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Application of cell sorting for enhancing the performance of the cytokinesis-block micronucleus assay

Ayumi Nakamura[1], Satoru Monzen[1], Yuki Takasugi[1], Andrzej Wojcik[2] and Yasushi Mariya[1]*

[1]Department of Radiological Life Sciences, Division of Medical Life Sciences, Hirosaki University Graduate School of Health Sciences, Hirosaki, Aomori 036-8564, Japan
[2]Centre for Radiation Protection Research, MBW Department, Stockholm University, SE-106 91, Stockholm, Sweden
*Corresponding author. Department of Radiological Life Sciences, Division of Medical Life Sciences, Hirosaki University Graduate School of Health Sciences, Hirosaki, Aomori 036-8564, Japan. Tel/Fax: +81-172-39-5917; Email: mariya@hirosaki-u.ac.jp
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ABSTRACT

Among the numerous methods available to assess genotoxicity, the cytokinesis-block micronucleus (CBMN) assay is very popular due its relative simplicity and power to detect both clastogenic and aneugenic compounds. A problem with the CBMN assay is that all DNA damaging agents also inhibit the ability of cells to progress through mitosis, leading to a low number of binucleated cells (BNCs). One method to resolve this issue is to ensure a sufficient proportion of BNCs in the samples. In the current study, the applicability of a cell sorting system capable of isolating cell fractions containing abundant BNCs was investigated. Furthermore, to investigate the relationship between the cell division delay due to radiation exposure and the generation of BNCs and micronuclei (MN), we assessed a series of lag times between radiation exposure and addition of cytochalasin-B (Cyt-B). Cells from the human chronic myelogenous leukemia cell line K562 were exposed to X-rays (2 Gy and 4 Gy), and Cyt-B was subsequently added at 0, 6 and 12 h following irradiation. After treatment with Cyt-B for 24 h, the percentage of BNCs, the MN frequency and the cell cycle distribution were analyzed. In addition, cells displaying the DNA contents corresponding to BNCs were isolated and analyzed. The results indicate that applying the cell sorter to the CBMN assay increased the percentage of BNCs compared with the standard method. Thus, this technique is a promising way of enhancing the capacity of the CBMN assay.

KEYWORDS: cytokinesis-block micronucleus assay, cell cycle delay, ionizing radiation, cell sorting, binucleated cells

INTRODUCTION

The cytokinesis-block micronucleus (CBMN) assay is employed to determine the level of cytogenetic damage in cells exposed to various cytotoxic agents [1–3]. The assay relies on estimating the frequency of micronuclei (MN) in binucleated cells (BNCs) that are formed in the presence of cytochalasin-B (Cyt-B) and represent cells that have divided once [4, 5].

A problem with the CBMN assay is that all DNA damaging agents also inhibit the ability of cells to progress through mitosis [6, 7]. When scoring chromosomal aberrations, this problem can be mitigated to some extent by analyzing prematurely condensed chromosomes induced by treating cells with phosphatase inhibitors [8]. No such mitigation exists for the CBMN assay, and alternatives for analyzing MN in BNCs were sought, such as scoring nuclear division indices as a marker of exposure [9, 10]. Automation of MN analysis can also be of help because it allows analyzing of a large number of cells on microscopic slides [11–14]. However, in order to avoid scanning a large number of microscopic slides, it would be advantageous to be able to prepare slides by dropping a cell population enriched in the fraction of BNCs. Such approach is the aim of the present study.

We used a flow cytometer equipped with a cell sorting system that can enrich the cell population with BNCs or selectively isolate BNCs. We investigated the ability of this cell sorting system to improve the performance of the CBMN assay using the human chronic

myelogenous leukemia cell line K562. Furthermore, we investigated the influence of radiation-induced cell cycle delay and the timing of Cyt-B treatment after radiation exposure on both the proportion of BNCs and the production of MN. The results show that our approach is a promising solution for mitigating the problem of low numbers of BNCs in the CBMN assay.

