Long-term testosterone treatment prevents gonadal and adrenal tumorigenesis of mice transgenic for the mouse inhibin- α subunit promoter/simian virus 40 T-antigen fusion gene

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Abstract

We have developed a transgenic (TG) mouse model for tumorigenesis of gonadal somatic cells using a 6 kb fragment of the mouse inhibin- α subunit promoter (Inh- α) fused with the simian virus 40 T-antigen (Tag) coding sequence. Gonadal tumors, of Leydig or granulosa cell origin, develop in the TG mice with 100% penetrance by the age of 5–8 months. Conspicuously, if the mice are gonadectomized, they develop adrenal tumors. Gonadal and adrenal tumorigenesis in these mice seem to be gonadotropin dependent. On the other hand, testosterone stimulates the proliferation of a cell line (C α 1) established from one of the adrenal tumors. The purpose of the present study was therefore to investigate further whether testosterone affects the growth of these gonadal and adrenal tumors *in vivo*.

Two experimental models were used: (1) Tag TG/hypogonadotropic (*hpg*) double mutant mice and (2) castrated Tag TG mice. Both were treated between 1–2 and 7–8 months of age with Silastic rods (length 2 cm) containing testosterone. None of the control or testosterone-treated Tag/*hpg* mice developed gonadal or adrenal tumors. The castrated Tag TG mice displayed, upon microscopical examination, early stages of adrenal

tumors, whereas those receiving testosterone did not show such changes. Testosterone increased the weights of gonads in the Tag/*hpg* mice, and those of uteri and seminal vesicles in both groups. In contrast, the adrenal weights were significantly reduced in both groups by testosterone treatment. Gonadal histology of the testosterone-treated mice showed hyperplasia of testicular Leydig cells and ovarian stroma. Spermatogenesis was induced by testosterone in the Tag/*hpg* mice. Adrenal histology of the testosterone-treated animals demonstrated the disappearance of the X-zone. Serum levels of FSH in testosterone-treated Tag/*hpg* mice were significantly increased, while those of serum LH were decreased.

In conclusion, the present result indicate that the suppression of gonadotropins by testosterone implants in castrated Inh- α /Tag TG mice prevents the tumorigenesis of their adrenals. In intact Tag/*hpg* mice, testosterone implants were not able to induce gonadal or adrenal tumorigenesis. Although testosterone treatment was able to induce interstitial cell hyperplasia in gonads of the Inh- α /Tag mice, direct gonadotropin action is responsible for gonadal and adrenal tumorigenesis.

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Introduction

Elevated levels of gonadotropins may promote the development of ovarian and testicular cancer (Biskin & Biskin 1944, Matzuk *et al.* 1992). Accordingly, a number of studies support a causal relationship between high levels of gonadotropins and development of cancer in human ovarian epithelium (Gardner 1955, Murphy & Beamer 1973, Parmar *et al.* 1988, Manetta *et al.* 1995), breast (Yano *et al.* 1992) and prostate (Korkut *et al.* 1991, Szepeshazi *et al.* 1991). In accordance, the suppression of circulating gonadotropins by gonadotropin-releasing hormone (GnRH) agonist or antagonist inhibits tumor growth in nude mice bearing ovarian tumor xenografts (Peterson

& Zimniski 1990, Yano *et al.* 1994), in W^x/W^y mice (Blaakaer *et al.* 1995), in T-antigen (Tag)-expressing transgenic (TG) mice, as well as in crosses of the latter with gonadotropin-deficient (*hpg*) mice (Kananen *et al.* 1997). Gonadotropin dependence of certain gonadal tumors is also supported by *in vitro* studies demonstrating that gonadotropins stimulate the proliferation of Leydig (Czerwicz *et al.* 1989), ovarian (Simon *et al.* 1983), and adrenal (Rilianawati *et al.* 1998) tumor cells.

