Growth Repression in Diethylstilbestrol/ Dimethylbenz[a]anthracene–Induced Rat Mammary Gland Tumor Using Hecate-CGβ Conjugate

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Recently, we have shown that Hecate-CGβ conjugate, which is a fusion of the lytic peptide Hecate and a 15–amino acid fragment of the β-chain of chorionic gonadotropin (CGβ), selectively destroys mammary gland carcinoma cells that possess luteinizing hormone receptors (LHR) *in vitro*. We induced mammary gland tumors using combined prenatal exposure to synthetic diethylstilbestrol (DES) and additional postnatal exposure to dimethylbenz[a]anthracene (DMBA). Rats with tumors were equally randomized (10/group) and treated with either sham (control) or 12 mg/kg body wt of either Hecate or Hecate-CGβ once a week for 3 weeks by tail vein injections. One week after the last injection, rats were killed. Reverse-transcription–nested polymerase chain reaction/Southern blotting revealed alternatively spliced mRNA for LHR in tumor tissues of 5 of 30 females, which was further confirmed by Western blot analysis. The percentage of tumor volume increase was lowest in the group treated with Hecate-CGβ (45.3 ± 27.6), compared with Hecate- and sham-treated, control group (324.8 ± 78.1 and 309.9 ± 51.2, respectively; $P < 0.001$). Hecate-CGβ induced a significant decrease in tumor burden compared with controls (9.5 ± 2.1 mg/g body wt vs. 21.6 ± 2.9; $P < 0.01$). A smaller reduction in tumor burden was also observed in Hecate-treated females (17.6 ± 1.6 mg/g body wt vs. 21.6 ± 2.9; $P < 0.05$). Our results prove the principle that Hecate-CGβ conjugate is able to repress mammary gland tumor growth, even in tumor tissues that lack or have very low levels of LHR. The mechanism of Hecate-CGβ conjugate action in repression of DES/DMBA-induced tumor growth needs to be further analyzed to clarify the molecular mechanisms of Hecate-CGβ conjugate action *in vivo*. Exp Biol Med 229:335–344, 2004

Key words: mammary gland tumor; LH receptor; Hecate-CGβ conjugate; cancer treatment

Breast cancer is the most common malignancy among women in the United States and other developed countries and is the second most common cause of cancer death (1). Until recently, the therapeutic treatment of breast cancer has been dominated by endocrine-based drugs (estrogen receptor antagonists, aromatase inhibitors, etc.) and conventional cytotoxins (doxorubicin, cyclophosphamide, 5-fluorouracil, etc.; Ref. 2). Classical cancer chemotherapy is limited when tumor cells have intrinsic or acquired drug resistance and when the drug is also toxic to normal cells (3). More selective delivery of toxic agents to the primary tumor and their metastases would allow dose escalation and reduce peripheral toxicity (3). Therefore, a continuous search for novel and selective methods of treatment for the management of breast cancer is essential.

Diethylstilbestrol (DES), the first synthetic orally active estrogen, was first used during the late 1940s to prevent spontaneous abortion and preterm delivery (4). However, a few years later, the broad transplacental, teratogenic

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activity of DES on both male and female offspring was discovered (5–9). Animal studies have suggested that DES might increase the transgenerational susceptibility to malignant tumors of the female reproductive tract and mammary glands (10), presumably from damage to germ cells and abnormal imprinting (11). We used the DES/DMBA-induced mammary gland rat tumor model, which was easy to induce and monitor the tumor growth during treatment trials.

