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Correlation between serum prolactin levels and hepatocellular tumorigenesis induced by 3'-methyl-4-dimethylaminoazobenzene in mice

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Summary Ovariectomy at 1 month of age promotes development of hepatocellular adenomatous nodules in female C57BL/6 × DS-F_1 mice treated neonatally with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB). Implantation of oestradiol-17β (E_2) pellets at 1 month of age suppresses nodule development. Since E_2 increases serum levels of prolactin, high serum levels of prolactin in mice that have received implants of E_2 pellets may play a role in the suppression of hepatocellular tumorigenesis. Therefore, to investigate the role of prolactin in hepatocellular tumorigenesis, we examined development of adenomatous nodules in female mice that had been treated neonatally with 3'-Me-DAB and had undergone ovariectomy at 1 month of age, under various serum levels of prolactin. Treatment of these mice with perphenazine (dopamine antagonist) from 6 months of age or transplantation of pituitary glands under the renal capsule at 6 months of age markedly increased serum levels of prolactin and significantly suppressed the incidence of adenomatous nodules at 12 months of age. Implantation of E_2 pellets at 1 month of age increased serum levels of prolactin to a greater extent and further decreased the incidence of adenomatous nodules. Treatment of mice that had received implants of E_2 pellets at 1 month of age with bromocriptine (dopamine agonist) from 6 months of age decreased serum levels of prolactin, and was accompanied by an increase in the incidence of nodules. The present results showed that an increase in serum levels of prolactin was accompanied by a decrease in incidence of liver tumours induced by 3'-Me-DAB in mice, suggesting a suppressive effect of prolactin on liver tumorigenesis in mice. Thus, it is possible that the suppressive effect of oestrogen on liver tumorigenesis in mice is mediated, at least in part, by prolactin.

Keywords: prolactin; 3'-methyl-4-dimethylaminoazobenzene; oestrogen

Administration of carcinogens to prepubertal mice induces development of hepatocellular tumours. Male mice are more susceptible than females (Klein and Weisburger, 1966; Vesselinovitch and Mihailovich, 1967; Vesselinovitch, 1969; Roe *et al.*, 1971; Vesselinovitch *et al.*, 1972, 1980; Rao and Vesselinovitch, 1973; Moore *et al.*, 1981; Kemp *et al.*, 1989). This sex difference in susceptibility is due in part to the promotive effect of androgens secreted by the testes after puberty (Vesselinovitch *et al.*, 1980; Moore *et al.*, 1981; Kemp *et al.*, 1989; Weghorst and Klaunig, 1989). Furthermore, several studies (Vesselinovitch and Mihailovich, 1967; Vesselinovitch *et al.*, 1980; Goldfarb and Pugh, 1990; Yamamoto *et al.*, 1991; Tsutsui *et al.*, 1992) have shown that ovariectomy after administration of carcinogens shortens the latent period in the development of hepatocellular tumours and increases their incidence, indicating that the ovaries suppress hepatocellular tumorigenesis. These findings suggest that an ovarian hormone, oestrogen or progesterone, suppresses hepatocellular tumorigenesis in mice.

Administration of 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) to neonatal female mice induces hepatocellular tumorigenesis (Roe *et al.*, 1971; Yamamoto *et al.*, 1991), and ovariectomy promotes development of hepatocellular tumours (Yamamoto *et al.*, 1991, 1993*a*; Tsutsui *et al.*, 1992). We have shown that the ovarian hormone oestradiol-17β (E_2) suppresses hepatocellular tumorigenesis, but that the other ovarian hormone, progesterone, does not (Yamamoto *et al.*, 1991, 1993*b*). Moreover, we suggested that the suppressive action of E_2 is due not to its direct action on the liver but to its indirect action on tissues other than the liver (Yamamoto *et al.*, 1993*b*).

Since oestrogen increases secretion of prolactin by the pituitary gland (Chen *et al.*, 1970; Meites, 1974), and since mouse liver contains prolactin receptors (Harigaya *et al.*, 1988; Davis and Linzer, 1989), it is conceivable that prolactin is an extrahepatic mediator of oestrogen's suppression of hepatocellular tumorigenesis. Therefore, to investigate the role of prolactin in hepatocellular tumorigenesis in mice, we examined hepatocellular tumorigenesis induced by 3'-methyl-4-dimethylaminoazobenzene in mice under various serum levels of prolactin. We produced high serum levels of prolactin by daily injections of perphenazine, a dopamine antagonist (Wicha *et al.*, 1980; Shinha and Gilligan, 1982; Singtripop *et al.*, 1991), or transplantation of pituitary glands under the renal capsule (Chen *et al.*, 1970; Lam *et al.*, 1976), and decreased the oestrogen-induced high serum levels of prolactin by daily injections of bromocriptine, a dopamine agonist (Mori and Nagasawa, 1984; Wood *et al.*, 1991).

