Suppressive effect of oestriadiol on chemical hepatocarcinogenesis in rats

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Abstract
Aims—To examine the effects of oestriadiol and testosterone on the early carcinogenic changes expressed in rat liver from the diethylnitrosamine (DEN), 2-acetylaminofluorene (AAF), partial hepatectomy (PH) model of hepatocarcinogenesis.

Methods—Prenoeplastic liver lesions were evaluated using immunohistochemical analysis of glutathione-S-transferase placental form (GST-P) expression; oestrogen and androgen receptor levels were measured by radioimmunoassay.

Results—Oestriadiol administration to non-castrated DEN-AAF-PH treated males resulted in a decrease in the area of GST-P positive foci, while testosterone increased the serum oestriadiol level and reduced the area. In males, castration alone and castration with oestriadiol replacement significantly reduced the GST-P positive area, and increased the hepatic oestrogen receptor level. In DEN-AAF-PH treated females, castration with testosterone replacement was associated with a significant increase in the GST-P positive area and the hepatic androgen receptor level.

Conclusion—These findings suggest that exogenous and endogenous oestriadiol can suppress chemical hepatocarcinogenesis. It appears that oestrogen receptors may be involved in the inhibition of malignant transformation of preneoplastic liver cells, while androgens and androgen receptors are involved in hepatocarcinogenesis.

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Keywords: hormone receptor; testosterone; diethylnitrosamine; glutathione-S-transferase placental form; antioxidant

The incidence of hepatocellular carcinoma (HCC) in humans has been reported to be higher in men than in women.1 2 Although both men and women have a higher incidence of HCC in cirrhosis, the development of HCC in cirrhotic livers occurs with much greater frequency in men than in women (approximate ratio 11:1).3 Moreover, carcinomas induced experimentally using carcinogens, as well as the appearance of spontaneous neoplasms, occur at a higher incidence in male rats and mice.4 Androgens have been implicated in hepatic adenomas.5 Substituted androgens have been associated with the development of HCC in patients with Fanconi’s anaemia,6 and aplastic anaemia.7 In rat studies, testosterone appears to be a growth factor for Morris hepatoma 7787.8 These findings suggest that androgens may be implicated in the aetiology of HCC, and that oestrogens may have a suppressive role in the development of human liver cancer. However, oral contraceptive agents are reported as hyperplastic agents in liver and are thought to be implicated in the development of hepatic adenoma and focal nodular hyperplasia in both sexes.9–11

We examined the effects of the sex hormones oestriadiol and testosterone on the early carcinogenic changes expressed in rat liver using a diethylnitrosamine (DEN), 2-acetylaminofluorene (AAF), partial hepatectomy (PH) model of hepatocarcinogenesis.12 This model utilises DEN for the initiation step, and dietary AAF and PH for the promotion step and has been shown to cause an increased number of preneoplastic liver lesions, including hyperplastic nodules or γ-glutamyl transferase (GGT) or glutathione-S-transferase placental form (GST-P) positive foci.13–15 To exclude the interaction of intrinsic sex hormones, young castrated rats of both sexes were also studied. The eventual changes in preneoplastic liver lesions were judged by histochemical analysis of GST-P16 expression. GST-P has been shown to be a very accurate marker enzyme for detection of initiated cells during hepatocarcinogenesis; it is hardly detectable in normal rat liver, but is strongly expressed in preneoplastic liver lesions.13 Furthermore, GST-P positive staining of these foci has been shown to correlate with (GGT positivity with the advantage of far less background hepatocyte staining.16–18

