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Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis

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Tamoxifen is a non-steroidal anti-estrogen used for the treatment of breast cancer and, more recently, as a chemopreventive agent in healthy women at high risk of developing breast cancer. On the other hand, tamoxifen is a potent hepatocarcinogen in rats, with both tumor-initiating and tumor-promoting properties. There is substantial evidence that hepatic tumors in rats are initiated as a result of formation of tamoxifen–DNA adducts; however, events subsequent to DNA adduct formation are not clear. Recently, it has been demonstrated that genotoxic carcinogens, in addition to exerting genotoxic effects, often cause epigenetic alterations. In the current study, we investigated whether or not the mechanism of tamoxifen-induced hepatocarcinogenesis includes both genotoxic and epigenetic components. Female Fisher 344 rats were fed a 420 p.p.m. tamoxifen diet for 6, 12, 18 or 24 weeks. Hepatic tamoxifen–DNA adduct levels, as assessed by high-performance liquid chromatography and electrospray tandem mass spectrometry, were 580 adducts/10^8 nt at 6 weeks, and increased to ~1700 adducts/10^8 nt by 18 weeks. Global liver DNA hypomethylation, as determined by an HpaII-based cytosine extension assay, was increased at all time points, with the maximum increase (~200%) occurring at 6 weeks. Protein expressions of maintenance (DNMT1) DNA methyltransferase and *de novo* DNA methyltransferases DNMT3a and DNMT3b were decreased at all time points. Likewise, trimethylation of histone H4 lysine 20 was significantly decreased at all time points. In contrast, non-target tissues (i.e. mammary gland, pancreas and spleen) did not show any changes in global DNA methylation or DNA methyltransferase activity. These data indicate the importance of genotoxic and epigenetic alterations in the etiology of tamoxifen-induced hepatocarcinogenesis.

Abbreviations: dG-DesMeTam, (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen; dG-Tam, (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen; F344, Fisher 344; GST-P, glutathione *S*-transferase placental form; MRM, multiple reaction monitoring; PCNA, proliferating cell nuclear antigen.

Introduction

Tamoxifen is a non-steroidal anti-estrogen that has been used in the treatment of breast cancer for >25 years (1) and lately as a long-term chemopreventive agent for breast cancer in healthy women at high risk of developing breast cancer (1,2). Although beneficial for preventing the occurrence or re-occurrence of breast cancer, the use of tamoxifen has been shown to increase the incidence of endometrial cancer and venous thromboembolism (3,4). Additionally, tamoxifen is a potent hepatocarcinogen in rodents (5,6), with both initiating (7) and promoting (8) properties. There is substantial evidence that hepatic tumors in rats are due to a genotoxic mechanism resulting from formation of tamoxifen–DNA adducts (9–13); however, events subsequent to DNA adduct formation are not clear.

Recently, it has been demonstrated that genotoxic carcinogens, in addition to exerting genotoxic effects, often cause epigenetic alterations (14). Furthermore, it is becoming increasingly evident that these induced epigenetic changes may play a role in mechanisms of carcinogenesis (14,15). Specifically, progressive loss of cytosine DNA methylation (i.e. DNA hypomethylation) induced experimentally by different carcinogens often takes place at early stages of tumor development (16–20). However, it has not yet been established whether epigenetic changes induced by carcinogens and found in tumors play a causative role in carcinogenesis or are merely a consequence of the transformed state (14,21). In our previous studies using a methyl-deficient model of hepatocarcinogenesis in rats, we showed that stable irreversible DNA hypomethylation is a key step in carcinogenic process induced by methyl deprivation and is specific only to the carcinogenesis target tissue (22,23). A recent study by Yamada *et al.* (24), showing that hypomethylation of DNA caused the development of multiple liver tumors in $APC^{-/+}$ mice, provided additional evidence of the importance of epigenetic alterations, specifically hypomethylation of DNA, in the origin of cancer. Based on these considerations, the present study was undertaken to determine whether the mechanism of tamoxifen-induced hepatocarcinogenesis includes both genotoxic and epigenetic components, and whether the epigenetic alterations observed early in liver tissue in the methyl-deficient model of hepatocarcinogenesis also occur with a genotoxic carcinogen and are specific to target tissue only.

We demonstrate that long-term exposure of female Fisher 344 (F344) rats to tamoxifen-containing diet leads to the formation of tamoxifen–DNA adducts, a substantial decrease in cytosine DNA methylation, and a decrease histone H4 lysine 20 trimethylation in liver tissue accompanied by altered activity and expression of maintenance and *de novo* DNA methyltransferases. These epigenetic alterations are specific for liver tissue only and are unaffected in non-target tissues such as pancreas, spleen and mammary glands. The results of the study provide the first conclusive evidence of the

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importance of both genotoxic and epigenetic alterations in the etiology of tamoxifen-induced hepatocarcinogenesis.

