Mitosis of rotenone-induced endoreduplication in Chinese hamster cells

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ABSTRACT

Endoreduplication was induced by rotenone with an extremely high frequency (approximately 90% of all the metaphases) in cultured Chinese hamster cells. Endoreduplicated cells were fixed without colchicine or hypotonic treatment, and chromosomal configurations were examined in various mitotic stages. The two sister chromosomes of each diplochromosome at late prophase were widely separated except the centromeric region, but they became gradually paired along the total length as the cell cycle progressed to metaphase. The anaphase cells underwent multipolar division, resulting in three or four aneuploid daughter cells. Indirect immunofluorescence staining using anti- β tubulin antibody revealed tripolar or tetrapolar spindles and unusual equatorial plates.

1. INTRODUCTION

Endoreduplication is a specific type of polyploidization that arises from endomitotic chromosome duplication (Levan and Hauschka, 1953). In endoreduplicated cells, diplochromosomes consisting of two paired homologous chromosomes appear at metaphase. In spite of many studies on the structure of diplochromosomes and on the mechanism of the induction of endoreduplication using a variety of tissue cultures in plants and animals (Levan and Hauschka, 1953; D'Amato, 1965; Sutou and Tokuyama, 1974; Sutou, 1981; Goyanes and Schvartzman, 1981), it is still unclear how endoreduplicated cells undergo mitosis. It has been generally thought that endoreduplicated cells produce two tetraploid daughter cells after normal bipolar division (Levan and Hauschka, 1953; Schwarzacher and Schnedl, 1965; Lafi and Parry, 1988). On the other hand, Schmid (1966) suggested that endoreduplicated cells underwent multipolar division.

The major difficulty in studying mitosis of endoreduplicated cells has been a low incidence of endoreduplication. We recently developed a method to induce endoreduplication at a remarkably high frequency (approximately 90% of all the metaphases) in Chinese hamster CHL cells by rotenone treatment (Matsumoto and Ohta, 1992). This makes it possible to observe chromosomal configurations effectively not only at metaphase but also at other mitotic phases. In the present study, we investigated the processes of endoreduplication mitosis and the formation of the spindle body in endoreduplicated cells.

2. MATERIALS AND METHODS

Cell line and chemicals

A pseudo-diploid clone with 24 chromosomes of the Chinese hamster CHL cell line was used. The cells were cultured in Eagle's minimum essential medium supplemented with 2.0 mM L-glutamine and 10% newborn bovine serum (Flow Laboratories) in an atmosphere of 5% CO₂ at 37°C. Under these conditions, cells were karyotypically stable and 86.9% of cells had 24 chromosomes. The frequency of spontaneous polyploidy was 1.2%. Doubling time of the cells was approximately 15 h. Rotenone (Aldrich) and the mitotic poison TN-16 (Wako) were dissolved in dimethyl sulfoxide. The final concentration of solvent in culture medium was 0.5%.

Selective induction of endoreduplication

The most sensitive period for the induction of endoreduplication by rotenone is mitotic metaphase (Matsumoto and Ohta, 1992). In order to arrest the cells in metaphase, TN-16 was used. In comparison with colcemid or colchicine, TN-16 has low cytotoxicity and it allows the cells immediately and synchronously to reform the mitotic spindle after removal of TN-16 from the culture medium (Hashimoto et al., 1972). Moreover, TN-16 itself never induces any endoreduplications in CHL cells (Matsumoto and Ohta, 1992). Cells were seeded in 100-mm dishes and grown to 80–90% confluence. Then, they were treated with TN-16 at a final concentration of 1.5 µg/ml for 1 h. The medium was removed, and the cells were quickly rinsed twice with phosphate-buffered saline (PBS) and recovered for 20 min in a fresh medium. Rotenone was added at a final concentration of 10 µg/ml to the cells, which were reforming a normal mitotic apparatus. Thirty minutes after rotenone addition, metaphase cells were selectively detached from the monolayer by gentle pipetting. A small portion of the cell suspension was sampled and immediately fixed with Carnoy's solution (methanol:acetic acid=3:1) for chromosome preparations. It was microscopically confirmed that over 99.5% of the cells in these preparations were at metaphase. The remaining cell suspensions containing rotenone were incubated 2.5 h more. They were then collected by centrifugation, washed twice with PBS to remove residual rotenone, and recultured on 24×24-mm coverslips or 35-mm dishes in a fresh medium. The coverslips were taken out 24 h after recultivation and divided into two groups. One group was fixed with Carnoy's solution, air-dried, and stained with 4% Giemsa solution in 1/15 M phosphate buffer (pH=6.8). The other group was fixed for 1 min with 3% formaldehyde diluted in PBS and permeabilized with cold methanol (−20°C) for 5 min for immunofluorescence examination. The cells cultured in 35-mm dishes were harvested for chromosome preparations at 24, 48, and 72 h after recultivation. The number of chromosomes was counted in the 48- and 72-h preparations.

