Role of the centrosome in mitosis: UV micro-irradiation study

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Abstract

Ultraviolet micro-irradiation (UV-MI) of the PK (pig kidney embryo) cell centrosome (A\textsubscript{max} = 280 nm, spot diameter 1.6 mm, exposure time 5-15 s) at metaphase and anaphase resulted in functional damage of the centrosome. After UV-MI of the centrosome at early metaphase, chromosomes quickly (in 1-3 min) moved away from the irradiated pole and then encircled the non-irradiated pole. Within 10 min after UV-MI the spindle disassembled and chromosomes remained unseparated. The minimal dose inducing this effect in 90% of cells was accumulated in 5 s. After the same UV-MI at late metaphase, chromosomes shifted towards the non-irradiated pole; however, anaphase started and chromosome motion towards the non-irradiated pole continued normally.

UV-MI of the centrosome at early anaphase for 5-15 s slowed down and then stopped chromosome motion towards the irradiated pole. This was a result of rapid (within 2-3 min) disorganization of the half-spindle. Chromosomes continued to move towards the opposite pole normally, while cytokinesis was significantly retarded.

No visible lesion was revealed by electron microscopy after 5 s UV-MI, while 15 s irradiation resulted in the truncation of the microtubule bundles 1.5-2 µm from the centrosome.

We concluded that UV-MI inactivates the centrosome and induces disaggregation of microtubule initiation sites. The critical point (checkpoint) in mitosis up to which this damage induces mitotic arrest is mid-metaphase.

Keywords: Centrosome; Mitosis; Micro-irradiation; Microtubules

1. Introduction

In animal cells the mitotic spindle is organized by the centrosome containing a pair of centrioles. The centrioles duplicate at S phase and at the onset of mitosis two centrosomes serving as mitotic poles initiate microtubule assembly and form the spindle where chromosomes are captured, aligned and then separated via kinetochore-microtubule interaction [1]. However, despite numerous studies, the role of the centrosome in the control of mitosis remains far from being understood. In some cells mitosis occurs without a prominent centrosome. Centrosome core structures — centrioles — are absent in higher plants, diatoms [2] and even in one Drosophila cell line [3]. To gain insight into the role of the centrosome in mitosis, direct experiments are essential. Such experiments have been performed using the micro-irradiation technique. Two main set-ups, namely UV microbeam and laser systems, were used.

Experiments on UV micro-irradiation of the centrosome (spindle pole) performed in the 1960s gave some controversial results. Irradiation of the mitotic pole in telophase spermatocytes of Bombyx mori resulted in a rapid shift of chromosomes from the irradiated pole [4]. However, Zirkle found the mitotic blockage induced by UV irradiation in newt pneumocytes to be independent of the presence or absence of the centrosome in the irradiated area [5].

In a series of studies, Berns and coworkers used an argon laser microbeam for centrosome irradiation [6-9]. They found irradiation of the mitotic pole in prophase after acridine orange treatment to inhibit anaphase onset [7], but in some cases mitosis was completed despite the damage to the centrosome and even in the absence of centrioles in the mitotic poles [6]. More recently, re-evaluating these studies, Vorobjev et al. showed that the cell behaviour after centrosome micro-irradiation with an argon laser microbeam (with and without acridine orange pretreatment) depended strongly on the stage of mitosis at which irradiation had been performed [10]. Irradiation of the centrosome at prophase and early prometaphase always blocked mitosis at metaphase; when performed at metaphase, the irradiation had no effect on the course of mitosis. In all cases no lesion was observed by electron microscopy [10].

To gain a better insight into the role of the centrosome in mitosis, we have undertaken a study of mitosis in PK (pig...
kidney embryo) cells using a UV microbeam. A UV microbeam was chosen for several reasons. First, a UVC microbeam reversibly severs microtubules in a living spindle [11], so we were able to compare the effect of centrosome damage with spindle destruction. Second, UV micro-irradiation gives rapid visible alterations of the living spindle, being much more convenient than laser microbeam irradiation. Third, a UV micro-irradiation device based on the high pressure mercury lamp is compact and more easily adjustable as compared with an argon laser micro-irradiation system.

