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ICRF-193

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Abnormal chromosome migration and chromosome aberrations in mouse oocytes during meiosis II in the presence of topoisomerase II inhibitor ICRF-193

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Abstract

Using a mouse parthenogenetic system, effects of ICRF-193, a noncleavable complex-forming topoisomerase II inhibitor, on female meiosis II chromosomes and pronuclear chromosomes were studied. Eggs were exposed to the inhibitor (10 μ M) at various times after parthenogenetic stimulation, and chromosomes of them were analyzed at the first cleavage metaphase. When eggs were exposed to the inhibitor during the period from metaphase II to anaphase II, a significant increase in incidences of structural chromosome aberrations (51.1% vs. 1.3% in the control) and aneuploidy (30.3% vs. 0.7% in the control) was found. Structural chromosome aberrations were observed in 10-20% of eggs following treatments during telophase II, but there was no increased incidence of aneuploidy in treatments during this meiotic stage. When pronuclear eggs at S phase were targeted by the inhibitor, no significant increase in chromosome aberrations was found.

Interestingly, when chromatids moved to each pole during anaphase II in the presence of ICRF-193, most of them oriented their centromeres toward the spindle equator as if moving backwards. Moreover, lagging chromatids with the centromeres present were observed in more than 50% of treated eggs. However, chromosomal bridges that resulted from chromosome stickiness did not appear in any egg.

These findings indicate that ICRF-193 can induce structural chromosome aberrations and aneuploidy in mouse secondary oocytes in meiotic stage-dependent manner. The induction of aneuploidy is due to disruption of the separation of sister centromeres at anaphase II. There appears to be mechanism(s) other than cleavable complex formation or chromosome stickiness behind the induction of structural chromosome aberrations by ICRF-193.

Key words: chromosome aberrations, topoisomerase II inhibitor, ICRF-193, mouse oocytes,
meiosis II

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1. Introduction

Topoisomerase II is a ubiquitous nuclear enzyme that is involved in chromosome condensation and chromosome segregation during mitosis and meiosis [1, 2]. Therefore, 5 topoisomerase II interactive agents have potential clastogenic and aneugenic actions on meiotic cells. Etoposide, a specific topoisomerase II inhibitor, caused both structural chromosome aberrations and aneuploidy in primary oocytes of the mouse [3, 4] and the Chinese hamster [5]. Similar chromosomal effects of the inhibitor were found in mouse 10 primary spermatocytes [6, 7]. Another topoisomerase II inhibitor, merbarone, caused aneuploidy in mouse primary spermatocytes without formation of structural chromosome aberrations [8]. Studies also revealed that the sensitivity of these meiotic cells to 15 topoisomerase II inhibitors was stage-dependent. Oocytes from late dictyate to diakinesis [5], and spermatocytes from pachytene to diakinesis [6-8] were highly sensitive.

On the other hand, it is premature to conclude that inhibition of topoisomerase II 15 activity during meiosis II can induce chromosome aberrations. Kallio and Lähdetie [6] found a significant increase of micronuclei in mouse spermatids following treatment with etoposide during meiosis II. However, meiosis II chromosomes were much less sensitive to the inhibitor than meiosis I chromosomes. Subsequently, the investigators failed to find a significant increase of spermatid micronuclei after treatment with merbarone [8]. Mailhes et 20 al. [4] found in the mouse that incidence of chromosome aberrations was higher in 1-cell zygotes than in MII oocytes when etoposide was administered during preovulatory stage. The investigators have suggested that the inhibitor may cause chromosome aberrations during female meiosis II. However, no attempt has been made to obtain direct evidence that 25 topoisomerase II inhibitors can induce chromosome aberrations in mammalian secondary oocytes.

In this study, we examined clastogenic and aneugenic effects of a noncleavable complex-forming topoisomerase II inhibitor, ICRF-193, on mouse oocytes during meiosis II in order to evaluate the heritable risk of topoisomerase II inhibitors and to understand the mechanism of chromosome aberration formation. Our interest further extended to the chromosomal sensitivity of pronuclear eggs. For these purposes, we utilized a mouse parthenogenetic system since mouse oocytes are easily activated by physical and chemical stimulation, and parthenogenetically activated eggs normally complete the second meiotic division and develop beyond the first cleavage mitosis [9].

