Micronucleus Test Using Cultured New Born Rat Astrocytes

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Abstract: Micronuclei is induced in cytoplasm as a consequence of the formation of chromosomal fragments or remaining chromosomes during cell division by the cause of clastogens or spindle poisons, and is used as an indicator of genotoxicity screening tests. There are few short-term genotoxicity screening tests using brain cells. We attempted to establish a new *in vitro* micronucleus test (MN test) system by use of central nervous system cells. Primary cultured astrocytes were prepared from newborn male Sprague-Dawley (SD) rats. In growth curve of astrocytes, doubling time was determined to be 31 h. In time study, the highest frequency of micronuclei was observed at 48 h, 72 h and 6 h-exposure-66 h-recovery by vincristine (VCR), mitomycin C (MMC) without metabolic activation system and cyclophosphamide (CPM) with metabolic activation system, respectively. Dose-response relationships between micronucleus frequency and concentrations of MMC, VCR and CPM were observed, respectively. It is suggested that the *in vitro* MN test using new born rat-astrocytes could be used as a screening test of environmental and occupational genotoxic chemicals in the central nervous system cells.

Key word: Micronuclei, Rat-astrocyte, Mitomycin C, Vincristine, Cyclophosphamide

Introduction

We are exposed to environmental and occupational hazardous chemicals in daily life. The carcinogenicity assay with experimental animals have been used to estimate the carcinogenic potential of chemicals¹⁾. But it costs much expense, and it takes a few years. Since recent progress in cancer and mutation research has proven a high correlation between mutagenicity and carcinogenicity, mutagenicity testing on chemicals has become more significant in evaluating their carcinogenicity²⁾. Therefore many low cost and short-term genotoxicity screening tests such as the Ames *Salmonella* mutagenicity assay, chromosomal aberration test and *in vivo* micronucleus test (MN test), are widely used to detect mutagenicity of chemicals predicting of carcinogens³⁾.

Both epidemiological studies and carcinogenicity tests in Sprague-Dawley (SD) rats suggested that some of environmental and occupational hazardous chemicals induced not only brain tumors but also genotoxic potential^{4–6)}. As brain tumors usually arise from glial cells or meningeal cells⁷⁾, it may be useful to establish a genotoxic assay system using cultured astrocytes in order to prevent brain tumors and to study the mechanism of generation of brain tumors.

Micronuclei is induced in cytoplasm as a consequence of the formation of chromosomal fragments or remaining chromosomes during cell division by the cause of clastogens or spindle poisons, and is used as an indicator of genotoxicity screening methods⁸⁾. The aim of this experiment is to establish an *in vitro* MN test using new born rat cultured astrocytes.

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Materials and Methods

Experimental animals

Four-five day-old male SD rats used to prepare primary cultured astrocytes and seven-week-old male SD rats used to prepare 9,000 g supernatant (S9) were obtained from Charles River Japan Inc. Rats were kept in a clean animal room (constant temperature 24–25°C, humidity 45–55%, lights on 8 a.m. to 8 p.m.). The rats were given chow (FR2, Funabashi Farm Co., Ltd., Japan) and tap water *ad libitum*. All animal experiments were performed in accordance with Animal Experiments Guidelines of the Jikei University School of Medicine.

Chemicals

Dulbecco's phosphate-buffered saline (PBS), minimum essential medium with Earle's salts (MEM), penicillin-streptomycin, HEPES buffer solution, and fetal bovine serum (FBS) were obtained from GIBCO BRL, Life Technologies, Inc., Rockville, Md., USA. DNase I, trypsin solution, trypsin/EDTA solution, NADP, and poly-D-lysine were obtained from Boehringer Mannheim GmbH, Germany. Rabbit polyclonal anti-cow glial fibrillary acidic protein (GFAP) antibody, and rhodamine-conjugated swine polyclonal anti-rabbit immunoglobulin were obtained from DAKO A/S, Denmark. Acridine orange, and glucose-6-phosphate were purchased from Sigma, St. Louis, Mo., USA. Phenobarbital sodium, and β -naphthoflavone were purchased from Wako Pure Chemical Industries. Ltd., Japan. Mitomycin C (MMC) was obtained from Kyowa Hakko Kogyo Co., Ltd., Japan. Vincristine (VCR) sulfate was obtained from Eli Lilly Japan K. K. Cyclophosphamide (CPM) was obtained from Shionogi and Co., Ltd., Japan.