The development and growth of many cancers depends on paracrine and autocrine hormonal regulation. Recent evidence has shown that human chorionic gonadotropin/luteinizing hormone (CG/LH) can be synthesized ectopically and exert paracrine effects that regulate the growth of various cell types. The role of these hormones in the occurrence and development of various cancers has been extensively discussed (12–17). The synthesis of CGβ by a variety of cancer cell lines of various origins has been demonstrated (17, 18). Specific binding sites for LH have been found in healthy mammary tissues and mammary cancer cells (19–21). Pregnancy (especially early pregnancy) has been shown to be protective against breast cancer carcinogenesis (22, 23). A protective effect of CG on the development of DMBA-induced mammary tumor in rats has been shown (12–14), but the mechanism of action was not precisely explained. It has been reported that CG could exert a direct antiproliferative effect on human breast epithelial cells in culture (24), which might be mediated through the secretion of inhibin (25, 26).

The targeting of cytotoxic agents to cancer cells represents a modern approach to the treatment of human breast cancer and other cancers, which could increase the inhibition of tumor growth and reduce peripheral toxicity (27). This new approach, which is based on the selectivity of certain carrier molecules for specific binding sites in tumor tissues, has been proposed in a number of experimental treatments. Leuschner *et al.* (28) and Hansel *et al.* (29) reported that the conjugate of the 23–amino acid amphipathic lytic peptide Hecate and a 15–amino acid segment (81–95) of the CGβ subunit (Hecate-CGβ) targets prostate cancer cells that express the LH receptor (LHR) both *in vitro* and *in vivo*. It was further demonstrated that Hecate-CGβ killed ovarian epithelial cancer cells (OVCAR-3) *in vitro* and stopped their growth in xenografts *in vivo* (30). Recently, the ability of Hecate-CGβ to destroy human breast cancer cell lines (MCF-7 and MDA-MB-435S) *in vitro* and MDA-MB-435S cell xenografts in nude mice was demonstrated (31). Our recent studies have proved the inhibitory potential of the Hecate-CGβ conjugate to LHR, because the Hecate-CGβ conjugate selectively and in a dose-dependent manner destroyed cells possessing LHR *in vitro*. Hecate-CGβ was effective in lower concentrations than Hecate alone, and its cytotoxic effect was strongly correlated with the number of LHRs (32, 33). In the present study, we investigated the efficacy of Hecate-CGβ conjugate *in vivo* in the selective inhibition of DES/DMBA-induced rat mammary gland tumors. Moreover, we checked the LHR expression status of the DES/DMBA-induced mammary gland tumor cells and the cytotoxic effect of the *in vivo* treatment.

Materials and Methods

Animals. All the experiments were carried out using Wistar rats maintained in pathogen-free conditions with controlled lighting (12:12-hr light:dark cycle) and temperature (21°C ± 1°C). The animals received water and food *ad libitum*. The local Ethical Commission, in compliance with the national guidelines for laboratory animal care, approved all procedures involving animals in our experiment.

Tumor Establishment and Treatment. Mammary tumors were induced according to the method described by Boylan and Calhoon (34), with some modifications. In brief, pregnant Wistar rats were given injections of DES (Sigma-Aldrich Co., St. Louis, MO) dissolved in corn oil on days 15 and 18 of gestation (two injections of 0.6 μg in 0.3 ml of corn oil). Animals were allowed to deliver and raise their offspring until weaning. Litters were reduced to seven pups by removing male offspring. Female offspring were separated from dams 30 days after delivery and housed five to a cage. All animals received two gastric intubation of DMBA (60 mg/kg body wt, each) at 50 and 57 days of age. DMBA (Sigma-Aldrich Co.) was dissolved in corn oil, stirred in the dark overnight to achieve complete solubilization, and used the following day. Beginning 4 weeks after the first intubation with DMBA, mammary tumor volumes were determined weekly with microcalipers, and we continued to measure tumors until the end of drug treatment. Tumor volume was calculated according to the method of Tomayko and Reynolds (35), using the formula $V\,(mm^3) = 0.5234\,(\text{length} \times \text{width} \times \text{height})$. Palpable tumors appeared 10 weeks after the second intubation with DMBA. Animals with tumor volumes of 500–800 mm^3 were randomly divided into three treatment groups of 10 per group and treated as follows: (1) control (sham-treated; vehicle), (2) Hecate at a dose of 12 mg/kg body wt, or (3) Hecate-CGβ at a dose of 12 mg/kg body wt. We also had an additional group of 10 non–tumor-bearing, untreated rats. In preliminary studies, we determined that the maximum nontoxic dose of Hecate-CGβ is 12 mg/kg body wt. Animals were treated once per week for 3 weeks by tail vein injections. One week after the last treatment, animals were killed, and complete necropsies were done. Blood samples were collected for hormone assays, and body, tumor and organ weights were recorded. The tissues were fixed in 10% neutral buffered formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. For Western blotting reverse-transcription (RT)–nested polymerase chain reaction (PCR) and Southern hybridization, a portion of each tumor was collected immediately after death, snap frozen in liquid nitrogen and stored at –70°C.

