A Modified Protocol for Accurate Detection of Cell Fusion-Mediated Premature Chromosome Condensation in Human Peripheral Blood Lymphocytes

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Summary When interphase cells are fused with mitotic cells, loosely distributing chromatins in nuclei are induced to form prematurely condensed chromosomes. In this paper we report a modified protocol to unequivocally detect prematurely condensed chromosomes in human peripheral blood lymphocytes that were fused with mitotic Chinese hamster ovary (CHO) cells. To examine cell fusion-mediated premature chromosome condensation (PCC), we conducted morphological analysis by differential interference contrast microscopy and molecular cytogenetic analysis by fluorescence in situ hybridization using pan-centromeric and telomeric peptide nucleic acid (PNA) probes. These modified procedures may serve to improve the usefulness of the technique of PCC in cytogenetic investigations.

Key words Premature chromosome condensation, Cell fusion, Radiation, PNA-FISH.

Premature chromosome condensation (PCC) is a phenomenon that occurs in eukaryotic interphase cells when they are fused with mitotic cells. The technique that induces PCC facilitates the visualization of interphase chromatin as a structure of condensed chromosome. The appearance of resulting prematurely condensed chromosomes varies depending on the cell-cycle position of the interphase at the time of PCC induction by cell fusion. While G0/G1 cells exhibit single-chromatid chromosomes, S cells show pulverized chromosomes and G2 cells yield elongated double-chromatid chromosomes. Studies on cell fusion-mediated PCC started in the early 1970s (Rao and Johnson 1970, Johnson and Rao 1970, Sperling and Rao 1974, Waldren and Johnson 1974). The search for substances that induce cell-fusion mediated PCC began, and a heterodimeric protein composed of cyclin B and cyclin-dependent kinase was identified as a substance that induces PCC (Nurse 1990, Freeman and Donoghue 1991). Since then, explorations for factors involved in cell cycle regulation have progressed greatly (Cheng et al. 1993). In the cells cycling in culture, inhibition of the DNA phosphorylation using Okadaic acid or calyculin A can also induce PCC (Pantelias and Maillie 1984, Durante et al. 1998, Prasanna et al. 2000, Gotoh et al. 2005, Gotoh 2009). This chemically induced PCC technique has been used for the analysis of the G2 PCC assay (Bezrookove et al. 2003, Kanda et al. 1999, Lamadrid et al. 2007, Lindholm et al. 2010). These PCC-inducing techniques enable analysis of the kinetics of chromatin formation in the early cell cycle stage (Rao et al. 1977).

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To analyze cytogenetic events occurring in human cells, peripheral blood lymphocytes (PBLs) are most commonly used in research. Chinese hamster ovary (CHO) cells exhibiting a distinctively different chromosomal constitution are used as mitosis inducer cells. PCC has been induced by cell fusion in the presence of fusing reagents, or fusogens, such as polyethylene glycol (PEG) (Pantelias and Maillie 1983). The cell fusion-mediated PCC technique has been used both in basic and applied cell sciences (Hittelman et al. 1979, Vyas et al. 1991, Garcia et al. 2001, Gotoh and Durante 2006, Stevens et al. 2010).


Although the cell fusion-mediated PCC assay has a wide range of potential applications, it has not been frequently utilized in cytogenetic researches compared to the chemically induced PCC assay. There were disadvantages in conducting the fusion-mediated PCC assay. One problem in the procedures for the PCC assay was that certain skills and experience were required to obtain fused cells efficiently. To solve this issue, improved methods have already been described in many references (IAEA 2011). The fuzzy appearance of prematurely condensed chromosomes poses another problem for accurate analysis of PCC. The prematurely condensed human chromosomes might also be confused with metaphase CHO chromosomes especially when the sister-chromatids of the CHO chromosomes are separated due to the excess effect of colcemid. Efforts have been made to find a way to overcome these problems concerning the difficulties in discerning between the chromosomes of two species. For instance, the 5-bromo-2′-deoxyuridine (BrdU) labeling technique has been used to differentially stain the chromosomes of mitosis inducer cells (Cornforth and Bedford 1983, Hittelman et al. 1988).

In this short technical report, we describe a newly modified protocol designed to accurately detect PCC in human PBLs.