MATERIALS AND METHODS

Reagents

The cell culture medium, RPMI 1640 supplemented with penicillin/streptomycin, was purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Japan Bio Serum (Hiroshima, Japan). Cyt-B and calcium- and magnesium-free phosphate-buffered saline (PBS) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Hoechst33342 was purchased from Sigma–Aldrich (Saint Louis, Missouri, USA). Membrane permeation reagent (Cytofix/Cytoperm) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). The mounting medium for vvslide preparation was purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

In vitro X-ray irradiation

Irradiation (150 kVp, 20 mA, 0.5 mm aluminum and 0.3 mm copper filters) was performed using an X-ray generator (MBR-1520R; Hitachi Medical Co. Ltd, Tokyo, Japan) at a distance of 45 cm between the beam focus and the target. The dose was monitored with a thimble ionization chamber placed next to the sample. The dose rate was 1 Gy/min. Irradiation was carried out at room temperature.

Cytokinesis-block micronucleus assay

The established cell line K562 was purchased from the RIKEN BioResource Center (Tsukuba, Japan). The doubling time of the cells was 24 h. The K562 cells were seeded at a concentration of 1×10^5 cells/ml in a 35 mm cell culture dish (Corning Life Sciences, Falcon, New York, NY, USA) (containing RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin) and then incubated in a humidified atmosphere at 37°C with 5% CO_2. Cells were irradiated and then treated with Cyt-B at a final concentration of 6 μg/ml. Cyt-B treatment was performed at three different lag times (0, 6 and 12 h) after exposure to X-irradiation (2 Gy and 4 Gy). Cells were then incubated for 24 h in the presence of Cyt-B. Cells were prepared for assessment by washing with a membrane permeation reagent and staining with a 1 μl/ml Hoechst33342 solution. This procedure was referred to as 'the standard method' in this study. The slides were scored at ×400 magnification using a fluorescence/bright-field microscope (IX71; Olympus, Tokyo, Japan). At least 500 BNCs were scored per slide. The parameters used in this study were the number of MN per BNC and the percentage of BNCs, which was defined as the proportion of BNCs in the total countable cells on the slides, including mono-, bi-, and poly-nucleated cells. Cells were analyzed according to the criteria described by the International Atomic Energy Agency [2].

Cell cycle analysis

The cells were analyzed just before treatment with Cyt-B and after 24 h incubation in the presence of Cyt-B. They were harvested, washed and resuspended in RPMI 1640 medium containing Hoechst 33342 solution (1μl/ml) to stain the cellular DNA. A cell cycle distribution analysis was performed using a Cell Lab QuantaTM SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA). Using sham-irradiated cells as controls, the ranges of DNA contents representing different cell cycle phases and nucleation states (SubG1, G1, S, G2/M, S2 and poly) were determined on the DNA histogram (Fig. 1A). Here, we defined 'poly' as the region of octoploid cells and 'S2' as the region between G2/M and 'poly', indicating cells with DNA content between tetraploid and octoploid. These range values were also applied to measurements after irradiation and treatment with Cyt-B, and the parameter 'G2/M + S2 fraction' then indicates the fraction of cells that contain BNCs (see the equation 1).

$$\text{G2/M + S2 fraction} = (\text{G2/M + S2})/(\text{subG1 + G1 + S} + G2/M + S2 + poly) \quad (1)$$

Cell cycle analysis was performed following the irradiation (2 Gy and 4 Gy) of cells using 6 different harvesting intervals between 0 and 36 h, and the G2/M + S2 fraction was obtained from the DNA histogram. Cyt-B was added to the cells after irradiation using the above-mentioned intervals. Cells were then incubated in the presence of Cyt-B for 24 h, which resulted in a total harvesting time ranging from 24 to 60 h.

Cell isolation

After treatment with Cyt-B for 24 h, the G2/M + S2 fraction, which is considered BNC-rich, was isolated using a flow cytometer (BD FACSAria) equipped with a cell sorting system (Becton Dickinson and Company, Japan). The G2/M + S2 fraction was gated on the histogram, the cells were collected in PBS using the cell sorter, fixed and dropped on microscopic slides. Cells were scored in conformity with the standard CBMN method. In the present study, we named this newly introduced procedure the 'cell sorter–assisted method'.