Materials and methods

Mice

Female C57BL/6 × DS-F_1 mice (bred in our laboratory) were housed at 25°C under controlled lighting (12 h light/12 h darkness) and allowed free access to water and food pellets. Ovariectomy was performed under pentobarbital sodium anaesthesia.

Administration of carcinogen

The carcinogen, 3'-Me-DAB (ICN Pharmaceuticals, Plainview, NY, USA) was suspended in an aqueous solution of 0.7% (w/v) gelatin at a concentration of 10 mg ml^{-1}; 0.05 ml of the suspension was injected intraperitoneally into mice 10, 12, 14, 16 and 18 days old.

Implantation of oestradiol-17β

Cylindrical cholesterol pellets containing 1% (w/v) E_2 were prepared. Ten milligram pellets were implanted sub-

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cutaneously (s.c.) in the interscapular space. Pellets were replaced every 3 months.

Injection of perphenazine or bromocriptine

Perphenazine (Sigma, St Louis, MO, USA) was dissolved in saline at a concentration of 1 mg ml^{-1}; 0.1 ml of the solution was injected s.c. daily. Bromocriptine (2-bromo-α-ergocriptine methanesulphonate salt) was dissolved in 10% (v/v) ethanol in saline at a concentration of 1 mg ml^{-1}; 0.1 ml of the solution was injected s.c. daily. Bromocriptine was kindly supplied by Sandoz Pharmaceuticals (Tokyo, Japan).

Transplantation of pituitary glands

Four pituitary glands obtained from 5 to 6-month-old female C57BL/6 × DS-F_1 mice that had received no treatments were transplanted under the kidney capsule of 6-month-old mice that had been treated with 3'-Me-DAB neonatally and undergone ovariectomy at 1 month of age.

Treatment of mice

The study consisted in two experiments (experiments I and II). All mice (318 mice) used in the experiments were treated neonatally with 3'-Me-DAB as described above, and underwent ovariectomy at 1 month of age. In experiment I (Figure 1), the mice were divided into four groups (groups 1, 2, 3 and 4). Group 1 mice ($n = 35$) received daily injections of saline (0.1 ml) from the age of 6 months. Group 2 mice ($n = 50$) received daily injections of perphenazine (0.1 mg) dissolved in 0.1 ml of saline. Group 3 mice ($n = 48$) received transplants of four pituitary glands under the kidney capsule at 6 months of age. Group 4 mice ($n = 53$) received implants of E_2 pellets (10 mg) at 1 month of age. In experiment II (Figure 2), the mice were divided into three groups (groups 1, 2 and 3). Group 1 mice ($n = 54$) received daily injections of 0.1 ml of vehicle (10% ethanol in saline) from the age of 6 months. Both groups 2 and 3 received implants of E_2 pellets (10 mg) at 1 month of age. In addition, group 2 mice ($n = 38$) received daily injections of bromocriptine (0.1 mg) dissolved in 0.1 ml of vehicle, while group 3 mice ($n = 40$) received injections of vehicle (0.1 ml) only from the age of 6 months. At 12 months of age, blood was taken from the inferior vena cava of all mice under pentobarbital sodium anaesthesia, after which the mice were killed by cervical dislocation and their livers were promptly removed.

In experiment I, we examined the effects on hepatocellular tumorigenesis of high serum levels of prolactin, produced by either daily injections of perphenazine (Wicha *et al.*, 1980; Shinha and Gilligan, 1982; Singtripop *et al.*, 1991) or transplantation of pituitary glands (Chen *et al.*, 1970; Lam *et al.*, 1976) (Figure 1).

In experiment II, we investigated the effects of bromocriptine, which decreases serum levels of prolactin (Mori and Nagasawa, 1984; Wood *et al.*, 1991), on the suppressive action of oestrogen on hepatocellular tumorigenesis (Figure 2). Since our previous studies suggested that oestrogen exerts its suppressive effect after 6 months of age (Tsutsui *et al.*, 1992; Yamamoto *et al.*, 1993*a*), injections of perphenazine or bromocriptine were started and pituitary glands were transplanted at that time.

