Mitotic slippage underlies the relationship between p53 dysfunction and the induction of large micronuclei by colcemid

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Received on January 15, 2013; revised on March 4, 2013; accepted on March 17, 2013

Micronuclei induced by aneugens are larger than those induced by clastogens in both in vitro and in vivo micronucleus (MN) assays. p53 dysfunction increases the formation of large micronuclei following treatment with aneugens; this study sought to identify the mechanisms responsible for this. Treatment with colcemid, both a mitotic inhibitor and an aneugen, induced MN containing two or more chromosomes more frequently in NH32 cells, in which p53 function is compromised, than in TK6 cells, in which p53 is functional. This indicates that p53 dysfunction enhances aneugen-induced chromosome loss or perturbs apoptosis, resulting in the formation of large MN. To examine the former hypothesis, the incidence of chromosome malsegregation in colcemid-treated TK6 and NH32 cells was compared using the cytokinesis-block MN assay. The incidence of chromosome non-disjunction was higher in NH32 cells than in TK6 cells, whereas the incidence of MN containing two or more chromosomes was similar between the two cell lines. To address the involvement of apoptosis in cell cycle progression, examination of chromosome 8 distribution revealed that more mononuclear NH32 than TK6 cells were tetraploid after prolonged mitotic inhibition, which indicated that the more number of NH32 cells may have bypassed the spindle assembly checkpoint via mitotic slippage and progression into the next interphase. Cells that underwent mitotic slippage were likely to contain lagging chromosomes formed via chromosome malsegregation, resulting in MN separated from the main nucleus. The number of TK6 cells containing large MN following colcemid treatment was increased by treatment with a caspase inhibitor in a dose-dependent manner, indicating that TK6 cells with MN normally undergo apoptosis. In conclusion, these findings indicate that mitotic slippage and perturbed apoptosis contribute to the induction of large MN in p53-compromised cells following treatment with colcemid.

Introduction

Perturbed spindle formation often leads to unequal segregation of sister chromatids (chromosome malsegregation), resulting in an unequal number of chromosomes in the daughter cells, a condition termed aneuploidy (1). Mitotic inhibitors cause aneuploidy via two major mechanisms: chromosome nondisjunction (NDJ) and chromosome loss. Chromosome NDJ occurs when sister chromatids attach to only one kinetochore and so both chromatids migrate to only one centriole during anaphase. Chromosome loss is caused by a lagging chromosome(s) that does not attach to the spindle. Chromosome NDJ is observed at low concentrations where chromosome loss is not observed, and chromosome loss arises at higher concentrations where a greater number of spindles are damaged and more chromosomes fail to attach to the spindle (1,2). Lagging chromosomes are decondensed and surrounded by a nuclear envelope, resulting in a micronucleus (MN) that is separated from the main nucleus (3–5). Clastogens induce chromosome breakage, whereas aneugens induce chromosome loss. Therefore, aneugens can induce micronuclei containing an entire chromosome(s), which are likely to be larger than those induced by clastogens both in vitro and in vivo (6,7). The spindle assembly checkpoint (SAC) is activated to prevent chromosome malsegregation following treatment with mitotic inhibitors by preventing cells progressing through mitosis (8). After prolonged activation of the SAC, cells can eventually escape mitosis and re-enter G1 phase without dividing, resulting in tetraploid cells. This process is known as ‘mitotic slippage’ (9). In the cytokinesis-block MN assay (CBMN), aneugens activate the SAC and thereby enable cells to undergo mitotic slippage to generate tetraploid mononuclear cells containing MN derived from lagging chromosome. Depending on the p53 status, these cells may eventually arrest, die by apoptosis or continue cycling (10).

Aneugens are discriminated from clastogens in the in vitro MN assay by immunochemical labelling of kinetochore proteins or by fluorescence in situ hybridisation (FISH) using a centromeric probe (11–13). We suggest that this could instead be simply and reliably determined by calculating the proportion of cells containing large MN among micronucleated mononuclear cells. This proposal is based on the observation that treatment with aneugen increases the proportion of p53-compromised Chinese hamster lung cells containing large MN and centromere-containing MN (14). Although treatment with aneugen also increases the proportion of NH32 cells containing large MN (in which p53 function is compromised), this is not always increased in TK6 cells (in which p53 is functional) (15). These findings suggest that p53 dysfunction is involved in the formation of large MN.

