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Effects of Colcemid-Block on Chromosome Condensation in Metaphase Analysis and Premature Chromosome Condensation Assays

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The frequency of chromosome aberrations correlates with the radiation dose, therefore measuring chromosome aberrations in peripheral lymphocytes obtained from exposed persons is considered the most reliable, specific, and sensitive biomarker for dose estimation. The influence of radiation is evaluated by analyzing chromosome aberrations in dicentric and premature chromosome condensation (PCC) assays. It has been suggested that the degree of chromosome condensation may have significant effects on the detection of ring-chromosomes, small fragments, and translocations. In order to investigate the effects of colcemid treatment on chromosome condensation, we compared the relative length of chromosome 2, which identification is easy and contains approximately 8.1% of genome DNA, in lymphocytes treated with 0.01, 0.03, 0.05, and 0.1 μg/ml colcemid for 2, 24, and 48 h each. Our data showed that the most elongated chromosomes were obtained from the lymphocytes treated with 0.01 μg/ml colcemid for 2 h. Furthermore, it has been reported that calyculin A treatment results in induction of PCCs with over-condensed chromosomes, then, we analyzed the effect of colcemid-block on chromosome condensation in PCC assay. PCC cells with the over-condensed chromosomes were observed frequently in PCC assay combined with colcemid-block. Since colcemid treatment has large influence on chromosome condensation, it is necessary to choose the optimal conditions for the biodosimetry analysis of each chromosome aberration.

Key words: chromosome length; colcemid-block; premature chromosome condensation; human blood culture, biodosimetry

1.INTRODUCTION

Biological dosimetry is based on the analysis of conventional Giemsa-stained dicentric chromosomes and has been used since the mid-1960s. The analysis of dicentric chromosomes has become a routine component of radiological protection programs [1)]. During the investigation of radiation accidents, it is important to estimate the exposure dose of whom persons are received. In the case of high dose of acute

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exposure of more than 1 Gy, this information assists in the planning of therapy and in alerting physicians to possible health consequences that could arise in the weeks and months following the exposure. Biological dosimetry can be also valuable in the early phases soon after an accident where many people may have been exposed. At this time, triage of casualties using biological and clinical endpoints can rapidly give an approximate estimate of the exposure dose.

Chromosome aberrations in lymphocytes are used to estimate the absorbed dose of radiation in over-exposed people[1, 2]. The aberrations scored in the lymphocytes are interpreted using a dose-response calibration curve, produced by *ex vivo* exposure of blood to increasing doses of the appropriate type of radiation. Three cytogenetic techniques: dicentric assay [1], cytokinesis-blocked micronucleus (CBMN) assay[1, 3, 4], and premature chromosome condensation (PCC) assay[5-7], are currently used for biodosimetry. Among these 3 assays, the dicentric assay is most common. Therefore, preparation of chromosome spreads suitable for observation is needed in dicentric and PCC assays. For the dicentric assay, phytohemagglutinin (PHA)-stimulated peripheral lymphocytes are synchronized in metaphase by treatment with colcemid.

In order to analyze the first round of mitosis after PHA-stimulation, lymphocytes are cultured in the presence of colcemid [1]. Over-condensed chromosomes are often observed in colcemid-blocked metaphase [8]. When detecting dicentric chromosomes, the appearance of over-condensed chromosomes is not a serious problem. However, when analyzing other abnormalities in chromosome structure, the presence of over-condensed chromosomes reduces the sensitivity of detecting chromosome aberrations in metaphase analysis and PCC assay. Miura and Blakely reported that prolonged treatment with calyculin A, a phosphatase inhibitor, induced shortened PCC in an irradiation dose-dependent manner in human peripheral lymphocytes [9]. Furthermore, in some laboratories, colcemid treatment is combined with calyculin A for induction of metaphase PCC [10]. Over-condensed chromosomes are observed also in chemical-induced PCC assays in the human blood culture model. In this study, we analyzed the effects of colcemid treatment on chromosome condensation in human peripheral lymphocytes in metaphase assay and PCC assay using calyculin A.

2. Materials and methods

Volunteers and blood collection

Peripheral blood was obtained from adult two male healthy persons and one male healthy donor in metaphase assay and PCC assay, respectively. After informed consent was obtained, whole peripheral blood was collected by venipuncture into the vacutainers containing lithium heparin as an anticoagulant. Peripheral blood lymphocytes were isolated using CPT tubes (BD Biosciences, USA) or Histopaque 1077 (Sigma Aldrich, USA).

