Microtubule-organizing Centers Abnormal in Number, Structure, and Nucleating Activity in X-irradiated Mammalian Cells

CHIKAKO SATO, RYOKO KURIYAMA, and KIMIKO NISHIZAWA
Laboratory of Experimental Radiology, Aichi Cancer Center Institute, Nagoya 464, and National Institute for Basic Biology, Okazaki 444, Japan; and Laboratory of Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT Microtubule-organizing centers (MTOCs) in irradiated cells were visualized by immunofluorescence using antibody against tubulin. From two to ten reassembly sites of microtubules appeared after microtubule depolymerization at low temperature in an irradiated mitotic cell, in contrast to nonirradiated mitotic cells, which predominantly show 2 MTOCs. A time-course examination of MTOCs in synchronously cultured cells revealed that the multiple MTOCs appeared not immediately after irradiation but at the time of mitosis. Those multiple MTOCs formed at mitosis were inherited by the daughter cells in the next generation.

The structure and capacity of the centrosomes to nucleate microtubules in vitro were then examined by electron microscopy of whole-mount preparations as well as by dark-field microscopy. About 70–80% of the centrosomes derived from nonirradiated cells were composed of a pair of centrioles and pericentriolar material, which initiated >100 microtubules. The fraction of fully active complete centrosomes decreased with time of incubation after irradiation. These were replaced by disintegrated centrosomal components such as dissociated centrioles and pericentriolar cloud, a nucleating site with a single centriole, or only an amorphous structure of pericentriolar cloud. Assembly of <20 microtubules onto the amorphous cloud without centrioles was seen in 54% of the initiating sites in mitotic cells 2 d after irradiation. These results suggest that x-irradiation causes disintegration of centrosomes at mitosis when the structural and functional reorganization of centrosomes is believed to occur.

The comprehensive term microtubule-organizing centers (MTOCs) has been given to sites from which microtubules radiate (1). Centrosomes, composed of a pair of centrioles and amorphous pericentriolar material, are the centers for the highly ordered assembly of spindle microtubules in the mitotic apparatus as well as the cytoplasmic microtubule network in mammalian cells. Originally, MTOCs were recognized in thin-section electron micrographs as foci on which large numbers of microtubules converged. Subsequent immunofluorescence studies showed that MTOCs are active sites of in vivo regrowth of microtubules when cells are removed from microtubule-depolymerizing agents (2, 3). The ability of the centrosome to nucleate microtubules has been directly tested in vitro by incubation of lysed cells with purified exogenous microtubule protein (4, 5). Moreover, whole-mount electron microscopy of cell lysates, combined with negative staining, offered rapid and accurate examination of the whole structure of the centrosome in association with its nucleating capacity (6, 7). We applied these available methods in a study of radiation effects on mitosis as a model system to investigate the function of MTOCs in abnormal mitosis.

After x-irradiation with moderate doses up to 1,000 rad, proliferating cells lose their colony-forming ability according to dose. Mitotic cells are far more sensitive to radiation than interphase cells (8). Some early studies on irradiated tissues and cultured cells indicated that irradiation did not kill cells immediately, but that cell death was delayed until the first postirradiation mitotic peak (9, 10). Direct evidence of the association of cell death with mitosis has been documented by a detailed analysis of time-lapse cinemicrographs of irradiated cells. Cell death, as indicated by blebbing, collapse, fragmentation, or disintegration of cells, was observed mostly in cells
in the rounded mitotic state (11). Multipolar and retarded incomplete mitosis followed by cell fusion have been thought to account for a significant proportion of nonsurviving clones (12). We showed an abnormal organization of the mitotic spindles of irradiated cultured mammalian cells as visualized with antitubulin antibody (13). In that study, a good statistical correlation was obtained between the fraction of cells having spindle abnormalities and the fraction of dead rounded cells lacking dye-exclusion ability (13). Cell death in the rounded state without the completion of the mitosis was also suggested. Since centrosomes are key control points for microtubule organization, radiation effects on centrosomes were investigated next.