2. Materials and methods

2.1. Topoisomerase II inhibitor

ICRF-193, *meso*-4,4'-(2,3-butanediyl)bis(2,6-piperazinedione), was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). It was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, divided into 5 μ l aliquots and stored at -20°C until use. Approximately 2 h before use, the inhibitor was diluted with an appropriate medium to a final concentration of 10 μM . The final concentration of DMSO was 0.1%.

2.2. Media

CZB medium supplemented with 5.56 mM D-glucose [10] was used for culturing parthenogenetic mouse eggs. Oocytes were collected in Heps-buffered CZB containing 20 mM Heps-HCl, 5 mM NaHCO_3 , and 0.1 mg/ml polyvinyl alcohol in lieu of bovine serum

albumin (BSA). The pH was adjusted to approximately 7.4 using 1 N HCl. The CZB was used under 5% CO₂ in air, and Hepes-buffered CZB was used under 100% air.

2.3. *Animals*

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B6D2F1 hybrid female mice 7-12 weeks of age were used in this study. They were maintained under a 14 h-light/10 h-dark photoperiod at a temperature of 23 ± 2 °C and humidity of 40-60%. Food and water were given *ad libitum*.

10 2.4. *Parthenogenetic activation of oocytes*

Females were induced to superovulate by an intraperitoneal injection of 8 iu pregnant mare's serum gonadotropin (PMSG) followed 48 h later with an injection of 8 iu human chorionic gonadotropin (hCG). Oocytes at metaphase II were collected from oviducts between 15 h and 16 h after hCG injection and were treated with 0.1% hyaluronidase for 3-5 min in Hepes-buffered CZB to remove cumulus cells. Strontium chloride (SrCl₂) was used to induce parthenogenetic activation [11] because it does not affect chromosome segregation in mouse oocytes during meiosis II [12]. Cumulus-free oocytes were incubated in 5 mM SrCl₂ in Ca-free CZB for 2 h. The activated eggs were then transferred to CZB for further cultivation.

2.5. *Progression of meiosis II and timing of DNA synthesis in recently activated eggs*

Prior to treatment with ICRF-193, the time-course progression of meiosis II and the timing of DNA synthesis in recently activated eggs were determined. As mentioned above,

oocytes were collected from 6 females and parthenogenetically activated. At 0.5 h, 1.0 h, 2.0 h, 4.0 h and 6.0 h after parthenogenetic stimulation, 30-40 eggs were taken from the culture and treated with 0.5% protease (Kaken Pharmaceuticals, Tokyo, Japan) for 10 min at room temperature (25-26 °C) to remove the zona pellucida. They were fixed and placed on
5 microscopic glass slides by gradual-fixation/air-drying method [13]. The slides were stained with 2% Giemsa (Merck, Darmstadt, Germany) in phosphate buffered solution (pH 6.8). To determine S phase, eggs were labelled with 10 µM bromodeoxyuridine (BrdU) for the periods between 4 h and 6 h, and between 6 h and 8 h after parthenogenetic stimulation. As described by Aoki and Schultz [14], eggs were washed with PBS containing 0.3% BSA
10 (PBS/BSA), and fully fixed with 3.7% formaldehyde. After washing with PBS/BSA, the DNA was denatured by incubating the eggs in 2N HCl for 1 h at 37 °C. The eggs were then neutralized with borate buffer (pH 8.6) for 15 min. To detect incorporation of BrdU, the eggs were incubated with mouse anti-BrdU monoclonal antibody (Roche, Mannheim, Germany) for 45 min at 37 °C. The eggs were then incubated with fluorescein-conjugated
15 anti-mouse IgG (Roche) for 45 min at 37 °C. They were then mounted on poly -L-lysine coated-glass slides, and covered with Vectashield mounting medium (Vector, CA) for fluorescent microscopy.

