Targeted therapy for adrenocortical tumors in transgenic mice through their LH receptor by Hecate-human chorionic gonadotropin β conjugate

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Abstract

Novel strategies are needed for the treatment of adrenocortical tumors that are usually resistant to chemotherapy. Hecate, a 23-amino acid lytic peptide, was conjugated to the 15-amino acid (81–95) fragment of the human chorionic gonadotropin β (CGβ) chain, which would selectively kill cancer cells expressing the LH receptor (LHR) sparing the normal ones with LHR. To prove the principle that Hecate-CGβ conjugate may eradicate tumors ectopically expressing plasma membrane receptors, transgenic (TG) inhibin α-subunit promoter (inhα)/Simian Virus 40 T-antigen mice, expressing LHR in their adrenal gland tumors, were used as the experimental model. Wild-type control littermates and TG mice with adrenal tumors were treated with either Hecate or Hecate-CGβ conjugate at the age of 6.5 months for 3 weeks and killed 7 days after the last treatment. The Hecate-CGβ conjugate reduced the adrenal tumor burden significantly in TG male but not in female mice, in comparison with Hecate-treated mice. Hecate-CGβ conjugate treatment did not affect normal adrenocortical function as the serum corticosterone level between Hecate and Hecate-CGβ conjugate groups were similar. The mRNA and protein expressions of GATA-4 and LHR colocalized only in tumor area, and a significant downregulation of gene expression was found after the Hecate-CGβ conjugate in comparison with Hecate- and/or non-treated adrenal tumors by western blotting. This finding provides evidence for a selective destruction of the tumor cells by the Hecate-CGβ conjugate. Hereby, our findings support the principle that Hecate-CGβ conjugate is able to specifically destroy tumor cells that ectopically express LHR.

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Introduction

Adrenocortical carcinomas (ACCs) are rare and aggressive types of human malignancies with poor prognosis as they often are diagnosed late and the survival rate remains quite low (Schulick & Brennan 1999b). ACCs tend to occur in the first and fifth decades of life and are relatively more common in women than men (Schulick & Brennan 1999a,b). There are still no efficient forms of therapy for adrenal tumors. The only effective treatment at this moment is complete adrenalectomy since with partial adrenalectomies the survival rate remains quite poor. However, even in patients undergoing complete surgical resection, recurrent and metastatic disease is common (Reincke et al. 1994, Bornstein et al. 1999). Chemotherapy with mitotane (o,p'-DDD) or cisplatin is indicated for advanced stages of disease, although the outcome remains often poor with plenty of side effects including adrenal insufficiency (Ahlman et al. 2001). Thus, there is a great need for new curative
methods as well as novel markers for early detection of these rare ACCs. Besides the normal gonadal expression of luteinizing hormone receptor (LHR), there are reports about malignant androgen-producing LH/human chorionic gonadotropin (hCG)-dependent adrenal tumors (de Lange et al. 1980, Leinonen et al. 1991, Lacroix et al. 1999). The LHR is expressed at low levels in normal human adrenal cortex (Pabon et al. 1996), and hCG stimulates dehydroepiandrosterone sulfate synthesis of human fetal adrenals (Jaffe et al. 1981). LHR expression has been shown in different kinds of human adrenal pathologies i.e., virilizing or Cushing’s syndrome (CS)–related adrenocorticotropin-independent macronodular adrenal hyperplasias (AIMAH; Lacroix et al. 1981). LHR expression has also been shown in aldosterone- (Saner-Amigh et al. 2006) or androgen-producing LH-dependent adrenal adenomas (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 in ACCs (Wy et al. 2002, Barbosa et al. 2004). Thus, this abundant/upregulated LHR expression in the adrenal hyperplasia/adenomas and adenocarcinomas makes them a susceptible target to ligand as a strategy for tumor eradication through Hecate-human chorionic gonadotropin β (CGβ) conjugate.

During the last few years, a novel targeted treatment approach for hormonally active, LHR-bearing tumors has been developed using the Hecate-CGβ conjugate (Leuschner et al. 2001). Hecate is a synthetic lytic peptide based on the structure of melittin, the principal toxin of honeybee venom. It rapidly destroys the outer membrane shell of only negatively charged cells such as bacteria and cancer cells by making small pores to the membrane (Leuschner & Hansel 2004, Rivero-Muller et al. 2007). Cancer cells have been shown to be negatively charged due to the changed distribution of phosphatidyserines in the outer cell membrane and thus, in comparison with the healthy cells, only negatively charged cells are destroyed by this lytic peptide (Utsugi et al. 1991). To target the action of this lytic peptide directly to the cancer cells through a membrane receptor, Hecate can be conjugated to a ligand or its receptor-binding domain, such as, in our case, to the 15-amino acid fragment (81–95) of the human CGβ chain (Hecate-CGβ conjugate). This allows the Hecate-CGβ conjugate to bind specifically and disrupt cells bearing the LHR. The same principle should apply to the plasma membrane receptors ectopically expressing in cancer cells. The mechanism of destruction is necrosis without activation of apoptosis (Bodek et al. 2005b) and Hecate-CGβ conjugate appears to selectively kill only cancer cells with LHR and spare all other gonadal/non-gonadal healthy cells (even with LHR) due to negative/positive charge changes in their membrane potential (Leuschner & Hansel 2004, Bodek et al. 2005b). Hecate-CGβ conjugate has previously been studied in specific cancer cell lines and xenografts of LHR-bearing carcinomas of prostate (Leuschner et al. 2001, 2003b, Bodek et al. 2005a), mammary gland (Bodek et al. 2003, Leuschner et al. 2003a), and ovary (Gawronska et al. 2002). In vivo studies have so far been carried out in transgenic (TG) mice bearing ovarian and testicular tumors (Bodek et al. 2005b).

