Hecate-CG β conjugate and gonadotropin suppression shows two distinct mechanisms of action in the treatment of adrenocortical tumors in transgenic mice expressing Simian Virus 40 T antigen under inhibin- α promoter

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Abstract

Lytic peptide Hecate (23-amino acid (AA)) fused with a 15-AA fragment of human chorionic gonadotropin- β (CG- β), Hecate-CG β conjugate (H-CG β -c) selectively binds to and destroys tumor cells expressing LH/chorionic gonadotropin receptor (Lhcgr). Transgenic mice (6.5 month old) expressing SV40 T-antigen under the inhibin- α promoter (inh α /Tag) presenting with *Lhcgr* expressing adrenal tumors were treated either with H-CG β -c, GnRH antagonist (GnRH-a), estradiol (E₂; only females) or their combinations for 1 month. We expected that GnRH-a or E₂ in combination with H-CG β -c could improve the treatment efficacy especially in females by decreasing circulating LH and eliminating the potential competition of serum LH with the H-CG β -c. GnRH-a and H-CG β -c treatments were successful in males (adrenal weights 14 ± 2.8 mg and 60 ± 26 vs 237 ± 59 mg in controls; $P < 0.05$). Histopathologically, GnRH-a apparently destroyed the adrenal parenchyma leaving only the fibrotic capsule with few necrotic foci. In females, H-CG β -c was totally ineffective, whereas GnRH-a (19 ± 5 mg) or E₂ (77 ± 50 mg) significantly reduced the adrenal weights compared with controls (330 ± 70 mg). Adrenal morphometry, cell proliferation markers, post-treatment suppression of serum progesterone, and quantitative RT-PCR of GATA-4, *Lhcgr*, and GATA-6 further supported the positive outcome. H-CG β -c selectively killed the *Lhcgr* expressing tumor cells, whereas GnRH-a blocked tumor progression through gonadotropin suppression, emphasizing the gonadotropin dependency of these adrenocortical tumors. If extrapolated to humans, H-CG β -c could be considered for the treatment of gonadotropin-dependent adrenal tumors in males, whereas in females gonadotropin suppression, but not H-CG β -c, would work better.

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Introduction

Adrenocortical tumors are rare and aggressive as they often are diagnosed late and have poor survival rate (Schulick & Brennan 1999a). These tumors are 1.5-fold more common in females than males with peak incidences in the first and fifth decades of life (Schulick

& Brennan 1999a,b), and high probability around menopause (Mijnhout *et al.* 2004). The incidence of these malignancies increases during chronic gonadotropin overload, e.g. in pregnancy (Sheeler 1994) or after menopause (Mijnhout *et al.* 2004), resulting in ACTH-independent macronodular adrenocortical

hyperplasia and Cushingoid symptoms maintained by ectopic adrenal *Lhcgr* expression (Lacroix et al. 1999, Bourdeau et al. 2001, Miyamura et al. 2002, Feelders et al. 2003, Goodarzi et al. 2003). Also LH-dependent adrenal adenomas producing aldosterone (Saner-Amigh et al. 2006) or androgens (Werk et al. 1973, Givens et al. 1975, Larson et al. 1976, Smith et al. 1978, Takahashi et al. 1978, de Lange et al. 1980, Leinonen et al. 1991) and adrenocortical carcinomas (Wy et al. 2002, Barbosa et al. 2004) with abundant *Lhcgr* expression have been described. Moreover, the normal human adrenal cortex has been shown to express low levels of *Lhcgr* (Pabon et al. 1996). The only effective cure for adrenocortical tumors so far is their total surgical removal (Kuruba & Gallagher 2008). Adjuvant therapies such as radiotherapy and mitotane after surgery have been tested (Fassnacht et al. 2006, Terzolo & Berruti 2008), but there is still the need for better curative methods of treatment.

Hecate is a 23-amino acid (AA) amphipathic, positively charged lytic peptide, a synthetic analogue of melittin, which is the principal toxic component of natural honeybee venom (Arrowood et al. 1991a,b, Henk et al. 1995, Baghian et al. 1996). Its mechanism of action depends on the formation of pores or channels in cell membranes by wedge-shaped insertion of monomers of the lytic peptide. This causes aggregation of the membrane proteins and expulsion of phospholipids (Leuschner & Hansel 2004, Bodek et al. 2005a), leading to osmotic cytolysis and cell death through necrosis. The binding of lytic peptides is mainly subjected to negatively charged membranes typical of the outer leaflet of tumor cells due to altered distribution of their phosphatidylserines (Utsugi et al. 1991). In order to specifically target the action of Hecate, its 23 AA sequence was fused with a 15-AA segment (81–95) of the β -subunit of human chorionic gonadotropin (CG- β), which possesses high affinity for *Lhcgr* (Leuschner et al. 2001). Hecate-CG β conjugate (H-CG β -c) selectively kills tumor cells expressing *Lhcgr* but spares the healthy receptor-positive and -negative cells (Leuschner et al. 2001, Bodek et al. 2005b). The cytotoxic activity of the H-CG β -c induces plasma membrane disruption within minutes (Chen et al. 2003, Bodek et al. 2005b, Hansel et al. 2007b), it is not antigenic, and it has not been shown to have any clear side effects (Bodek et al. 2005b, Hansel et al. 2007b, Vuorenoja et al. 2008). H-CG β -c has been found to selectively kill *Lhcgr* expressing prostate (Hansel et al. 2001, Leuschner et al. 2001, 2003, Bodek et al. 2005a), mammary gland (Bodek et al. 2003, Leuschner et al. 2003, Zaleska et al. 2004, Leuschner & Hansel 2005, Hansel et al. 2007a,b), ovarian (Gawronska et al. 2002, Bodek et al. 2005b),

and testicular cancer cells (Bodek et al. 2005b). H-CG β -c has been shown to induce dose-dependent rapid and cell-specific membrane permeabilization of *Lhcgr* expressing cells *in vitro*, resulting in necrotic cell death without activation of apoptosis (Bodek et al. 2005b). More generally, our model is able to provide the proof of principle for the efficacy of receptor-mediated targeting of toxic molecules in the abolition of tumors.