Statistical analysis

Statistical analyses were performed using the Excel 2013 software (Microsoft Corp., Redmond, WA, USA) equipped with the Statcel 3 add-in software package. Data were expressed as the mean ± standard deviation (SD) of three independent experiments. The statistical significance of differences were determined using Student's *t*-test and the multiple comparison test. Differences resulting in a value of $P < 0.05$ were considered statistically significant.

RESULTS

Cell cycle analysis

The size of the G2/M + S2 fraction was measured after an incubation time ranging from 0 to 36 h. The highest accumulation of cells in G2/M + S2 was observed 12 h after irradiation, with a gradual decrease thereafter (Fig. 1B). The G2/M + S2 fractions after incubation for 0, 6 and 12 h after irradiation with 2 Gy were 29.8 ± 9.3% (mean ± SD), 44.5 ± 6.8% and 62.8 ± 0.3%, respectively. The G2/M + S2 fractions after incubation for 0, 6 and 12 h after irradiation with 4 Gy were 30.4 ± 7.8%, 40.4 ± 10.3% and 71.6 ± 1.5%, respectively. Statistically significant differences between the values were observed after irradiation with both 2 Gy and 4 Gy at 0 h and 12 h, as well as 6 h and 12 h.

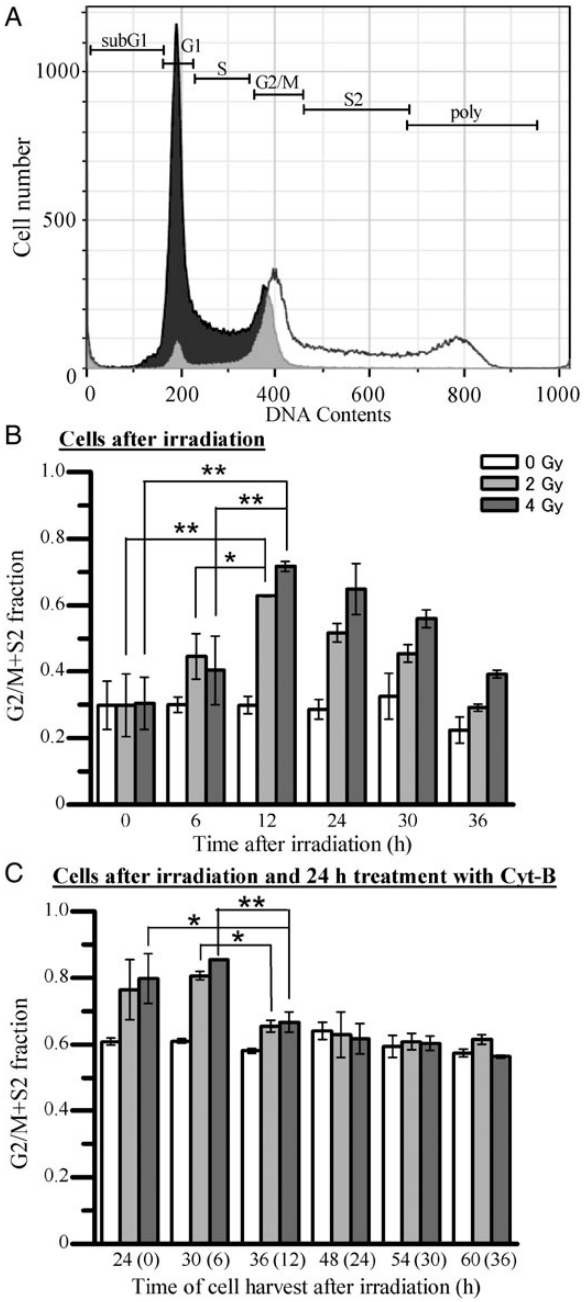

Fig. 1. Distribution of cell cycle. (A) DNA histogram obtained using a flow cytometer. The horizontal bars labeled subG1, G1, S, G2/M, S2 and poly indicate the cell cycle fractions of subG1, G1, S, G2/M, S2 (the region between G2/M and poly) and poly (the region of octoploid cells), respectively, on the DNA histogram. Grey: histogram of sham-irradiated cells used to determine the width of each fraction. White: histogram of cells treated with Cyt-B for 24 h. (B) The proportion of the cells in the G2/M + S2 fraction in the total number of cells (G2/M + S2 fraction) according to the lag time after exposures of 0, 2 and 4 Gy. (C) The G2/M + S2 fraction of cells treated with Cyt-B for 24 h according to the lag time until treatment after the exposures of 0, 2 and 4 Gy. Numbers in parentheses show the lag time between irradiation and addition of Cyt-B. Data are expressed as the mean ± SD. Statistical significance level: *$P < 0.05$ and **$P < 0.01$.