TG mice expressing the inhibin α promoter/Simian virus 40 (SV40) T-antigen (inhα/Tag) were originally found to produce gonadal tumors with 100% penetrance at the age of 6 months, and if gonadectomy was prepubertally, they produced discernible adrenal tumors at the same age, but never in intact mice (Kananen et al. 1996a, Rilianawati et al. 1998, Rahman et al. 2004). The adrenal tumors and a tumor-derived cell line (Cz1) express very high levels of LHR (Kananen et al. 1996a, Rilianawati et al. 1998, Rahman et al. 2004), and the growth and steroidogenesis of the adrenal tumors were shown to be dependent on LH stimulation (Mikola et al. 2003). The post-castration elevation of LH levels apparently induced the ectopic LHR expression, which together with the potent oncogene Tag expression triggered adrenocortical tumorigenesis (Mikola et al. 2003). LH dependence of the tumors was proven by findings that they failed to appear if the post-castration increase in gonadotropins was blocked by either treating the mice with a gonadotropin-releasing hormone antagonist or crossbreeding them to the gonadotropin-deficient hpg (hypergonadotropic) genetic background (Cattanach et al. 1977, Kananen et al. 1997). The adrenocortical tumors tend to grow in prepubertally gonadectomy mice by hyperplasia–adenoma–carcinoma sequence, as hyperplasia of the adrenal cortex/adrenal adenoma could be observed at the age of 4 months, whereas discernible gonadal tumors were seen only at 6 months with low metastatic incidence (Rahman et al. 2004). In this study, we took the advantage of this inhα/Tag TG adrenocortical tumor model in order to prove the principle that adrenocortical tumor cells expressing ectopically a hormone receptor are sensitive to the Hecate-CGβ conjugate treatment in vivo, sparing simultaneously healthy cells.
Materials and methods

Experimental animals

In order to induce adrenal tumors, we gonadectomized inhz/Tag TG mice prepubertally as described previously (Kananen et al. 1996a). As these mice have earlier been extensively characterized (Kananen et al. 1996a, Rilinanawati et al. 1998, 2000, Kero et al. 2000, Rahman et al. 2001, 2004), the discernible adrenocortical tumors appear at the age of 6 months with 100% penetrance. We started all the treatments at the age of 6.5 months, in order to make sure that any treatment effect will be only due to the anti-tumoral effect, but not by prevention of the tumor development. Gonadectomy was performed under Avertin anesthesia and postoperative buprenorphine analgesia was administered on a routine basis. Seven to ten mice per treatment group were selected for the experiments. Wild-type (WT) control littermate mice (C57Bl/6N) were used as controls. For routine genotyping, PCR analysis was carried out using DNA extracts from ear biopsies, as previously described (Kananen et al. 1995). After weaning at the age of 21 days, the mice were housed two to four per cage, females and males in separate cages, in a room of controlled light (12 h light: 12 h darkness) and temperature (21 °C ± 1 °C). They were fed with mouse chow SDS RM-3 (Whitham, Essex, UK) and tap water ad libitum. The mice were kept in a specific pathogen-free surrounding and were routinely screened for common mouse pathogens. The University of Turku Ethics Committee on Use and Care of Animals approved the animal experiments.

Preparation of drugs

Hecate and the Hecate-CGβ conjugate were synthesized and purified in the Peptide and Protein Laboratory, Department of Virology, Hartman Institute, University of Helsinki, as described previously (Bodek et al. 2003).