TG mice of both sexes expressing the inhibin- α promoter/Simian Virus (SV40) T-antigen (inh α /Tag) were originally found to produce gonadal tumors with 100% penetrance by the age of 6 months, but when gonadectomized prepubertally they produced adrenal tumors by the same age (Kananen et al. 1996a, Rilianawati et al. 1998, Rahman et al. 2004). The TG adrenal tumors and a tumor-derived cell line (C α 1) were found to express high levels of *Lhcgr* (Rilianawati et al. 1998, Rahman et al. 2004), and their growth was found to be gonadotropin dependent (Kananen et al. 1997). Adrenal tumors failed to appear if the post-gonadectomy increase in gonadotropin secretion was blocked either by administration of GnRH antagonist (GnRH-a) or by cross-breeding the TG mice into the hypogonadotropic *hpg* genetic background (Cattanach et al. 1977, Kananen et al. 1997). Hence, the post-gonadectomy elevation of LH levels apparently induced ectopic *Lhcgr* expression in the adrenal cortex, which together with co-expression of the potent oncogene Tag triggered the tumorigenesis (Rahman et al. 2001, 2004, Mikola et al. 2003). The adrenocortical tumorigenesis occurs in a slow hyperplasia-adenoma-carcinoma sequence following prepubertal gonadectomy of the TG mice; hyperplasia is seen at 4 months and discernible tumors at 6 months (Rahman et al. 2004). The chronically elevated LH level (Kananen et al. 1996a) and ectopic/up-regulated *Lhcgr*, with low (5–10%) metastasis frequency (Rahman et al. 2004, Vuorenoja et al. 2008), makes these TG mice a relevant experimental model for human adrenal tumors.

This study represents continuation to our previous work on the use of H-CG β -c in treating the *Lhcgr* expressing adrenal tumors of the inh α /Tag TG mice, where the treatment effectively killed tumor cells in males but was ineffective in females (Vuorenoja et al. 2008). We hypothesized that treatment of the mice with estradiol (E₂) or GnRH-a, alone or in combination with H-CG β -c, would decrease the circulating serum LH and consequently eliminate the potential competition between H-CG β -c and LH. This could increase the efficacy of the H-CG β -c treatment in particular in the female TG mice. We also compared the effects of GnRH-a treatment with H-CG β -c in TG males.

Materials and methods

Experimental animals

Adrenal tumors were induced by prepubertal gonadectomy of *inhα*/Tag TG mice as described earlier (Kananen *et al.* 1996a). Discernible adrenocortical tumors with 100% penetrance appear in these mice by the age of 6 months (Kananen *et al.* 1996a, Rilianawati *et al.* 1998, 2000, Kero *et al.* 2000, Rahman *et al.* 2001, 2004). Gonadectomy was performed under Avertin anesthesia (Hogan *et al.* 1994) prepubertally between 21 and 24 days of life, and buprenorphine (Schering-Plough, Brussels, Belgium) was administered as post-operative analgesia. Six to eight mice per treatment group were used for the experiments. Wild-type (WT) control littermate mice (C57Bl/6N) were used as controls ($n=6$). For routine genotyping, PCR analysis was carried out as previously described (Kananen *et al.* 1995) using DNA extracts from ear biopsies. After weaning at the age of 21 days, the mice were housed two to four per cage, females and males separately, in a room of controlled light (12 h light:12 h darkness) and temperature (21 ± 1 °C). The mice were fed with mouse chow SDS RM-3 (Whitham, Essex, UK) and tap water *ad libitum*, kept in a specific pathogen-free surrounding and routinely screened for common mouse pathogens. Ethics Committees for animal experimentation of the Turku University and the State Provincial Office of Southern Finland approved all the animal experiments.

Preparation of drugs

Hecate and H-CG β -c were synthesized and purified in the Peptide and Protein Laboratory, Department of Virology, Hartman Institute, University of Helsinki as described earlier (Bodek *et al.* 2003). Silastic implant tubes, 8 mm in length (inner diameter 1.58 mm and outer diameter 2.41 mm) were filled with 8 mg of E₂ powder (Sigma Chemical Co.) and sealed at both ends with silastic adhesive (Elastosil RTV-1 Silicone Rubber, Wacker-Chemie GmbH, Munich, Germany) as described earlier (Pakarainen *et al.* 2005). GnRH-a (cetrotelix acetate, Cetrotide) was purchased from Merck Serono.