The present experimental system was designed to assay, first, the number and distribution of MTOCs in an irradiated mitotic cell by immunofluorescence. Subsequently, the nucleating capacity and the structure of each nucleating site were examined by incubating extracted dividing cells with exogenous microtubule protein purified from porcine brain. Immediate dark-field microscopy gave a rough estimation of the initiating activity, and electron microscopy on whole-mount samples displayed the relationship between structure and the nucleating capacity of each site. Exposure to 1,000 rad was chosen to allow only a few cycles of cell division in the majority of cells and to cause a high frequency of abnormal mitosis (13). These experiments revealed the formation of MTOCs that were abnormal in number, structure, and nucleating activity in the x-irradiated cells.

MATERIALS AND METHODS

Cells: A cultured cell line B16-C2W (14) originally derived from a mouse melanoma was used. Cells were grown on Falcon plastic dishes (Falcon Plastics, Oxnard, CA) in Ham's F12 medium supplemented with 10% fetal calf serum in a CO₂ incubator and were used only at the logarithmic phase of growth. The generation time was 19 h. Spread cells attached firmly to the dish wall during interphase but became rounded with the onset of mitosis. The rounded mitotic cells were harvested by gentle pipetting (8). Cells grown on a coverslip were used for the immunofluorescence study of interphase cells.

Irradiation: Cells in a 3-mm deep layer of culture medium in a Falcon plastic dish were irradiated with an x-ray dosage of 1,000 rad at room temperature (15°-20°C). The physical factors of exposure were: 200 kVp kilovolt peak, 20 mA, 0.5 mm Al + 0.5 mm Cu filter added, 50 cm target-sample distance, and dose rate 1000 rad/min.

Indirect Immunofluorescence Microscopy to Visualize MTOCs: Mitotic cells suspended in phosphate-buffered saline were held in an icebox for 30 min to depolymerize microtubules and then transferred to a water bath set up at 30°C to allow repolymerization of microtubules for 30 s. Formaldehyde was added to give a final concentration of 3.7% for the fixation of mitotic cells. The fixation was performed in a 10-mm deep layer of culture medium in a Falcon dish. Mitotic cells suspended in phosphate-buffered saline were held in an icebox for 30 min to depolymerize microtubules, as well as from the increase in

confirmation of cell lysis with a phase microscope, an aliquot of the lysate was incubated with microtubule protein (~0.5 mg of protein/ml) at 35°C for 5 min in 0.5 mM PIPES, 1 mM EGTA, 0.5 mM MgCl₂, and 1 mM glycerol at pH 6.7. Microtubule protein was purified from porcine brain by two cycles of temperature-dependent polymerization-depolymerization (17, 18) and stored at -80°C before use, with no loss of polymerizing ability. Polymerization of microtubules was terminated by addition of 0.5 vol of 3% glutaraldehyde in 10 mM PIPES, 1 mM EGTA, 0.5 mM MgCl₂ at pH 6.7. The sample was processed for dark-field microscopy and electron microscopy.

Microscopy: A drop of the fixed sample of lyse containing assembled microtubules was spread under a coverslip and was immediately examined with a Nikon Fluophot VFD-TR microscope equipped with a dark-field condenser. The number of microtubules radiating from each nucleating site, except for the kinetochores of the chromosomes, was counted roughly. Whole mounts of samples for electron microscopy were prepared as described by Gould and Borisy (6), with some modification (16). Several drops of a fixed sample were slide-mounted, at 500-1,000 g for 5-10 min, onto an ionized Formvar-coated 400-mesh grid that had been coated with carbon. After washing with distilled water, the grids were negatively stained with 2% phosphotungstic acid, adjusted to pH 6.5 with NaOH, and examined in a JEM 100 CX or Hitachi H-500 electron microscope operated at 75–100 kV.

RESULTS

Number of MTOCs in a Cell

MTOCs were visualized as bright fluorescent foci with short microtubules radiating outward by indirect immunofluorescence using antitubulin antibody (Fig. 1). This was obtained by treatment of mitotic or interphase cells at 3°C for 30 min, which caused almost complete disruption of the original immunofluorescent array of microtubules. Only homogeneous fluorescence and some weakly fluorescent fibers were observed in the controls. Subsequent incubation of the cell at 30°C for 15–30 s allowed the partial reassembly of microtubules onto MTOCs.

