Tamoxifen Induces Hepatic Aneuploidy and Mitotic Spindle Disruption after a Single in Vivo Administration to Female Sprague-Dawley Rats

Linda M. Sargent, Yvonne P. Dragan, Nicole Bahnub, John E. Wiley, Carol A. Sattler, Pauline Schroeder, Gerald L. Sattler, V. Craig Jordan, and Henry C. Pitot


Abstract

Tamoxifen has found extensive use in the treatment of all stages of human breast cancer. The efficacy of tamoxifen treatment for the prevention of second primary tumors and its chemosuppressive action in animal models have led to initiation of clinical trials to test its efficacy for prevention of this disease in women. Recently, tamoxifen has been shown to induce hepatocellular carcinomas in rats. For determination of the mechanism of induction of these tumors and assessment of the possibility of risk of human cancer development from tamoxifen treatment, female Sprague-Dawley rats (five rats per treatment) were administered tamoxifen at doses ranging from 0.3 to 35 mg/kg. One day after treatment, the rats were sacrificed, and the hepatocytes were isolated and cultured for 50 h. Colcemid was added 3 h prior to harvest, and the hepatocytes were then prepared for karyotypic evaluation. One hundred metaphase spreads were examined per animal. Tamoxifen treatment resulted in the induction of aneuploidy in approximately 70% of the examined hepatocytes at the doses used. In addition, premature condensation (2–10%) and endoreduplication (5–10%) were observed in hepatocytes of rats treated with tamoxifen. Furthermore, exchanges between chromosomes as well as chromosome breakage were observed. Examination of the cultured hepatocytes from rats treated with tamoxifen by electron microscopy demonstrated both unipolar spindles and incompletely elongated spindles. Exposure of rats to a single in vivo dose of tamoxifen produced multiple changes in rat hepatocytes including clastogenic damage at doses comparable to that administered to humans. The occurrence of aneuploidy induction, premature condensation, chromosome breakage, and improper mitotic spindle formation indicates that risk versus benefit of tamoxifen treatment should be carefully evaluated.

Introduction

The antiestrogen, tamoxifen, has been used extensively to prevent the recurrence of human breast neoplasms, as well as the appearance of further primary lesions (1). The efficacy of tamoxifen treatment for all stages of breast cancer (1, 2) and the low incidence of acute side effects associated with its use (3) have led to the institution of further primary lesions (1). The efficacy of tamoxifen treatment for the recurrence of human breast neoplasms, as well as the appearance of further primary lesions (1). The efficacy of tamoxifen treatment for all stages of breast cancer (1, 2) and the low incidence of acute side effects associated with its use (3) have led to the institution of clinical trials to test its efficacy for prevention of this disease in women. Recently, tamoxifen has been shown to induce hepatocellular carcinomas in rats. For determination of the mechanism of induction of these tumors and assessment of the possibility of risk of human cancer development from tamoxifen treatment, female Sprague-Dawley rats (five rats per treatment) were administered tamoxifen at doses ranging from 0.3 to 35 mg/kg. One day after treatment, the rats were sacrificed, and the hepatocytes were isolated and cultured for 50 h. Colcemid was added 3 h prior to harvest, and the hepatocytes were then prepared for karyotypic evaluation. One hundred metaphase spreads were examined per animal. Tamoxifen treatment resulted in the induction of aneuploidy in approximately 70% of the examined hepatocytes at the doses used. In addition, premature condensation (2–10%) and endoreduplication (5–10%) were observed in hepatocytes of rats treated with tamoxifen. Furthermore, exchanges between chromosomes as well as chromosome breakage were observed. Examination of the cultured hepatocytes from rats treated with tamoxifen by electron microscopy demonstrated both unipolar spindles and incompletely elongated spindles. Exposure of rats to a single in vivo dose of tamoxifen produced multiple changes in rat hepatocytes including clastogenic damage at doses comparable to that administered to humans. The occurrence of aneuploidy induction, premature condensation, chromosome breakage, and improper mitotic spindle formation indicates that risk versus benefit of tamoxifen treatment should be carefully evaluated.

