Lack of Genotoxicity of Tamoxifen in Human Endometrium

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Abstract

The potential for the anti-breast cancer drug tamoxifen [(S)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butenyl] to induce genotoxic damage (DNA adducts) in the human endometrium was investigated in vivo and in vitro. Endometria from hysterectomy patients who were not on tamoxifen were sectioned and maintained in short-term organ culture. The cultures were treated with either solvent vehicle (DMSO), tamoxifen, α-hydroxytamoxifen [((E)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butenyl-3-ol; the major DNA-reactive metabolite in the rat], or benzo(a)pyrene. DNA was isolated and analyzed by 32P postlabeling. Chromatography on polyethyleneimine-cellulose TLC plates revealed DNA adducts in endometria treated with α-hydroxytamoxifen identical to those seen previously in the rat liver. However, no adducts were seen from treatment with tamoxifen itself. The viability of the enzyme-metabolizing systems of the endometrial samples was demonstrated by the detection of expected DNA adducts induced by benzo(a)pyrene. Examination by liquid chromatography-mass spectrometry of the explant culture media from endometria treated with tamoxifen revealed the presence of the α-hydroxy metabolite in a dose-dependent manner, although apparently at levels insufficient to produce detectable DNA adducts. Endometrial DNA obtained from 18 patients undergoing daily treatment with 10–40 mg tamoxifen for 3 months–9 years was also analyzed. No evidence for any DNA adducts induced by tamoxifen was found in any of the patients examined. These data suggest that the genotoxic events observed with tamoxifen in the rat may not apply to the human endometrium.

Introduction

The antiestrogen tamoxifen [(S)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butenyl] is effective in both the treatment of primary breast cancer and in the prevention of contralateral breast cancer in women who have already developed first tumors (1). Its success in treating cancer patients has led to chemoprevention trials of primary breast cancer and in the prevention of contralateral breast cancer (2) and who are at a greater risk of developing the disease. However, there have been concerns about long-term safety, because of the potent hepatocarcinogenicity of tamoxifen in the rat (3). Indeed, evidence exists for the genotoxicity of tamoxifen both in vivo and in vitro. Tamoxifen gives rise to DNA adducts in the livers of rats when administered i.p. (4) or by gavage (5), and it induces micronucleus formation in MCL5 cells (5, 6), a genetically engineered human cell line that expresses five human cytochrome P-450s and epoxide hydrolase (7). It has also been demonstrated that rat and human liver microsomal fractions metabolize tamoxifen to reactive intermediates that bind covalently to protein (8) and DNA (9). Furthermore, low doses of tamoxifen have been shown to induce aneuploidy and chromosone exchanges in rat hepatocytes (10), and tamoxifen-induced liver tumors in rats have mutations in the p53 gene, clustered at two specific codons (11). However, most importantly, evidence has emerged that women treated with tamoxifen have an increased risk of developing endometrial cancer (12, 13), with relative risks ranging as high as 7.5. The increase has been attributed, by some, to hormonal influences due to the partial estrogenic effect of tamoxifen in the endometrium. However, as stated recently (14), the question arises, “Does tamoxifen cause tumors by a genotoxic or nongenotoxic mechanism, or are both mechanisms involved? Most importantly, are tumors induced in humans by the same processes?” Hence, with regard to the potent genotoxicity of tamoxifen in the rat, it has become paramount to assess whether a genotoxic mechanism of action occurs in the human endometrium and the implications that such a mechanism could have for other human tissues. In this study, we have sought evidence for such a genotoxic event by determining the presence or absence of DNA adducts (15) in the endometria of women treated with tamoxifen and in in vitro experiments using endometria exposed in organ culture to tamoxifen.

Materials and Methods

Chemicals. Tamoxifen was purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). α-Hydroxytamoxifen [(E)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenyl-1-butenyl-3-ol; Ref. 16] was generously provided by Professor Michael Jarman (Institute of Cancer Research, Sutton, United Kingdom). Reagents and materials for 32P postlabeling, explant culture, and LC-MS were obtained from the suppliers mentioned previously (5, 17).

Ethical Approval. Informed consent for the investigations described herein was obtained from all patients, and approval for the study was given by the local research ethics committee.

Tissue Accrual. The human endometrium intended for explant culture was sectioned directly from tissue removed at hysterectomy. Patients were selected from cases receiving no drug therapy, in which the indications for hysterectomy were for benign menstrual dysfunction and fibroid. The endometrium was placed immediately in a dry vessel and kept on ice until further sectioning and culture as described below. The time period between the surgical removal and explant culture was less than 2 h in all cases.

