Hecate-CGβ 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-CGβ conjugate (H-CGβ-c) selectively binds to and destroys tumor cells expressing LH/chorionic gonadotropin receptor (Lhcg). Transgenic mice (6.5 month old) expressing SV40 T-antigen under the inhibin-α promoter (inhα/Tag) expressing with Lhcg expressing adrenal tumors were treated either with H-CGβ-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-CGβ-c could improve the treatment efficacy especially in females by decreasing circulating LH and eliminating the potential competition of serum LH with the H-CGβ-c. GnRH-a and H-CGβ-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-CGβ-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, Lhcg, and GATA-6 further supported the positive outcome. H-CGβ-c selectively killed the Lhcg 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-CGβ-c could be considered for the treatment of gonadotropin-dependent adrenal tumors in males, whereas in females gonadotropin suppression, but not H-CGβ-c, would work better.

Endocrine-Related Cancer (2009) 16 549–564

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...
and testicular cancer cells (Bodek et al. 2005b). H-CGβ-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 (inhα/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 (Cz1) 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-CGβ-c in treating the Lhcgr expressing adrenal tumors of the inhα/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-CGβ-c, would decrease the circulating serum LH and consequently eliminate the potential competition between H-CGβ-c and LH. This could increase the efficacy of the H-CGβ-c treatment in particular in the female TG mice. We also compared the effects of GnRH-a treatment with H-CGβ-c in TG males.
Materials and methods

Experimental animals

Adrenal tumors were induced by prepubertal gonadectomy of inhz/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 burprenorphine (Schering-Plough, Brussels, Belgium) was administrated 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-CGβ-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 (cetrorelix acetate, Cetrotide) was purchased from Merck Serono.

H-CGβ-c, GnRH-a, and E2 treatments

Male and female mice (inhz/Tag TG; n = 6–8 per group) were treated at the age of 6.5 months with either H-CGβ-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-CGβ-c by adding hCG to Hecate, both Hecate and H-CGβ-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-CGβ-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 μm 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; Wallac, Turku, Finland) as described previously. Serum progesterone was measured using the Delfia Progesterone Kit (Wallac). The approximate assay sensitivity for LH was 0.1 μg/l, for FSH 0.03 μg/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 μm) 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 ×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 denaturate the template, 10 s in 94 °C to denaturation, 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 Quide 3.0 program (SAS Institute, Inc., Cary, NC, USA).

Table 1 Oligonucleotides used in quantitative RT-PCR analysis

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<th>Gene</th>
<th>Primers</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>Temperature (annealing) (°C)</th>
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<tr>
<td>L19</td>
<td>F</td>
<td>5'-GGACAGAGTCTTGATGATCTC-3'</td>
<td>195</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CTGAAGGTCAAAGGGAATGTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-4</td>
<td>F</td>
<td>5'-TCTCACTATGGCCACAGCAG-3'</td>
<td>246</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CGAGCAGGAATTGAAGAAGG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-6</td>
<td>F</td>
<td>5'-GAGCTGCTGGTCTCAACAGG-3'</td>
<td>193</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TGCAAAAGCCCATCTCTTCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LHR</td>
<td>F</td>
<td>5'-CAATGGGACAGCAGCTAATCT-3'</td>
<td>204</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CTGGAGGCGAGTTTCAG-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 ± S.E.M.

Results

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

Treatments with GnRH-a or H-CGβ-c alone were highly effective with significant antineoplastic effect in male TG mouse adrenal glands (Fig. 1A). Combination of H-CGβ-c with GnRH-a did not show further improvement of treatment efficacy in males compared with H-CGβ-c or GnRH-a treatment alone (Fig. 1A). Following a 1-month treatment of males with H-CGβ-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-CGβ-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 inhα/Tag TG mice, a common phenomenon for SV40/Tag effects (Hanahan 1989, Kananen et al. 1995, Rahman et al. 1998), we also analyzed the tumor burden (tumor weight/body weight). It also decreased significantly after combination treatment of H-CGβ-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-CGβ-c (Vuorenoja et al. 2008) was confirmed (330 ± 65 vs 249 ± 64 mg, control versus H-CGβ-c treatment). GnRH-a either alone or combined to H-CGβ-c drastically reduced the tumor weight in females by 95% (249 ± 64 vs 10 ± 0.4 mg or 12 ± 1 mg; H-CGβ-c versus GnRH-a or H-CGβ-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-CGβ-c (\( P < 0.001 \); Fig. 1B). The treatment did not affect body weights in any of the groups (data not shown).

H-CGβ-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-CGβ-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-CGβ-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-CGβ-c versus control; \( P < 0.01 \)).

Endocrine consequences of the treatments

The adrenal tumors and Cz1 cells derived from them secrete progesterone (Kananen et al. 1996a, Vuorenoja et al. 2008). In TG males, treatment with either H-CGβ-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-CGβ-c, significantly reduced the progesterone levels in comparison with control- or H-CGβ-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-CGβ-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-CGβ-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-CGβ-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.
In TG females, the expression of Lhcgr and GATA-4 remained high in the H-CGβ-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-CGβ-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 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 ± s.e.m. c, control; conj, Hecate-CGβ 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 ± s.e.m. c, control; conj, Hecate-CGβ 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 ± s.e.m. c, control; conj, Hecate-CGβ conjugate; E2, estradiol. 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 lose 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...
zona fasciculata was preserved and no residual tumorous tissue could be detected (Fig. 4A3). After H-CGβ-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-CGβ-c treatment seemed to cure the tissue sparing the normal adrenal structure in the males. GnRH-a alone or combined to H-CGβ-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-CGβ-c, unlike H-CGβ-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-CGβ-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-CGβ-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
further proof of efficacy of the treatments. In control tumors of both sexes, after Hecate treatment in males and H-CGβ-c treatment in females, over 90% of the adrenal mass composed of tumor cells, fibrosis or blood-filled cysts (Table 2). In males, H-CGβ-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-CGβ-c almost all of the tissue (91.3 ± 4%) was healthy, but after the H-CGβ-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-CGβ-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-CGβ-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-CGβ-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-CGβ-c in females, showed similar distribution of proliferating cells throughout the whole tissue with both markers (Fig. 5). H-CGβ-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-CGβ-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).
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\(\beta\)-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 nodulus 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 conditions.