Materials and methods

PREPARATION OF ANIMALS
Male and female Fischer 344 rats (Japan SLC, Hamamatsu, Japan) three weeks old, weighing 42 (4) g (male) or 36 (3) g (female), were housed in air conditioned animal quarters with lighting from 08.00–20.00, and had unrestricted access to a basal diet (CE-2; Nihon Clea, Tokyo, Japan) and water. Thirty five male rats were divided into six groups (groups 1–6), and 23 female rats were divided into four groups (groups 7–10) (fig 1). Bilateral orchectomy and ovariectomy were performed in males (groups 5 and 6) and females (groups 9 and 10), respectively, as described previously.19 Sham operated males (groups 1–4) and females (groups 7 and 8) were similarly treated, but the testes or ovaries were not removed. Eight experimental groups received the complete carcinogenic treatment (DEN-AAF-PH); rats in the experimental groups (groups 2–6 and 8–10) were initiated with a single intraperitoneal...
and underwent a standard two thirds PH at the midpoint of this treatment, to activate the rapid growth of DEN altered hepatocytes not suppressed by AAF. Rats in control groups (groups 1 and 7) were treated with saline, fed the basal diet, and subjected to a two thirds PH. Animals alive one week after the PH treatment were returned to the basal diet. Oestradiol valerate (Mochida Pharmaceutical Co., Tokyo, Japan) in olive oil was administered intraperitoneally to the orchietomised three week old males (group 6) at a dose of 3.3 mg/kg body weight/day for three weeks beginning on the day of the orchietomy, followed by a dose of 10 mg/kg body weight twice a week for four weeks. Rats in group 3 received oestradiol (3.3 mg/kg body weight/day) for two weeks during the period of AAF feeding. Likewise, testosterone propionate (Teikokuzoki Pharmaceutical Co., Tokyo, Japan) in olive oil was given to the ovariectomised females (group 10) for three weeks after ovariectomy using the same protocol as that described for group 6. Animals in group 4 received two weeks of testosterone (3.3 mg/kg body weight/day) injections during the period of AAF feeding. All groups received injections of the same volume of olive oil. All animals were deeply anaesthetised with sodium pentobarbital (40 mg/kg body weight, intraperitoneally) and killed after terminal exsanguination two weeks after the PH. Blood samples were drawn from the inferior vena cava for radioimmunoassay analysis of serum oestradiol and testosterone, and liver tissue specimens were taken for light microscopy and immunohistochemistry. The remaining liver tissue was promptly removed and was frozen in liquid nitrogen and stored at −80°C for cytosol preparation for oestrogen and androgen receptor studies. All animals were treated humanely in accordance with the Japanese National Guidelines on animal care and use.

**Light microscopy and immunohistochemistry**

The liver specimens were fixed overnight in phosphate buffered formaldehyde, embedded in paraffin wax, and stained with haematoxylin and eosin. Polyclonal antisera to rat GST-P21 (MBL Co., Nagoya, Japan), produced against GST-P purified from hyperplastic nodule bearing rat livers, was determined to be specific for the rat liver GST-P using two dimensional electrophoresis, immunofinity chromatography, and western blot analysis. The avidin–biotin complex (Vectastain R ABC reagent, Vector Laboratories, Burlingame, California, USA) immunohistochemical method was used with minor modifications. Briefly, tissue block sections were mounted on slides, deparaffinised in xylene, and rehydrated in alcohol. Endogenous peroxidase was blocked with 1 ml of hydrogen peroxide per 100 ml of methanol. Incubation with anti-GST-P antibody was preceded by digestion with 0.1 g of trypsin in 100 ml of phosphate buffer saline (PBS) at 37°C for 30 minutes. The antigen–antibody complexes were localised with the use of an ABC-peroxidase-diaminobenzidine (DAB) method. As a negative control to
confirm the specificity of the anti-GST-P antibody, non-immune rabbit serum was substituted for the antisera.

The numbers and areas of GST-P positive liver foci over 0.2 mm in diameter were measured using a microcomputer based image analysis system (Imaging Research Inc., Tokyo, Japan). The number of GST-P positive foci was expressed as number/cm² and the area of GST-P positive foci was expressed as area (mm²)/cm².

OESTROGEN AND ANDROGEN RECEPTOR ASSAYS

Table 1 Liver and body weights, serum hormone concentrations, and cytosolic hormone receptor concentrations in DEN-AAF-PH treated rats

