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

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ABSTRACT Microtubule-organizing centers (MTOCs) in x-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

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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 CO_2 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 100 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 the cells for 5 min at room temperature. Aliquots of the fixed and washed cells were then centrifuged at 900 *g* onto a microscopic glass slide using a cytocentrifuge (Shandon-Elliot, Cheshire, England). The glass slides were previously coated with poly-L-lysine to obtain firm attachment of the rounded cells to the slides. These cells were treated with 95% ethanol for 5 min at room temperature, then stained successively with antitubulin antibody and with fluorescein-conjugated goat antibody against rabbit IgG (Miles-Yeda, Rehovot, Israel). Specific antitubulin antibody was a generous gift from Dr. Yahara (15). Spread interphase cells on a coverslip were treated first at 3°C, then transferred to 30°C, fixed, and stained by the same procedure as used on mitotic cells. Fluorescence microscope observations were carried out with Fluophot VFD-TR (Nikon Optics Co., Tokyo, Japan). Photographs were taken with Kodak Tri-X film with an exposure time of 3–10 s. Prior incubation of tubulin antibody with brain tubulin gave rise to no fluorescent fibers in mitotic cells.

Cell-free Polymerization of Microtubules onto the Centrosomes: The detailed procedure was described in a previous paper (16). The mitotic cells were chilled on ice for 30 min to ensure the complete depolymerization of in situ microtubules. The pelleted cells were then suspended in distilled water (10:1 vol/vol) and allowed to stand for 1–2 min at room temperature, then mixed with an equal volume of 0.5% Triton X-100 in 10 mM PIPES buffer. After 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 polymerization buffer (100 mM PIPES, 1 mM EGTA, 0.5 mM $MgCl_2$, and 1 mM guanosine triphosphate, 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 $MgCl_2$ at pH 6.7. The sample was processed for dark-field microscopy and electron microscopy.

Microscopy: A drop of the fixed sample of lysate 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 sedimented, 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 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 G_1 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