We have produced a TG mouse model for tumorigenesis of gonadal somatic cells, using a 6 kb fragment of the mouse inhibin- α subunit promoter (Inh- α) fused with the simian virus 40 (SV40) Tag coding sequences (Kananen *et al.* 1995, 1996a). Gonadal tumors, originating

from Leydig or granulosa cells, develop by the age of 5–8 months with 100% penetrance. Recently, we have shown that (1) gonadectomy of the TG mice results in adrenocortical tumorigenesis (Kananen *et al.* 1996b), (2) testosterone stimulates the proliferation of an established cell line, Cα1, originating from one of the adrenal tumors (Rilianawati *et al.* 1998) and (3) gonadal tumorigenesis and tumor progression in the TG mice are dependent on gonadotropins, as revealed by their inhibition by GnRH-antagonist treatment or by crossbreeding of the Tag mice to the hypogonadal mutant (*hpg*) mouse genetic background (Kananen *et al.* 1997). Besides dependence on gonadotropins, the possibility also exists that gonadotropin-stimulated steroidogenesis, e.g. production of testosterone, or another gonadal factor, is ultimately responsible for the gonadal and adrenal tumorigenesis of the TG mice. Therefore, the purpose of the present study was to investigate whether testosterone itself, in the absence of high gonadotropin levels, could stimulate gonadal and adrenal tumor growth *in vivo* in the TG mice. Two *in vivo* models were used: (1) Tag/*hpg* double mutant mice (low gonadotropin levels with intact gonads) and (2) castrated Tag TG mice (high gonadotropin levels without gonads). Both were implanted with Silastic rods containing testosterone, to achieve blockage of gonadotropin secretion and high circulating testosterone levels.

Materials and Methods

Experimental animals

The mice used in these studies were males and females of IT6-M and IT6-F lines of the *Inh-α*/Tag TG mice, as described before (Kananen *et al.* 1995), and male and female *hpg* mice (Cattanach *et al.* 1977). The *hpg* mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Genotyping of the mice was performed by tail DNA PCR as described earlier (Lang 1991, Kananen *et al.* 1995). The mice were housed four to six per cage, after weaning at the age of 21 days, in a room with controlled light (12 h light:12 h darkness) and temperature (21 ± 1 °C). They were fed with mouse chow SDS RM-3 (Special Diet Service; E, soy-free; Whitman, Essex, UK) and tap water *ad libitum*. In addition, the mice were maintained in specific pathogen-free conditions. All the procedures using mice were approved by the University of Turku Ethical Committee on Use and Care of Animals.

Rod implants (length 2 cm) filled with testosterone (from Sigma, St Louis, MO, USA or Fluka, Buchs, Switzerland) were prepared from Silastic tubing (inner diameter 1.98 mm; outer diameter 3.18 mm; Dow Corning, Midland, MI, USA, catalog No. 602–305) and sealed at both ends with Silastic adhesive (Dow Corning, 7–2947, catalog No. 890). Avertin anesthesia (Hogan *et al.* 1994) was used during the surgical procedures and, for

postoperative analgesia, buprenorphine (0.3 mg/kg, s.c.) was administered.

Hpg experiment

In the first mating, TG males of IT6-M and IT6-F lines were crossbred with females heterozygous for the *hpg* mutation (HT females). TG males heterozygous for *hpg* (Tag/HT males) derived from the first mating were further crossbred with HT females to produce the hypogonadal transgenic (Tag/*hpg*) double mutant mice, as described earlier (Kananen *et al.* 1997). These mice (12 females and 15 males) were implanted with the Silastic rods (see above) containing testosterone at 2 months of the age, and killed 6 months after implantation. Non-treated Tag/*hpg* mice (10 females and 10 males) served as controls.

Experiment with castrated TG mice

TG mice of both lines, IT6-F and IT6-M (16 females and 14 males), were castrated at approximately 4 weeks of age and inserted at the same time with testosterone implants. Castrated non-treated TG mice served as controls (9 females and 8 males).