Indirect immunofluorescence

Anti-tubulin indirect immunofluorescence was carried out according to the method of Sakakibara et al. (1991) with minor modification. The coverslip preparations fixed with formaldehyde and methanol were treated with 1% Triton X-100 in PBS for 20 min and incubated with 1% bovine serum albumin in PBS for 30 min at room temperature. They were briefly washed in PBS and overlaid with 0.5 ml each of mouse monoclonal anti- β tubulin antibody (Amersham; diluted 1:250 in PBS). They were then incubated in a moist chamber for 45 min at 37°C and washed in PBS for 30 min. The preparations were overlaid with 0.5 ml each of FITC-conjugated sheep anti-mouse IgG₁ antibody (Binding Site; diluted 1:20 in PBS) and incubated in a moist chamber for 45 min at 37°C. Following a 30-min rinse in PBS, the wet coverslips were mounted on glass slides in a glycerol:PBS (9:1) solution containing 10 mg/ml 1,4-diazabicyclo[2,2,2]octane to retard quenching. Cells were observed and photographed using a Nikon BIOPHOTO fluorescence microscope.

Chromosome preparations

With the exception of cells obtained by mitotic pipetting, cultures intended for chromosome preparation were treated with colchicine at a final concentration of 0.5 μ g/ml for 2 h prior to harvesting. Cells were harvested by trypsinization and exposed to a hypotonic solution (0.075 M KCl) for 5 min. They were then fixed with Carnoy's solution and air-dried under a humidified atmosphere to prevent the chromosomes from scattering. Chromosome preparations were stained with 2% Giemsa solution.

3. RESULTS

Mitosis of endoreduplicated cells

Figure 1 shows the air-dried preparation made 24 h after the end of rotenone treatment. The frequency of endoreduplicated cells with diplochromosomes among 200 metaphases observed was 86%, and that of diploid and ordinary tetraploid cells were 1% and 13%, respectively. These results indicated that endoreduplications were preferentially induced by rotenone. The endoreduplicated cells in various mitotic stages were thus examined by direct fixation without colchicine or hypotonic treatment.

In early prophase cells, diplochromosomes could not be distinguished because they are highly stretched and entangled one another (Figure not shown). In late prophase cells, 46–50 chromosomes could be observed and they were in pairs, not distributed at random (Fig. 2A), indicating that these late prophases were of endoreduplicated cells. The sister chromosomes of diplochromosomes were widely separated along the total length of the chromosome, but the centromeric

Fig. 1. Endoreduplicated metaphases of CHL cells in air-dried preparations made 24 h after rotenone treatment. All chromosomes are diplochromosomes. Bar represents 20 μm .

regions were close to each other especially in long sized chromosomes. The sister chromatids of each sister chromosome, in contrast, were tightly paired along the total length, as are those of ordinary prophase chromosomes, so that individual chromatids could not be distinguished. The distance between sister chromosomes gradually shorten during late prophase to prometaphase (Fig. 2B), and the chromosomes finally paired along their length at metaphase (Fig. 2C). Individual chromatids of diplochromosomes could not be discriminated during these periods. In late metaphase cells, however, each diplochromosome was clearly seen to be composed of four sister chromatids (Fig. 2D).