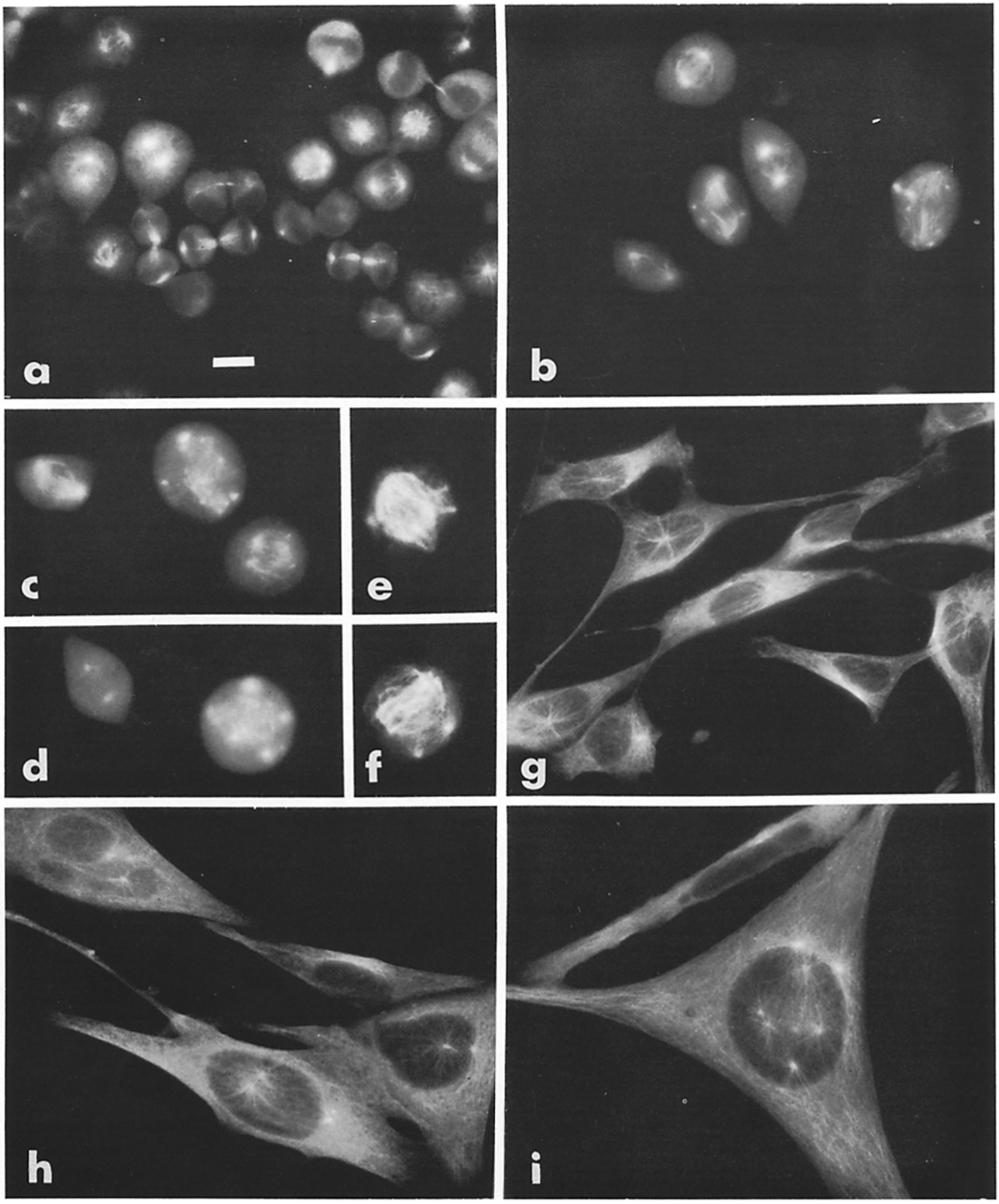

FIGURE 1 Microtubule-organizing centers (MTOCs) visualized by indirect immunofluorescence using antitubulin antibody. Immunofluorescence staining was performed after 30 s of repolymerization of microtubules at 30°C following a 30-min depolymerization at 3°C. Generally two MTOCs were observed in nonirradiated mitotic cells (*a*), and 2–14 MTOCs appeared as asters and dots in mitotic cells harvested 24 (*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 G_1 phase (*g*), and one to eight MTOCs appeared in the vicinity of the nuclear membrane 24 (*h*) or 48 h (*i*) after the irradiation. Bar, 10 μm.

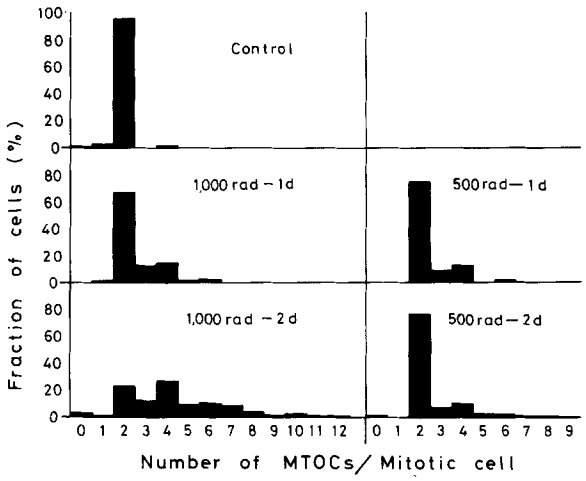

FIGURE 2 Histogram of the number of MTOCs in a mitotic cell after irradiation. Mitotic cells were harvested by gentle pipetting from a nonirradiated dish of a random culture (control), from dishes 24 h after irradiation with 1,000 or 500 rad, or from dishes 48 h after the irradiation. The number of immunofluorescent foci per cell was counted on >400 cells/experiment. The mean values of MTOCs/mitotic cell in the three repeated experiments are 2.223 (control), 2.692 (*1,000 rad-1 d*), 4.295 (*1,000 rad-2 d*), 2.561 (*500 rad-1 d*) and 2.453 (*500 rad-2 d*), respectively.

