Immunohistochemical localization and semi-quantitation of hepatic tamoxifen–DNA adducts in rats exposed orally to tamoxifen

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Introduction

The non-steroidal anti-estrogen tamoxifen (TAM) is widely used as adjuvant therapy for all stages of breast cancer. In addition, it is administered as a prophylactic drug for the prevention of breast cancer in high-risk women (1,2). However, TAM therapy is associated with an increase in incidence of both premalignant and malignant lesions of the human endometrium (3–5). Chronic oral exposure to TAM has been shown to induce tumors in rodents (6–8) and these observations have raised concerns regarding use of the drug in large-scale prophylactic trials involving healthy women considered to be at high risk of developing breast cancer.

The association of TAM–DNA adduct formation with TAM rodent tumorigenicity has been widely documented (9–12) as TAM–DNA adducts have been detected in target organs of exposed experimental animal models. However, localization and visualization of TAM–DNA adducts in target tissues have not been previously described. The 32P-post-labeling, accelerator mass spectrometry, HPLC and immunoblot methods used currently for quantitating TAM–DNA adducts require the isolation of genomic DNA and therefore do not allow direct morphological localization of TAM–DNA adducts. The immunohistochemical approach offers localization of TAM–DNA adducts at the cellular level and takes into account the tissue architecture.

In the present study we report detection and visualization of TAM–DNA adducts in liver tissue, a target for TAM-induced carcinogenesis in the rat. Polyclonal antibodies elicited against TAM–DNA (13) were used for direct semi-quantitation of TAM–DNA adducts in sections of paraaffin-embedded liver from rats exposed to TAM. The localization of TAM–DNA adducts was achieved by immunohistochemistry and semi-quantitation was achieved using an Automated Cellular Imaging System (ACIS). The ACIS is a robotic bright field microscope module equipped with advanced color space transformation software that allows sensitive and specific identification of the color of interest in nuclei or cytoplasm of cells or tissue sections. A dose–response relationship for hepatic TAM–DNA adducts determined by ACIS has been validated by quantitative comparison of TAM–DNA adducts in extracted rat liver DNA using a chemiluminescence immunoassay (CIA) (13). We also investigated TAM–DNA adduct formation in hepatic glutathione S-transferase, placental form (GST-p)-altered foci, which increased in number with TAM dose level.

Materials and methods

Rat maintenance and exposure

Timed pregnancy, late gestation Sprague–Dawley rats were obtained from Harlan Sprague Dawley. At 4 weeks of age neonatal female pups were retained for the study described. At the time of weaning, 28 days of age, female animals were fed a Teklad mouse/rat powdered basal diet containing 4% fat (Teklad Test Diets; Harlan Teklad, Madison, WI). Fifteen animals per group were fed this basal diet, to which TAM (Sigma-Aldrich Chemical Co., St Louis, MO) was added at levels of 0, 5, 50, 150 or 500 mg TAM/kg diet.
The animals were exposed continuously until the experiment was terminated after 45 weeks of TAM feeding. The tissue slices were processed, embedded in low melting point paraffin and sectioned with a microtome at a thickness of 5 µm thickness were obtained. Sequential sections were stained for hematoxylin and eosin, GST-p and TAM-DNA adducts.

**TAM-DNA staining by immunohistochemistry**

Liver sections were deparaffinized as follows: xylene, two changes of 3 min each; 100% ethanol, two changes of 1 min each; 90% ethanol, 1 min; 80% ethanol, 1 min; 70% ethanol, 1 min; 50% ethanol, 1 min; 25% ethanol, 1 min; deionized water, 1 min; phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺, two changes of 1 min followed by one change of 30 min. For antigen retrieval by microwaving, slides were incubated with 10 µg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) at room temperature (22°C) for 10 min, rinsed in deionized water and placed in a plastic staining holder. Empty slots in the holder were filled with blank slides. The holder was placed in a microwave-resistant container containing 250 ml of 1 × Antigen Retrieval Citra solution (BioGenex, San Ramon, CA). Microwaving was performed at high power for 2 min to bring the solution to a quick boil, followed by 15 min on low power (50% level) to simmer the slides. During simmering, a glass beaker containing 500 ml of deionized water was kept along with the slide container to absorb excess heat and maintain a constant temperature. At the end of microwaving the container holding the slides was allowed to reach room temperature and the slides were rinsed with several changes of deionized water followed by equilibration in PBS for 3 min at 22°C. Non-specific binding was blocked by incubation of slides with 0.25% casein (containing ultra low alkaline phosphatase; Tropix, Bedford, MA) for 20 min. Slides were then incubated with anti-TAM-DNA rabbit antiserum (13) diluted 1:5000 in 0.25% casein in PBS with 0.05% Tween 20, applied (by hand and incubated for 12 h at 4°C. Amplified color development was achieved with one cycle of amplification using a peroxidase-linked anti-rabbit IgG antibody (1:2000 dilution, 30 min at 22°C), biotinylated tyramide and H₂O₂ (Dako Corp., Carpinteria, CA), streptavidin linked to alkaline phosphatase (30 min at 22°C (BioGenex), fast red as a substrate (6–10 min at 22°C (BioGenex) and aqueous hematoxylin (BioGenex) as a counterstain (20 s).

