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# Sex Hormone Dependency of Diethylnitrosamine-induced Liver Tumors in Mice and Chemoprevention by Leuprorelin

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**The prevalence of liver tumors throughout the world makes it imperative to seek chemopreventive agents. This tumor appears to be hormone-responsive and hormonal manipulations may therefore be beneficial. On this basis, both sexes of 12-day-old B6C3F$_1$ mice were injected i.p. with diethylnitrosamine (DEN) at the dose of 2.5 $\mu$g/g body weight and observed for 32 weeks (males) or 36 weeks (females). In 100% of male mice, liver tumors were observed with an average diameter of 2.72 mm and multiplicity of 60.8. Orchidectomy at 6 weeks of age in these mice inhibited the incidence, multiplicity and size to 63%, 5.6 and 1.54 mm, respectively. By further implantation with an E$_2$ pellet at monthly intervals, these parameters were reduced to 26%, 0.6 and 0.61 mm, respectively. Administration of a gonadotropin-blocking chemical, leuprorelin, to DEN-treated male mice significantly reduced the multiplicity and size of tumors to 18.3 and 2.54 mm ($P<0.01$ compared to those of DEN only). In female mice, the incidence of liver tumor was significantly smaller than that of males. However, ovariectomy and/or testosterone supplement significantly increased the occurrence of liver tumor. An anti-estrogen, toremifene, caused a marked further decrease of liver tumors. Mitotic indices with bromodeoxyuridine in tumor tissues paralleled the occurrence of liver tumors. Serum testosterone levels were significantly reduced by orchidectomy or by leuprorelin administration. These results further confirm that liver tumor is testosterone-responsive and hormonal manipulation by surgical orchidectomy or by chemical orchidectomy i.e. by leuprorelin, could substantially prevent the appearance of liver tumors.**

Key words: Liver tumor — Testosterone — Leuprorelin — DEN — Mice

Hepatocellular carcinoma (HCC) is one of the major cancers throughout the world. It shows male predominance, and in Asian countries, the male-to-female ratio ranges from 2.4 to 4.3.[1)] It ranks fourth highest (10%) among the sites of cancer in males in Japan, albeit its 4% contribution to cancers in females is not negligible.[2)] More than 80% of the cases of HCC occur in males in other continents. The geographic distribution of HCC is highly uneven, with well recognized but variable risk factors such as male sex, increasing age, viral infection, cirrhosis, etc. However, no substantial difference between areas with low or high incidence in male prevalence was noted.[3)] Sex hormones have long been known to play a role in mouse hepatocarcinogenesis.[4, 5)] Gonadectomy in male mice decreased and ovariectomy in female mice increased the incidence of liver tumors, both spontaneous and chemically initiated.[6)] Thus, the role of sex hormones in hepatocarcinogenesis has been well established.[7)] Recently, androgen and its receptor (AR) have been suggested to be involved in the male predominance. Yu *et al.* found an increase in both AR protein and its messenger RNA in liver tumors among 70% of male patients compared with 37% of female cases.[8)] A human hepatic tumor line (SH10) showed androgen-responsive growth, and androgen ablation inhibited tumor growth.[9)] An androgen blocking agent, leuprorelin, showed anti-tumor activity against hormonally responsive mammary tumors in rats.[10, 11)] It suppresses hormonally controlled mammary and prostatic cancers in humans.[12–14)] Although anti-androgen therapy seemed a reasonable approach, genetic variability was observed in the results of different experiments.[15–17)] The present study was performed to elucidate the effects of gender, castration and especially of hormone replacement therapy using leuprorelin, on the occurrence of liver tumors in both sexes of B6C3F$_1$ mice.

## MATERIALS AND METHODS

**Animals** Male C3H/HeN and female C57BL/6N mice were purchased from Charles River Japan, Inc. (Kanagawa) and were allowed to mate to produce offspring (B6C3F$_1$) in our laboratory. All mice were given MF diet (Oriental Co., Ltd., Tokyo) and tap water *ad libitum*. Mice were maintained under the guidelines set forth in the 'Guide for the Care and Use of Laboratory Animals' by Hiroshima University.

