DNA replication occurs in all lamina positive micronuclei, but never in lamina negative micronuclei

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A micronucleus is a small nucleus-like structure found in the cytoplasm of dividing cells that suffered from genotoxic stress. It is generally hypothesised that micronuclei content is eventually lost from cells, though the mechanism of how this occurs is unknown. If DNA located within the micronucleus is not replicated, it may explain the loss of micronuclei content. Because there had been no compelling evidence for this issue, we have addressed whether DNA located within the micronucleus is replicated this issue. Pulse labelling of bromodeoxyuridine revealed that DNA synthesis takes place in a portion of micronuclei that contain nuclear lamina B protein. By using iodine 3'-deoxyuridine/chlorodeoxyuridine double labelling, we found that all micronuclei containing lamin B are replicated during one cell cycle, whereas micronuclei lacking lamin B are never replicated. This result suggests that the content of lamin B-negative micronuclei is lost during cell division. Furthermore, we simultaneously visualised sites of DNA synthesis, lamin B and the extrachromosomal double minutes chromatin, which contain amplified oncogenes. We found that although the replication timing of double minutes was generally preserved in micronuclei, at times it differed greatly from the timing in the nucleus, which may perturb the expression of the amplified oncogenes. Taken together, these findings uncovered the DNA replication occurring inside micronuclei.

Introduction

The micronucleus is a small nucleus-like structure that forms within cytoplasm, separately from the main nucleus (1,2). It can be detected in almost all growing mammalian cells in response to a wide variety of stimuli that cause genome damage. Therefore, the presence of micronuclei is widely used as a hallmark of genotoxic stress. Micronuclei are generated by several different mechanisms. They can be generated post-mitotically from the lagging chromatid, and such ‘chromatin laggards’ can be derived from (i) an acentric chromosome fragment generated by drugs or radiation that induce DNA breaks, (ii) a whole chromosome that was not bound to or merotically bound to the spindle microtubule or (iii) a broken chromatin bridge connecting the separating anaphase chromosome. Furthermore, micronuclei can be generated from aggregates of extrachromosomal elements such as double minutes (DMs) chromatin bodies, which is a cytogenetic manifestation of gene amplification (3). This type of micronucleus is referred to as DM-type, while the other micronuclei are known as chromosome type (4). In addition to post-mitotic generation, micronuclei are also formed during interphase by nuclear budding (5). The nuclear buds may form through the lamina break, which is coupled to cytoplasmic membrane blebbing (Koh-ichi Utani, Atsushi Okamoto and Noriaki Shimizu, submitted for publication). On the other hand, there are micronuclei that lack the lamin B protein, a constituent of nuclear lamina, as well as others that contain lamin B. It has been suggested that micronuclei lacking lamin B are generated by broken chromatin bridges (6) and/or by nuclear budding through the lamina break (Koh-ichi Utani, Atsushi Okamoto and Noriaki Shimizu, submitted for publication).

It is commonly hypothesised that the content of micronuclei is ultimately eliminated from cells, though the mechanism of how this occurs remains unknown. The micronuclei may be degraded in situ in cytoplasm (7). Alternatively, they may be extruded out of cells since extracellular micronuclei have been detected in culture fluid (8). Another possibility is that no DNA replication occurs in micronuclei, leading to the dilution of the unreplicated micronuclei DNA during cell division. However, previous studies using short-term bromodeoxyuridine (BrdU) pulse labelling revealed that at least a portion of micronuclei underwent DNA synthesis, though there were also micronuclei that did not incorporate BrdU (5,9,10). Since DNA replication inside micronuclei has not been well studied (reviewed in ref. 7), we used several modern in situ techniques to examine this process more extensively.

Materials and methods

Cell and cell culture

Human colorectal tumour COLO 320DM (CCL 220) cells (11) and human cervical tumour HeLa cells (6) were obtained and maintained as described previously. For the experiments shown in Figure 2, HeLa cells at the mitotic phase were collected by the mitotic shake-off. The cells were cultured for 14 h in normal conditions and then chlorodeoxyuridine (CldU) and cytochalasin B (4 μg/ml; Sigma, St Louis, MO, USA) were added to the culture.