Hecate and Hecate-CGβ conjugate treatments

Male and female mice (inhz/Tag or WT C57Bl control littersmates; n = 7–10 per group) were treated at the age of 6.5 months with either Hecate-CGβ conjugate (12 mg/kg b.w.) or Hecate (12 mg/kg b.w.) by i.p. injections. Since no substantial weight changes could be observed between Hecate and Hecate-CGβ conjugate by adding hCG to Hecate, both Hecate and Hecate-CGβ conjugate were given in the same dose (Leuschner et al. 2001). The mice were injected once per week for three consecutive weeks, according to an earlier protocol for in vivo treatment for nude mice xenografts (Leuschner et al. 2001) and TG mice with gonadal tumors (Bodek et al. 2005b). Seven days following the last injection, mice were killed by cervical dislocation and blood was collected by cardiac puncture. Weights of body, tumor, and different organs were recorded. Tissues were either snap-frozen in liquid nitrogen, or fixed in Bouin’s solution or 4% paraformaldehyde and embedded in paraffin. Paraffin sections of 5 μm thickness were stained for further histological analysis with hematoxylin–eosin or used for immunohistological analysis. For each tissue, at least five independent specimens were examined from each group.

Northern hybridization analysis

Total RNA was isolated from cells using the single-step guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). Twenty micrograms of RNA per lane were resolved on 1.2% denaturing agarose gel and transferred onto Hybond-XL nylon membranes (Amersham, Amersham International). Membranes were prehybridized overnight at 65 °C in a solution containing 5 × sodium chloride/sodium citrate (SSC), 5 × Denhardt’s solution, 0.5% SDS, 50% formamide, and 5 g/l of denatured calf thymus DNA. A complementary RNA probe for the rat LHR generated from a fragment of the LHR cDNA, spanning nucleotides 441–849 of its extracellular domain, subcloned into the pGEM-4Z plasmid was used for hybridization (LaPolt et al. 1990). The [32P]-dUTP (800 Ci/mmol, Amersham) labeled probe was generated using a Riboprobe system II kit (Promega). The probes were purified with NickColumns (Pharmacia Biotech). Hybridization was carried out at 65 °C for 20 h in the same prehybridization solution after an addition of labeled probe. After hybridization, the membranes were washed twice in 2 × SSC with 0.1% SDS at room temperature for 10 min, followed by two washes in 0.1 × SSC with 0.1% SDS at 65 °C to remove most of the background. For GATA-4, the membranes were prehybridized overnight at 42 °C in a solution containing 5 × SSC, 5 × Denhardt’s, 0.5% SDS, 50% formamide, and 5 g/l denatured calf thymus DNA. The GATA-4 cDNA probes were cut out from pMT2-GATA4 (Arceci et al. 1993, Ketola et al. 1999), using EcoRI/PstI and SmaI restriction enzymes respectively labeled with Prime-a-Gene kit (Pharmacia) using [α-32P]-dCTP for 4 h at 37 °C and purified with NickColumns. Hybridization was carried out at 42 °C for 20 h in the same prehybridization solution after an addition of the denatured labeled cDNA probe. After hybridization,
the membranes were washed twice in 2× SSC and 0.1% SDS at room temperature for 10 min, followed by two washes in 0.1× SSC and 0.1% SDS at 42 °C to remove most of the background. Finally, the membranes were exposed to Kodak X-ray films (Kodak XAR-5; Eastman Kodak) at −70 °C for 4–7 days or to phosphor-imager (Fujiﬁlm BAS-5000, Fujiﬁlm IzI, Tokyo, Japan) for 4–24 h. The intensities of speciﬁc bands were quantiﬁed using the Tina software (Raytest, Stranbenhardt, Germany) which came as an integrated program with the phosphor-imager (Fujiﬁlm BAS-500; El-He˘fnawy & Huhtaniemi 1998, El-He˘fnawy et al. 2000, Manna et al. 2001, Strassburg et al. 2002, Moran et al. 2006), and related to those of the 28S rRNA in the gel stained with ethidium bromide. The molecular sizes of the mRNA species were estimated by comparison with the mobility of the 18S and 28S rRNAs.

Hormone measurements
Serum levels of LH were measured by immunoﬂuorometric assay for rat (Delfia; Wallac, Turku, Finland) as described previously (Haavisto et al. 1993). Corticosterone levels were measured from diethyl ether extracts of the sera by RIA using a kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA) and progesterone was measured by Delfia Progesterone Kit (Wallac). The approximate assay sensitivities for LH, progesterone, and corticosterone are 0.75 pg/tube, 50 fmol/tube, and 0.05 M Tween, the incubation with the phosphor-imager (Fujiﬁlm BAS-500; El-He˘fnawy & Huhtaniemi 1998, El-He˘fnawy et al. 2000, Manna et al. 2001, Strassburg et al. 2002, Moran et al. 2006), and related to those of the 28S rRNA in the gel stained with ethidium bromide. The molecular sizes of the mRNA species were estimated by comparison with the mobility of the 18S and 28S rRNAs.