Hormone Measurement. Blood collected immediately after death was placed in heparinized plastic tubes on ice and centrifuged for 15 mins at 1000 *g* at 4°C. Plasma was removed and stored at –20°C until it was analyzed.

Estradiol concentrations were measured by radioimmunoassay, with the addition of antibodies from immunized rabbits against conjugate of 17β-estradiol(6-O-carboxymethyl)-oxime-bovine serum albumin (36) and [2,4,6,7-^{3}H] estradiol (PerkinElmer Life Sciences, Inc., Boston, MA) as a tracer. Antiserum was used in a titer of 1:20 000. The sensitivity of the assay was 5 pg per tube. Intra- and interassay coefficients of variation were 2.6% and 4.7%, respectively.

Follicle-stimulating hormone (FSH) and LH levels were measured by a supersensitive immunofluorometric assay (Delfia; Wallac OY, Turku, Finland), according to methods described elsewhere (37, 38). The assay sensitivities for FSH and LH were 0.85 and 0.75 pg/tube, respectively. Intraassay coefficients of variation were 3.2% and 2.3%, respectively. Interassay coefficients of variation for these assays were below 15%.

Thyroid-stimulating hormone (TSH) and prolactin (PRL) were determined by radioimmunoassays, as described elsewhere (39). The assay sensitivities for TSH and PRL were 50 and 30 fmol/tube, respectively. Intraassay coefficients of variation were 1.9% and 1.8%, respectively. Interassay coefficients of variation for these assays were <15%.

Assessment of LHR mRNA by RT–Nested PCR. Total RNA was extracted from rat mammary cancer tissues collected at necropsy with the Total RNA Prep Plus kit (A&A Biotechnology, Gdansk, Poland), according to the manufacturer's recommendations. The RNA concentration was measured spectrophotometrically, and purity was determined by agarose-formaldehyde gel electrophoresis (40). To detect the LHR expression in rat mammary gland tumors, the sense primer corresponding to nucleotides 176–195 (5′-CTCTCACCTAT CTCCCTGTC-3′) and the antisense primer (5′-TCTTTCTTCGGCAAATTCCTG-3′) corresponding to nucleotides 878–858 of the rat LHR cDNA were used (Fig. 1). The RT and PCR reactions were performed sequentially in the same tube. Fifty microliters of RT-PCR mixture contained 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.001% gelatin, 40 pmol of each primer, 0.2 m*M* dNTP, 20 U of RNase inhibitor RNasin (Sigma-Aldrich Co.), 8 U of avian myeloblastosis virus (AMV) RT (Promega, Madison, WI), and 2 U *Taq* polymerase (Sigma-Aldrich Co.). Two micrograms of total RNA were reverse transcribed at 50°C for 50 mins. Then, after 3 mins of primary denaturation at 97°C, the reverse-transcribed cDNA was amplified for 35 cycles, including denaturation at 96°C for 1 min, primer annealing at 57°C for 1 min, extension at 72°C for 2 mins, and a final extension for 10 mins. As a control for RNA quality, a fragment of the β-actin gene was coamplified with each sample (sense, 5′-TCTACAATGAGCTGCGTGTG-3′; antisense, 5′-GGTCAGGATCTTCATGAGGT-3′). To exclude the possibility of genomic DNA contamination in the RNA samples and cross-contamination between samples, the reactions were also run on rat kidney RNA, on blank-only buffer samples and in absence of the reverse-transcriptase enzyme. Ovary RNA was used in positive control amplifications. To further verify the specificity of the amplified products, a second round of PCR was performed on the products from the first reaction. The oligonucleotide primers, which were designed to correspond to nucleotide sequence 254–268 (5′-CTCAGAGTGATTCCC-3′) at the 5′-end and 849–835 (5′-GCAGTGGCTGGGTA-3′) at the 3′ end of cDNA rat LHR were used (Fig. 1). The second round of amplification started at 95°C for 3 mins and ran for 35 cycles (1 min at 94°C, 1 min at 55°C, and 2 mins at 72°C). The PCR products (20 μl) were subjected to electrophoresis on 1% agarose gel and photographed under UV light.

