Excision of Tamoxifen-DNA Adducts by the Human Nucleotide Excision Repair System

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Abstract

The antiestrogen tamoxifen is used in the treatment of breast cancer and has recently been recommended as a chemopreventive drug for women at high risk for breast cancer. However, women treated with the drug have an increased incidence of endometrial cancer. It has been suggested that this endometrial cancer might result from mutagenic DNA adducts, which are formed by electrophilic tamoxifen species generated by metabolic activation of the drug. Because the frequency of damage-induced mutations is strongly dependent on the repairability of the lesion, we investigated the repair of the major tamoxifen-DNA adducts by the human nucleotide excision repair system. Using the reconstituted human excision repair system and synthetic DNA substrates, we found that the four types of tamoxifen-DNA adducts detected in the endometrium were repaired with moderate to poor efficiency by nucleotide excision repair. It is concluded that individual variations in repair capacity may play a role in the development of tamoxifen-induced endometrial cancer.

Introduction

Tamoxifen is a synthetic antiestrogen that has been used as an adjuvant to surgical and chemotherapeutic treatment of breast cancer. Inclusion of tamoxifen in the treatment regimen reduces the rate of recurrence by nearly a factor of two compared with surgery plus chemotherapy alone (1), and at present, more than 1 million women worldwide with breast cancer are being treated with tamoxifen (2). Moreover, a chemoprevention trial projected for 5 years was recently stopped ahead of schedule when it became apparent that tamoxifen reduced the breast cancer incidence in high risk women by 49% (3). This finding will likely result in an increase in the number of women taking tamoxifen to several million in the United States alone. However, tamoxifen has a rare but potentially fatal side effect. The drug increases the incidence of endometrial cancer by a factor of 2–10, depending on the duration of treatment and the age of the patient, as well as other factors (3–5).

The mechanism of induction of endometrial cancer by tamoxifen is not known. However, there are two possible mechanisms: either tamoxifen acts as a tumor promoter because of its weak estrogen agonist activity on the endometrium or it acts as a tumor initiator via its metabolites, which act as weak genotoxicants that damage DNA (6). In rats, tamoxifen causes DNA damage in liver at a relatively high level and induces liver cancer at a correspondingly elevated rate (7), and there is a consensus in the field that liver cancers in rats are caused by differential specificity or activity of xenobiotic metabolizing enzymes. Tamoxifen induces low and negligible levels of DNA damage in the liver of mice and humans, respectively (6); hence there is no increased incidence of liver cancer in mice or in women receiving high doses of the drug. Initial measurements with human endometrial tissue of tamoxifen-treated women showed a minor putative adduct [0.27 adducts/10³ nucleotides (8)] or failed to detect any evidence of tamoxifen genotoxicity to endometrial DNA (9). However, recent studies using highly sensitive methods have detected tamoxifen adducts in endometrial DNA of women receiving the drug at levels of 1.5–13.1 adducts/10⁸ nucleotides (10). Although these levels are 40–230-fold lower than the adduct levels induced in rat liver (11), they are considered high enough to cause mutation and cancer when present in the DNA of women treated with the drug for many years.

Two factors important in the mutagenicity of a DNA adduct are its miscoding properties and its susceptibility to repair. The major DNA adducts detected in the endometrium of tamoxifen-treated women are the cis and trans isomers of TAM (10, 12–15). Furthermore, both the cis and trans forms have two diastereoisomers, which can be separated by analytical methods to yield four species, which are denoted TAM1 through -4, according to the order of elution from the HPLC column (10, 13–15). The structures of these species are shown in Fig. 1. Mutagenesis studies with site-specifically located adducts demonstrated that the TAM4 form is the most mutagenic and that the TAM1 form is the least mutagenic species in primate cells (16), raising the possibility that they may contribute to mutagenesis in the endometrium in that order. Because the other mutagenicity determinant factor is repair, we were interested in the repair of these adducts by the human nucleotide excision repair system. In particular, we were intrigued by the finding that when endometrial DNA samples of women taking tamoxifen were analyzed, dG-N²-TAM adducts were detected at a frequency of 1.5–13.1 adducts/10⁸ nucleotides in six patients, whereas no TAM adducts were detected in the other seven patients in the study group (10). This raises the possibility that individual variability in repair capacity may play a role in the long-term presence of adducts in endometrial DNA.

Human excision nuclease is the enzymatic activity resulting from the combined actions of six repair factors, and it is the only known repair system for removing bulky adducts from DNA (17, 18). The enzyme makes dual incisions bracketing the lesion and excises the damaged base(s) in the form of a 24–32 nucleotide long oligomer (19). To test the susceptibility of the tamoxifen-DNA adducts to excision nuclease, the various tamoxifen-guanine adducts (TAM1 through -4) were incorporated into 143-bp duplexes and tested with the human excision nuclease reconstituted with XPA, RPA, XPC, ERCC1, and Rep protein (20). The results presented here demonstrate that compared with the (6-4) photoproduct, the four tamoxifen-
DNA adducts detected in the endometrium are repaired with moderate to poor efficiency by either cell extracts or the basal excision repair system in the absence of other cellular proteins.

Materials and Methods

Materials. Tamoxifen was obtained from Aldrich Chemicals (Milwaukee, WI). Oligonucleotides for excision repair substrates were purchased from Operon Technologies (Alameda, CA). Mammalian cell cultures used for the preparation of cell extracts or the purification of repair factors were obtained from Lineberger Comprehensive Cancer Center (Chapel Hill, NC) or the National Cell Culture Center (Minneapolis, MN). CHO cell lines were purchased from the American Type Culture Collection (Manassas, VA).