Immunoblotting analysis for GATA-4 and LHR
Western blotting was used to determine the concentration changes in GATA-4 and LHR due to the different treatments (Hecate or Hecate-CGβ conjugate). Tissue samples were subjected to homogenization and protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockﬁeld, IL, USA). Electrophoresis was carried out through a 12.5% SDS-PAGE gel (1 mg protein/well). Dual Color Precision Plus Protein Standards (Bio-Rad) were used as standards. After electrophoresis, proteins were electrotransferred to Hybond-P PVDF Membrane (Amersham Biosciences) using Trans-Blot SD cell (Bio-Rad) by 20 V for 60 min. After blocking the membrane with TBS containing 2% fat-free milk powder +0.05 M Tween, the incubation with the primary antibody was performed at +4 °C overnight. Anti-GATA-4 (goat polyclonal antibody, dilution 1:100; C-20, sc-1237; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-LHR (rabbit polyclonal antibody, dilution 1:4000; Acris Antibodies, Hiddenhausen, Germany) were used separately. As secondary antibody, either bovine anti-goat IgG-HRP (sc-2350, Santa Cruz Biotechnology) or ECL Anti-rabbit IgG, HRP-linked whole AB (Amersham Biosciences) was used in dilution 1:10.000. Signals were visualized using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech) and finally exposed to Fuji X-ray ﬁlm (Super RX, Fuji Photo Film Ltd, Bedford, UK). The intensities of speciﬁc bands were quantiﬁed using the Tina Software (Raytest).

Laser pressure catapulting (LPC)
LPC was performed by Laser Microbeam Microdissection (PALM Microlaser Technologies, Bernried, Germany) using a protocol described previously (Westphal et al. 2002). Briefly, for laser microdissection, cryosections (5 μm) were prepared from three independent specimens. Subsequent dehydration was achieved using graded alcohol and xylene treatment as follows: 95% ethanol for 30 s (twice), 100% ethanol for 30 s (twice), and xylene for 5 min (twice). The slides were dried under laminar ﬂow for 10 min and then stained with hematoxylin using RNase-free conditions and kept on dry ice until LPC. LPC was performed using a Zeiss inverted microscope PALM Laser Micro-Beam System u.v. laser at 337 nm, linked to a PC with the required software programs. The tumor and non-tumor areas to be dissected were selected by means of high-precision microcuts (the specimens may range from as little as 1 to 1000 μm in diameter). LPC dissection was performed using a few shots each of 100 μm in diameter from both tumor and non-tumor areas and from each slide. After catapulting similar amounts of tumor and non-tumor area, the material was removed from the caps for further analysis. Total RNA was extracted from tumor tissues using RNeasy kit (Qiagen).

RT-PCR and Southern hybridization
One microgram of total RNA was reverse transcribed using 20 IU of avian myeloma virus reverse transcriptase (RT), 50 IU RNase inhibitor, 25 pmol random primers, and 10 mmol of each dNTP (all from Promega), in 25 μl ﬁnal volume for 1 h at room temperature. For amplification of LHR and GATA-4 cDNA, speciﬁc primers were used as described previously (Morrisey et al. 1997, Kero et al. 2000). PCR was performed in a 25 μl ﬁnal volume, 4 μl RT solutions were mixed with 2 IU of the DyNAzyme II DNA polymerase (Finzymes Oy, Espoo, Finland), 10 pmol of each primer, and 10 mmol of each dNTP
(Zhang et al. 1994). The RT and PCRs were carried out sequentially in the same assay tube. First, the RT reaction was carried out (50 °C for 10 min), followed by a denaturation period of 3 min at 97 °C. Thereafter, a PCR with 40 cycles (96 °C for 1.5 min, 57 °C for 1.5 min, and 72 °C for 3 min, with a final extension period of 10 min at 72 °C) was performed. The sense primer for LHR corresponded to nucleotides 176–195 (5'-CTCTCACTATCTCCTGTC-3') and the antisense primer to nucleotides 875–885 (5'-TCTTTCCTCGCAATTCTTG-3') of the mouse 700 bp fragment of the LHR cDNA. The sense primer for GATA-4 corresponded to sense nucleotides 1527–1550 (5'-AAACGGAAAGCCCAAGAACCTGAAT-3') and the antisense primer to 1935–1953 (5'-GGCCCCCAGCTCCCAAGTC-3') (expected size: 427 bp) of mouse GATA-4 cDNA (Morrisey et al. 1997). As a control for RNA quality, a 395 bp fragment of the L19 ribosomal protein gene was co-amplified with each sample (sense primer, 5'-GAAATCGCCTATGCCAACTC-3'; antisense primer, 5'-TCTTAGACCTGCGAGCCTCA-3'). Kidney RNA was used as a negative control. After RT-PCR, 20 μl aliquots of the reaction mixtures were loaded on 1% agarose gel containing ethidium bromide (0.4 mg/l), to identify the amplified DNA fragments. Southern hybridization was used, according to standard techniques, to confirm the specificity of these PCR products. Hybridization was carried out with the 5'-end labeled oligonucleotide 5'-TGGAGAAGATGCA-TCTTAGACCTGCGAGCCTCA-3', corresponding to nucleotides 641–660 of LHR cDNA and for GATA-4 with the 5'-AGTGGGCACGATAGCGGCGAGGAC-3', corresponding to nucleotides 1676–1699. The membranes were washed according to the manufacturer's instructions and then exposed to Kodak X-ray film (XAR 5; Eastman Kodak).