Two MTOCs per cell were observed in a large majority of the nonirradiated mitotic cells (Fig. 1-a) located at the poles of the mitotic spindle. In mitotic cells harvested 24 or 48 h after irradiation, however, 2–14 MTOCs were recognized by focusing the lens to different depths of the cell (Fig. 1-b–d). When the cells were allowed to continue regrowth for 15 min at 30°C, these mitotic cells displayed multicenter mitotic spindles with a complicated array of microtubules running in various directions (Fig. 1-e and f). The number of regrowing sites for microtubules was two in 96% of unirradiated cells (Fig. 2) but markedly increased up to 14 in irradiated cells. Mitotic cells with four MTOCs were the most frequent 2 d after irradiation with 1,000 rad when the cell underwent the second postirradiation mitosis. The time course of accumulation of mitotic figures after administration of colchicine, as well as the cell count in asynchronous cultures indicated that mitotic cells harvested 24 and 48 h after irradiation were mostly of the first and second mitosis, respectively. After irradiation with 500 rad, mitotic cells having two MTOCs decreased to 75% but maintained that level thereafter (Fig. 2).

The relationship between the appearance of multiple MTOCs and mitosis was investigated on synchronously cultured interphase cells (Fig. 3). The cells were irradiated with 1,000 rads at the G1 phase of the cell cycle (2-h culture after harvest of mitotic cells), and their MTOCs were stained serially after irradiation and progression through the cell cycle. As shown in Fig. 3-a, for up to 8 h of incubation, both nonirradiated and irradiated cells displayed predominantly one prominent initiation site. The number of MTOCs in the nonirradiated cells transiently increased around 14 h and decreased thereafter. From the accumulation of the mitotic cells after administration of colchicine, as well as from the increase in

SATO, KURIYAMA, AND NISHIZAWA Centriolar Complex in Irradiated Cells 777
Microtubule-organizing centers (MTOCs) visualized by indirect immunofluorescence using antitubulin antibody. Immunofluorescence staining was performed after 30 s of repolymerization of microtubules at 30°C following a 30-min depolymerization at 3°C. Generally two MTOCs were observed in nonirradiated mitotic cells (a), and 2–14 MTOCs appeared as asters and dots in mitotic cells harvested 24 h (b) or 48 h (c and d) after irradiation with 1,000 rad. Prolonged polymerization of microtubules for 15 min at 30°C resulted in multicenter mitotic spindles with a complicated array of microtubules in the irradiated cells (e and f). One MTOC was present in most interphase cells 3 h after irradiation at G₁ phase (g), and one to eight MTOCs appeared in the vicinity of the nuclear membrane 24 h (h) or 48 h (i) after the irradiation. Bar, 10 μm.
cell number in the dishes, it was concluded that the changes in MTOC numbers corresponded to the separation of centrosomes at G2 phase and their distribution into daughter cells after mitosis. In the irradiated cells, multiple MTOCs started to appear 12 h after irradiation. However, the increase in the number of MTOCs was not transient but kept increasing up to 24 h. This increase did not result from the failure of cell division, as was demonstrated by the increase in cell number (Fig. 3b). This result suggests that multiple MTOCs were formed at mitosis and inherited by the daughter cells. The number of MTOCs per cell increased up to 12 during the subsequent incubation for 2 d. These multiple initiation sites were observed mostly in the vicinity of the nuclear membrane as was usual in the nonirradiated cells (Fig. 1h, and i).

**Activity of Nucleating Sites Detected by Dark-field Microscopy**

Mitotic cells were serially harvested after irradiation, lysed, and kept on ice, and then incubated with exogenous microtubule protein at 30°C for 5 min. The in vitro assembly of microtubules onto a centriolar complex was immediately examined by dark-field microscopy. Although precise counting was impossible because of Brownian movement, an approximate count of the number of microtubules nucleated from each assembly site was made (Fig. 4). About 73% of the nucleating sites isolated from nonirradiated cells revealed numerous microtubules radiating in every direction like an aster. The fraction of fully active nucleating sites decreased with time of incubation after irradiation. Fewer than 20 microtubules assembled, often in an asymmetric manner, onto 55% of the nucleating sites isolated from cells irradiated 2 d earlier.

**Structure and Activity of Nucleating Sites Detected by Electron Microscopy**

After the in vitro incubation of mitotic cell lysates with microtubule protein, grids of whole-mount preparations were negatively stained and examined in the electron microscope.
High beam illumination and high magnification allowed a determination of the presence or the absence of centrioles in the densely stained focal region of the microtubules, with reasonable confidence.