Histological examination of the liver

The liver was fixed in Zamboni's solution and cut into 4-mm-thick serial strips. One section (5 μm) of each strip was stained with haematoxylin and eosin; all such sections were examined for nodular lesions, i.e. adenomatous nodules and carcinomas. An adenomatous nodule of hepatocellular origin was defined with reference to previous reports (Vesselinovitch *et al.*, 1978; Frith *et al.*, 1980; Lipsky *et al.*, 1981) as described previously (Yamamoto *et al.*, 1991) as a mixture of eosinophilic, basophilic, vacuolated and foamy hepatocytes in various proportions that compresses the adjacent parenchyma but does not contain a carcinomatous lesion with a trabecular structure. Hepatocellular carcinoma was defined as a nodular lesion with a trabecular structure, as described previously (Yamamoto *et al.*, 1991).

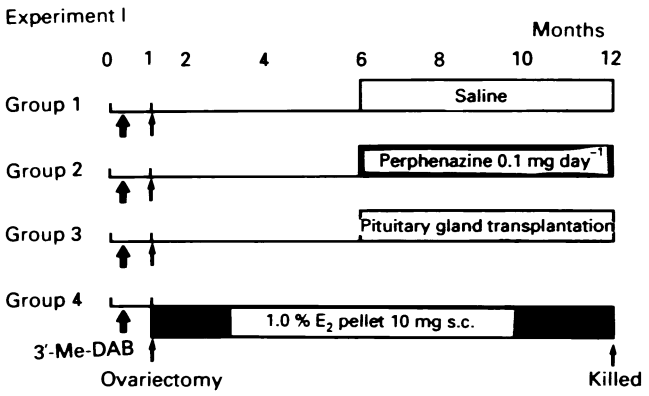

Figure 1 Design of experiment I. All female mice were treated with 3'-Me-DAB neonatally and underwent ovariectomy at 1 month of age. Groups 1 and 2 received daily injections of saline (0.1 ml) and perphenazine (0.1 mg in 0.1 ml of saline) from the age of 6 months. Group 3 received transplants of four pituitary glands under the kidney capsule at 6 months of age. Group 4 received implants of E_2 pellets (10 mg) at 1 month of age.

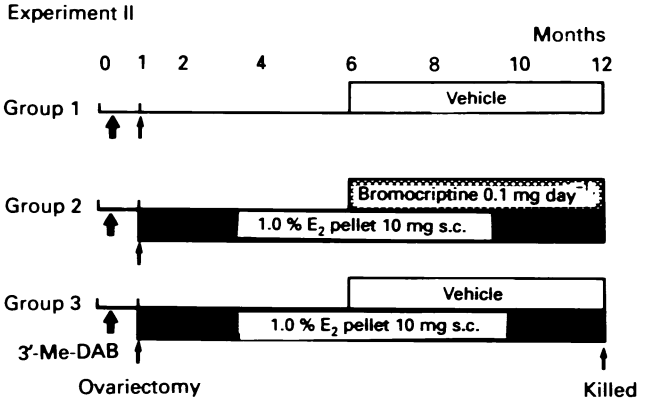

Figure 2 Design of experiment II. All female mice were treated with 3'-Me-DAB neonatally and underwent ovariectomy at 1 month of age. Group 1 received daily injections with 0.1 ml of vehicle (10% ethanol in saline) from the age of 6 months. Groups 2 and 3 received implants of E_2 pellets at 1 month of age. Group 2 received daily injections of bromocriptine (0.1 mg in 0.1 ml of vehicle), and group 3 received vehicle (0.1 ml) only from 6 months of age.

Number of adenomatous nodules per mouse

All sections of the liver prepared as described above were examined, and the number of adenomatous nodules was counted. An adenomatous nodule found in two adjacent sections was counted as one lesion.

Assay of serum prolactin

Serum was obtained by centrifugation of blood at 1000 *g* for 10 min, and was stored at −80°C until assay. Serum prolactin was determined by double-antibody radioimmunoassay with materials and protocols supplied by AF Parlow (Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA). Results are expressed in terms of standard AFP6476C. All samples were assayed in duplicate. The intra-assay and inter-assay variations were less than 8% and 10% respectively.

Statistical analysis

Statistical analysis was performed with the χ^2 test or Student's *t*-test. A *P*-value below 0.05 was considered significant.

