Jpn. J. Cancer Res. (Gann), 79, 1335–1339; December, 1988

Inhibitory Effect of Tamoxifen on Diethylstilbestrol-promoted Hepatic Tumorigenesis in Male Rats and Its Possible Mechanism of Action

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Male Sprague-Dawley rats were given a single intraperitoneal injection of diethylnitrosamine (DEN, 200 mg/kg body weight). Two weeks later the rats were divided into 4 groups; DEN-C group rats were given no further treatment; DEN-DES group rats were fed diethylstilbestrol (DES, 0.5 mg/day); DEN-TMX group rats were given tamoxifen (TMX, 1.0 mg/ day) orally; DEN-DES TMX group rats were fed both DES and TMX for 8 months. Rats of the DEN-DES group developed grossly visible hepatic tumors. On the other hand, tumor development was significantly inhibited in rats of the DEN-DES TMX group. Total area of γ-glutamyl transpeptidase-positive lesions and the mean area per lesion were significantly larger in rats of the DEN-DES group than those of the DEN-C, DEN-TMX or DEN-DES TMX group. Estrogen receptor (ER) content of liver cytosol assayed by enzyme immunoassay (EIA) was significantly greater in rats of the DEN-DES group than in those of the DEN-C group and smaller in rats of the DEN-TMX and DEN-DES TMX group than in the DEN-C group. On the contrary, ER content of liver nuclei was significantly greater in rats of the DEN-TMX and DEN-DES TMX group than in those of the DEN-C or DEN-DES group. These results suggest that the promotive action of DES and the inhibitory action of TMX on DES-promoted hepatic tumorigenesis are, at least in part, mediated by ER in the rat.

Key words: Diethylstilbestrol — Tamoxifen — Hepatic tumorigenesis — Estrogen receptor

It has been reported that hepatic benign[1] and malignant[2] tumors occasionally occur in patients taking oral contraceptives. It has also been shown that exogenous estrogen promotes hepatocarcinogenesis in rats.[3–5] Assuming that estrogen has a promotive action, it may exert its effect through the same mechanism by which estrogen acts in other target tissues such as the uterus and pituitary gland. It has been reported that rat[6] and human[7] livers contain estrogen receptor (ER), like other target tissues. Little work,[8] however, has been done to examine the relationship between hepatic ER and hepatocarcinogenesis.

In our previous studies, we demonstrated that a synthetic estrogen, diethylstilbestrol (DES), promoted hepatic tumorigenesis in male rats and that cytosolic ER of the liver was increased by DES administration.[9] In this study, we examined the effect of an anti-estrogen, tamoxifen (TMX), on the promotive action of DES and studied the possible mechanism of TMX action by assaying cytosolic and nuclear ER of the liver.

The abbreviations used are: DEN, diethylnitrosamine; DES, diethylstilbestrol; TMX, tamoxifen; ER, estrogen receptor; EIA enzyme immunoassay; EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; H-E, hematoxylin and eosin; γ-GTP, γ-glutamyl transpeptidase.

MATERIALS AND METHODS

Chemicals TMX was supplied by ICI Pharma, Osaka. Diethylnitrosamine (DEN) and DES were purchased from Tokyo Chemical Industry Co. Ltd., Tokyo, Tris, ethylenediaminetetraacetate (EDTA) disodium salt from Nakarai Chemicals Co., Kyoto, and bovine serum albumin (BSA) from Sigma Chemical Co., St Louis, Mo. All other reagents used were of analytical grade.

Animals and Treatment Group Male Sprague-Dawley rats (Charles River Japan, Inc., Atsugi) were housed in an air-conditioned room at 24° with a 12 hr-12 hr light-dark cycle and were given Oriental M powdered basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*. All rats were given a single intraperitoneal injection of DEN (200 mg/kg body weight) at eight weeks of age. Two weeks later they were divided into four

groups. Rats of the DEN-C group were given only olive oil in the diet, those of the DEN-DES group were fed DES (0.5 mg/day) in olive oil, those of the DEN-TMX group were fed TMX (1.0 mg/day) and those of the DEN-DES TMX group were fed both DES and TMX. DES and/or TMX were given for eight months. The administration of DES or TMX was stopped 72 hr before sacrifice and all rats were fasted for 24 hr prior to sacrifice.

