Fragmentation of centromeric DNA and prevention of homologous chromosome separation in male mouse meiosis in vivo by the topoisomerase II inhibitor etoposide

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The mechanism of action of the topoisomerase II inhibitor etoposide (VP-16) was investigated in male mouse meiosis using the spermatid micronucleus (MN) test and two molecular cytogenetic approaches: (i) fluorescence in situ hybridization (FISH) with a mouse centromere specific minor satellite DNA probe; and (ii) immunolabelling of kinetochore proteins with CREST autoimmune serum. VP-16 caused significant increases in the frequencies of MN at all meiotic stages studied. VP-16 induced MN showed significantly elevated frequencies of centromeric hybridization signals compared to the controls. Similarly, after CREST immunostaining the majority of MN induced by the drug showed kinetochore signals when meiotic S phase and diplotene-diakinesis were treated. This would suggest that most induced MN were due to lagging of whole chromosomes. However, more than 80% of the small MN observed were signal-positive and a large pool of minute MN almost exclusively (92%) contained a kinetochore or centromere-DNA signal. This indicates that VP-16 causes chromosome fragmentation at centromeres. In addition, arrested first division (MI) anaphase figures with stretched bivalent(s) at the spindle equator were observed when diplotene-diakinesis and MI were targeted. Moreover, many small and medium size MN had two centromere or centro- chore signals at opposite sides, suggesting that inhibition of topo II at MI causes lagging of whole bivalents. Together, these results indicate that VP-16 acts by several genotoxic mechanisms at male meiosis: (i) fragmentation of centromeres possibly as a result of inhibition of the DNA strand religation reaction in a topoisomerase II mediated decatenation process of sister centromeres; and (ii) the induction of aneuploidy as a result of failures in separation of homologous chromosome arms possibly due to disturbances of chiasma resolution and decatenation processes during MI. Our results indirectly suggest that topoisomerase II plays an important role in male meiosis and its activity is needed at the metaphase-anaphase transition of both meiotic divisions for proper chromosome disjunction.

Introduction

DNA topoisomerase II (topo II) is an essential enzyme for DNA integrity due to its ability to untangle sister DNA strands that are topologically linked after DNA replication. In mammalian cells, there are two types of topo II that are differentially expressed and regulated (Drake et al., 1989a). Topo IIα, a 170 kDa form, is found in proliferating cells and is regulated during the cell cycle (Heck et al., 1988; Woessner et al., 1991), while the 180 kDa β-form is less regulated and is found in both proliferating and quiescent cells (Woessner et al., 1991). Experiments with yeast (Holm et al., 1985, 1989; Uemura et al., 1987), frog egg extract (Shamu and Murray, 1992) and mammalian cell studies in vitro (Downes et al., 1991) all show that topo II is required at the time of sister chromatid segregation. Moreover, Rose et al. (1990) and Rose and Holm (1993) have suggested a role for topo II in the resolution of recombined homologous chromosomes and in resolving tangles between nonhomologous chromosomes during meiosis I of yeast. In addition, topo II activity is also required for chromosome condensation (Uemura et al., 1987; Adachi, 1991).

Topo II, especially in its α-form, is thought to be an important structural component of the mitotic chromosomal scaffold (Earnshaw et al., 1985) and is associated with the chromatin and synaptonemal complex of pachytene and diplotene chromosomes of male chickens (Moens and Earnshaw, 1989). Whether the localization of topo II in mitotic and meiotic chromosomes denotes strictly its structural role (Gasser and Läänemäe, 1987) or not (Hirano and Mitchison, 1993) or is more an indication of requirement for the enzyme during chromosomal condensation and segregation remains to be established.

The use of inhibition of topo II plays a major role in recent development of cancer chemotherapy. Many potent clinical drugs in use such as epipodophyllotoxins (VP-16 and VM-26), anthracyclines (doxorubicin and daunorubicin), acridines (m-amsacrine) and anthracenediones (mitoxantrone) target topo II by stabilizing the enzyme-mediated DNA cleavage complex and, thus, inducing a covalent complex of topo II and DNA, which blocks DNA religation (Chen et al., 1984; Robinson and Osheroff, 1991). Recently, many new topo II-directed agents such as bis(2,6-dioxopiperazine) derivates (ICRF-159, ICRF-187 and ICRF-193) and the barbiturate derivative Merbavone have been demonstrated to inhibit normal chromosome segregation (Clarke et al., 1993; Gorbsky, 1994; Chen and Beck, 1995) by inhibiting topo II without stabilizing topo II-DNA covalent complexes (Drake et al., 1989b; Tanabe et al., 1991), but rather by affecting some unidentified catalytic step in a topo II-mediated reaction. This demonstrates that there are different mechanisms of inhibition of topo II function in target tissues which are not fully understood.

