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Live cell imaging of micronucleus formation and development

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ABSTRACT

The micronucleus (MN) test is widely used to biomonitor humans exposed to clastogens and aneugens, but little is known about MN development. Here we used confocal time-lapse imaging and a fluorescent human lymphoblastoid cell line (T105GTCH), in which histone H3 and α-tubulin stained differentially, to record the emergence and behavior of micronuclei (MNi) in cells exposed to MN-inducing agents. In mitomycin C (MMC)-treated cells, MNi originated in early anaphase from lagging chromosome fragments just after chromosome segregation. In γ-ray-treated cells showing multipolar cell division, MN originated in late anaphase from lagging chromosome fragments generated by the abnormal cell division associated with supernumerary centrosomes. In vincristine(VC)-treated cells, MN formation was similar to that in MMC-treated cells, but MNi were also derived from whole chromosomes that did not align properly on the metaphase plate. Thus, the MN formation process induced by MMC, γ-rays, and VC, were strikingly different, suggesting that different mechanisms were involved. MN stability, however, was similar regardless of the treatment and unrelated to MN formation mechanisms. MNi were stable in daughter cells, and MN-harboring cells tended to die during cell cycle progression with greater frequency than cells without MN. Because of their persistence, MN may have significant impact on cells, causing genomic instability and abnormally transcribed genes.

1. Introduction

The micronucleus (MN) test is widely used to biomonitor humans exposed to clastogens and aneugens [1–4] and has recently become a useful tool for predicting cancer risk [5–8]. Micronuclei (MNi), which consist of chromatin (chromosomes and chromosome fragments), are formed dose-dependently in parallel with increasing concentrations of clastogens and aneugens both *in vitro* and *in vivo* [9–11].

The two basic mechanisms that give rise to MNi during M-phase are chromosome breakage and spindle apparatus defects (for review, see [12]). MNi originate as lagging acentric chromosome fragments and/or as whole chromosomes that fail to bind to the mitotic spindle during cell division. In addition, some MNi are formed from fragments induced by broken anaphase bridges [13,14]. Such mechanisms, however, are speculations based on the analysis of fixed cells. Direct observations of the active process are few [15–17]. Moreover, the fate of MNi after they first appear at M-phase remains uncertain. If they persist in the cytoplasm during cell cycle progression [16], they might have significant impact. Genes on MN, for example, may be transcribed extrachromosomally [18–20] and influence the cell's phenotype.

Since MN formation is dynamic and rapid (lasting a few minutes) and may occur unseen behind a main nucleus, live-cell analysis at long intervals without confocal recording is inadequate to capture the event and also cannot distinguish whether a MN originates from a chromosome fragment or a whole chromosome. Here we used multi-fluorescent cells and three-dimensional, high-resolution imaging over short intervals to accurately record when, where, and how MN originates and concludes in live cells. We constructed for the study dual-color fluorescent T105GTCH cells in which histone H3 and α-tubulin were differentially expressed as fusion to monomeric Cherry (mCherry) and enhanced green fluorescent protein (EGFP), respectively. Using those cells and a high-resolution imaging system, we investigated the life cycle of MN induced by exposure to mitomycin C (MMC; a crosslinking agent), γ-rays (a strand-breaking agent), and vincristine (VC; a spindle poison).

Abbreviations: MN, micronucleus; MNi, micronuclei; MMC, mitomycin C; VC, vincristine; GFP, green fluorescent protein; CFP, cyan fluorescent protein.

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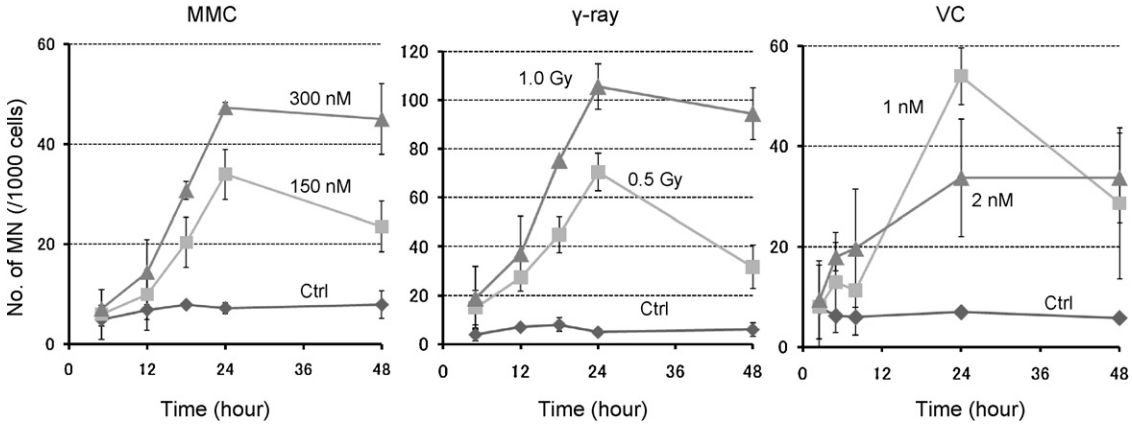


Fig. 1. *MN induction by MMC, γ-rays, and VC*: MMC, γ-rays, and VC induced MNi in T105GTCH cells dose-dependently and peaked 24 h after treatment. Control cell MN frequencies were ~5 MNi per 1000 cells.