Materials and methods

Animals, treatments and tissue preparations

Female weanling F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed two per cage in a temperature-controlled (24°C) room with a 12-h light/dark cycle, and given *ad libitum* access to water and NIH-31 laboratory diet. At 6 weeks of age, the rats (body weight 150 g) were allocated randomly to receive either NIH-31 diet containing 420 p.p.m. tamoxifen (Dyets, Bethlechem, PA) or control NIH-31 diet. Diets were stored at 4°C and given *ad libitum* with biweekly replacement. Five rats per diet group and five rats per control group were sacrificed at 6, 12, 18 and 24 weeks after diet initiation. The livers, pancreas, kidneys, spleens and mammary glands were excised, frozen immediately in liquid nitrogen and stored at −80°C for subsequent analyses.

Determination of tamoxifen–DNA adduct levels

DNA was isolated from rat liver tissue using standard digestion with proteinase K, followed by standard phenol–chloroform extraction and ethanol precipitation (25). The DNA samples were hydrolyzed to nucleosides and analyzed for (*E*)-α-(deoxyguanosin-N^2-yl)-tamoxifen (dG-Tam) and (*E*)-α-(deoxyguanosin-N^2-yl)-*N*-desmethyltamoxifen (dG-DesMeTam) by electrospray ionization tandem mass spectrometry (ES-MS/MS) coupled with online sample preparation and high-performance liquid chromatography [HPLC; (26)] as described in Schild *et al*. (27).

Determination of methylation changes at CCGG sites in DNA by cytosine extension assay

Genomic DNA was isolated from frozen liver, pancreas, kidneys, spleen and mammary gland by digestion with proteinase K, followed by standard phenol–chloroform extraction and ethanol precipitation (25). The extent of the global DNA methylation was evaluated with a radiolabeled [^{3}H]-dCTP extension assay as described previously (28). Briefly, 1 µg of genomic DNA was digested with 20 U of methylation-sensitive HpaII or AciI restriction endonucleases (New England Biolabs, Beverly, MA) for 16–18 h at 37°C. A second DNA aliquot (1 µg) was digested with methylation-insensitive isoschizomer MspI, which cleaves CCGG sites in DNA regardless of CpG methylation status, to serve as a control for the digestion efficiency. Undigested DNA served as background control. The single nucleotide extension reaction was performed in a 25 µl reaction mixture containing 1.0 µg DNA, 1× PCR buffer II, 1.0 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 0.1 µl of [^{3}H]dCTP (57.4 Ci/mmol; Perkin Elmer Life and Analytical Sciences, Boston, MA) and incubated at 56°C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M sodium phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. [^{3}H]dCTP incorporation into DNA is expressed as mean disintegrations per minute (d.p.m.) per µg of DNA after subtraction of the d.p.m. incorporation in undigested samples (background).

Determination of DNA methyltransferase activity

The determination of DNA methyltransferase activity in tissue lysates was performed as described previously (29).

Analysis of expression of maintenance DNA methyltransferase (DNMT1) and de novo (DNMT3a and DNMT3b) DNA methyltransferases

Expression of DNMT1, DNMT3a, DNMT3b and proliferating cell nuclear antigen (PCNA) was determined by western immunoblotting analysis. Briefly, tissue samples were homogenized, sonicated in 500 µl of ice-chilled 1% sodium dodecyl sulphate (SDS) and boiled for 10 min. Protein concentrations were determined by the Bradford assay (Pierce, Rockford, IL). Equal amounts of proteins (10 µg) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) using slab gels of 12% polyacrylamide, made in duplicate, and transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were incubated with primary antibodies against DNMT1 (1:1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, Abgent, San Diego, CA) and PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with a horseradish peroxidase-conjugated secondary antibody, antibody binding was detected with an ECL Plus Immunoblotting Detection System (Amersham Biosciences). Chemiluminescence was detected by Biomax MR Films (Eastman Kodak, New Haven, CT). To confirm equal protein loading, unaltered PVDF membranes were stained with Coomassie Blue. Signals were quantified using NIH ImageJ 1.63 Software and normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Histone extraction

Acidic cell extracts were prepared from frozen liver tissues using a lysis buffer containing 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 500 µM DTT, 1.5 mM PMSF, followed by addition of HCl to a final concentration of 200 mM according to manufacturer's protocol (Upstate, Charlottesville, VA). Cell lysates were centrifuged at 14 000× *g* for 10 min at 4°C, and the acid-insoluble pellets were discarded. The supernatant fractions, which contain the acid soluble proteins, were purified by sequential dialysis against 100 mM acetic acid, then H_2O. Protein concentrations were determined by the Bradford assay and aliquots of total histones were stored at −80°C.

Analysis of trimethylation of histone H3 lysine 9 (H3K9) and histone H4 lysine 20 (H4K20)

Equal amount of total histones (40 µg) were mixed with two volumes of gel loading buffer [250 mM Tris–HCl (pH 8.0), 20% β-mercaptoethanol, 40% glycerol, 8% SDS, 1.2 mg/ml bromophenol blue], heated for 5 min at 95°C and resolved on 15% polyacrylamide gel. Proteins were transferred onto PVDF membranes. The membranes were blocked for 4 h in Tris-buffered saline (TBS) containing 5% non-fat dry milk and 0.1% Tween-20. Anti-trimethyl-histone H3K9 and anti-trimethyl-histone H4K20 primary antibodies were diluted 1:1000 and 1:2000, respectively, according to manufacturer's recommendations (Upstate). Primary antibody binding was performed at 4°C overnight with constant shaking. A secondary donkey anti-rabbit antibody, labeled with alkaline phosphatase (Santa Cruz Biotechnology), was applied at 1:5000 dilutions and binding was carried out at room temperature for 1.5 h. Chemifluorescence detection was performed with the ECF substrate for western blotting (Amersham Biosciences) and measured directly by a Storm Imaging System (Molecular Dynamics, Sunnyvale, CA). Images are representative of three independent immunoblots and were analyzed by ImageQuant software. All membranes were stained with Coomassie Blue and with anti-histone H3 and anti-histone H4 antibodies to confirm equal protein loading.