2.6. Treatment with ICRF-193

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To investigate the effects of ICRF-193 on eggs at various stages from metaphase II through S phase, eggs were treated by incubating them in medium supplemented with 10 µM ICRF-193 at predetermined periods after parthenogenetic stimulation. Time and duration of the treatment were determined as mentioned earlier (see Table 1). Eggs exposed
25 to 0.1% DMSO alone served as the control for the solvent.

2.7. Chromosome preparation and analysis

Eight hours after parthenogenetic stimulation, eggs were transferred to CZB
5 containing 0.008 $\mu\text{g/ml}$ vinblastine and cultured until they reached the first cleavage
metaphase. Between 18 h and 20 h post-stimulation, metaphase eggs were treated with
0.5% protease for 5-6 min at room temperature to loosen the zona pellucida. They were
then exposed to a hypotonic solution (1:1 mixture of 1% sodium citrate and 30% fetal
bovine serum) for 10 min at room temperature. Chromosome preparations were made by
10 the gradual-fixation/air-drying method [13]. The chromosome spreads were stained with
2% Giemsa for 8 min for conventional chromosome analysis. Subsequently, chromosomes
were C-banded to identify the centromeres according to Sumner [15] with some
modifications. In brief, the slides were heated at 50 °C for 2-3 h to harden the eggs. They
were then treated with 0.2N HCl for 30 min at room temperature, 5% Ba(OH)₂ for 3-4 min
15 at 45 °C, and 2×SSC for 5 min at 45 °C, followed by staining with 4% Giemsa for 10 min.

2.8. Statistical analysis

Fisher's exact probability test was used to compare results between control and
20 experimental groups. Differences at $p < 0.05$ were considered significant.

3. Results

3.1. Meiosis II and DNA synthesis in recently activated eggs

At 0.5 h post-stimulation, a total of 35 eggs were examined: 63% were at anaphase II, 31% were at telophase II, and the rest were at metaphase II. At 1.0 h post-stimulation, 97% of 36 eggs were at telophase II, and the rest was at anaphase II. At 2.0 h post-stimulation, all of 35 eggs had a second polar body. They were still at telophase II because their
5 chromosomes remained condensed. At 4.0 h post-stimulation, 59% of 34 eggs displayed telophase II figures with condensed chromatin, and 41% had an early pronucleus. At 6.0 h post-stimulation, 94% of 32 eggs had a well-developed pronucleus, and the rest still had condensed chromatin. Incorporation of BrdU was found in 28% of 51 pronuclear eggs labelled between 4.0 h and 6.0 h post-stimulation, and in all of 61 pronuclear eggs labelled
10 between 6.0 h and 8.0 h post-stimulation. From these results, ICRF-193 treatment periods were determined to correspond with meiosis II stages and the timing of pronuclear formation and DNA synthesis (Table 1).

3.2. *Chromosome analysis*

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In all experimental groups, more than 97% of oocytes successfully underwent parthenogenetic activation, and almost all (93-99%) of the activated eggs were able to proceed to the first mitotic metaphase. The findings indicate that ICRF-193 at a concentration of 10 μ M does not prevent activation, second polar body formation
20 (cytokinesis), chromosome decondensation, pronuclear formation, DNA synthesis and chromosome condensation in mouse eggs.

Eggs exposed to DMSO alone, whether during incubation for parthenogenetic activation or after, showed similarly low incidences of chromosome aberrations. Hence, data from both groups were combined as the non-treated control (Table 2). While the
25 inhibitor caused structural chromosome aberrations in eggs at any stage in meiosis II, a

significant increase of aneuploid eggs occurred when ICRF-193 treatment was conducted between 0 h and 0.5 h (metaphase II - anaphase II). The aneuploid eggs consisted of nearly equal number of hyperhaploids (n=32) and hypohaploids (n=38). The highest incidence of structural chromosome aberrations was also found in this treatment period. The treatments
5 between 0.5 h and 1.0 h (anaphase II - telophase II), between 1.0 h and 2.0 h (telophase II when cytokinesis takes place), and between 2.0 h and 4.0 h (telophase II when chromosomes decondense) were also fairly effective in inducing structural chromosome aberrations. However, when the treatment was done from 4.0 h to 6.0 h (telophase II – G₁ phase when pronucleus is formed), the aberrations were considerably reduced. No significant increase
10 was observed during treatment between 6.0 h and 8.0 h (S phase).

