Mutation Research 800–801 (2016) 35–39

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Short communication

Optimization of specimen preparation from formalin-fixed liver tissues for liver micronucleus assays: Hepatocyte staining with fluorescent dyes

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ARTICLE INFO

Article history:
Received 19 September 2015
Received in revised form 24 February 2016
Accepted 9 March 2016
Available online 14 March 2016

Keywords:
Liver micronucleus assay
Formalin-fixed method
Collagenase
Hepatocyte
Fluorescent dye

ABSTRACT

The liver micronucleus (MN) assay is an effective and important *in vivo* test for detecting genotoxic compounds, particularly those that require metabolic activation. For this assay, hepatocytes (HEPs) can be isolated by collagenase treatment but without requirement for *in situ* liver perfusion. Consequently, the liver MN assay can be integrated into a general repeated-dose (RD) toxicity study. The method is also applicable to liver MN assays involving partial hepatectomy or the use of juvenile rats. Here, we propose an improved method for staining HEPs prepared from formalin-fixed liver tissues for MN assays, without collagenase treatment. HEP suspensions are prepared by treating the tissues with concentrated KOH and a fluorescent dye, SYBR® Gold (SYGO), is used for staining. Visualization of the MN in SYGO-stained HEPs is clearer than with Wright-Giemsa staining. We compared the induction of MN as measured with our new method *versus* the conventional method using collagenase dispersion. Our method not only enables the integration of the liver MN assay into a general RD toxicity study but also allows it to be conducted retrospectively.

1. Introduction

The liver micronucleus (MN) assay can detect genotoxic rodent hepatocarcinogens [1], including compounds (such as diethylnitrosamine and 2,4-diaminotoluene) that require metabolic activation to exert genotoxicity [2,3] and are not detectable in rodent erythrocyte MN assays [4,5]. The liver MN assay usually requires isolation of hepatocytes (HEPs), typically carried out by collagenase digestion of fresh tissues. Modified liver MN assays involving partial hepatectomy [6–8], juvenile rats [9–11], or repeated dosing in young adult rats [1,12] may be used to obtain larger numbers of dividing cells. However, all of these methods require the use of collagenase for dispersion of HEPs from tissues on the day of euthanasia/necropsy, for preparation of HEP specimens.

In an alternative method for the preparation of HEP specimens, HEPs are isolated from formalin-fixed liver tissues by treatment with a concentrated solution of KOH [13]. We refer to this method as the "formalin-fixed method". The formalin-fixed method enables retrospective isolation of HEPs from fixed liver tissues. In previous studies with the formalin-fixed method [13,14], Wright-Giemsa staining was used to prepare the HEP specimens. One of these reports stated that acridine orange (AO), a fluorescent dye, is not appropriate for staining HEP specimens after formalin fixation [13]. However, we find that it is more difficult to discern MN in Wright-Giemsa-stained HEPs than in those stained with a mixture of AO and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), commonly used for staining fresh tissue in the collagenase dispersion method for liver MN assays [1]. The difficulty in recognizing MN in HEP specimens with the Wright-Giemsa staining method is a limitation of the formalin-fixed method for liver MN assays.

To improve the identification of MN in HEPs, we have investigated staining formalin-fixed specimens with various fluorescent dyes and compared the measured frequencies of micronucleated HEPs (MNHEPs) [12].

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http://dx.doi.org/10.1016/j.mrgentox.2016.03.004
1383-5718/

2. Materials and methods

2.1. Preparation of hepatocyte suspensions

Liver tissues fixed with 10% phosphate-buffered formalin were used to prepare HEP specimens for microscopic observation. Liver tissues fixed in 10% phosphate-buffered formalin for 5 years were obtained from specimens in one of our previous reports [12,15]. In that work, male Crl:CD(SD) rats were purchased from Charles River Japan, Inc. (Yokohama, Japan); the rats were 6 weeks of age and weighed approximately 200–250 g at the beginning of the experiment. The animals were housed two or three per cage in an air-conditioned room with a 12-h light/dark cycle and access to food and drinking water *ad lib*. The experimental protocol was approved by the Institutional Animal Care and Use Committee prior to its implementation. The rats (n = 5/group) were administered diethyl nitrosamine (DEN, CAS No. 55-18-5, >99.0% purity) by oral gavage in a repeated dosing regimen at 0, 6.25 or 12.5 mg/kg/day for 5, 14, or 28 consecutive days. For each time point, rats were euthanized under thiopental anesthesia, 24 h after the last administration. The livers were removed and a part of each liver was used for the MN assay as previously reported [12,15]. Residual tissues were immersed in 10% phosphate-buffered formalin, and they were used for the present investigation. The HEP specimens were prepared from the fixed-liver tissues with a slightly modified version of a previously reported method [13]. Briefly, a small portion of tissue was cut into about 3 mm cubes with a razor and washed well with water. About ten cubes were incubated in KOH solution (about 15 mL, 12 M; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at room temperature for 16 h and then washed well with water. The tissue cubes were then mashed, filtered through a cell strainer (pore size: 100 μm), and suspended with water to disperse HEPs. The HEP suspensions were centrifuged at 50*g* for 5 min and washed with 10% phosphate-buffered formalin. Centrifugation and washing steps were repeated three times or more. The pellet of HEPs was mixed with 10% phosphate-buffered formalin to prepare the HEP suspension.

