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Genotoxic Effects of Nitrosamine Compounds in a Human Liver Cell Line (FLC-4)

Akira Takahashi, Yuichi Miyakoshi, Hironari Sano,
Yoshimitsu Toyama, and Yuji Suzuki

Department of Public Health and Environmental Medicine,
The Jikei University School of Medicine

ABSTRACT

To evaluate the genotoxicity of the nitrosamine compounds dimethylnitrosamine (DMN), diethylnitrosamine (DEN), and dibutylnitrosamine (DBN), which induce hepatic tumors in experimental animals, we used the *in vitro* micronucleus test system with a human hepatocellular carcinoma cell line (FLC-4). The number of micronuclei increased significantly in FLC-4 cells exposed to nitrosamine compounds. The highest frequencies of micronuclei in FLC-4 cells induced by DMN, DEN, and DBN at concentrations of 6.75, 9.75, and 1.25 mM were 1.93, 3.67, and 2.40%, respectively. This micronucleus test system could be used to evaluate the genotoxic effects of chemicals that induce hepatic cancer without the use of a metabolic activation system because FLC-4 cells partly retain the enzyme system for metabolizing hepatic drugs. Recently, the *in vitro* micronucleus test, especially with human cells, has been recommended to assess the risks of cancer from environmental mutagens without the use of experimental animals. Almost all chemicals reveled of those mutagenic and/or carcinogenic activities after metabolizing with enzymes. Human hepatic cell lines are useful for use in *in vitro* mutagenicity tests because they retain the drug-metabolizing enzyme system. (Jikeikai Med J 2002 ; 49 : 143-8)

Key words : micronuclei, hepatocellular carcinoma, nitrosamines, human

Introduction

The *in vivo* bone marrow erythroblast-erythrocyte micronucleus test was developed to detect mutagens and carcinogens[1,2]. The frequency of micronuclei increases with cellular genotoxic damage, such as chromosome aberrations and interference with spindle function[3]. Recently, the *in vitro* micronucleus test, especially with the use of human cells, has been recommended to assess the risks of cancer from environmental mutagens without the use of experimental animals. The induction of micronucleus in human peripheral lymphocytes[4-6], urothelial cells[7], and liver cells[8] has been used to evaluate the mutagenic effects of chemicals.

We established a sensitive new *in vitro* micronucleus test using the FLC-4 cell line[8-10], which is derived from human hepatocytes, to assess the genotoxic effects of chemicals[8]. The aim of this study was to investigate whether the micronucleus test using the FLC-4 cell line could be determine the genotoxic effects in hepatic cells of the nitrosamine compounds dimethylnitrosamine (DMN), diethylnitrosamine (DEN), and dibutylnitrosamine (DBN), which are considered tumorigenic agents on the basis of Registry of Toxic Effects of Chemical Substances criteria[11] and

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高橋　明，宮越　雄一，佐野　浩斎，冨山　吉光，鈴木　勇司
Mailing address : Yuji Suzuki, Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.

by the International Agency for Research on Cancer (IARC)[12]. These chemicals did not induce micronuclei in the original *in vivo* bone marrow erythroblast-erythrocyte micronucleus test[13,14].

MATERIALS AND METHODS

Preparation of exposure chemicals

DMN (CAS 62759, purity>90%; Wako Pure Chemical Industries, Tokyo), DEN (CAS 55185, purity>99%; Tokyo Kasei Kogyo, Tokyo), and DBN (CAS 924163, purity>95%; Tokyo Kasei Kogyo) were dissolved in phosphate-buffered saline (PBS) as soon as possible before addition to the FLC-4 culture medium.

Cell cultures

FLC-4 cells were cultured in T75 flasks or 60-mm Petri dishes in ASF-104 medium (Ajinomoto Co., Tokyo) supplemented with 2% normal human serum (Anapure Bioscientific Co., Beijing, China). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air, and the medium was renewed twice a week. FLC-4 cells synthesize albumin, alpha-fetoprotein, and retinal conjugate protein, metabolize ammonia into urea, and retain intracellular enzyme activities such as LDH and γ-GTP. Hepatitis B and C viral antigens are not detected in FLC-4 cells[15-17]. FLC-4 cells have 65 chromosomes with the following karyotype: 65, XX, add (1)(p11), +2, −5, +6, add (7)(p11), +add (7)(p32), add (12)(p13), −13, add (14)(p11) +der (15) t (15; 21)(p11; q11), +16, +I (17)(q10), +20, −22, +16mar[18].

The FLC4 cell line was kindly provided by Prof. Seishi Nagamori, M.D., of the Kyorin University School of Medicine, Tokyo.

Growth inhibition study

FLC-4 cells at a concentration of 1.0×10^5 cells per 5 ml of medium were plated on 60-mm Petri dishes. After growing for 48 hours[8], the FLC-4 cells were exposed to DMN at concentrations of 0.84, 1.69, 3.38, 6.75, 13.50, 27.0, and 54.0 mM for an additional 48 hours. FLC-4 cells were washed several times with PBS, then exposed to PBS containing 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA; trypsin-EDTA solution) for 5 minutes at 37°C, collected, centrifuged at 1000 rpm for 10 minutes, and resuspended in PBS. The cells were counted with a hemocytometer under an inverted microscope (Nikon Corp., Tokyo).