### TABLE 1

<table>
<thead>
<tr>
<th>Group treatment number</th>
<th>Oestradiol (pg/ml)</th>
<th>Testosterone (ng/ml)</th>
<th>Oestrogen (fmol/mg protein)</th>
<th>Androgen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Body (g)</strong></td>
<td><strong>Liver (g)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 PH control</td>
<td>273 (4)</td>
<td>9.0 (0.8)</td>
<td>59 (8)</td>
<td>1.3 (0.5)</td>
</tr>
<tr>
<td>2 DEN-AAF-PH</td>
<td>220 (14)†</td>
<td>9.1 (0.9)</td>
<td>43 (8)†</td>
<td>1.2 (0.5)</td>
</tr>
<tr>
<td>3 DEN-AAF-PH + oestradiol</td>
<td>170 (11)‡‡</td>
<td>7.0 (1.7)</td>
<td>238 (13)†‡</td>
<td>0.4 (0.1)‡</td>
</tr>
<tr>
<td>4 DEN-AAF-PH + testosterone</td>
<td>192 (15)‡</td>
<td>7.6 (1.8)</td>
<td>79 (11)‡</td>
<td>0.8 (0.4)</td>
</tr>
<tr>
<td>5 Orchiectomy + DEN-AAF-PH</td>
<td>184 (15)‡</td>
<td>8.4 (0.6)</td>
<td>41 (10)†‡</td>
<td>0.8 (0.6)</td>
</tr>
<tr>
<td>6 Orchiectomy + Oestradiol+ replacement</td>
<td>148 (8)†‡</td>
<td>6.9 (0.3)†‡</td>
<td>147 (59)†‡</td>
<td>&lt;0.2‡‡</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 PH control</td>
<td>161 (3)</td>
<td>3.9 (0.6)</td>
<td>110 (8)</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>8 DEN-AAF-PH</td>
<td>144 (4)</td>
<td>4.0 (1.0)</td>
<td>79 (15)†</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>9 Orchiectomy + DEN-AAF-PH</td>
<td>153 (3)‡‡</td>
<td>6.3 (0.6)∗</td>
<td>72 (21)‡</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>10 Orchiectomy + AAF-PH + testosterone replacement</td>
<td>160 (8)†‡</td>
<td>6.9 (0.4)∗</td>
<td>40 (9)†‡</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SD).

*p<0.05 versus corresponding value for DEN-AAF-PH in the same sex group.

††p<0.05 versus corresponding value for PH control in the same sex group.

‡‡p<0.05 versus corresponding value for castration + DEN-AAF-PH in the same sex group (two tailed Mann-Whitney U test).
males. Testosterone administration to the DEN-AAF-PH treated males significantly decreased the body weights, while testosterone administration to the ovariectomised females significantly increased the body and liver weights. These findings suggest that exogenous oestradiol has a suppressive effect on body and liver weights, and that the weight enhancing effect of exogenous testosterone is at least partly downregulated by oestrogen production in the ovaries and by increased peripheral conversion of androgens and oestrogen precursors into oestrogens.

CHARACTERISTICS OF SEX STEROID HORMONES IN THE DEN-AAF-PH MODEL

Table 1 shows the mean levels of hepatic oestrogen and androgen receptors and serum sex steroid hormones in the DEN-AAF-PH treated rats with or without castration and/or exogenous hormone treatment. The DEN-AAF-PH treated rats showed diminished levels of the hepatic oestrogen receptor and serum oestradiol, but there was no decline in levels of the hepatic androgen receptor or serum testosterone in male or female rats. Significant differences were found for all values measured in DEN-AAF-PH treated rats as compared with PH controls except for the hepatic oestrogen receptor level in females. Hepatic oestrogen receptor levels in non-castrated DEN-AAF-PH treated males were significantly lower than those for PH only control animals, but no significant difference was seen between castrated, DEN-AAF-PH treated males, and PH controls. Castration in females was associated with higher hepatic androgen receptor levels compared with those of non-castrated females, but was not associated with a significant change in the oestrogen receptor levels. Exogenous oestradiol increased the serum oestradiol concentration and decreased the serum testosterone concentration in the DEN-AAF-PH treated males (both non-castrated and castrated); exogenous oestradiol increased the hepatic oestrogen receptor levels in castrated, but not non-castrated males, and did not significantly affect the hepatic androgen receptor levels. In response to testosterone administration, the serum oestradiol level in the DEN-AAF-PH treated males increased to the PH control level, and declined in the castrated females. Moreover, exogenous

Figure 2 GST-P positive liver foci in non-castrated male rats induced using the DEN-AAF-PH model (A) given oestradiol (B) or testosterone (C). Original magnification \( \times 5 \).

Figure 3 Effect of castration and opposite sex steroid hormone on the development of GST-P positive liver foci in male and female rats induced using the DEN-AAF-PH model. Original magnification \( \times 5 \).
testosterone was associated with an increase in the hepatic androgen receptor levels in the castrated females to the male PH control values.