Table I summarizes the number of centrosomal structures with assembled microtubules detected by whole-mount electron microscopy. About 80% of them, derived from nonirradiated cells, were composed of a pair of centrioles and pericentriolar material (Fig. 5 a). After irradiation, the fraction of complete centriolar complexes decreased to 30% and the nucleating sites with no centriole or a single centriole increased. In Fig. 5 c and d, microtubule nucleation from the amorphous cloud is shown. The dissociation of centrosomes into a pair of centrioles (Fig. 5 b) or a centriole (Fig. 5 e) and pericentriolar material also increased after irradiation. These results indicate a tendency of the centrosomal components to separate from each other in irradiated cells.

Fig. 6 illustrates the relationship between the structure and activity of centrosomes. The complete centrosome, composed of a pair of centrioles and a pericentriolar cloud (open column), always initiated more than 100 microtubules even after irradiation. However, the total number of centrosomes in this category greatly diminished in irradiated cells. Numerous nucleating sites are formed in irradiated cells that do not have any centrioles (hatched column in Fig. 6) or have one centriole (dotted column in Fig. 6). The sites without any centriole could initiate from a few to over a hundred microtubules, while those with one centriole mostly initiated 25–50 microtubules. Dissociated centrosomes, composed of a pair of centrioles and a pericentriolar cloud (shaded column in Fig. 6), showed high nucleating activity.

**DISCUSSION**

The results presented above show that x-irradiation results in the formation of multiple MTOCs in B16-C2W cells at mitosis. Abnormal centrosomal structures and reduced activity were found at the sites of microtubule nucleation in vitro. It is not clear whether the immunofluorescent foci within the cells exactly correspond to the nucleating sites for in vitro assembly of microtubules from exogenous brain tubulin. The structure of multiple MTOCs in nonlysed mitotic cells should be exam-
after irradiation with 1,000 rad. Whole-mount preparations of lysed mitotic cells after incubation with exogenous microtubule protein were examined by electron microscopy. The structure was classified into four categories as follows: a complete centrosome consisting of a pair of centrioles and electron-dense pericentriolar material (open column), centrosomes dissociated into a pair of centrioles and adjacent electron-dense material (shaded column), the site containing a single centriole (dotted column), and a nucleating site lacking centrioles (hatched column).

The authors are grateful to Mr. Terashima for photographs and to Mrs. Tokumori for the preparation of the manuscript.

This study was supported in part by a grant-in-aid for cancer research from the Ministry of Education of Japan.

Received for publication 2 August 1982, and in revised form 1 November 1982.

REFERENCES

1. Pickett-Heaps, J. D. 1969. The evolution of the mitotic apparatus: an attempt at comparative ultrastructural cytology in dividing plant cells. Cytobios. 1:257-280.

2. Osborn, M., and K. Weber. 1976. Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane. Proc. Natl. Acad. Sci. USA. 73:867-871.

3. Britikley, B. R., G. M. Fuller, and D. P. Higfield. 1976. Tubulin antibodies as probes for microtubules in dividing and nondividing mammalian cells. In Cell Motility. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Cold Spring Harbor Laboratory, New York 435-445.

4. McGill, M., and B. R. Britikley. 1975. Human chromosomes and centrosomes as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin. J. Cell Biol. 67:189-199.

5. Snyder, J. A., and J. R. McIntosh. 1975. Initiation and growth of microtubules from mitotic centers in lysed mammalian cells. J. Cell Biol. 67:744-760.

6. Gould, R. R., and G. G. Borisy. 1977. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. J. Cell Biol. 73:601-615.

7. Teferi, B. R., and J. L. Rosenbaum. 1979. Cell cycle-dependent, in vitro assembly of tubulin onto the pericentriolar material of HeLa cells. J. Cell Biol. 81:484-497.

8. Terashima, T., and L. J. Tolmach. 1963. Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. Exp. Cell Res. 30:544-562.

9. Spear, F. G., and A. Glucksman. 1958. The effect of gamma radiation on cells in vivo. Single exposures of the normal tadpole at room temperature. Br. J. Radiol. 31:533-535.

10. Watanabe, K., and S. Okada. 1966. Study of mechanisms of radiation-induced reproductive death of mammalian cells in culture: estimation of stage at cell death and biological description of processes leading to cell death. Radiat. Res. 27:290-306.