Next, the cell cycle distribution after 24 h of treatment with Cyt-B followed by a lag time ranging from 0 to 36 h was analyzed (Fig. 1C). The aim of this analysis was to find the optimal lag time for the cell sorter–assisted CBMN method. The G2/M + S2 fractions after a radiation dose of 2 Gy followed by 24 h of treatment with Cyt-B, which was added at 0, 6 and 12 h after exposure, were 76.5 ± 9.0%, 80.6 ± 1.2% and 65.3 ± 1.7%, respectively. The G2/M + S2 fractions after a dose of 4 Gy, followed by 24 h of treatment with Cyt-B, which was added at 0, 6 and 12 h after exposure, were 79.7 ± 7.3%, 85.5 ± 0.1% and 66.6 ± 0.3%, respectively. At later harvest times, the G2/M + S2 fractions remained constant at a level of ~60%, and differences between control and irradiated cells disappeared.

Generally, all values of the G2/M + S2 fractions in irradiated cells remained higher than 65% between 24 and 36 h of incubation (Fig. 1C). Further, there was a statistically significant difference between the G2/M + S2 accumulation observed in cells analyzed 6 h after irradiation and the corresponding accumulation observed in cells analyzed 12 h after irradiation (Fig. 1B and C). Therefore, in the following experiments, we focused on a lag time of 6–12 h before treatment with Cyt-B.

Percentage of BNCs and MN frequency obtained using the standard method

To estimate the genotoxic effects induced by X-ray irradiation of the cells, the CBMN assay was conducted. When employing the standard method, the percentage of BNCs significantly decreased in a dose-dependent manner for each lag time (Fig. 2A). With respect to the cells exposed to a radiation of 2 Gy and 4 Gy, those receiving Cyt-B at 6 and 12 h after irradiation showed significantly higher values for percentage of BNCs than those receiving Cyt-B immediately after irradiation.

Next, the number of radiation-induced MN per BNC (MN/BNC) was counted based on time and dose. The MN/BNC increased in a dose-dependent manner. With respect to the cells exposed to a radiation of 4 Gy, those receiving Cyt-B at 6 and 12 h after irradiation showed significantly higher values of MN/BNC than those receiving Cyt-B immediately after irradiation. The MN/BNC values after irradiation with 2 Gy and 4 Gy, followed by treatment with Cyt-B for 24 h with lag times of 6 and 12 h, are shown in Fig. 2B.

Percentage of BNCs and MN frequency obtained using the cell sorter–assisted method

By adding a cell sorting function to the flow cytometer, we could isolate cells in the G2/M + S2 fraction and analyze them for MN under a fluorescence microscope in the same way as the standard method. With our method, the percentage of BNCs after exposure to 0, 2 and 4 Gy followed by 24 h treatment with Cyt-B, which was added at 6 h after exposure, was 69.4 ± 5.0, 47.2 ± 4.2 and 32.0 ± 2.8, respectively. Further, the percentage of BNCs after exposure to 0, 2