Etoposide (VP-16) is one of the most studied anticancer drugs with widespread clinical use (Henwood and Brogden, 1990). It has improved the treatment of germ cell tumours, small-cell and non-small-cell lung carcinomas, and acute lymphocytic leukaemia. However, secondary leukaemias have been reported in patients treated with etoposide-containing therapy (Pui et al., 1991; Nichols et al., 1993; Winick et al., 1993). The molecular mechanism of action of VP-16 in vitro and the stereo-configuration of the cleavable complex is only partially known (reviewed in Anderson and Berger, 1994). Moreover, recent data obtained from different groups show some discrepancy in the mechanism of action of the drug in vivo; VP-16 has been shown to induce chromosomal fragmentation in mammalian cells in vitro (Sumner, 1992) and...
in vivo (Agerwal et al., 1994), while induction of aneuploidy is suggested by germ cell studies (Kallio and Lähdetie, 1993; Mařilíe et al., 1994). We addressed the question, are there different mechanisms of action or different cellular targets in meiotic systems compared with mitotic cells?

We used two molecular cytogenetic approaches, fluorescence in situ hybridization (FISH) with a mouse minor satellite DNA probe and immunofluorescent labelling of the kinetochore with calciosis-Raynaud’s phenomenon-oesophageal dysmobility-sclerodactyly-telangiectasia syndrome of scleroderma (CREST) autoimmune serum, to investigate whether the formation of meiotic micronuclei (MN) during divisions after VP-16 treatment is due to non-disjunction and lagging of a whole chromosome(s) with assembled kinetochores or is an indication of breakage of pericentromeric DNA of meiotic chromosomes of the male mouse. In our previous work (Kallio and Lähdetie, 1993), where the mouse major satellite DNA probe was utilized, we could establish the effects of etoposide only on pericentromeric DNA that flanks the actual centromeric heterochromatin. Now by using the mouse minor satellite DNA probe, that localizes to the actual centromere (Wong and Rattner, 1988), we have further evaluated the VP-16 induced damage in this region.

Materials and methods

Animals and exposures

Outbred male Han/NMRI mice (age 9–16 weeks, weight 42–53 g, purchased from the Animal Department of the Institute of Biomedicine, Turku, Finland) were treated with a single i.p. injection of 10 or 20 mg/kg of VP-16 (Delta West Pty Ltd, Bentley, Australia). Four mice per each exposure group and four to five untreated control animals were used for both FISH and CREST experiments. VP-16 was dissolved in sterile 0.9% NaCl solution immediately prior to injection. After methanol fixation (−20°C for 20 min) and air-drying the slides were incubated in 2x0.3 M sodium citrate, 0.1 M Na2HPO4, 0.1% Tween 20, pH 8.0 for 15 min each at 39°C. Slides with Cy3 labelled minor probe were directly counter stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) at 0.2 μg/ml in ddH2O for 30 s. After final rinses in ddH2O for 2×8 min the slides were mounted with antifade solution (2% diazobicyclo[2,2,2]octane (DABCO) in glycerol:PBS 9:1). In experiments with digoxigenin labelled probes, fluorescent staining of the hybridized probe was performed by preincubating slides in 50 μl aliquots of PMN buffer (PN buffer, pH 8.0, 5% non-fat dry milk, 0.05% sodium azide) for 20 min at 37°C. The slides were overlaid with 25 μl of anti-digoxigenin fluorescent Fab fragments (33 μg/ml in PBS. Boehringer), covered with plastic coverslips, and incubated in a moist box for 45 min at 37°C. After two washes in PN buffer at 37°C for 10 min each, the slides were stained with DAPI and mounted.

Slide preparation for meiotic division analysis in spermatocytes

The testes were decapsulated and incubated with 0.005% colchicine (Sigma) in TIM for 15–30 min. Segments of stage XII of the seminiferous epithelium were isolated as described above and placed on hypotonic medium (0.56% KCl, 1% sodium citrate, 3:1) for 30 min. Methanol-acetic acid (3:1; for 10 min). The isolated segments were fixed in a drop of 95% ethanol-acetic acid and placed onto a clean objective slide. The slides were air-dried and mounted with antifade solution.