2. Materials and methods

2.1. General

We obtained MMC from Kyowa Hakko Bio Co., Ltd. (Tokyo) and VC from Wako Pure Chemical Industries, Ltd. (Tokyo) and dissolved them in phosphate-buffered saline (Takara Bio Inc., Shiga, Japan) just before use. We delivered γ-ray irradiation with a Gammacell 40 Exactor (MDS Nordion, Canada). We purchased RPMI1640 medium, penicillin, and streptomycin from Invitrogen Corp. (USA), horse serum from JRH Biosciences (USA), sodium pyruvate from Sigma–Aldrich Corp. (USA), Dulbecco's modified Eagle's medium from Nacalai Tesque Inc. (Kyoto, Japan), and fetal calf serum from MP Biomedicals Inc. (USA).

2.2. Cell culture

We grew T105GTCH cells (derived originally from human lymphoblastoid cell line TK6 [21,22]) in RPMI1640 medium supplemented with 10% heat-inactivated horse serum, 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained them at 10^5–10^6 cells/ml at 37 °C in a 5% CO_2 atmosphere with 100% humidity. We maintained fluorescent MDA-435 cells (constructed by Sugimoto [23,24]) in Dulbecco's modified Eagle's medium containing 10–15% fetal calf serum at 37 °C in a 5% CO_2 atmosphere with 100% humidity.

2.3. Preparation of pEGFP-Tub and pmCherry-H3 plasmid

We obtained pEGFP-Tub containing human α-tubulin cDNA from BD Biosciences, Clontech. We constructed pmCherry-H3 by replacing the EGFP cDNA of pEGFP-H3 [23] with mCherry cDNA that we amplified by PCR using pRSET-B mCherry [25] (provided by Prof. Roger Y. Tsien, University of California, San Diego) as a template.

2.4. Construction of dual-color fluorescent cell line T105GTCH

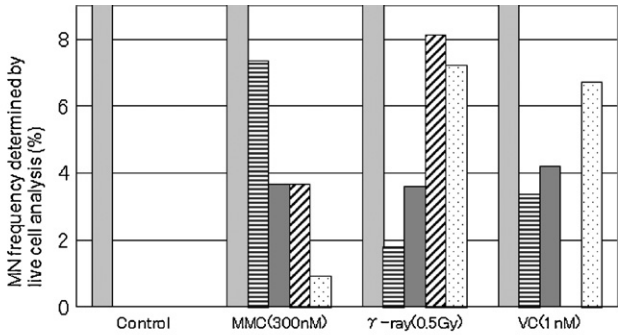
pEGFP-Tub and pmCherry-H3 expression vectors, 20 μg each, were mixed with 5×10^6 TK6 cells in 100 μl Nucleofector Solution V, containing 18% Supplement 1 (Amaxa Inc., USA) and tranfected into the cells with Amaxa Nucleofector (Amaxa Inc., USA) at a setting of A-30. The vector-integrated cells were selected in the presence of G418 (750 μg/ml) and collected 10 days later. We selected single colonies of stably transfected cells with the aid of a fluorescent microscope and cultured them for 2 more weeks.

2.5. MN test

We carried out the MN test on 5×10^5 T105GTCH cells at 0, 5, 12, 18, 24, and 48 h after treatment with MMC (150 or 300 nM for 4 h followed by a PBS rinse), γ-rays (0.5 or 1 Gy), or VC (1 and 2 nM). We suspended approximately 10^6 treated cells in hypotonic KCl solution (75 mM), incubated them for 10 min at room temperature, fixed them twice with ice-cold glacial acetic acid in methanol (1:3), and resuspended them in methanol containing 1% acetic acid. We placed a drop of the suspension on a clean glass slide and allowed it to air-dry. We then stained the cells with 40 μg/ml acridine orange solution and observed them immediately with the aid of a fluorescence microscope (Olympus Corp., Tokyo). We examined at least 1000 intact interphase cells for each treatment and scored the cells containing MNi, which we defined by size as equal to or less than one-third the size of the main nucleus.

2.6. Live cell imaging for capturing MN formation

We cultured 5×10^5 T105GTCH cells in 2 ml RPMI1640 medium with a 35 mm BAM-coat dish (NOF Corp., Tokyo), which retained the cells for ~4 h on the surface, in a humid chamber set at 37 °C and 5% CO_2 on the stage of a fluorescent microscope in FV1000 system (Olympus Corp., Tokyo) equipped with



	Control	MMC(300nM)	γ-ray(0.5Gy)	VC(1 nM)
Normal cell division	100.0	84.4	80.2	85.7
MN formation during bipolar division	0.0	7.3	1.8	3.4
Multipolar cell division	0.0	3.7	3.6	4.2
MN formation during multipolar division	0.0	3.7	8.1	0.0
Cell death	0.0	0.9	7.2	6.7

Fig. 2. *MN formation in T105GTCH cells analyzed by live cell imaging*: From 22 to 25 h we recorded moving images of 109 cell divisions following treatment with MMC (300 nM), 111 following irradiation with γ-rays (0.5 Gy), and 119 following treatment with VC (1 nM). MN frequencies (%) are presented by category. All control cells divided normally with no evidence of MN formation.