Preparation of primary cultured astrocytes

The whole brains were prepared from four-five day-old SD rats according to the method of McCarthy *et al.*⁹⁾, and were incubated in PBS containing 0.25% trypsin, 40 μ g/ml DNase for 30 min at 37°C. After inactivation of trypsin activity, the cell suspensions were centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in MEM containing 5% FBS, 5 mg/ml glucose, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The suspended brain cells were dissociated into single cells by gently pipetting. The cell suspension was divided into 75 cm²-culture flasks (Falcon). Then they were incubated in a 5% CO₂/95% air incubator at 37°C. Two-three days after, the culture flasks were shaken on an orbital shaker (Bel-Art products, USA)

for about 12 hours at 37°C to separate astrocytes from mixed glial cells, neurons, fibroblasts and other cells⁹⁾. After 3–4 wk, cultured cells were used for the doubling-time study, for the cell growth inhibition test, and for the micronucleus assay.

Preparation of S9

S9 for metabolic activation was prepared from the livers of male SD rats treated with sodium phenobarbital and 5,6-Benzoflavone as enzyme inducers¹⁰⁾. The ingredients of S9mix and the final concentration of S9 in medium (5%) of *in vitro* micronuclei assay⁹⁾, similarly applied to this study. S9mix was filtered through 0.22- μ m pore size cellulose acetate membranes before usage.

Staining of astrocytes

The cells growing on the coverslips were rinsed in PBS, and then were fixed with cold methanol for 10 min. The fixed cells were washed in PBS containing 0.1% triton X-100, and then incubated with rabbit polyclonal anti cow GFAP antibody (1:100) for 60 min at 37°C. Then cells were washed in PBS, and were incubated with rhodamine-conjugated swine polyclonal anti-rabbit immunoglobulin for 30 min at room temperature. To recognize main nuclei and micronuclei, the cells were stained with 1.25 mM acridine orange in distilled water for 5 min at room temperature. After the cells were washed in PBS, coverslips were mounted on glass slides in PBS. Observations of astrocytes were made under an Olympus microscope equipped with phase contrast, fluorescein, and rhodamine optics at 300x magnification. Astrocytes were identified as GFAP-positive nucleated cells. The ratios of astrocytes were determined by counting 1000 nucleated cells.

Calculation of the cell numbers

The nucleated cells plated on 6-well-plate (Falcon) with MEM containing 15% FBS, and 5 mg/ml glucose (15% FBS/MEM) in growth inhibition ratio study were washed several times with physiological saline, and then fixed in 10% formalin neutral buffer solution for 10 min at room temperature. The fixed cells were washed with distilled water, and stained with 0.1% crystal violet in distilled water for 10 min at room temperature. Using a Monocellator (Olympus, Japan) at the absorbance of 555 nm, the growth inhibition ratio was calculated from the intensity of solvent-treated cells as 100% and intensity without cells as 0%. Then the cell growth inhibition ratio for triplicated wells was estimated.

The concentration of 50% growth inhibition ratio was calculated from the linear function found two values before and behind 50% growth inhibition ratio.

Determination of doubling time

To estimate the induction of micronuclei in astrocyte in logarithmic growth phase, doubling time was determined. In growth curve, culture time was plotted at the X axis, and cell numbers was plotted at the Y axis in logarithmic scale. In logarithmic growth phase, the numerical formula of regression was expressed by the relationship between cell plating time and cell numbers. The doubling time of nucleated cells was determined from this formula.