Results

Table I shows the effects of perphenazine or transplantation of pituitary glands on development of adenomatous nodules induced by neonatally administered 3'-Me-DAB. The incidence of adenomatous nodules in mice that had undergone ovariectomy at 1 month of age was 71.4% at 12 months of age. Treatment with perphenazine from 6 months of age and transplantation of pituitary glands at 6 months of age significantly decreased the incidence of adenomatous nodules to 16.0% and 20.6% respectively, while implantation of E_2 pellets at 1 month of age decreased the incidence to 1.9%. However, the mean number of adenomatous nodules per mouse was not significantly affected by treatment with perphenazine or transplantation of pituitary glands. Treatment with perphenazine and transplantation of pituitary glands markedly elevated serum levels of prolactin, and implantation of E_2 pellets raised prolactin levels to an even greater extent.

Table II shows the effects of bromocriptine on development of adenomatous nodules in mice that had received implants of E_2 pellets. Implantation of E_2 pellets at 1 month of age markedly decreased the incidence of adenomatous nodules at 12 months of age to 5.0%, while the incidence was 66.7% in mice that did not receive E_2 pellets. When mice that had received E_2 pellets were treated with bromocriptine from 6 months of age, the incidence of adenomatous nodules increased to 23.7%. This incidence was significantly lower than that in mice that did not receive E_2 pellets, and was similar to that in mice treated with perphenazine or transplanted pituitary glands, as shown in Table I. The number of adenomatous nodules was not significantly affected by either implantation of E_2 pellets or treatment with bromocriptine. Serum prolactin levels in mice that received E_2 pellets were extremely high, in agreement with data shown in Table I. Treatment with bromocriptine significantly decreased serum prolactin to levels similar to those in mice treated with perphenazine or pituitary gland transplants, as shown in Table I. No carcinomas were found in any groups. No adenomatous nodules or carcinomas developed in the livers of 21 female mice at 12 months of age which had not been treated neonatally with 3'-Me-DAB, but had instead undergone ovariectomy at 1 month of age.

Discussion

Increases in serum levels of prolactin produced by injections of perphenazine (dopamine antagonist) and by pituitary grafts were accompanied by decreases in the incidence of adenomatous nodules. The greater the increase in serum levels of prolactin produced by implantation of E_2 pellets, the greater was the decrease in the incidence of nodules. Furthermore, bromocriptine (dopamine agonist) decreased the high serum levels of prolactin induced by implantation of E_2 pellets and increased the incidence of adenomatous nodules. Dopamine agonists and antagonists produce changes in serum levels of prolactin (Shinha and Gilligan, 1982; Mori and Nagasawa, 1984; Singtripop *et al.*, 1991; Wood *et al.*, 1991), but have also been reported to exert other effects. For example, it is reported that dopamine antagonists stimulate aldosterone and corticosterone secretion in rats (Goebel *et al.*, 1992) and that bromocriptine increases serum levels of growth hormone in normal human subjects (Wood *et al.*, 1991). Pituitary grafts secrete growth hormone as well as prolactin (Blanck *et al.*, 1984). Moreover, a recent report by

Table I Effects of perphenazine or transplantation of pituitary glands on serum prolactin levels and development of adenomatous nodules in the liver

Treatment	*Adenomatous nodules* Incidence	*Number per mouse*[a]	*Serum prolactin* (ng ml^{-1})[a]
Saline	25/35 (71.4%)[b]	2.5 ± 0.4	16.6 ± 3.3[c] ($n = 11$)
Perphenazine	8/50 (16.0%)[b,d]	1.5 ± 0.3	178.6 ± 17.2[c,e] ($n = 20$)
Transplantation of pituitary glands	10/48 (20.6%)[b,d]	1.4 ± 0.2	203.0 ± 22.2[c,e] ($n = 17$)
Implantation of E_2 pellets	1/53 (1.9%)[d]	1.0	1320.5 ± 158.9[e] ($n = 15$)

The experimental design is shown in Figure 1. Serum concentrations of prolactin were measured in mice, the number of which is shown in parentheses. Incidence indicates the number of mice with adenomatous nodules out of number of mice examined, and percentage incidence is shown in parentheses. Number per mouse indicates the number of adenomatous nodules in each mouse with adenomatous nodules. [a]Mean ± s.e. [b,c]$P < 0.05$, significant difference from the value of mice implanted with E_2 pellets ([b]by χ^2 test; [c]by Student's *t*-test). [d,e]$P < 0.05$, significant difference from the value of mice injected with saline alone ([d]by χ^2 test; [e]by Student's *t*-test).