H-CG β -c, GnRH-a, and E₂ treatments

Male and female mice (*inhα*/Tag TG; $n=6-8$ per group) were treated at the age of 6.5 months with either H-CG β -c or Hecate (12 mg/kg b.w.) by i.p. injections as described earlier (Vuorenoja *et al.* 2008). As no significant weight changes of the mice could be observed between Hecate and H-CG β -c by adding hCG to Hecate, both Hecate and H-CG β -c were given in the same dose (Leuschner *et al.* 2001). The mice

were injected once per week for three consecutive weeks, according to earlier protocols for *in vivo* treatment for nude mouse xenografts (Leuschner *et al.* 2001), TG mice with gonadal tumors (Bodek *et al.* 2005b), and for adrenal tumors (Vuorenoja *et al.* 2008). In addition to H-CG β -c treatment, a group of TG males and females and WT controls were injected subcutaneously with GnRH-a (10 mg/kg) every 84 h according to a previously established protocol (Kananen *et al.* 1997). Another group of TG females, and controls, were treated with E₂ silastic implants. After 4 weeks of treatment, blood was collected by cardiac puncture under Avertin anesthesia and the mice were killed by cervical dislocation. Total body weights, adrenal tumor, and selected organ weights were recorded. Tissues were either snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections of 5 μ m thickness were stained for histological analysis after hematoxylin–eosin (HE) staining, or used for immunohistochemical analysis. For each tissue and treatment group, at least four independent specimens were examined.

Hormone measurements

Serum levels of LH (Haavisto *et al.* 1993) and FSH (van Casteren *et al.* 2000) were measured by immunofluorometric assays (Delfia; Wallace, Turku, Finland) as described previously. Serum progesterone was measured using the Delfia Progesterone Kit (Wallace). The approximate assay sensitivity for LH was 0.1 μ g/l, for FSH 0.03 μ g/l, and for progesterone 0.5 nmol/l. The intra- and interassay coefficients of variations for these assays were below 10%.

Immunohistochemistry

The paraffin sections (5 μ m) of tumors and wild-type control adrenals were deparaffinized and rehydrated. Three percent H₂O₂ in water was used to block endogenous peroxidases, and the sections were boiled by microwave treatment for 10–15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. Two washes were carried out after each step with 0.05 M Tris and 150 mM NaCl with 0.1% Tween (TBS-T). Serial sections were subjected to immunohistochemistry (IHC). To detect GATA-6, slides were incubated with the primary goat polyclonal anti-GATA-6 antibody (dilution 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. After incubation and washings with TBS-T buffer, the slides were incubated with the anti-goat (dilution 1:400; Santa Cruz Biotechnology) secondary antibody for 30 min. The avidin–biotin immunoperoxidase system

was used to visualize bound antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) with 3,3'-diaminobenzidine (Sigma) as a substrate. For Ki-67, a rabbit monoclonal anti-Ki-67 SP6-clone (dilution 1:2000; NeoMarkers, Fremont, CA, USA) with EnVision + System-HRP labeled Polymer (Dako-Cytomation, Inc., Carpinteria, CA, USA) was used. In this case, the microwave treatment was done in 10 mM Tris-EDTA buffer (pH 9.0), and the primary antibody was diluted in 3% BSA. Novolink Polymer Detection System Kit (Novocastra, Benton Lane, UK) was used for the detection of GATA-4 with a rabbit polyclonal anti-GATA-4 IgG (dilution 1:800; Santa Cruz Biotechnology) and PowerVision + Poly-HRP IHC Kit for goat (ImmunoVision Technologies, Hague, The Netherlands) for the detection of p53 using a goat polyclonal antibody anti-p53 IgG (dilution 1:100; Santa Cruz Biotechnology). The protocols followed the manufacturer's instructions except for endogenous peroxidase blocking that was done after the first antibody incubation. The antibodies were diluted to PBS with 0.1% Triton X-100 (anti-GATA-4) or TBS (anti-p53). As a control for the antibodies, adjacent sections were incubated with either 1% normal goat serum in PBS or rabbit IgG instead of primary antibody to differentiate unspecific from specific staining (data not shown).

Morphometric analyses

Serial sections ($n=6$ per group) of HE-stained slides from each treatment group were analyzed morphometrically by a point counting technique as described earlier (Haapasalo et al. 1990, Howard & Reed 1998) in order to quantify the histological differences between the study groups. A grid of orthogonal lines was placed on top of the tissue section (magnified under light microscope $\times 5$). The volume fraction estimation (in %) was done by counting the number of crossing points of the whole section and points that hit

the specific types of tissue of interest (i.e., tumor, healthy, fibrotic/necrotic, cyst-type formation) and thereafter by dividing the points of each tissue type of interest by the total number of points.

Quantitative RT-PCR

We extracted total RNA from snap-frozen whole adrenal tumors after different treatments ($n=5$ for each treatment group) for the quantitative (q)RT-PCR analysis, as described earlier (Kero et al. 2000, Rahman et al. 2004). Total RNA was extracted with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and treated with amplification grade DNaseI (Invitrogen). For cDNA synthesis and subsequent qRT-PCR, the SYBR Green DyNAmo HS qRT-PCR kit (Finnzymes, Espoo, Finland) was used with 1:50 diluted aliquots. qRT-PCR analysis was performed using the DNA Engine Thermal Cycler (BioRad) with continuous fluorescent detection. The program was by the manufacturer's recommendations as follows: 15 min in 95 °C to activate the hot start DNA polymerase and to denature the template, 10 s in 94 °C to denature, 30 s in 56–61 °C, depending on the primers for annealing, 30 s in 72 °C for extension, and 1 s in 78 °C for data acquisition. The above protocol was repeated for 44 times after which there was 10 min incubation at 72 °C for final extension. Melting curve was between 72 and 90 °C, in 0.5 °C intervals for 1 s. The protocol ended by 5 min in 72 °C to re-anneal. Primer pairs and annealing temperatures are shown in Table 1, and the samples and standards were run in triplicates. The housekeeping gene L19 was analyzed to normalize the results between samples. Amplification products were separated on 1% agarose gel and stained with ethidium bromide.

Statistical analysis

Statistical analyses were carried out by ANOVA with the post-hoc Bonferroni test, using SAS Enterprise Guide 3.0 program (SAS Institute, Inc., Cary, NC, USA).

