Tamoxifen Induces Short-Term Cumulative DNA Damage and Liver Tumors in Rats: Promotion by Phenobarbital


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ABSTRACT

Tamoxifen administered in the diet (420 ppm) to Wistar rats (TOX:P) for only 3 months caused cumulative hepatic DNA damage as assessed by 32P-postlabeling, consistent with the proposal that tamoxifen is a genotoxic carcinogen in this species. Promotion of tumor development with phenobarbital after discontinuation of dietary tamoxifen resulted in the formation of liver carcinomas after 9 months. At 12 and 20 months in this study, the majority of these rats had liver carcinomas. Rats treated with tamoxifen for 3 months but not promoted with phenobarbital also developed liver tumors over a longer period of time. These tumors were predominantly adenomas, with one carcinoma, and occurred at a lower incidence than the tumors produced by promotion with phenobarbital. Rats treated with phenobarbital alone did not develop tumors after 20 months. Tamoxifen-induced DNA adducts were relatively persistent, with only a 38% decrease 3 months after tamoxifen treatment had been discontinued. This demonstrates that, in a susceptible species (the rat), tamoxifen can cause initiation of liver cancer after only 3 months exposure. It is proposed that the persistence of such DNA adducts may account for the ability of phenobarbital to promote a high incidence of liver carcinoma, even after discontinuation of tamoxifen treatment. These data are relevant to the concern for women given prophyphylic tamoxifen for long periods in that even if there is a relatively small amount of cumulative tamoxifen-induced liver DNA damage, liver tumors could be promoted by other agents, even after the cessation of tamoxifen treatment.

INTRODUCTION

Tamoxifen has been successfully used as an adjunct therapy for breast cancer in women for over 15 years (1). More recently, it has been proposed as a prophylactic therapy in disease-free women, who may have a greater risk of developing this disease (2, 3). A number of clinical trials is currently under way worldwide, despite the finding of liver cancers in rats induced by tamoxifen, both in regulatory and experimental studies (4–7). Toremifene, a closely related analogue of tamoxifen, does not induce liver cancers in rodents (7).

A major difference between the genotoxic effects of the two drugs is the presence of liver DNA adducts in tamoxifen-exposed rats, whereas little or no 32P-postlabeled adducts could be detected in the livers of rats exposed to toremifene (7, 8). Using rats of three different strains, the formation of DNA adducts in the liver has been shown to be cumulative with time (9), although the subsequent time to the development of liver tumors was strongly influenced by the amount of liver cell proliferation in rats treated with tamoxifen (10). All strains of rats treated with tamoxifen to date have developed liver cancer. Wistar, Lewis, and Sprague-Dawley rats develop liver tumors in less than 1 year, whereas Fischer rats take up to 20 months (4–7, 10). This has been explained in terms of the lower proliferation index found in the Fischer rats after 6 months of tamoxifen treatment, compared to the Wistar and Lewis rats. (10) In particularly responsive strains of rats, the proliferative effect that tamoxifen has on liver cells would seem to cause rapid promotion of the foci of altered gene expression in the liver to give multiple liver carcinomas.

In contrast, mice have been found to be refractory to the induction of liver tumors by tamoxifen (11). The interpretation of the negative bioassay in mice is confounded by the different biological effects of tamoxifen in mice, where in some organs it acts as an agonist, rather than an antagonist of estrogen as in the rat (11). Previous studies have shown that tamoxifen given as a single dose does not act as a tumor initiator in the liver but is able to promote lesions caused by the initiator diethyl nitrosamine (12). The present study addresses several questions with regard to the carcinogenicity of tamoxifen in rats: (a) can a three-month, short-term exposure to tamoxifen cause sufficient cumulative liver DNA damage to lead to subsequent tumor development, or does such a short-term exposure require a subsequent promoting stimulus, such as phenobarbital? and (b) what is the amount of DNA damage required, with or without promotion, in quantitative terms to lead to tumor formation? The inclusion of interim sacrifices has allowed us to determine the progression of tamoxifen-induced liver pathology and the relative persistence of the induced liver DNA adducts after tamoxifen treatment has been discontinued.