**Immunohistochemistry (IHC)**

Paraformaldehyde-fixed paraffin sections (5 μm) of mouse adrenal tumors were deparaffinized and rehydrated. For immunostaining, 3% H2O2 in water was used to block endogenous peroxidases and all sections were boiled by microwave treatment for 15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. Two washes were done after each step with 0.05 M Tris and 150 mM NaCl (TBS) with 0.1% Tween (TBS-T). Serial sections of adrenal gland and testes as a positive control were subjected to immunohistochemistry with an anti-LHR antibody (dilution 1:1000, a rabbit polyclonal antiserum directed against human peptide sequence, KKLPSRETFVLNLLEA; a gift of Dr A Fazleabbes) and/or commercial rabbit polyclonal anti-GATA-4 IgG (dilution 1:800, Santa Cruz Biotechnology). IHC for anti-steroidogenic factor (SF)-1 (goat polyclonal antibody, 1:400, Santa Cruz Biotechnology) was also carried out. The slides were incubated with primary antibody at 4 °C overnight, and after incubation and washings with TBS–Tween buffer the slides were incubated with the anti-rabbit (Vector Laboratories, Inc., Burlingame, CA, USA) or anti-goat (Santa Cruz Biotechnology) secondary antibody. The avidin–biotin immunoperoxidase system was used to visualize bound antibody (Vectastain Elite ABC Kit, Vector Laboratories) with 3,3'-diaminobenzidine (Sigma) as a substrate. As a control for antibodies, adjacent sections were incubated with either 1% normal goat serum in PBS or rabbit IgG as a primary antibody to differentiate unspecific staining from specific staining.

**Statistical analysis**

Statistical analyses were carried out by ANOVA and/or the non-parametrical Mann–Whitney Rank test using SAS Enterprise Guide 3.0 program (SAS Institute Inc., Cary, NC, USA). Non-parametrical tests were used when the variances were not equal. ANOVA was used in other cases. P values <0.05 were regarded as statistically significant. All the values are presented as mean ± S.E.M.

**Results**

**Hecate-CGβ conjugate treatment reduced adrenal tumor volume**

Hecate-CGβ conjugate had an antineoplastic effect on the adrenal tumors of inhaz/Tag TG mice. We have earlier characterized the ontogeny of adrenocortical tumorigenesis in these mice and observed tumors weighing 20- to 30-fold more than normal adrenals (290 ± 69 mg) at 6–6.5 months of age following pre-pubertal gonadectomy (Rahman et al. 2004). Following a 1-month treatment with Hecate-CGβ conjugate, adrenal tumor weight was reduced by an average of 59% in males compared with Hecate treatment (364 ± 147 vs 150 ± 130 mg; Hecate versus Hecate-CGβ conjugate; P < 0.01; Fig. 1A). In females, the difference was only 18% (292 ± 156 vs 240 ± 225 mg; Hecate versus Hecate-CGβ conjugate; P > 0.05; Fig. 1B). As the age-related individual tumor progression rate was variable between inhaz/Tag TG mice and has been observed already earlier in inhaz/Tag and Tag mice (Hanahan 1989, Kananen et al. 1995, 1996b, Rahman et al. 1998), we also analyzed the
tumor burden (tumor weight/body weight). This parameter also decreased after Hecate-CGβ conjugate treatment significantly in males in comparison with Hecate treatment. In females, the treatment effect remained non-significant (Fig. 1A and B). We did not observe any statistically significant differences between the total body weights of the mice between the treatment groups (data not shown). No statistically significant differences in tumor weights/burden between the sexes could be observed in the present study, which was in line with the earlier observations (Rahman et al. 2004). The adrenal tumors metastasized in 10% \((n=10\) for both male and/or female inhz/Tag mice) cases in the Hecate-treated group to liver (proven by histopathological analysis, data not shown), but never in Hecate-CGβ conjugate-treated groups. According to earlier publications and observations, these tumors metastasized only occasionally (around 10% cases) to liver, more commonly in females than males (Kananen et al. 1996a). LHR expression is higher in the adrenal tumors of male mice

To find out why Hecate-CGβ conjugate treatment was less effective in female adrenal tumors, we quantified LHR and GATA-4 mRNA expressions by northern hybridization in male and female non-treated tumors (Fig. 2). GATA-4 was included in the study due to its close association with LHR expression and adrenocortical tumorigenesis (Kiiveri et al. 1999, Rahman et al. 2004). We also measured LHR and GATA-4 expressions in the established Cz1 cell line derived from inhz/Tag adrenocortical tumor (Kananen et al. 1996a, Rilianawati et al. 1998) and from non-treated adrenocortical tumors. A significantly higher level of LHR mRNA expression was found in the male than in the female in adrenal non-treated tumors \((P<0.01;\text{ Fig. 2})\). No significant differences in GATA-4 mRNA levels between the female and male adrenocortical tumors could be observed.