**Chemicals** Diethylnitrosamine (DEN) was purchased

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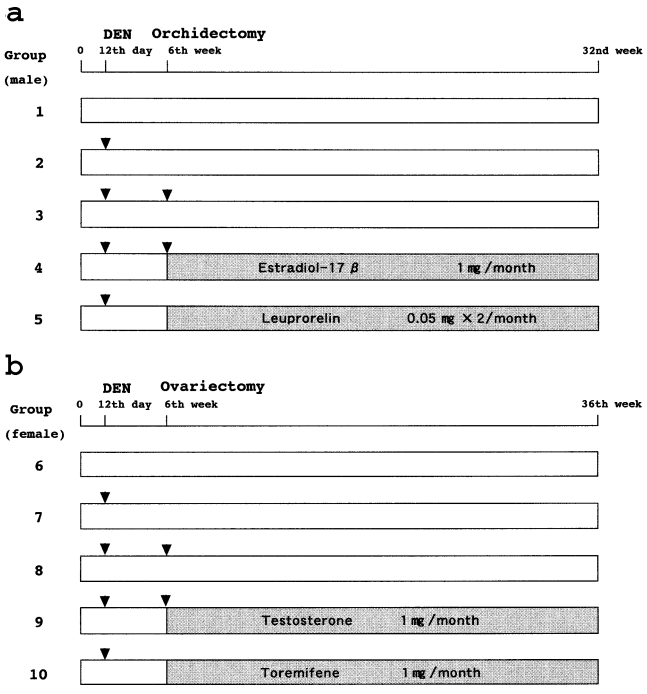


Fig. 1. a. Experimental schedule of the male model. On the 12th day, mice of all groups were given a single i.p. injection of DEN at a dose of 2.5 $\mu$g/g body weight, except group 1. At the 6th week, groups 3 and 4 were subjected to orchidectomy. Estradiol-17β or leuprorelin was given to groups 4 and 5 from the 6th week to the end of the experiment, respectively. b. Experimental schedule of the female model. On the 12th day, mice of all groups were given a single i.p. injection of DEN at a dose of 2.5 $\mu$g/g body weight, except group 1. At the 6th week, groups 3 and 4 were subjected to ovariectomy. Testosterone or toremifene was given to groups 4 and 5 from the 6th week to the end of the experiment, respectively.

from Kantou Kasei Co., Ltd. (Tokyo), dissolved in physiological saline and injected i.p. at a dose of 2.5 $\mu$g/g body weight. Testosterone (T), 17β-estradiol ($E_2$) and toremifene (analogue of tamoxifen, Nihon Kayaku, Ltd., Tokyo) were made into cholesterol pellets by heating the steroid hormones and cholesterol powder until the mixture fused. Pellets were individually weighed and cut to size so that each contained 0.5 or 1 mg of steroid hormone. These pellets were implanted s.c. at an interval of a month throughout the experimental period. Leuprorelin is a gonadotropin releasing hormone antagonist and a synthetic antagonist to testosterone, and is used for therapeutic purposes in human prostate and breast cancer patients.[18–20] It desensitizes the pituitary gland by inhibiting follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion and disturbs sexual activities. Its molecular weight is 1269.47 and it is composed of 9 amino acids. It was reported that serum $E_2$ and testosterone levels were decreased by the continuous supply of leuprorelin.[21] The drug was kindly provided by Takeda Pharmaceutical Co., Ltd., Osaka, and injected s.c. at the dose of 0.05 mg/mouse every 2 weeks throughout the experimental period.

**Surgery** At 6 weeks of age, both male and female mice were subjected to orchidectomy in groups of 3 and 4 and ovariectomy in groups of 8 and 9 under light ether anesthesia.

