Mutation Research 762 (2014) 10–16

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Chromosome loss caused by DNA fragmentation induced in main nuclei and micronuclei of human lymphoblastoid cells treated with colcemid

Mika Yamamoto[a,b], Akihiro Wakata[a], Yoshinobu Aoki[a], Yoichi Miyamae[a], Seiji Kodama[b,*]

[a] Drug Safety Research Laboratories, Astellas Pharma Inc., 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan
[b] Laboratory of Radiation Biology, Department of Biological Science, Graduate School of Science, Osaka Prefecture University, 1-2, Gakuen-cho, Naka-ku, Sakai, Osaka 599-8570, Japan

ARTICLE INFO

Article history:
Received 2 September 2013
Received in revised form
19 December 2013
Accepted 18 February 2014
Available online 27 February 2014

Keywords:
Aneuploidy
Chromosome loss
DNA fragmentation
Micronucleus
FISH
TUNEL assay

ABSTRACT

Aneuploidy, a change in the number of chromosomes, plays an essential role in tumorigenesis. Our previous study demonstrated that a loss of a whole chromosome is induced in human lymphocytes by colcemid, a well-known aneugen. Here, to clarify the mechanism for colcemid-induced chromosome loss, we investigated the relationship between chromosome loss and DNA fragmentation in human lymphoblastoid cells treated with colcemid (an aneugen) compared with methyl methanesulfonate (MMS; a clastogen). We analyzed the number of fluorescence *in situ* hybridization (FISH) signals targeted for a whole chromosome 2 in cytokinesis-blocked binucleated TK6 cells and WTK-1 cells treated with colcemid and MMS, and concurrently detected DNA fragmentation by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Results revealed that DNA fragmentation occurred in 60% of all binucleated TK6 cells harboring colcemid-induced chromosome loss (30% of micronuclei and 30% of main nuclei). DNA fragmentation was observed in colcemid-induced micronuclei containing a whole chromosome but not in MMS-induced micronuclei containing chromosome fragments. In contrast, colcemid-induced nondisjunction had no effect on induction of DNA fragmentation, suggesting that DNA fragmentation was triggered by micronuclei containing a whole chromosome but not by micronuclei containing chromosome fragments or nondisjunction. In addition, the frequency of binucleated cells harboring chromosome loss with DNA fragmentation in micronuclei or main nuclei was higher in wild-type p53 TK6 cells than in mutated-p53 WTK-1 cells treated with colcemid. Taken together, these present and previous results suggest that colcemid-induced chromosome loss is caused by DNA fragmentation, which is triggered by a micronucleus with a whole chromosome and controlled by the p53-dependent pathway.

1. Introduction

Aneuploidy, an abnormal chromosome number resulting from gain or loss of one or more whole chromosomes during cell division, is a common characteristic of tumor cells [1]. Approximately 70% of solid and hematopoietic cancers in humans are aneuploid with a near-diploid number of chromosomes. In contrast, aneuploid tumors with near-tetraploid karyotypes are rarely observed in solid or hematopoietic cancers [2]. While aneuploidy is known to lead to chromosomal instability by destabilizing the karyotype, promoting tumor initiation and progression [3], an increased rate of aneuploidy (*i.e.*, a high level of chromosomal instability) operates as a suppressor rather than an initiator of tumorigenesis [3,4].

Aneugens induce micronuclei containing whole chromosomes in addition to nondisjunction caused by chromosome segregation error. Crasta et al. [5] examined micronuclei in nocodazole-treated U2OS cells by living cell time-lapse imaging and showed that 62% of micronuclei persisted in the second-generation cells, while 38% of micronuclei were reincorporated into daughter nuclei. Further, they also observed that chromosomes in a micronucleus reincorporated into a main nucleus were pulverized, suggesting that micronuclei containing a whole chromosome caused chromosomal structural aberrations. These previous findings demonstrated that micronuclei arising from segregation error may induce not only

* Corresponding author. Tel.: +81 72 254 9855; fax: +81 72 254 9855.
E-mail address: kodama@riast.osakafu-u.ac.jp (S. Kodama).

http://dx.doi.org/10.1016/j.mrfmmm.2014.02.002
0027-5107/

chromosomal numerical aberrations but also chromosomal structural aberrations, suggesting that aneugens may produce genome rearrangements leading to cancer development.

In a previous study, we revealed that colcemid significantly induced the loss of a whole chromosome in primary lymphocytes and TK6 cells [6]. Although the mechanism for inducing chromosome loss remains unclear, several studies have explored the relationship between aneugen treatment and chromosome loss. For example, Haaf et al. [7] demonstrated that micronuclei derived from lagging chromosomes triggered DNA fragmentation, and were subsequently eliminated. Elhajouji et al. [8] reported that induction of centromere-positive micronuclei by aneugens showed a clear threshold while centromere-negative micronuclei induced by clastogens showed no threshold. The authors speculated that the observed threshold for inducing micronuclei through aneugen exposure is due to the elimination of cells harboring centromere-positive micronuclei by apoptosis. Further, Decordier et al. [9] demonstrated that human lymphocyte cells harboring centromere-positive micronuclei induced by carbendazim and nocodazole showed early apoptotic events, which turned out to be annexin-V positive, in higher frequency than cells exhibiting nondisjunction. This study suggested that the early apoptosis of cells with micronuclei correlates with the specific elimination of aneuploid cells. Several other studies showed that DNA fragmentation occurs in aneuploid human cells resulting from merotely induced nondisjunction, indicating that the induction of aneuploidy triggers apoptosis [10,11]. Taken together, these previous results indicate that DNA fragmentation occurs in micronuclei and main nuclei after aneugen exposure, suggesting that DNA fragmentation leads to chromosome loss.