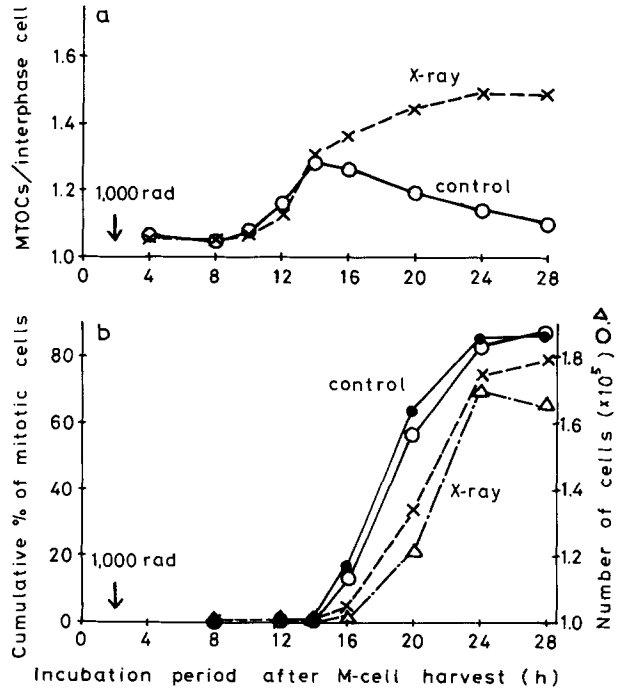

FIGURE 3 Time course of appearance of multiple MTOCs and mitosis in synchronously cultured cells. Cells were irradiated with 1,000 rad at G_1 phase (2 h after the harvest of mitotic cells). At different incubation times, MTOCs of spread interphase cells were counted by immunofluorescence (1,000 rad, ×; 0 rad, ○) as shown in the upper figure (*a*). MTOCs/cell represents the mean value of MTOCs in an interphase cell on >200 cells. The lower figure (*b*) indicates the number of cells in the (0 rad, ○; 1,000 rad, ×) at each incubation time, and the accumulation of mitotic cells after administration of 0.1 μg/ml colchicine (0 rad, ○; 1,000 rad, △). Comparison of the upper and the lower panels shows that the increased MTOCs at 12–14 h of incubation (G_2 phase) decreased thereafter as the cells divided in control cells but progressively increased as the cells divided in irradiated cells. The whole experiment was repeated three times.

cell number in the dishes, it was concluded that the changes in MTOC numbers corresponded to the separation of centrosomes at G_2 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. 3*b*). 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. 1 *h*, 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 evern 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.

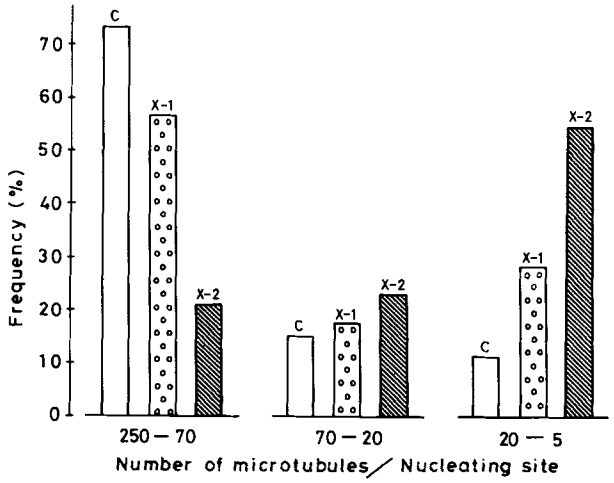

FIGURE 4 Decrease in the initiating capacity of the nucleating sites isolated from mitotic cells after irradiation. Mitotic cells were harvested from nonirradiated cultures (*C*), or from 24- (*X-1*), and 48-h (*X-2*) cultures after irradiation with 1,000 rad. The number of microtubules generated from each site was counted roughly by dark-field microscopy immediately after a 5-min incubation at 30°C with exogenous microtubule protein. The frequency was obtained by counting ~200 sites in four repeated experiments.

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.

