Tamoxifen-induced DNA Adducts in Endometrial Samples from Breast Cancer Patients

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Abstract

Tamoxifen-induced DNA adducts were analyzed with the ³²P-postlabeling method using high-performance liquid chromatography (HPLC)-radioactivity detection from endometrial tissue of breast cancer patients and controls. Liver DNA from tamoxifen-treated rats was used as a positive standard. In blind analysis, five of the seven samples from tamoxifen-treated patients showed DNA adducts; none of the five controls were positive. The identity of the tamoxifen adduct was confirmed by using different chromatographic systems, isolating the HPLC fractions and running them on TLC, with or without spiked rat liver samples. The level of adducts in the treated patients was 2.7 adducts/10⁹ nucleotides in the HPLC analysis.

Introduction

Tamoxifen is an antiestrogen successfully used in adjuvant therapy of breast cancer since the 1970s. It is effective in the treatment of primary breast cancer by decreasing recurrence rates and increasing 5-year survival rates (1). Because the adjuvant treatment lasts several years, hundreds of thousands of women currently receive tamoxifen. Tamoxifen is also used in several chemoprevention trials being carried out among healthy women who have a family history of breast cancer. Tamoxifen has recognized and anticipated side effects. It has been shown to cause liver cancer (2-5) and DNA adducts in several carcinogenic hepatocytes and human microsomes in vitro (11-13). Endometrial cancer and other serious side effects of therapy have been reported in tamoxifen-treated patients (1, 14-16). In 1996, a working group of the IARC (17) concluded that tamoxifen is carcinogenic in humans, i.e., tamoxifen was added to the IARC list of some 50 known human carcinogens. Thus, it is of large clinical and mechanistic interest to investigate the levels of DNA adducts in humans and their possible utility in risk assessment and selection of therapeutic agents. The studies thus far reported have found no tamoxifen-induced DNA adducts in humans (18-20). Using ³²P-postlabeling coupled to HPLC³ and flow-through radioactivity detection (21), we report here for the first time identification of tamoxifen-induced DNA adducts in human samples.

Materials and Methods

Patients, DNA Isolation, and Rat Liver Standard. Samples from a total of 11 patients were obtained in quantities enabling analysis of DNA adducts. From one patient, both an endometrial D&C and a hysterectomy sample were obtained. The patients were referred to the gynecological departments usually with the indication of uterine bleeding. They underwent endometrial D&C; a sample of sparse endometrium was immediately frozen for this study. Two patients had been diagnosed with endometrial cancer and underwent a hysterectomy. A small endometrial section of the uterus was obtained for the study and maintained frozen. Some blood and stromal tissue were present in the samples obtained, but the main source of DNA was endometrium.

The mean age of the tamoxifen-treated and control patients was 67 and 59 years, respectively. Of the 11 patients, 4 had received 40-mg/day tamoxifen p.o. and 2 had received 20 mg/ day for at least 3 months (range, 3 months-5 years). Of the five control (nontamoxifen-treated) gynecological patients, two had breast cancer; their gynecological diagnosis included hyperplasia, atrophy, and endometrial cancer. All of the samples were coded for blinded analysis.

DNA from endometrial D&C samples was isolated by first lysing the cells with 0.5% Triton X-100 and recovering the crude nuclear fraction (11). The nuclear fraction was then treated with RNase A and T1 followed by proteinase K and extraction with phenol and chloroform-isooamyl alcohol. DNA was precipitated and washed with ethanol. This procedure ensured efficient removal of RNA, as evident in the HPLC analysis of the labeled products. DNA from hysterectomy samples was isolated as described previously (11). In brief, the tissue (0.1-1.0 g) was homogenized in 5 ml of 1 mM MgCl₂, 10 mM Tris-HCl, and 0.15 M NaCl (pH 8.0), after which the suspension was treated with 5 ml of 0.5% Triton X-100. The nuclei were collected by centrifugation at 3000 rpm for 10 min at 20°C. DNA was isolated with RNase A and RNase T1 treatment followed by proteinase K digestion, as described above.

Rat liver DNA was used as a standard in the analyses (11). The animals had received 45 mg/kg tamoxifen p.o. The DNA isolation was carried out as described above for hysterectomy samples.