Human lymphoblastoid cell lines TK6 and WTK-1 are derived from one donor, and TK6 has wild-type p53 while WTK-1 has mutated-type p53. Because the p53 protein is involved in the regulation of apoptosis, the status of p53 may cause a difference in inducing DNA fragmentation. Therefore, we examined DNA fragmentation in both TK6 cells and WTK-1 cells.

Here, to determine the relationship between DNA fragmentation and chromosome loss, we examined whether or not DNA fragmentation occurred in human lymphoblastoid binucleated cell lines TK6 and WTK-1 having chromosome missegregation detected *via* whole chromosome painting (WCP) fluorescence *in situ* hybridization (FISH) targeted for chromosome 2 by treatment with colcemid (aneugen) and methyl methanesulfonate (MMS; clastogen).

2. Materials and methods

2.1. Chemicals and solvents

Cells were treated with colcemid (CAS No. 477-30-5; Nacalai Tesque, Inc., Kyoto, Japan) as an aneugen and methyl methanesulfonate (MMS; CAS No. 66-27-3; Sigma, St. Louis, MO, USA) as a clastogen. Both colcemid and MMS were dissolved in physiological saline.

2.2. Cell culture

Human lymphoblastoid TK6 cells and WTK-1 cells, which respectively expressed wild-type p53 and mutated p53 were obtained from the National Institute of Health Sciences (Tokyo, Japan) and cultured in RPMI 1640 medium (Invitrogen Corp., Grand Island, NY, USA) supplemented with 10% heat-inactivated (at 56 °C for 60 min) horse serum (SAFC Biosciences, Lenexa, KS, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM sodium pyruvate (Invitrogen Corp.). Cells (2.5×10^4 cells/mL) were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for approximately 48 h before treatment with chemicals.

2.3. Treatment and slide preparation

TK6 cells or WTK-1 cells (2×10^5 cells/mL) were suspended in fresh medium after pre-culture for 48 h, and 7 mL of cell suspension was seeded in each flask (25 cm^2). Test chemical solutions (1%) and 2 mg/mL cytochalasin B (0.15%) were then applied simultaneously to TK6 and WTK-1 cells for 15 h to examine missegregation at first cell division.

After harvesting, the cells were centrifuged at 100 g for 5 min, re-suspended in 0.1 mL of PBS (−), and fixed 3 times using ice-cold fixative (methanol:glacial acetic acid, 3:1, v/v). A drop of cell suspension was applied on a slide and air-dried. Two slides were prepared at each concentration of test chemicals. One was stained with 0.005% acridine orange for *in vitro* MN assay, and the other was stained for TdT-mediated dUTP nick end labeling (TUNEL) assay and then further used for WCP FISH. The rate of cell growth inhibition was calculated using the following formula:

$$\text{Cell growth inhibition rate } (\%) = \frac{\text{the number of binucleated cells in vehicle control group} - \text{the number of binucleated cells in the test article group}}{\text{the number of binucleated cells in vehicle control group}} \times 100$$

The top concentration of test chemicals was set as the highest dose under a 50% cell growth inhibition dose.

2.4. TdT-mediated dUTP nick end labeling (TUNEL) assay

For the *in situ* detection of DNA fragmentation in nuclei and micronuclei, TUNEL assay (*In Situ* Cell Death Detection Kit, Fluorescein; Roche, Basel, Switzerland) was conducted in accordance with the manufacturer's instructions, with slight modifications. The slides were rinsed in PBS (−), permeabilized in ice-cold 0.1% sodium citric acid/0.1% Triton X-100 for 2 min, washed twice in PBS (−) for 1 min, and then air-dried. A 100-μL aliquot of 10% terminal deoxynucleotidyl transferase enzyme end-labeling solution (fluorescein-dUTP) was applied to each slide, and the slides were then covered with parafilm immediately and incubated for 60 min at 37 °C in a pre-warmed humidified box. After incubation, the slides were washed 3 times for 1 min each in PBS (−) and finally counter-stained with DAPI II (Vysis; Downer, Grove, IL, USA). Images of slides were captured using an ArrayScan (Thermo Fisher Scientific, Waltham, MA, USA) equipped with band pass filters that permit detection of DAPI and FITC.

2.5. Fluorescence in situ hybridization (FISH)

WCP FISH was applied using a probe targeting for a whole chromosome 2 in accordance with the manufacture's instructions (Cambio; Dry Drayton, Cambridge, UK). Slides stained by TUNEL assay were rinsed in PBS (−) at room temperature for 5 min; denatured in 70% formamide in 2× SSC (pH 7.4) at 65 °C for 2 min; quenched in ice-cold 70% ethanol for 4 min; dehydrated by serial washing in 70% ethanol (2 min, twice), 90% ethanol (2 min, twice), and 100% ethanol (5 min); and then air-dried. The WCP probe mixture was prepared by adding 3 μL probe to 12 μL hybridization buffer, denatured at 65 °C for 10 min, and then kept at 37 °C for 30–60 min. Denatured WCP probe mixtures were applied to each slide on a 38 °C slide warmer. The slides were immediately covered with coverslips, sealed with rubber cement, placed into a pre-warmed humidified box, and incubated overnight at 37 °C. After removing coverslips, the slides were washed twice in 50%