Analysis of expression of Suv-39h1 and Suv2-40h2 histone methyltransferases

Liver tissue lysates were prepared by homogenization of 50 mg of tissue in 500 µl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 µg/ml each aprotinin, leupeptin, pepstatin; 1 mM Na_3VO_4, 1 mM NaF), sonication and incubation at 4°C for 30 min, followed by centrifugation at 10 000× *g* at 4°C for 20 min.

Extracts containing equal quantities of proteins were separated by SDS–PAGE on 10% polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with antibodies specific for Suv-39h1 (Upstate) and Suv4-20h2 (Abcam) histone methyltransferases (HMTs) at 1:1000 and 1:1500 dilutions, respectively. Alkaline phosphatase-coupled donkey anti-rabbit secondary antibodies were used for visualization.

Immunohistochemistry

The status of glutathione *S*-transferase placental form (GST-P) expression in liver was determined in liver sections after 24 weeks of feeding the rats the tamoxifen-containing diet. Formalin-fixed paraffin-embedded liver sections were deparaffinized and re-hydrated. Endogenous peroxidase was inhibited by incubation with freshly prepared 3% hydrogen peroxide with 0.1% sodium azide for 10 min at room temperature. Non-specific staining was blocked with normal goat 10% serum (Sigma, St Louis, MO) for 20 min at room temperature. The sections were then incubated with rabbit polyclonal anti-human GST-P (DAKO, Carpinteria, CA) at the dilution of 1:100 (10 µg/ml) for 1 h at room temperature. The specificity of the DAKO antibody is similar to that reported for other polyclonal antibodies to GST-P (30), and the cross-reactivity of this antibody with rodent pi-class glutathione *S*-transferases has been demonstrated earlier (31). After incubation with primary antibody, tissue sections were incubated with biotinylated goat anti-rabbit IgG (ExtrAvidin Kit, Sigma) at a dilution of 1:30 for 30 min at RT, and later with streptavidin-conjugated horseradish peroxidase (ExtrAvidin Kit, Sigma) at the dilution of 1:30 for 30 min at room temperature. Staining was developed with diaminobenzidine substrate for 5 min at room temperature, and sections were counterstained with hematoxylin and mounted with Permount (Fisher Scientific, Pittsburgh, PA). For a negative control, 10 µg/ml rabbit IgG (Jackson Immunoresearch) or phosphate-buffered saline replaced the primary antibody. For the positive control, rat liver sections containing previously confirmed diethylnitrosamine-initiated foci were immunostained for GST-P. All sections were examined by light microscopy (BX40, Olympus, Japan).

Statistical analysis

The extent of DNA methylation, DNA methyltransferase activity, was assessed by two-way ANOVA, using treatment and weeks as fixed factors. Pair-wise comparisons were conducted with Student-Newman-Keuls test. *P*-values <0.05 were considered significant.

Results

Hepatic tamoxifen–DNA adduct levels

The presence of dG-Tam and dG-DesMeTam in liver DNA was assessed by HPLC-ES-MS/MS after feeding rats 420 p.p.m. tamoxifen for 6, 12, 18 or 24 weeks. Representative multiple reaction monitoring (MRM) chromatograms are shown in Figure 1. Chromatograms A, B and C present the traces corresponding to the MRM of the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for 25 pg of the internal standard dG-Tam-d_6 (m/z 322 → 264). Chromatogram D is the trace corresponding to the MRM of the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for 25 pg of the synthetic standard dG-TAM (m/z 319 → 261). Identical MRM transitions are presented in Chromatograms E and F for rat liver DNA samples from a rat fed tamoxifen for 6 weeks and a control rat. In Chromatogram E a peak is clearly evident that corresponds to dG-TAM, whereas this peak in not present in Chromatogram F. Chromatogram G is the trace corresponding to the MRM of the transitions from the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for 50 pg of the synthetic standard dG-DesMeTam (m/z 312 → 254). Chromatograms H and I are the same MRM transitions for the rat liver DNA samples; dG-DesMeTam is clearly evident in Chromatogram H and is not present in the control sample (Chromatogram I).

The total levels of tamoxifen–DNA adducts increased with time until a plateau was reached after 18 weeks of feeding (Table I). dG-Tam accounted for 45% of the total tamoxifen–DNA adducts after 6 weeks of feeding, and decreased to 38% of the total tamoxifen–DNA by 18 weeks of feeding. dG-Tam and dG-DesMeTam were not detected (<3 adducts/10^8 nt) in hepatic DNA from control rats at any time point.