Micronucleus assay

Ten thousand nucleated cells suspended with 15% FBS/MEM were plated on poly-D-lysine coated plastic 30-mm rounded coverslips in 6-well-plate. The culture plates were incubated in a 5% CO₂ incubator at 37°C.

The nucleated cells were rinsed with PBS. And the cells on the coverslips fixed with cold methanol were stained with GFAP and acridine orange. The frequency of micronucleated astrocytes was determined by counting 1000 GFAP-positive nucleated cells.

The identification of micronucleated cells used the following criteria; counted cells should have normal cell morphology with cytoplasmic borders, the diameter of the micronuclei should be no larger than one-third of the main nucleus, and bi- or polynucleated cells should be not scored⁶⁾.

Triplicated coverslips were assayed for each time and dose. The experimental results were analyzed statistically using the Kastenbaum-Bowman table¹¹⁾.

Results

Growth curve

The cells cultured in 75 cm²-culture flasks were washed several times with PBS, and then treated with 0.25% trypsin and 0.02% EDTA in PBS for 20 min at 37°C. After inactivation of trypsin activity, the cell suspensions were centrifuged at 1000 rpm for 5 min. The cell pellet was resuspended in 15% FBS/MEM. The nucleated cell density was adjusted to 5×10^5 cells/dish using a hemocytometer with 0.2% trypan blue staining, and the cells were plated on 60-mm petri dishes with 15% FBS/MEM. The nucleated cell density of dishes were counted after 24, 48, 72, 96, 120, 144, and 168 h after cells plating. Triplicated dishes were counted at each time. The ratio of GFAP-positive cells in

nucleated cells was determined at 168 hours after plating.

The number of nucleated cells was decreased at 24 h after plating, and increased until 72 h in logarithmic growth phase (Fig. 1). The cell number reached a plateau (1×10^6 cells/dish) after 96 h. The relationship between cell plating time (from 24 h to 72 h) and cell numbers was determined by the following formula:

$$X = 102.5 \times \log(Y) - 536.7$$

X: culture time (h), Y: cell numbers/plate.

Consequently, doubling time of nucleated cells was determined to be 31 h.

The ratio of GFAP-positive cells (astrocytes) in nucleated cells at 168 h after cell plating was $93.0 \pm 2.0\%$.

Growth inhibition ratio

The one hundred thousand of the nucleated cells were plated on 6-well-plate (Falcon) with 15% FBS/MEM for 48 h, and the culture plates were exposed to MMC, VCR without metabolic activation system, and CPM with metabolic activation system in a 5% CO₂ incubator at 37°C. The exposure time of chemicals in growth inhibition ratio study was chosen to above twice of the doubling time, 72 h.

The concentrations of MMC exposed for 72 h were 8×10^{-5} , 1.6×10^{-4} , 3.2×10^{-4} , 6.4×10^{-4} , and 1.28×10^{-3} mM, and VCR were 8.5×10^{-6} , 1.7×10^{-5} , 3.4×10^{-5} , 6.8×10^{-5} , and 1.35×10^{-4} mM. The 50% growth inhibition ratio of MMC was calculated to 5.7×10^{-4} mM (Fig. 2). And the 50% growth inhibition ratio of VCR was calculated to $3.2 \times$

Fig. 1. Growth curve of cultured nucleated cells prepared from new born rat brain.

Triplicated dishes were counted at each points, and standard deviation per points was shown.

Fig. 2. Growth in cultured nucleated cells prepared from new born rat brain inhibited by 72 h exposure of MMC.

Triplicated plates were counted at each points, and standard deviation per points was shown.

Fig. 3. Growth in cultured nucleated cells prepared from new born rat brain inhibited by 72 h exposure of VCR.

Triplicated plates were counted at each points, and standard deviation per points was shown.