11. Hurwitz, C., and L. J. Tolmach. 1969. Time lapse cinematographic studies of x-irradiated HeLa S3 cells. I. Cell progression and cell disintegration. Biophys. J. 9:607-632.

12. Marks, G., and M. A. Bender. 1966. Radiation-induced mammalian cell death: lapse-time cinematographic observations. Exp. Cell Res. 43:413-423.

13. Sato, C., K. Nishizawa, and I. Yahara. 1980. Abnormal organization of mitotic spindles in cultured mammalian cells after X-irradiation as visualized with anti-tubulin antibody. Cell Struct. Funct. 5:93-103.

14. Claussen, C., A. Okawa, T. T. Chen, and F. H. Advances in Biology of Skin. The Pigmentary System. W. Montague and F. H. editors. Pergamon Press, Oxford. 847-868.

15. Yahara, I., and F. Kakinoto-Samcheko. 1978. Microtubule organization of lymphocytes and its modulation by patch and cap formation. Cell. 15:251-259.

16. Kuczykarn, A., and G. G. Borisy. 1981. Microtubule-nucleating activity of centrosomes in Chinese hamster ovary cells is independent of the centriole cycle but coupled to the mitotic cycle. J. Cell Biol. 91:822-836.

17. Borisy, G. G., J. M. Marrain, J. B. Olmsted, D. B. Murphy, and K. A. Johnsen. 1975. Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly in vitro. Ann. NY Acad Sci. 253:107-132.

18. Kuczykarn, A. 1975. Further studies on tubulin polymerization in vitro. J. Biochem. (Tokyo) 77:23-31.

19. Britikley, B. R., S. M. Cox, D. A. Pepper, L. Whitle, S. L. Brenner, and R. L. Purdie. 1981. Tubulin assembly sites and the organization of cytoplasmic microtubules in cultured mammalian cells. J. Cell Biol. 90:554-562.

20. Watt, F. M., and H. Harris. 1980. Microtubule-organizing centres in mammalian cells in culture. J. Cell Sci. 44:103-121.

21. Speigelman, B. M., M. A. Lopata, and M. W. Kirschner. 1979. Multiple sites to the initiation of microtubule assembly in mammalian cells. Cell. 16:239-252.
initiation sites preceding neurite outgrowth in mouse neuroblastoma cells. Cell. 16:253-263.
23. Hochkiss, S. K., and J. E. Sisken. 1979. Multiple mitosis produced by mitotic arrest in
HeLa cells. J. Cell Biol. 79(2, Pt. 2):298a (Abstr.).
24. DeBrabander, M., G. Genens, R. Nuydens, R. Willebroeeds, and J. DeMey. 1981. Taxol
induces the assembly of free microtubules in living cells and blocks the organizing capacity
of the centrosomes and kinetochores. Proc. Natl. Acad. Sci. USA. 78:5608-5612.
25. Schatten, G., H. Schatten, T. Bestor, and R. Balezim. 1981. Taxol blocks the pronuclear
migration at fertilization and induced asters in unfertilized eggs. J. Cell Biol. 91(2, Pt. 2):185a (Abstr.).
26. Ring, D., R. Hublie, C. Caput, and M. Kirschner. 1980. Isolation of microtubule
organizing centers from mouse neuroblastoma cells. In Microtubules and Microtubule
Inhibitors. M. DeBrabander and J. DeMey, editors. Elsevier/North-Holland, Amsterdam.
297-310.
27. Sharp, G. A., M. Osborn, and K. Weber. 1981. Ultrastructure of multiple microtubule
initiation sites in mouse neuroblastoma cells. J. Cell Sci. 47:1-24.
28. Dirksen, E. R. 1961. The presence of centrioles in artificially activated sea urchin eggs. J.
Biophys. Biochem. Cytol. 11:244-247.
29. Rieder, C. L., and G. G. Borisy. 1981. Asymmetric distribution and cell-cycle changes of
pericentriolar material. J. Cell Biol. 91(2, Pt. 2):319b (Abstr.).
30. Kuriyama, R., and G. G. Borisy. 1981. Centriole cycle in Chinese hamster ovary cells as
determined by whole-mount electron microscopy. J. Cell Biol. 91:814-821.