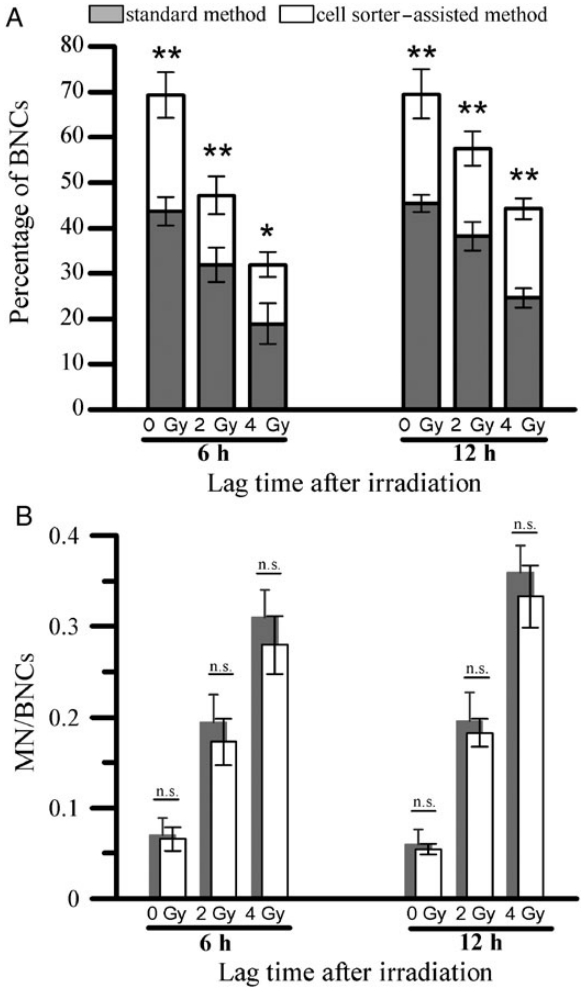

Fig. 2. Comparison of results obtained with the standard method and the cell sorter-assisted method. (A) The proportion of BNCs in the total number of countable cells, indicated as the percentage of BNCs. (B) The MN frequency, indicated as MN/BNCs. Data are expressed as the mean ± SD. Statistical significance level: *$P < 0.05$, **$P < 0.01$, n.s. = not significant vs standard method (same dose and lag time).

and 4 Gy followed by 24 h treatment with Cyt-B, which was added at 12 h after exposure, was 69.5 ± 5.5, 57.5 ± 3.7 and 44.3 ± 2.3, respectively (Fig. 2A). Compared with the standard method, the fraction of BNCs increased 1.55-fold with this method. The MN/BNC frequency increased in a dose-dependent manner. With respect to MN frequency, there were no significant differences between lag times of 6 and 12 h, as observed in the standard method (Fig. 2C).

DISCUSSION

The aim of the current study was to increase the fraction of BNCs in cell populations analyzed for the frequency of MN in order to improve the performance of the CBMN assay. To this end, we used a flow cytometer equipped with a cell sorting system. In addition, we analyzed the percentage of BNCs and the MN frequency with respect to the treatment time and the effects of radiation-induced cell cycle delay.

First, our results confirmed that the cell sorter–assisted method leads to an enrichment of BNCs on microscopic slides, as compared with the standard method. We were able to effectively eliminate cells of less than tetraploid and those of more than octoploid. Therefore, in our experimental setup the enrichment equaled 55%. This value could possibly be further increased by isolating a pure S2 fraction of cells instead of G2/M + S2. However, such a strategy would eliminate those BNCs in which both nuclei are in the G1 phase of the cell cycle and are indistinguishable from mononuclear G2 cells. BNCs with both nuclei in the G1 phase at the time of cell harvest would have been at a late phase of the cell cycle during treatment, and it is known that cellular sensitivity to radiation dramatically increases as cells approach mitosis [15]. Hence, this procedure would lead to an artificial reduction of the MN frequency, biasing the outcome of the experiment.