At the age of 7–8 months, i.e. 6 months after insertion of the testosterone implants, the mice were anesthetized with avertin, and the body weights were recorded. Blood samples were collected into heparinized syringes by cardiac puncture. Blood was allowed to clot overnight at 4 °C and centrifuged (300 g) at room temperature to separate serum. The sera were stored at –20 °C until analyzed. The gonads, adrenal glands, uteri and seminal vesicles were dissected out, weighed, and snap-frozen in liquid nitrogen for RNA isolation, or fixed in Bouin's solution for histological analyses.

Pituitary and serum hormone measurements

Luteinizing hormone (LH) levels in sera and pituitary homogenates were measured by an immunofluorometric assay (IFMA) for rat LH (Delfia; Wallac OY, Turku, Finland), as described previously (Haavisto *et al.* 1993). The sensitivity of the assay was 0.02 ng/ml. Follicle-stimulating hormone (FSH) levels in sera and pituitary homogenates were measured by an IFMA for rat FSH, essentially as described before (Hakola *et al.* 1997), with a new pair of antibodies: an anti-human FSHβ monoclonal antibody (FSH 56 A) as capture antibody and a polyclonal anti-human α-subunit antibody (R93–2705) as signal antibody (van Casteren *et al.* 2000). Both were donated by Dr W Schoonen (Organon, Oss, The Netherlands). The intra-assay coefficient of variation (C.V.) of the assay was less than 3% (at rat FSH concentration 4 ng/ml), and the interassay C.V. 9.0% (at rat FSH concentration 4 ng/ml). The analytical sensitivity of the assay (signal at 0 standard $\pm$ 2 s.d.) was 0.02 ng/tube which corresponds to

Table 1 Weights of gonads, uterine/seminal vesicles, and adrenal glands of the Tag/hpg and castrated TG mice with and without testosterone (T) implants. Values are means $\pm$ S.E.M.

	<i>n</i>	Gonadal weight (mg)	Uterine/seminal vesicle weight (mg)	Adrenal gland weight (mg)
Tag/hpg experiment				
Females				
Without T implant	10	1.40 $\pm$ 0.28	5.18 $\pm$ 1.13	9.34 $\pm$ 0.84
With T implant	12	4.03 $\pm$ 0.86 ^a	57.5 $\pm$ 9.05 ^c	6.38 $\pm$ 0.37 ^b
Males				
Without T implant	10	8.37 $\pm$ 1.99	66.0 $\pm$ 6.00	8.28 $\pm$ 0.50
With T implant	15	133.6 $\pm$ 21.6 ^c	799 $\pm$ 168 ^b	5.49 $\pm$ 0.36 ^c
Castrated TG mice				
Females				
Without T implant	9		3.08 $\pm$ 1.46	9.54 $\pm$ 0.94
With T implant	16		83.1 $\pm$ 13.8 ^c	4.53 $\pm$ 0.16 ^c
Males				
Without T implant	8		132 $\pm$ 110	7.92 $\pm$ 0.23
With T implant	14		670 $\pm$ 135 ^b	4.39 $\pm$ 0.52 ^c

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs the respective group without T implant.

a concentration of 0.8 ng/ml at a sample volume of 25 μ l. The cross-reactivity of the assay was $<0.3\%$ with rat LH and $<0.06\%$ with rat growth hormone. Both gonadotropin assays demonstrated parallel dilution curves for mouse serum and pituitary extracts and the respective rat gonadotropin standards (NIH RP-2; results not shown), which was considered evidence for suitability of the assays developed for rat gonadotropins to monitor the same hormones in mouse samples.

Serum levels of testosterone were measured from diethyl ether extract by RIAs, as described earlier (Huhtaniemi *et al.* 1985).

Histology

Bouin-fixed paraffin sections (5 μ m thick) of gonads and adrenal glands were stained with hematoxylin/eosin for histological analyses.

Statistical analysis

A MacIntosh version of the superANOVA program (Abacus Concepts, Inc., Berkeley, CA, USA) was used to perform one-factor analysis of variance, followed by factorial test, Duncan's new multiple range and Fisher's protected LSD post-hoc tests. Because the hormone values were skewed they were analysed using non-parametric Mann-Whitney tests. A P value below 0.05 was chosen as the limit of statistical significance.