Table 1 Oligonucleotides used in quantitative RT-PCR analysis

Gene	Primers	Sequence	Product size (bp)	Temperature (annealing) (°C)
L19	F	5'-GGACAGAGTCTTGATGATCTC-3'	195	56
	R	5'-CTGAAGGTCAAAGGGAATGTG-3'		
GATA-4	F	5'-TCTCACTATGGGCACAGCAG-3'	246	61
	R	5'-CGAGCAGGAATTTGAAGAGG-3'		
GATA-6	F	5'-GAGCTGGTGCTACCAAGAGG-3'	193	61
	R	5'-TGCAAAAGCCCATCTCTTCT-3'		
LHR	F	5'-CAATGGGACGACGCTAATCT-3'	204	56
	R	5'-CTGGAGGGCAGAGTTTTCAG-3'		

F, forward; R, reverse.

Logarithmic transformations were carried out for the analysis of groups with unequal variations. *P* values <0.05 were regarded as statistically significant. All values are presented as mean $\pm$ S.E.M.

Results

H-CG β -c and GnRH-a treatments were effective in males, while in females only GnRH-a but not H-CG β -c was successful

Treatments with GnRH-a or H-CG β -c alone were highly effective with significant antineoplastic effect in male TG mouse adrenal glands (Fig. 1A). Combination of H-CG β -c with GnRH-a did not show further improvement of treatment efficacy in males compared with H-CG β -c or GnRH-a treatment alone (Fig. 1A). Following a 1-month treatment of males with H-CG β -c, the adrenal tumor weights were reduced from 237 ± 59 mg in controls to 60 ± 26 mg in treated ones ($P < 0.01$), but after GnRH-a alone or together with H-CG β -c the weights were further reduced down to 14 ± 2.8 and 13 ± 0.9 mg respectively ($P < 0.001$; Fig. 1A). Owing to the age-related variability of tumor progression rate between inh α /Tag TG mice, a common phenomenon for SV40/Tag effects (Hanahan 1989, Kananen *et al.* 1995, 1996b, Rahman *et al.* 1998), we also analyzed the tumor burden (tumor weight/body weight). It also decreased significantly after combination treatment of H-CG β -c and GnRH-a or GnRH-a alone in males, in comparison with control or Hecate treatments ($P < 0.001$; Fig. 1A). In TG females, the poor response to H-CG β -c (Vuorenoja *et al.* 2008) was confirmed (330 ± 65 vs 249 ± 64 mg, control versus H-CG β -c treatment). GnRH-a either alone or combined to H-CG β -c drastically reduced the tumor weight in females by 95% (249 ± 64 vs 10 ± 0.4 mg or 12 ± 1 mg; H-CG β -c versus GnRH-a or H-CG β -c and GnRH-a; $P < 0.001$; Fig. 1B). As Hecate alone previously had no effects in females (Vuorenoja *et al.* 2008), we did not include an additional Hecate only treatment group for them. Also the treatment effect for tumor burden in TG females was significant in the GnRH-a treatment groups in comparison with controls or H-CG β -c ($P < 0.001$; Fig. 1B). The treatment did not affect body weights in any of the groups (data not shown).

H-CG β -c did not enhance the antitumor effect of E₂ in females

Groups of TG female mice ($n = 6$ –8 per group) were treated with E₂ alone or with a combination of E₂ and

H-CG β -c. E₂ alone reduced the adrenal weight significantly compared with controls (77 ± 50 vs 330 ± 70 mg, $P < 0.05$), probably due to its negative feedback on gonadotropin secretion. The adrenal weights and tumor burden decreased significantly ($P < 0.01$) in the groups receiving E₂, and the additional effect of H-CG β -c was not significant (Fig. 1C). As a sign of effects of the E₂ treatment, the uterine weights in the E₂ treatment groups increased (160 ± 16 vs 63 ± 30 mg, E₂ versus control; or 173 ± 29 vs 63 ± 30 mg, E₂ and H-CG β -c versus control; $P < 0.01$).

Endocrine consequences of the treatments

The adrenal tumors and C α 1 cells derived from them secrete progesterone (Kananen *et al.* 1996a, Vuorenoja *et al.* 2008). In TG males, treatment with either H-CG β -c, GnRH-a or their combination significantly decreased serum progesterone in comparison with control- or Hecate-treated mice ($P < 0.01$), reaching the levels of gonadectomized WT mice (Fig. 2a). The same phenomenon could be seen in females: both GnRH-a and E₂ treatments, either alone or combined to H-CG β -c, significantly reduced the progesterone levels in comparison with control- or H-CG β -c-treated groups ($P < 0.01$), where GnRH-a and E₂ blocked progesterone production apparently by blocking gonadotropin secretion. The progesterone levels correlated with the decrease of tumor weight and thus were helpful in monitoring the treatment outcome. In comparison, elevated LH level in the WT group was due to the lack of negative feedback from the gonads. GnRH-a treatment effectively blocked gonadotropin secretion especially in males, and the levels of LH in all the GnRH-a-treated groups were nearly unmeasurable ($P < 0.05$; Fig. 2a). Serum FSH showed similar pattern to LH (Fig. 2a and b).

H-CG β -c selectively destroyed the *Lhcgr* expressing adrenal tumor cells

In TG males, qRT-PCR analysis revealed a concomitant significant decrease in *Lhcgr* and *GATA-4* expression in the H-CG β -c and GnRH-a groups in comparison with control- and/or Hecate-treated tumors ($P < 0.01$). *Lhcgr* mRNA was undetectable in the groups treated with H-CG β -c (Fig. 3), indicating selective destruction of *Lhcgr* and *GATA-4* positive adrenocortical tumor cells by this lytic peptide (Fig. 3). In mice treated with GnRH-a alone, low levels of *Lhcgr* and *GATA-4* message could be detected. In WT control male adrenals, *Lhcgr* and *GATA-4* mRNA expression were hardly detectable.