In anaphase and telophase cells, multipolar divisions were observed with very high frequency. As shown in Table 1, 93% each of anaphase and telophase cells exhibited multipolar division, and the remaining 7% showed normal bipolar division in these phases. Multipolar dividing cells were either tripolar or tetrapolar with roughly equal frequencies. A notable feature of multipolar anaphase cells was an uneven distribution of chromosomes, suggesting the frequent occurrence of chromosomal nondisjunction (Fig. 2E). In fact, lagging chromosomes or chromosome bridges were often observed in multipolar anaphase cells (Table 1; Fig. 2E, arrow). Also, most multipolar telophase cells had daughter nuclei with

Fig. 2. Various mitotic stages of endoreduplicated CHL cells. The cells were grown on coverslips and fixed without colchicine or hypotonic treatment. (A) Late prophase, The sister chromosomes of each diplochromosome are situated a short distance from each other. The centromeres have already divided into two daughter centromeres. (B) Prometaphase, The two sister chromosomes are coming together. (C) Metaphase, Sister chromosomes are completely paired. (D) Late metaphase, All the sister chromosomes constituting the diplochromosomes divide into two sister chromatids, and it first becomes apparent that diplochromosomes consist of four sister chromatids. (E) Anaphase, A tripolar division with a lagging chromosome (arrow). The chromosomes are unevenly divided among three daughter cells. (F) Telophase, A tetrapolar division with two micronuclei (arrows). The four daughter nuclei clearly differ in size. Bar represents 10 μ m.

Table 1. Frequencies of multipolar divisions in anaphase and telophase cells at 24 h after the induction of endoreduplication by rotenone

Stage	No. of cells analyzed	Multipolar division		
		Tripolar	Tetrapolar	Total
Anaphase	100	48 (7)	45 (8)	93 (15)
Telophase	100	40 (11)	53 (18)	93 (29)

Numerals in parentheses represent the number of cells with lagging chromosomes at anaphase or micronuclei at telophase.

uneven diameter, which was consistent with the anaphase observations, and approximately 30% of them contained micronuclei (Table 1; Fig. 2F, arrows).

Indirect immunofluorescence

The cells similarly prepared to those shown in Figure 2 were stained by means of an indirect immunofluorescence technique using anti- β tubulin antibody. When 200 mitotic cells were analyzed, 91% of them revealed tripolar or tetrapolar spindles (Fig. 3A). No mitotic cells having five or more spindle poles were observed. In metaphase cells with multipolar spindles, the chromosomes (seen here as darkly stained material) were disposed on unusual multiple equatorial plates (Fig. 3B). Three equatorial plates were apparent in the tripolar spindles, and four equatorial plates in the tetrapolar spindles.

Fig. 3. Endoreduplicated mitoses stained by indirect immunofluorescence using anti- β tubulin antibody. (A) Most mitoses exhibit tripolar or tetrapolar spindles. (B) A mitotic cell with tripolar spindle. Chromosomes (darkly stained material) are seen on three equatorial plates forming between three spindle poles. Bar represents 10 μ m.

Aneuploid cells after endoreduplication

The frequencies of diploid, aneuploid and tetraploid cells in an untreated CHL culture were 86.9%, 11.9%, and 1.2%, respectively. At 48 h after rotenone treatment, however, 91.5% of the mitoses analyzed were of aneuploid cells. Endoreduplicated mitoses were not observed. The number of chromosomes per cell was broadly distributed from 21 to over 61 (Fig. 4A). The distribution was also broad 72 h after treatment, but two peaks, one near diploidy and one near tetraploidy, became apparent (Fig. 4B).

Fig. 4. Distribution of chromosome numbers in the CHL cells 48 h (A) or 72 h (B) after the end of the induction of endoreduplication by rotenone treatment. At least 150 metaphases were analyzed in each time.

4. DISCUSSION

The present results showed that the two sister chromosomes of a diplochromosome were loosely associated with some distance between them at late prophase. The typical configuration of diplochromosomes as two sister chromosomes closely paired along the total length did not appear until metaphase. It has been suggested in an electron microscopic study of whole-mounted diplochromosomes that the two sister chromosomes constituting a diplochromosome are physically joined together by chromatin fiber loops connecting the two neighboring chromatids of sister chromosomes (Goyanes and Schwartzman, 1981). If such fiber loops actually exist, it is considered that they are shortened during prophase to metaphase as the chromosome condensation proceeds.