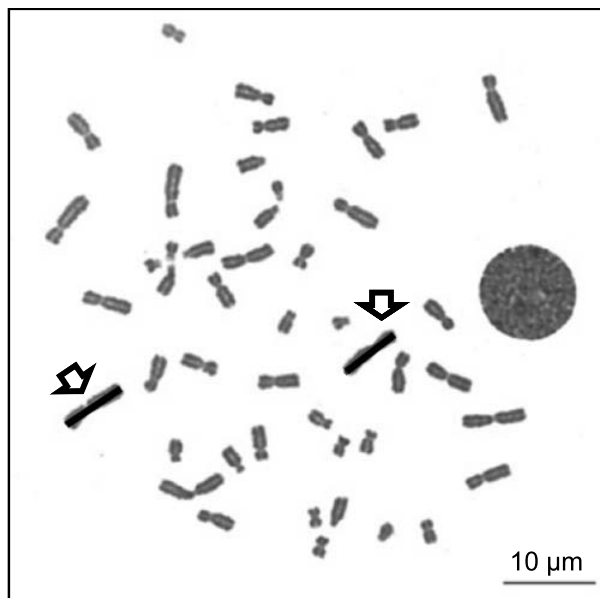

Fig. 1. Measurements of the length of chromosome 2 in metaphase spreads of human peripheral lymphocyte cultures by image analyzer. Scale bar shows 10 μm. Open arrows homologous human chromosome 2.

Metaphase assay

Isolated lymphocytes were cultured in RPMI1640 medium (Invitrogen, USA) supplemented with 20% fetal bovine serum, PHA, and kanamycin. Increasing concentrations (0.01, 0.03, 0.05, and 0.1 μg/ml) of colcemid (Invitrogen, USA) were added into the culture medium at 2, 24, or 48 h each before harvesting.

PCC assay

The chemical-induced PCC assay was performed as described by Miura [9]. Calyculin A (Calbiochem, USA) was used to induce PCC in treated cells. Colcemid (0.05 μg/ml) was added to the culture medium 24 h before harvesting. Calyculin A at a final concentration of 50 nM was added 15 min before harvesting the 48-h time-point.

Cell harvest, metaphase spreads, and staining

After 48-h culture, lymphocytes were centrifuged at 300 × g for 8 min at 20°C. Supernatants were removed by aspiration, cell pellets were suspended in 75-mM potassium chloride for 20 min for hypotonic treatment, and subsequently fixed in cold fixative solution (3:1 methanol: glacial acetic acid). A temperature-humidity-controlled chamber (Hanabi metaphase spreader, ADSTEC, Japan) was used to prepare chromosome spreads on pre-cleaned glass slides in a controlled microenvironment. The slides were then stained with Giemsa solution and mounted with cover slips.

Measurement of chromosome length

Images of 82 to 102 metaphase spreads were captured

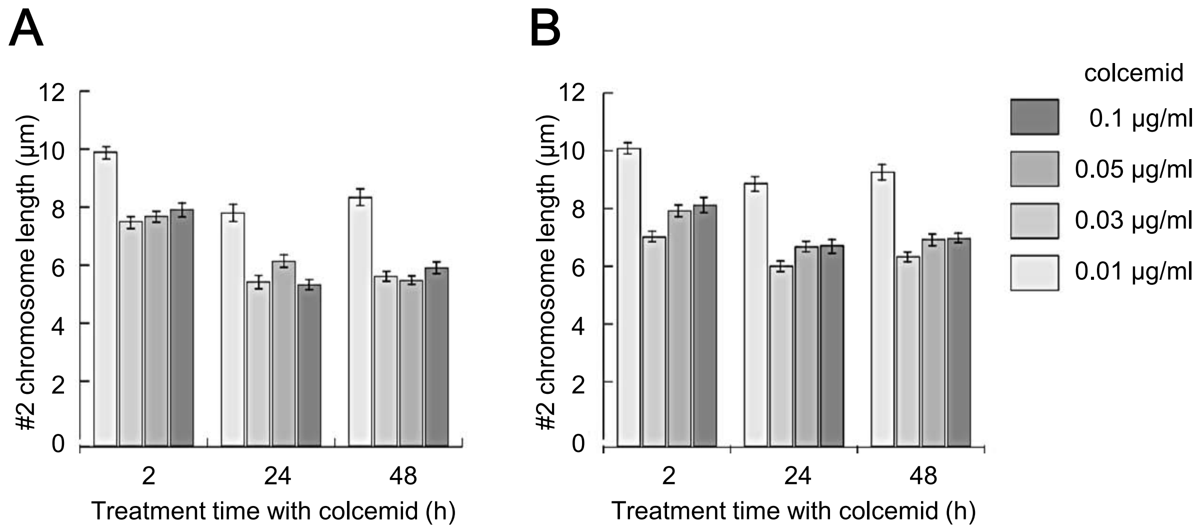

Fig. 2. Effect of colcemid-block on the condensation of human chromosome 2. Human peripheral lymphocytes obtained from two adult male persons aged thirties (A) and fifties (B).