Slide preparation for micronucleus scoring in early spermatids

The time intervals (13 days, 40 h, 18 h and 6 h) between the injection and the cell harvest were chosen to correspond to injection at meiotic DNA synthesis (to detect errors in solving DNA tangling that arises during DNA replication), diplotene-diakinetic phase (to detect defects in late chromosome condensation and chiasma displacement), first meiotic division (to detect disturbances in non-homologous or homologous chromosome) and second meiotic division (to detect errors in sister chromatid separation due to failure in decatenation of centromeric DNA), respectively (Onkberg, 1956). At the selected time intervals the animals were killed by cervical dislocation and slides were prepared essentially as described by Lähdetie and Parvinen (1981). In brief, the testes were isolated and decapsulated in testis isolation medium (TIM) (Dietrich et al., 1983). The early post-meiotic cells (stage I spermatids) were collected by microdissection of seminiferous tubules under a stereomicroscope using transmitted light. Two to three short (1–2 mm) segments from different tubules were placed on one objective slide. After fixation with methanol:acetic acid (3:1) at room temperature for 20 min (FISH slides) or with methanol at −20°C for 20 min (CREST slides), the slides were air-dried overnight and stored at −20°C under N2 atmosphere when necessary.

Fluorescence in situ hybridization

A stock of mouse minor satellite DNA clone into a Smal site of pTZ19U) was a kind gift from Dr I.D. Adler. The plasmid was transformed into competent Escherichia coli cells. The minor satellite DNA probe was generated by random primed method or by nick-translation with Cy3-dUTP (BDS, Pittsburgh, PA) or digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) as a label. Final probe DNA was stored without further purification at −20°C for the presence or absence of signals in MN. A MN with a clear centromere (CREST), indicative of successful hybridization/immunoreaction, were scored as a normal hybridization pattern (FISH) or a normal kmetochore labelling (CREST), respectively. The frequency of induced MN was determined by counting 1000 round spermatids in five slides per mouse. The frequency of signal-positive MN for each dose was scored among all spermatids with a normal hybridization/labeling pattern in good quality slides. In all, between 70 000–160 000 cells were scored per dose. Within a group all data were pooled. Differences between groups were compared pairwise with Pearson’s χ2 test using BMDP software.

For size comparison the MN were classified into four categories according to their diameter (minute <1 μm; small 1–2 μm; medium 2-4 μm, large >4 μm) measured using a ×1 00 objective and an eyepiece graticule. The diameters of the round spermatids nucleus is ~11 μm. The minute micronuclei were not included in calculations of MN frequencies or of percentages of signal-positive MN, since it would have prohibited comparisons of the data with our earlier studies where MN of this size were excluded as propidium iodide staining artefacts. The MN diameter measurements in FISH and CREST experiments could not be compared due to different fixation procedures.

Results

To study the VP-16 induced aneuploidy and chromosome damage we monitored MN induction by harvesting the early post-meiotic spermatids after various time intervals that correspond to treatment of mice at premeiotic DNA synthesis,
VP-16 induced disturbances of meiotic divisions

Fig. 1. Schematic presentation of the meiotic cell cycle progression during the experiments. Mouse spermatogenesis is a highly synchronized differentiation process where specific meiotic events occur in a stage dependent fashion. Meiotic DNA synthesis (mainly stage VIII) occurs in preleptotene-leptotene spermatocytes 13 days before early post-meiotic round spermatids (stage I) evolve from meiotic division (stage XII). Diploctene-diakinesis (stage XI) takes place ~40 h before completion of the second meiotic division (Oakberg, 1956). The mean duration of stage XII during which the first and the second meiotic division occur is ~21 h (Oakberg, 1956). Etoposide (VP-16) injections 18 and 6 h before the harvest of early post-meiotic spermatids are targeted to late prometaphase-to-metaphase I and late prometaphase-to-metaphase II respectively.

diplotene-diakinesis, and meiotic divisions (Figure 1). At these times we scored MN frequencies and determined presence of centromeres and kinetochores in the MN.

Presence of centromeric DNA in meiotic micronuclei after VP-16 treatment

First, the specificity of the minor satellite probe (Wong and Rattner, 1988) was confirmed by performing the FISH on meiotic chromosome preparations. The probe hybridizes to the centromere region of all chromosomes except Y (Joseph et al., 1989) (Figure 2a). In control animals the mean frequency of MN observed was 0.86/1000 spermatids (Table I and Figure 3). A total of 154 MN were analysed and 80 (47%) of them were centromere positive. A total of 15 MN (9.7%) with two signals were seen. In animals treated with the lower dose of VP-16, the highest MN frequency, 9.2/1000 spermatids, was observed at 18 h time interval. At this time point, 64% of MN observed were centromere positive and 30 MN (9%) with two signals were detected (Table I). At the 6 h time interval, the mean frequency of MN was only slightly higher than the control level of 1.85/1000 spermatids; 62% of MN analysed were centromere positive and four of them had two signals (3%). At the 40 h time interval only a slightly higher frequency of centromere-positive MN (65%) was seen in animals treated with 10 mg/kg of VP-16 (Table I and Figure 2b). Interestingly, three MN with three (0.7%) and two MN with four (0.5%) centromere signals were observed at this time point. The mean frequency of MN at 40 h, 4.7/1000 spermatids, is somewhat lower compared with the 18 h time interval.

