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진보된 세포분열억제 소핵 평가법을 활용한 체외 유전독성검사 및 3차원 핵 이미지 분석

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Three Dimensional Nucleus Imaging analysis & *in vitro* Genotoxicity Test through Advanced Cytokinesis-Block Micronucleus Assay

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ABSTRACT. The cytokinesis-block micronucleus cytome assay is a measuring of DNA damage in once-divided binucleated (BN) cells scoring micronuclei (MNi) that are widely used as biomarkers of cancer risk in humans on drugs or chemicals. However, this assay is still challenging due to the speed of sample processing and the toxicity of organic chemicals on researcher. To address these problems, we developed a new and convenient method for the micronucleus cytome assay. This method by DAPI stained micronucleus detection is non-toxic to the researcher and is being simplified because it does not require the use of methanol and acetic acid for fixing cell. Especially, three dimensional imaging of nucleus by formalin fixed DAPI staining provides more detailed information for testing the effects of chemical and bio-materials, compared to the classic method. In this study, we examine the spatial distribution of the TANNylated GFPs by advanced cytokinesis-block micronucleus (ACBMN) assay. The TANNylated GFPs were precisely observed in the inner nucleus by three dimensional imaging, whereas GFPs were not detected in any regions of cells. This micronucleus assay can readily be used in genetic based toxicity and efficacy assay to the various bio-chemicals and pharmaceutical evaluation.

KEY WORDS: The cytokinesis-block micronucleus cytome assay, micronuclei (MNi), advanced cytokinesis-block micronucleus (ACBMN) assay DAPI staining, TANNylated GFPs, Paraformaldehyde (PFA), Three dimensional image

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Introduction

Micronucleus is a small nucleus that the chromosome is not integrated into one of the daughter nuclei during cell division. It is usually the maker of genotoxic events and chromosomal instability in cancer cells (Heddle, 1973).

The cytokinesis-block micronucleus (CBMN) assay was developed for measuring micronucleus in cultured human and/or mammalian cells, which was the reliable evidence for cancer risk by exposure to physical and chemical agents (Stich et al., 1982; Fenech, 2000; Fenech, 2007). Micronuclei (MNi) were expressed in once-divided binucleated (BN) cells after blocking cytokinesis with cytochalasin-B (Cyt-B) that is an inhibitor of microfilament ring assembly (Fenech et al., 1985; Fenech et al., 1985). It results from break of unrepaired double strands in DNA or chromosome loss (Savage et al., 1993). Its reliability and reproducibility were made to the standard cytogenetic tests for genotoxicity tests in human and mammalian cells.

The classical cytogenetic assay provides the precise observation in metaphases and the most detailed analysis for carcinogenic chemicals. However, this classical method requires an alternative and simpler approach because of the complexity and toxicity. In the CBMN assay, the detection of micronucleus takes long time through the complex sampling procedures. Furthermore, organic chemicals for fixing cells are toxic to researcher who works with this assay due to evaporation. We have proposed the best resolution that micronucleus assay by formalin fixed DAPI staining was not used organic solvent for fixing cells. It was significantly simplified and non-toxic to the sampling process. For example, the cultured cells are directly fixed with PFA and then DAPI is used to stain the nucleus and micronucleus. Therefore, PFA fixing can reduce 37 to 20 steps for micronucleus assay procedures, compared to the classical method, and exposure to organic solvents.

Toxicologists have long appreciated to analyze drug toxicity based on two dimensional results. However, there were limitations on data analysis from two dimensional image because biological samples are intrinsically three dimensional and damaged during sectioning. For these reasons, there is an increasing the need for volumetric imaging to study the three-dimensional structure of cells and tissues. The most critical example is neuronal network, which neurons extend in many regions in space and their nature cannot be ascertained by a thin section. Therefore, we have applied volumetric image conception to more accurate evaluation in nucleus interaction with chemical and bio-materials.

In the present study, we demonstrate that the formalin fixed DAPI staining reduces the steps for micronucleus assay procedures, and the exposure to organic solvents. We also show that the detection of micronucleus by the ACBMN that we named is remarkably similar to the cytokinesis-block micronucleus cytome assay. However, the ACBMN assay is more convenient and less-toxic to the researcher for micronucleus assay. Furthermore, we found that it gives more information by three dimensional bio-image of nucleus related to chemical and bio-materials. Therefore, our study shows that the ACBMN assay is useful a new protocol in future genetic based toxicity.