Materials and Methods

In order to determine whether tamoxifen induced aneuploidy or chromosomal damage in rat liver in vivo, we administered tamoxifen citrate (Sigma Chemical Co., St. Louis, MO) to groups containing five female Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) in doses of 0.3, 3.0, and 35.0 mg/kg by gavage. Alternatively, the solvent, trioctanoin (Pfaltz and Bauer, Waterbury, CT), or the positive control for induction of aneuploidy (15), 35 mg TCPOBOP, synthesized and provided as a gift by Dr. James Miller, McArdle Laboratory, was administered. Twenty-four hours after dosing, the rat livers were perfused with a collagenase solution as described previously (16). Hepatocytes were separated from litorral cells by a Percoll (Pharmacia, LKB Biotechnology, Piscataway, NJ) isodensity centrifugation and immediately plated in 75-cm collagen-coated flasks (16) at a density of 2.6 × 10^5 cells in 15 ml of serum-free "supplemented" Dulbecco's modified Eagle's medium/Ham's F-12 medium (17). The medium was supplemented with bovine serum albumin (0.2%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (18 mM), and sodium pyruvate (5 mM). The medium was changed after 3 h, and 10 ng epidermal growth factor (Collaborative Biomedical Products, Bedford, MA) was added per ml of medium to stimulate cell division. After 47 h of culture, 40 ng/ml Colcemid was added to each flask, and the cells were incubated for an additional 3 h. Parallel cultures were treated with 25 μm 5-bromo-2'-deoxyuridine (Sigma) for the last 6 h of culture to permit analysis of late replication banding (18). Cultures were randomly selected, and the cells were prepared for examination by electron microscopy by methods described previously (19) or harvested for karyotypic analysis. The hepatocytes were removed from the dish with 0.01% collagenase and harvested by hypotonic treatment (0.075 M KCl) for 8.5 min. Once removed from the flask, the hepatocytes were fixed with methanol/acetic acid (3:1, v/v), and slides were prepared as described by Sargent et al. (20). One hundred metaphases of good morphology were randomly selected from cultures of each rat.

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3 The abbreviations used are: DES, diethylstilbestrol; TCPOBOP, 1,4-bis[2-(3,5-di-chloropyridylo)]benzene.
In the animals examined, approximately 70% of the hepatocytes were tetraploid (chromosome number is 84), and 30% were diploid (chromosome number is 42). A spread was considered aneuploid if it deviated from the established diploid or tetraploid number of chromosomes. The metaphases were analyzed blindly for ploidy, breakage, exchanges, premature condensation, and endoreduplication. The significance of differences between groups was determined by nonparametric $\chi^2$ statistics; evidence of a dose-response relationship was tested by regression analysis. The alpha level was adjusted to take into account the number of comparisons.

**Results**

Karyotypic analysis of metaphase spreads from Sprague-Dawley female rats was performed to determine the effect of a single tamoxifen treatment on the number and integrity of hepatic chromosomes. Only a low level of breakage and aneuploidy similar to that reported previously for control untreated rats was observed in the solvent-treated rats (Table 1). TCPOBOP, which has previously been shown to induce aneuploidy in the rat liver in vivo (15), was used as a positive control to demonstrate that aneuploidy could be detected with this model. The present study showed that 35 mg TCPOBOP/kg body weight resulted in substantial aneuploidy (Table 1). Tamoxifen also induced a marked level of aneuploidy compared with the solvent control ($P < 0.05$). The level of aneuploidy was extremely high compared with the control. Aneuploidy was significantly increased for all doses of tamoxifen tested ($P < 0.05$). Since the level of aneuploidy noted was independent of the dose of tamoxifen used, doses lower than 0.3 mg/kg may be necessary to demonstrate a dose-dependent effect for induction of aneuploidy by tamoxifen. Tamoxifen treatment induced a dose-related increase in premature condensation and chromosome breakage (Table 1) that was significantly greater than that observed in solvent-treated rats ($P < 0.05$). Additionally, a dose-dependent induction of endoreduplication was observed with tamoxifen treatment. Furthermore, a significant level ($P < 0.05$) of aneuploidy, chromosomal exchanges, and endoreduplication were observed for all doses of tamoxifen administered (Table 1).

Fig. 1 is a photomicrograph of a metaphase of a type typically observed in rats treated with 35 mg tamoxifen citrate/kg. The spread is aneuploid. The presence of diplochromosomes (paired homologous chromosomes) indicates that the spread has undergone endoreduplication. In addition, chromosomes without a homologue can be observed (Fig. 1, arrow). Also, two exchanges are shown in this metaphase spread (Fig. 1, double arrows). All of the observed exchanges were between homologous chromosomes, indicating that the exchanges occurred during S synthesis (21). Since the mechanisms of induction of aneuploidy and endoreduplication can be quite varied (22), two factors potentially responsible for these effects were examined. In order to determine whether the tamoxifen-induced aneuploidy and endoreduplication are associated with an inhibition of chromosome synthesis, we examined metaphase spreads by late replication banding (18). In these studies, all metaphase spreads investigated showed a normal banding pattern with a light-staining, late-replicating X chromosome (data not shown), indi-