Results

Effect of testosterone implants on gonadal and adrenal tumorigenesis in castrated and hypogonadal TG mice

None of the control or testosterone-treated Tag/hpg mice developed macroscopically discernible gonadal or adrenal tumors. Neither did the testosterone-treated castrated TG mice develop adrenal tumors, whereas histological analysis demonstrated clear adrenal tumors in the castrated, non-treated, control TG mice (see below). Unlike in our earlier studies (Kananen *et al.* 1996b, Rilianawati *et al.* 1998), the adrenal tumors detected in the mice had not reached macroscopic stage at the time of killing. The difference from our earlier findings is apparently due to biological variation and slower tumor progression to macroscopic stage in the currently studied groups of mice. The weights of gonads, uteri and seminal vesicles were significantly increased by testosterone in comparison with non-treated animals ($P < 0.001$). In contrast, the adrenal weights were significantly reduced by testosterone treatment ($P < 0.001$) in all animals (Table 1). Although some differences were found between mean body weights of the different treatment groups, expression of organ weights per body weight did not change the findings (not shown).

Gonadal and adrenal histology

The histological picture of ovarian sections of the non-treated Tag/hpg females showed complete arrest of folliculogenesis at the primary/secondary stage (Fig. 1A).

Figure 1 Histological analysis of ovarian tissue of 8-month-old control and testosterone-treated Tag/hpg mice. (A) Ovarian section of a non-treated Tag/hpg mouse showing arrest of follicular development at the stage of primary/secondary follicles. (B) Ovarian section of a testosterone-treated Tag/hpg mouse with development of follicles up to the early antral stage. In addition, stromal hyperplasia is apparent. The bars in the panels are 10 μ m.

More advanced folliculogenesis was observed in the testosterone-treated Tag/hpg mice, up to the early antral stage, but not beyond (Fig. 1B). Surprisingly, stromal hyperplasia was found in the ovaries of the testosterone-treated females. The testes of the non-treated Tag/hpg mice showed complete spermatogenic arrest and lack of development of the interstitial compartment (Fig. 2A). The testosterone-treated Tag/hpg males displayed hyperplasia of testicular interstitial cells, and spermatogenesis was induced, even though only partly (Fig. 2B).

Histological analysis of adrenal glands of the castrated non-treated TG mice showed early stages of tumorigenesis in the cortex (Fig. 3A). It originated from the innermost layer of the adrenal cortex, apparently the X-zone. The non-treated Tag/hpg mice, as a consequence of functional gonadectomy, clearly showed persistence of the X-zone,

Figure 2 Histological analysis of testis tissue of 8-month-old control and testosterone-treated Tag/hpg mice. (A) Testis section of non-treated Tag/hpg mouse showing arrested spermatogenesis and lack of interstitial cell development. (B) Testis section of testosterone-treated Tag/hpg mouse showing hyperplasia of interstitial cells and induced spermatogenesis. The bars in the panels are 10 μ m.

but no signs of tumorigenesis (Fig. 4A). The medulla was intact in all animals examined. In contrast, in castrated (Fig. 3B) and Tag/hpg (Fig. 4B) mice, testosterone treatment appeared to induce involution of the X-zone, and also the other zones of the adrenal cortex were less distinct. This result indicated that, in the testosterone-treated animals, adrenal tumorigenesis was inhibited.

Pituitary and serum hormone levels

Testosterone treatment did not affect the pituitary contents of FSH and LH in the Tag/hpg and castrated Tag mice (Tables 2 and 3). Interestingly, the serum levels of FSH in the testosterone-treated Tag/hpg mice were significantly increased in both sexes, whereas, in castrated testosterone-treated males and females, serum FSH was