Histological Study The livers were weighed and cut into 5 mm thick sections from each lobe. Tissue blocks were fixed in cold acetone and stained with hematoxylin and eosin (H-E) by the routine method and stained for γ-glutamyl transpeptidase (γ-GTP) by the method of Rutenburg *et al.*[10] The most commonly used marker for the identification of preneoplastic lesions in rat is the appearance of γ-GTP activity in focal areas of hepatocytes. Most hyperplastic nodules and hepatocellular carcinomas have elevated levels of γ-GTP.[11] So we examined this enzyme in liver lesions in the rat. Numbers, total areas per cm^2 and mean areas of γ-GTP-positive lesions (enzyme-altered foci or hyperplastic nodules) were determined using the Mop-VIDEOPLAN (Kontron Bildanalyse).

Cytosolic ER Assay All procedures were performed at 0°. The liver was minced and homogenized with a glass homogenizer in 6 volumes of TED buffer [0.01*M* Tris-HCl, 1.5m*M* EDTA, 0.5 m*M* dithiothreitol, pH 7.4, 0°]. The resultant homogenate was centrifuged for 20 min at 25,000*g*. The supernatant was centrifuged for 60 min at 105,000*g*. The supernatant (cytosol) was carefully removed to avoid lipid contamination. Protein concentration in the cytosol was assayed by the method of Lowry *et al.*[12] using BSA as the standard.

Enzyme immunoassay (EIA) was performed to quantitate cytosolic ER by using an Abbott ER EIA monoclonal kit (Abbott Laboratories) with monoclonal antibodies to human ER. EIA were done in duplicate.

Nuclear ER Assay Nuclear extract was obtained by the method of Okulicz *et al.*[13] Liver was homogenized in TED buffer. The homogenate was centrifuged at 800*g* for 15 min and the nuclear fraction was washed four times by resuspension in TED buffer and centrifugation at 800*g* for 15 min. Nuclear receptor was extracted from the washed pellet by resuspension in TED buffer containing 0.5 *M* NaSCN. The nuclear suspension was incubated for 60 min with mixing at 15 min intervals, and centrifuged at 105,000*g* for 60 min.

EIA was performed for the resultant supernatant (nuclear extract) as for the cytosol.

Statistical Analysis Results were expressed as mean ± SE. Statistical analysis was performed using Student's *t* test.