Effect of tamoxifen on DNA methylation in liver and non-target tissues

DNA methylation status was assessed with a sensitive cytosine extension assay that measures the proportion of unmethylated CpG sites in DNA. The assays are based on the ability of the methylation-sensitive restriction enzymes HpaII or AciI to cleave unmethylated CCGG (HpaII), CCGC and GCGG (AciI) sequences and leave a 5′ guanine overhang that can be used for the subsequent single nucleotide extension with labeled $[^3H]$dCTP (28). The extent of $[^3H]$dCTP incorporation is directly proportional to the number of unmethylated CpG sites. In livers of control rats, the extent of DNA methylation did not change over the 24 week period (Figure 2). In livers of rats fed tamoxifen, DNA became significantly hypomethylated after 6 weeks on diet (Figure 2). At that time, the incorporation of $[^3H]$dCTP into HpaII- or AciI-digested DNA isolated from livers of tamoxifen-treated rats was, respectively, 2.0 and 1.7 times greater than in the age-matched control animals. At later times (12, 18 and 24 weeks) the extent of DNA hypomethylation in livers of tamoxifen-treated rats decreased slightly compared to the 6 week value, but still remained 1.5–1.6 times greater than the control values. Treatment with tamoxifen did not change the extent of DNA methylation in the mammary gland, pancreas or spleen. In the kidney, tamoxifen treatment caused a 1.1- to 1.2-fold increase in DNA hypomethylation, with the difference being significant after 24 weeks of feeding (data not shown).

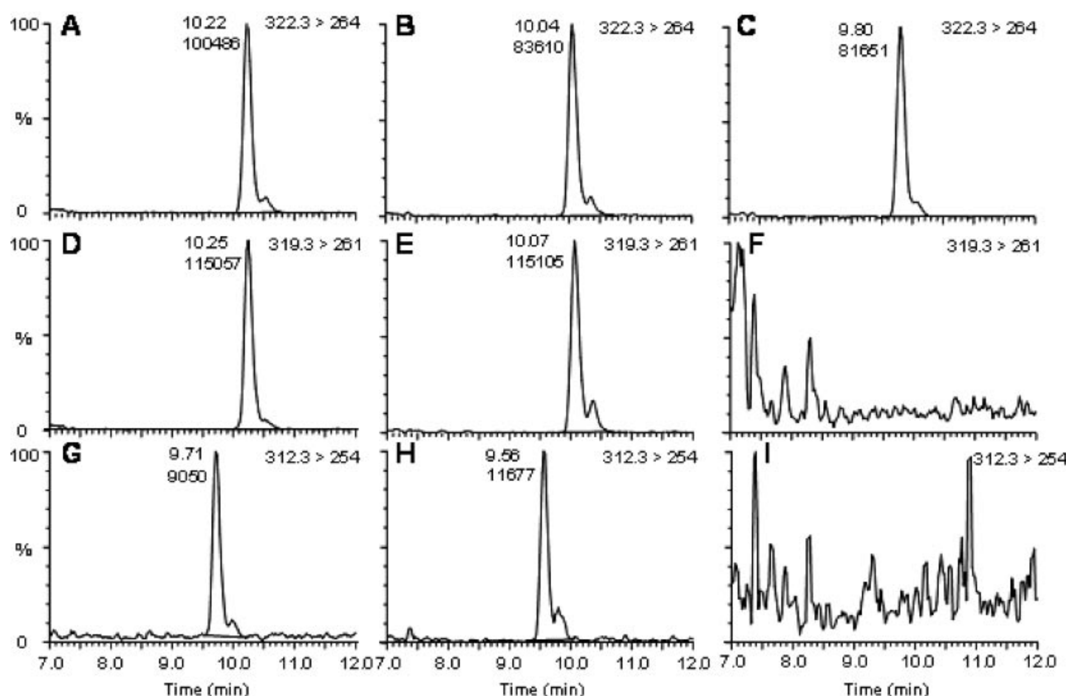

Fig. 1. HPLC-ES-MS/MS analyses of tamoxifen–DNA adducts. (**A–C**) MRM (relative signal intensity versus time) chromatograms for the doubly charged protonated nucleoside molecule $[(M + 2H)^{2+}]$ to the doubly charged protonated purine base $[(BH + 2H)^{2+}]$ for 25 pg of the internal standard dG-Tam-d_6 (m/z 322 → 264). MRM chromatograms for $[(M + 2H)^{2+}]$ to $[(BH + 2H)^{2+}]$ for 25 pg of synthetic dG-Tam (**D**) and DNA from tamoxifen-treated (**E**) and control (**F**) rats (m/z 319 → 261). MRM chromatograms for $[(M + 2H)^{2+}]$ to $[(BH + 2H)^{2+}]$ for 50 pg of synthetic dG-DesMeTam (**G**) and DNA from tamoxifen-treated (**H**) and control (**I**) rats (m/z 312 → 254). The retention times and areas are indicated for the most prominent peak is each chromatogram.

Table I. Hepatic tamoxifen–DNA adduct levels in rats fed 420 p.p.m. tamoxifen for 6, 12, 18 or 24 weeks[a]

Weeks of feeding	dG-TAM	dG-DesMeTAM	Total tamoxifen–DNA adducts
6	265 ± 13	318 ± 16	583 ± 21
12	493 ± 28	769 ± 43	1262 ± 66
18	631 ± 22	1020 ± 36	1651 ± 57
24	639 ± 56	1073 ± 87	1712 ± 143

[a]The tamoxifen–DNA adduct levels are expressed as adducts/10^8 nt (n = 5, mean ± SEM).