Fig. 4. Growth in cultured nucleated cells prepared from new born rat brain inhibited by 6 h-exposure time of CPM with metabolic activation system and 66 h-recovery time.

Triplicated plates were counted at each points, and standard deviation per points was shown.

10^{-5} mM (Fig. 3).

The nucleated cells exposed to CPM with 5% S9mix for 6 h (exposure time)⁵⁾, were exchanged to fresh 15% FBS/MEM, and cultured in 15% FBS/MEM for 66 h (recovery time). The concentrations of CPM were 0.14, 0.22, 0.29, 0.36, 0.43, 0.50, 0.58 and 0.65 mM. Physiological saline was used as a solvent control for the three chemicals. The

Table 1. Frequency of micronuclei in cultured new born rat astrocytes induced by 3.2×10^{-4} mM MMC in time study

Exposure time (h)	Frequency of micronuclei	
	Control (% $\pm$ SD)	MMC (% $\pm$ SD)
24	8.0 $\pm$ 0.00	15.3 $\pm$ 0.58*
48	6.3 $\pm$ 1.53	18.7 $\pm$ 3.51**
72	8.0 $\pm$ 1.73	32.7 $\pm$ 8.50**
96	6.7 $\pm$ 2.08	23.0 $\pm$ 2.65**
120	6.7 $\pm$ 2.08	17.0 $\pm$ 2.00**

*: $p < 0.05$. **: $p < 0.01$. MMC: mitomycin C, SD: standard deviation.

50% growth inhibition ratio of CPM was calculated to 0.54 mM (Fig. 4).

Frequency of micronuclei induced by MMC

Time study was performed at the concentrations of 3.2×10^{-4} mM MMC for 24, 48, 72, 96, and 120 h. Significant increased frequencies of micronuclei compared with control were observed at 24 h ($p < 0.05$), 48, 72, 96, and 120 h ($p < 0.01$). The highest frequency of micronuclei was obtained at 72 h (Table 1). Dose-response study of MMC was done at 72 h exposure of MMC. The concentrations of MMC were shown on Table 2. From dose-response study at 72 h exposure time, significant increased frequencies of

Table 2. Frequency of micronuclei in cultured new born rat astrocytes induced by MMC at 72 h exposure in dose-response study

Concentration of MMC ($\times 10^{-4}$ mM)	Frequency of micronuclei (% $\pm$ SD)
0.0	8.7 $\pm$ 0.58
0.8	14.3 $\pm$ 4.16*
1.6	14.7 $\pm$ 1.53*
3.2	17.3 $\pm$ 2.52**
6.4	26.7 $\pm$ 4.93**
12.8	10.7 $\pm$ 2.08

*: $p < 0.05$. **: $p < 0.01$. MMC: mitomycin C, SD: standard deviation.

Fig. 5. Micronuclei induced by 1.6×10^{-4} mM MMC in astrocytes at 72 h exposure.

Nucleated cells were double labeled with acridine orange to identify main nuclei and micronuclei (A), and with GFAP antibody to identify astrocytes (B). Arrowhead indicates micronuclei. Scale bar=20 μ m.

micronuclei were observed at all doses except the highest concentration of 12.8×10^{-4} mM (Table 2). The dose-response relationship of micronucleus frequencies was

Table 3. Frequency of micronuclei in cultured new born rat astrocytes induced by 3.4×10^{-5} mM VCR in time study

Exposure time (h)	Frequency of micronuclei	
	Control (% $\pm$ SD)	VCR (% $\pm$ SD)
24	6.7 $\pm$ 0.58	11.3 $\pm$ 1.15
48	8.0 $\pm$ 1.00	22.3 $\pm$ 2.08**
72	8.3 $\pm$ 2.52	21.3 $\pm$ 1.53**
96	7.3 $\pm$ 2.52	18.3 $\pm$ 2.52**
120	6.7 $\pm$ 1.15	17.0 $\pm$ 1.00**