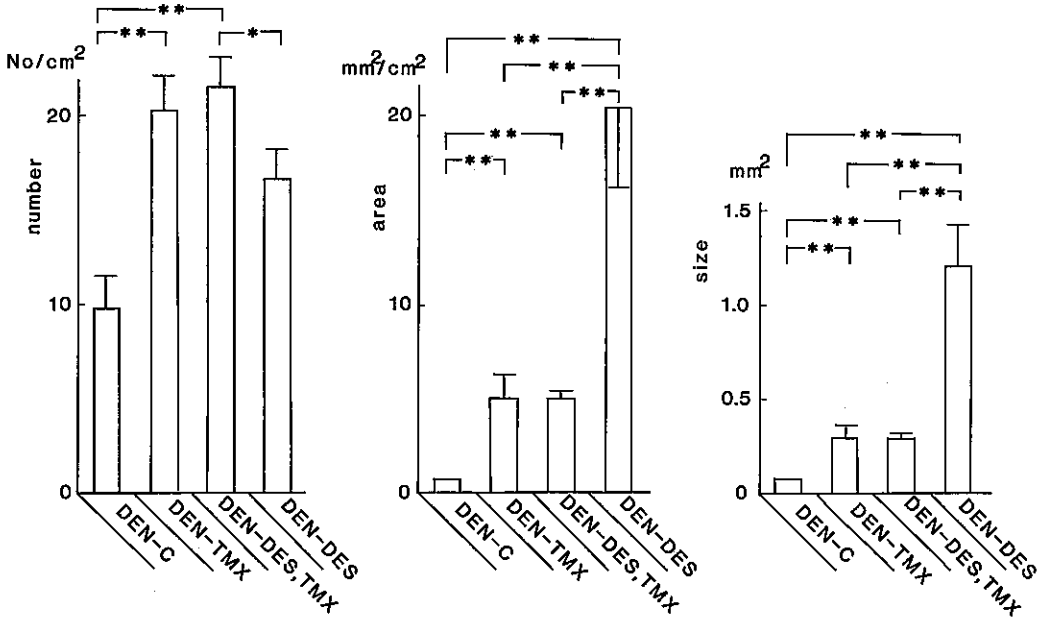

Fig. 1. Numbers (left), areas (middle) and sizes (right) of γ-GTP-positive lesions in the rat liver in each experimental group. Mean±SE, *, $P<0.05$; **, $P<0.01$.

Results

Numbers of rats studied were eight in the DEN-DES group, 11 in the DEN-DES TMX group, 12 in the DEN-TMX group and four in the DEN-C group. The body weight was decreased most remarkably in the DEN-DES group and moderately in the DEN-TMX group. On the other hand, the liver weight and the ratio of liver to body weight were significantly larger in the DEN-DES group than in the other three groups.

All livers of the DEN-DES group were large and had many nodules of various sizes, but livers of the DEN-DES TMX group had only a few very small nodules. The livers of the DEN-TMX and DEN-C groups showed no macroscopic changes.

Microscopically, nodules were defined as clusters of hepatocytes which were delineated from the surrounding cells. The major abnormality of cells within nodules was vacuolation of the cytoplasm. The foci and nodules were not accompanied with fibrosis or oval cell proliferation. Most of the nodules were histologically similar to those classified as "neoplastic nodules" in a workshop report.[14]

Figure 1 shows the result of quantitative analysis for γ-GTP-positive lesions. The number of lesions was larger in the DEN-DES TMX and DEN-TMX groups than in the DEN-C group and rather smaller in the DEN-DES group than in the DEN-DES TMX group. Total area and mean area of lesions were, however, much larger in the DEN-DES group than in the other three groups and moderately larger in the DEN-DES TMX and DEN-TMX groups than in the DEN-C group.

Table I shows the results of cytosolic and nuclear ER assay by EIA. The ER content of the cytosol was larger in the DEN-DES group than in the DEN-C group, and less in the DEN-TMX and DEN-DES TMX groups than in the DEN-C group. The ER content of the nuclear extract was significantly larger in the DEN-TMX and DEN-DES TMX groups than in the DEN-C or DEN-DES group. Cytosolic and nuclear ER in the DEN-C group were almost the same as those in untreated control rats (data not shown).

Table I. Cytosolic and Nuclear ER in Male Rat Liver in Each Experimental Group

Group	Cytosolic ER (fmol/mg protein)[a)]	Nuclear ER (fmol/g liver)[a)]
DEN-C	7.9±1.2	53±14
DEN-TMX	5.1±0.5[b,d)]	430±111[b,d)]
DEN-DES TMX	5.3±0.3[c,d)]	366±85[b,d)]
DEN-DES	15.5±2.2	62±20

a) Mean±SE.
b) Significantly different from DEN-C group; $P<0.05$.
c) Significantly different from DEN-C group; $P<0.01$.
d) Significantly different from DEN-DES group; $P<0.01$.

