Note

Visualization of Aberrant Perinuclear Microtubule Aster Organization by Microtubule-Destabilizing Agents

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Received October 21, 2008; Accepted January 7, 2009; Online Publication, May 7, 2009

[doi:10.1271/bbb.80754]

In vitro experiments have shown that microtubule-destabilizing agents such as nocodazole and Vinca alkaloids depolymerize microtubules, and that stabilizing agents such as paclitaxel and epothilones promote microtubule assembly.1) These drugs disturb spindle architecture during mitosis and therefore inhibit cell proliferation in vivo.2–4) Since spindle formation is a complicated and highly dynamic process in the cell cycle, live cell imaging with fluorescent proteins is used to analyze the molecular behaviors of spindle components.5,6) However, attention was paid only to the behavior of one or two mitotic components such as the nuclear envelope and microtubules in those studies. We have constructed a multicolor cell line in which the centrosomes and chromosomes could be simultaneously visualized,7) and using this cell line, we recently characterized the in vivo effect of the microtubule-stabilizing agents taxanes and epothilones on centrosomes at low concentrations that do not cause mitotic arrest.8,9) Since centrosomes are the dominant microtubule organizing centers (MTOCs) for spindle formation, it is thought that microtubule-targeting agents disturb the spindle architecture by inhibiting microtubule organization. In the present study, to characterize in detail the involvement of microtubule organization in spindle assembly disturbance, we constructed another multicolor MDA-MB-435 cell line in which the centrosomes, spindle microtubules, and chromosomes can be simultaneously visualized with EGFP-Aurora-A, mCherry-α-tubulin, and ECFP-histone H3. Our multicolor live cell imaging showed that both microtubule-destabilizing and -stabilizing agents induced aberrant spindles in the M phase, as previously reported, but revealed that only microtubule-destabilizing agents induced additional microtubule asters in the perinuclear region in the early M phase.

First, we constructed multicolor human MDA-MB-435 cells. pmCherry-α-tubulin, pmKO-AF8,10) and pECFP-histone H37) were separately introduced into MDA-GFP-Aurora-A cells7) with selection plasmids, pTK-Hyg, pPur, and pCAGGS-bsr respectively. Stable transformants were sequentially selected by hygromycin B, puromycin, and blasticidin to establish MDA-Auro\(\text{A}/\text{tub/AF8/H3}\) (clone 7-1). mCherry-α-tubulin was incorporated into interphase and mitotic microtubules, while EGFP-Aurora-A localized to the centrosomes in the interphase and to the spindle poles in the M phase. ECFP-histone H3 was used as a marker for interphase chromatin and mitotic chromosomes.7) Live cell imaging and fluorescence microscopy were performed as described previously.7,8) Time-lapse images of mitosis of this cell line are shown in Fig. 1. The two centrosomes separated in the late G2 phase and aster microtubules developed from the centrosomes, as chromatin condensation occurred (Fig. 1, −12 to −3 min). Mitotic chromosomes were aligned to the spindle equator (Fig. 1b, 21 min), and the chromosomes were segregated into two daughter cells (63 min). Thus, untreated MDA Auro\(\text{A}/\text{tub/AF8/H3}\) cells showed a normal mitotic process.

In this study, the microtubule-destabilizing agents nocodazole and vinblastine and the stabilizing agent paclitaxel were used. Minimum concentrations, enough to induce mitotic arrest, were determined from mitotic indices (not shown). Mitotic arrest was observed by adding nocodazole at 100–nM, vinblastine at 6–nM, and paclitaxel at 10–nM.

Fixed and living cell images of mitotically arrested cells are shown in Fig. 2. Treatment of multicolor MDA cells with the microtubule-destabilizing agents nocodazole and vinblastine primarily induced multi-aster phenotypes (Fig. 2a, b, d, e). In addition to the two large asters emerging from the centrosomes indicated by

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Abbreviations: MTOC, microtubule organizing center; NEB, nuclear envelope breakdown
bright Aurora A dots, mini-asters were observed. Those asters contained γ-tubulin at their center (Fig. 2i). In contrast, in cells treated with paclitaxel, the microtubules loosely aggregated in the cell center without obvious alignment and formed a single large cluster. Mitotic chromosomes were placed around the microtubule cluster (Fig. 2c, f).

The aberrant multi-aster spindle formation induced by microtubule-destabilizing agents was analyzed by live cell imaging microscopy. Since the bi-aster and multi-aster formation processes in the nocodazole- and vinblastine-treated cells were quite similar to each other, only time-lapse images in vinblastine-treated cells are presented (Fig. 3a–d). The interphase microtubule array was hardly observed in late G2 vinblastine-treated cells, probably because of its depolymerization (Fig. 3c, -9 min). Coincident with chromatin condensation, the fluorescence intensity of the centrosomes and the centrosomal asters increased (Fig. 3b–d, -3 min). Subsequently, small microtubule foci or mini-asters appeared at the periphery of the nucleus just before nuclear envelope breakdown (NEB) (Fig. 3c, -1.5 min). The mini-asters remained through mitosis, or were incorporated into the large centrosomal asters in the case of diaster formation (not shown). The mitotic chromosomes were not aligned at the spindle equator and the cells were mitotically arrested (Fig. 3b, 33 min).

When the cells were treated with the microtubule-stabilizing agent paclitaxel, as chromatin condensed in prophase, cytoplasmic microtubules gradually accumulated at the periphery of the nucleus (Fig. 3e, -2 min). In contrast to nocodazole- and to vinblastine-treated cells, no asters were observed in the perinuclear region or at the centrosomes. After NEB, the microtubules moved inward to the unseparated centrosomes and formed a large cluster of microtubules, instead of forming a typical bipolar spindle (Fig. 3e, 40 min). Based on these results, a schematic diagram of aberrant spindle formation induced by these microtubule-targeting drugs is presented (Fig. 3f).