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**Table 1** Effect of a single oral dose of tamoxifen on rat hepatic chromosomal number and integrity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aneuploidy</th>
<th>Breakage</th>
<th>Premature Condensation</th>
<th>Exchanges</th>
<th>Endoreduplication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent control</td>
<td>3 ± 3.0</td>
<td>5.0 ± 2.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>35 mg TCPOBOP/kg</td>
<td>65 ± 6.5&quot;</td>
<td>10.0 ± 5.0&quot;</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>0.3 mg Tam/kg</td>
<td>71 ± 8.0&quot;</td>
<td>8.0 ± 2.0</td>
<td>2.0 ± 1.0</td>
<td>8.0 ± 3.0&quot;</td>
<td>5.0 ± 1.0&quot;</td>
</tr>
<tr>
<td>3 mg Tam/kg</td>
<td>70 ± 5.0&quot;</td>
<td>9.4 ± 3.0</td>
<td>4.0 ± 2.3&quot;</td>
<td>5.0 ± 2.0'</td>
<td>6.0 ± 2.0&quot;</td>
</tr>
<tr>
<td>35 mg Tam/kg</td>
<td>85 ± 7.0&quot;</td>
<td>23.0 ± 4.0&quot;</td>
<td>10.0 ± 5.0&quot;</td>
<td>10.0 ± 2.0</td>
<td>10.0 ± 2.0&quot;</td>
</tr>
</tbody>
</table>

* Significantly different from the control treatment ($P < 0.05$); Tam, tamoxifen.
The metaphase spread in (A) has a spindle apparatus of normal length and contains two poles. The metaphase cell in (B) contains a monopolar spindle. In addition, the organelles rat following trioctanoin intubation (A) or treatment with 3 mg/kg tamoxifen Citrate (B). Single pair of centrioles in this spindle apparatus. Are not excluded from the metaphase plate in the spread provided in (B). B, arrow, the were observed. Of the 36 informative spindles examined, 9 were cultures from tamoxifen-treated rats, monopolar spindles (Fig. 2B) noted in control cultures (Fig. 2A), while in a significant number of the normally tightly regulated coordination of spindle formation makes the presence of tamoxifen-induced DNA adducts (10–12) may result in premature condensation, exchanges between homologous chromosomes, and chromosomal breakage. Since the exchanges were observed in this study only between homologous chromosomes, the exchanges occurred during S (21). Thus, tamoxifen-induced DNA damage results in changes in cell cycle progression and further karyotypic instability.

Tamoxifen administration in vivo results in aneuploidy, an alteration in the number of chromosomes, in cell cycle progression, which is the result of two or more synthesis cycles in the absence of an intervening mitosis. A block in DNA synthesis or the inhibition or delay of spindle formation can result in endocycles and in aneuploidy. The presence of a light-staining, late-replicating X chromosome indicates that the endoreduplication and aneuploidy that occurred after tamoxifen treatment were not due to a block in S synthesis (18). The presence of monopolar spindles and incompletely elongated spindles from which the organelles were not excluded suggests that the aneuploidy and endoreduplication observed with tamoxifen treatment may be due to the induction of spindle aberrations. A structural analogue of tamoxifen, DES, has also been shown to induce micronuclei (25), aneuploidy (26), and cellular transformation (27). In addition, DES-treated cells have been shown to undergo mitotic arrest (24) and to contain monopolar spindles (28). Therefore, this class of agents appears to have multiple effects on the cell that result in the disruption of the normally tightly regulated coordination of spindle formation and chromosomal integrity.

Multiple factors are involved in the maintenance and proper formation of the mitotic spindle apparatus. Although the mechanism by which tamoxifen induces aneuploidy and karyotypic instability is unknown, tamoxifen is known to alter cellular calcium levels (29) and acts as a calmodulin antagonist (30). Although the role of calmodulin in spindle assembly is not known, calmodulin is associated with the spindle pole body and is required for proper formation of the spindle apparatus (31). Calmodulin-defective mutants in yeast contain a single spindle pole body and is required for proper formation of the spindle apparatus in vivo (14). Since the blockage of cell cycle progression can result in premature condensation, this action of tamoxifen should be explored to uncover the mechanism by which tamoxifen causes premature condensation. The presence of tamoxifen-induced DNA adducts (10–12) may result in premature condensation, exchanges between homologous chromosomes, and chromosomal breakage. Since the exchanges were observed in this study only between homologous chromosomes, the exchanges occurred during S (21). Thus, tamoxifen-induced DNA damage results in changes in cell cycle progression and further karyotypic instability.

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are often associated with malignant conversion (33). In addition, the hallmark of the stage of progression is one of an evolving karyotypic instability, including an increase in aneuploidy and chromosome breakage with gene deletions, rearrangements, and duplications (34). Since tamoxifen possesses clastogenic action as evidenced by the induction of micronuclei, aneuploidy, and chromosomal breakage, its carcinogenic action may include effectiveness during the stage of progression. This supposition is supported by the induction of transformation of Syrian hamster embryo cells by tamoxifen (35). Retrospective studies have found an increased incidence of endometrial cancers in tamoxifen-treated women (36). The present study performed in rat hepatocytes indicates that tamoxifen alters the number of chromosomes, increases the incidence of chromosomal aberrations, and may disrupt mitotic spindle integrity. The carcinogenic action of tamoxifen for the rat liver, coupled with the occurrence of aggressive breast and uterine tumors, albeit at a low incidence in women treated with tamoxifen, suggests that a careful assessment of the risk as well as the benefit of chronic tamoxifen administration should be considered in the nonmalignant use of this drug.

References
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