With the higher dose of 20 mg/kg of VP-16, the MN frequencies were clearly raised at the 18 and 40 h time interval compared with the lower dose, being 12.6/1000 spermatids and 18.2/1000 spermatids respectively (Table I). Again, the MN frequency was considerably lower at 6 h (1.8/1000 spermatids). The frequency of centromere-positive MN ranged from 63% at 18 h to 72% at 6 h (Table I and Figure 3). At all time points, MN with two signals were detected, at 6 h 12 MN (13%), at 18 h 36 MN (9%) and at 40 h 135 MN (13%) (Figure 2c and d). Again, MN with more than two signals were observed at the 40 h time interval, two large MN with three signals and one large MN with four signals were detected.

Most of the MN induced in animals treated by 20 mg/kg of VP-16 at 13 days were centromere positive (74%), the mean frequency of MN was 5.8/1000 spermatids. In all, 44 MN with two signals (12%) and two large MN with three signals (0.6%) were detected.

From this we conclude that the meiotic DNA synthesis, diplotene-diakinesis and the first meiotic division are more severely affected by VP-16 than the second meiotic division. In addition, most MN induced by VP-16 contain centromere-signals suggesting that the drug causes non-disjunction and lagging of whole chromosome(s).

Presence of kinetochore proteins in meiotic micronuclei after VP-16 treatment

In order to further investigate the hypothesis of aneuploidy induction we repeated VP-16 treatments (40 h and 13 days) and investigated the presence of kinetochores in the MN induced by the drug using immunofluorescense with CREST autoimmune serum. Simultaneously, we studied whether the drug had an effect on kinetochore structure which again could cause lagging of whole chromosomes.

In control animals the frequency of kinetochore-positive MN corresponds well with the FISH results, 81 MN (59%) of a total 137 analysed contained kinetochore signal(s) (Figure 3). Similarly, in animals treated with 20 mg/kg of VP-16 40 h or 13 days earlier, both the MN frequencies and the percentages of signal-positive MN are only slightly lower than with FISH (Table I). Likewise, after CREST staining MN with two or more signals were observed (Figure 2e). In animals treated 40 h or 13 d before harvest with VP-16, 30 and 31% of all kinetochore-positive MN had two signals, respectively (Table I). At the 40 h time interval, three large MN with three signals and two large MN with four signals were seen.

We conclude that VP-16 induces kinetochore-positive MN and centromere-positive MN with the same frequency after treatment of meiotic S phase and diplotene-diakinesis. Moreover, the similar percentages of centromere and kinetochore
Fig. 2. Fluorescent micrographs showing two first meiotic metaphases with univalent pairs in a control animal after DAPI staining (A) and in situ hybridization (A') with minor satellite DNA probe. In the upper division the sex chromosomes are as univalents (small arrows in A), Y chromosome lacks the minor satellite DNA signal while X chromosome possess a clear hybridization signal (small arrows in upper division of A'). The lower division has one autosome univalent pair (arrowheads in A), in the X/Y bivalent (small arrow in A), the Y chromosome lacks the minor satellite DNA signal (small arrow in lower division of A'). The chiasmata are stabilized and the homologous chromosomes are held together via connections at their q-arm telomeres. (B and B') DNA staining and minor satellite DNA pattern of early post-meiotic spermatids in a mouse treated with 10 mg/kg of etoposide (VP-16) 40 h before harvest, one with a centromere-positive spermatid micronucleus (MN) is shown (arrow) (C and C') A secondary spermatocyte MN with two centromere signals (small arrows) in an animal treated with VP-16 40 h before the harvest. The size of the MN corresponds well with the size of bivalents seen in (A), the distance between centromere signals in the MN is the same as in a bivalent. (D and D') A spermatid MN with two centromere signals (small arrows) in an animal treated with VP-16 40 h before harvest. The MN is somewhat larger than the bivalents seen in (A) probably due to decondensation of the chromatin but the distance between centromere signals is the same as in a bivalent. (E and E') A medium size MN with two kinetochore signals after CREST staining in a mouse treated at diplotene-diakinesis (40 h before harvest) (F and F') A minute MN (small arrow) in a mouse treated with 20 mg/kg of VP-16 at metaphase I (18 h before harvest). The minor satellite DNA covers the whole area of the MN. The magnification is same in all micrographs (X1000) Bar = 10 μm.
VP-16 induced disturbances of meiotic divisions