Endocrine consequences of the treatments

As sham-treated group (castrated TG mice treated with vehicle) shows identical hormonal values with the Hecate-treated group, which has been demonstrated earlier for testicular and ovarian tumors (Bodek et al. 2005b), ovarian xenograft model (Gawronska et al. 2002), and also rat mammary gland tumor model (Zaleska et al. 2004), in this study the sham-treated group was not taken into account in order to concentrate on the differences between the Hecate and Hecate-CGβ conjugate groups. In the WT groups, gonadectomy-induced elevated levels of LH were clear due to the lack of negative feedback from the gonads. After 1 month of treatment with Hecate-CGβ conjugate, no statistically significant differences could be seen in Hecate and Hecate-CGβ conjugate treatment groups in the LH, corticosterone, or progesterone levels in either sex (Fig. 3), although progesterone levels were twofold lower, but not statistically significant in Hecate-CGβ conjugate-treated males in comparison with Hecate.
group (in females no differences in hormonal values seen, data not shown), due to the high individual variations between the serum progesterone values (Fig. 3). Significant change in LH levels was seen between gonadectomized WT mice and the TG mice for both Hecate and Hecate-CGβ conjugate-treated groups ($P < 0.05$; Fig. 3). Serum progesterone levels were three- to four-fold higher in the TG mice than in WT mice, which might explain the lower level of LH in adrenal tumor-bearing TG mice ($P < 0.05$; Fig. 3). This finding is in agreement with the earlier findings in adrenal tumors and adrenal tumor-derived Cα1 cells (Kananen et al. 1996a) indicating their steroidogenic activity, e.g., progesterone secretion.

Hecate-CGβ conjugate selectively destroyed the LHR possessing adrenal tumor cells

To assess whether the Hecate-CGβ conjugate treatment can eradicate specifically the LHR-expressing adrenocortical tumor cells, we measured the LHR and GATA-4 protein expression levels in Hecate-CGβ conjugate-treated adrenals in comparison with the Hecate-treated and non-treated adrenocortical tumor LHR protein levels. Western blotting analysis revealed that there was a concomitant significant decrease in LHR and GATA-4 in the Hecate-CGβ conjugate-treated adrenal tumors ($P < 0.01$ Hecate-CGβ conjugate versus non-treated or Hecate-treated adrenal tumors; Fig. 4), indicating selective destruction of LHR and GATA-4 positive adrenocortical tumor cells. Higher GATA-4 expression compared with LHR after Hecate-CGβ conjugate might reflect the previously described phenomenon that these adrenocortical cells might express GATA-4 without LHR, but not vice versa and their expression pattern was higher/broader (Kiiveri et al. 1999, 2002). The LHR or GATA-4 protein expression in WT control mouse adrenals could not be detected.

Histopathological analysis demonstrating antitumoral effects of Hecate-CGβ conjugate treatment

Histopathological analysis revealed that Hecate-CGβ conjugate treatment of TG adrenocortical tumor mice

(B) The upper panel shows GATA-4, middle panel LHR, and lower panel β-actin protein expressions in western blotting analysis. Each lane represents three independent experiments, each time three different tumors for both sexes and from three different cell extracts. Each band shows a different tumor/mouse. Cα1, adrenal tumor cell line derived from a transgenic mice expressing (inhα)/Simian Virus 40 T-antigen (Tag) adrenal tumor; tu, tumor; on the right side, the molecular weights of the proteins in kilodaltons are indicated.
induced a definite reduction of the adrenocortical tumor mass where residual tumor tissue could be observed in a reduced area of the zona glomerulosa in hematoxylin–eosin-stained sections (Fig. 5B). This finding in the representative sample was reproducible as the same phenomenon was observed in all stained samples of the same treatment groups ($n=5$ from each group). The adrenal tumors metastasized in 10% ($n=10$ for both males and/or female inh$_2$/Tag mice) cases in the Hecate-treated group to liver (proven by histopathological analysis, data not shown), but never in Hecate-CG$_b$ conjugate-treated groups. According to earlier publications and observations, these tumors metastasized only occasionally (around 10% cases) to liver, more commonly in females than males (Kananen et al. 1996a).

LHR and GATA-4 expressions are colocalized in the tumors

We next demonstrated that LHR and GATA-4 are co-localized in the same adrenocortical tumor region. The data obtained by LPC of the tumors and non-tumorous tissues from the same slice demonstrated the co-localization of GATA-4 and LHR mRNA messages in adrenal tumor tissue and their absence in normal adrenal cortex (Fig. 6). The finding in the representative sample was reproducible as the same phenomenon was observed in all other three samples.