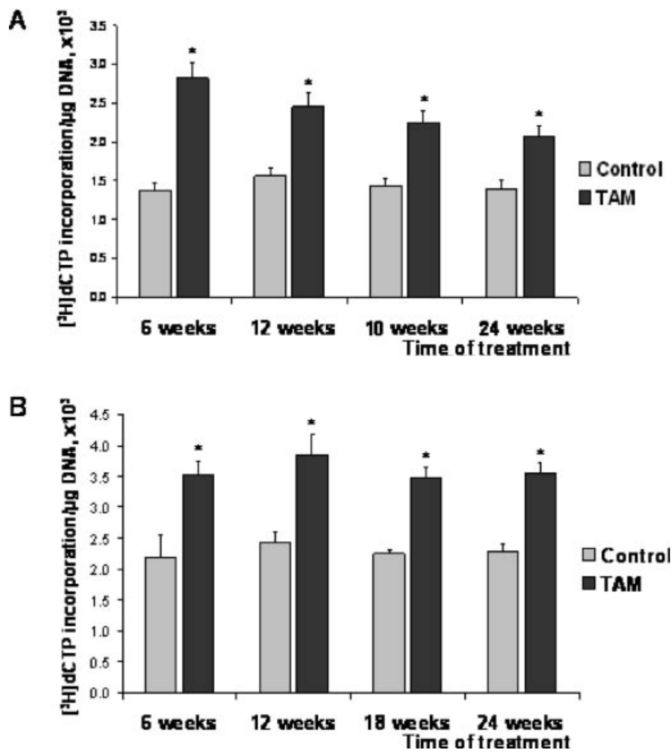

Fig. 2. Level of DNA methylation in liver of control rats and rats fed tamoxifen-containing diet. DNA methylation in liver of tamoxifen-treated and age-matched control rats were measured by the cytosine extension assay after treatment of DNA with methylation-sensitive restriction endonuclease HpaII (**A**) that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands, or after treatment of DNA with methylation-sensitive restriction endonuclease AciI (**B**) that has a non-palindromic recognition site and cleaves CCGC and GCGG sequences when cytosine residues are unmethylated on both strands. *Significantly different from control at the same time point (n = 5, mean ± SEM).

Effect of tamoxifen on activity of DNA methyltransferase in liver and non-target tissues

Since global loss of cytosine DNA methylation could be linked to altered activity and/or expression of DNA methyltransferases, the activity of endogenous DNA methyltransferase was analyzed in control and tamoxifen-fed rats. In rats fed tamoxifen, there was time-dependent increase in endogenous DNA methyltransferase activity in liver tissue with the increase becoming significant after 24 weeks of feeding (Figure 3). DNA methyltransferase activity was also measured in the kidneys, mammary gland and spleen after 12 and 24 weeks of feeding tamoxifen. The activity did not differ between the tamoxifen-exposed and age-matched control rats (data not shown).

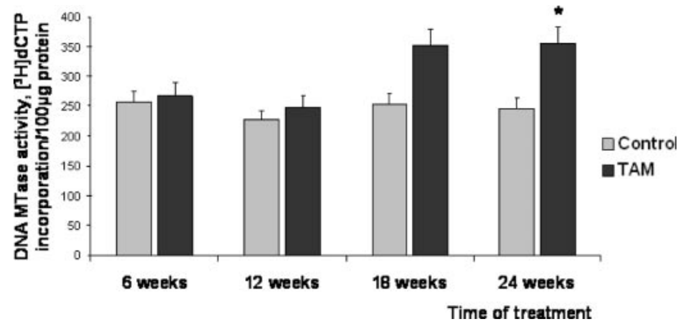

Fig. 3. DNA methyltransferase activity in liver of control and tamoxifen-treated rats. *Significantly different from control at the same time point (n = 5, mean ± SEM).

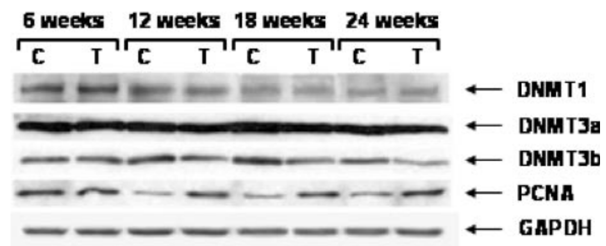

Fig. 4. Expression of DNA methyltransferases in liver of control rats and rats fed tamoxifen-containing diet. Liver tissue lysates were separated by SDS–PAGE and subjected to immunoblotting using specific antibodies against DNMT1, DNMT3a, DNMT3b and PCNA. Equal sample loading was confirmed by immunostaining against GAPDH. These results were reproduced in two independent experiments. Representative immunoblot images are shown.