Discussion

Some previous studies[4,5] demonstrated that synthetic estrogens could act as promoters in hepatocarcinogenesis induced by initiators, but the mechanism of their action is still unknown. In our previous studies, we showed that DES, a synthetic estrogen, promoted hepatic tumorigenesis in male rats initiated by DEN.[9] Assuming that DES acted through the binding with ER, we planned to observe the effect of TMX, an antiestrogen, on the promotive action of DES in DEN-initiated hepatic tumorigenesis. As in the previous studies, DEN and DES produced grossly visible hepatic tumors. When TMX was given along with DEN and DES, however, the tumor development was remarkably inhibited. Quantitative analysis of γ-GTP-positive lesions, which were enzyme-altered foci or hyperplastic nodules, clearly demonstrated that the promotive action of DES was significantly inhibited by TMX. These results are in agreement with the previous reports that an antiestrogen, clomifene citrate, was effective in inhibiting hepatic and pituitary tumorigenesis associated with DES treatment[15] and that the simultaneous administration of antiestrogens, such as nafoxidine or enclomifene, with DES completely suppressed the induction by estrogen of renal carcinoma in the hamster.[16] In the latter experiment, the ability of these antiestrogens to block renal carcinogenesis in the presence of estrogen correlated well with the antiestrogen competition for the renal ER in DES-treated hamsters.[16]

Recently, Yager *et al.*[17] reported that TMX inhibited the induction of DNA synthesis caused by ethinyl estradiol and mestranol but

not by phenobarbital, and that TMX inhibited the appearance of γ-GTP-positive foci promoted by ethinyl estradiol. These reports suggest that ER have a role in the mechanism of action of synthetic estrogen.

The EIA system using two monoclonal antibodies against human breast cancer ER was reported to be applicable for quantitative analysis of rat uterine ER,[18] so we used this assay procedure for analysis of rat hepatic ER. Our observation that cytosolic ER were increased in the DEN-DES group is in agreement with the reports that ER in renal[16] and hepatic[8] cytosol were increased in DES-treated hamsters. In contrast, cytosolic ER in the DEN-DES TMX and DEN-TMX groups were decreased, and nuclear ER in these groups were increased as compared with that in the DEN-C or DEN-DES group. The significance of the decrease in cytosolic ER and the increase in nuclear ER induced by TMX is still unknown, since the localization and turnover of ER in intact cells have not been completely elucidated. Recent studies using monoclonal antibodies to ER suggest that ER are present only in the nucleus.[19] If this is the case, ER in the cytosol fraction after sequestration can be regarded as artifactual, arising from transfer from the nucleus to the cytosol. However, the possibility that cytosolic ER can not be detected by the monoclonal antibodies is still not completely excluded.

Recently, Nakao *et al.*[18] observed that TMX injection resulted in a prolonged accumulation of ER in the nuclear extract of rat uterus and suggested that TMX induces a new class of ER-related molecules which are different from putative ER. Jasper *et al.*[20] also postulated that antiestrogen binding to ER could cause a conformational change in ER which is different from that caused by estrogens, and which explains the different biological responses. Our present observation could be explained by assuming that different conformational changes of ER induced by TMX and DES might cause different distributions of ER between cytosol and nuclear fraction and might inhibit hepatic tumorigenesis.

It must be pointed out that TMX showed a weak promotive action, since the total area of γ-GTP-positive lesions was significantly larger in the DEN-TMX group than in the DEN-C group. Although TMX is a non-steroidal antiestrogen, it acts as a partial agonist depending on the animal species, target organs or endocrinological environment. Wei *et al.*[21] showed that TMX alone increased uterine weight to a level lower than that induced by estradiol, although TMX inhibited the increase of uterine weight stimulated by estrogen. This observation in a target organ is concordant with our observation in liver tissue. All these results indicate that both DES and TMX act through ER, thus modifying hepatic carcinogenesis. Further studies, such as time course studies of ER during carcinogenesis, dose-response studies of TMX action in the carcinogenesis and immunocytochemical assays, are required to draw a firm conclusion.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Special Project Research in Cancer-Bioscience from the Ministry of Education, Science and Culture, Japan. The authors thank Dinabott Inc. (Tokyo) for providing the Abott ER-EIA monoclonal kit and ICI Pharma (Osaka) for providing TMX.

(Received Aug. 3, 1988/Accepted Oct. 25, 1988)

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