We then investigated whether the expression patterns of these proteins were affected by the Hecate or Hecate-CG$_b$ conjugate treatment. GATA-4 expression was found to directly localize to the

![Figure 4](https://www.endocrinology-journals.org/642)

**Figure 4** Western blot analysis for LHR and GATA-4. Protein extracts from male inh$_2$/Tag TG mice treated with Hecate or Hecate-CG$_b$ conjugate or from non-treated tumor samples were analyzed by western blotting. The upper panel shows LHR, GATA-4, and b-actin expressions and the lower panel their densitometric analysis. The representative band data shown are from one of the four different adrenal tumors/mouse per group/independent experiments ($n=4$). Each bar in densitometric quantification shows the quantification of four separate independent tumor samples/experiments. Each bar represents the mean ± S.E.M. of four independent experiments, each time different tumor sample. Hecate-CG$_b$ conjugate downregulated significantly both LHR and GATA-4 expressions compared with non-treated control adrenal tumor or Hecate-treated tumor (**$P<0.001$). No detectable LHR or GATA-4 was detected in WT C57Bl mouse adrenals. On the right side, the molecular weights of the proteins in kilodaltons are indicated.
H–E-stained tumor area that was in line with our previous findings (Rahman et al. 2004; Fig. 7A and C). In Hecate-CGβ conjugate-treated sections, no LHR staining could be detected even in the same H–E-stained, marginalized, and reduced tumorous area (Fig. 7A and D). We also found SF-1 expression in the same tumor areas where GATA-4 expression could be observed (Fig. 7B and C).

Discussion

The lack of a suitable animal model for adrenocortical tumorigenesis has been a major obstacle for unraveling the molecular mechanisms involved in their pathogenesis, as well as for improving their diagnostic and therapeutic strategies. Thus, establishment of suitable animal models is of extremely high importance in order to study human adrenal tumorigenesis. Chronically elevated LH, ectopic/upregulated LHR expression, hyperplasia–adenoma–adenocarcinoma sequence tumor formation, slow tumor growth, late discernible tumor incidence (analogous to human postmenopausal/andropausal age), low metastases frequency (5–10%); these characteristics make the inhxA/Tag TG murine model a good tumor relevance model for human ACCs studies. Our inhxA/Tag TG mouse is at the moment one of the rare and unique murine models in which adrenocortical tumorigenesis and its molecular mechanisms or any treatment strategies can be investigated. Although the physiologic significances of extragonadal LHR expression in reproductive function is questioned (Pakarainen et al. 2005, 2007), some recent studies (Rao Ch et al. 2004, Alevizaki et al. 2006, Vuorenoja et al. 2007) suggest that the adrenal cortex expresses low levels of LHR (Pabon et al. 1996) whose function becomes significant when LH levels are elevated, as after gonadectomy or postmenopausally. In light of the present study, we could postulate that the upregulated LHR expression in adrenal tumors is functionally relevant and can be used to target the tumors by the cytotoxic Hecate-CGβ conjugate. This model serves as an example demonstrating how ectopically expressed receptors in tumors can be used as therapeutic target.

We used in the present study an established inhxA/Tag TG mouse model for post-gonadectomy adrenocortical tumorigenesis, where the pathophysiological and endocrine responses induced by tumorigenesis are well characterized. We found that Hecate-CGβ conjugate treatment reduced the total adrenocortical tumor weights without detectable side effects, e.g., adrenal
insufficiency. Curiously, the treatment was, for unknown reason, ineffective in female with adrenocortical tumors. The adrenal response to elevated LH in inhα/Tag mice was probably more sensitive in females and we believe it was also more aggressive, which could explain why immortalization of Cz1 cells from a female inhα/Tag founder mouse tumor was possible (Kananen et al. 1996a, Rilianawati et al. 1998). It has been shown that the castrated male mice developed adrenocortical tumors more slowly than gonadectomized females along with the ectopic LHR expression (Bielinska et al. 2003). Moreover, the elevated LH levels induced abundant ectopic LHR expression and acted as a tumor promoter selectively only in the intact female double-positive TG mice, where bLHβ-CTP mice producing constitutively elevated levels of LH (Risma et al. 1995) were crossbred with inhα/Tag TG mice (Mikola et al. 2003). This never happened in the male double-positive mice (Mikola et al. 2003). In humans, ACCs are also more common in postmenopausal women than in aging males (Schulick & Brennan 1999a,b). Therefore, it could be an established phenomenon that female adrenals respond more readily to high LH and tumorigenesis might be more aggressive in them, which might imply that the suppression of LH action needed to block tumorigenesis in females should be more complete/stronger, whereas male tumorigenesis can be blocked with less-effective suppression. It is likely that this mechanism occurred in our Hecate-CGβ conjugate-treated inhα/Tag mice where, with the applied dose of the drug, we achieved much a better response in the males than in the females. Probably, this phenomenon was additionally helped by the higher concentration of LHR in the tumor mass in the male tumors attracting larger amounts of Hecate-CGβ conjugate, exerting consequently a more severe lytic effect. The reason for the higher LHR expression in male adrenal tumors remains unknown. The putative difference in tumor cell membrane charges between the male and females could also be one of the potential mechanistic reasons behind this differential treatment effects, which needs to be further evaluated in future studies.