**Experimental design** At 12 days old, male and female mice were given a single i.p. injection of DEN. The mice were sub-divided into the following groups. In males (Fig. 1a), they are group 1—untreated, group 2—DEN only, group 3—DEN + orchidectomy, group 4—DEN + orchidectomy + $E_2$ and group 5—DEN + leuprorelin. In females (Fig. 1b), they are group 6—untreated, group 7—DEN only, group 8—DEN + ovariectomy, group 9—DEN + ovariectomy + testosterone and group 10—DEN + toremifene. All mice were routinely inspected and body weights were recorded at intervals of a month until the end of the experiment. Since liver tumors are predominant in males and less frequent in females, the observation period was set for 32 weeks for males and 36 weeks for females.

**Pathology** Six to 8 mice from each experimental group were injected i.p. with 0.1 ml of 10% bromodeoxyuridine (BrdU, Sigma Chemicals Co., St Louis, MO) prepared in physiological saline on the last day of the experiment and they were killed under ether anesthesia at 1 h post-injection. Body and organ weights were recorded and major organs, liver and other tumors, if any, were fixed in 10% buffered formalin and prepared for pathological studies by staining with hematoxylin and eosin (HE).

At the time of autopsy, all liver tumors more than 1 $mm^2$ in diameter were macroscopically enumerated at the surface of liver. The individual liver tissues including tumor were cut into 2 to 3 pieces and stained with HE and monoclonal mouse anti-BrdU antibody (Dako Japan Co., Ltd., Kyoto). The percent of BrdU incorporated cells (labeling index) was expressed by counting 1000 parenchymal cells from whole lobes of liver. Since we did not make a thorough study on all developed tumors, the classification of liver tumors into hyperplasia, adenoma and carcinoma was not performed.

**Hormone assay** Blood was collected only at the time of sacrifice of mice from the cervical vein at 32 weeks of age in males and 36 weeks in females. Sera were separated and kept at −20°C until assay. An aliquot was added to the antibody-coated tubes together with $^{125}$I-labeled testosterone. After 3-h incubation at 37°C, the tubes were washed with saline and the activity ratio in each tube was counted. The antibody used is a poly-clonal anti-human testosterone and its cross reactivity is 0.8% to 11β-hydroxytestosterone, 0.2% to triamcinolone, and 3.3% to 5α-dihydrotestosterone, but undetectable to progesterone. Serum testosterone and $E_2$ levels were calculated as ng/ml and pg/ml

values with standard curves. Average values of duplicate individual samples were taken.

## RESULTS

Effective numbers, and body and major organ weights in various experimental groups for both male and female mice are depicted in Table I. At the termination of the experiment, effective numbers of male mice were greater than 90% in all experimental groups except 76.7% in group 4, indicating that $E_2$ is toxic when administered to DEN-treated castrated mice, as the body weight was also significantly reduced. Leuprorelin on the other hand had no toxic effects. The body weight of the ovariectomized female mice treated with testosterone (group 9) was significantly higher than that of the respective control in group 6. Toremifene did not show any toxic effects or cause any reduction in body weight in DEN-treated female mice. Liver weight in group 2 increased more than twofold in comparison to that of group 1. The increased liver weight is correlated with proliferation leading to the development of liver tumor. When DEN-treated mice were castrated and supplemented with $E_2$ (group 3 and 4) or supplemented with leuprorelin (group 5), liver weight increase was significantly reduced in comparison to group 2. This result may be attributed to the reduction of liver tumorigenesis.[22)]

In group 1, a very small tumor 0.2 mm in size was found in one out of 18 male mice (Table II). In group 2, the frequency of tumors was 100%, with an average multiplicity of 60.8 and an average diameter of 2.72 mm. Orchidectomy decreased the incidence of tumor significantly to 63% (5.6 multiplicity and 1.54 mm average diameter). Further, decrease in the incidence of liver tumors was noted when orchidectomized mice were additionally treated with $E_2$. The continuous administration of leuprorelin to DEN-treated mice caused a significant decrease of average tumor number and diameter compared to those of group 2. These results clearly indicated that the deprivation of male hormone was effective for decreasing liver tumors.