GST-P POSITIVE FOCI

GST-P is present in bile duct epithelial cells but not in hepatocytes, in the livers of normal and PH control rats of both sexes. When male and female rats were treated with DEN-AAF-PH, numerous GST-P positive foci in the liver were seen (figs 2 and 3). The area of GST-P positive foci induced in the DEN-AAF-PH treated rats was sevenfold higher in males than in females (figs 3 and 5). Oestradiol administration resulted in a significant decrease in the number (61 (14)/cm²) and area (4 (1) mm²/cm²) of GST-P positive foci as compared with DEN-AAF-PH values (81 (6)/cm² and 52 (5) mm²/cm², respectively) in non-castrated male rats; however, when non-castrated males were given testosterone, GST-P positive foci were reduced in terms of area (29 (9) mm²/cm²) but not number (106 (21) cm²) (fig 4). In DEN-AAF-PH treated males, the area of GST-P positive foci was reduced in animals that underwent ovariectomy alone (27 (9) mm²/cm²) and in those that underwent orchietomy and oestradiol replacement (10 (3) mm²/cm²) (fig 5). In DEN-AAF-PH treated females, the GST-P positive area was threefold higher in animals that underwent ovariectomy and testosterone replacement, but was not significantly different in animals that underwent ovariectomy only (fig 5). The apparent differences in the effect of exogenous testosterone on non-castrated males compared with castrated females may be due to a feedback effect on oestrogen formation in the ovaries and peripheral organs by the male sex hormone. These findings suggest that oestradiol suppresses the development of GST-P positive liver foci in this model of hepatocarcinogenesis.

Discussion

In this study we found that oestradiol administration resulted in a significant decrease in the number and area of GST-P positive liver foci in non-castrated male animals that underwent the DEN-AAF-PH hepatocarcinogenic treatment, and that testosterone administration to non-castrated males resulted in an increased serum oestradiol level, and decreased area of GST-P positive foci. In DEN-AAF-PH treated males, treatment with castration only or with castration
and oestradiol replacement was associated with a significant reduction in the area of GST-P positive foci, and an increase in the oestrogen receptor level. In DEN-AAF-PH treated female rats, the area of GST-P positive foci and the androgen receptor level were significantly greater in animals that underwent ovariectomy and testosterone replacement than in those that did not. These findings suggest that exogenous and endogenous oestradiol can suppress chemical hepatocarcinogenesis, that oestrogen receptors may be involved in the inhibition of malignant transformation of preneoplastic liver cells distinguished as GST-P positive foci, and that androgens and androgen receptors are involved in hepatocarcinogenesis. Moreover, our preliminary study indicated that oestradiol had the ability in a dose dependent manner to reduce the area of GST-P positive foci in non-castrated males (data not shown).

Much controversy exists with respect to the effectiveness of oestrogens as promoters of hepatocarcinogenesis, particularly after induction by chemical carcinogens. Taper showed that ovariectomised female rats given 17b-oestradiol phenylpropionate and oestradiol benzoate developed an increased number of transformed hepatic foci and HCC initiated by N-nitrosomorpholine. Camm et al showed that ethinyloestradiol administration enhanced these foci in Fischer rats after exposure to DEN. In intact rats after PH, Yager and Yager showed that animals fed mestranol and norethynodrel containing diets had greater numbers of transformed foci. These findings are contradictory to our results showing suppression of DEN initiated preneoplastic liver foci by oestradiol. This discrepancy may be a result of differences in the sex steroid compounds and experimental procedures used. In particular, it should be noted that the doses of ethinyloestradiol and mestranol used in these studies were 200 times the usual doses found in oral contraceptive steroids. In contrast, studies by others with several hepatic promoters, including phenobarbital, clofibrate, and ethinyloestradiol at non-hepatotoxic doses, have shown that following an initial, transient increase in liver growth, continued exposure causes inhibition of basal and/or induced growth. Abanobi et al found that while DNA synthesis was stimulated by feeding a hepatotoxic choline devoid diet, simultaneous treatment with phenobarbital was inhibitory. Barbazon et al and Yager et al reported that treatment with phenobarbital or ethinyloestradiol inhibited hepatocyte proliferation induced by PH. Yager et al also have shown in intact female rats that while ethinyloestradiol increased hepatocyte proliferation during the first seven days of treatment, the basal level of liver growth was dramatically inhibited after 28 and 42 days of ethinyloestradiol treatment. Mishkin et al have found that exogenous 17b-oestradiol exerted inhibitory effects on the growth and malignant transformation of liver foci produced by AAF in male Fischer 344 rats. Moreover, they reported that combined administration of tamoxifen and oestradiol also inhibited liver foci, malignant transformation, and mortality, suggesting that tamoxifen is acting as an oestrogen agonist.