As we did not find significant changes in the serum endocrine profiles of the Hecate- or Hecate-CGβ conjugate-treated mice, the treatment outcome could not be monitored by serum hormone measurements. The progesterone levels are decreased in Hecate-CGβ conjugate male in comparison with Hecate group but the high individual variations made them non-significant. LH levels were higher in gonadectomized WT than in TG males, which indicated increased endocrine activity of the tumors. The hormone with increased production by the tumors exerting a negative feedback action of LH secretion is probably progesterone. However, the adrenal tumors can also produce sex steroids (androgens or estrogens; Leinonen et al. 1991, Kananen et al. 1996a, Lacroix et al. 1999, Bielinska et al. 2005). No effect of the treatment could be seen on corticosterone levels between Hecate and Hecate-CGβ conjugate treatment groups, which indicates that the Hecate-CGβ conjugate treatment does not destroy normal adrenal tissue or induce adrenocortical hypofunction. This additionally supports the principle that this peptide attacks only the negatively charged tumor cells and spares the healthy ones.

We have shown earlier a precise co-initiation of GATA-4 along with the LHR expression during adrenocortical tumorigenesis ontogeny (Rahman et al. 2004). GATA-4 has been proposed to be a potential marker for adrenocortical tumorigenesis (Kiiveri et al. 1999), along with LHR (Rahman et al. 2004). Further proof is needed to determine whether GATA-4 works as a specific co-activator for LHR (or even vice versa) in adrenocortical tumorigenesis. Thus, we wanted to see whether GATA-4 expression would also diminish due to the Hecate-CGβ conjugate treatment, which could lead us to further mechanistic studies in the future. As there are some difficulties with the availability of good LHR antibodies, GATA-4 immunohistochemistry could also additionally serve as a marker for treatment efficacy. We found that both LH and GATA-4 protein expressions decreased significantly after Hecate-CGβ conjugate treatment compared with Hecate. By immunohistochemistry, we could detect GATA-4 in residual tumor area indicating that some tumor steroidogenic cells are still left, as GATA-4 is expressed.
only in tumor adrenocortical steroidogenic cells (Kiiveri et al. 1999, 2002, Peterson et al. 2004), but LHR staining was no longer detectable in the Hecate-CGβ conjugate-treated samples. These results indicated that Hecate-CGβ conjugate killed specifically the tumor cells expressing LHR. This finding is well in line with the adrenal tumor weight results. Even after the Hecate-CGβ conjugate treatment, the adrenal has not returned to totally normal size. However, GATA-4 and SF-1 were colocalized in the same tumor area. SF-1 is known to be another factor activating adrenocortical steroidogenesis and it is shown to transactivate both GATA-4 and LHR activities (Tremblay & Viger 1999). The expression pattern of SF-1 may implicate its role as a co-activator of GATA-4 and LHR in adrenocortical tumorigenesis of the inhα/Tag TG mice.

Human adrenal LH/hCG responsive tumors have been well described (Leinonen et al. 1991, Lacroix et al. 1999). Higher basal levels of corticosteroids in postmenopausal women are also associated with higher circulating LH and LHR expressions in adrenals (Pabon et al. 1996, Alevizaki et al. 2006). A recent publication confirms that ectopic LHR may induce adrenal hyperplasia- and Cushing’s syndrome-like symptoms as well as hCG-mediated steroidogenic activity (Mazzuco et al. 2006). Ectopic expression of LHR in the adrenal gland has been shown to be associated with not only adrenocorticotropic-independent Cushing’s syndrome but also benign aldosterone-producing adenomas (Saner-Amigh et al. 2006). There are also reports about malignant androgen-producing LH-dependent adrenal tumors (de Lange et al. 1980, Leinonen et al. 1991). Hence, LH/hCG-dependent mechanisms of adrenal pathogenesis do exist and they may be clinically important in circumstances with elevated LH levels, e.g., in connection with polycystic ovarian syndrome and after menopause in women.

Taken together, the present data provide novel evidence that targeted destruction of adrenocortical tumors expressing ectopically LHR by the Hecate-CGβ conjugate is possible. In addition to the currently used TG mouse model, our findings prove the more general principle that receptors expressed ectopically in malignant cells can be exploited in targeted cytotoxic therapies without affecting the normal healthy cells.

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