2.2. Fluorescent dyes and reagents

Three fluorescent dyes were used to stain the isolated HEPs from the formalin-fixed tissue. SYBR® Gold (SYGO; 10,000× concentrate in dimethyl sulfoxide) was purchased from Life Technologies, Inc. (Carlsbad, CA, USA). Acridine orange (AO) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tris-hydrochloride (Tris-HCl; pH 7.5, 1 M) and ethylenediamine tetraacetic acid (EDTA; pH 8.0, 0.5 M) were also purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and these solutions were mixed to prepare a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0). SYGO was diluted into the TE buffer at 2× concentrate. AO and DAPI were dissolved in water.

2.3. Comparison of staining methods of hepatocyte-specimens

Just before microscopic observation, the HEP suspension was mixed and stained with an equal volume of a solution containing SYGO at 1× concentrate and DAPI, 50 μg/mL (SYGO-DAPI), a solution containing AO, 500 μg/mL and DAPI, 10 μg/mL (AO-DAPI), or SYGO at 2× concentrate alone. The mixtures were dropped onto clean glass slides and spread with coverslips. Each of the slide specimens was observed under a fluorescence microscope (BX51N-34-FL-1-D; Olympus Corporation, Tokyo, Japan) with B excitation filter (420–490 nm) and emission filter (510 nm) for SYGO-DAPI and SYGO alone or UV excitation filter (330–385 nm) and emission filter (420 nm) for AO-DAPI.

The non-stained HEP suspension was transferred and smeared or dropped onto a clean glass slide and dried to prepare the slide specimen stained using the Wright-Giemsa method, as previously described [13]. Briefly, the slide was stained with Wright's staining solution for 3 min followed by staining with Wright-Giemsa mixture containing 50 mL Giemsa solution, 50 mL Wright's solution, 100 mL Dulbecco's phosphate buffered saline (PBS, pH 6.4), and 300 mL water for 3 min. The slide was rinsed with water for 2 min and air-dried prior to cover-slipping. Each of the slide specimens was observed under a light microscope (BX51N-34-FL-1-D; Olympus Corporation, Tokyo, Japan).

2.4. Calculation of micronucleus incidences and mitotic indices, and statistics

For the HEP specimens stained with SYGO using our improved method, the incidence of micronucleated HEPs (MNHEPs) was calculated by counting 2000 parenchymal HEPs for each animal. The parenchymal hepatocytes scored in this experiment were identified by their morphology. The cells scored were comparatively large, circular or oval, with circular or oval main nuclei, and included cells with one, two or more nuclei. In contrast, stromal cells such as Kupffer's cells were irregular in shape and smaller than parenchymal hepatocytes, and were largely removed by the centrifugation step. In addition, the number of mitotic phase cells was scored in 2000 HEPs to determine the mitotic index (MI). Differences in the incidence of MNHEPs between the DEN-treated and control groups were analyzed using the conditional binomial test of Kastenbaum and Bowman [16]. The proportions of MI between the treated and control groups were analyzed using Dunnett's test. These data obtained from our improved method were compared to those reported by Narumi et al. [12] using Student's *t*-test.

3. Results and discussion

3.1. Comparison of staining methods of hepatocyte-specimens

Photos of the specimens observed under a microscope at magnification with 100× with the following staining methods are shown in Fig. 1: SYGO-DAPI (Fig. 1A), the Wright-Giemsa method (Fig. 1B) as previously described [13], and AO-DAPI (Fig. 1C) generally used in a liver MN assay as previously described [1]. Photos of the HEPs and a MNHEP at large magnification (400×) with the following staining methods are shown in Fig. 2: SYGO-DAPI (Fig. 2A), SYGO alone (Fig. 2B) and the Wright-Giemsa method (Fig. 2C). Among the four methods, staining with SYGO-DAPI or SYGO alone was the most appropriate (Figs. 1 and 2). With SYGO-DAPI or SYGO alone, the main nucleus in the cytoplasm was clearly defined in each of the HEPs (Figs. 1A and 2A,B), and a MN was recognized in a MNHEP (Fig. 2A and B). In the previous reports of the MN assay using the collagenase method [11], the AO-DAPI double staining was superior to staining with AO alone or DAPI alone, because the HEPs that were relatively large and thick could be observed in three dimensions by the AO-DAPI double staining. The same effect was expected for SYGO-DAPI double staining; however, there were no substantial differences in staining between SYGO alone and SYGO-DAPI. When using the Wright-Giemsa staining method, the main nuclei and cytoplasm were well defined (Figs. 1B and 2C), but a MN was not as clearly defined as compared to the SYGO method (Fig. 2C). Even if a clear MN was defined, it was poorly reproducible. The difficulty of scoring Wright-Giemsa stained preparations is one reason why there are few reports concerning the liver MN assay using the formalin-fixed method [13,14]. In contrast, the components in the HEPs stained with AO-DAPI (micronuclei and even main nuclei) were not recognized in the cytoplasm (Fig. 1C).