MATERIALS AND METHODS

Animals and Treatments. Female Wistar (Alderley Park) rats (TOX:P) were obtained from Harlan Olac (Bicester, United Kingdom). Seventy-two rats were housed in negative pressure isolators and fed tamoxifen at 420 ppm in powdered RM1 diet (SDS) for 3 months from 10 weeks of age. This level of exposure to tamoxifen has been shown previously to produce a high incidence of liver tumors in Wistar rats (5). An additional seventy-two rats were isolated housed and fed powdered RM1 diet from the same age as controls. At the end of the 3-month tamoxifen treatment, the first group was divided into two groups of 36 animals. One of these groups of 36 rats was subsequently given water supplemented with 0.1% w/v phenobarbital, while the remaining 36 were allowed normal drinking water. At this time, the second group of 72 control rats was also divided into two; 36 of them were given a 0.1% w/v phenobarbital supplement in the drinking water, and the remaining 36 rats had no phenobarbital water supplement. The groups with their corresponding treatment regime are summarized in Fig. 1. At 3, 6, 9, and 12 months, groups of six animals were sacrificed from all of the treatment groups, leaving 15 rats in each of the 4 groups to complete the lifetime bioassay.

Tissue Preparation. Blood was collected by cardiac puncture. Livers were removed and weighed, and a sample was frozen in liquid nitrogen for DNA isolation. Sections of all major lobes of the liver and other organs were fixed in formal buffered saline for routine histological examination and in Carnoy's fluid and ice-cold acetone for immunohistochemical studies.

Immunohistochemical Detection of GST-P.2 Paraffin sections (5 μm) from acetone-fixed livers were rehydrated, and GST-P proteins were detected immunohistochemically using an anti-GST-P polyclonal antibody (1:100 dilution) followed by an anti-rabbit alkaline phosphatase conjugated second antibody (1:50 dilution; Sigma Chemical Co.). Bound antibody was detected by incubation with naphthol AS/BI phosphate and Fast Red TR using methods described by Green et al. (13). Single GST-P foci (defined as groups of five or more cells) were counted on at least 1 sq cm of liver sections from animals at each time point. Areas of section were calculated using an Analytical Mea-

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2 The abbreviations used are: GST-P, placental form of glutathione S-transferase; PCNA, proliferating cell nuclear antigen.

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Liver Cell Proliferation Assessed by PCNA Expression. Paraffin sections (5 μm) from Carnoy’s-fixed liver were rehydrated. For PCNA detection, a monoclonal mouse anti-PCNA antibody was used (1:25 dilution; Novocastra, United Kingdom) and foci were expressed as the number per sq cm. For PCNA detection, slides prepared and stained with hematoxylin and eosin for examination.

Liver Cell Proliferation with Tamoxifen Treatment. Liver cell proliferation, measured by the index of PCNA expression, was in the group treated with tamoxifen (group 2) and the group treated with phenobarbital (group 4) was increased at 3 months of tamoxifen treatment, compared to the control group (Table 1). At the 9-month sacrifice, the proliferation index in the group treated with tamoxifen and in the livers of rats treated with tamoxifen for 3 months and their subsequent stability. Hepatic DNA damage, determined by 32P-postlabeling in Wistar-AP rats after 3 months dietary administration of tamoxifen, was 721 ± 420 adducts/10⁶ nucleotides (mean ± SE; n = 3). The mean numbers of DNA adducts in control rat livers was low, ranging between 80 and 75 adducts/10⁶ nucleotides at 3 and 6 months. The pattern of 32P-postlabeled adduct spots in both groups of treated rats did not change between the 3- and 6-month time points and closely resembled the pattern reported previously (9). Four major spots were visualized, but up to 8 other minor spots were also detected.

Liver Pathology after Dietary Tamoxifen and Phenobarbital Treatment. There was a significant increase in the number of GST-P-expressing foci in the livers of rats treated with tamoxifen for 3 months and their subsequent stability. Hepatic DNA damage, determined by 32P-postlabeling in Wistar-AP rats after 3 months dietary administration of tamoxifen, was 721 ± 420 adducts/10⁶ nucleotides (mean ± SE; n = 3). The mean numbers of DNA adducts in control rat livers was low, ranging between 80 and 75 adducts/10⁶ nucleotides at 3 and 6 months. The pattern of 32P-postlabeled adduct spots in both groups of treated rats did not change between the 3- and 6-month time points and closely resembled the pattern reported previously (9). Four major spots were visualized, but up to 8 other minor spots were also detected.