Table II Effects of bromocriptine injections on serum prolactin levels and development of adenomatous nodules

Treatment	*Adenomatous nodules* Incidence	*Number per mouse*[a]	*Serum prolactin* (ng ml^{-1})[a]
Vehicle	36/54 (66.7%)[b]	1.9 ± 0.3	14.9 ± 3.1[c] ($n = 20$)
Bromocriptine and implantation of E_2 pellets	9/38 (23.7%)[b,d]	1.3 ± 0.2	164.5 ± 21.0[c,e] ($n = 15$)
Implantation of E_2 pellets	2/40 (5.0%)[d]	1,2	818.5 ± 158.1[e] ($n = 15$)

The experimental design is shown in Figure 2. Serum concentrations of prolactin were measured in mice, the number of which is shown in parentheses. Incidence indicates the number of mice with adenomatous nodules out of number of mice examined, and percentage incidence is shown in parentheses. Number per mouse indicates the number of adenomatous nodules in each mouse with adenomatous nodules. [a]Mean ± s.e. [b,c]$P < 0.05$, significant difference from the value of mice implanted with E_2 pellets ([b]by χ^2 test; [c]by Student's *t*-test). [d,e]$P < 0.05$, significant difference from the value of mice injected with vehicle alone ([d]by χ^2 test; [e]by Student's *t*-test).

Ishibashi *et al.* (1994) that bromocriptine inhibits growth of human small-cell lung cancer through tumour dopamine receptors raises the possibility that dopamine agonists and antagonists influence 3'-Me-DAB-induced tumorigenesis through a direct effect on hepatocytes. Therefore, we cannot exclude the possibility that the influences of perphenazine, pituitary grafts and bromocriptine on liver tumorigenesis are irrelevant to influences on serum levels of prolactin. However, our finding that the increase in serum levels of prolactin was accompanied by a suppression of liver tumorigenesis in mice supports the possibility that high serum levels of prolactin suppress development of 3'-Me-DAB-induced hepatocellular tumours in mice. If this is true, then it is likely that the suppressive action of oestrogen on hepatocellular tumorigenesis in mice is mediated, at least in part, by prolactin secreted by the pituitary glands.

In rats prolactin has been shown to induce hepatic ornithine decarboxylase and plasminogen activator activity and specific enzyme markers expressed early in the G_1 phase of the cell cycle (Crowe *et al.*, 1991), to cause hypomethylation of DNA in the liver (Reddy and Reddy, 1990), to stimulate DNA synthesis by hepatocytes (Buckley *et al.*, 1986) and to produce hepatomegaly (Buckley *et al.*, 1985). Furthermore, it is reported that prolactin promotes development of diethylnitrosamine-induced preneoplastic γ-glutamyltranspeptidase-positive foci in the liver of female rats (Buckley *et al.*, 1985). Blank *et al.* (1987) reported that prolactin did not suppress growth in male rats of γ-glutamyltransferase-positive hepatic foci induced by diethylnitrosamine followed by treatment with 2-acetylaminofluorene. Thus, the effects of prolactin on the development of carcinogen-induced hepatocellular tumours in rats may be opposite to those in mice, although it is unknown whether the effects of prolactin on normal hepatocytes differ in mice and rats.

The effects of oestrogens on hepatocellular tumorigenesis in rats are opposite to those in mice. In mice, a natural oestrogen, E_2, and a synthetic oestrogen, ethinyloestradiol, have been reported to suppress carcinogen-induced hepatocellular tumorigenesis (Lee *et al.*, 1989; Yamamoto *et al.*, 1993*a*,*b*). In contrast, in rats several synthetic oestrogens have been shown to promote carcinogen-induced hepatocellular tumorigenesis (Metzler and Degen, 1987; Campen *et al.*, 1990; Mayol *et al.*, 1992). A synthetic oestrogen has been shown to increase serum levels of prolactin (Campen *et al.*, 1990). Synthetic oestrogens may exert toxic effects on the liver that contribute to the promotion of hepatocellular tumorigenesis (Metzler and Degen, 1987). However, if the promotive effects of synthetic oestrogens on hepatocellular tumorigenesis are due to the high serum levels of prolactin which they produce, the difference in the effects of oestrogens on hepatocellular tumorigenesis in mice and rats might be due to the difference in the effects of prolactin on hepatocellular tumorigenesis.

In carcinogen-induced hepatocellular tumorigenesis in rats, the difference in the secretory pattern of growth hormone is due, at least in part, to the lesser susceptibility of females to carcinogens; higher basal levels of serum growth hormone in females suppresses hepatocellular tumorigenesis (Blank *et al.*, 1987; Hallstrom *et al.*, 1991). The role of growth hormone in suppression of hepatocellular tumorigenesis in female mice is unknown, but the present results suggest that the pituitary gland plays a role in hepatocellular tumorigenesis in mice.

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