TABLE I

Number of Microtubule-nucleating Sites Examined by Electron Microscopy of Whole-mount Preparations of Lysed Mitotic Cells

Treatment	Centriole(+)		Centriole(−)	Dissociated centrosome	Total
	Single	Pair			
rad	n(%)		n(%)	n(%)	n(%)
0	13 (8.0)	131 (80.4)	17 (10.4)	2 (1.2)	163 (100)
1,000, 24 h	19 (11.2)	84 (49.7)	59 (34.9)	7 (4.1)	169 (100)
1,000, 48 h	35 (15.6)	69 (30.8)	95 (42.4)	25 (11.2)	224 (100)

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-

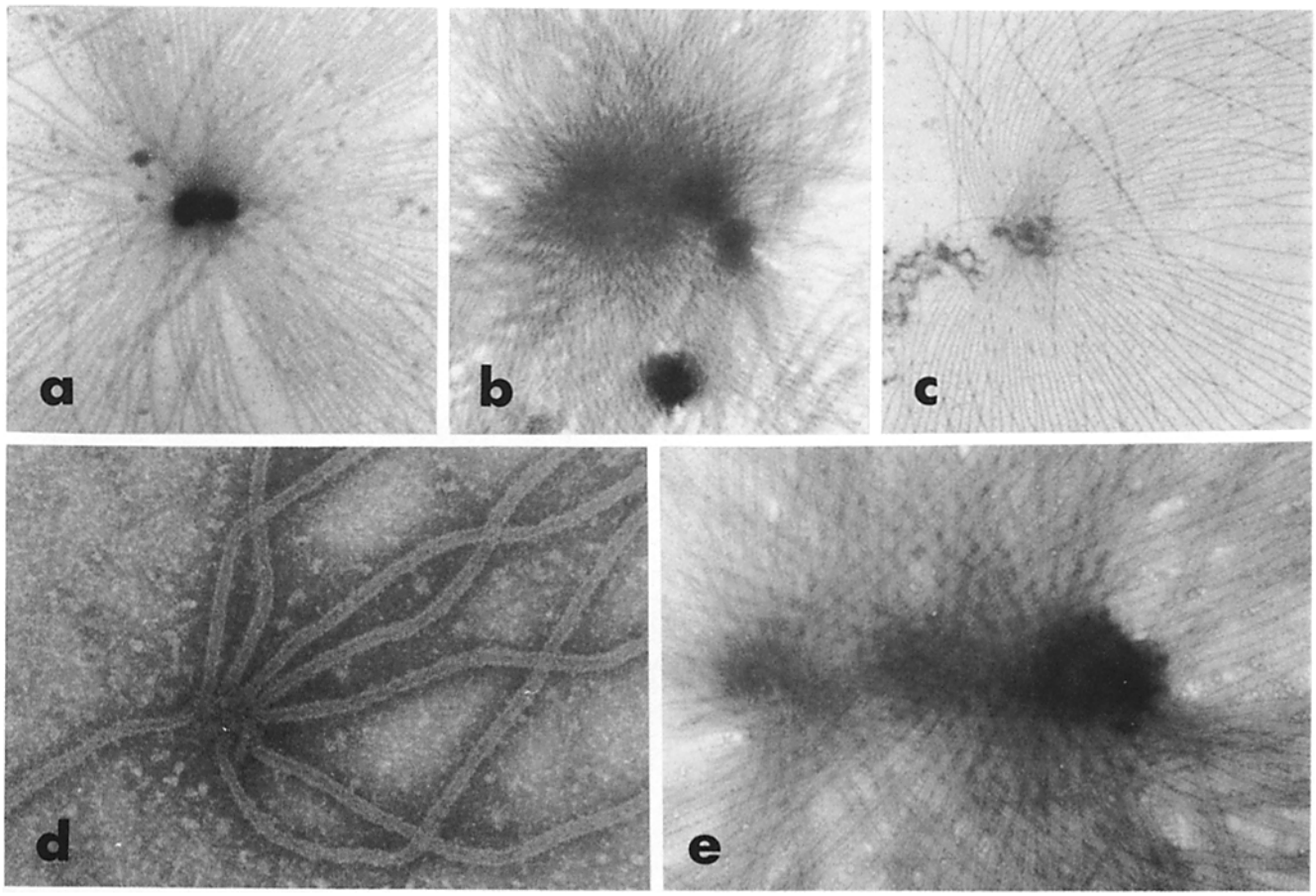

FIGURE 5 Four categories of whole-mount electron micrographs of a microtubule-nucleating site after incubation with brain tubulin in vitro. A complete centrosome (*a*), a centrosome dissociated into a pair of centrioles (*b*) or single centriole (*e*) and pericentriolar cloud (*b*), amorphous cloud lacking centrioles (*c* and *d*), or single centriole. (*a*) × 9,600, (*b*) × 12,000, (*c*) × 13,700, (*d*) × 26,500, and (*e*) × 16,000.