Cytochemical procedure

BrdU (Sigma), iodine 3'-deoxyuridine (IdU) (Sigma) or CldU (ICN pharmaceuticals, Inc., Irvine, CA, USA) is a halogenated thymidine analogue, and it was used to label the sites of DNA synthesis. Indirect immunofluorescence detection of BrdU or the simultaneous detection of IdU and CldU was performed using antibodies and the protocols described in our previous paper (12). The incorporated CldU was detected with a monoclonal rat anti-BrdU antibody (6 μg/ml; OBT0030; Oxford Biotechnology), and IdU was detected with a monoclonal mouse anti-BrdU antibody (1.5 μg/ml, cat. no. 347580; Becton Dickinson). The former antibody binds to both BrdU and CldU but not to IdU, while the latter antibody strongly binds to both BrdU and IdU and weakly to CldU. Therefore, samples were first incubated with the rat anti-BrdU antibody for 1 h at 37°C to detect CldU and then incubated with the mouse anti-BrdU antibody for 1 h at 37°C to detect IdU. These primary antibodies were detected by Alexa Fluor
647-conjugated donkey anti-mouse IgG (Invitrogen, Co.) and Alexa Fluor 488-conjugated donkey anti-rat IgG (Invitrogen, Co.).

In the experiment shown in Figures 3 and 4, DNA synthesis was detected by EdU (5-ethynyl-2'-deoxyuridine) labelling. Because fluorescent detection of EdU employs only a chemical reaction, it enabled the simultaneous detection of lamin B by immunofluorescence and of DMs by fluorescence in situ hybridization, both of which require multiple antibody reactions. Labelling and detection of EdU was performed as described previously (13). In short, EdU (Invitrogen) was added to the culture to a final concentration of 10 μM and incubated for the indicated times. After labelling EdU, the cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde (PFA) for 10 min at room temperature. Then, the cells were cytacentrifuged onto poly-L-lysine-coated slides. The slides were reacted with 1 mM CuSO4, 100 mM ascorbic acid and 10 μM Alexa Fluor 647 azide (Invitrogen) for 30 min at room temperature and were washed with PBS. Denaturation of DNA, hybridisation with DIG-labelled probe prepared from c-myc cosmid DNA, and simultaneous detection of the hybridised probe and the lamin B protein was performed using the same procedure as described previously (10).

Immunofluorescent detection of lamin B protein used 5 μg/ml Goat anti-Lamin B (M-20; Santa Cruz Biotechnology, Inc.) and 10 μg/ml Texas red-conjugated rabbit anti-goat IgG (EY Laboratories).

**Microscopy**

The images appearing in Figures 1 and 2 were obtained with an Olympus FV10-ASW confocal system on FV1000D-IX81 with a ×60 objective (UPLSAPO NA 1.35, oil). The images appearing in Figure 3 were obtained with a Nikon inverted microscope (TE2000-E; Nikon, Tokyo) with a ×60 objective (Nikon, Tokyo, Plan Apo VC 1.40, oil). All images were processed and assembled using Adobe Photoshop CS version 8.0.1 (Adobe Systems Inc.).

**Results and discussion**

**BrdU pulse labelling revealed that DNA replication occurs in lamin B-positive micronuclei**

About 5% of logarithmically growing cultures of cells (HeLa or COLO 320DM) contained micronuclei. We treated these cultures with BrdU for 2 h to label the DNA replication sites, fixed the cells and visualised the BrdU-labelled DNA and the nuclear lamin B protein simultaneously. Representative images of HeLa cells are shown in Figure 1A–F. We observed both lamin B-positive and negative micronuclei. Presence of both type was reported for chromosome-type (6,14–16) or DM-type micronuclei (10,16). While we were able to detect DNA replication in some lamin B-positive micronuclei (Figure 1A and B), we did not detect it in another portion of lamin B-positive micronuclei (Figure 1C and D) nor in any lamin B-negative micronuclei (Figure 1E and F), despite the fact that DNA replication occurred within the main nucleus. To address whether this was due to cells being in different cell cycle stages, we classified the cells into early, middle and late S phase as well as G1/G2 phase according to the nuclear distribution.