In this paper we report the effect of centrosome damage induced by UV micro-irradiation at various stages of cell division. Our initial premise was that the centrosome is an essential part of the spindle at the beginning of cell division but becomes a passive "passenger" at the later stages. Therefore we made efforts to get direct evidence for the centrosome-dependent critical point(s) in mitosis. Some evidence for this supposition comes from a previous laser microbeam study [10]. To tackle the problem, we performed UV irradiation of the centrosome at various stages of mitosis and followed the fate of the cells. Observations of living cells were supplemented with immunofluorescent staining of microtubules and with ultrastructural study. Some similarities of our results to those of previous laser microbeam studies [6,9,10,12] as well as some striking differences in cell behaviour immediately after irradiation were revealed.

2. Materials and methods

2.1. Cell culture

PK cells were used. They were grown on 199 tissue culture medium (Biomedical Drugs and Chemicals Factory, Moscow) supplemented with 10% bovine serum (Medical Drugs and Chemicals Factory, Moscow) and antibiotics (penicillin and streptomycin, 100 units ml⁻¹ each). The cells were subcultured on to round quartz coverslips (d = 25 µm, 0.17 µm thickness) 48-72 h before use. For the experiments the coverslips were mounted in modified Dvorak-Stottler chambers, where the cells continued dividing for 6-8 h without change of the medium.

2.2. Microscope and micro-irradiation instruments

A modified transmission light microscope was used (Fig. 1). It was supplemented with a UV epi-illumination device. The quartz objective used for irradiation was a Zeiss Ultrafluar 100 X/1.25 (glycerol immersion). The incident light was collected from an HBO-100 lamp through a high numerical aperture (NA) quartz collector lens, passed through a UV wide bandpass filter, then through a UV bandpass interference filter (Aₚₛₚ = 280 nm, 50% transmission, bandwidth +10 nm) and focused by a quartz condenser lens in the rear focal plane of the objective. A field diaphragm was placed in the front focal plane of the condenser lens to cut a small area in the objective focal plane. To measure the diameter of the beam, a uranium slide was put on the microscope stage and the diameter of the fluorescent spot was measured either using a video camera or from a photograph. The diameter of the beam in the front focal plane of the objective lens was 1.6 µm.

The average power of the UV light directed on to the specimen in all experiments was approximately 0.04 erg µm⁻² s⁻¹ (as measured under the objective). The dose depended on the exposure time (see Section 3). Cells were observed using eyepieces with a graticule to focus the microbeam. After irradiation, cells were either observed with Plan-neofluar 40 X/0.9 phase-contrast objective lens (glycerol immersion) using a charge-coupled device (CCD) video camera (400 TV lines) or photographed using a mm single-lens reflex (SLR) camera.

2.3. Light microscopy: immunofluorescence

Cells were briefly rinsed in warm phosphate-buffered saline (PBS), then lysed on the coverslips with microtubule stabilizing buffer containing 50 mM imidazole (pH 6.8), 5 mM MgCl₂, 1 mM EGTA, 0.1 mM EDTA, 35% glycerol and 0.5% Nonidet P-40 (Sigma) and fixed with 0.5% glutaraldehyde in PBS for 30 min-2 h. The coverslips were rinsed in PBS and placed in 2% sodium borohydride for 20 min, then washed twice in PBS and incubated with mouse monoclonal anti-a-tubulin antibodies (clone DM-1A, Sigma) for 30 min at 37° C. The antibodies were rinsed off with three changes of PBS and the coverslips were incubated in secondary FITC-labeled rabbit-antimouse IgG (Sigma) for 30 min at 37° C. The stained specimens were mounted in Elvanol (Sigma) or Moviol (Calbiochem). The cells were examined in a Zeiss Photomicroscope 3 equipped for phase-contrast and epiflu
Table 1
Results of micro-irradiation of centrosome at early metaphase (exposure time 5s)

