Extensive Chromosomal Breaks Are Induced by Tamoxifen and Estrogen in DNA Repair-Deficient Cells

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ABSTRACT

Tamoxifen (TAM) possesses antiestrogen activity and is widely used for the treatment or prevention of breast cancer. However, it is also carcinogenic in the human uterus and rat liver, highlighting the profound complexity of its actions. To explore the molecular mechanisms of TAM-induced mutagenesis, we analyzed the effects of this drug on gene-disrupted chicken B lymphocyte (DT40) clones deficient in various DNA repair pathways. Rad18, Rev3, and Polε are involved in translesion DNA synthesis (TLS), which facilitates recovery from replication blocks on damaged template strands. DT40 cells deficient in TLS were found to be hypersensitive to TAM, exhibiting an increase in chromosomal breaks. Furthermore, these mutants were also hypersensitive to 4-hydroxyestradiol, a physiological metabolite of estrogen. These data suggest a contribution of TLS to the prevention of chromosomal breaks by TAM and estrogen, and they therefore indicate that such error-prone DNA synthesis underlies mutagenesis induced by these agents.

INTRODUCTION

Tamoxifen (TAM) manifests an antiestrogen activity and is widely used for the treatment of breast cancer as well as for the prevention of this condition in high-risk women. Paradoxically, however, TAM is also carcinogenic in the human uterus and rat liver, highlighting the profound complexity of its actions. To explore the molecular mechanisms of TAM-induced mutagenesis, we analyzed the effects of this drug on gene-disrupted chicken B lymphocyte (DT40) clones deficient in various DNA repair pathways. Rad18, Rev3, and Polε are involved in translesion DNA synthesis (TLS), which facilitates recovery from replication blocks on damaged template strands. DT40 cells deficient in TLS were found to be hypersensitive to TAM, exhibiting an increase in chromosomal breaks. Furthermore, these mutants were also hypersensitive to 4-hydroxyestradiol, a physiological metabolite of estrogen. These data suggest a contribution of TLS to the prevention of chromosomal breaks by TAM and estrogen, and they therefore indicate that such error-prone DNA synthesis underlies mutagenesis induced by these agents.

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Materials and Methods

Chemicals. TAM, 4-OHTAM, and estrogens (E2, 2-OHE2, and 4-OHE2) were obtained from Sigma (St. Louis, MO). α-OHTAM was synthesized as described previously (11). All drugs were dissolved in ethanol and were added to the cells at final concentrations ranging from 0.2 to 15 μM; the final concentration of ethanol in cultures was 0.2% for all drug concentrations and controls.

Cell Culture. The phenotypes of the rad18, rev3, polk, xpa, rad54, and rad70 DT40 cells were described previously (7, 9, 12). The cells were cultured as described previously (12).

Growth Curve. The DT40 cells were cultured in 35-mm dishes for 5 days, and cell density was maintained at ~1 × 106 cells/dish by daily dilution. Cell proliferation was monitored with a FACScanLibor (Becton Dickinson, Mountain View, CA) and with the use of propidium iodine staining and a fixed number of plastic beads (3 × 106/ml), as described previously (12).

Cell Cycle Analysis. Cells (1 × 106) were labeled for 10 min with 20 μM 5-bromo-2′-deoxyuridine (Nacalai Tesque, Kyoto, Japan) 24 h after the addition of...
the fixed cell suspension was then transferred to an ice-cold and wet glass microscope slide and immediately dried with a flame. The slides were stained with 3% Giemsa solution at pH 6.4 for 1 h. Chromosomal aberrations were assessed 100 mitotic cells for each treatment by microscope.

RESULTS AND DISCUSSION

To evaluate DNA damage caused by TAMs in vivo, we examined the cellular growth response to these agents in various DNA repair mutants derived from DT40. rad18 cells exhibit a normal growth rate and divide every 8 h. We continuously exposed wild-type and rad18 cells to TAMs at concentrations of 0.4–12 μM, a concentration range similar to that (1–4 μM) apparent in the serum of individuals treated with TAM (13). Continuous exposure of rad18 cells to TAM, α-OHTAM, or 4-OHTAM began to interfere with cell growth after 24 h, whereas these agents had little effect on the growth of wild-type cells (Fig. 1A). The extent of the growth inhibition in rad18 cells was dependent on the concentration of TAMs (Fig. 1B–D). rad18 cells also exhibited an increased sensitivity to 4-OHE2 compared to wild-type cells, whereas sensitivity to E2 or 2-OHE2 did not differ between the two cell types (Fig. 1E–G). These results thus suggested that Rad18 is required for tolerance to the DNA lesions induced by 4-OHE2 as well as to those induced by TAMs.