**Fig. 1. BrdU pulse labelling revealed that DNA replication occurs in micronuclei.** HeLa cells (A–G) or COLO 320DM cells (H) were pulse treated with 100 μM BrdU for 2 h and then harvested and fixed with PFA. The location of BrdU incorporation and the lamin B protein was visualised simultaneously, and confocal images were obtained (A–F). Micronuclei were classified according to lamin B status and BrdU incorporation. After examination of 149 (HeLa) or 115 (COLO 320DM) micronuclei, each type of micronuclei were quantified and plotted in G and H.

**Fig. 2. DNA replication occurs in all lamin B-positive micronuclei but not in lamin B-negative micronuclei.** (A) Outline of the experimental procedure. Logarithmically growing COLO 320DM or cell cycle-synchronised HeLa cells were cultured in the presence of 30 μM CldU and 4 μg/ml cytochalasin B for 12 h. Then, 100 μM IdU was added during the last 20 min. The cells were harvested and fixed with PFA. Incorporated CldU and IdU as well as lamin B protein were simultaneously detected, and DNA was counterstained by 4′,6-diamidino-2-phenylindole (DAPI). The representative images are shown in B–G. Mononucleated cells that were CldU-labelled, but not IdU-labelled (D–F), were designated as G2 phase cells. The COLO 320DM and the HeLa culture contained 5.5 and 32.5% G2 phase cells, respectively. The number of micronuclei in G2 phase cells, as classified by the presence or absence of CldU signal and lamin B within micronuclei, were plotted (H and I).
increased when the BrdU labelling time increased (data not shown). However, labelling the cells with BrdU for an entire cell cycle (>24 h) resulted in the delay of cell cycle progression, perhaps due to the cytotoxic effect of BrdU. Furthermore, continuous BrdU labelling induced DNA damage in the labelled chromatin, which generated BrdU-positive micronuclei after mitosis that hindered analysis. To overcome this technical obstacle, we used a double labelling technique in the following section.

DNA replication occurs in all lamin B-positive micronuclei but not in lamin B-negative micronuclei

To determine whether every micronucleus is replicated during S phase (~10 h), we performed a double pulse-chase experiment using IdU and CldU, instead of a single BrdU pulse labelling. Namely, we cultured logarithmically growing COLO 320DM cells in the presence of CldU and cytochalasin B for 12 h, added IdU during the last 20 min of culture (Figure 2A) and then harvested the cells. We fixed the cells with PFA and detected the incorporated CldU, IdU and lamin B protein simultaneously by using specific antibodies. CldU or IdU, as BrdU, was a halogenated thymidine analogue that label the site of DNA replication. Its labelling pattern among the nucleus correlates to the position in the S phase, as shown above for BrdU-labelling. When cells in late S phase were harvested, the IdU label showed a late S phase pattern and the CldU labelling represented DNA in the main nucleus that was replicated during the 12 h prior to harvest (Figure 2B). If the cells passed through mitosis, they appeared as binucleated cells since cytochalasin B inhibited cytokinesis (Figure 2C). Therefore, G2 phase cells were identified as cells containing a CldU signal in the absence of an IdU signal. The representative image shown in Figure 2D shows that a CldU signal was detected in the laminn B-positive micronucleus. Out of the 85 lamin B-positive micronuclei (Figure 2H), 84 were labelled with CldU, while only one was not (Figure 2F). It is possible that S phase progression was partly delayed in that the cell lacking a CldU signal and thus required >12 h of CldU labelling. Apart from this exception, we concluded that all lamin B-positive micronuclei are replicated during S phase. By contrast, lamin B-negative micronuclei in G2 phase were not labelled with CldU (Figure 2E and H), suggesting that DNA replication does not occur in lamin B-negative micronuclei. Similar results were obtained for cell cycle-synchronised HeLa cells (Figure 2I).

DNA replication timing of DM-type and chromosome-type micronuclei

During the above experiment, we noticed that some micronuclei actively incorporated IdU despite the fact that its neighbouring nucleus had already completed DNA synthesis (Figure 2G). Therefore, we examined the replication timing of not only the usual chromosome-type micronuclei but also DM-type micronuclei in COLO 320DM cells. DMs are extra-chromosomal, transcriptionally active euchromatin (16,17) that are replicated during early S phase within the nucleus (18). To detect DMs, lamin B and the DNA replication sites simultaneously, we labelled DNA replication sites with EdU. We used EdU instead of halogenated thymidine analogue, because detection of EdU required only chemical reaction, and it enabled the simultaneous detection of lamin B and DMs. The representative images are shown in Figure 3A–E, and all the images contain lamin B-positive DM-type micronuclei. In Figure 3A and B, DNA replication was detected in the micronucleus when the nucleus was at early S phase (A) or middle S phase (B). As