As the mechanism underlying the relationship between p53 dysfunction and the increased formation of large MN following aneugen treatment has not been clarified, we formulated two hypotheses to explain this phenomenon: (i) the NDJ model, in which spindle disruption induces chromosome NDJ more frequently in p53-compromised cells than in p53-functional cells, leading to chromosome loss and the formation of large MN containing multiple whole chromosomes (Figure 1A); and (ii) the mitotic slippage model, in which MN containing one or more unsegregated chromosomes form in mononuclear tetraploid cells during mitotic slippage. Undivided tetraploid cells arrest after mitosis and are thereby prevented from re-entering S phase; therefore, they undergo apoptosis in a p53-dependent manner (16,17). These tetraploid cells can escape apoptosis if p53...
function is compromised; thus, the proportion of mononuclear tetraploid cells with large MN is higher in p53-compromised cells than in p53-functional cells (Figure 1B). In this study, we tested these two hypotheses using an in vitro MN assay. TK6 and NH32 cells were treated with colcemid, a typical spindle disruptor, and the proportion of cells showing lagging chromosomes and chromosome NDJ were analysed by FISH using centromeric probes. In addition, to clarify the involvement of apoptosis in the elimination of cells following treatment with colcemid, the proportions of TK6 and NH32 cells with large MN or tetraploidy were examined in the presence or absence of a caspase inhibitor.

Materials and methods

Cell lines and culture conditions

The human B lymphoblastoid cell lines TK6 (wild-type p53) and NH32 (double p53 knockout) (18) were both derived from the human lymphoblastoid cell line, WI-L2. TK6 cells were generously provided by Dr M. Honma (National Institute of Health Science, Tokyo, Japan) and were originally distributed by Dr J. B. Little (Harvard School of Public Health, Boston, MA, USA). NH32 cells were generously provided by Dr Z. Darzynkiewicz (New York Medical College, New York, NY, USA) and were originally distributed by Dr H. L. Liber (Colorado State University, Fort Collins, CO, USA). Both cell lines were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l sodium pyruvate (Invitrogen Corporation, Grand Island, NE, USA). Cells were incubated in a humidified incubator at 37°C under 5% CO2 in air. Under these culture conditions, the doubling time was approximately 12–14 h for TK6 cells and 13–15 h for NH32 cells.

Chemical treatments and cell harvesting

Colcemid (Wako Pure Chemical Industries Ltd, Osaka, Japan; CAS number 477-30-5) was dissolved and diluted in dimethyl sulphoxide (Wako). Exponentially growing cell cultures were adjusted to a density of 1×10⁶ cells/ml, and 3 ml of these cultures were seeded in six-well plates. Cells were treated with colcemid just after seeding. The final concentration of dimethyl sulphoxide was 1% (v/v). The highest concentration of colcemid used was 12 ng/ml. This was based on a previous study, which reported that this concentration markedly decreases the relative population doubling and increases the proportion of cells with MN (15). In the CBMN assay, cytochalasin B (CyB) was added at 6 h after colcemid treatment (to a final concentration of 3 μg/ml) and cells were cultured for a further 18 h. In the MN assay without CyB, cells were cultured for 24 h. In the caspase inhibition assay, cells were preincubated with the pan-caspase inhibitor, Z-VAD-fmk (BioVision Research Products, Mountain View, CA, USA) for 1 h prior to the addition of colcemid, after which the cells were treated with colcemid for a further 24 h in both the CBMN and the MN assays. Following these treatments, the cells were collected by centrifugation and resuspended in 3 ml of 75 mmol/l hypotonic KCl solution for 10 min at room temperature. In the MN assay, cells were fixed with cold fixative [methanol:acetic acid = 3:1 (v/v)] and then resuspended in methanol containing 5% (v/v) acetic acid. For the CBMN assay, the cells were resuspended in methanol containing 5% (v/v) acetic acid. Cell suspensions were placed onto a clean glass slide and air-dried for MN counting or FISH analysis.