Materials and Methods

1. Reagents

- MEM medium without L-glutamine (Gibco, Cat. No. 10370)
- Heat-inactivated, sterile, Fetal Bovine Serum (FBS; Gibco, Cat. No.10082139)
- L-Glutamine (200 mM, Gibco Cat. No. 25030)
- Penicillin-Streptomycin (10,000 U/mL, Gibco, Cat. No. 15140122)
- Ca^{++}-Mg^{++}-free Dulbecco's phosphate buffered saline (CMF D-PBS, Gibco, Cat.No. 14190235)

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- 0.05% Trypsin-EDTA (Gibco, Cat.No. 25300054)
- Post-mitochondrial supernatant (S9, Molecular Toxicology Inc, Cat. No. 11-01L)
- Cofactor-I (Oriental Yeast Co Ltd, Cat. No. 309-50611)
- Cyclophosphamide (Sigma, Cat. No. C3250000)
- Cytochalasin B (Sigma, Cat. No. C6762)
- 75 mM potassium chloride (KCL, CAS No. 7447-40-7)
- 4% paraformaldehyde solution in PBS (Affymetrix, Cat. No. AAJ19943K2)
- Gurr buffer tablets, pH 6.8 (Gibco, Cat. No. LS10582013)
- Giemsa staining solution (Sigma, Cat. No. 48900)
- DAPI solution (Life science, Cat.No. R37606)
- Tannin (Sigma, Cat. No. 403040)

2. Expression and purification of GFP

The *GFP* gene was cloned into a modified pET28a_Tev vector and GFP was expressed in *E. coli* BL21RILP strain cells in *N*-terminal his-tag fusion. The protein was purified with Ni-NTA resin and the his-tags were removed with TEV protease. GFP was further purified using size exclusion chromatography equilibrated with a buffer containing 100 mM NaCl and 50 mM Tris HCl.

3. Preparation of TANNylated GFP

TA solution (10 mM) and GFP solution (5.1 μg, stock in PBS, pH 7.4) were prepared. They were then mixed vigorously at a volumetric ratio of 1:1 for GFP TANNylation (pH 7.4). All samples were incubated at room temperature for 30 min, and then treated to CHL cells for 4h.

4. Culture medium

Prepare by MEM medium supplement with 10% FBS, 2 mM L-Glutamine, 1% Penicillin-Streptomycin

5. Cell strain

CHL/IU (Chinese hamster lung cells, ATCC, CRL-1935)

6. Cytokinesis-block micronucleus assay

- Prepare a T-25 flask of CHL (1.5 x 105 cells/5 mL) cells culture in 5 mL MEM with 10% FBS, L-glutamine, and penicillin-streptomycin and grow in a 37 ℃, 5% CO2 tissue culture incubator for 48 hr
- Change fresh medium after 48 hr
- Add S9 (final concentration, 2% v/v)
- Treatment of vehicle and CPA
- Incubation at 37 ℃ for 3 hr
- When the end of treatment period, replace the fresh medium with 3 ㎍/mL of cytochalasin B
- about 21 hr additional cell culture
- Remove culture medium, wash cells twice in DPBS and collect cells by trypsinization followed by Centrifugation 1,000 rpm for 5 min at 4 ℃
- Discard of supernatant
- Add 75 mM of KCL and mix
- Incubation for 5 min at RT
- Add ice cold Fixative I solution [acetic acid: methanol=1:5 (v/v)], concurrently mix (Primary fix)
- Incubation for 20 min at 4 ℃
- Centrifugation 1,500 rpm for 5 min at 4 ℃ (Eppendorf, Cat. No. 5920R)
- Discard of supernatant
- Add ice-cold Fixative I solution, concurrently mix (Primary fix)
- Incubation for 30 min at 4 ℃
- Centrifugation 1,500 rpm for 5 min at 4 ℃
- Discard of supernatant
- Add ice-cold Fixative II solution [acetic acid: methanol=1:19 (v/v)], concurrently mix (Secondary fix)
- Incubation for 30 min at 4 ℃
- Centrifugation 1,500 rpm for 5 min at 4 ℃
- Discard of supernatant (remain about 2 mL of Fix solution)

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- Vortex
- Drop the suspended sample onto the microscope-slide
- Air dry
- Staining with 5% Giemsa
- MN scoring (Nikon, Eclipse Ni)

7. Advanced cytokinesis-block micronucleus assay

- Prepare a 8-well slide chamber (8 well, ibid.i, Cat. No. 80826) of CHL (1.0 x 10^4 cells/ 0.2 mL) cells culture in 5 mL MEM with 10% FBS, L-glutamine, and penicillin- streptomycin and grow in a 37 ℃, 5% CO_2 tissue culture incubator for 48 hr
- Incubation at 37 ℃ for 48 hr
- Change fresh medium
- Add S9 (final concentration, 2% v/v)
- Treatment of vehicle and CPA
- Incubation at 37 ℃ for 3 hr
- When the end of treatment period, replace the fresh medium with 3 μg/mL of cytochalasin B
- About 21 hr additional cell culture
- Discard treatment medium and wash with PBS
- Fix the cells by incubating in 4% paraformaldehyde in PBS for 10 min at RT
- Permeabilize the fixed cells by incubating in 0.2% Triton X-100 in PBS for 2 min at RT
- Staining with DAPI (fluorescence)
- MN scoring (Leica, TCS SP8)