RESULTS

Concentration of Tamoxifen and Its Metabolites in the Liver and Serum of Rats following Dietary Administration. After 3 months exposure to dietary tamoxifen, hepatic concentrations of this drug, N-desmethyltamoxifen and 4-hydroxytamoxifen, were 34 ± 10, 50 ± 13, and 11 ± 3 nmol/g, respectively (mean ± SE; n = 4). The corresponding mean serum concentration of tamoxifen after 3 months was 666 ± 16 ng/ml. No tamoxifen or its metabolites could be detected in the livers or sera of dosed rats 3 or 6 months after the cessation of dosing, precluding the possibility of any further tamoxifen-induced cumulative DNA damage occurring.

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months (Table 2). However, at 6 months, after tamoxifen administration had been discontinued, livers did not have significantly increased numbers of GST-P foci relative to controls (group 1). In contrast, rats which had been exposed to tamoxifen for 3 months and then promoted with phenobarbital (group 4) had increased numbers of GST-P liver foci when compared to the control groups of phenobarbital (group 3) or untreated rats (group 1). After 9 months, the numbers of GST-P liver foci were significantly increased in both tamoxifen-treated groups (2 and 4) compared to their respective controls (Table 2). Table 3 shows that 3 months exposure to dietary tamoxifen, followed by phenobarbital promotion, produced liver tumors at 9 months. After 20 months, of the 14 rats remaining in group 4, 12 had liver tumors, and 11 of these were hepatocellular carcinomas of a trabecular or glandular type, and one adenoma. None of the controls or controls promoted with phenobarbital had any liver tumors after 20 months.

Liver Tumors in Rats after Dietary Tamoxifen for 3 Months, without Subsequent Promotion. None of the rats treated with tamoxifen for 3 months and then returned to the control diet had liver tumors at the 6-, 9-, or 12-month time points. However, when animals in this group were sacrificed after 20 months, five of 15 had liver tumors. One of these liver tumors was a hepatocellular carcinoma (glandular type), and the other four animals had adenomas. The details of the liver tumor incidence in the various groups and their classification is shown in Table 3.

DISCUSSION

Treatment of Wistar-AP female rats with dietary tamoxifen for 3 months was sufficient to cause cumulative hepatic DNA damage, as measured by 32P-postlabeling. When only the basal diet was given, this DNA damage persisted with some decrease in DNA adduct levels over the next 3 months (721 to 443 adducts/10^8 nucleotides). Following cessation of dosing, tamoxifen is rapidly cleared from the body, with a single dose 1/2 of about 12 h (19). Tamoxifen was not detected in the livers of rats 3 months after dosing was discontinued, indicating that tamoxifen-induced DNA adducts are relatively persistent. An earlier study also showed that, following dosing for 7 days, DNA damage persisted for 20 days with only a small decrease (8).

When tamoxifen was administered for 3 months and phenobarbital was used as a promoter, liver tumors, including carcinomas, were found as early as 9 months after commencing treatment. This was very similar to the time scale for tumor formation in rats given tamoxifen continuously (9) and is consistent with the hypothesis that phenobarbital promotes at least some of the same foci that tamoxifen itself is capable of promoting after initiation (12). At 12 months in the study, 5 of 7 rats treated with phenobarbitaline after 3 months exposure to tamoxifen had liver carcinomas; while after 20 months of phenobarbital promotion, 11 of 14 rats had liver carcinomas. We propose that the persistence of the DNA adducts may account for the ability of phenobarbital to promote a high incidence of liver carcinoma after discontinuation of the tamoxifen dosing.