ICRF-193 predominantly caused chromosome-type structural aberrations, with breaks and fragments occurring most frequently. Frequencies of interchromosomal exchanges such as translocation and formation of dicentric chromosomes, as well as intrachromosomal exchanges such as ring formation were also relatively high (Figure 1).
15 While etoposide frequently induces centromeric breaks in mouse primary oocytes [3] and mouse primary spermatocytes [6, 7], these were not predominantly shown in this study using ICRF-193.

To observe chromosomal behavior during anaphase II/telophase II in the presence of ICRF-193, cytological preparations of oocytes during these stages (approximately 0.5 h
20 post-stimulation) were made by the gradual-fixation/air-drying method [13]. In this procedure, hypotonic treatment was omitted to avoid chromosome scattering. A total of 225 and 162 anaphase II/telophase II figures were successfully analyzed in treated and control eggs, respectively. Control eggs always showed normal anaphase II figures in which chromatids moved to each pole with their centromeres leading ahead (Figure 2a).
25 Interestingly, however, treated eggs displayed abnormal anaphase II figures in which most

of the chromatids oriented their centromeres toward the spindle equator (Figure 2b). Furthermore, lagging chromatids were also observed at the spindle equator in 55.1% of anaphase II/telophase II figures. All of the lagging chromatids were C-band positive, indicating that they have the centromere. No recognizable chromosomal bridges appeared in
5 the anaphase II/telophase II chromosomes.

4. Discussion

The present results demonstrated that inhibition of topoisomerase II activity by
10 ICRF-193 during meiosis II is responsible for induction of structural chromosome aberrations and aneuploidy in mouse eggs. In addition, it was found that sensitivity to the inhibitor was stage-dependent, as the cell stage during ICRF-193 treatment clearly determined the incidence of aberrant eggs (Table 2 and Figure 3).

Mechanisms behind the chromosome aberrations caused by topoisomerase II
15 inhibitors have been proposed in studies with somatic cells. It is generally accepted that the induction of numerical aberrations is due to incomplete separation of sister chromatids at the onset of anaphase by inhibition of topoisomerase II decatenation activity [16-24]. In our anaphase II/telophase II assay of treated eggs, lagging chromatids were frequently observed at the spindle equator (Figure 2b). Since ICRF-193 does not interact with spindle fibers [21],
20 it seems likely that lagging chromatids are generated when the inhibitor disrupts the separation of sister centromeres at anaphase II, thus resulting to aneuploidy.

There is considerable mystery in connection with mechanism(s) behind the formation of structural chromosome aberrations by ICRF-193. It is believed that the clastogenic property of topoisomerase II inhibitors depends on whether they are capable of
25 forming cleavable complexes, since stabilized cleavable complexes cause structural

chromosome aberrations. For example, complex-forming agents including amsacrine, doxorubicin, daunorubicin, etoposide, mitoxantrone and teniposide have been demonstrated to cause structural chromosome aberrations in various types of somatic cells in S-independent manner [24-26]. Undoubtedly, etoposide can cause structural chromosome
5 aberrations in mammalian meiotic cells [3-7]. In contrast, a noncomplex-forming inhibitor, such as merbarone, does not generate structural chromosome aberrations in mouse spermatocytes [8] and DNA breaks in mouse leukemia cells [27]. Like merbarone, ICRF-193 can inhibit topoisomerase II activity without forming a cleavable complex [28, 29]. It has no ability to generate DNA strand breaks [19]. However, a significant increase in
10 structural chromosome aberrations has been found in Chinese hamster V79 cells exposed to ICRF-193 from late S phase to G₂ phase [30]. The present results also demonstrated that the inhibitor had clastogenic effects on mouse oocytes during meiosis II. Hence, ICRF-193 can probably cause chromosome breaks through mechanisms other than the stabilization of cleavable complexes.