As described above, after treatment with nocodazole or vinblastine, the interphase microtubule array was hardly observed in the late G2 phase, probably because of depolymerization of microtubules by microtubule-destabilizing agents, but mini-asters arose in the perinuclear region just before NEB in the MDA cells (Fig. 3c). In the present study, we treated cells with microtubule-destabilizing agents at the lowest concentrations sufficient to induce mitotic arrest. At higher concentrations (200–nm nocodazole, 10–nm vinblastine), microtubules and asters were not observed in MDA cells (not shown), as reported in living11) and fixed19) HeLa cells. These results suggest that microtubule-destabilizing agents did not prevent microtubule

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**Fig. 1.** Live Cell Imaging of Mitosis in MDA AuroA/tub/AF8/H3 Cells without Drugs.

(a) Merged images of ECFP-histone H3 (b, blue), mCherry-γ-tubulin (c, red), and EGFP-Aurora A (d, green). Elapsed time is shown at the top left; time is relative to nuclear envelope breakdown (NEB). The time of NEB was estimated from the collapse of the nucleus after chromatin condensation. Asterisks indicate a dead cell straying into the observation field.
organization at the entry to mitosis at lower concentrations, and suggest that disturbance of spindle architecture by multi-aster formation was sufficient to inhibit mitosis.

The aster microtubules seemed to be newly synthesized rather than elongated from pre-existing microtubules, because the interphase microtubule array disappeared in the late G2 phase, and because the γ-tubulin signal was located at the center of the asters. Interestingly, a very few mini-asters were occasionally observed in the perinuclear region before NEB in untreated MDA AuroA/tub/AF8/H3 cells (38.5%, n = 26), and they were rapidly absorbed into the centrosomal asters (not shown). Therefore, it is likely that MDA-MB-435 cells have an intrinsic ability to organize microtubules in the perinuclear region in the onset of mitosis. However, this

Fig. 2. Mitotic Phenotypes Induced by Microtubule-Targeting Agents in MDA AuroA/tub/AF8/H3 Cells.

(a–c) Mitotically arrested MDA AuroA/tub/AF8/H3 cells in fixed and living states. Images were photographed 6–12 h after drug addition at the concentrations noted, and are presented as maximum intensity projection images. (a, b) Nocodazole (NOC) and vinblastine (VBL) induced additional mini-asters (arrowheads). Encircled regions are presented in (g) and (h) as sectioned images. (c) Cells treated with paclitaxel. A single microtubule cluster was evident, and no obvious alignment of microtubules was observed in it. (d–f) Percentage of mitotic phenotypes. After treatment with drugs for 6–24 h, the rounded-up mitotic cells were counted using an inverted microscope. A minimum of 50 mitotic cells were counted per sample in at least three independent experiments. (g, h) Sectioned images showing the morphology of the asters in NOC- (g) and VBL-treated (h) cells. In (g), since the mini-aster was located in a different Z-plane from the large asters emerging from the centrosomes, only centrosomal asters appear in the images. (i) VBL-treated parental MDA-MB-435 cells stained with anti α- (green in merged image) and γ-tubulin (red) antibodies. The γ-tubulin signal was observed at the center of the microtubule asters (arrowheads). DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI, blue).
Fig. 3. Aberrant Spindle Formation Induced by Microtubule-Targeting Drugs.
(a–d) Time-lapse images of cells treated with vinblastine at 6 nM for 17 h. (a) Merged images of ECFP-histone H3 (b, blue), mCherry-α-tubulin (c, red), and EGFP-Aurora A (d, green). Time point 0 min is relative to NEB. Arrowheads indicate mini-asters emerging in the perinuclear region just before NEB. (e) Microtubule cluster formation induced by the microtubule-stabilizing agent paclitaxel. Time-lapse images were acquired after treatment with 10 nM paclitaxel for 11 h. Merged images of ECFP-histone H3 (blue), mCherry-α-tubulin (red), and EGFP-Aurora A (green) are shown. (f) Schematic representation of aberrant mitotic spindle formation in MDA AuroA/tub/AF8/H3 cells. Microtubules are depicted by thin lines, the centrosomes are indicated by black dots, and chromatin is drawn in gray. (A) In untreated cells, the bipolar spindle is normally formed from the centrosomes until the metaphase. (B) Microtubule-destabilizing agents nocodazole and vinblastine stimulated mini-aster formation in the perinuclear region just before NEB. The cells were arrested in the prometaphase-like stage, and several mini-asters remained. (C) In cells treated with the stabilizing agent paclitaxel, cytoplasmic microtubules, presumably remnants of the interphase array, moved inward at NEB and formed a spherical cluster of microtubules around the centrosomes. Mitosis did not proceed from the prometaphase-like stage.
activity may be weak or overwhelmed by the microtubule-organizing activity at the centrosomes under normal conditions.

In this study, EGFP-Aurora A, mCherry-α-tubulin, and ECFP-histone H3 were used as markers of centrosomes/spindle poles, microtubules, and chromatin respectively. Simultaneous visualization of those proteins clearly showed that microtubule-organizing activity at the nuclear periphery was accelerated by the microtubule-destabilizing agents nocodazole and vinblastine, while aster formation was suppressed by the microtubule-stabilizing agent paclitaxel. Further characterization of the different effects of these microtubule-targeting drugs on microtubule-organizing activity might yield an understanding of the mechanism of mitotic inhibition by these drugs, and might provide insights into the complicated spindle assembly process.

Acknowledgment

We thank Dr. Takashi Fukada and Ms. Kumi Nishida, Osaka Prefecture University, for helpful discussion.

References