Table I. Induction of meiotic micronuclei by a single injection of etopside (VP-16)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Meiotic stage treated/ time interval</th>
<th>No. of animals/ method used</th>
<th>No. of MN scored</th>
<th>Frequency of MN/1000 spermatids (mean ± SEM)</th>
<th>No. of s+ MN (of them 2×s+ )</th>
<th>s+ MN (%)</th>
<th>Frequency of s+ MN/1000 spermatids a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>MII/6 h</td>
<td>4/FISH</td>
<td>128</td>
<td>1.85 ± 0.72 b</td>
<td>79 (4)</td>
<td>61.7</td>
<td>1.14</td>
</tr>
<tr>
<td>10</td>
<td>MI/18 h</td>
<td>4/FISH</td>
<td>337</td>
<td>9.15 ± 6.49 f</td>
<td>216 (30)</td>
<td>64.1</td>
<td>5.86</td>
</tr>
<tr>
<td>20</td>
<td>MII/6 h</td>
<td>4/FISH</td>
<td>423</td>
<td>4.65 ± 1.07 c</td>
<td>273 (36)</td>
<td>64.5</td>
<td>3.00</td>
</tr>
<tr>
<td>20</td>
<td>MI/18 h</td>
<td>4/FISH</td>
<td>94</td>
<td>1.80 ± 0.60 b</td>
<td>68 (12)</td>
<td>72.3</td>
<td>1.30</td>
</tr>
<tr>
<td>20</td>
<td>diplotene/40 h</td>
<td>4/FISH</td>
<td>417</td>
<td>12.60 ± 3.61 c</td>
<td>256 (36)</td>
<td>61.4</td>
<td>7.74</td>
</tr>
<tr>
<td>20</td>
<td>diplotene/40 h</td>
<td>4/FISH</td>
<td>1073</td>
<td>18.20 ± 10.60 f</td>
<td>698 (135)</td>
<td>65.1</td>
<td>11.84</td>
</tr>
<tr>
<td>20</td>
<td>diplotene/40 h</td>
<td>4/CREST</td>
<td>655</td>
<td>9.85 ± 9.38 c</td>
<td>385 (115)</td>
<td>58.8</td>
<td>5.79</td>
</tr>
<tr>
<td>20</td>
<td>meiotic S-phase/13 d</td>
<td>4/FISH</td>
<td>359</td>
<td>5.80 ± 3.73 c</td>
<td>267 (44)</td>
<td>74.4</td>
<td>4.31</td>
</tr>
<tr>
<td>20</td>
<td>meiotic S-phase/13 d</td>
<td>4/CREST</td>
<td>202</td>
<td>3.50 ± 0.33 c</td>
<td>129 (40)</td>
<td>63.9</td>
<td>2.24</td>
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</table>

Fig. 3. Effects of etopside (VP-16) on the frequencies of spermatid micronucleus (MN) and on the percentages of centromere/kinetochore-positive and centromere/kinetochore-negative MN after various time intervals (mean ± SEM). Asterisks indicate statistically significant differences in MN frequencies when compared with controls (*P < 0.05; ***P < 0.001; s+ signal-positive (FISH/CREST).

signals in analysed MN suggests that VP-16 does not affect kinetochore plate structures.

Size categorization of MN induced after VP-16 treatments

The size of MN induced in animals treated with 20 mg/kg of VP-16 was measured (Table II). MN were observed to have different diameters, ranging from <1 μm to >5 μm. Four size categories were created (see Materials and Methods) since an exceptionally high number of minute MN were observed (Figure 2f). These minute MN almost always contain centromere or kinetochore signal(s) and were clearly distinguishable from staining artefacts as a result of using different wavelengths for detecting DNA staining (DAPI) and DNA probe/CREST antibodies (Cy3), instead of the same wavelength (i.e. propidium iodide and Cy3). Interestingly, in our earlier study (Kallio and Lähdetie, 1993) minute MN were occasionally observed in animals treated with VP-16 but not with the other chemical mutagens tested (mitomycin C and vinblastine). MN of this size were all negative for pericentromeric heterochromatin after FISH with mouse major satellite DNA probe and, consequently, were excluded as propidium iodide staining artefacts.