Staining and FISH

For MN counting, the caspase inhibition assay was performed in the absence of CyB and cells were stained with Giemsa solution using a standard method (15). FISH was performed on cells subjected to the MN and CBMN assays using the Posedon™All Human Centromere (AHC) red probe (KREATECH Diagnostics, Amsterdam, Netherlands), which comprises a mixture of aliphid repetitive sequences present on each human chromosome, to detect the centromeres of all chromosomes. Each slide was pretreated with 2× saline–sodium citrate (SSC)/0.5% Igepal (pH 7.0) at 37°C for 15 min followed by dehydration in 70, 85 and 100% ethanol for 1 min each. Samples were air-dried at room temperature, after which 10 μl of the probe was applied and the sample was covered with a glass cover slip. The slides were placed on a hot plate at 75°C for 10 min for denaturation. For in situ hybridisation, the slides were incubated at 42°C overnight. After hybridisation, the cover slip was removed and the slide was washed with 0.4× SSC/0.3% Igepal at 72°C for 1 min and then with 2× SSC/0.1% Igepal at room temperature for 1 min. The slides were then dehydrated in 70, 85 and 100% ethanol for 1 min each. Finally, the slides were mounted in Vectashield® mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA) to counterstain the nuclei.

For cells that underwent the CBMN assay, an additional FISH analysis was performed with chromosome enumeration probes containing chromosome 8-specific tandem-repeat DNA sequences (CEP8) (CEP® Chromosome Enumeration DNA FISH Probes; Abbott Molecular Inc., Des Plaines, IL, USA). The slides were pretreated as described for the AHC probe, and 10 μl of the probe mixture (7 μl CEP hybridisation buffer, 1 μl probe and 2 μl purified water) were applied. The samples were then covered with a glass cover slip. The slides were placed on a hot plate at 73°C for 5 min for denaturation. For in situ hybridisation, slides were incubated at 42°C overnight. After hybridisation, the cover slip was removed, and the slide was washed with a solution of 50% formamide/2× SSC at 46°C for a few seconds. The slides were dehydrated in 70, 85 and 100% ethanol.
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Figure 2A. After treatment with 10 or 12 ng/ml colcemid, 100 micronucleated mononuclear interphase cells were examined and classified as either +single CEN MN, +multi-CEN MN or −CEN MN (Figure 2B). No +multi-CEN MN were observed in either cell line following treatment with the vehicle (DMSO) (Figure 2C). After colcemid treatment, the proportion of +multi-CEN MN increased to approximately 10% and >20% in TK6 cells and NH32 cells, respectively. The proportion of +single CEN MN was similar in the TK6 and NH32 cell lines after colcemid treatment.

Fig. 2. Number of centromeric signals in MN after treatment of TK6 and NH32 cells with colcemid. (A) FISH using a pan-centromeric probe against a TK6 chromosome spread. (B) A typical MN containing single (top) or multiple (bottom) centromeric signals (indicated by arrowheads) following colcemid treatment. (C) TK6 and NH32 cells were hybridised with a pan-centromeric probe and counterstained with DAPI. The proportions of MN that contain single or multiple centromeric signals are shown. The proportion of +multi-CEN MN following colcemid treatment was significantly higher in the NH32 cell line than in the TK6 cell line. **P < 0.01 (Fisher’s exact test).
Next, cells were treated with lower concentrations of colcemid, 1–8 ng/ml, to determine the threshold concentration of colcemid needed to induce chromosome NDJ in TK6 and NH32 cells. The number of TK6 and NH32 cells showing chromosome NDJ significantly increased following treatment with ≥6 ng/ml colcemid (Figure 3D), whereas treatment with ≤5 ng/ml colcemid did not significantly induce chromosome NDJ in either cell line. This indicates that the threshold concentration of colcemid required to induce chromosome NDJ is 6 ng/ml for both cell lines. Following treatment with colcemid above this threshold concentration, the number of NH32 cells showing chromosome NDJ markedly increased; however, the increase was less marked in TK6 cells. These results suggest that cells harbouring dysfunctional p53 were more susceptible to chromosome NDJ following colcemid treatment than cells harbouring functional p53.