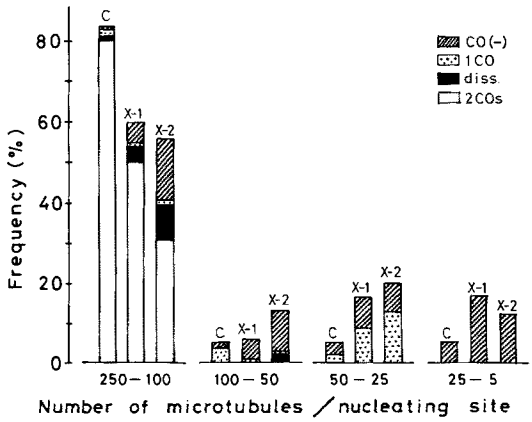

FIGURE 6 Relationship between the nucleating capacity of a nucleating site and its structure. Mitotic cells were harvested from nonirradiated cultures (*C*), or from 24- (*X-1*) and 48-h (*X-2*) cultures after irradiation with 1,000 rad. Whole-mount preparations of lysed mitotic cells after incubation 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).

ined by serial sections to reconstruct three-dimensional images by electron microscopy for further clarification of this point.

The number of nucleating sites for microtubule assembly has been reported to be predominantly 1–2/cell in the majority of cell lines (19, 20), as in B16-C2W presented in this paper. On the other hand, multiple organizing centers were demonstrated in several kinds of organisms including human and mouse fibroblast cells (21) and undifferentiated neuroblastoma cells (22). Moreover, it is possible to induce artificial multiasters in cultured mammalian cells or in sea urchin eggs by treatment with antimicrotubular drugs such as colchicine or Taxol (23–25). The present experiments demonstrate that x-rays are another agent capable of producing multiple sites for initiation of microtubules.

Centrioles were exhibited in each of the multiple MTOCs in undifferentiated neuroblastoma cells (26, 27) and in cytasters induced within artificially activated unfertilized sea urchin eggs (28). Multiple microtubule-nucleating sites in irradiated cells, however, frequently showed a dissociation of centrosomes into centrioles and a pericentriolar cloud or, in some cases, only an amorphous cloud of pericentriolar material, as seen in the centers of many asters derived from Taxol-treated, unfertilized sea urchin eggs (25) or cultured mammalian mitotic cells (24). Thus, x-irradiation seems to cause an inhibition of normal assembly of centrosomes or a fragmentation of centrosomes into smaller pieces. It is necessary to note that disruption of the centrosomal complex seems to occur during preparation of lysed cells for in vitro nucleation. In our experiments, ~10% of the nucleating sites isolated from nonirradiated mitotic cells lacked centrioles and showed a reduced activity. Marked increase in nucleating sites without centrioles after irradiation may be explained partially by the disruption of the centriolar complexes during preparation, if we assume enhanced fragility of the complex after irradiation. Another problem in the sample preparation is the discrepancy between the results obtained by the two different methods to detect in vitro nucleating activity (Figs. 4 and 6). A higher fraction of nucleating sites was detected to have reduced activity by dark-field microscopy than by electron microscopy. This was due, at least in part, to the different mounting processes. The incubation mixture was observed directly by dark-field microscopy, whereas electron microscopy was done on whole-mount samples after centrifugation. An important point of the present experiment is that the appearance of multiple MTOCs in irradiated cells was tightly coordinated with mitosis. It has been noticed previously that a drastic change in the microtubular array occurs from the cytoplasmic microtubule complex to the mitotic spindle at the transition from interphase to mitosis. Evidence for structural (29) and functional (30) changes of centrosomes during the transition to mitosis have been obtained. It might therefore be concluded that x-irradiation disturbs the normal rearrangement of centrosomes that usually takes place at the onset of mitosis. Identification of the molecular nature of the substance(s) functioning during this rearrangement of centrosomes would help our understanding of abnormal MTOCs as well as abnormal mitosis.

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