Effect of tamoxifen on the expression of DNA methyltransferases in liver tissue

The observed changes in the level of DNA methylation and endogenous DNA methyltransferase activity in liver of tamoxifen-fed rats prompted us to investigate further the expression of maintenance (DNMT1) and *de novo* (DNMT3a and DNMT3b) DNA methyltransferases in liver of tamoxifen-exposed and control rats. DNMT1, DNMT3a and DNMT3b are the three main functional enzymes responsible for setting and maintaining DNA methylation patterns in mammalian cells. They have distinct abilities to catalyze maintenance and *de novo* DNA methylation. Deregulation of any or all of their expression may result in perturbations of DNA methylation patterns. Expression of the maintenance DNA methyltransferase DNMT1 in liver of tamoxifen-fed rats did not significantly change over 24 weeks of experiment (Figure 4, Table II). The expression of *de novo* DNMT3a showed slight, but statistically significant, decrease beginning after 18 weeks of feeding tamoxifen (Table II), and the expression of DNMT3b was substantially lower after 12 weeks of feeding tamoxifen (Table II). In contrast, the expression of PCNA in liver of tamoxifen-fed rats was significantly higher beginning after 6 weeks of feeding (Table II).

Considering the well-established link between expression of DNA methyltransferases and DNA replication (32,33), and fact that tamoxifen increased cell replicative activity (34,35), to determine precisely tamoxifen-induced changes in DNMTs expression in liver, we adjusted the protein level

Table II. Protein expression of DNMT1, DNMT3a, DNMT3b and PCNA in liver of F344 rats fed 420 p.p.m. tamoxifen for 6, 12, 18 or 24 weeks[a]

Weeks	DNMT1		DNMT3a		DNMT3b		PCNA	
	Control	TAM	Control	TAM	Control	TAM	Control	TAM
6	171 ± 12	201 ± 15 (117)	186 ± 13	178 ± 14 (95)	121 ± 9	133 ± 10* (110)	140 ± 12	127 ± 11* (91)
12	170 ± 11	125 ± 10 (74)	182 ± 12	183 ± 15 (101)	176 ± 13	119 ± 9* (68)	65 ± 5	117 ± 9* (180)
18	112 ± 8	96 ± 6 (86)	212 ± 16	184 ± 13* (87)	189 ± 14	110 ± 8* (58)	73 ± 6	112 ± 9* (154)
24	101 ± 7	122 ± 11 (121)	179 ± 12	152 ± 12* (85)	116 ± 8	44 ± 4* (38)	84 ±7	129 ± 10* (153)

[a]The protein expression levels are presented in digital light units (DLU) $\times 10^3$ ($n = 5$, mean ± SEM) and, in parentheses, the percentage of the control at the same time.
*Significantly different from control at the same time.

Table III. Protein expression of DNMT1, DNMT3a and DNMT3b normalized to PCNA level in liver of F344 rats fed 420 p.p.m. tamoxifen for 6, 12, 18 or 24 weeks

Weeks	DNMT1/PCNA		DNMT3a/PCNA		DNMT3b/PCNA	
	Control	TAM	Control	TAM	Control	TAM
6	1.22 ± 0.08	1.58 ± 0.12* (129)	1.33 ± 0.09	1.40 ± 0.09	0.86 ± 0.05	1.05 ± 0.06* (122)
12	2.62 ± 0.17	1.08 ± 0.07* (41)	2.81 ± 0.18	1.57 ± 0.11* (56)	2.73 ± 0.15	1.03 ± 0.07* (38)
18	1.54 ± 0.11	0.87 ± 0.05* (56)	2.93 ± 0.17	1.65 ± 0.10* (56)	2.60 ± 0.16	0.99 ± 0.04* (38)
24	1.21 ± 0.10	0.96 ± 0.06* (79)	2.13 ± 0.15	1.19 ± 0.08* (56)	1.38 ± 0.11	0.34 ± 0.01* (25)

[a]The data are expressed as the protein expression levels of DNMT1, DNMT3a and DNMT3b, as present in Table II), divided by the protein expression level of PCNA ($n = 5$, mean ± SEM) and, in parentheses, the percentage of the control at the same time.
*Significantly different from control at the same time.

of DNA methyltransferases to PCNA expression. The data presented in Table III show that long-term exposure of F344 rats to tamoxifen resulted in a decreased expression of DNMT1, DNMT3a and DNMT3b beginning after 6 weeks of feeding. This finding corresponds to the observation reported by Kimura *et al.* (36) showing decrease of DNMTs expression relative to cell proliferation in transitional cell carcinoma.

Effect of tamoxifen on histone H3K9 and H4K20 trimethylation and on expression of HMTs Suv-39h1and Suv2-40h2 in liver

Considering tight interaction between DNA methylation and modifications of histones (37,38) and the results of recent studies showing that aberrant histone modifications play an important role in carcinogenesis (38), we examined the alterations in the trimethylation of histones H3K9 (H3K9me3) and H4K20 (H4K20me3) in livers of rats exposed to tamoxifen. Figure 5A shows that feeding rats tamoxifen-containing diet resulted in rapid and sustained loss of histone H4K20me3. The level of histone H4K20me3 in liver of animals exposed to tamoxifen for 6 weeks was ~28% lower than in control group. After 12, 18 and 24 weeks of experiment, level of histone H4K20me3 in the livers of the rats fed tamoxifen was decreased by 32, 34 and 45%, respectively, compared to the age-matched control animals. In contrast, the extent of histone H3K9me3 in the livers of tamoxifen-treated animals increased after 6 weeks of feeding and remained elevated until 18 weeks (Figure 5B). The expression of HMTs Suv-39h1 and Suv2-40h2, which are responsible for trimethylation of histones H3K9 and H4K20, did not change significantly over the 24 week experiment (Figure 6).