FLC-4 cells were exposed to DEN (1.22, 2.44, 4.88, 9.75, 19.5, 39.0 and 78.0 mM) or DBN (0.04, 0.08, 0.16, 0.31, 0.63, 1.25 and 2.50 mM) for 48 hours and tested by the same method as for DMN. Triplicate cultures were incubated at each concentration.

Micronucleus test

Cell number gradually increased from 48 to 168 hours after plating. The relationship between incubation time (X) and cell number (Y) could be determined with the following formula: $Y=0.3446\ X + 15.32$.

The doubling time of FLC-4 cells is 28 hours[8]. The number of micronucleated cells increases with exposure to genotoxic chemicals or spindle poisons for more than one cell cycle and then peaks near two cell cycles. The cells were exposed to chemicals for 48 hours. The present experiment was based on the results of a dose-response and time-course study using mitomycin C as a mutagen[8].

Scott et al.[19] reported that high concentration of some chemical substances induce chromosome aberration (include micronuclei) indirectly through non-physiologic osmolality or pH, or ion unbalance. We usually applied the under limited concentration of 10 mM for the in vitro chromosome aberration test and *in vitro* micronucleus test using CHL cell line[20].

FLC-4 cells were exposed to DMN at concentrations of 0.84, 1.69, 3.38, 6.75 and 13.50 mM for 48 hours starting from day 2, when the cell growth had reached an exponential stage. Triplicate cultures were incubated for each concentration. FLC-4 cells were exposed to DEN (1.22, 2.44, 4.88, 9.75, and 19.5 mM) or DBN (0.04, 0.08, 0.16, 0.31, 0.63, 1.25, and 2.50 mM) and tested with the same method as that for DMN.

Slide preparation and statistical analysis

After being briefly washed in PBS, cultured cells were exposed to trypsin-EDTA solution for 5 minutes at 37°C, collected, and centrifuged at 1000 rpm for 10 minutes. Collected cells were treated with a hypotonic solution (75 mM KCl) for 10 minutes, fixed in methanol containing 2% acetic acid, spread onto clean slides, air dried, and stained with 3.5% Giemsa solution (1/150 M Sörensen's PBS, pH 6.4) for 10 minutes. All procedures were conducted at room temperature. The numbers of micronucleated cells were counted under a light microscope at 1000x magnification. Cells were considered micronucleated if they had clear cytoplasmic borders with micronuclei whose diameter was not larger than one-third that of the nucleus. Binucleated and polynucleated cells were not counted. The experimental results were analyzed statistically using the Kastenbaum-Bowman table[21].

Results

Growth inhibition

The relationship between the growth rate of FLC-4 cell (Y) and concentration of DMN, DEN, and DBN (X), respectively are expressed with the following formulae: $Y=-15.15\ \log(X)+78.49$ (correlation coefficient=0.9215), $Y=-25.18\ \log(X)+116.99$ (correlation coefficient=0.8835), and $Y=-16.39\ \log(X)+50.38$ (correlation coefficient=0.9057) (Fig. 1). The 50% growth inhibition concentrations of DMN, DEN, and DBN were calculated to be 6.56, 14.3, and 1.0 mM, respectively.

Micronucleus test

In the micronucleus test, the highest concentration of chemicals to which FLC-4 cells exposed were higher than the 50% growth-inhibition concentrations.

A linear dose-response relationship between concentrations of DMN, DEN, and DBN, and the frequency of micronucleated cells was observed in FLC-4 cells exposed to DMN, DEN, or DBN without a metabolic activation system for 48 hours (Table 1).

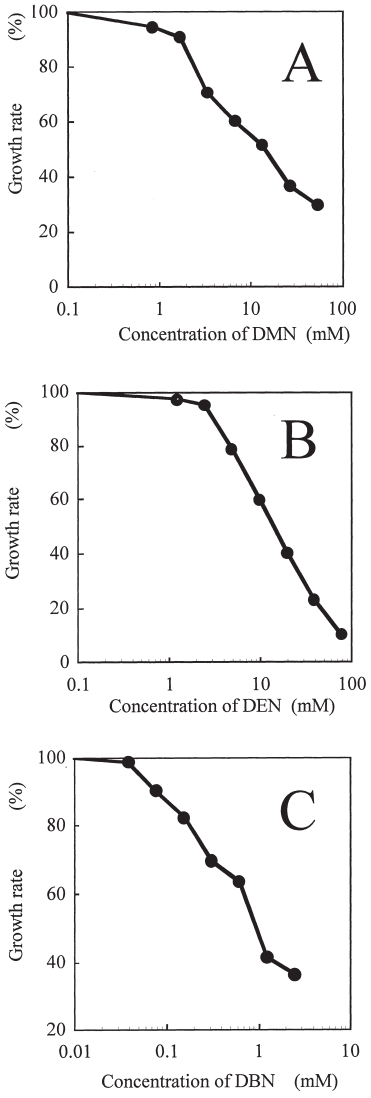

Fig. 1. Effect of DMN (A), DEN (B), and DBN (C) on the growth of FLC-4 cells.