<table>
<thead>
<tr>
<th>Site of irradiation</th>
<th>Number of cells irradiated</th>
<th>Normal division</th>
<th>Delayed division</th>
<th>Scattering of chromosomes</th>
<th>Abnormal cytokinesis</th>
<th>Anaphase blockage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrosome</td>
<td>75</td>
<td>0</td>
<td>15</td>
<td>74</td>
<td>15</td>
<td>60</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>17</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chromosome</td>
<td>7</td>
<td>0</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Several alterations of mitosis were sometimes observed in one and the same cell after irradiation. Thus the sum of the last five numbers in each line can exceed the total number of irradiated cells.

Fluorescence optics using a 63 X/1.4 Planapochromate phase-contrast objective. Photomicrographs were taken on RF-3 film (1500 ASA).

2.4. Electron microscopy

Cells were fixed with 2.5% glutaraldehyde (Merck) on 0.1 M K-Na phosphate buffer (pH7.2) for 1-2 h, then rinsed in PBS, postfixed with osmium tetroxide, stained with uranyl acetate, dehydrated and embedded in Epon 812 mixture. Serial ultrathin (70 nm) sections were obtained parallel to the substrate plane using an LKB-III ultramicrotome (LKB, Sweden) and mounted on single-slot grids. Sections were examined and photographed in HU-11B and HU-12 electron microscopes (Hitachi, Japan) operating at 75 kV.

3. Results

Initial experiments were conducted to determine the minimal dose of irradiation of the pole sufficient for a prominent change in mitosis. We found that upon irradiation of the centrosome at metaphase the minimum time it took for mitotic blockade accompanied by gradual disassembly of the spindle to be established in more than 90% of the cells was 5 s (Table 1). After 15 s irradiation the effect was identical. However, 3 s irradiation of the pole blocked anaphase onset in roughly 50% of cells treated. It was concluded that 5 s irradiation (dosage 0.2 X 10^{-7} J) sufficed to damage the centrosome at metaphase.

Irradiation of the pole for 5 s (low dose) or even 15 s (high dose) at early anaphase did not stop mitotic progression but caused some changes in chromosome movement (see below). Irradiation for 30 s or more inflicted gross damage on the cell. In the subsequent experiments the anaphase cells were irradiated for 15 s as a maximum dose giving specific effects.

3.1. Control irradiations

Different regions of mitotic cells were irradiated at metaphase and anaphase to determine whether the effects of micro-irradiation of the pole were specific. Cells were irradiated in the cytoplasm outside the spindle and in the spindle area between chromosomes and one pole. Chromosomes were also irradiated.

Irradiation of the cytoplasm at metaphase and anaphase for as long as 30 s had little effect on mitotic progress. The spindle appeared normal and the chromosome plate was not displaced. In some instances anaphase onset and cytokinesis were retarded for 10-15 min (Table 1), but when started they ran normally. The two daughter cells were of the same shape and had equivalent nuclei and a normal microtubule system (data not shown).

Chromosomes are far more sensitive to UVC light than are cytoplasmic organselles [13]. Thus the most effective control of non-specific damage was irradiation of chromosomes in the metaphase plate. Indeed, irradiation of chromosomes for 15 s resulted in visible lesions; however, this only postponed anaphase onset but did not inhibit mitotic progression and cytokinesis (Table 1).

Irradiation of the spindle between chromosomes and the pole was described in detail elsewhere [11,14-16]. Our results are consistent with the previous data. Irradiation of this area locally destroyed the spindle for some minutes (data not shown) and anaphase progression slowed down. After recovery the cell behaviour returned to normal mode.