One could argue that, with the automated MN analysis methods now available [11–14], an enrichment of the BNCs yield by 55% is not worth the effort associated with cell sorting. Here it should be recalled that the automated MN analysis systems generally detect a lower frequency of MN as compared with manual scoring. Therefore, the advantage of the cell sorting method is that it will increase BNC fractions without decreasing MN frequency, where analysis by the standard CBMN method would require preparing many parallel microscopic slides. In other words, our newly introduced method maintains the quantitative relationship between the radiation dose and the MN frequency. A large proportion of BNCs was easily observed on the microscopic slides, being a clear improvement of the CBMN assay efficiency at scoring MN under conditions of low cell proliferation. Thus, we believe that our sorting method will be a useful tool for genotoxicity testing using the CBMN test.

It is well known that the genome monitoring mechanisms that respond to DNA damage caused by radiation include the G2/M checkpoint, which gives cells time to repair DNA damage before entering mitosis [16]. In accordance with this mechanism, we observed a dose-dependent accumulation of cells in the G2/M + S2 fraction 12 h post irradiation, with a subsequent decline. This bell-shaped response illustrates the phenomenon of cell accumulation in the G2 block and its release. In order to find the relationship between the cell cycle block and the level of G2/M + S2 cells in the presence of Cyt-B, the actin spindle inhibitor was added to the culture medium at 0, 6, 12, 24, 30 and 36 h after irradiation and the condition was retained until fixation 24 h later. The highest level of G2/M + S2 fraction was observed when Cyt-B was added 6 h post irradiation. An interesting observation is the apparent lack of correlation between the peak times for the G2/M + S2 fractions. Thus, when the percentage of BNCs was analyzed on microscopic slides, a higher level of binucleation was seen after a lag time of 12 h as compared with 0 and 6 h, demonstrating that the G2/M + S2 fraction does not fully represent the binucleation state of a cell population following addition of Cyt-B. This seeming discrepancy is due to the fact that, in the presence of Cyt-B, the G2/M + S2 fraction contains BNCs in G1 that have the same DNA content as mononuclear cells in G2 and are therefore indistinguishable from one another. Also, it suggested that the influence of the radiation-induced cell cycle delay still existed after a lag time of 6 h.

When the MN frequency was analyzed as a function of fixation time after irradiation, a steady increase of MN frequency with fixation time was seen, with the highest level of damage after a lag time of 12 h. This result is interesting and requires a more detailed discussion. When asynchronously proliferating cells are harvested for analysis of chromosomal aberrations following accumulation of mitotic cells by the colcemid-block method, the interpretation of yield-time curves for chromatid aberrations induced by low linear energy transfer radiation is very complex [15, 17–20]. The major problem is that cells progress through the cell cycle with variable speed, hence a simple relationship between the time of treatment plus the adding of colcemid and the phase of the cell cycle during which the treatment occurred is not possible. Nevertheless, when aberrations are analyzed on a series of slides prepared after increasing times post treatment, the mean yield of chromatid aberration declines with post-treatment time [21–23]. This result is interpreted as a reflection of the increasing radiosensitivity of cells as they progress from S-phase towards mitosis [24]. It could be expected that similarly, as in the chromosomal aberration assay, a decreasing frequency of MN with time between irradiation and harvest will be seen. This, however, is not the case, as demonstrated in the present investigation and by others using TK6 cells [25]. What is the reason for this discrepancy? One problem is that the aberration frequency assessed in cells harvested after a short colcemid block represent an instantaneous average, whereas MN scored after a long treatment time with Cyt-B represent a running average [26]. No direct relationship between the two can be expected. A second problem is the influence of Cyt-B on cell cycle progression. It is well known that cancer cells are particularly sensitive to Cyt-B–induced cell cycle inhibition at concentrations used in the present study [27]. It can therefore be assumed that the increase of MN frequency with harvest time is related to the release of cells from the Cyt-B–induced cell cycle arrest rather than from cell cycle arrest induced by DNA damage from radiation exposure. Needless to say, this assumption needs to be tested in separate experiments that are beyond the scope of the present study.

In conclusion, our results confirm the idea that the cell sorter–assisted CBMN method is superior to the standard CBMN assay in that it leads to a higher fraction of BNCs on microscopic slides. Importantly, the scored frequency of MN is same with both methods, demonstrating that the use of the cell sorter–assisted method does not lead to an under- or overestimation of the response to a genotoxic agent.

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