15 Interestingly, Gaulden [31] has proposed 'chromosome stickiness' as a possible cause of mutagen-induced structural chromosome aberrations without DNA interaction. She hypothesized that some deleterious changes in topoisomerase II and chromosomal peripheral proteins are responsible for the stickiness. The mechanical stresses imposed on such catenated chromosomes by mitotic spindle force can cause structural chromosome
20 aberrations during anaphase. In mammalian somatic cells, massive chromosome bridges were found during anaphase following treatment with etoposide [16, 24] and ICRF-193 [19]. In the present results, however, no recognizable chromosomal bridges appeared in the anaphase II/telophase II chromosomes following ICRF-193 treatment. Therefore, it is unlikely that ICRF-193 causes chromosome breaks in mouse secondary oocytes through
25 chromosome stickiness.

We found that treatment with ICRF-193 from metaphase II through anaphase II was most effective in inducing structural chromosome aberrations, and many of the induced aberrations were of chromosome-type (Table 2). This suggests that ICRF-193-induced some harmful changes in topoisomerase II can be converted into double-strand breaks in chromosome DNA. It is well known that topoisomerase II plays a role as scaffold protein of metaphase chromosomes, and chromatin fiber is associated with the chromosome scaffold [2]. It seems likely that ICRF-193 affects the chromosome scaffold and makes chromatin fiber unstable, thus resulting in formation of double-strand breaks when chromosomes (chromatids) decondense and form pronuclei. Further studies are required for a better understanding of the mechanism(s) behind formation of ICRF-193-induced structural chromosome aberrations during mammalian female meiosis II.

The most interesting phenomenon found in the present study is that when chromatids moved toward each pole in the presence of ICRF-193, the terminal region (telomere) of each chromatid behaved as if it were the centromere (Figure 2b). How do chromatids move toward the poles? Is the connection of spindle microtubules to the kinetochores at centromere region normally maintained? These questions remain to be tackled.

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Legends

Figure 1

Interchromosomal and intrachromosomal exchanges found at the first cleavage
5 metaphase of mouse parthenogenetic eggs treated with ICRF-193 during meiosis II.
(a) Two dicentric chromosomes (fine arrows) and a translocation (thick arrow).
Arrowheads indicate derivative acentric fragments. (b) A ring chromosome (arrow). Bar,
10 μm .

10 Figure 2

Anaphase II figures of recently activated eggs.
(a) Normal anaphase II figure of a control egg. Chromatids normally move to each pole
with the centromere leading ahead, as shown positively stained by C-banding. (b)
Abnormal anaphase II figure in the presence of ICRF-193. Most of the chromatids
15 oriented their centromere toward the spindle equator as if moving backwards. There are
two lagging chromatids in the middle zone (arrows). Bar, 10 μm .

Figure 3

Occurrence of eggs with structural chromosome aberrations and aneuploidy following
20 treatment with ICRF-193 at various times during meiosis II and pronuclear stage.

Table 1 Cell stage of mouse parthenogenetic eggs during a period of treatment with ICRF-193

Period (h)	Cell stage (major cytological event)
0.0-0.5	metaphase II – anaphase II
0.5-1.0	anaphase II – telophase II
1.0-2.0	telophase II (cytokinesis: extrusion of a polar body)
2.0-4.0	telophase II (chromosome decondensation)
4.0-6.0	telophase II – G ₁ phase (pronuclear formation)
6.0-8.0	S phase

Table 2

Chromosome analysis at the first cleavage metaphase of mouse parthenogenetic eggs treated with ICRF-193 at different times during meiosis II and pronuclear stage

Period (h) of treatment with ICRF-193 after stimulation	No. of animals used	No. of eggs analyzed	No. (%) of eggs with aneuploidy	No. (%) of eggs with structural chromosome aberrations	No. of structural chromosome aberrations						
					chromosome-type					chromatid-type	
					break / fragment	dicentric	trans-location	ring	deletion	break / fragment	exchange
non-treatment (control)	11	452	3 (0.7)	6 (1.3)	2	0	0	0	0	2	0
0.0-0.5	6	231	70 (30.3) ^a	118 (51.1) ^a	95	32	9	21	1	29	1
0.5-1.0	8	230	5 (2.2)	44 (19.1) ^a	21	16	4	7	1	2	0
1.0-2.0	6	206	3 (1.5)	21 (10.2) ^a	10	7	2	2	1	1	0
2.0-4.0	7	221	0	40 (18.1) ^a	30	10	0	1	0	4	1
4.0-6.0	5	206	4 (1.9)	11 (5.3) ^b	8	0	0	0	0	2	1
6.0-8.0	6	223	1 (0.4)	6 (2.7)	3	0	0	0	0	2	1