Figure 1 Adrenal weights and tumor burden (total adrenal weight/body weight) after the treatments. (A) Total adrenal weights of male GnRH antagonist treated *inhα*/Tag TG and wild-type (WT) mice after 1 month treatment ($n=6-8$ per group). Tumor burden, tumor weight/body weight in grams. The values are mean $\pm$ s.e.m. c, control; conj, Hecate-CG β conjugate; hec, Hecate; g, GnRH antagonist. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$). (B) Total adrenal weights of female GnRH antagonist treated *inhα*/Tag TG and WT mice after 1 month treatment ($n=6-8$ per group). Tumor burden, tumor weight/body weight in grams. The values are mean $\pm$ s.e.m. c, control; conj, Hecate-CG β conjugate; hec, Hecate; g, GnRH antagonist. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$). (C) Total adrenal weights of female E₂ treated *inhα*/Tag TG and WT mice after 1 month treatment ($n=6-8$ per group). Tumor burden, tumor weight/body weight in grams. The values are mean $\pm$ s.e.m. c, control; conj, Hecate-CG β conjugate; E₂, estradiol. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$).

In TG females, the expression of *Lhcgr* and *GATA-4* remained high in the H-CG β -c-treated groups, apparently because of the lack of effect of these treatments. By contrast, GnRH-a and E₂ treatments decreased significantly *Lhcgr* and *GATA-4* ($P<0.05$). The suppression of *Lhcgr* expression with GnRH-a was greater than with E₂ ($P<0.05$; Fig. 3), suggesting better treatment response with the former. *GATA-6* expression is normally abundant only in WT adrenal

cells, but never in tumor cells (Kiiveri et al. 1999). We found concomitant reappearance of *GATA-6* mRNA expression in the H-CG β -c and GnRH-a-treated groups in comparison with control and Hecate-treatments. Hence, along with the disappearance of *GATA-4* expression as treatment response there was a trend of reappearance of apparently healthy cells expressing *GATA-6*; the difference reached statistical significance in males ($P<0.05$; Fig. 3).

Figure 2 Serum hormonal levels of the treated mice. (a) Serum progesterone (A), LH (B), and FSH (C) concentrations in transgenic *inhα/Tag* TG male mice after a 1 month treatment with wild-type (WT), control (c), Hecate-CGβ conjugate (conj), Hecate (hec), GnRH-antagonist (g) or a combination of them; Hecate-CGβ conjugate and GnRH antagonist (conj+g) or Hecate and GnRH antagonist (hec+g). The values are mean $\pm$ S.E.M.; $n=5-6$. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$). (b) Serum progesterone (A), LH (B), and FSH (C) concentrations in transgenic *inhα/Tag* TG female mice after a 1 month treatment with WT, control (c), Hecate-CGβ conjugate (conj), Hecate (hec), GnRH-antagonist (g), estradiol (E₂), Hecate-CGβ conjugate, and GnRH antagonist (conj+g) or Hecate-CGβ conjugate and estradiol (conj+E₂). The values are mean $\pm$ S.E.M.; $n=5-6$. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$).

Two distinct mechanisms of antitumoral action of H-CGβ-c and GnRH-a

Histopathological analysis revealed that H-CGβ-c treatment induced a definite reduction of the adrenocortical tumor mass, where the residual tumor tissue could only be observed in a reduced area of zona glomerulosa, supporting our previous report (Vuorenoja *et al.* 2008). The histology of control adrenal samples fulfilled the criteria of nodular endocrine tumors with loose endothelium and lack of stromal tissue (Fig. 4A1 and B1). Most of the tumor

cells seemed to be of the zona fasciculata type, and the tumor tissue was blood-filled and appeared in cyst-like formation (Fig. 4A1 and B1). The TG male tumors treated with only Hecate or the TG female tumors treated with H-CGβ-c, in line with earlier observation (Vuorenoja *et al.* 2008), did not respond to the treatment, which could be monitored by the histopathological analysis where the structure did not differ from the control-treated group (Fig. 4A2 and B2).

The treatment of TG males with H-CGβ-c reduced the adrenal tumor volume; the sinusoidal structure of

Figure 3 mRNA levels of LH/human chorionic gonadotropin receptor (Lhcgr), GATA-4, and GATA-6 by qRT-PCR in males (left panel) and females (right panel). The presented results are the mRNA values divided by the housekeeping gene L19 values, in order to normalize the results between the samples. The values are mean $\pm$ S.E.M.; $n=5-6$. Different letters above the bars indicate that the difference between them is statistically significant ($P<0.05$).

zona fasciculata was preserved and no residual tumorous tissue could be detected (Fig. 4A3). After H-CG β -c, zona glomerulosa diminished in males, but in some tumors (two out of six) it could be seen as an area of nodular hyperplasia (Fig. 4A3). In general, H-CG β -c treatment seemed to cure the tissue sparing the normal adrenal structure in the males. GnRH-a alone or combined to H-CG β -c drastically reduced the tumor volume, but it also seemed to destroy the normal adrenal structure, leaving only a thick outer capsule with matured fibrosis/necrotic tissue with hemosiderosis-like remnants (Fig. 4A4 and A5). Inside the capsule only zona fasciculata-type cells were left in the GnRH-a groups in males (Fig. 4A4 and A5). GnRH-a alone ($n=4/6$) or combined to Hecate ($n=5/6$) left abundant disorganized cells and some tumor-like nodules (Fig. 4A5 and A6).