Morphological changes in liver of rats fed tamoxifen-containing diet

The expression of GST-P, a marker for initiated cells (39,40), was evaluated in control and tamoxifen-treated rats after 24 weeks of feeding. In most sections from rats fed tamoxifen, immunostaining for GST-P was not detected, and only few samples contained single cells that were GST-P-positive (data not shown). Diethylnitrosamine-initiated liver foci, which were used as a positive control, were intensely stained for GST-P. No staining was observed in livers from control rats.

Discussion

The results of this study show that the mechanism of tamoxifen-induced hepatocarcinogenesis in rats includes both genotoxic and epigenetic components. The hepatocarcinogenic effects of tamoxifen may be attributed to its tumor-initiating and tumor-promoting activities (7,8,41). The mechanisms involved in liver tumor induction by tamoxifen include its metabolic activation to genotoxic derivatives by hepatic cytochrome P450-dependent monooxygenases through α-hydroxylation (13), which leads to DNA adduct formation (9–13,42); however, events subsequent to DNA adduct formation are not clear.

Recent studies have suggested the link between long-term carcinogenic exposure, epigenetic alterations and the following malignant transformation (20,43,44). In light of these considerations, we examined whether or not tamoxifen, in addition to being genotoxic, altered the cellular epigenetic status of liver cells. The results of our study provided the first experimental evidence that feeding the F344 rats tamoxifen-containing diet led to substantial epigenetic changes in liver tissue comprising a rapid and sustained global DNA hypomethylation, altered activity and expression of DNA methyltransferases, and stable loss of histone H4K20 trimethylation. In contrast, non-target tissues displayed no changes in global DNA methylation status or DNA methyltransferase activity.

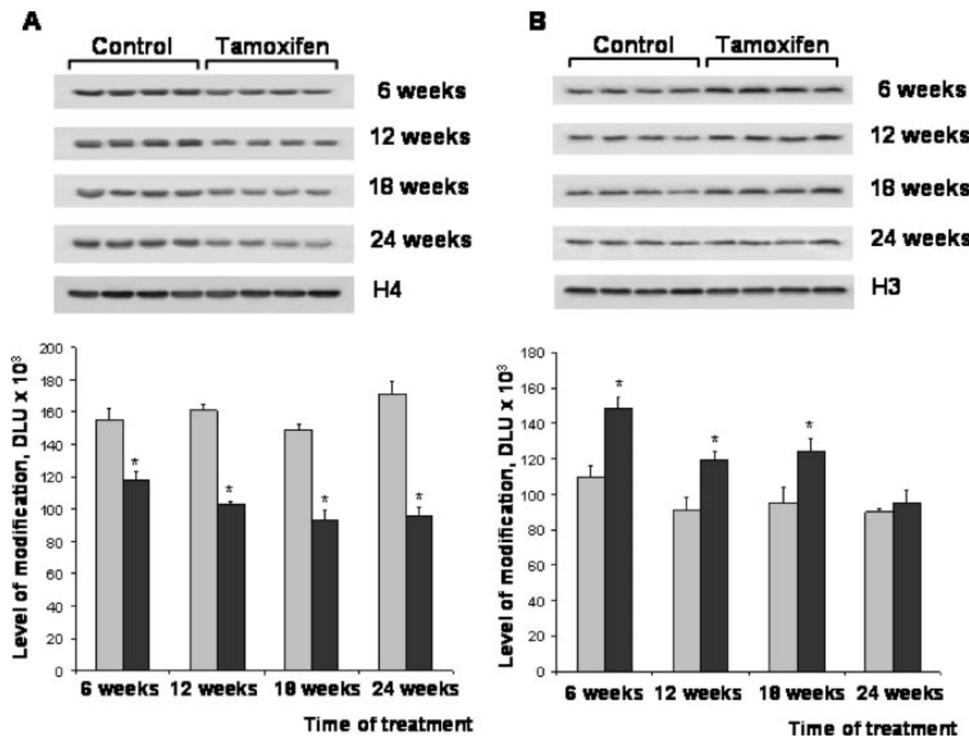

Fig. 5. Western blot analysis of histone H4K20me3 and H3K9me3 in liver of control rats and rats fed tamoxifen-containing diet. Acid extracts of total histones were separated by SDS–PAGE and subjected to immunoblotting using specific antibodies against histone H4K20me3 (**A**) and H3K9me3 (**B**). Results are presented as change in methylation relative to control animals. Equal sample loading was confirmed by immunostaining against histone H3 and histone H4. Representative immunoblot images are shown. *Significantly different from control at the same time point ($n = 5$, mean ± SEM).