To investigate the cause of the growth retardation of rad18 cells treated with TAMs, we performed cell cycle analysis after exposure of the cells for 24 h to 2 μM TAM, 12 μM α-OHTAM, and 0.4 μM 4-OHTAM. Each of these agents at these concentrations inhibited the growth of rad18 cells almost completely (Fig. 1A). Pulse-labeling

![Graphs showing relative growth of TAM, α-OHTAM, and 4-OHTAM treated cells.](image)

Table 1 Percentage of wild-type or rad18 cells in each cell cycle after 24 h treatment with 2 μM TAM, 12 μM α-OHTAM, and 0.4 μM 4-OHTAM

<table>
<thead>
<tr>
<th>Sub-G1</th>
<th>G2/M</th>
<th>M</th>
<th>G2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>7.32</td>
<td>18.5</td>
<td>1.96</td>
</tr>
<tr>
<td>WT + TAM</td>
<td>16.8</td>
<td>14.4</td>
<td>1.16</td>
</tr>
<tr>
<td>WT + α-OHTAM</td>
<td>17.8</td>
<td>16.1</td>
<td>1.82</td>
</tr>
<tr>
<td>WT + 4-OHTAM</td>
<td>18.4</td>
<td>16.2</td>
<td>2.41</td>
</tr>
<tr>
<td>rad18</td>
<td>4.89</td>
<td>21.5</td>
<td>1.27</td>
</tr>
<tr>
<td>rad18 + TAM</td>
<td>13.1</td>
<td>39.8</td>
<td>0.84</td>
</tr>
<tr>
<td>rad18 + α-OHTAM</td>
<td>17.7</td>
<td>39.1</td>
<td>2.04</td>
</tr>
<tr>
<td>rad18 + 4-OHTAM</td>
<td>15.2</td>
<td>38.4</td>
<td>1.96</td>
</tr>
</tbody>
</table>

α-TAM, tamoxifen; α-OHTAM, α-hydroxytamoxifen; 4-OHTAM, 4-hydroxytamoxifen; WT, wild-type.

![Graphs showing relative growth of TAM, α-OHTAM, and 4-OHTAM treated cells.](image)

CHROMOSOMAL BREAKS BY TAMOXIFEN AND ESTROGEN
with 5-bromo-2′-deoxyuridine and determination of the mitotic index revealed that the proportion of cells that accumulated in G2 phase after treatment of TAMs was greater for rad18 cells than for wild-type cells, whereas the sub-G1 fraction did not differ between the two cell types (Table 1). Similarly, rad18 cells treated with 0.8 μM 4-OHE2 accumulated in G2 phase to a greater extent than did wild-type cells (data not shown). These observations suggest that an increase in the amount of unrepaired DNA damage in rad18 cells treated with TAMs or 4-OHE2 might activate the DNA damage checkpoint and thereby result in transient cell cycle arrest at the G2 phase.

To identify other molecules that contribute to TAM tolerance, we examined the sensitivity of various gene-disrupted DT40 clones to TAMs. Exposure of wild-type or mutant cells to TAMs for 120 h revealed that rev3 and polκ cells also exhibited an increased sensitivity to these agents (Figs. 1B–G and 2), consistent with the notion that TLS plays an important role in the tolerance to TAM-induced DNA damage. In contrast, xpa cells, which are defective in the elimination of bulky adducts from genomic DNA (5), exhibited a sensitivity to TAMs similar to wild-type cells. We also examined rad54 and ku70 cells, which have defects in the two major pathways for DSB repair, homologous DNA recombination, and nonhomologous end joining (12), respectively. Whereas rad54 cells showed an increased sensitivity to TAM (but not to α-OHTAM and 4-OHTAM), ku70 cells exhibited increased sensitivity to all three agents.

To identify the cause of the TAM-induced growth retardation of rad18 or rev3 cells, we measured structural abnormalities of chromosomes in mitotic cells. Wild-type and mutant cells were treated with various concentrations of TAMs for 30 h. Consistent with previous observations (8), chromosomal aberrations were virtually undetectable in either wild-type, rad18, polκ, xpa, rad54, or ku70 cells during the cell cycle, whereas rev3 cells exhibited the spontaneous generation of such aberrations (Fig. 3A). After exposure to TAMs, wild-type or xpa cells showed only a few chromosomal aberrations, whereas rad18, rev3, or polκ cells manifested a marked, dose-dependent increase in the frequency of such aberrations.

Fig. 3. Increased frequency of chromosomal aberrations in translesion DNA synthesis mutants treated with tamoxifens (TAMs) or estrogen metabolites. Chromosomal aberrations were evaluated in wild-type and mutant cells treated with (A) TAMs or (B) estrogen metabolites for 30 h. Mitotic cells were enriched by exposure to colcemid for the last 3 h of the incubation. Data are derived from 100 metaphase cells for each treatment.
Similarly, exposure of rad18, rev3, or polk cells to 4-OHE2, but not to E2 or 2-OHE2, resulted in a substantial increase in the number of chromosomal breaks (Fig. 3B). Given the correlation that exists between the extent of growth inhibition and the level of induced chromosomal breaks, we conclude that the increased number of chromosomal breaks may be responsible for inhibition of the growth of rad18, rev3, or polk cells by TAMs or 4-OHE2.