The hormonal treatments alone or combination with H-CG β -c, unlike H-CG β -c alone, appeared effective in the TG females (Fig. 4B3–B6). E₂ treatment alone reduced tumor volume, but still some nodular tumor structures persisted ($n=3/6$; Fig. 4A5), and its combination with H-CG β -c did not change the outcome (Fig. 4B6). After GnRH-a treatment, only disorganized layers and areas of hyperplasia were left ($n=4/6$; Fig. 4B3 and B4). The combination of H-CG β -c and GnRH-a reduced the tumor volume leaving mainly zona glomerulosa-like hyperplastic areas ($n=5/6$; Fig. 4B4).

Morphometric analysis

Volume fractions/densities of the different cell compartments of the adrenal glands were assessed as

Figure 4 Histopathological analysis of the male (A) and female (B) adrenal tumor after different treatments. (A) Hematoxylin–eosin staining of male adrenal tumors after treatments: control (1), Hecate only (2), Hecate-CG β conjugate (3), Hecate-CG β conjugate and GnRH antagonist (4), GnRH antagonist only (5), and Hecate and GnRH antagonist (6). (B) Hematoxylin–eosin staining of female adrenal tumors after treatments: control (1), Hecate-CG β conjugate (2), GnRH antagonist (3), Hecate-CG β conjugate and GnRH antagonist (4), E₂ (5), and Hecate-CG β conjugate and E (6).

further proof for efficacy of the treatments. In control tumors of both sexes, after Hecate treatment in males and H-CG β -c treatment in females, over 90% of the adrenal mass composed of tumor cells, fibrosis or blood-filled cysts (Table 2). In males, H-CG β -c alone or in combination to GnRH-a significantly reduced the proportion of tumor tissue to around only 10% in comparison with tumor tissue in control (63%), Hecate (77%) or GnRH-a alone (35%) treatment ($P < 0.05$). It is noteworthy that in males, after the H-CG β -c almost all of the tissue ($91.3 \pm 4\%$) was healthy, but after the H-CG β -c and GnRH-a combination treatment, 40% of the tissue was fibrotic. GnRH-a alone caused less fibrosis (17%), but left about 35% of tumorous tissue.

In females the proportions of tumor did not change significantly following the treatments due to the high variation on the volume between cyst formation and tumorous tissue (Table 2). GnRH-a and E₂ alone or combined to H-CG β -c reduced the tumor volume and increased the proportion of the healthy tissue, although the hyperplasia/tumor tissue still represented 30–40% of the whole tissue (Table 2). The combination of GnRH-a and H-CG β -c in females, in comparison with the same treatment in males, left only a tiny fraction of fibrotic tissue (46% compared with 44% in males). E₂ alone or in combination with H-CG β -c restored around 40% of healthy tissue but still 55 or 39% of residual tumor tissue were left respectively (Table 2). We also analyzed the morphometric data taking into account the tumor weights, which showed identical results (data not shown).

Ki-67 and p53 as markers for treatment efficacy

The proliferation markers Ki-67 and p53 were used in order to monitor the tumor residues after the treatments. The control treatment in both sexes with tumors, as well as Hecate in males (not shown) and H-CG β -c in females, showed similar distribution of proliferating cells throughout the whole tissue with both markers (Fig. 5). H-CG β -c in males showed both Ki-67 and p53 expression in patchy areas, whereas after GnRH-a the proliferating areas were seen as tumor islets between the fibrotic areas (Fig. 5). After combined treatments of H-CG β -c and GnRH-a, the staining pattern was more scattered and very few cells were stained. The same phenomenon could be seen in females in the case of GnRH-a treatments (Fig. 5). The abundance of Ki-67- and p53-positive cells after E₂ treatment supports lower treatment efficacy by E₂ compared with GnRH-a in the females (data not shown).

Table 2 Results of morphometrical analysis in percentages $\pm$ S.E.M.

	Healthy	Tumor	Fibrosis	Cysts
Treatment (males)				
Wild-type	100	0	0	0
Control	1.3 $\pm$ 1.3 ^a	62.6 $\pm$ 5.8 ^a	19 $\pm$ 11.9 ^a	17.1 $\pm$ 5.8 ^a
Hecate + GnRH antagonist	50.4 $\pm$ 5.5 ^b	34.2 $\pm$ 10.4 ^{ab}	15.4 $\pm$ 5.5 ^a	0 ^b
Hecate-CG β conjugate + GnRH antagonist	44.1 $\pm$ 14 ^b	11.5 $\pm$ 5.3 ^b	44.4 $\pm$ 17.9 ^a	0 ^b
Hecate	0.5 $\pm$ 0.5 ^a	76.6 $\pm$ 13.1 ^{ab}	2.8 $\pm$ 2.8 ^a	20 $\pm$ 9.7 ^a
Hecate-CG β conjugate	91.3 $\pm$ 4 ^b	6.7 $\pm$ 4.6 ^b	2 $\pm$ 1.4 ^a	0 ^b
GnRH antagonist	48.3 $\pm$ 12.5 ^b	34.7 $\pm$ 2.9 ^{ab}	17 $\pm$ 11.6 ^a	0 ^b
Treatment (females)				
Wild-type	100	0	0	0
Control	0.8 $\pm$ 0.3 ^a	57.7 $\pm$ 35.6 ^a	4.6 $\pm$ 4.6 ^a	36.9 $\pm$ 31.2 ^a
Hecate + GnRH antagonist	24.4 $\pm$ 12.1 ^{bc}	36.6 $\pm$ 23.1 ^a	33.6 $\pm$ 16.9 ^a	5.5 $\pm$ 5.5 ^a
Hecate-CG β conjugate + GnRH antagonist	66 $\pm$ 9.4 ^b	29.4 $\pm$ 9.9 ^a	4.6 $\pm$ 0.5 ^a	0 $\pm$ 0 ^a
Hecate-CG β conjugate	4 $\pm$ 2.6 ^{ac}	65.2 $\pm$ 16.9 ^a	5.4 $\pm$ 3.1 ^a	25.4 $\pm$ 11.2 ^a
GnRH-antagonist	38.4 $\pm$ 11.2 ^{bc}	35.9 $\pm$ 6.9 ^a	25.7 $\pm$ 14.9 ^a	0 ^a
Estradiol	31.3 $\pm$ 7.4 ^{bc}	55.3 $\pm$ 6.5 ^a	8.5 $\pm$ 3.4 ^a	4.8 $\pm$ 4.4 ^a
Estradiol + Hecate-CG β conjugate	39.6 $\pm$ 8.3 ^{bc}	38.8 $\pm$ 2.8 ^a	21.6 $\pm$ 5.9 ^a	0 ^a