In addition, tissue intended for direct 32P-postlabeling analysis was obtained following surgical removal and included endometria sectioned from both hysterectomy tissue and biopsy samples. In these cases, all tissue taken at surgery was placed in a dry vessel and frozen immediately, where it remained (at −80°C) until thawing and DNA extraction as below. Patients for these samples were selected to include both tamoxifen-treated patients and control individuals. The age of the patient, tamoxifen dose, duration of treatment, concurrent drug therapy, and smoking status were documented in each case, the details of which are presented in Table 1.

Culture and Treatment of Human Endometrium. The endometrium from each patient was partitioned into equal-size sections of approximately 5 mm2 and maintained in short-term organ culture by immersion in 1 ml DMEM containing 10% FCS, 110 μg sodium pyruvate, 100 μg streptomycin sulfate, and 60 μg benzylpenicillin. One section from each patient was used as an internal control and treated with 10 μl of the solvent vehicle (DMSO). The remaining sections (one from each patient) were treated with tamoxifen (20–500 μM), α-hydroxytamoxifen (20–500 μM), or benzo(a)pyrene (250 μM; positive control). The explant cultures were then kept in a humidified incubator.
at 37°C with a 5% CO2 atmosphere for 24 h. DNA was then isolated using the extraction procedures described below.

DNA isolation. DNA isolation was performed essentially as described by Gupta (18). Endometrial samples were homogenized using an Ultra-Turrax instrument (Sartorius Instruments, Belmont, Surrey, United Kingdom) in 10 mM EDTA (1 ml) to which 1% SDS and 1 mg proteinase K were added, and incubation was performed for 1 h at 37°C. The mixture was then extracted sequentially with equal volumes of phenol, phenol:chloroform:isoamylalcohol (25:24:1), and chloroform:isoamylalcohol (24:1). Sodium chloride (0.1 volume) and cold ethanol (2 volume). Precipitated DNA was reprecipitated twice with chloroform:isoamylalcohol (24:1), and the DNA was redissolved in 1 mM EDTA (500 µl). to which was added 50 mM Tris (pH 7.4, 5 M) and 2 volume cold ethanol were then added. Precipitated DNA was redissolved in 1 mM EDTA (1 ml) to which 1% SDS and l mg proteinase K were added, and incubation was performed for 1 h at 37°C. The mixture was then extracted twice with chloroform:isoamylalcohol (24:1), and the DNA was reprecipitated by the addition of 5 M NaCl (0.1 volume) and cold ethanol (2 volume).

32P-Postlabeling Analysis. 32P-Postlabeling analysis, using the nuclease P1 digestion method of sensitivity enhancement, was carried out as described previously (15). This DNA adduct comigrated on TLC and high-performance liquid chromatography with the major DNA adduct formed in endometrial explant cultures treated with the polycyclic aromatic hydrocarbon, a carcinogen that requires bioactivation prior to DNA binding (see example 32P-postlabeling.

Results

Explant Culture of Human Endometrium. Eight endometrial samples were cultured as described above, and in each case, the active metabolizing capability of the samples was demonstrated by the detection of the expected major benzol(a)pyrene-DNA adduct in culture media. The formation of the adduct was concentration dependent and was observed at 0-25 ng/ml (r > 0.999).

32P-Postlabeling Analysis. 32P-Postlabeling analysis, using the nuclease P1 digestion method of sensitivity enhancement, was carried out as described previously (5), except that apprase was not used to terminate the labeling reaction. Labeled adducts were resolved by PEI-cellulose TLC (5) [solvent system: D1: 2.3 M sodium phosphate (pH 5.8); D2: 2.28 mM lithium formate and 5.53 mM urea (pH 3.5); and D3: 0.52 M LiCl, 0.33 mM Tris-HCl, and 5.53 mM urea (pH 8.0); D4 was omitted from the procedure] and reverse-phase, high-performance liquid chromatography (19). For the latter, cis-9,10-dihydroxy-9,10-dihydroxyphenanthrene and 9,10-dihydroxyphenanthrene were used as an internal elution marker. In some experiments, the sensitivity of the 32P-postlabeling assay was increased by replacing the nuclease P1 digestion step with a procedure involving sorbent extraction of the DNA digest prior to labeling. The DNA digest was loaded onto a Bond Elut C8 extraction cartridge (Varian; obtained from Anachem, Ltd., Luton, Hertfordshire, United Kingdom) that had been equilibrated with methanol (3 ml) followed by deionized water (3 ml). The column was then washed with deionized water (5 × 1 ml) to remove normal nucleotides, and the adducts were then eluted with methanol:ammonia (9:1; 2% aqueous solution of 0.88-specific gravity ammonia; 1.5 ml). The solvent was removed under reduced pressure, and the residue was redissolved in water (10 µl) for 32P-postlabeling.