The intact female mice of group 6 did not show liver tumors at all in the observation period (Table II). In the DEN-administered group 7, tumor incidence, multiplicity and average diameter were 38%, 1.2 and 0.9 mm, respectively. By ovariectomy, these values were significantly increased to 63%, 6.6 and 1.31 mm, respectively. Further treatment with testosterone in group 9 resulted in 100% frequency, a multiplicity of 45.4 and a diameter of 2.25 mm, comparable to those of group 2. Administration of toremifene in female mice resulted in parameters somewhat similar to those of group 8.

Representative macroscopic findings of liver tumors are shown in Fig. 2. The numbers of tumor nodules were markedly reduced by orchidectomy or by administration of leuprorelin in male mice. In female mice, the numbers of tumor nodules were increased in the ovariectomized group. Further enlargement of liver tumors and increase of multiplicity were seen upon the addition of testosterone. In both male and female groups, light microscopic observa-

Table I. Experimental Groups, Body and Organ Weights in Mice

| Experimental groups | Treatment | Effective No. | BW (g) | Liver weight (g) | Liver weight /BW[a)] | Kidney (g) | Testis (g) | Ovary (g) | Uterus (g) | Spleen (g) |
|---|---|---|---|---|---|---|---|---|---|---|
| Male | | | | | | | | | | |
| 1 | Control | 18/18 | 40.9±3.0[b)] | 1.5±0.3[b)] | 0.04±0.00[b)] | 0.50±0.06[b)] | 0.23±0.02[b)] | — | — | 0.15±0.21[b)] |
| 2 | DEN | 19/19 | 41.9±3.2 | 3.8±1.1 | 0.09±0.03 | 0.56±0.05 | 0.23±0.01 | — | — | 0.12±0.03 |
| 3 | DEN+Orex | 30/30 | 42.4±2.1 | 1.9±0.8[d)] | 0.05±0.02 | 0.38±0.05[d)] | — | — | — | 0.11±0.02 |
| 4 | DEN+Orex+$E_2$ | 23/30[c)] | 33.5±3.3[d)] | 1.6±0.2[d)] | 0.05±0.01[d)] | 0.55±0.05 | — | — | — | 0.18±0.02[d)] |
| 5 | DEN+Leuprorelin | 28/30[c)] | 42.8±3.0 | 2.4±0.9[d)] | 0.06±0.02[d)] | 0.43±0.05[d)] | 0.18±0.02[d)] | — | — | 0.11±0.02 |
| Female | | | | | | | | | | |
| 6 | Control | 17/17 | 33.2±5.0[b)] | 1.1±0.1[b)] | 0.04±0.00[b)] | 0.36±0.07[b)] | — | 0.04±0.03[b)] | 0.22±0.10[b)] | 0.10±0.03[b)] |
| 7 | DEN | 21/21 | 35.3±5.1 | 1.3±0.2 | 0.04±0.00 | 0.36±0.03 | — | 0.03±0.01 | 0.26±0.12 | 0.11±0.05 |
| 8 | DEN+Ovex | 30/30 | 37.6±6.0 | 1.4±0.5 | 0.04±0.01 | 0.34±0.03[e)] | — | — | 0.09±0.08[e)] | 0.10±0.02 |
| 9 | DEN+Ovex+T | 29/30 | 39.8±4.4[e)] | 2.1±0.7[e)] | 0.05±0.02[e)] | 0.48±0.05[e)] | — | — | 0.05±0.02[e)] | 0.09±0.02[f)] |
| 10 | DEN+Toremifene | 29/30 | 34.7±4.7 | 1.1±0.2 | 0.03±0.04 | 0.30±0.03[e)] | — | 0.02±0.01[e)] | 0.09±0.01[e)] | 0.10±0.01 |

*a*) BW, body weight.
*b*) Mean±SD.
*c*) One mouse died of leukemia in group 5, and others died of surgery.
*d*) $P<0.01$ compared with group 2.
*e*) $P<0.01$ compared with group 7.
*f*) $P<0.05$ compared with group 7.

