays into general toxicity studies; (2) even in adult rat liver tissues with low mitotic activity, micronucleus induction is detectable with satisfactorily high sensitivity by administering repeated doses of the test chemical for 14 days or more; (3) hepatocytes micronucleated by genotoxic hepatocarcinogens may undergo selective removal; nevertheless, they persist for a long period in a certain proportion and repeated administration results in their accumulation and increased frequencies. These findings strongly suggest that the liver MN assay is suitable for use in comparatively long-term repeated administration protocols.

The repeated-dose liver MN assay developed in this study will reduce the number of animals required and provide a method for comprehensive evaluation of toxicological risks of a test compound over a relatively low exposure range.
References


References


341–349.


References