Results

1. The Detection of Specific Nuclear Anomaly by CBMN and ACBMN assay.

In an in vitro experimental setting, we used culture cell container in ACBMN instead of the conventional slide method in CBMN to observe the micronucleus. Figure 1 shows three representative photographic images of the morphology of nuclear anomaly. Nuclear anomaly which is an indication for genotoxic effect (MNi) is as shown in Figure 1 (A and B); their morphology is similar to those observed by CBMN and ACBMN assay. In this respect, ACBMN assay can be a new protocol to detect micronucleus by geno- toxic carcinogens.

2. Detection for TANNylated GFP interaction with Nucleus.

New bio-chemical materials were investigated by ACBMN assay whether they are carcinogens or not. TANNylated green fluorescent proteins (GFP) were prepared as the previous paper (Shin, 2018). To test the genetic toxicity of this material in vitro, we applied 5.1 μ g of GFPs with TANNylated or unTANNylated individually to CHL cells. Three dimensional images of GFP and DAPI in each condition were obtained using confocal microscope to confirm more accurately whether or not the TANNylated GFP and nucleus were interacted (Fig. 2). For the un-TANNylated control GFP treatment, the fluorescence emission was not observed in the cytoplasm or nucleus of CHL cells (Fig. 2, top). For the TANNylated GFP treatment, the fluorescence emission was interestingly observed in the nucleus, but not in the cytoplasm (Fig. 2, bottom). However, Micronucleus by DAPI image were not detected in both of TANNylated or unTANNylated GFP treatments. These results suggest that TANNylated or unTANNylated GFP did not induce genetic toxicity, and a useful strategy for gene targeting by bio-chemical material.

Discussion

MNi assay has developed for a robust assay for cancer risk assessment with application in ecotoxicology (Gauthier et al., 1999), nutrition (Fenech et al., 1995), radiation (Scott et al., 1998), and importantly testing of new pharmaceuticals and other chemicals (Lorge et al., 2006). We now demonstrate that ACBMN assay can detect the micronucleus that is genetic damage maker as CBMN assay. In addition, our method does not only show the reduction of the experimental procedures and inhalation of organic chemical exposure, but also show three dimensional image

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that leads to increase the accuracy of analysis in bio-chemical material application in vitro system.

CBMN assay using organic solvent has been well demonstrated in protocol for the detection of micronucleus (Heddle et al., 1973; Schmid et al., 1975), but there is a need for quicker and more safe protocol. In the present study, we developed advance in the CBMN assay by the culture cell container and 4% PFA fixing that has reduce the experiment steps and inhalation for organic solvent. Although we did only tested Cyclophosphamide (CPA) for the positive control to detect the micronucleus, we showed that the morphology of nuclear anomaly in the ACBN assay was comparable with the classical CBMN assay (Fig. 1). However, to accurate the ACBMN assay is required to further studies and test more carcinogenic chemicals.

Two-dimensional sections have been a common strategy to analyze pathology and micronucleus, but recently, there is a growing trend to inquire three dimensional images to get more information about structure and morphology (Richardson et al., 2015). In this study, we performed the ACBMN assay with three- dimensional image by confocal microscope. The carcinogenic tests of TANNylated or unTANNylated GFPs did not detect micronucleus in three dimension images, which indicate that these bio-chemical materials are not carcinogen (Fig. 2). Furthermore, volumetric image indicates that TANNylated GFP was directly interacted with nucleus in cells (Fig. 2; bottom). The results suggest that ACBMN assay