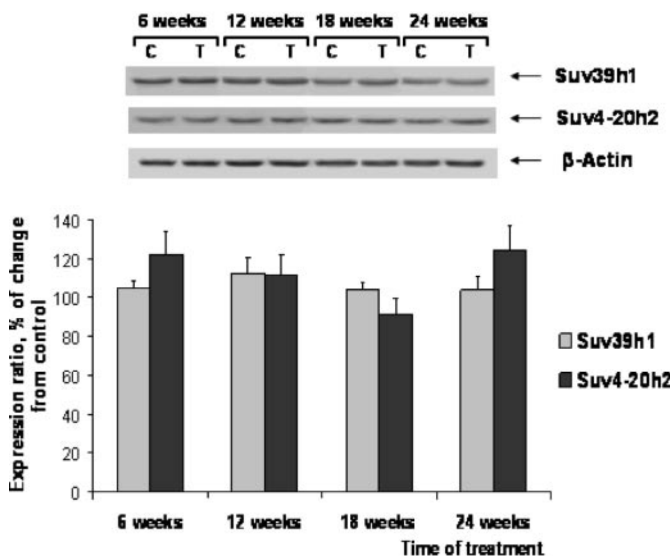

Fig. 6. Expression of Suv-39h1 and Suv4-20h2 HMTs in liver of control rats and rats fed tamoxifen-containing diet. Liver tissue lysates were separated by SDS–PAGE and subjected to immunoblotting using specific antibodies against Suv4-20h2 and Suv-39h1. Equal sample loading was confirmed by immunostaining against β-actin. The upper part of the figure shows representative western immunoblot images from two independent experiments. The lower part of the figure shows a quantitative evaluation of the Suv4-20h2 and Suv-39h1 expression in liver of tamoxifen-fed animals relative to those of control animals. Data are presented as percent fractions of controls. Control values at each time point were considered as 100%.

We reported previously that stable DNA hypomethylation is an important step in the transition of normal cells to tumor cells during hepatocarcinogenesis induced by methyl-deficient diet (22,23). Similar observations have been found in response to variety of genotoxic hepatocarcinogens, such as ethionine (16), hydrazine sulfate (45) and sodium arsenite (18); however, in these studies, alterations in global and regional DNA methylation were analyzed at a single time point after formation of liver lesions. As such, it is impossible to determine clearly whether the observed epigenetic changes were induced by carcinogens and play a causative role in neoplastic cell transformation or are merely a consequence of the formed lesions. In the present study, alterations in global DNA methylation status occurred in the absence of evident morphological changes in liver tissue, which demonstrates clearly that they are directly associated with the effects of tamoxifen and are not a consequence of the morphological lesions.

Several possible explanations exist for the mechanism of DNA hypomethylation after exposure to tamoxifen. First, the formation of tamoxifen–DNA adducts may lead to hypomethylation of DNA. The major DNA adducts induced by tamoxifen, dG-Tam and dG-DesMeTam, take place at GC base pairs. The presence of these or other adducts may profoundly diminish the ability of DNA methyltransferases to methylate a target cytosine resulting in hypomethylation of DNA. Second, dG-Tam is a miscoding DNA adduct (46–48), that causes a variety of sequence alterations preferentially at GC base pairs (47,48), which could lead to a loss of either 5-methyldeoxycytosine or deoxyguanosine from CpG sites in DNA. dG-DesMeTam may have similar characteristics.

Third, nucleotide excision repair, which plays a significant role in the removal of tamoxifen-induced DNA lesions, could also lead to genome-wide hypomethylation (48,49). Decreased expression of DNMT1, DNMT3a and DNMT3b DNA methyltransferases relative to DNA replication may further contribute to hypomethylation of DNA. Indeed, the results from the studies on partial or complete DNMT1 knockout cells or mice demonstrating that inadequate levels of DNMT1 cause genome-wide hypomethylation (50) support this suggestion.

Tamoxifen-induced hypomethylation of DNA was accompanied by progressive loss of histone H4K20me3 in liver tissues. Recently, it has been found that histone H4K20me3 is present through much of the genome but does not appear concentrated at any single area and trimethylation of histone H4K20 does not have any apparent role in the regulation of gene expression, instead it plays a crucial role in the DNA damage checkpoint control (51). Loss of histone H4K20me3 compromises the ability of cells to maintain cell cycle arrest resulting in genomic and chromosomal instabilities in a variety of somatic cells and is associated with increased tumor risk (51,52).

It has been suggested that exposure of cells to sustained stress environment results in emergence of epigenetically reprogrammed cells with specific tumor-related epigenetic alterations leading to succeeding mutations and malignant transformations (20). The result of our study shows that chronic exposure to tamoxifen leads to global demethylation of the genome, diminished expression of DNA methyltransferases and compensatory increased DNA methyltransferase activity, sustained loss of histone H4K20me3 or, in other words, epigenetic reprogramming of the liver cells. This could result in permanent phenotypic changes in liver cells, subsequently leading to a genomic instability, variety of mutational effects and neoplasia.

The results of the study provided evidence of the importance of epigenetic alterations in the etiology of tamoxifen-induced hepatocarcinogenesis. The early appearance of epigenetic changes in the absence of the evident morphological abnormalities confirms that these alterations are directly related to effect of tamoxifen and are not consequence of morphological changes. In addition, these results provide experimental support for epigenetic theories of carcinogenesis, specifically of the role of epigenetic reprogramming of the genome and emergence of tumor-related epigenetically altered cells as response to chronic carcinogenic insults.

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