Control experiments, carried out in order to ensure that the observed staining was due to the presence of TAM-DNA adducts, included: immunohistochemical staining with TAM-DNA antiserum preabsorbed with the immunogen TAM-DNA; treatment of sections with DNase before immunostaining; incubation with normal rabbit serum or irrelevant secondary antibodies. To prepare preabsorbed serum, TAM–DNA antiserum was used in combinations with biotinylated anti-mouse IgG, an irrelevant secondary antibody (1:10 dilution in PBS), giving a final concentration of 250 µg TAM-DNA/ml antiserum. Two preabsorptions, one for 4 h at 22°C and one at 4°C overnight, were performed with centrifugation (3000 r.p.m.) after each incubation. The resulting supernatant was diluted to the same extent as the specific antiserum and used routinely as a control. In another control experiment the sections were pretreated with DNase (100 U/ml for 1 h at 37°C (Promega, Madison, WI)) and rinsed with water, followed by applying the 10× objective of PBS before incubation with the specific antiserum. In a third type of control experiment, normal rabbit antiserum was used at a dilution equivalent to that of the TAM-DNA antiserum to determine the level of non-specific staining generated by the detection system. Finally, the TAM–DNA rabbit antiserum was used in combination with biotinylated anti-mouse IgG, an irrelevant secondary antibody, as an additional control.

**Quantitative imaging using ACIS**

Sections were scanned and images were captured using ACIS (ChromaVision, San Juan Capistrano, CA). The instrument uses an Olympus bright field microscope equipped with an automated robotic slide moving platform, 4×–60× objectives, a charge coupled display (CCD) camera and ACIS 1.81 Microdensitometry Software (ChromaVision). The CCD camera produces a voltage signal proportional to transmitted light intensity, which is then converted into a numerical density (intensity) measurement. Entire tissue sections or designated regions were scanned using the 10× objective and images were captured using the 40× objective. Typically 10 000–20 000 hepatic nuclei were counted and the average nuclear intensity for all nuclei in a section or defined circle/square/rectangle of tissue was expressed in arbitrary units. The ACIS advanced color space transformation software provides sensitive and specific identification of the color of interest and the parameters recorded for imaging technology allow sensitive and specific identification of morphological changes. Therefore, the instrument has the capacity to select a specific color of interest (hue) within the morphological feature of interest and to express the intensity of nuclear staining (luminosity). Thresholds for nuclear color (hue) were set on a slide containing visually negatively stained cells with hematoxylin (blue) nuclear staining (hue range 160–195). The nuclear threshold in cells with no adducts was able to mask the blue nuclei in positive cells and therefore only the presence of the pink color of interest was detected. The non-specific cytosolic light pink background (hue range 1–20) was filtered using the appropriate color (hue) threshold. The color threshold for TAM-DNA adducts was high enough to detect faint to intense pink color (hue range 200–246) staining in nuclei positive for adduct. Thresholds for intensity (luminosity) for light pink cytosolic background and intense pink nuclear staining were set at the ranges 180–215 and 45–170, respectively. Using a 7 µm morphological filter it was possible to eliminate the contribution of cytosol intensity (luminosity) to nuclear staining. The luminosity thresholding range chosen took into consideration the slight differences in counterstaining between slides, to which ACIS is very sensitive. After the thresholds were set, the entire section or specific defined areas were scanned using the 10× objective and an intensity profile was obtained.

**GST-p immunohistochemistry**

Staining with a GST-p antiserum was used to localize enzyme-altered and non-altered regions of the liver. After deparaffinization and antigen retrieval, as described above, sections from between three and five rats of each experimental group were incubated with anti-GST-p antibody (Biogenex), followed by biotinylated anti-rabbit antibody and streptavidin–alkaline phosphatase, with new fuchsin as substrate and hematoxylin as counterstain. Control experiments were carried out with normal rabbit serum. The slides were scored for number of foci positive for cytoplasmic GST-p staining and the results are expressed as number of foci per 20 000 cells (measured using ACIS) in the tissue section.