The filter set we used transmitted about 7% of UV light at wavelengths above 300 nm. To make sure that such UV irradiation of the centrosome was without effect, we repeatedly the experiment with cells grown on regular (glass) coverslips. The glass coverslips used transmitted more than 90% of light with A>310 nm (UVB and UVC) but were not transparent to light of wavelength below 295 nm. In these cells, mitosis was not affected by 1 min irradiation of the spindle pole.

3.2. Cell behaviour after irradiation of the centrosome at metaphase

During the first 10 min after irradiation the metaphase plate drifted towards the non-irradiated pole. Chromosomes near the spindle axis moved slower than those on the edges and after several minutes all chromosomes surrounded the non-irradiated pole in a semicircle or almost a complete circle. The average velocity (20 cells measured) of central chromosomes was 0.095 + 0.026 µm min^{-1}, while on the sides it was 0.73 + 0.14 µm min^{-1} at the beginning and declined...
Fig. 2. Chromosome shift after irradiation at metaphase: (a), before irradiation; (b), 10 min after irradiation; (c), 40 min after irradiation. Arrow indicates irradiated pole (bar, 5 µm).

to 0.27 ± 0.21 µm min⁻¹ (10 min after irradiation). On average the total shift of the metaphase plate 10 min after irradiation of the centrosome was 0.95 ± 0.26 µm in the centre and 5.38 ± 0.21 µm on the sides. Later on, different chromosomes came out of the plate and were randomly distributed in the central part of the cell (Fig. 2).

As mentioned above, initial experiments showed that the cell behaviour after micro-irradiation of the pole depended strongly on the moment irradiation had been performed. We constructed a time scale taking as zero point the visible dissolution of the nuclear envelope at the end of prophase. In PK cells the period between the beginning of prometaphase and anaphase onset lasted for 45 ± 3 min. Prometaphase continued for approximately 10 min and metaphase took the rest of the time (about 35 min). Irradiation was performed at various times after the beginning of prometaphase. Fifty cells were irradiated in the first 10 min of metaphase (with the low dose). All of them were blocked in metaphase and did not enter anaphase. Anaphase was observed in 15 out of 25 cells irradiated 35 min after prometaphase onset, while in the other 10 it was blocked. All 25 cells with the pole irradiated 40 min after the beginning of prometaphase (i.e. 5 min prior to normal anaphase onset) and all 50 cells irradiated in early anaphase divided into two daughter cells.

3.3. Behaviour of the spindle after centrosome irradiation at metaphase

Within 1 min after irradiation of the pole the half-spindle at the opposite pole shortened; 2-3 min after irradiation the half-spindle at the irradiated pole disorganized (Fig. 3); 5-8 min after irradiation the whole spindle was disrupted and several microtubule convergence centres appeared. The metaphase plate dissolved within 10-20 min after irradiation of the pole. At that time no spindle was visible. The number of microtubule convergence centres increased with time and ranged up to 10 or more. They were located on or very close to chromosomes. One hour after irradiation the overall picture did not change (Fig. 4). Sometimes the spindle partially rehabilitated — two microtubule convergence centres dominated over the others, but the metaphase plate was not re-established.

3.4. Cell behaviour after irradiation of the centrosome at anaphase

Micro-irradiation of the spindle pole was performed within 50-60 s after chromosome splitting. During this time chromosomes had separated by a distance of 1.96 +
Immediately after irradiation the chromosome movement towards the irradiated pole slowed down while that towards the opposite pole continued normally (Fig. 5). At telophase the distance between two groups of chromosomes was 30% less than in control cells (Fig. 5C).

Immediately after irradiation of the centrosome no difference between the two half-spindles was observed; however, within 3 min after irradiation a distinction became evident. On the site of the irradiated pole the spindle disassembled and only short bundles of kinetochore microtubules remained in a non-convergent array (Fig. 6). Within 8-15 min after irradiation numerous centres of microtubule convergence appeared on this site, while the opposite half-spindle was still arranged in normal fashion.