Table II. The Incidence of Liver Tumors in Mice

| Experimental groups | Treatment | Effective No. | Incidence (%) | Multiplicity | Average diameter (mm) |
|---|---|---|---|---|---|
| Male | | | | | |
| 1 | Control | 18/18 | 6 ( 1/18) | 0.1±0.2[a)] | 0.22±0.94[a)] |
| 2 | DEN | 19/19 | 100 (19/19) | 60.8±19.5 | 2.72±0.62 |
| 3 | DEN+Orex | 30/30 | 63 (19/30)[c)] | 5.6±8.0[c)] | 1.54±1.49[b)] |
| 4 | DEN+Orex+$E_2$ | 23/30 | 26 ( 6/23)[c)] | 0.6±1.7[c)] | 0.61±1.16[c)] |
| 5 | DEN+Leuprorelin | 28/30 | 96 (27/28) | 18.3±12.6[c)] | 2.54±1.12 |
| Female | | | | | |
| 6 | Control | 17/17 | 0 | 0 | 0 |
| 7 | DEN | 21/21 | 38 ( 8/21) | 1.2±1.9 | 0.90±1.24 |
| 8 | DEN+Ovex | 30/30 | 63 (19/30) | 6.6±9.9[e)] | 1.31±1.14 |
| 9 | DEN+Ovex+T | 29/30 | 100 (29/29)[e, f)] | 45.4±26.1[e, f)] | 2.25±0.55[e, f)] |
| 10 | DEN+Toremifene | 29/30 | 55 (16/29) | 3.9±5.9[d)] | 1.23±1.28 |

*a*) Mean±SD.
*b*) $P<0.05$ compared with group 2.
*c*) $P<0.01$ compared with group 2.
*d*) $P<0.05$ compared with group 7.
*e*) $P<0.01$ compared with group 7.
*f*) $P<0.01$ compared with group 8.