**CIA of TAM-DNA adducts**

DNA was extracted by a non-organic extraction method (Stratagene, La Jolla, CA) followed by digestion with 1 U/ml amyleglosucidase (Boehringer Mannheim). Subsequently, DNA (3–10 µg) was sonicated (15 s) and heat denatured (5 min at 95°C) before being subjected to adduct quantitation by TAM-DNA CIA (13). Sample quantitation was achieved by comparison with a TAM-DNA standard curve in which 0.5 ± 0.12 fmol TAM in TAM–DNA gave 50% inhibition. Since up to 20 µg DNA could be analyzed, the lower limit of detection was calculated as ~10 amol adduct/µg DNA, or ~3 adducts/10⁹ nt.

**Results**

**Immunohistochemical detection and semi-quantitation of TAM-DNA adducts in livers of TAM-exposed rats**

A dose-dependent increase in TAM–DNA adduct nuclear staining was determined by ACIS in hepatic nuclei from rats (n = 3) administered 5, 50, 150 and 500 p.p.m. TAM/kg diet for 45 weeks (Table I). Representative specific staining for TAM–DNA adducts in livers of rats exposed to 0, 150 and 500 p.p.m. TAM for 45 weeks is shown in Figure 1. The presence of TAM-DNA adducts (pink-red staining) is evident in livers from TAM-fed rats (Figure 1D and F), but not in the unexposed control (Figure 1B). The TAM–DNA adduct staining was homogeneously distributed throughout the hepatic lobes, although a slight intralobular difference in color intensity was occasionally observed. The staining appeared primarily in

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**Table I. TAM-DNA adduct levels determined in livers of rats exposed to dietary TAM, at the indicated doses, for 45 weeks**

<table>
<thead>
<tr>
<th>TAM dose (p.p.m.)</th>
<th>TAM-DNA adducts</th>
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<tbody>
<tr>
<td></td>
<td>CIA (adducts/10⁸ nt)</td>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>5</td>
<td>16.7 ± 9.0</td>
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<tr>
<td>50</td>
<td>71.5 ± 2.7</td>
</tr>
<tr>
<td>150</td>
<td>122.6 ± 13.0</td>
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<tr>
<td>500</td>
<td>212.2 ± 26.5</td>
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The table shows a comparison between TAM-DNA immunostaining by ACIS and TAM-DNA adduct determination in DNA by CIA. Values represent means ± SD of three animals.
Fig. 1. Representative immunohistochemical staining for TAM–DNA adducts (pink-red color) in liver sections from rats fed 0 (A and B), 150 (C and D) and 500 p.p.m. TAM (E and F) in the diet for 45 weeks. Staining with rabbit TAM–DNA antiserum is shown in (B), (D) and (F), while staining with normal rabbit antiserum is shown in (A), (C) and (E). Counterstaining with aqueous hematoxylin (blue color) reveals localization of the nuclei. 600× magnification.

Parenchymal cells and no staining was observed in bile duct cells. Nuclear staining was very intense in liver sections from rats fed 150 and 500 p.p.m. TAM and at these doses a weaker cytosolic pink staining was observed.

Substitution of the TAM–DNA antiserum by normal rabbit serum (Figure 1A, C and E) or substitution of the secondary antibody by non-specific secondary antibody (data not shown) abrogated the pink nuclear staining, while the blue hematoxylin
staining of the nuclei remained. The specificity of the immunostaining was further confirmed by preabsorption of the TAM–DNA antiserum with an excess of the immunogen TAM–DNA. Incubation of slides with preabsorbed anti-TAM–DNA produced a very weak staining of hepatic cytosol but no nuclear staining (data not shown). In addition, pretreatment of slides with DNase virtually abolished nuclear TAM–DNA staining (data not shown).

Validation of the ACIS for TAM–DNA adduct determination

In order to validate the numbers obtained with ACIS, a quantitative TAM–DNA CIA (13) was used to determine TAM–DNA adducts in additional rats (n = 3 per dose) fed the same TAM doses (0, 5, 50, 150 and 500 p.p.m.) in the diet for 45 weeks. Table I lists the values obtained using both ACIS and CIA and Figure 2 demonstrates the correlation of the data obtained by the two methods. There was a strong correlation (r = 0.9236) between the arbitrary intensity units obtained by immunohistochemistry/ACIS and values generated by quantitative CIA. Since the rats were subjected to chronic TAM feeding for 45 weeks, the TAM–DNA adduct values at each dose reflect both DNA adduct formation and DNA adduct removal.