Even in the absence of a promoter, 3 months exposure to tamoxifen followed by a return to basal diet was sufficient to cause liver tumor formation. By 20 months, 5 of 15 rats in the group fed tamoxifen for 3 months had developed liver tumors. One of these was a liver carcinoma, and the four adenomas could have progressed to carcinomas with time, based on the accepted temporal relationship between adenomas and their subsequent progression to carcinomas after treatment with carcinogens (20). This progression may involve mutations of the p53 gene, as has been reported for rats given tamoxifen daily for 12 months (21).

The findings of liver tumors in rats exposed to tamoxifen for only a short period of time and not subsequently given a promoter may be a cause for concern. The relationship between DNA adduct formation and the subsequent development of tumors in rats is not currently understood, in terms of either dose response for tumorigenesis or time to tumor. It is known, however, that a single dose of tamoxifen in rats, which would be expected to give rise to about 30 adducts/10^8 nucleotides of hepatic DNA damage (8), measured by 32P-postlabeling, does not lead to the development of liver tumors following phenobarbital promotion (12). With rats given tamoxifen for 3 months the extent of hepatic DNA damage, around 400 adducts/10^8 nucleotides, is very much greater than the value of 36 adducts/10^8 nucleotides found in the livers of women being treated with tamoxifen. It has been proposed that women will be at considerably lower risk than rats for developing tamoxifen-induced liver tumors. Women treated with tamoxifen for longer periods than have been examined thus far may accumulate greater amounts of liver DNA adducts, and this would cause concern in its prophylactic use. This could be clarified by the examination of liver biopsies using the 32P-postlabeling technique. Although the data acquired thus far indicates a much lower level of liver DNA adduct formation in tamoxifen-exposed human livers than has been found in rats, in a polymorphic human population there may be a greater range of response to tamoxifen due to individual variations in metabolism. This could be particularly important, since tamoxifen requires metabolic activation in the rat for the subsequent formation of covalently bound protein adducts (22).

The relative contribution of initiation of DNA damage by tamoxifen and the subsequent promotion by tamoxifen itself, or by agents such as phenobarbital, remains to be established. Liver cell proliferation would be a major factor in converting DNA adducts to heritable genetic alterations, which could eventually result in tumor formation. At low doses of carcinogens, the time for tumor development in experimental studies is particularly prolonged (23). A major concern

| Table 2 | GST-P foci in livers of Wistar-AP rats at different intervals (numbers of foci/cm²) |
|---|---|---|---|
| Group | Treatment | 3 mos | 6 mos | 9 mos |
| 1 | Control | 3.1 ± 1.7 | 3 ± 0.2 | 1.6 ± 0.3 |
| 2 | TAM | 9.6 ± 2.3^b | 19 ± 10 | 3 ± 2.2 |
| 3 | PB | 3 ± 2.2 | 1 ± 0.6 |
| 4 | TAM+PB | 20 ± 6.6 | 54 ± 14^b |

^a TAM, tamoxifen; PB, phenobarbital.

^b Statistically significantly increased compared to its respective control at the 5% level.

| Table 3 | Liver tumor formation in the tamoxifen/phenobarbital promotion study |
|---|---|---|---|---|---|
| Group | Treatment | 6 mos | 9 mos | 12 mos | Lifetime |
| 1 | Control | 0/6 | 0/6 | 0/6 | 0/15 |
| 2 | TAM | 0/6 | 0/6 | 0/6 | 5/15^a |
| 3 | PB | 0/6 | 0/6 | 0/6 | 0/15 |
| 4 | TAM+PB | 0/6 | 3/6^c | 5/7^d | 12/14^e |

^a TAM, tamoxifen; PB, phenobarbital.

^b One rat with a hepatocellular carcinoma and four rats with an adenoma.

^c Two rats with hepatocellular carcinomas and one rat with an adenoma.

^d Five rats with hepatocellular carcinomas (and one or more adenomas).

^e Eleven rats with hepatocellular carcinomas (and one or more adenomas) and one rat with an adenoma.
for women given prophylactic tamoxifen for long periods would be that, even if there is a relatively small amount of cumulative tamoxifen-induced DNA damage (equivalent to a low dose of carcinogen), liver tumors could be promoted by other agents, even after the cessation of tamoxifen treatment. The probability that this could take much longer to occur, because of the effective low dose, means that this possible hazard will not become clinically apparent until late in the prophylactic trials.

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