Most of the MN induced by VP-16 in the FISH experiments were of medium size (48%) and preferentially centromere-positive (64%) especially at 6 h time interval (Table II). Minute MN were almost exclusively centromere-positive (<95%); at 40 h time interval 20% of all MN analysed were min MN, and 98% of them were centromere-positive. Interestingly, at 6 h time interval minute MN were not observed at all (Table II).

The majority of MN analysed in the CREST experiments were also medium size (48%) and preferentially kinetochore-positive (56%) (Table II). However, only 27% of large MN were kinetochore-positive at all time intervals analysed. In contrast, almost all of the small MN analysed were signal-positive both in the FISH (81%) and CREST (84%) experiments.

We conclude that VP-16 induces mainly medium size MN, more than half of them are signal-positive. In addition, the drug induces a large number of minute and small MN with centromere and kinetochore signals after treatment of meiotic S phase, diplotene-diakinesis and first meiotic division. In controls and in animals where second meiotic division was targeted this fraction is significantly smaller. This implies that VP-16, in addition to its aneugenic mode of action also causes fragmentation of centromeric DNA prior to and at the first meiotic division.
Table II. Size categorization of spermatid micronuclei (MN) induced by 20 mg/kg of etoposide (VP-16)*

<table>
<thead>
<tr>
<th>Time interval/method used</th>
<th>No. of large MN</th>
<th>No. of medium MN</th>
<th>No. of small MN</th>
<th>No. of minute MN</th>
<th>Total no of MN analysed</th>
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<tr>
<td></td>
<td>s+ s-</td>
<td>s+ s-</td>
<td>s+ s-</td>
<td>s+ s-</td>
<td></td>
</tr>
<tr>
<td>control/FISH</td>
<td>7 8</td>
<td>28 17</td>
<td>15 10</td>
<td>2 1</td>
<td>88</td>
</tr>
<tr>
<td>6 h/FISH</td>
<td>14 8</td>
<td>41 14</td>
<td>13 4</td>
<td>0 0</td>
<td>94</td>
</tr>
<tr>
<td>18 h/FISH</td>
<td>16 14</td>
<td>91 49</td>
<td>67 13</td>
<td>25 2</td>
<td>277</td>
</tr>
<tr>
<td>40 h/FISH</td>
<td>19 36</td>
<td>120 89</td>
<td>110 26</td>
<td>98 2</td>
<td>500</td>
</tr>
<tr>
<td>13 d/FISH</td>
<td>11 8</td>
<td>92 40</td>
<td>60 15</td>
<td>21 3</td>
<td>250</td>
</tr>
<tr>
<td>Total no of FISH MN (%)</td>
<td>60 (5.4)</td>
<td>66 (5.9)</td>
<td>192 (17.2)</td>
<td>250 (22.3)</td>
<td>58 (5.2)</td>
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<td></td>
<td>144 (12.8)</td>
<td>7 (0.6)</td>
<td>1121</td>
<td></td>
<td></td>
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<tr>
<td>control/CREST</td>
<td>11 23</td>
<td>57 27</td>
<td>13 6</td>
<td>0 2</td>
<td>139</td>
</tr>
<tr>
<td>40 h/CREST</td>
<td>28 85</td>
<td>187 151</td>
<td>170 34</td>
<td>60 10</td>
<td>725</td>
</tr>
<tr>
<td>3 d/CREST</td>
<td>11 21</td>
<td>63 42</td>
<td>55 10</td>
<td>5 0</td>
<td>207</td>
</tr>
<tr>
<td>Total no of CREST MN (%)</td>
<td>39 (4.2)</td>
<td>106 (11.4)</td>
<td>250 (26.8)</td>
<td>193 (20.7)</td>
<td>225 (24.1)</td>
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<tr>
<td></td>
<td>65 (7.0)</td>
<td>10 (1.1)</td>
<td>932</td>
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</table>

* Diameter ranges for MN size categories are described in Materials and methods.

Fig. 4. Anaphase defects in mice treated with etoposide (VP-16). (A) Many bivalents lag behind while the rest of them are already at the poles. Some of the bivalents are slightly stretched (small arrows) and seem to be properly orientated while the other are more condensed and maloriented. (B) Numerous spermatid micronuclei (MN) near the first division figures and interphase nuclei, notice also two small fragments, possibly centromeric DNA, in the right hand division (small arrows). The size of these small fragments corresponds well with the size of the observed signal-positive min MN (Figure 2f). The left hand MI division has one stretched bivalent and one compact bivalent at the spindle mid-zone (arrowhead). (C and C') DNA staining and CREST labelling of late anaphase I. A mass of chromosomal material has lagged behind while some homologous chromosome have segregated and moved to the poles. Kinetochore signals can be seen both at the spindle mid-zone in the bivalents (small arrows) and at the spindle poles (arrows). (D and D') DNA staining and CREST labelling of late anaphase I. One extremely stretched bivalent is lagging in the spindle mid-zone (arrow). Kinetochore signals localize to the opposite ends of the bivalent (small arrows) implying that microtubules have tried to separate the homologues but failed probably due to improper chiasma resolution at the q-arm telomeres. The magnification is same in all micrographs (X1000). Bar = 10 μm.