Labeling and Separation. DNA (5 or 10 μg) was enzymatically digested to ³'-mononucleotides as described (11), first by incubating for 2 h at 37°C with micrococcal nuclease [80 million units DNA in 3 ml biene (pH 9.0) and 0.5 mM CaCl₂] and then for 2 h at 37°C with spleen phosphodiesterase (1.6 million units/mg DNA) in added 20 mM ammonium acetate, pH 5.0. P1 nuclease (1 mg/ml; 1 h at 37°C) was used for dephosphorylation of normal nucleotides. In one experiment, DNA up to 40 μg was used in the HPLC analysis. This was achieved by combining four 10-μg labeling mixtures.

The modified nucleotides were converted to ³²P-postlabeled diphosphates in labeling mixture (2 μl containing 2.4 units T4 polynucleotide kinase and 2.3 pmol ATP (7 μCi [³²P]ATP, 3000 Ci/mmol). The reaction was carried out at pH 9.6. For HPLC analysis, the mixtures were diluted to 20 μl with water, and the whole sample was injected into the Beckman HPLC System Gold equipped with a Phenomenex Kromasil C₁₈ (12 × 250 mm; particle size, 5 μm) column. A precolumn filter was installed in front of the analytical column. The volume of the sample loop was 20 μl.

Radioactivity was measured on-line with a Beckman 171 radioisotope detector. The size of the Teflon sample loop in the flow cell was 75/100 μl, which was then folded into a scintillation tube containing scintillation liquid (Ready Safe; Beckman Instruments). The adducts were quantified by integration of the peak area after subtraction of background radioactivity.

Separations were carried out at an ambient temperature using a binary gradient with methanol and 0.2 M ammonium formate (pH 5.4) adjusted to pH

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³ The abbreviations used are: HPLC, high-performance liquid chromatography; D&C, dilation and curettage.
4.2 with phosphoric acid (resulting in a final concentration of 20 mM with respect to phosphoric acid). Labeled samples were analyzed using two different gradients. Gradient A had initial conditions of 2% methanol for 5 min, after which the proportion of methanol increased linearly to 70% in 65 min and then further to 100% in 5 min. The 100% methanol was maintained for 10 min before a linear decrease to 2% in 10 min. In gradient B, the proportion of methanol increased to 40% in 40 min, to 45% in 10 min, and was maintained isocratic for 15 min, during which time the tamoxifen fraction eluted. After that the methanol concentration was increased to 100% in 20 min. The flow rate was 0.25 ml/min.

TLC analyses were performed on 10 x 10-cm polyethyleneimine-cellulose TLC plates (Macherey-Nagel), as described (11), but developed only in directions 2 and 3. The plates were washed by flotation in water between each dimension. The adducts were detected in a Fuji X BAS 2000 phosphoimager.

Analysis of Results. Adduct levels were calculated from the radioactivity of the samples, background subtracted, assuming 100% recovery, and labeling efficiency. Because these are never 100% (21, 22), the apparent adduct levels are underestimates. In the HPLC analysis, the background was obtained by taking the average of radioactivity eluting before and after the tamoxifen peak; in the TLC analysis, the background was an empty area of the same TLC plate. The approximate detection limit using such a background definition was 1 adduct/10^6 nucleotides in the HPLC analysis and 0.2/10^6 in the TLC analysis. For evaluation of statistical significance, Student's t test with Levine’s adjustment for variance was applied.

Results

Endometrial DNA from a control and a tamoxifen-treated patient was analyzed using HPLC (Fig. 1). An adduct peak coeluting with liver DNA from tamoxifen-treated animals was observed at 56 min. In control samples, a small amount of radioactivity was observed at that elution time.

When a mixing experiment was carried out by spiking a liver DNA sample from a tamoxifen-treated rat (Fig. 2A) with an equal amount of radioactivity from a human endometrial sample of a tamoxifen-treated patient (Fig. 2B), a single peak was obtained (Fig. 2C), suggesting that the material is identical.

The tamoxifen adduct peak collected from HPLC was further analyzed in TLC and a single main spot was obtained comigrating with the rat liver standard. The one from a control patient was faint, indicating the presence of some background material, whereas a dominant spot was obtained from a treated patient (Fig. 3).