Figure 3 Histological analysis of adrenal glands of castrated non-treated and testosterone-treated TG mice at 8 months of age. (A) Adrenal gland of a non-treated mouse showing early stage of adrenocortical tumorigenesis in the adrenal cortex, apparently arising from the X-zone. (B) Adrenal section of a castrated testosterone-treated TG mouse showing no adrenal tumorigenesis and near-total disappearance of the X-zone. The bars in the panels are 10 µm. m, medulla; ZF, zona fasciculata; ZG, zona glomerulosa; X, X-zone.

not affected by the steroid implants. However, compared with intact mice (males 11.3 ± 0.69 , females 1.22 ± 1.05 (s.d.) ng/ml), the post-gonadectomy FSH levels were significantly increased. Testosterone did not affect serum LH in the Tag/hpg mice, whereas in castrated testosterone-treated males and females, these concentrations were significantly reduced. As expected, the testosterone concentration was significantly increased in Tag/hpg and castrated testosterone-treated males and females (Tables 2 and 3).

Discussion

The main aim of the present study was to find out whether testosterone is able to mediate the action of gonadotropins

Figure 4 Histological analysis of adrenal glands of control and testosterone-treated Tag/hpg mice at 8 months of age. (A) Adrenal section of a non-treated Tag/hpg mouse showing a prominent X-zone. (B) Adrenal section of a testosterone-treated Tag/hpg mouse, showing disappearance of the X-zone and less clear zonation of the adrenal cortex. The bars in the panels are 10 µm. m, medulla; X, X-zone; ZF, zona fasciculata; ZG, zona glomerulosa.

in gonadal and adrenal tumorigenesis of the Tag TG mice. In addition, the study further elucidated, in these mice, the inhibition of gonadal and adrenal tumor growth by suppression of gonadotropin secretion. The suppression of gonadotropins by testosterone treatment totally inhibited gonadal and adrenal tumorigenesis in hypogonadal (hpg) and castrated TG mice. The concomitantly elevated testosterone levels demonstrated that increased production of this hormone in response to gonadotropin stimulation is not sufficient to evoke gonadal tumorigenesis of intact TG mice. The tumorigenic effect of high gonadotropin levels is therefore apparently direct.

None of the testosterone-treated Tag/hpg mice developed macroscopically or microscopically detectable gonadal or adrenal tumors. However, the weights of gonads,

Table 2 Serum (S) concentrations and pituitary (Pit) contents of gonadotropins and testosterone (T) in female mice. Values are median (interquartile range; number of samples analyzed); the number of samples analyzed per group was determined by their availability

	S FSH (ng/ml)	Pit FSH (ng/gland)	S LH (ng/ml)	Pit LH (ng/gland)	S T (nmol/l)
Tag/hpg experiment					
Without T implant	<0.8 (<0.8; 6)	36.2 (17.6–48.7; 3)	<0.02 (<0.02–0.17; 8)	38.0 (17.7–43.1; 9)	0.32 (0.27–0.40; 12)
With T implant	1.52 (1.30–1.84; 6) ^a	33.4 (11.2–52.0; 6)	<0.01 (<0.02–0.02; 14)	34.1 (25.4–44.5; 10)	21.6 (19.2–29.3; 7) ^c
Castrated TG mice					
Without T implant	31.1 (24.7–43.1; 6)	419 (179–673; 5)	1.03 (0.63–6.95; 3)	99.7 (62.0–124.0; 5)	0.31 (0.26–0.42; 5)
With T implant	47.3 (26.3–79.2; 8)	589 (102–665; 6)	0.13 (0.09–0.14; 8)	104.0 (100.0–120.0; 7)	16.7 (8.15–27.9; 8) ^b

^a*P*<0.05, ^b*P*<0.01, ^c*P*<0.001 vs the respective group without T implant.**Table 3** Serum (S) concentrations and pituitary (Pit) contents of gonadotropins and testosterone (T) in male mice. Values are median (interquartile range; number of samples analyzed); the number of samples analyzed per group was determined by their availability