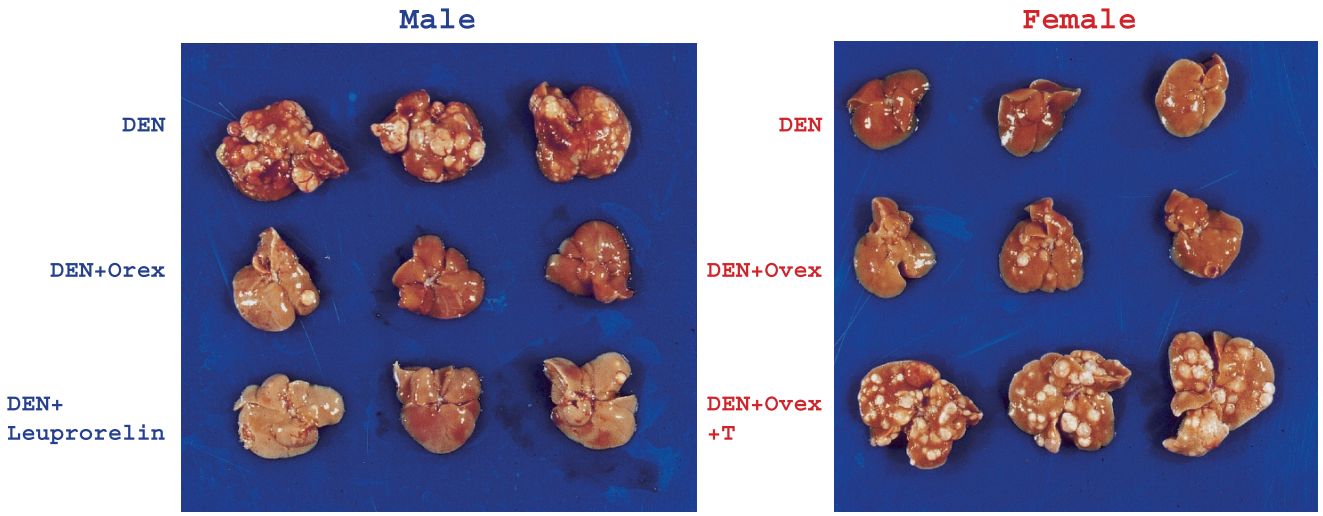


Fig. 2. Gross appearance of liver tumors. The number of nodules was reduced markedly after orchidectomy or administration of leuprorelin in male mice. The number of nodules was increased by ovariectomy, and further increased by testosterone administration in female mice.

tion confirmed neoplastic changes, with macroscopic appearance of grayish-white nodules. Most liver tumors were well demarcated with occasional appearance of periosis hepatis. Tumor cells were vacuolated, eosinophilic or basophilic. We did not classify the tumors in terms of malignancy.

DNA labeling indices obtained by BrdU staining showed a significantly higher proliferative activity within liver tumors, but no positive cells in the surrounding liver tissues. The activity was particularly marked at the marginal region of tumor tissues. Moreover, there was a significant reduction in the mean value of labeling index in the tumors of the DEN plus orchidectomy group and DEN plus leuprorelin group compared to that of the DEN alone group in males. On the other hand, a significant increase in the mean value of labeling indices was caused by testosterone administration to ovariectomized mice, indicating a role of testosterone in promoting tumors (Fig. 3).

Serum testosterone and $E_2$ levels under various experimental conditions were measured at 32 weeks of age in males and 36 weeks in females (Table III). In group 2, testosterone level was reduced to 0.45 ng/ml from 1.05 ng/ml in control mice. Following orchidectomy in group 3 and supplement with leuprorelin in group 5, it was reduced to 0.2 ng/ml. Supplement with testosterone, resulted in a value comparable to that of group 2. In female mice, the testosterone level was very low (0.20 ng/ml) irrespective of DEN treatment or ovariectomy, being comparable to that of orchidectomized male mice. Addition of testosterone or toremifene in ovariectomized mice significantly increased the serum testosterone level.


Fig. 3. BrdU labeling indices in the tissues of liver tumors. Significant reduction of labeling indexes was noted in the tumors of the Orex and Leuprorelin groups of male mice compared to that of the control. The significant reduction in tumors of the Ovex group was reversed by testosterone administration in female mice.

## DISCUSSION

Hepatocellular carcinoma is one of the most common cancers throughout the world and is predominant in human and murine males, with about 4 times higher incidence than in females.[23] Similar patterns of incidence have also been reported in spontaneous,[24] radiation-induced[25] and DEN-induced cancers of $B6C3F_1$ mice.[26] Data from the present experiments also showed that the incidence of DEN-induced liver tumors was 100% in male mice against only 38% in females, indicating a differential response between sexes. These results may be attributed to sex-related differences in liver metabolism, especially differences in carcinogen-metabolizing enzymes.[27, 28] After orchidectomy of DEN-treated mice, significantly reduced occurrence of liver tumors was found, suggesting a definite involvement of male hormone in liver tumorigenesis. Orchidectomy removes the source of testosterone, but the serum level of male hormone is retained more or less intact. Therefore, complete inhibition of tumorigenesis was not evident. When orchidectomized mice were further supplemented with estrogen, the tumor incidence was reduced to a minimum, indicating a negative role of estrogen in liver tumorigenesis in the male. This was supported by the fact that tumor growth in ovariectomized female mice was enhanced compared to that of intact female mice. When spayed mice were treated with testosterone, serum testosterone level rose and the tumor incidence became 100% in female mice, confirming that testosterone potentiated the growth of liver tumors.[29] Similar decreased incidence of carcinogen-induced hepatomas in orchidectomized male rats and increased incidence of chemically induced liver tumors in ovariectomized female rats were