To elucidate a link between chromosome NDJ and chromosome lagging, which leads to the formation of +multi-CEN MN, the specimens prepared in the CBMN assay were hybridised with a pan-centromeric probe and the micronuclei in 100 binucleated cells were examined. The proportion of cells with +single CEN MN or +multi-CEN MN was calculated. The proportions of binucleated TK6 and NH32 cells containing two or more CEP8 signals in the MN were counted. NS: not significant.

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Colcemid treatment induces tetraploidy in NH32 cells

To further investigate the mechanism underlying the difference in the number of cells with large MN in the TK6 and NH32 cell lines following colcemid treatment, we examined whether mitotic slippage is involved in chromosome loss and in the formation of large MN. In the CBMN assay, the frequency of mononuclear tetraploid TK6 and NH32 cells was compared and the proportion of tetraploid cells containing MN was determined. Five hundred mononuclear interphase cells were imaged and the number of diploid (containing two CEP8 signals) or tetraploid (containing four CEP8 signals) cells was counted. The number of untreated mononuclear TK6 and NH32 cells showing tetraploidy was <10 (<2%) (Figure 4A), indicating that spontaneous mitotic slippage is rare, regardless of whether p53 function is compromised. After treatment with 10 or 12 ng/ml colcemid, the number of tetraploid mononuclear TK6 and NH32 cells increased; however, more mononuclear NH32 cells than mononuclear TK6 cells were tetraploid (Figure 4A). Following treatment with lower concentrations of colcemid (1–7 ng/ml), we found that the number of mononuclear tetraploid NH32 cells increased significantly with treatment with 1 ng/ml colcemid or higher, whereas the number of mononuclear tetraploid TK6 cells only increased significantly after treatment with 7 ng/ml colcemid (Figure 4B). Thus, the threshold concentration of colcemid required to induce mitotic slippage is lower for NH32 cells than for TK6 cells, suggesting that NH32 cells are more susceptible to colcemid-induced mitotic slippage than TK6 cells.

Chromosome loss was analysed by further classifying the tetraploid cells into three categories according to the distribution of the CEP8 signals: (i) type I, all four CEP8 signals in the main nucleus; (ii) type II, one CEP8 signal present in the MN and three in the main nucleus and (iii) type III, two CEP8 signals in the MN and two in the main nucleus (Figure 5). The combined proportion of tetraploid TK6 cells that were type II or III following treatment with 10 and 12 ng/ml colcemid was 0 and 3.9%, respectively, and the combined proportion of tetraploid NH32 cells was 4.1 and 4.8%, respectively, (Table I). Thus, type II and III tetraploid cells were induced by colcemid treatment irrespective of whether p53 function was compromised; however, a higher proportion of these types of tetraploid cells was observed in the NH32 cell line (p53-compromised) than in the TK6 cell line (p53-functional). These observations demonstrate that colcemid induces mononuclear tetraploid cells containing MN via mitotic slippage and suggest that these cells are normally eliminated by apoptosis.

Perturbation of apoptosis increases the formation of TK6 cells with large MN or tetraploidy following colcemid treatment

Next, the effect of perturbing p53-dependent apoptosis on the formation of large MN following colcemid treatment was clarified by examining the effects of the pan-caspase inhibitor, Z-VAD-fmk, on the formation of TK6 cells with large MN following colcemid treatment. TK6 cells were pretreated with 0, 50, 100 and 200 µmol/l Z-VAD-fmk followed by treatment with 10 or 12 ng/ml colcemid in the presence of CyB. Table II shows the proportion of cells with MN, and among them, the proportion of micronucleated cells with large MN was scored. The proportion of large MN was increased by Z-VAD-fmk in a dose-dependent manner when combined with 12 ng/ml colcemid. Next, the effects of caspase inhibition on tetraploidy in TK6 cells were examined in the CBMN assay by pretreating cells with or without 200 µmol/l Z-VAD-fmk (Table I). Of the 500 mononuclear, interphase TK6 cells counted, the number

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Fig. 4. Hybridisation of cells with the CEP8 probe and counterstaining with PI following exposure to colcemid in the presence of CyB. (A) TK6 and NH32 cells were treated with 10 or 12 ng/ml colcemid in the presence of CyB. 500 mononuclear interphase cells were imaged and the number of tetraploid cells was counted. **P < 0.01. (B) Comparison of the threshold concentration of colcemid required to significantly increase the number of tetraploid TK6 and NH32 mononuclear cells. **P < 0.01.