Anaphase defects in cells treated with VP-16

Arrested anaphase/telophase figures with multiple lagging chromosomes at the midzone (Figure 4a and b) were observed at stage I among the post-meiotic spermatids where normally no division figures are found. This "out of stage" location suggests that these divisions were arrested during the first division at stage XII. These cells were determined to be arrested at the first meiotic division since: (i) the DNA content of the cells was approximately double compared with that of second division cells; (ii) the diameter of the cells was larger compared with the second division cells. Some of the lagging chromosomes observed in these cells were clearly stretched.
probably due to microtubule-mediated pulling forces and improper chiasma resolution or a lack of untangling between non-homologous chromosome arms. After CREST staining, clear kinetochore signals could be detected in all lagging chromosomes and in chromosomes already at the poles (Figure 4c' and d').

Arrested anaphase/telophase divisions with bivalents at the spindle mid-zone were frequently observed in animals injected with VP-16 18 or 40 h before harvest while in control mice and in animals injected with the drug 6 h or 13 days before harvest such divisions were only occasionally observed.

Divisions of this kind were probably already directed into apoptosis since the DNA staining pattern was much brighter compared to normal telophases and because there were membrane and/or DNA structure changes that prevented hybridization of the minor satellite DNA probe into the cells. Such phenomena have been related to the initial stages of programmed cell death (Schwartzman and Cidlowski, 1993).

From this we conclude that VP-16 causes lagging of whole bivalents at first meiotic division. Arrested anaphase figures with stretched bivalents at the spindle equator suggest VP-16-induced malfunction of the segregation machinery.

Discussion

VP-16 induces various types of structural chromosome aberrations and numerical lesions in mammalian somatic cells in vitro (Pommier et al., 1988; Marashchin et al., 1990) and in vivo (Sieber et al., 1978). In addition, VP-16 and ICRF-193, which acts by a different mechanism, both inhibit mitotic sister chromatid separation in vitro (Downes et al., 1991; Shamu and Murray, 1992). However, observations on meiotic cells have been limited mainly due to lack of suitable test methods. Therefore, we performed a molecular cytogenetic study in order to answer the following questions: (i) was VP-16 capable of inducing structural or numerical aberrations or both in male mammalian germ cells in vivo? (ii) what were the effects of VP-16 on homologous chromosome disjunction at anaphase I? and (iii) how did it affect sister chromatid separation at anaphase II? Both FISH with the centromere-specific mouse minor satellite DNA probes and immunolabelling of certain kinetochore proteins using CREST autoimmune serum were successfully utilized for this study. We demonstrated that VP-16 induced fragmentation of centromeric DNA. In addition, the drug affected separation of homologous chromosomes at anaphase I of mouse spermatocytes based upon the lagging of stretched bivalents in the arrested first division anaphase figures and the frequent formation of MN with two signals.

MN arise when either a whole chromosome or chromosome fragment(s) fails to segregate to the daughter cell nucleus during cell division. Both FISH with centromere detecting DNA probes and CREST staining in combination with MN analysis have been shown to be efficient tools in distinguishing the induction of aneuploidy from clastogenic mechanisms (chromosome breakage) by chemical mutagens in human and mouse mitosis (Eastmond and Tucker, 1989; Miller et al., 1991; Gudi et al., 1992) and in mouse meiosis (Kalio and Lahdetie, 1993).

Fragmentation of the centromeric DNA after VP-16 treatments can be seen leading to the formation of minute and small centromere/kinetochore-positive MN. They constituted a major fraction of all MN induced at 18 and 40 h, i.e. at first meiotic division. At diplotene-diakinesis, (40 h) according to

the duration of the cycle of mouse seminiferous epithelium (Oakberg, 1956), 86% of MN of this size showed one or two centromere or kinetochore signals. The results are also in agreement with similar studies in oocytes; Mailhes et al. (1994) have reported increases in hyperploidy and in chromatid breaks at the centromere region of C-banded mouse oocyte metaphase II preparations after VP-16 injections. However, C-banding is not able to show whether a breakage occurred at pericentromeric heterochromatin or on the actual centromere. This distinction can be made by using the mouse major and minor satellite DNA probes. Our FISH results presented here are the first direct evidence for such an action on the true mouse centromeric DNA constructed of minor satellite sequences.