![Figure 3. Simultaneous imaging of DNA replication sites, lamin B and DMs revealed the timing of replication in DM-type and chromosome-type micronuclei. Logarithmically growing COLO 320DM cells were treated with EdU for 2 h and fixed with PFA. The incorporated EdU, lamin B and DMs were simultaneously detected by different fluorescent colours, and DNA was counterstained with 4’,6-diamidino-2-phenylindole (DAPI). The representative images are shown in A–E. COLO 320DM (F) or HeLa (G) cells were cultured in the presence of 4 μg/ml of cytochalasin B for 24 h. The cells were treated with 100 μM BrdU for 30 min and then fixed with PFA. BrdU was detected as in above and counterstained with DAPI. The binucleated cells showing differential replication timing are shown.](https://academic.oup.com/mutage/article-abstract/27/3/323/1055360)
shown in Figure 3C, DNA replication was not detected in the micronucleus, when the nucleus was in late S phase. Strikingly, there were other micronuclei that replicated while the nucleus was in late S phase (Figure 3D) and in G1/G2 (no nuclear EdU-labelling; Figure 3E). These events were quantified, and the frequencies were plotted in Figure 4A. The micronuclei containing EdU signals were most frequently associated with nuclei in early or middle S phase, because DMs are early replicating euchromatin in the nucleus (18), the results suggest that the timing of replication in the nucleus was similar to the one in the micronuclei. However, a portion of DM-type micronuclei replicated while the nucleus was in late S to G1/G2 phase (Figures 3D and E and 4A), consistent with Figure 2G, suggesting that the replication timing of some DMs is not synchronised with nuclear replication. On the other hand, the replication timing of the chromosome-type micronuclei appeared to be distributed more randomly because approximately half of the micronuclei replicated at each time point examined (Figure 4B).

Differentiation of replication timing between the micronucleus and the nucleus

As described above, we demonstrated that the timing of micronuclei replication differed from that of nuclear replication, despite being within the same cytoplasm. Interestingly, we also found that the timing of DNA replication also differed between two daughter nuclei within the same cytoplasm, i.e. binucleated cells, generated by cytochalasin B treatment (Figure 3F and G). When 180 binucleated cells that contained at least one nucleus in S phase were examined, we found that the replication timing was identical between sister nuclei in 162 cells but was different in 18. A similar phenomenon has been reported previously (19,20). Thus, it appears that the difference in replication timing between the micronucleus and the nucleus parallels the difference in replication timing between the two nuclei of binucleated cells. Importantly, the time when DMs are replicated may differ from the time when the same DMs are replicated in the nucleus, i.e. early S phase. This difference in replication timing may affect transcriptional control and may ultimately influence tumour cell phenotype.

Implications of this study

Here, we showed for the first time that lamin B-positive micronuclei replicate during the cell cycle and likely prevents the dilution of micronuclear content during cell division. DNA replication within a micronucleus does not necessarily mean that the resulting chromosome fragments will segregate properly to the daughter cells after the cell division. Thus, the next task will be to determine whether the sister chromatids generated in micronuclei are segregated and distributed equally to the daughter cells during the next mitosis. However, this may be difficult to determine since we previously showed that cells containing micronuclei frequently produced cells with additional micronuclei after undergoing mitosis (6).

By contrast, DNA replication was not detected in lamin-negative micronuclei, indicating that their chromatin may be lost during cell division. These lamin-negative micronuclei were likely generated from broken chromatin bridges during anaphase (6) or from interphase nuclear budding through the lamina break (Koh-ichi Utani, Atsushi Okamoto and Noriaki Shimizu, submitted for publication). Notably, transcription was not detected inside these micronuclei (16). We reported that the nuclear localisation signal-bearing protein did not enter the lamin-negative micronuclei (16). Therefore, the protein required for replication and transcription may be missing in these micronuclei. Understanding the nature of DNA replication and segregation within micronuclei will be important for determining how the entrapment of genetic material within inert micronuclei may contribute to the malignant nature of human cancer cells.

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