In cells with the irradiated pole, cytokinesis was postponed for 10-15 min. Sometimes lobes of the cytoplasm were separated near the cleavage furrow by additional constrictions (Table 2).

3.5. Fine structure of the centrosome after irradiation with low (5 s) and high (15 s) doses

Irradiation with the low dose leaves the centrosome unchanged within the first 5 min. The two centrioles remained perpendicular to each other. Numerous microtubules emanated from the mitotic halo. The irradiated pole looked exactly as the opposite one did (data not shown).

Irradiation with the high dose resulted in severing of spindle microtubules around the centrosome. With 1-3 min after irradiation, bundles of kinetochore microtubules terminated at a distance of 1.5-2 µm from the centrosome (Fig. 7, arrowheads). The distance between chromosomes and the irradiated pole exceeded that between chromosomes and the opposite pole (Fig. 7). Only a few short, randomly oriented microtubules remained around the centrioles (Fig. 7, inset). Sometimes centriole lesions were also observed — occasionally triplets of microtubules appeared destroyed (data not shown).

4. Discussion

In the micro-irradiation studies the mechanism of damage and the diameter of lesions depend on the wavelength of the light used, the intensity of the incident light and the diameter of the focused beam.
When using visible light (400-700 nm), one needs to employ extremely high power densities or to sensitize cells with dyes [9]. Using an argon laser, Berns and coworkers obtained certain effects on mitosis with a power density of $10^{12}$ W m$^{-2}$ ($10^7$ erg $\mu$m$^{-2}$ s$^{-1}$) in the focal plane of the objective lens. With acridine orange or psoralens they managed to slightly reduce the power used, but it still was very high [7,8].

UV light at wavelengths of 260-280 nm is strongly absorbed by cells and a power density of only $10^2$-$10^4$ Wm$^{-2}$ ($10^{-3}$-$10^{-1}$ erg $\mu$m$^{-2}$ s$^{-1}$) is sufficient to produce specific lesions [11,13]. The molecular targets for UVC irradiation are proteins and nucleic acids. Photochemical damage of proteins occurs via a variety of mechanisms as a result of high absorption by tryptophan and tyrosine at 280 nm [17]. This gives strong evidence for a local and direct effect of UVC micro-irradiation. Another line of evidence for determination of lesion area comes from disruption of micro-tubules. They are truncated by a UV microbeam, with a mercury lamp as light source, exactly in the irradiated region [11,18,19]. It is worth noting that a pulsed UV laser (266 nm) produces in interphase cells a lesion area much larger than the beam spot [20] and has little effect on mitotic progression even at higher power [2].

In 1965 Forer described the formation of an area of reduced birefringence (ARB) by UV micro-irradiation of the spindle [14]. Subsequently it was shown that formation of the ARB corresponds to local depolymerization of microtubules [19,21,22]. Recently Spurck et al., using a high pressure mercury lamp as UV light source, made it clear that the area of microtubule depolymerization is strictly identical with the irradiated spot [11]. Quantitative data provided by various authors show that microtubules are destroyed by irradiation doses of 0.2—0.6 erg $\mu$m$^{-2}$, which are achieved by 3 s [11] to 90 s [15] exposure. Microtubules in the irradiated area rehabilitate within a few minutes and the ARB disappears [11,18,23]. The action spectrum of ARB formation in living cells and that of microtubule truncation in vitro are nearly identical [19].

In our micro-irradiation experiments the beam was applied to the centrosome area, thus affecting the centrosome as well as microtubules (MTs) emanating from it. The influence of centrosome irradiation on mitosis observed in our experiments is much stronger than that described for irradiation of spindle MTs [16,18,19,23-25] and is irreversible when performed at metaphase. It is worth noting that the dose of irradiation required for centrosome damage was three times less than that necessary for truncating microtubules. This means that specific targets located in the centrosome essential for its function in mitosis are more sensitive to UV damage than are MTs.