Different letters next to the value indicate that the difference between them is statistically significant ($P < 0.05$).

Reciprocal expression of GATA-4 and GATA-6 in healthy and adrenal tumor tissue

We finally analyzed the expression patterns of GATA-4 and GATA-6 proteins by IHC in the healthy (WT), tumorous (control), and treated adrenal tissues. In males, the WT and H-CG β -c-treated tumors had

abundant GATA-6 (Fig. 6A and C) but no GATA-4 expression (Fig. 6D and F), whereas an opposite observation was made with the control adrenals (Fig. 6B and E). GATA-4 could be detected in the nodule formations of E₂-treated female tumors, and those treated with GnRH-a alone in both sexes, whereas GATA-6 expression was equal in all treatment

Figure 5 Immunohistochemistry for Ki-67 (upper panel) and p53 (lower panel) for both males and females. The order from the left: control (c), Hecate-CG β conjugate (conj), GnRH antagonist (g), and Hecate-CG β conjugate and GnRH antagonist (conj+g).

Figure 6 Immunohistochemistry for GATA-6 (left panel) and GATA-4 (right panel). Wild-type (WT) (A and D), control (B and E), and Hecate-CG β conjugate (C and F) in male adrenal tumor sections.

groups with effective treatment response (data not shown). These reciprocal expression patterns of GATA-4 in tumor and GATA-6 in healthy adrenal tissues are in line with the mRNA data (Fig. 3) and also with an earlier publication (Kiiveri *et al.* 1999).

Discussion

H-CG β -c has been shown to provide a strong and tumor cell-specific antineoplastic effect towards the *Lhcgr*-bearing endocrine tumors (Hansel *et al.* 2001, Gawronska *et al.* 2002, Leuschner *et al.* 2003, Bodek *et al.* 2005b, Vuorenoja *et al.* 2008). In our previous study (Vuorenoja *et al.* 2008), following a 1-month treatment with H-CG β -c adrenal tumor weight could be reduced by an average of two-thirds in *inh α /Tag* TG males compared with Hecate treatment, whereas in TG females the reduction rate was only a non-significant, 18%. There was no good explanation for this sex difference in treatment effects, but as for unknown reason a higher *Lhcgr* expression was found in the male tumors, a plausible explanation could be higher *Lhcgr* concentration in the tumor mass attracting larger amounts of H-CG β -c, with a consequently more severe lytic effect (Vuorenoja *et al.* 2008). Owing to negative results in females, it was found important to improve

the treatment efficacy of the TG female mice. This improved treatment strategy could be helpful for possible future applications in humans, as women are more prone to develop adrenocortical tumors (Schulick & Brennan 1999a,b). We tested two approaches in order to improve the treatment efficacy by reducing the circulating LH levels, i.e., by GnRH-a and E₂ treatment, expecting that they would augment binding of the H-CG β -c to *Lhcgr* following reduced competition by endogenous LH.

In clinics, GnRH-a are generally and successfully used in IVF protocols, where a fast blockage of endogenous gonadotropins are required (Detti *et al.* 2008, Lainas *et al.* 2008, Huhtaniemi *et al.* 2009). The effectiveness of GnRH-a in cancer treatment, namely in prostate cancer and mammary gland tumors, was first established in animal models already 25 years ago (Redding *et al.* 1982, Redding & Schally 1983, Schally *et al.* 1983). The first studies with the cetrorelix (used in this study) were performed in the 1990s (Srkalic *et al.* 1990, Szende *et al.* 1990, Korkut *et al.* 1991). There are also reports showing the effectiveness of GnRH-a in benign prostate hyperplasia treatment (Lepor 2006) and they have been recently clinically tested in the treatment of human prostate cancer (Gittelman *et al.* 2008, Klotz *et al.* 2008, Huhtaniemi *et al.* 2009). The GnRH agonist, leuprolide acetate, has been successfully used for treating gonadotropin-dependent *Lhcgr*-bearing adrenal adenoma/Cushing syndromes (Lacroix *et al.* 1999) and the findings of the present study support the idea of treating the gonadotropin-dependent adrenal tumors in females by GnRH-a.