Table III. Serum Testosterone and $E_2$ Levels in Mice

| Experimental groups | Treatment | Effective No. | Testosterone (ng/ml) | $E_2$ (pg/ml) |
|---|---|---|---|---|
| Male | | | | |
| 1 | Control | 4 | 1.05±0.72 | <10 |
| 2 | DEN | 8 | 0.45±0.53 | <10 |
| 3 | DEN+Orex | 8 | <0.2 | 16.8±7.6[b] |
| 4 | DEN+Orex+T | 7 | 0.54±0.28[a] | 13.2±3.3[b] |
| 5 | DEN+Leuprorelin | 8 | <0.2 | <10 |
| Female | | | | |
| 6 | Control | 5 | <0.2 | <10 |
| 7 | DEN | 6 | <0.2 | 11.2±2.0 |
| 8 | DEN+Ovex | 9 | <0.2 | <10 |
| 9 | DEN+Ovex+T | 10 | 0.73±0.58[c] | 11.1±3.2 |
| 10 | DEN+Toremifene | 6 | 0.85±0.25[c] | <10 |

*a*) $P<0.01$ compared with group 3.
*b*) $P<0.05$ compared with groups 1 and 2.
*c*) $P<0.05$ compared with groups 6, 7 and 8.

suggested to be mostly mediated by the respective sex hormones via effects on carcinogen metabolism and activation.[30–32] Hormonal responsiveness of tumors, however, is also related to the presence of the relevant receptor.[33] Administration of toremifene, an estrogen antagonist, in female mice produced a condition similar to that of spayed mice. Vesselinovitch *et al.* suggested that orchidectomy of male B6C3F$_1$ mice after various periods of DEN administration significantly decreased the occurrence of liver tumors compared to the respective control mice.[5] Considering all available results, anti-androgen therapy appears to be a logical approach to the treatment of liver tumor. Among hepatoma patients treated with an anti-androgen, cyproterone acetate, a modest response was obtained in 20% of patients, which is comparable to that expected with conventional cytotoxic chemotherapeutic agents.[34–37] However, the mechanism underlying these events has not been well elucidated. The results suggest that hormonal manipulation may be effective in the treatment of hepatoma if free plasma or serum testosterone level can be reliably reduced.[32]

Kemp and Drinkwater reported that a DEN model in testicular feminization mutant (Tfm) mice showed a significant decrease of liver tumors compared to wild-type mice.[38, 39] In our previous studies, incidence and multiplicity of liver tumors were significantly reduced in male B6C3F$_1$ mice given DEN after orchidectomy compared to those of control mice.[40, 41] The result clearly indicates the importance of testosterone for liver tumorigenesis. In the present study, BrdU labeling indices showed a significant difference in the mean number of BrdU-labeled cells between intact and orchidectomized mice and ovariectomy and ovariectomy plus testosterone treated mice. Tumor growth represents the balance between cell proliferation and cell death, including apoptosis, and hormone ablation may readily cause cell loss in such hormone-dependent tumors as prostate and breast cancers.[42] In this study, the removal of testosterone reduced the cell proliferation in liver tumors. The physiological concentration of serum testosterone may be enough to support liver tumorigenesis, and androgen ablation by orchidectomy could cause inhibition of the tumor growth in the liver, but might not be a preferred choice for patients. Leuprorelin has been extensively used for therapeutic purposes in human prostate and breast cancer patients.[11, 12] It was found in the present and previous studies that serum $E_2$ and testosterone levels were decreased by continuous supply of leuprorelin.[9, 10] Our results demonstrate that although leuprorelin only marginally inhibited the incidence of liver tumors, their multiplicity and size were significantly reduced.

In our previous studies, the non-steroid anti-androgen flutamide was shown to inhibit liver tumors by reducing androgen receptor levels.[41] Despite data suggesting that liver tumors may be androgen-dependent or androgen-responsive, flutamide appears to have no significant clinical anti-cancer effect in hepatoma patients.[15–17] Our results indicated that leuprorelin is an effective and less toxic agent that could be suitable for first line endocrine treatment for hepatoma patients.

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