Mass Spectrometry. The medium from endometrial explant cultures (1 ml) was extracted with 2% ethanol in hexane (2 × 5 ml). The organic fractions were combined and concentrated to dryness. The dry sample was reconstituted in acetonitrile (50 µl), and a 10-µl aliquot was used for analysis. LC-MS analysis was performed on a Finnigan (San Jose, CA) MAT TSQ mass spectrometer as described previously (15). The full-scan mass spectrum was acquired over the mass range of m/z 200–600 atomic mass units in the positive ion mode. Tandem mass spectrum for α-hydroxytamoxifen was produced by using argon gas in the collision cell at a pressure of 0.3 millitorr. The linearity of a standard curve was confirmed by plotting the ratio of the drug metabolite and internal standard peak areas versus the ratio of the drug metabolite and internal standard concentrations. The calibration curve was linear over the range of 0-25 ng/ml (r > 0.999).

Results

Explant Culture of Human Endometrium. Eight endometrial samples were cultured as described above, and in each case, the active metabolizing capability of the samples was demonstrated by the detection of the expected major benzo(a)pyrene-DNA adduct in cultures treated with the polycyclic aromatic hydrocarbon, a carcinogen that requires bioactivation prior to DNA binding (see example 32P-postlabeling map in Fig. 1). Treatment of endometrial samples with α-hydroxytamoxifen gave rise to a distinct adduct spot migrating close to the center of the TLC plates in all cases (see example maps in Fig. 1), and the formation of the adduct was concentration dependent (Fig. 2). This DNA adduct comigrated on TLC and high-performance liquid chromatography with the major DNA adduct formed in rat liver hepatocytes following treatment with either tamoxifen or α-hydroxytamoxifen (15; data not shown). However, treatment of
endometrial samples with tamoxifen at the same concentrations, or with the solvent vehicle, did not generate any similar DNA adducts detectable by the $^{32}$P-postlabeling procedures used here (examples in Fig. 1). Nevertheless, LC-MS analysis of the explant culture media from samples treated with tamoxifen revealed the presence of a major hydroxylated metabolite at m/z 388 atomic mass units. This metabolite had an identical retention time and mass spectrum to that of $\alpha$-hydroxytamoxifen, and structural confirmation was obtained by tandem mass spectrometry generating the product-ion spectrum. Table 2 demonstrates that the formation of $\alpha$-hydroxytamoxifen in the culture media was proportional to the concentration of tamoxifen, although, it would seem, not at levels sufficient to generate DNA adducts. Low background levels of $\alpha$-hydroxytamoxifen detected in control sample media (Table 2) seem to be due to oxidative processes incurred during the sample workup, because no increase in $\alpha$-hydroxytamoxifen levels was seen in incubations carried out in the absence of endometrial tissue.

**Table 2: Formation of $\alpha$-hydroxytamoxifen, determined by LC-MS, in the media of cultured endometrium treated with tamoxifen**