Materials and methods

Cell fusion

A subclone of the CHO-K1 cell line, supplied from American Type Culture Collection (Summit Pharmaceuticals International Corporation, Tokyo, Japan), was used as the mitotic inducer for the PCC assay. The modal number of chromosomes in our CHO cells was 20. Mitotic CHO cells were harvested by the shake-off procedure 4–5 h after adding 0.1 µg/ml colcemid. Mitotic cells suspended in a cryopreservation medium (CELLBANKER, Zenoaq, Fukushima, Japan) (1×10⁶ cells/ml) were poured in 1-ml vials and kept at −80°C until use. Blood samples were collected by venipuncture in heparinized tubes from healthy donors after obtaining their informed consent.
Mononuclear cells, consisting of more than 90% lymphocytes, were isolated from whole peripheral blood using Ficoll-Hypaque sedimentation (Axis Shield, Oslo, Norway) and washed with medium RPMI-1640. Some of the isolated lymphocyte samples were exposed to 2-Gy gamma rays at a dose rate of 0.5 Gy/min to examine radiation-induced chromosome aberrations.

PCC was induced according to the procedure of IAEA (IAEA 2001, 2011). In brief, mitotic CHO cells were thawed to defreeze, suspended in medium RPMI-1640 and centrifuged for 7 min at 1,200 rpm. Then lymphocytes (5×10⁶) and CHO cells (1×10⁶) were mixed in a round-bottomed culture tube and washed again with RPMI-1640. After centrifugation (1,200 rpm, 7 min), the supernatant was discarded. Polyethylene glycol (PEG; 150 μl, 50% w/v, MW 1500) (Roche, Mannheim, Germany) was added onto the cell pellet and gently mixed. After a 1-min PEG treatment, 3 ml RPMI-1640 was added to dilute PEG and cells were washed. Subsequently 800 μl complete medium (90% RPMI-1640 and 10% fetal bovine serum) containing colcemid (final concentration: 150–200 ng/ml) was added and incubated for 1 h at 37°C. Hypotonic treatments with 0.075 M KCl and methanol–acetic acid (3:1, v/v) fixation were performed according to the standard chromosome preparation procedure.

**Differential interference contrast microscopy**

Air-dried chromosome preparations were weakly stained with 3% Giemsa solution (pH=6.8) for 3 min, instead of standard 25-min staining, and rinsed in clean water. These chromosomes were observed under a light microscope equipped with a differential interference contrast (DIC) apparatus (Olympus, Tokyo, Japan). The optical axis was adjusted to produce distinct images.

**Fluorescence in situ hybridization**

FISH using peptide nucleic acid (PNA) probes (PNA-FISH) was conducted as described previously (Suto et al. 2012), with modifications for PCC detection. Chromosome preparations were dried in an oven for 30 min at 64°C, fixed in 4% paraformaldehyde solution at 4°C for 2 min, followed by washing in phosphate buffered saline (PBS) and dehyrdration with an ethanol series. Then, chromosomes were denatured in an alkaline solution composed of 0.1 N NaOH (70%) and ethanol (30%) at room temperature for 40 s followed by dehydration in an ethanol series. Synthesized Cy3-conjugated pan-centromeric and carboxyfluorescein-aminohexyl (FAM)-conjugated telomeric PNA probes (Panagene, Daejon, South Korea) were used for chromosome identification. A total of 60 μl probe mix (60% formamide in 2×SSC, 5 ng salmon sperm DNA, 5 ng of each PNA probe) was denatured at 90°C for 5 min, dropped onto a slide to hybridize to alkaline-denatured chromosomes, covered with a piece of plastic film and kept at room temperature for 0.5–2 h in the dark. Then, the slide was washed in a post-hybridization solution (2×SSC/0.1% Tween-20) for 10 min at 57°C, air-dried, and counterstained with 125 ng/ml 4,6-diamidine-2-phenylindole (DAPI). When background noise was observed, the amount of each PNA probe was reduced to one fifth. For the analysis of fluorescent signals on chromosomes, images obtained from filter sets specific for DAPI, FITC and Cy3 were merged using image-processing software. Multiplex fluorescence in situ hybridization (M-FISH) was conducted using a commercially available multicolor probe (MetaSystems, Altlussheim, Germany) following the manufacturer’s instructions with a slight modification. To economize on using the expensive commercially available probe, we diluted it to half of the recommended concentration with a hybridization buffer [50% formamide (v/v), 10% dextran sulfate (w/v) and 20% bovine serum albumin (v/v) in 2×SSC].