Fig. 5. Localisation of chromosome 8 in mononuclear interphase cells in the CBMN assay. Cells exposed to colcemid in the presence of CyB were hybridised with the CEP8 probe and counterstained with PI. Examples of NH32 cells showing mononuclear diploidy and tetraploidy containing two and four CEP8 signals, respectively, are shown. Examples of type I, II and III tetraploid cells are shown. Arrowheads indicate CEP8 signals in MN.
showing tetraploidy following treatment with 10 or 12 ng/ml colcemid in the absence of Z-V AD-fmk was 49 and 153, respectively. The number in the presence of Z-V AD-fmk was 64 and 197, respectively. Furthermore, the frequency of tetraploid cells with MN containing a CEP8 signal(s) (types II and III) following treatment with 10 or 12 ng/ml colcemid in the absence of Z-V AD-fmk was 0 and 3.9%, respectively. The frequency in the presence of Z-V AD-fmk was 3.1 and 5.1%, respectively. Thus, pretreatment with a pan-caspase inhibitor increased the number of tetraploid TK6 cells with MN containing multiple chromosomes. The combined proportion of TK6 cells that were type II and III following treatment with 12 ng/ml colcemid in the presence of Z-V AD-fmk was comparable to that observed following treatment of NH32 cells with the same concentration of colcemid. This suggests that perturbation of p53-dependent apoptosis increases the formation of MN containing multiple chromosomes following colcemid treatment.

Discussion

Large MN induced by aneugens contains a large amount of DNA, which is assumed to be due to the enclosure of multiple chromosomes within the MN (19–21). This assumption was supported by the FISH analysis conducted in this study using a pan-centromeric probe to examine the number of whole chromosomes in MN; large MN containing two or more chromosomes were present in TK6 and NH23 cells following colcemid treatment. However, the proportion of cells with MN that contained multiple whole chromosomes was higher in the NH32 cell line, in which p53 function is compromised, than in the TK6 cell line, in which p53 is functional. This suggested that p53 dysfunction may increase the incidence of chromosome lagging following mitotic inhibition.

Chromosome NDJ arises following the failure of sister chromatids to separate in human lymphocytes (22–25). We hypothesised that susceptibility of the cells to chromosome NDJ is associated with that to chromosome loss and lagging, which leads to the formation of large MN containing multiple whole chromosomes in the daughter cells, and that p53-compromised cells are prone to chromosome NDJ following treatment with colcemid. This hypothesis was tested in the CBMN assay using the CEP8 probe (Figure 3), which revealed that colcemid induces chromosome NDJ irrespective of whether p53 is functional, and that p53 dysfunction increases the susceptibility of cells to chromosome NDJ following colcemid treatment. However, in the CBMN assay using a pan-centromeric probe, the number of MN containing two or more signals was the same in the TK6 and NH32 cell lines, which suggests that the frequency of chromosome loss is not increased by p53 dysfunction. We concluded that the malsegregation of chromosomes via NDJ is not directly associated with the occurrence of chromosome lagging in p53-compromised cells following treatment with colcemid.