Sex hormones exert their effects on the liver through interaction with specific intracellular receptors. The oestrogen receptor is well characterised in both rat and human liver. Several groups have shown the presence of oestrogen receptors in HCC specimens as well as in adjacent normal human liver. The data suggested that oestrogen receptor levels were lower in HCC samples than in normal liver, but the differences reported were not significant. However, it is difficult to correlate or compare the data from these various reports because of the limited numbers of human tumours examined, and the wide range of binding values obtained in each study. In this study, we found cytosolic oestrogen receptors in preneoplastic liver lesions produced by DEN-AAF-PH. The oestrogen receptor level in the preneoplastic liver in male rats was significantly lower than that in control livers treated with PH only, and was not significantly different from the PH control level in animals that received oestradiol alone or oestradiol and orchiectomy. Female oestrogen receptor levels were lower, but not significantly, in DEN-AAF-PH treated animals compared with the PH controls, but were not changed with hormone treatments. Recently, Villa et al have reported variant oestrogen receptor transcripts lacking the hormone binding domain in cancerous liver tissues, but not in pericancerous or normal liver tissues in men. They suggested that the variant oestrogen receptors lose the dependence on hormone control, and may offer a potential mechanism for the escape of the tumour from hormone control. These findings and our data implicate oestrogen dependency for anticarcinogenic regulation in hepatocarcinogenesis.

Androgens have been associated with liver disease as well. Most studies involving cytosolic and nuclear androgen receptor levels of HCC samples have suggested increased androgen receptor positivity or androgen receptor levels in HCC as compared with normal liver tissue. These findings are in accordance with our data indicating a significant decrease in serum testosterone levels in non-castrated males after oestradiol administration and a significant increase in hepatic cytosolic androgen receptor levels in females that received castration and testosterone, in association with the development of preneoplastic liver lesions. However, testosterone administration to non-castrated males in this study reduced the area of transformed GST-P positive foci with an increased serum oestriol level. This could be due to a feedback effect on the hypothalamic-pituitary axis responsible for oestrogen formation in the testes and peripheral organs by the administered testosterone, suggesting that endogenous oestradiol might be a more important factor involved in the regulation of malignant transformation of preneoplastic liver cells than exogenous testosterone. It remains to be elucidated, however, whether a larger quantity of testosterone than
that used in this study can inhibit malignant transformation of liver foci, and how hepatic receptor levels and serum levels of sex steroid hormones will respond after administration of sex hormones to non-castrated humans.

The mechanism whereby sex hormones are involved in hepatocarcinogenesis is unknown. However, oestrogen is a strong endogenous antioxidant that inhibits serum and liver lipid peroxide levels.\(^1\)\(^6\)\(^7\) Our preliminary studies have also shown that oestradiol inhibits lipid peroxidation in rat liver mitochondrial membranes induced by adenosine 5'-diphosphate (ADP) and Fe\(^2+\).\(^8\) There is increasing evidence that naturally occurring and synthetic antioxidants involved in hepatocarcinogenesis in rats.\(^9\)\(^10\) An alternative possibility to account for the sex difference in the growth rate of GST-P positive foci in rats treated with DEN-AAF-PH would be a sexual dimorphism in growth hormone secretion.\(^11\) Blanck \(et\ al\)\(^12\) reported that castration of male rats and continuous infusion of growth hormone to males during AAF-PH treatment in the DEN-AAF-PH model decreased the focal growth rate towards that in female rats. All manipulations leading to a "feminised" pattern of growth hormone secretion were characterised by a higher basal serum level of growth hormone than in the normal male.\(^13\) Although a possible causative role for oxygen radical interactions and growth hormone regulated mitoinhibition during the promotion stage in the present protocol has not been confirmed, such mechanisms merit further consideration.

Recently, our preliminary study using a hepatic fibrosis model induced in male rats by a single dose of dimethylnitrosamine (DMN) has indicated that oestradiol treatment resulted in repaired hepatic fibrosis, whereas treatment with a neutralising antirat oestradiol antibody has been confirmed, such mechanisms merit further consideration.

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