	S FSH (ng/ml)	Pit FSH (ng/gland)	S LH (ng/ml)	Pit LH (ng/gland)	S T (nmol/l)
Tag/hpg experiment					
Without T implant	<0.8 (<0.8; 3)	53.8 (26.1–74.3; 3)	0.02 (<0.02–0.03; 4)	23.5 (13.9–34.2; 3)	0.27 (0.25–0.41; 4)
With T implant	1.51 (0.96–2.79; 13) ^b	44.4 (40.4–55.5; 13)	<0.02 (<0.02–0.02; 14)	34.5 (26.2–48.7; 12)	23.4 (12.3–29.5; 15) ^c
Castrated TG mice					
Without T implant	55.8 (23.3–80.1; 13)	480 (290–690; 14)	1.83 (0.54–4.68; 14)	98.7 (62.0–124.0; 16)	0.52 (0.29–0.70; 11)
With T implant	55.3 (36.7–91.1; 14)	649 (279–838; 16)	0.12 (0.05–0.23; 15) ^c	106.0 (100.0–120.0; 16)	15.9 (7.17–28.1; 16) ^c

^b*P*<0.01, ^c*P*<0.001 vs the respective group without T implant.

uteri and seminal vesicles were significantly increased (*P*<0.001), and the testes displayed activation of spermatogenesis. While the effects in the male animals are apparently caused by testosterone, the ovarian and uterine weight increases are best explained by aromatization of a part of the testosterone released from the implants. Gonadal growth response in the testosterone-treated Tag/hpg mice was caused by stromal hyperplasia in ovaries, and that of interstitial cells and seminiferous tubules in testes. The exact mechanism of these gonadal responses remains unclear since Tag protein could not be detected in the hyperplastic cells (result not shown). It is apparent that, in non-testosterone-treated TG mice, very low levels of the transgene expression, in conjunction with normal or increased gonadotropin action, are able to promote the tumorigenesis (Kananen *et al.* 1997). The presence of high levels of testosterone can apparently induce hyperplastic changes but not tumor growth, either alone or in synergy with low levels of Tag expression. Hence, the current data are not in support of testosterone-stimulated tumor growth in gonads or adrenals of the TG mice. In contrast, Rajpert-de Meyts & Skakkabaek (1993) showed that low levels of circulating androgen may contribute to testicular tumorigenesis, whereas high levels of androgen stimulate growth of ovarian tumors (Nash *et al.* 1989, Godwin *et al.* 1992). In accordance, ovarian Sertoli–Leydig tumor tissues may produce high levels of testosterone (Abrahamsson *et al.* 1995). Another study showed that in inhibin-deficient/*tfm* mutant mice, the androgens are not required

for testicular tumor development but may play a regulatory role in tumor progression (Shou *et al.* 1997).

The serum levels of FSH in testosterone-treated Tag/hpg males and females were significantly increased, while those of LH were decreased. In contrast, and as demonstrated before (Rodin *et al.* 1990), the pituitary and serum levels of FSH were unaltered in the castrated mice. This finding suggests that testosterone has a direct stimulatory effect on FSH synthesis and secretion. *In vivo* and *in vitro* studies have also shown that testosterone, in the absence of GnRH stimulation, has a positive effect on FSH gene expression, synthesis and secretion (Gharib *et al.* 1990, Perheentupa & Huhtaniemi 1990, Wierman & Wang 1990). Although testosterone can directly influence the FSH synthesis and secretion, there is more evidence on the role of inhibin as a gonadal feedback inhibitor of FSH secretion. The positive FSH response to testosterone treatment was very similar to that observed in our previous studies with GnRH antagonist- and testosterone-treated rats (Perheentupa *et al.* 1993a,b). Also, in these animals, testosterone was able to stimulate FSH gene expression, synthesis and secretion, in the absence of apparent changes in serum inhibin levels. Mice with genetically disrupted inhibin α -genes showed increased FSH secretion due to the lack of negative feedback of inhibin at the pituitary level. This elevation of serum FSH levels may contribute to, but is not directly responsible for, the development of gonadal tumors in these mice (Matzuk *et al.* 1994). In accordance, postmenopausal women developing

ovarian epithelial cancer exhibit increased serum LH and FSH (Godwin *et al.* 1992). The present results show that testosterone, either alone or in synergy with the slightly elevated FSH levels, may induce hyperplasia in gonadal interstitial cells, but is not able alone to cause tumorigenesis.