The above HPLC analyses were carried out using gradient A. Another system, gradient B, was devised to elute a tamoxifen adduct in an isocratic part of the program to maximize separation. A somewhat broader tamoxifen adduct, constituting multiple fractions, was noted in this system as compared to system A (Fig. 4).

The adduct was found only in tamoxifen-treated patients and not in control patients.

Among the 12 samples analyzed in this study, 7 were from tamoxifen-treated patients. When analyzing coded samples with HPLC using gradient A, five samples were considered positive (i.e., radioactivity was over twice the background in at least 50% of the analyses), and they were all obtained from the patients who had indeed received the drug. None of the controls were positive. The difference in the adduct levels between the tamoxifen-treated...
Fig. 3. TLC analysis of endometrial DNA from a control patient (A) and a tamoxifen-treated patient (B). The samples were first analyzed using HPLC, and the tamoxifen-DNA adduct fraction was collected and then analyzed using TLC in directions D2 and D3. The adduct spots comigrating with the main rat liver adducts are marked (a and b). The TLC plates were analyzed by a phosphorimager.

patients, 8.2 adducts/10⁹ (n = 7; SD, 3.9), and the controls, 2.4 adducts/10⁹ (n = 5; SD, 3.9) is statistically significant (P < 0.05, t test). The adduct levels were lower when gradient B was used: 2.7/10⁹ in the tamoxifen-treated patients and nondetectable (i.e., below 1/10⁹) in the controls.

Discussion

This is the first report identifying tamoxifen-induced DNA adducts in humans. The identification was based initially on HPLC analysis of coded samples. Of 12 samples, 5 were considered positive and 7 negative using the rat liver adduct as a standard in a blinded analysis. All five were tamoxifen-treated patients. Thus, no false positives but two false negatives were detected. After opening the code, several studies were carried out to confirm the result, including analysis in two HPLC systems, spiking experiments with rat liver DNA samples in two systems, and HPLC-TLC analysis. These data should leave little doubt about the identification of true tamoxifen adducts. However, because endometrial samples contain some blood and stromal cells, the adducts detected cannot be ascribed to endometrial DNA alone.

The apparent level of endometrial adducts was 2.7/10⁹ nucleotides, which is somewhat lower than that recently observed for leukocytes of different breast cancer patients, approximately 5/10⁹. However, since the adducts have not been characterized chemically and thus the total recovery of the method cannot be assessed, the present adduct level is most likely an underestimate (22, 23). The work on active intermediates and binding sites in DNA facilitates the final characterization, which would then allow comparisons to other types of adducts (8–14, 24, 25).

In previous work, no adducts have been detected in human liver (17), leukocytes (19), or endometrium (18). The main difference in the present work is that we used HPLC whereas the others used TLC in the separation of the adducts. We have recently been able to demonstrate tamoxifen-related leukocyte adducts in breast cancer patients also using the HPLC but not the TLC method. The HPLC method helps to reduce background radioactivity, thus boosting the effective level of detection to about 1 adduct/10⁹.

Although a similar or higher sensitivity can be achieved using TLC (19–21, 26), the high background may reduce the effective sensitivity of the TLC method (18). Thus, we assume that identification of adducts in endometrial samples was only possible using the HPLC method. Other advantages of the HPLC method are its reproducibility and the possible applicability to a large number of clinical samples.

Tamoxifen binds to DNA, causing adducts and conforming to a common mechanism by which chemicals are thought to cause mutations and cancer. However, due to its hormonal effects, tamoxifen may well have other carcinogenic mechanisms (16). Because the level of DNA adducts in populations is usually indicative of a risk of cancer (22), adduct studies in humans would be informative with regard to the cancer risks posed by this drug. Conversely, the role of DNA adducts in cancer, thus far supported largely by mechanistic and circumstantial evidence, can be tested in systems such as tamoxifen treatment. The present results on the identification of DNA adducts of a specific human carcinogen using the IARC criteria in target tissue are unique, assuming that the adducts are indeed derived from endometrial cell DNA. Using such a strict definition, the only other single agent causing specific DNA adducts in human target tissues is benzidine, recently identified in bladder cells from benzidine-dye workers (27).

DNA adduct determinations should have a natural application in comparative risk estimation in the search for safe drugs. Because the extent of DNA adduct formation among structural analogues may be related to their carcinogenic potency, it would be informative to
compare DNA-binding properties of analogues with similar therapeutic potencies.

References

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