Mitotic slippage is another mechanism that could underlie the formation of MN containing multiple chromosomes (Figure 6B), which rarely occurs without spindle poison (Figure 6A). Mitotic arrest is induced when cells are treated with a spindle inhibitor. Cells that escape mitotic arrest generate mononuclear tetraploid cells; thus, disruption of spindle formation can lead to the formation of MN containing a whole chromosome(s) (Figure 6B). The CBMN assay with the CEP8 probe revealed that the number of mononuclear cells that were tetraploid following colcemid treatment was at least 2-fold higher in the NH32 cell line than in the TK6 cell line (Figure 4 and Table I). Furthermore, the number of mononuclear tetraploid cells that contained MN with a whole chromosome(s) was higher in the NH32 cell line than in the TK6 cell line. Any chromosome can potentially lag; therefore, assays using other chromosome-specific probes, either alone or in combination with the CEP8 probe, would reinforce these conclusions.

Cells with MN and chromosome NDJ can be eliminated by apoptosis (10,26). Because p53 function is compromised in NH32 cells, meaning that p53-dependent apoptosis cannot occur, the proportion of micronucleated tetraploid cells containing a whole chromosome(s) generated following colcemid treatment was higher in the NH32 cell line than in the TK6 cell line. Tetraploid cells that aberrantly exit mitosis via mitotic slippage are arrested at G1 phase by the p53-dependent G1 checkpoint and are subsequently eliminated by apoptosis (Figure 6B) (27). This p53-dependent postmitotic arrest and subsequent apoptosis in the TK6 cell line likely eliminates mononuclear...
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tetraploid cells and cells with large MN following treatment with 12 ng/ml colcemid. Indeed, the proportion of these types of TK6 cells increased in a dose-dependent manner following pretreatment with the pan-caspase inhibitor Z-V AD-fmk. Interestingly, the proportion of TK6 cells with large MN following treatment with 10 ng/ml colcemid did not increase in the presence of Z-V AD-fmk (Table II). It is possible that p53 accumulates in the nucleus in TK6 cells following treatment with 12 ng/ml colcemid to induce apoptosis, whereas it does not reach the threshold level in the cells treated with 10 ng/ml colcemid. Chen et al. (28) reported that p53 accumulates in the nucleus and suddenly induces transcription of its target genes, such as those encoding PUMA, Bax, CD95 and BTG2, to trigger apoptosis following mitotic slippage in A549 human cells. It is also possible that sufficient mitotic slippage event occurred in TK6 cells treated with 12 ng/ml to generate tetraploid cells with large MN but not in the cells following treatment with 10 ng/ml colcemid (Table I). Dysfunction of p53 causes centrosome duplication defects during S phase, giving rise to more than two centrosomes in rodent cells (29,30). When cells with tripartite spindles undergo mitotic slippage following prolonged activation of the SAC, the resulting mononuclear tetraploid cells are likely to contain MN with a whole chromosome(s). Activation of Ataxia-Telangiectasia Mutated in rodent-derived tumour cell lines by mitotic stress suppresses centrosome amplification via a mechanism in which p53 and p21 are implicated (31). This indicates that p53 activation following prolonged activation of the SAC prevents centrosome hyperamplification; however, this pathway is blocked in p53-compromised NH32 cells, thereby enabling centrosome hyperamplification and inducing MN induction via chromosome lagging in the presence of colcemid (Figure 6B).

In summary, the formation of large MN induced by treatment with colcemid is associated with increased chromosome loss via mitotic slippage; this process occurs more frequently in p53-compromised cells than in p53-functional cells. This is because tetraploid cells that undergo mitotic slippage are not eliminated by p53-dependent apoptosis when p53 function is compromised.

Acknowledgements
The authors thank Drs Masamitsu Honma and Zbigniew Darzynkiewicz for kindly supplying the TK6 and NH32 cell lines, respectively. The authors also thank Drs Tsuneo Hashizume, Ryo Fukuda and Eric Spicer for reviewing the manuscript.

References

Fig. 6. Role of p53-dependent apoptosis in eliminating cells containing large MN generated by mitotic slippage following treatment with colcemid. (A) Normal cell division rarely gives rise to large MN spontaneously. (B) Chromosome loss following disruption of spindle formation can cause the formation of large MN, irrespective of p53 status. Following centrosome hyperamplification, cells exit mitosis and the lagging chromosome(s) generates large MN. TK6 cells with functional p53 undergo p53-dependent apoptosis, whereas NH32 cells with compromised p53 function do not.