4.1. Centrosome-dependent mitotic checkpoint is in mid-metaphase

The marked difference in cell behaviour following irradiation of the pole in early (first 10 min) and late (last 10 min) metaphase clearly demonstrates that during mid-metaphase a cell overcomes a certain checkpoint. At present it may be assumed that a centrosome-dependent mechanism essential for anaphase onset and subsequent cytokinesis occurs in PK cells in mid-metaphase.

Micro-irradiation of the mitotic pole with an argon laser revealed a centrosome-dependent checkpoint in PtK cells

Table 2

<table>
<thead>
<tr>
<th>Site of irradiation</th>
<th>Number of cells irradiated</th>
<th>Normal division</th>
<th>Altered chromosome separation</th>
<th>Delay of normal cytokinesis</th>
<th>Incomplete cytokinesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrosome</td>
<td>63</td>
<td>3</td>
<td>60</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>10</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Chromosome</td>
<td>20</td>
<td>2</td>
<td>12</td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

*Note:* Several alterations of mitosis were sometimes observed in one and the same cell after irradiation. Thus the sum of the last four numbers in each 1 can exceed the total number of irradiated cells.
Prometaphase or prometaphase-metaphase transition [9,10]. In PtK cells, prominent metaphase last for about 10 min, i.e. it is very short compared with metaphase in PK cells. Therefore, taken together, experiments on centrosome (mitotic pole) irradiation suggest that the centrosome is essential to mitosis until the last 10 min before chromosome separation. Following Picket-Heaps' [26] terminology, it may be inferred that 10 min prior to anaphase onset the centrosome becomes a "passenger" and plays no further part in mitotic progression.

4.2. UV micro-irradiation at anaphase results in asymmetric destruction of the spindle

As discussed above, irradiation of one pole at metaphase leads to a rapid shift of all chromosomes towards the opposite pole and a coordinated disassembly of the entire spindle. If the structure of the spindle is assumed to be the same at anaphase, then after irradiation of the pole at anaphase all chromosomes will be expected to shift and chromosomes moving towards the non-irradiated pole will speed up. Instead, no increase in the average speed of chromatids moving to the opposite pole was observed. Chromosomes continued moving to the irradiated pole but at reduced speed. This suggests that the two half-spindles at anaphase become independent of each other. Our data are contradictory to earlier observations [5,15] but consistent with the modern view on mechanisms guiding chromosome separation [27].

Comparing the data presented here with previous micro-irradiation experiments, two points should be emphasized.

(i) After laser irradiation of mitotic poles at anaphase no alterations in chromosome motions were described even when some pole structures were destroyed [7,12]. We are apt to explain this fact by only partial destruction of the essential centrosome structures by an extremely sharp microbeam about 0.25 μm in diameter [28].

(ii) In early experiments on UV irradiation of the pole at early telophase all chromosomes shifted towards the opposite pole and cytokinesis was abnormal [4]. Taking into account that the cells used were rather thick, the absorption of UV light by structures lying above the centrosome was high [13]. This result might thus be explained as non-specific damage of the whole cell.

Therefore we suppose that our instrumentation causes specific damage to the centrosome at mitosis. Our approach for studying cells lacking normal centrosomes offers a number of advances over other methods proposed by Karsenti et al. [29] and Maniotis and Schliwa [30]. For the method of Karsenti et al. [29] only cytoplasts without centrosomes were available. The cytoplasts had a short lifespan and could not be used for long-term experiments. Maniotis and Schliwa [30] could obtain only a restricted number of cultured cells.
since many of them did not survive after microsurgical dissection into two parts [30]. Our instrumentation may be used with any cultured cells that remain flat during mitosis, e.g. PtK2, newt lung epithelial cells, etc. Furthermore, in our method the sister cell with the normal centrosome serves as a natural control to the treated one.

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