**: $p < 0.01$. VCR: vincristine, SD: standard deviation.

Table 4. Frequency of micronuclei in cultured new born rat astrocytes induced by VCR at 48 h exposure in dose-response study

Concentration of VCR ($\times 10^{-5}$ mM)	Frequency of micronuclei (% $\pm$ SD)
0.0	7.3 $\pm$ 1.53
0.85	13.7 $\pm$ 1.15*
1.7	15.3 $\pm$ 3.51**
3.4	23.0 $\pm$ 5.57**
6.8	21.0 $\pm$ 1.00**
13.5	20.3 $\pm$ 0.58**

*: $p < 0.05$. **: $p < 0.01$. VCR: vincristine, SD: standard deviation.

observed until 6.4×10^{-4} mM MMC. Frequencies of micronuclei were decreased at 12.8×10^{-4} mM (Table 2). Fig. 5A and B show the morphological features of micronuclei in astrocytes following 72 h exposure to MMC at 1.6×10^{-4} mM.

Frequency of micronuclei induced by VCR

Time study was performed at the concentrations of 3.4×10^{-5} mM VCR for 24, 48, 72, 96, and 120 h. Significant increased frequencies of micronuclei in time study were observed at 48, 72, 96, and 120 h ($p < 0.01$). The highest frequency of micronuclei was obtained at 48 h (Table 3). Dose-response study of VCR was done at 48 h exposure of VCR. The concentrations of VCR were shown on Table 4. Table 4 shows a significant increase of micronucleus frequency at 48 h exposure in dose-response study at 8.5×10^{-6} ($p < 0.05$), 1.7×10^{-5} , 3.4×10^{-5} , 6.8×10^{-5} , and 13.5×10^{-5} mM ($p < 0.01$). The dose-response of micronucleus frequencies was observed up to 3.4×10^{-5} mM and then decreased tendency to 13.5×10^{-5} mM.

Table 5. Frequency of micronuclei in cultured new born rat astrocytes induced by 0.50 mM CPM with metabolic activation system in time study

Exposure time - recovery time (h)	Frequency of micronuclei	
	Control (% $\pm$ SD)	CPM (% $\pm$ SD)
6-18	7.3 $\pm$ 1.53	13.0 $\pm$ 6.25**
6-42	8.0 $\pm$ 1.73	16.7 $\pm$ 4.16**
6-66	7.7 $\pm$ 1.53	20.0 $\pm$ 1.73**
6-90	6.0 $\pm$ 2.00	13.7 $\pm$ 4.62**
6-114	6.3 $\pm$ 1.53	9.0 $\pm$ 3.00

** $p < 0.01$. CPM: cyclophosphamide, SD: standard deviation.

Frequency of micronuclei induced by CPM

In time study of 0.50 mM CPM with S9mix, the cells were exposed for 6 h (exposure time) and cells were cultured in fresh 15% FBS/MEM for an additional 18, 42, 66, 90, and 114 h (recovery time). Significant increases of micronucleus frequency in time study of 0.50 mM were observed at 6-18, 6-42, 6-66, and 6-90 h ($p < 0.01$). The highest frequency of micronuclei was obtained at 6-66 h (Table 5). Dose-response study of CPM at the concentrations of 0.036, 0.072, 0.18, 0.40, and 0.50 mM was carried out in 6 h-exposure-66 h-recovery (6-66 h). The frequencies of micronuclei obtained from a dose-response study in 6-66 h increased significantly at 0.036, 0.072, 0.18, 0.40, and 0.50 mM ($p < 0.01$) (Table 6). The dose-response relationship was observed to concentrations until 0.18 mM of CPM and then decreased to 0.50 mM (Table 6).