under the bright field microscope (objective: 100 ×). Image analyzing software ImageJ ver. 1.45 (NIH, USA) was used for the measurement of the length of chromosome 2 in each metaphase spread (Fig. 1).

3. Results and discussion

Effects of treatment time and concentration of colcemid on chromosome condensation in the metaphase assay

To obtain the accurate frequency of dicentric chromosomes for radiation dose estimation, it is important to identify the centromere on each chromosome. It has also been suggested that the degree of chromosome condensation may have significant effects on the analysis of abnormalities in chromosome structure. In order to investigate the effects of colcemid treatment on chromosome condensation, we measured the relative length of chromosome 2 in human peripheral lymphocytes treated with 0.01, 0.03, 0.05, and 0.1 μg/ml of colcemid for 2, 24, and 48 h each. Present data clearly showed that the longest chromosomes were obtained after treatment with 0.01 μg/ml colcemid for 2 h in male persons ages thirties (Fig. 2A) and fifties (Fig. 2B), whereas there was no significant difference in the relative length of chromosome 2 in other conditions (0.03, 0.05, and 0.1 μg/ml) at either 24 or 48 h.

Whereas in conventional metaphase analysis, long exposures to colcemid reduced the chromosome length in PHA-stimulated peripheral blood cultures [11], we did not find any significant difference in the degree of chromosome condensation by using a range of concentrations of colcemid (0.03–0.1 μg/ml). In order to analyze the first round mitosis after PHA-stimulation, IAEA recommended that lymphocytes were cultured in the presence of colcemid for whole culture time, and analyzed dicentric chromosome for dose estimation[1]. Although shortened chromosomes are induced under these conditions, we are still able to detect dicentric chromosomes. However, for a more precise estimation at low doses of irradiation or for the analysis of late effects, the use of lower concentrations (0.01 μg/ml) of colcemid should be chosen because the induction of the extended chromosomes increases the sensitivity of detecting rates of chromosome translocations, chromosome breaks as well as of dicentric chromosomes.

Effects of colcemid block on chromosome condensation in PCC assay

As mentioned above, colcemid-block affected the status of chromosome condensation in the blood culture stimulated with PHA. In some laboratories, colcemid treatment is combined with calyculin A for induction of PCCs [10]. In order to evaluate the effect of colcemid-block on the induction of over-condensed chromosomes in PCC assay, the chromosome relative length was compared in PCC assay with or without colcemid-block. Miura and Blakely reported that calyculin A treatment results in induction of PCCs with shortened chromosome length morphology [9]. Calyculin A treatment for 15 or 30 min before harvesting could induce extended length PCCs in the ex vivo radiation model of PHA-stimulated peripheral blood lymphocytes [9]. It is noteworthy to emphasize that the 15-min treatment with calyculin A in the absence of colcemid preserved good chromosome morphology suitable for the analysis of G_2/M-PCC spreads (Fig. 3A). In contrast, cells with shortened chromosome length morphology were observed frequently in the PCC assay when calyculin A was combined with colcemid (Fig. 3D). In these conditions, we also detected G_2/M-PCC cells with extended length morphology (Fig. 3C). Like calyculin A, okadaic acid, another protein phosphatase 2A (PP2A) inhibitor, influences the activity of cdc25 and p34cdc/cyclinB [12]. In PP2A inhibited cells, upon treatment with calyculin A, the inactive form of the