^a $P < 0.001$

^b $P < 0.01$

Figure 1

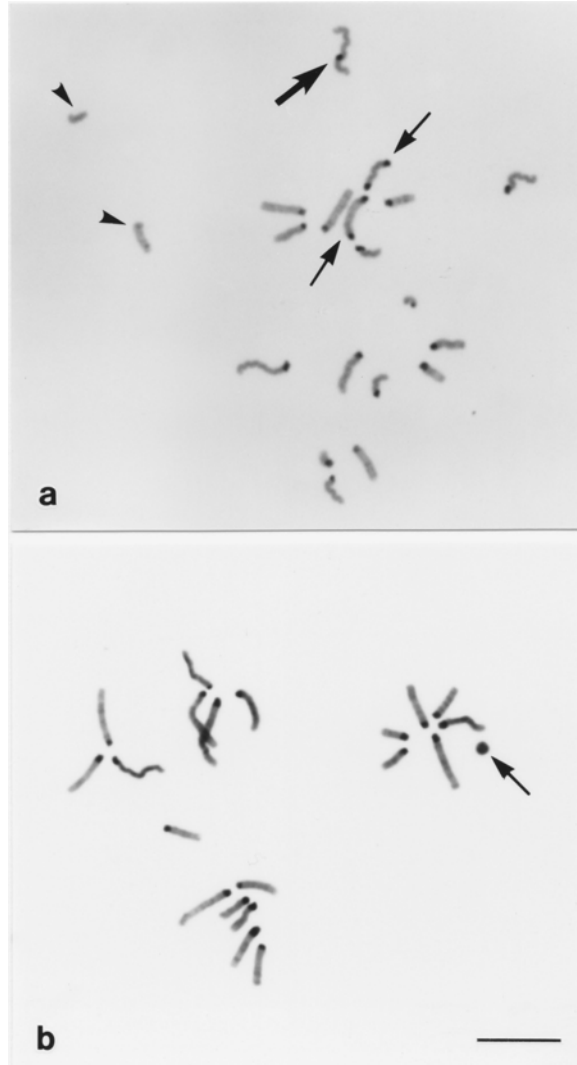


Figure 2

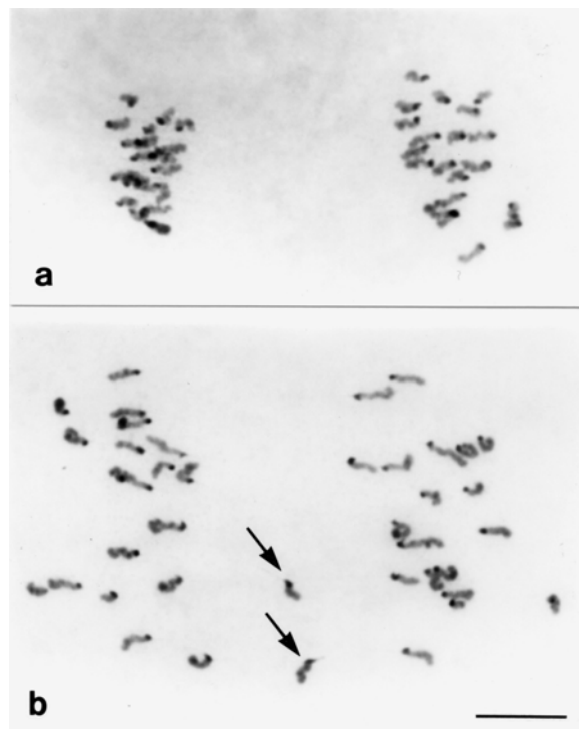


Figure 3