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>$\alpha$-Hydroxytamoxifen in medium (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometrium + DMSO, $t = 24$ h</td>
<td>0.01</td>
</tr>
<tr>
<td>Endometrium + 20 $\mu$M tamoxifen, $t = 24$ h</td>
<td>0.20</td>
</tr>
<tr>
<td>Endometrium + 100 $\mu$M tamoxifen, $t = 24$ h</td>
<td>1.10</td>
</tr>
<tr>
<td>Endometrium + 500 $\mu$M tamoxifen, $t = 24$ h</td>
<td>4.10</td>
</tr>
<tr>
<td>No endometrium + 500 $\mu$M tamoxifen, $t = 0$ h</td>
<td>0.15</td>
</tr>
<tr>
<td>No endometrium + 500 $\mu$M tamoxifen, $t = 24$ h</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$^{32}$P-Postlabeling of Endometrial DNA from Tamoxifen Patients. Endometrial DNA from 18 patients being treated with 10–40 mg tamoxifen for a period of 3 months–9 years and from 16 control patients (no drug treatment) was examined by $^{32}$P postlabeling (Table 1). Fig. 3 shows $^{32}$P-postlabeling maps from both controls and treated patients using both nuclease P1 and sorbent extraction methods. In each case, the maps were deliberately overexposed to enable the detection of DNA adducts at the detection limits of the technique (1 adduct/2.5 $\times$ 10$^9$ nucleotides). All maps displayed a general background of low level endogenous DNA damage, but those from tamoxifen patients were indistinguishable from those of control patients. Fig. 3I shows a positive control endometrial sample treated in culture with $\alpha$-hydroxytamoxifen as described above; the arrow denotes the position of the major tamoxifen-derived DNA adduct. The expected positions of the $\alpha$-hydroxytamoxifen adduct in the samples from patients are indicated by circles on the postlabeling maps in Fig. 3. No significant levels of radioactivity, indicative of the presence of the adduct, were found in this position in any of the chromatograms of postlabeled DNA from tamoxifen-treated patients.
LACK OF TAMOXIFEN GENOTOXICITY IN ENDOMETRIUM

Fig. 3. Autoradiographs of the PEI-cellulose TLC maps of 32P-postlabeled digests of endometrial DNA from control and tamoxifen-treated patients; A-C, example chromatograms from control patients; D-H, example chromatograms from tamoxifen-treated patients; A-F, samples analyzed using the nuclease PI method of 32P postlabeling; G-I, samples analyzed using the sorbent extraction method; I, cultured endometrium treated, as described in "Materials and Methods." with 100 μM α-hydroxytamoxifen; arrow, position of the major DNA adduct; circles in D-H, positions at which such an adduct would have been expected, had it been present. Autoradiography films were exposed for 3 days at -80°C.

Discussion

There is now substantial evidence that tamoxifen is carcinogenic in the human endometrium (12-14), and there has been a great deal of discussion concerning the possibility of the drug acting as a carcinogen in other tissues, such as the liver and gut (14). It has been shown that the major mechanism of carcinogenicity of tamoxifen in the rat is through metabolism via α-hydroxytamoxifen to a genotoxic and DNA-binding species (15, 20, 22). A similar mechanism of action and genotoxicity in the human endometrium could imply a significant risk to other tissues also capable of similar routes of tamoxifen bioactivation. However, as demonstrated in the current study, no evidence of α-hydroxytamoxifen DNA adducts and, therefore, no evidence of genotoxicity was seen in the endometrium of any of the tamoxifen-treated patients examined, regardless of the dose or length of treatment with the drug. In addition, tamoxifen did not form DNA adducts when introduced, at relatively high concentrations, to endometrial tissue maintained in explant culture. It is interesting to note that the metabolite implicated as a genotoxic intermediate in the rat liver, α-hydroxytamoxifen, was found to be formed by the human endometrium in a dose-dependent manner in the culture media. However, examination of the levels generated reveals the significant differences between the rat and human situations. A tamoxifen dose of 100 μM in the endometrial cultures gave rise to approximately 1.0 ng/ml α-hydroxytamoxifen, a level similar to that measured as a circulating metabolite in serum samples from some of the tamoxifen patients examined. We have shown previously that rat hepatocytes in culture can generate 20–40 times more α-hydroxytamoxifen at 10-fold lower doses of tamoxifen (21) than used here. No adducts were detected in cultures of human hepatocytes treated with tamoxifen, unless α-hydroxytamoxifen was used (21). In addition, the 1.0-ng/ml level of α-hydroxytamoxifen measured in endometrial cultures and human serum equates to approximately 2.5 nM α-hydroxytamoxifen. In the culture experiments presented here, the lowest dose of α-hydroxytamoxifen giving rise to the formation of detectable DNA adducts was 20 μM, a concentration 10,000 times higher than in the media of tamoxifen-treated cultures or circulating in the blood of patients. Therefore, it would seem that although the genotoxic metabolite α-hydroxytamoxifen may be generated by endometrial tissue and is detectable in serum, the levels formed in women are far too low to give rise to detectable DNA adducts. If any DNA adducts are formed, they are presumably at a level insufficient to pose a significant genotoxic risk in the manner seen in the rat. Alternative and nongenotoxic mechanisms of initiation of human endometrial cancer by tamoxifen now bear closer investigation. Furthermore, the enzymeology of tamoxifen activation is not fully understood and the possibility of some individuals being at higher risk due to genetic polymorphisms merits further research.

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References


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