Repair Factors. Cell extracts were prepared as described (20) and kept at \(-80^\circ C\). The human excision nuclease was reconstituted with six repair factors purified from HeLa cells or as recombinant proteins expressed in bacterial or insect cell systems using chromatographic schemes similar to those described (21, 22).

DNA Substrates. Unmodified oligonucleotides containing a single dG(5'-TCCTCCTCGCCTC-3') were reacted with (Z)-tamoxifen α-sulfate, and 15-mers containing a single stereoisomer of dG-N²-TAM were separated and isolated by high-pressure liquid chromatographic purification and gel electrophoresis (13–16, 23). High-pressure liquid chromatography fractions 1–4 are referred to as TAM1 through -4 when incorporated into DNA repair substrates. The site-specifically modified 15-mers were used in the preparation of 143-bp duplex molecules that serve as DNA repair substrates (24–26).

Excision/Incision Assays. Reaction conditions for the in vitro repair of tamoxifen-DNA adducts using the reconstituted human excision repair system or cell extracts were similar to those described previously (22, 25). After the repair reaction, DNA was deproteinized, ethanol precipitated, resuspended in formamide-dye mixture, and resolved on 10% polyacrylamide gels containing 7M urea to separate excision products from substrate DNA. DNA was visualized by autoradiography or by scanning on a Model 860 Storm PhosphorImager (Molecular Dynamics). The intensity of signal was analyzed with ImageQuant software (version 4.1 or 5.0; Molecular Dynamics) and the extent of repair for each reaction was determined from the percentage of signal migrating as 22–32-mers relative to the signal for full-length DNA [143 nucleotides for TAM substrates and 136 nucleotides for T(6-4)T]. The primary sites of incision were determined as described (26).

Results and Discussion

We found that dG-N²-TAM4 was repaired more efficiently than the other three lesions; therefore, we used this adduct for comparison of repair efficiency with a well-characterized substrate for the human excision repair system. The (6-4) photoproduct is a major lesion induced in DNA by UV and is among the best substrates for the human excision nuclease; it is removed very efficiently both in vivo and in vitro.
EXCISION REPAIR OF TAMOXIFEN-DNA ADDUCTS

Fig. 3. Kinetic analysis of excision of dG-N²-TAM adducts. A, substrate DNA (30 fmol) was incubated for 60 min in 25-µl reaction mixtures lacking (Lanes 1, 3, 5, 7, and 9) or containing (Lanes 2, 4, 6, 8, and 10) CHO AA8 cell extract at 2.4 mg/mL. A shows an autoradiograph obtained after resolution of DNA samples on a 10% sequencing gel. For kinetic analyses (B), reaction mixtures were increased proportionately, and 25-µl aliquots were removed at the indicated time points. Each data point is an average value from two independent experiments conducted under the same conditions using TAM1 (○), TAM2 (□), TAM3 (△), or TAM4 (■) substrate DNA. For comparison purposes, a 60-min data point for T(6-4)T substrate (▲) is shown. For the sake of clarity, the error bars are not shown, but the ranges of excision were ~20% of the average value for each time point.

(27, 28) and in vitro (29) and was used as a reference for repair efficiency. Fig. 2 shows that the repair of TAM4 by the human excision nuclease is less efficient than that of the (6-4) photoprod

uct, which is excised at a rate comparable to the in vivo rate of excision. Removal of both lesions requires the entire complement of excision nuclease factors because omission of selected factors abolished excision (Fig. 2A). Kinetic experiments with the two substrates (Fig. 2B) show that the (6-4) photoprod

uct is repaired 5–6 times more efficiently than the dG-N²-TAM4 adduct. The removal of the other three dG-N²-TAM adducts with the reconstituted excision nuclease was too inefficient for quantitative analysis.

We have found that mammalian whole-cell extracts are a good substitute for the reconstituted excision nuclease (22, 30). In particular, the CHO AA8 cell line reproducibly gives high-quality cell extracts appropriate for studying many aspects of mammalian excision repair (25). Hence, we tested all four TAM adducts with AA8 cell extract. Fig. 3 shows that, in agreement with the reconstituted system, TAM4 is repaired with moderate efficiency by the excision nuclease, whereas the other three adducts are repaired at lower rates comparable to those observed for the thymine cyclobutane dimer (25, 30). Kinetic analyses with TAM1 and TAM4 substrates were repeated with independent substrate prepa

rations, and the same moderately efficient excision of TAM4, relative to TAM1, was observed. With cell extracts, the repair efficiency of TAM4 was closer to that of the (6-4) photoprod

uct than was observed in the reconstituted system. This was the result of better overall efficiency of repair in cell extracts relative to the reconstituted system, which does not necessarily contain all of the repair factors in optimal ratios for the excision of all lesions. Interestingly, TAM4, which is the most efficiently repaired of all four adducts, is also the most mutagenic in primate cells (16). This would suggest that the mutagenicity of this adduct is so high compared with the other three that even its faster removal cannot compensate for its relatively high mutagenicity.

In conclusion, multiple factors are involved in the development of endometrial cancer, including the introduction and removal of DNA damage. Our data show that the tamoxifen-DNA adducts that have been found in the endometria of women treated with the drug (10) are removed by the human nucleotide excision repair system, although with poor to moderate efficiency. These adducts could potentially cause endometrial cancer, and the individual variations in overall repair capacities, which have been reported in human subjects (31, 32), may be true for the specific case of endometrial DNA repair. Thus, it might be prudent to factor in the repair capacity of individual patients when considering the benefit and risk of tamoxifen administration either for treating breast cancer or, perhaps more importantly, for chemoprevention.

Acknowledgments

We thank T. Bessho for critical reading of the manuscript, R. Hara for providing the repair substrate containing the (6-4) photoprod

uct, and members of the A. Sancar laboratory for purified repair factors.

References


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