It has been generally considered that a mitotic cell with diplochromosomes becomes two tetraploid daughter cells by a normal mitosis (Schwarzacher and Schnedl, 1965; Levan and Hauschka, 1953), and therefore, endoreduplication has been regarded as one of the important mechanisms of chromosomal polyploidization in polysomatic meristems (D'Amato, 1952) or in malignant tumor cells (Levan and Hauschka, 1953). However, our results indicated that endoreduplication was responsible for not only polyploidization but also aneuploid formation through

misdivisions. Since multipolar spindles were observed in about 90% of mitoses of the cell population in which about 90% of mitoses were of endoreduplicated cells, it is most probable that endoreduplicated cells are fated to form multipolar spindles and finally induce multipolar divisions. Similar interpretation was reported by Schmid (1966) from spontaneously occurring endoreduplication in primary kidney cultures of the European hamster.

In the endoreduplicated cell population, most cells complete endoreduplicated mitosis during 18 to 42 h after rotenone treatment (Matsumoto and Ohta, 1992). The result that aneuploid cells increased remarkably at 48 h after rotenone treatment (Fig. 4A) suggests that the endoreduplicated cell population shifted to the aneuploid cell population by multipolar division. The endoreduplicated mitoses with tripolar spindles must have yielded the aneuploid cells. Moreover, the fact that unequal chromosomal divisions often occurred in the anaphase cells with tetrapolar as well as tripolar spindles suggests that endoreduplicated mitoses with tetrapolar spindles also give rise to aneuploid cells. At 72 h after treatment, the percentage of aneuploid cells except near diploid and near tetraploid cells was decreased (Fig. 4B), suggesting the majority of the aneuploid cells were selected out.

It is of interest how the four chromatids constituting a diplochromosome are divided within a tripolar or tetrapolar spindle body. It has been demonstrated that the two sister chromosomes of an intact diplochromosome at metaphase do not lie in a plane, but overlap or orientate parallel in a three dimensional configuration (Takanari, 1985). This finding suggests that the kinetochores on the two inner chromatids of the diplochromosome, as well as those on the two outer ones, can actually connect with spindle fibers. Therefore, the diplochromosomes that are located in the center of multiple equatorial plates are probably divided into three or four parts separating between sister chromatids. At that time, some spindle fibers extending from the different poles would attach to one kinetochore. That is considered to be the reason why a number of lagging chromosomes and micronuclei were induced in endoreduplicated anaphase and telophase cells. On the other hand, the diplochromosomes that are located on the edge of the multiple equatorial plates may be divided into two parts separating between sister chromatids, not between sister chromosomes.

The primary cause of multipolar spindle formation is considered to be abnormal reduplication of the centrioles, though we did not observe them in this study. The cell cycle of rotenone-induced endoreduplication is supposed to be as follows (Matsumoto and Ohta, 1992): (1) rotenone affects cells at metaphase; (2) metaphase cells rapidly enter interphase (endoreduplication G_1 phase), skipping anaphase and telophase; (3) these interphase cells replicate their chromosomal DNA once again in a second S phase (S_2); (4) diplochromosomes appear in the next metaphase. The two sets of centrioles, which existed in metaphase at the time of rotenone treatment, necessarily stay together within one cell. Since these cen-

trioles would duplicate during S₂ phase, four sets of centrioles would appear and resulted in formation of a tetrapolar spindle. The frequent formation of tripolar spindles suggested that one of the two sets of centrioles failed to duplicate by unknown mechanism. Further studies on the structure of the multipolar spindle will provide additional insight into the mechanisms of mitotic spindle formation.

In conclusion, we propose that rotenone-induced endoreduplication will form multipolar spindles in mitosis, undergo multipolar division accompanied with nondisjunction or lagging chromosomes, and then give rise to aneuploid cells. Subsequently, most of aneuploid cells will be selected out. Endoreduplication induced chemically, physically, or occurred spontaneously has always thought to undergo two rounds of DNA synthesis (Schwarzacher and Schnedl, 1966; Rizzoni and Palitti, 1973; Caligo and Rainaldi, 1987), even though the mechanisms of induction are different. Since centrioles should also undergo two rounds of duplication during these two rounds of DNA synthesis, it is considered that multipolar division is a general characteristic phenomenon in endoreduplication mitosis.

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