The highest micronucleus frequencies were 2.23±0.15%, 4.63±0.40%, and 2.40±0.10% ($p<0.05$) after exposure to DMN, DEN, or DBN at concentrations of 13.5, 19.5, and 1.25 mM, respectively, for 48 hours ($p<0.05$; Table 1). The frequency of micronuclei induced by the three chemicals increased in a dose-dependent manner (Table 1). The frequency of micronuclei in FLC-4 cells induced by DMN, DEN, and DBN at concentration of 6.75, 9.75, and 1.25 mM (a concentration less than 10 mM) increased 2.6-, 4.8-, and 3.3-fold over that in control ($p<0.05$), respectively.

Table 1. Induction of micronuclei in FLC-4 cells by nitrosamine compounds

Chemicals	Concentration (mM)	Micronucleated cells (%)
DMN	0	0.73±0.06
	0.84	1.07±0.15
	1.69	1.27±0.15*
	3.38	1.57±0.15**
	6.75	1.93±0.21**
	13.50	2.23±0.15**
DEN	0	0.77±0.12
	1.22	2.03±0.25**
	2.44	2.30±0.20**
	4.88	2.90±0.20**
	9.75	3.67±0.15**
	19.50	4.63±0.40**
DBN	0	0.73±0.06
	0.04	0.83±0.15
	0.08	1.03±0.06
	0.16	1.33±0.06*
	0.31	1.47±0.15**
	0.63	2.13±0.06**
	1.25	2.40±0.10**
	2.50	1.93±0.06**

*; $p<0.05$ **; $p<0.01$

DISCUSSION

To evaluate the carcinogenic risk of environmental chemicals, IARC has used both epidemiologic studies and animal studies[10]. Ito et al.[22] have found that 34 of 65 carcinogenic chemicals evaluated by IARC induced liver tumors in experimental animals such as rat, hamster, and mouse. Accordingly, we established a short-term genotoxicity test system using a cell line derived from human hepatocytes to evaluate the cancer risk of environmental chemicals[8]. In this experiment, we investigated whether DMN, DEN, and DBN, which induce hepatocellular cancer in experimental animals[9,10], induce micronuclei in FLC-4 cells. Although DMN, DEN, and DBN did not induce micronuclei in a mouse bone marrow erythroblast-erythrocyte micronucleus assay system[11,12], DMN and DEN did induce micronuclei in mouse hepatectomized liver[24].

The *in vivo* bone marrow micronucleus test is commonly used to assess the cancer risk from test chemicals. However, this test has limitations for evaluating genotoxic chemicals. Goldberg et al.[23] have reported that 1, 2-dimethylhydrazine also did not induce micronuclei in mouse bone marrow cells but induced micronuclei in colonic epithelium cells. A test chemical may form micronuclei in specific organs rather than in whole body. These findings suggest that an in vitro micronucleus test using FLC-4 cells could be used to assess the risk of cancer from environmental mutagens without the use of experimental animals. Neither FT-207 (a metabolic antagonist with 5-fluorouracil in its chemical structure) nor cyclophosphamide (an alkylating agent, alkyl halides) formed micronuclei in the *in vitro* micronucleus test with erythroblast-erythrocyte, V79 Chinese hamster lung cells, and CHO Chinese hamster ovary cells without a metabolic activation system (a drug-metabolizing enzyme fraction prepared from rat liver treated with polychlorinated biphenyl or phenobarbital plus 5, 6-benzoflavone treated which is added to a cell-culture medium with nicotinamide adenine dinucleotide phosphate, nicotinamide adenine dinucleotide, and glucose-6-phosphate)[16,25,26], but they did form micronuclei in FLC-4 cells without a metabolic activation system[8]. Kawada et al.[27] have demonstrated that FLC-4 cells partly retain the hepatic drug-metabolizing enzyme system containing CYP1A1, 1B1, 2E1, and 3A. Kushida et al.[28] have shown that human CYP2E1 is mainly responsible for the metabolic activation of nitrosamines, including DMN, DEN, and DBN. The FLC-4 cell line expresses the properties of liver cells partially because it is of hepatic origin and can thus serve as a model system for normal human liver cells.

Darroudi and Natarajan[26] have shown that microsomal fractions (S9) from a human hepatoma cell line (HepG2) are useful for activating indirectly acting genotoxic carcinogens. The frequency of micronuclei in the HepG2 cell line was increased by exposure to 2-amino-3-methyl-imidazo [4, 5-f] quinoline and cadmium[29], methyl isothiocyanate[30], alpha-endosulfan and beta-endosulfan[31] or heterocyclic aromatic amines[32]. The HepG2 cell line is also useful for assessing the genotoxic effects of environmental chemicals on human hepatic cancer risk.

Our results suggest that the micronucleus test

using human hepatocytes (FLC-4) would be useful for evaluating the cytogenetic effects of nitrosamines without the addition of a metabolic activation system. We conclude that the *in vitro* micronucleus test using FLC-4 cells is suitable for evaluating the human hepatic cancer risk of chemicals.

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