**Results and discussion**

The objective of this work was to modify the existing protocols for visualizing prematurely condensed chromosomes in human PBLs with accuracy. We present here a protocol with two-fold
modifications. For morphological analysis using conventionally stained chromosome preparations, we applied DIC microscopy to visualize the distinct shape of chromosomes. DIC microscopy enables us to analyze transparent biological objects without staining. DIC images give a relief-like appearance corresponding to the variation of the optical density of the object, emphasizing its outlines and edges. The resolution and clarity of images obtained by DIC microscopy are unsurpassed among other standard optical microscopy techniques. This optical feature worked well with weakly stained objects as represented in Fig. 1. Images produced by a DIC microscope seem to have a three-dimensional physical relief with a shadow cast. The distinct coil-shaped structure of interphase chromosomes was observed. This may facilitate further studies such as the analysis of chromosomal condensation and compaction in the course of cell-cycle progression in relation to DNA replication processes (Rao et al. 1977, Gotoh and Durante 2006, Gotoh 2007, 2009).

The feasibility of the present protocol was assessed in radiation dosimetry. In the analysis of

Fig. 1. Examples of cells showing fusion-mediated premature chromosome condensation (PCC). (a) Metaphase Chinese hamster ovary (CHO) chromosomes and prematurely condensed human chromosomes in a fused cell stained weakly with Giemsa solution. Chromosomes were viewed with a differential interference contrast (DIC) microscope. Metaphase CHO and prematurely condensed human chromosomes exhibited double and single-chromatid structure, respectively. (b) DIC image of less condensed human chromosomes. The coil structure of prematurely condensed chromosomes is clearly demonstrated. Scale bar=10μm.

Fig. 2. Detection of prematurely condensed human chromosomes in fused cells by fluorescence in situ hybridization (FISH). (a) FISH with peptide nucleic acid probes (PNA-FISH) for pan-centromeric and telomeric repeat sequences. In this fused cell, all human chromosomes show centromeric alphoid signals (Cy3, red). Most CHO chromosomes exhibit hybridization signals with telomeric repeat sequences (FAM, green). Signals on amplified interstitial telomeric repeat sequences in CHO chromosomes are so intense that actual telomere signals on every chromosome are not displayed properly in this image. Note that the smallest chromosome in the CHO chromosomal complement can be identified because of its green hybridization signal (arrow). (b) Prematurely condensed human chromosomes in a fused cell identified by multicolor-FISH. This image was generated by merging separate images obtained with filters specific for the multicolor FISH probe. Scale bar=10μm.
radiation-induced chromosome aberrations using conventionally stained preparations, the coiled substructure of prematurely condensed chromosomes often causes difficulty in identifying fragments, especially when uncoiled portions of chromosomes are seemingly unstained. Therefore, DIC microscopy may lead to the accurate scoring of radiation-induced fragments (Fig. 3a). For molecular cytogenetic analysis using pan-centromeric and telomeric PNA probes, we improved the accuracy of discrimination between human and CHO chromosomes based on the differential hybridization property of the two probes (Fig. 2a). The pan-centromeric alphoid DNA probe solely hybridized with human chromosomes. On the other hand, there were large blocks of hybridization sites with amplified telomeric repeat sequences (TTAGGG)n in CHO chromosomes (Slijepcevic et al. 1997, Bolzán et al. 2001). The smallest CHO chromosome, or chromosome 21 according to the standard karyotype of CHO-K1 (Xu et al. 2011), is likely to be confused with prematurely condensed human chromosomes by the conventional method. In our modified method, these chromosomes could be identified because of their marked hybridization signals (Fig. 2a). Therefore, PNA-FISH using pan-centromeric and telomeric probes enabled us to accurately count radiation induced chromosomal fragments in excess of background frequency by identifying fragments without centromeric signals (Fig. 3b).

We introduced M-FISH to the detection of prematurely condensed human chromosomes (Figs. 2b and 3c). The M-FISH enables information to be obtained on the chromosomal territory deduced from the relative location of chromosomes in G0/G1 phase. Commercially available M-FISH probes are expensive. We diluted probes with a hybridization mixture to half the recommended concentration, and obtained sufficient signal intensity. The continued development of the PCC technique will open new research avenues in cytogenetics. Our modified protocol presented here may serve as a powerful tool for examining interphase chromosomes.

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