According to the hypothetical model of chromosome segregation during meiosis by Murray and Szostak (1985), topo II is needed both to resolve recombination-induced homologous chromosome intertwining at meiosis I, and daughter centromere catenations during anaphase II. However, this model presumes that replication of centromeric DNA occurs only after the completion of meiosis I. This assumption has been disputed, since there is no scientific evidence to support it, the available evidence leads to different predictions (Comings, 1966; McCarroll and Fangman, 1988; Collins and Newton, 1994).

Alternatives to explain the susceptibility of centromeric DNA to breakage through the disturbed action of topo II during meiosis are as follows. First, electron microscopic studies by Sumner (1991) clearly show that centromeric heterochromatin of human mitotic metaphase chromosomes does not split in two until anaphase. This phenomenon is probably related to differential coiling of the primary constriction during late stage condensation (Rattner and Lin, 1987). We suggest that cations between centromeres of sister chromatids result from centromere replication at S phase and that topo II cannot resolve these during anaphase I, since the centromere regions do not split in two at this point. The three-dimensional structure of closely connected and coiled centromeric DNA could block the access of topo II to this DNA region. Only after a short interphase, during which the DNA topology at centromeric regions is altered, topo II is able to catalyse decatenation of sister centromeres at anaphase II. Secondly, if centromeres after all do split in two before first anaphase or if topo II is able to operate at centromeres despite of different topology of the region, there must be other cohesive forces that hold centromeres together till second division even if they are fragmented. Then, only after this cohesion diminishes and the pulling forces of microtubules start to work at anaphase II, the breaks will appear in centromere regions. Such a cohesive interaction might be mediated through a centromeric filament observed in the first division chromosomes but not in the second division or mitotic chromosomes of many different vertebrate organisms (Solari and Tandler, 1991).

At all time intervals examined, VP-16 induced many small and medium size MN with two centromere or kinetochore signals at opposite sides. The distance of the two signals observed corresponds well with the distance of centromeres in a bivalent at MI. This suggests that lagging of whole bivalents was induced during the first meiotic division and this was further supported by observations of arrested MI divisions with bivalents at the spindle mid-zone. Failure in the chiasma resolution at anaphase I, i.e. decatenation of homologous chromosome arms that have been tangled during recombination events, prevents proper disjunction and may cause this detachment of whole bivalents from the spindle at
and to development of better antineoplastic strategies where cellular and molecular biology of male mammalian meiosis mechanisms is essential both to increase our knowledge of the characterization of different topo II forms and impact of targets another topo II mediated reaction or a totally different, satellite region. This is seen only after the first division when trimeric DNA during MI results in fragmentation of the minor centromeric DNA is late replicating, inhibition of topo II function by cleavable complex formation may have resulted of chromosomes. However, the frequency of MN with centromere/kinetochore signals was even higher than in animals which late meiotic phases were targeted. Microdissection of a small segment of seminiferous tubules containing the earliest postmeiotic stage of mice treated exactly 13 days (±1 h) before harvest gave us a narrow window (~10 h), to monitor effects on late DNA replication. We suggest that since centromeric DNA is late replicating, inhibition of topo II function by cleavable complex formation may have resulted in DNA strand breaks in centromeric regions during this period and the formation of signal-positive MN after meiotic divisions.

The results indicate that VP-16 causes both structural and numerical chromosome aberrations in male mouse meiosis in vivo. Several underlying mechanisms can be postulated. It seems that VP-16 inhibits the action of topo II by at least two different ways in meiosis. Inhibition of the topo II mediated strand religation reaction in the decatenation process of centromeric DNA during MI results in fragmentation of the minor satellite region. This is seen only after the first division when the topology of centromeric DNA has altered or cohesive forces between the sister centromeres have diminished. Lagging of bivalents at MI must be transmitted through another mechanism that may not necessary involve formation of cleavage complexes; possibly during resolution of chiasma VP-16 targets another topo II mediated reaction or a totally different, yet unidentified, topo II-like protein responsible of this action. Characterization of different topo II forms and impact of timing of topo II inhibition during cell cycle progression are next key questions to be elucidated. Understanding of these mechanisms is essential both to increase our knowledge of the cellular and molecular biology of male mammalian meiosis and to development of better antineoplastic strategies where the possible hazards to future generations have been minimized.

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