### Table 2 Results of morphometrical analysis in percentages ± S.E.M.

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<th>Treatment (males)</th>
<th>Healthy</th>
<th>Tumor</th>
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<th>Cysts</th>
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<td>Wild-type</td>
<td>100</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Control</td>
<td>1.3 ± 1.3(^a)</td>
<td>62.6 ± 5.8(^a)</td>
<td>19 ± 11.9(^a)</td>
<td>17.1 ± 5.8(^a)</td>
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<td>50.4 ± 5.5(^b)</td>
<td>34.2 ± 10.4(^a)</td>
<td>15.4 ± 5.5(^a)</td>
<td>0(^b)</td>
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<td>Hecate-CG(\beta) conjugate + GnRH antagonist</td>
<td>44.1 ± 14(^b)</td>
<td>11.5 ± 5.3(^b)</td>
<td>44.4 ± 17.9(^a)</td>
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</tr>
<tr>
<td>GnRH antagonist</td>
<td>48.3 ± 12.5(^b)</td>
<td>34.7 ± 2.9(^ab)</td>
<td>17 ± 11.6(^a)</td>
<td>0(^b)</td>
</tr>
<tr>
<td>Treatment (females)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0.8 ± 0.3(^a)</td>
<td>57.7 ± 35.6(^a)</td>
<td>4.6 ± 4.6(^a)</td>
<td>36.9 ± 31.2(^a)</td>
</tr>
<tr>
<td>Hecate + GnRH antagonist</td>
<td>24.4 ± 12.1(^b)</td>
<td>36.6 ± 23.1(^a)</td>
<td>33.6 ± 16.9(^a)</td>
<td>5.5 ± 5.5(^a)</td>
</tr>
<tr>
<td>Hecate-CG(\beta) conjugate + GnRH antagonist</td>
<td>66 ± 9.4(^b)</td>
<td>29.4 ± 9.9(^a)</td>
<td>4.6 ± 0.5(^a)</td>
<td>0 ± 0(^a)</td>
</tr>
<tr>
<td>Hecate-CG(\beta) conjugate</td>
<td>4 ± 2.6(^a)</td>
<td>65.2 ± 16.9(^a)</td>
<td>5.4 ± 3.1(^a)</td>
<td>25.4 ± 11.2(^a)</td>
</tr>
<tr>
<td>GnRH-antagonant</td>
<td>38.4 ± 11.2(^bc)</td>
<td>35.9 ± 6.9(^a)</td>
<td>25.7 ± 14.9(^a)</td>
<td>0(^a)</td>
</tr>
<tr>
<td>Estradiol</td>
<td>31.3 ± 7.4(^bc)</td>
<td>55.3 ± 6.5(^a)</td>
<td>8.5 ± 3.4(^a)</td>
<td>4.8 ± 4.4(^a)</td>
</tr>
<tr>
<td>Estradiol + Hecate-CG(\beta) conjugate</td>
<td>39.6 ± 8.3(^bc)</td>
<td>38.8 ± 2.8(^a)</td>
<td>21.6 ± 5.9(^a)</td>
<td>0(^a)</td>
</tr>
</tbody>
</table>

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\(\beta\)-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 nodulus 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 conditions.
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-CGβ-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-CGβ-c adrenal tumor weight could be reduced by an average of two-thirds in inhα/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-CGβ-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-CGβ-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 (Srkalovic 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 inhα/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-CGβ-c diminish the tumor weight almost by 95% compared with the control treatment. In TG males H-CGβ-c itself was as effective as GnRH-a. No significant additive effect of GnRH-a to H-CGβ-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-CGβ-c than H-CGβ-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-CGβ-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-CGβ-c treatment. H-CGβ-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-CGβ-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 inhα/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-CGβ-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-CGβ-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-CGβ-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-CGβ-c is an efficient treatment in inhα/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-CGβ-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-CGβ-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 thromboembolia, 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 inhα/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-CGβ-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.

Funding
This project was supported by the grants from Academy of Finland (I H, J T, N R), the Finnish Cultural Foundation at Varsinais-Suomi (S V), Ida Montin Foundation (S V), Turku University Foundations of Gerda and Aila Saarinen and Aili Salo (S V), Finnish Cancer Organizations (S V), the Finnish Medical Foundation (S V), Sigrid Juselius Foundation (I H, J T, I H), and Turku University Hospital (J T).

Acknowledgements
We thank Dr Harry Kujari for valuable advice with morphometrical analysis, Tero Vahlberg for the fruitful discussions with statistical matters and Juho-Antti Mäkelä with qRT-PCR analyses. The authors would also like to thank Taija Leinonen and Taina Kirjonen for skillful technical assistance. Heli Niittymäki, Nina Messner, and the personnel of Turku University Animal Facility are acknowledged for their help with mice.

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