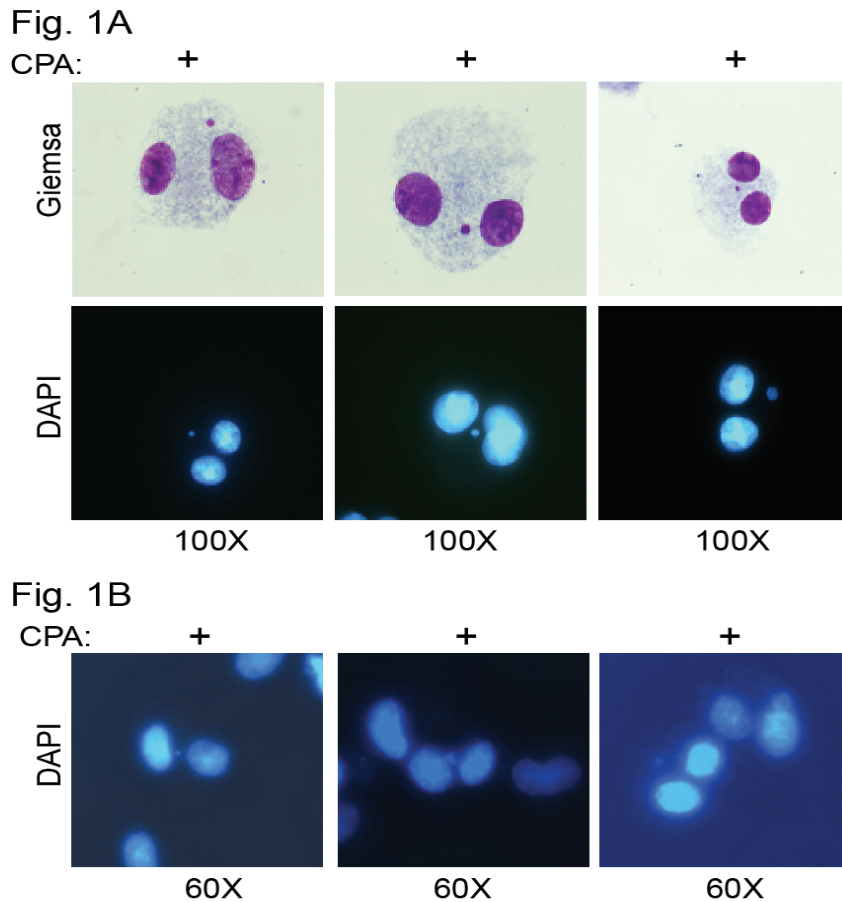

Fig 1. Photographic image of binucleated CHL cells with a micronucleus. Detection of MN after CBMN assays, (A) the detection of micronucleus by giemsa (top) and DAPI staining (bottom). The detection of micronucleus after Advanced micronucleus assay, (B) DAPI staining.

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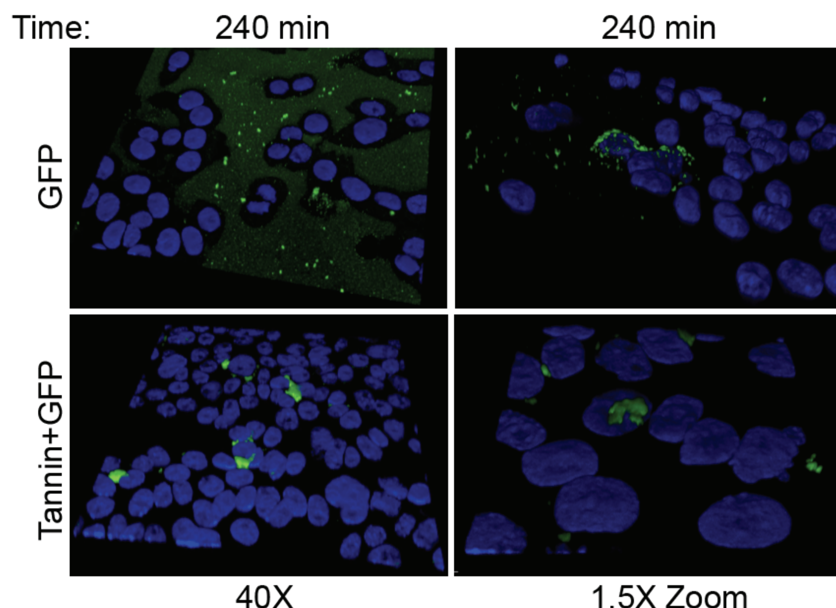

Fig 2. Nucleus-targeting bio-chemical materials for cellular uptake. Comparison of TANNylated complex by three dimensional DAPI image, (A) Left: Cellular uptake of un-TANNyleated (top) and TANNylated GFP complexes (bottom), Right: 1.5X Magnified nucleus and GFP fluorescence of un-TANNyleated (top) and TANNylated GFP complexes (bottom).

has a strong advantage to analyze MNi detection and interaction between bio-chemical materials and gene.

In summary, we have developed the ACBMN assay for sensing genetic damage on chemicals and bio-chemical materials. The experimental procedures of the ACBMN assay are simple, as it requires only 20 steps for detecting micronucleus in in-vitro system. Volumetric image could be useful for the accurate analysis between gene and bio-chemical materials. Our findings indicate that this assay can increase the prediction rate of genetic toxicity for new complex chemicals, bio and chemical materials. The ACBMN assay should be considered as a new protocol for micronucleus test.

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