We have shown earlier that prepubertally gonadectomized *inh α /Tag* TG mice develop large adrenal tumors by the age of 6 months (Rahman *et al.* 2004). The tumorigenesis is apparently induced by combined action of the oncogene SV40/Tag and elevated LH levels. In the present study, we found that GnRH-a in both sexes could either alone or together with H-CG β -c diminish the tumor weight almost by 95% compared with the control treatment. In TG males H-CG β -c itself was as effective as GnRH-a. No significant additive effect of GnRH-a to H-CG β -c or vice versa could be observed, even though the progesterone levels were equally decreased in the treatment groups and the cell proliferation markers Ki-67 and p53 showed better response after the combination treatment of GnRH-a and H-CG β -c than H-CG β -c alone. However, in histopathological evaluation and further quantitative morphometric analysis, GnRH-a in males caused a remarkable fibrotic and necrotic response in the tumorous adrenals, whereas after H-CG β -c treatment

the histology appeared more intact and more healthy cells could be seen. The fibrotic/necrotic tissue that filled the adrenal structure could possibly explain the decreased Ki-67 and p53 appearance of the combination treatment.

In TG females, the tumor reduction by GnRH-a was 95% and by E₂ 90%, in comparison with control or H-CG β -c treatment. H-CG β -c did not improve the outcome as compared with GnRH-a, and the progesterone and mRNA levels for *Lhcgr* and *GATA-4* after the treatment groups were equal. However, histopathologic and morphometric analyses in females showed more healthy tissue and less fibrosis after GnRH-a and H-CG β -c combination, and also the IHC for the proliferation markers was less prominent after the combination therapy compared with GnRH-a alone in females. As a whole, GnRH-a in females did not cause destruction to adrenal structure as it did in males – at the moment, there is no explanation to this. In E₂ treatment TG female group, although the tumor weight was significantly reduced, some tumor nodules could still be found in the histopathological analysis and significantly more *Lhcgr* mRNA was expressed after E₂ than GnRH-a. E₂ probably worked through negative feedback by blocking the gonadotropin secretion, causing similar effect as occurred after GnRH-a. The weaker response to E₂ could also be monitored by the hormonal status where GnRH-a blocked gonadotropin secretion but after E₂ treatment the gonadotropin blockage was less pronounced; E₂ was not either able to block FSH secretion. These results altogether suggest that E₂ treatment was not as effective as GnRH-a, where the near-total ablation of LH severely suppressed the tumor progression in TG females.

It is known that *GATA-4* is expressed in fetal mouse and human adrenals but disappears soon after birth (Kiiveri et al. 2002). However, *GATA-4* expression is upregulated again upon adrenocortical tumor formation, e.g. in *inh α /Tag* TG mice, where it is visible already 3 months after gonadectomy along with *Lhcgr* expression and correlates with adrenocortical tumorigenesis (Kiiveri et al. 1999, Rahman et al. 2004). We now found that *GATA-4* and *Lhcgr* were upregulated in the tumor cells, but H-CG β -c in males, GnRH-a, E₂ and their combinations downregulated their expression significantly. This result is in line with our former data, where we showed that H-CG β -c specifically eradicated *Lhcgr* expressing tumor cells overexpressing *GATA-4* (Vuorenoja et al. 2008). *GATA-6* expression has been shown in fetal and in the adult adrenal with a specific role in regulating the adrenal steroidogenesis (Kiiveri et al. 2005) and it has

also been shown to be dramatically downregulated along with the adrenal tumor formation and progression (Kiiveri et al. 2005). Here, we showed the novel phenomenon of the reappearance of *GATA-6* expression after the tumor treatment with H-CG β -c in males or with combinations with GnRH-a or E₂. This observation on *GATA-6* may become an additional prognostic marker for adrenocortical tumorigenesis and treatment response along with *GATA-4* and *Lhcgr*.

Taken together, we conclude that H-CG β -c is an efficient treatment in *inh α /Tag* TG males due to its selective efficacy in killing tumor cells bearing *Lhcgr* with no significant destruction of the normal tissue structure. GnRH-a combined to H-CG β -c caused severe damage to the histological structure of the adrenals and GnRH-a treatment left still some *Lhcgr*-bearing cells. In TG females, however, only gonadotropin suppression by GnRH-a or E₂ was effective, since both treatments reduced the tumor size significantly and did not affect severely the adrenal structure. H-CG β -c combined with GnRH-a did not improve the tumor reduction. Our findings support the idea of treating the gonadotropin-dependent adrenal tumors in females by GnRH-a. In fact, GnRH agonist (leuprolide acetate) has already been successfully used for treating gonadotropin-dependent *Lhcgr*-bearing adrenal adenoma/Cushing syndromes (Lacroix et al. 1999). E₂ treatment was also shown to be rather effective in TG females, although not as good as GnRH-a. However, the side effects (increased risk for thromboembolism, uterine cancer, and in some cases increased incidence of breast cancer) of prolonged E₂ treatment are of concern, while considering it as treatment in human. Our present data showed a novel phenomenon against the dogma of SV40/*Tag*-induced tumorigenesis that even severe adrenal tumorigenesis co-induced by the oncogene SV40/*Tag* could be reversible. Our data further showed that in *inh α /Tag* TG mice adrenal tumorigenesis, not only the tumor ontogeny, which have been shown earlier (Kananen et al. 1997), but also the tumor progression is gonadotropin dependent, which could be affected by GnRH-a treatment. Finally, we hereby have observed two different mechanisms of action underlying the treatment in adrenocortical tumors. As shown before, H-CG β -c caused tumor destruction by selective killing the tumor cells bearing *Lhcgr* (Hansel et al. 2001, Gawronska et al. 2002, Leuschner et al. 2003, Bodek et al. 2005b, Vuorenoja et al. 2008). GnRH-a inhibited tumor growth by blocking the gonadotropin-dependent tumor progression through the systemic effects.

Declaration of interest

We declare that there is no conflict of interest that would prejudice this manuscript's impartiality.

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