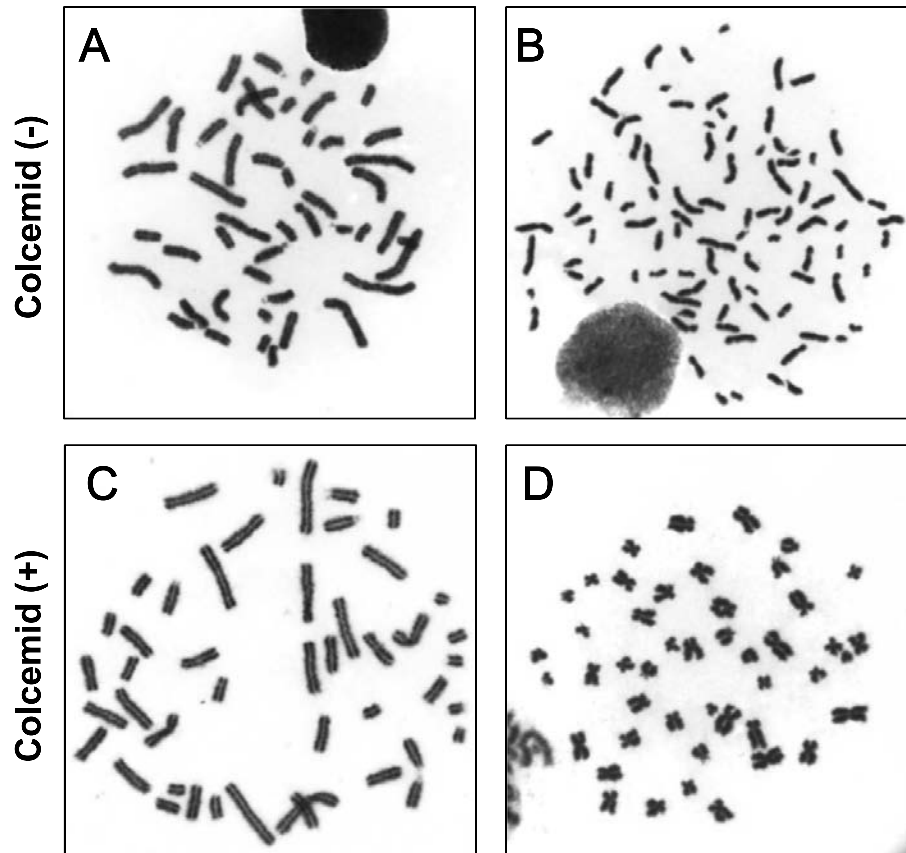

Fig. 3. PCC images of cell spreads prepared with or without colcemid-block. Cells were treated with calyculin A alone (A and B) or with a combination of calyculin A and colcemid (C and D). A and C: G2/M-PCC, B: M/A-PCC, and D: metaphase.

mitosis promoting factor (MPF) is activated and the cells progress through the cell cycle with resultant chromosome condensation [12]. In the PCC assay with human peripheral lymphocyte stimulated with PHA, it is possible to identify cells in all phases of the cell cycle (G_1-, S-, G_2/M-, and M/A-PCC). In our study, the frequency of the M/A-PCC (Fig. 3B) cells in the presence of colcemid was lower than the one in conventional PCC assay treated with calyculin A alone (data not shown). DNA double strand breaks (DSBs) caused by ionizing radiation activate the G_1/S checkpoint and induces G_1/S arrest[13]. DSBs are repaired both in a p53-dependent[14] manner during this block. However, the G_1/S checkpoint is inefficiently maintained, allowing cells to escape from the block and enter S phase[15]. The presence of DNA damage and chromosome breaks activates the G_2/M checkpoint and the cell cycle is rapidly arrested in G_2[16]. The presence of cells in different phases of the cell cycle observed in the PCC assay may reflect the cell cycle arrest induced by ionizing radiation. Since PCC assays performed in the presence of colcemid cannot give any information concerning cell cycle distribution, we recommended the usage of optimized calyculin A-induced PCC assay without colcemid-block for scoring of chromosome aberrations in biological dosimetry.

It was reported that calyculin A used in the conventional PCC assay caused fuzzy compact chromosomes [8, 9]. Shovman *et al.* described an improved assay for detecting radiation-induced chromatid breaks using a combination of colcemid-block and calyculin A-induced PCC in stimulated human peripheral blood T lymphocytes [17]. Their procedure, which combined with 1-h colcemid-block and a short (15 min) calyculin A treatment, eliminated the problem of centromere splitting present when calyculin A is used alone for a longer period (30 min) and improved the quality of the metaphase spreads. The chromosome compactness morphology in the PCC assay combined with colcemid-block was sharper than that in conventional PCC assays. In our present study, there was no remarkable difference in the frequency of the G_2/M-PCC and metaphase spreads with splitting centromere between combination with calyculin A (15 min) and colcemid (24 h) and calyculin A alone. Altogether, our data and data from other laboratories [17] indicate that it is necessary to prepare suitable chromosome spreads for better scoring of chromosome aberrations in biological dosimetry.

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