Discussion

Since a highly significant correlation has been reported between mutagenicity and carcinogenicity, genotoxicity screening for chemicals has been increasingly important for evaluating their carcinogenicity²⁾. There are some genotoxicity screening tests, such as *in vitro* MN test using organs and tissue-derived cells, which take from one wk to six months to estimate the genotoxicity of chemicals^{3, 12)}. The *in vitro* SD rat-astrocyte MN test developed by us would be important as a short-term screening test because: (1) it takes about six wk to estimate the genotoxicity of chemicals; (2) brain tumors usually arise from glial cells⁷⁾ and there are few genotoxicity tests using brain-derived cells⁶⁾; (3) SD rat-astrocyte might be sensitive to detect genotoxic chemicals since Maekawa *et al.* reported that the incidence of spontaneous astrocytomas in SD rats were higher than in other several strains of rat¹³⁾.

Table 6. Frequency of micronuclei in cultured new born rat astrocytes induced by CPM at 6 h-exposure-66 h-recovery in dose-response study

Concentration of CPM (mM)	Frequency of micronuclei (% $\pm$ SD)
0.0	7.0 $\pm$ 2.00
0.036	25.0 $\pm$ 1.00**
0.072	29.3 $\pm$ 5.13**
0.18	71.0 $\pm$ 8.72**
0.29	30.0 $\pm$ 5.57**
0.40	18.3 $\pm$ 3.21**
0.50	17.3 $\pm$ 2.08**

** $p < 0.01$. CPM: cyclophosphamide, SD: standard deviation.

We applied three anticancer agents (MMC, VCR and CPM) as mutagens, which have been known to produce micronuclei in a variety of cultured cells^{5, 14)}, to establish the *in vitro* rat-astrocyte MN test. The anticancer action of MMC reported caused severe fragmentation, depolymerization, irreversible cross-linking of DNA in the cells, and inhibition of cellular DNA synthesis¹⁵⁾. The anticancer action of VCR reported would be due to the inhibition of formation and to the disruption of mitotic spindle microtubules¹⁶⁾. The anticancer action of CPM reported was activated by hepatic mixed functions oxidases and the metabolites indicate anticancer effects. CPM is oxidized by P450 contained in S9, and is metabolized to phosphoramidate mustard, nornitrogen mustard, and acrolein. These three metabolites of CPM bind to DNA and form DNA adducts¹⁴⁾. In time study, the highest frequency of micronuclei was observed at 48 h, 72 h by VCR and MMC without S9mix, and 6 h-exposure-66 h-recovery by CPM with S9mix, respectively. The doubling time of rat-astrocyte was determined to be 31 h. It was suggested that clastogenic chemicals such as MMC, CPM induced structural chromosomal aberrations in the first cell cycle and then the micronuclei was directly derived from them in the second cell cycle¹⁶⁾. It was also suggested that spindle poison such as VCR induced micronuclei, which was caused by lagging of a whole chromosomes in the first cell cycle. And we have shown that micronucleus induction in rat-astrocyte is dose-dependent for these three genotoxic agents. Micronucleus frequency of astrocytes was decreased at higher concentrations for three chemicals, since it was suggested that micronucleus frequency was decreased as the cytotoxicity induced by chemicals was observed⁵⁾.

Our study has shown that micronuclei was induced by

use of both methods without S9mix (MMC, VCR) and with S9mix (CPM). It is suggested that this MN test using new born rats-astrocyte could be used as a screening test in the same manner as the Ames *Salmonella* mutagenicity assay, *in vitro* micronuclei assay using established cell lines and cultured human lymphocytes^{5,12}. The astrocytomas induced by chemicals have ever been investigated by carcinogenicity assay in experimental animals^{18–28}. Our study suggested that it might have a potential for detecting chemicals inducing astrocytomas which cannot be detected by other screening tests. Other anticancer drugs, mutagens and carcinogens should be estimated in order to confirm our method.

This experiment is the first step to develop and estimate our screening test of chemicals using new born rat primary cultured astrocytes, and might be applicable to detect new chemicals inducing astrocytomas, and to detect structural chromosomal aberrations, and DNA damages etc. Further study is needed.

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