There is evidence that testosterone alone, in the absence of FSH, may be sufficient for induction and maintenance of spermatogenesis. Singh *et al.* (1995) showed that androgen alone in the complete absence of FSH could initiate spermatogenesis in the hypogonadal (*hpg*) mouse. Some other studies have also demonstrated the same phenomenon in the rat (Ahmad *et al.* 1973, Awoniyi *et al.* 1989, 1992). In contrast, there are also studies emphasizing that both testosterone and FSH are needed for maintenance of spermatogenesis in the rat (Santulli *et al.* 1990, McLachlan *et al.* 1994). The present study showed that, when spermatogenesis was induced by testosterone treatment in *hpg* mice, there was a concomitant slight, but significant, increase in FSH secretion. Such an effect has not been detected earlier in studies attempting to induce spermatogenesis with testosterone treatment alone. Although the increase that we observed in FSH levels may not be high enough for major physiological effects, it calls for caution in interpretation of experiments where testosterone alone has been shown to induce spermatogenesis.

Previously, we detected adrenal gland tumorigenesis in castrated TG mice at the age of 6–8 months, but not in gonad-intact TG mice, suggesting that the presence of gonadal factors inhibits adrenal tumorigenesis (Kananen *et al.* 1995). However, the induction of adrenal LH responsiveness after gonadectomy was found to be the mechanism of adrenocortical tumorigenesis (Rilianawati *et al.* 1998). The suppression of gonadotropins by testosterone showed that adrenal tumorigenesis was inhibited in the absence of elevated gonadotropins. In the present study, the adrenal gland weights were significantly reduced ($P < 0.001$) and the X-zone disappeared in the testosterone-treated mice. The adrenocortical tumors appeared to originate from the juxtamedullary X-zone which has been shown to be stimulated by LH and inhibited by androgens (Ungar & Stabler 1980, Deacon *et al.* 1986). The X-zone is maintained after gonadectomy and may even reappear in adult male mice after castration (Deacon *et al.* 1986, Matsuura & Suzuki 1986). In adrenal tumors of the Tag mice, and in *Ca1* cells established from an adrenal tumor, co-expression of the LH receptor and Tag probably form the tumor-promoting mechanism (Rilianawati *et al.* 1998), and high LH action always seems to be related with the adrenal tumors. In accordance, the present results showed that when serum LH was suppressed by testosterone treatment, adrenal tumor growth was totally inhibited. Accordingly, the high gonadotropin levels after gonadectomy are the most obvious difference between gonadectomized and Tag/*hpg* mice or GnRH antagonist-treated mice. Although *in vitro* studies have

shown that testosterone stimulates the proliferation of adrenal tumor cell, *Ca1* (Rilianawati *et al.* 1998), the present *in vivo* study did not show a clear tumorigenic response to testosterone. The present mouse model is similar to the inhibin-deficient mouse model, which develops adrenal tumors after gonadectomy, following increased gonadotropin levels and lack of inhibin (Matzuk *et al.* 1994). In accordance, adrenal hyperplasia with LH receptor expression appears in TG mice constitutively overexpressing LH (Kero *et al.* 2000).

In conclusion, the present results suggest that testosterone, alone or in synergy with low levels of FSH, can induce hyperplasia of gonadal interstitial cells in the Tag-expressing TG mice. However, the progression from hyperplasia to tumor growth apparently needs strong LH action. In the adrenal glands, testosterone, as expected, caused involution of the X-zone, which is the likely origin of adrenal tumors in the *Inh- α* /Tag mouse model after castration. This response, in conjunction with suppressed LH levels, was apparently responsible for the absence of adrenal tumors in the testosterone-treated mice. The fact that suppression of gonadotropin secretion by testosterone implants inhibits gonadal and adrenal tumor growth excludes gonadotropin-stimulated androgen production as the cause of tumorigenesis.

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