GST-p-altered foci

The foci positive for expression of GST-p in liver sections from TAM-exposed rats (n = 5 per dose) were examined for TAM–DNA adduct formation. Representative GST-p staining of unexposed liver and TAM-exposed liver are shown in Figure 3A and B, respectively. No foci were observed in livers from five unexposed rats. Expression of GST-p is observed as red staining localized in the cytoplasm with intense nuclear membrane staining and faint nuclear staining. The number and size of the GST-p foci (Figure 3B) increased with increasing TAM dose with an average of 253 foci/liver, including all three liver lobes, observed in rats fed 500 p.p.m. TAM.

The levels of TAM–DNA adducts and GST-p foci increased with increasing TAM dose (Figure 4). In addition, examination of many foci revealed that there was virtually no TAM–DNA staining (Figure 3D) within the GST-p focal areas (Figure 3C and D). However, within some of the GST-p foci there were clusters of cells that were negative for GST-p (Figure 3C and E) and that showed intense staining for TAM–DNA adducts (Figure 3D and F). Similar to studies with other hepatocarcinogens, including aflatoxins and N-2-acetylaminofluorene (14), these data suggest that the GST-p foci induced by chronic TAM administration can either detoxify TAM more efficiently than normal liver or fail to activate the drug. In addition, the small adduct-positive focal areas within the GST-p foci have presumably undergone a second mutagenic event that alters the original change and results in the formation of TAM–DNA adducts.

Discussion

In this report we have described and validated a unique semi-quantitative immunohistochemical staining method for the determination of TAM–DNA adducts. This immunohistochemical semi-quantitation is made possible through the use of the ChromaVision ACIS, a novel bright field microscope and image analyzer that provides arbitrary numbers for the intensity of nuclear color staining for tissue sections and is able to count 10 000–20 000 cells within minutes. An entire tissue section is scanned, using an automated bright field microscope equipped with a robotic slide platform, and the program calculates an average color intensity score per nucleus. Photographic images are obtained using a CCD camera and stored for permanent record. The values determined by ACIS in liver sections from rats fed TAM at different levels in the diet were compared with quantitative determination (CIA) of TAM–DNA adducts in liver DNA from similarly exposed rats and results from the two assays were highly correlated. The data therefore suggest that semi-quantitative immunohistochemical staining for TAM–DNA adducts will be a powerful tool for many different types of studies where only small amounts of tissue are available.

In addition, these experiments demonstrated that, similar to a number of other chronically administered hepatocarcinogens (14–16), TAM induces the formation of hepatic enzyme-altered foci that have lost the capacity to metabolize the drug to DNA-binding species. In this study foci expressing GST-p were induced in a dose-related fashion and were lacking TAM–DNA adducts. Within some of the large GST-p-positive foci occasional islets of cells were negative for GST-p and positive for TAM–DNA adducts, suggesting that a further mutation may have altered expression and/or activity of drug metabolizing enzymes (17,18), resulting in a changed focus phenotype. Previous studies, both in the liver and in the skin, have demonstrated that malignant conversion of a benign tumor (skin) (19,20) or an enzyme-altered focus (liver) (21) may arise subsequent to a mutation that produces a malignant clonal expansion of cells within a larger benign focus of altered phenotype. Both in the liver (21) and in the skin (19,20) it has been possible to observe very small foci of more malignant phenotype located within the original benign clonal expansion.
The antibody used here to stain liver sections from rats given dietary exposure to TAM was elicited against TAM–DNA (13). In the CIA (13) the antibody showed cross-reactivity with TAM alone at concentrations 5000-fold higher than the TAM–DNA adduct concentration. The CIA 50% inhibition occurred at 5440 ± 100.2 fmol with TAM compared with 0.8 ± 0.13 fmol with the TAM–DNA adduct (13). In the present study there was some cytoplasmic staining that might have been the result of reactivity of antibody either with TAM alone, with TAM-modified mitochondrial DNA or with TAM-modified protein. A decrease in cytoplasmic background was observed when the sections were incubated with proteinase K for extended periods of time, but this treatment resulted in loss of cell structural integrity and was therefore not feasible to apply routinely. Fortunately, the sophistication of the ACIS software routines made it possible to subtract the contribution of cytoplasmic staining, thereby removing its influence from the nuclear intensity values.
the presence of TAM–DNA adducts in tissue from TAM-exposed women. Currently we are exploring the possibility of obtaining human endometrium for immunohistochemical studies.

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References


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