TAMs and 4-OHE2 might induce chromosomal breaks by two possible mechanisms: (a) they may induce DSBs directly. Indeed, certain TAM and E2 metabolites have been shown to elicit DSBs directly by oxidative DNA damage (14, 15). Because DSB repair mutants such as rad54 or ku70 cells showed hypersensitivity to TAM and 4-OHE2, metabolites of these agents could cause DSBs directly. However, this scenario alone does not explain increased chromosomal breaks induced by TAMs and 4-OHE2 in the absence of Polk because it is not directly involved in DSB repair (9). (b) Defective TLS at sites of TAMs- or 4-OHE2-induced damage may result in an increase in the number of gaps, some of which are then converted to DSBs. According to this latter scenario, TLS might prevent chromosomal breaks by releasing replication blocks, whereas Rad54-dependent homologous DNA recombination and Ku-dependent nonhomologous end joining may repair the DSBs caused by stalled replication forks. This latter scenario is in agreement with the present data that showed both TLS mutants and DSB repair mutants showed hypersensitivity to TAM and 4-OHE2. In summary, we conclude that TLS mediated by Rad18, Rev3, and Polk, as well as DSB repair pathways are responsible for tolerance to DNA damage induced by TAMs or 4-OHE2.

The markedly greater sensitivity of TLS mutants than of xpa cells to TAMs contrasts with the hypersensitivity of both nucleotide excision repair and TLS mutants to UV. Our data are in agreement with those of an in vitro study showing that TAM-DNA adducts are repaired by nucleotide excision repair with only low to moderate efficiency (10). Given that exposure to UV or TAMs is thought to result in the formation of bulky DNA adducts, it might be expected that these two types of DNA damage would be repaired by the same pathway. We therefore investigated the nature of the DNA damage induced by TAMs. However, we were unable to detect adducts in the genomic DNA of cells treated with high concentrations of TAMs, even with the use of a sensitive 32P-postlabeling/PAGE assay (11). This could be explained by the limits of detection of the assay (seven adducts/1 × 109 nucleotides), which is obviously not sensitive enough to identify the damage that causes a single chromosomal break/5 × 109 nucleotides (0.4 chromosomal breaks/chicken genome) (Fig. 3A). It is also possible that other DNA lesions such as base damage or single-strand gaps that are not readily detectable by this assay may contribute to the hypersensitivity of TLS mutants to TAMs in vivo.

We measured the kinetics both of chromosomal break formation during continuous exposure of rad18 cells to TAM as well as of the decrease in the number of breaks after drug removal. The number of chromosomal breaks was 0.02, 0.15, and 0.38/mitotic cell at 18, 24, and 30 h, respectively, after the addition of 2 μM TAM. These observations suggest that TAM damages DNA with slow kinetics in marked contrast to the rapid action of classical genotoxic agents such as UV, cross-linkers, and alkylating agents. Moreover, TAM-induced lesions were eliminated more slowly than are those induced by UV damage: 0.25 breaks/mitotic cell were still detectable 24 h after the removal of TAM. DNA damage induced by TAMs or estrogen-related molecules therefore appears markedly different from that caused by UV in terms of their half-life, repair pathway, and genotoxicity.

In the present study, we first identified DNA repair pathways that are responsible for tolerance to DNA damage induced by TAM or estrogen. These agents have been thought to increase the incidence of tumors by acting as promoters rather than as initiators. However, our data now show that TAM and estrogen derivatives posses a high mutagenic potential that is attained by two mechanisms. First, DNA replication blocks may cause chromosomal breaks and lead to translocation or large deletion. Second, the employment of error-prone TLS polymerases to bypass DNA damage is likely to result in an accumulation of point mutations. A substantial contribution of TLS to mutagenesis is apparent in yeast in which Rev3 is responsible for the generation of most point mutations not only after exposure to UV but also during normal cell cycles (reviewed in Ref. 6). The extent of the contribution of TLS polymerases to spontaneous mutagenesis as well as to that induced by sex hormones remains to be clarified in higher eukaryotes.

The similar sensitivities of TLS mutants to various TAMs apparent in the present study are consistent with previous analysis of the reactivity of these agents with chromosomal DNA. The main adducts detected in rats and humans treated with TAM are thus formed by α-OHTAM, whereas 4-OHTAM also efficiently forms DNA adducts by oxidation (4, 18). Likewise, 4-OHE2, which forms DNA adducts more efficiently than does E2 or 2-OHE2 (2), induced chromosomal breaks in a dose-dependent manner in the present study. These similarities between the results of these previous studies and our present observations indicate that DT40 mutants are a reliable model for analysis of the genotoxicity of estrogen- or TAM-related products. Furthermore, this model system might prove useful for characterization of the potential mutagenic activities of various agents that induce replication blocks. A better understanding of TAM-dependent mutagenesis may contribute to development of new drugs for the treatment or prevention of breast cancer with higher therapeutic efficacy and reduced genotoxicity.

REFERENCES


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