Southern Hybridization Analysis. The cDNA fragments generated from RT–nested PCR were resolved in 1% agarose gel and denatured in 1.5 *M* NaCl and 0.5 *M* NaOH, then neutralized in 1.5 *M* NaCl and 1 *M* Tris-HCl (pH 7.2) and transferred to nylon membranes (Hybond; Amersham Biosciences Ltd., Buckinghamshire, UK) by vacuum apparatus and fixed for 5 mins by cross-linking with UV light at 254 nm. The membranes were prehybridized for 2 hrs at 37°C in a solution that contained 5× standard saline citrate (SSC; 1× = 0.15 *M* NaCl and 0.015 *M* sodium citrate [pH 7.0]), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 50% deionized formamid, and 100 mg/l denatured salmon sperm DNA. The hybridization was performed at 30°C overnight in prehybridization solution with the addition of the nested ^{32}P-end-labeled oligonucleotide corresponding to nucleotides 641–660 of LHR cDNA (5′-TGGAGAAGATGCACAGTGGA-3′; Fig. 1). The oligonucleotides were labeled at the 5′ end using [^{32}P]ATP (6000 Ci/mmol; NEN). The blots were washed two times in 5× SSC and 0.1% SDS at room temperature, then exposed to X-ray films (MXG; Eastman Kodak, Rochester, NY) for 1–7 days.

Preparation of Membrane Fractions for Western Blotting. Membrane fractions for immunoblotting were obtained using a procedure described elsewhere (41), with some modifications. Tissues (tumors and ovaries) were placed in freshly made, ice-cold homogenization buffer (50 m*M* Tris-HCl [pH 8.0], 150 m*M* NaCl, 1% Triton X-100, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 20 m*M* phenylmethylsulfonyl fluoride) and homogenized on ice. Homogenates were then centrifuged for 15 mins at 1000 *g* at 4°C. The supernatant was centrifuged for 45 mins at 30,000 *g* at 4°C, and the sediment was suspended in 50 m*M* Tris-HCl buffer (pH 8.0). This suspension was stored at –70°C for further analysis. The protein level was determined using the Bradford method (42).

Western Blotting Analysis. Equal portions of protein (30–40 μg) were dissolved in SDS gel-loading buffer (50 m*M* Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, and 2% β-mercaptoethanol), heated to 95°C for 4 mins, and separated on 10% SDS–polyacrylamide gel electrophoresis (PAGE). Separated proteins were electroblotted onto a 0.45-μm nitrocellulose membrane in transfer buffer (20 m*M* Tris-HCl buffer [pH 8.2], 150 m*M* glycine, and 20% methanol). The nonspecific binding sites were blocked with 5% nonfat dry milk in TBS buffer (50 m*M* Tris-HCl [pH 7.4] and

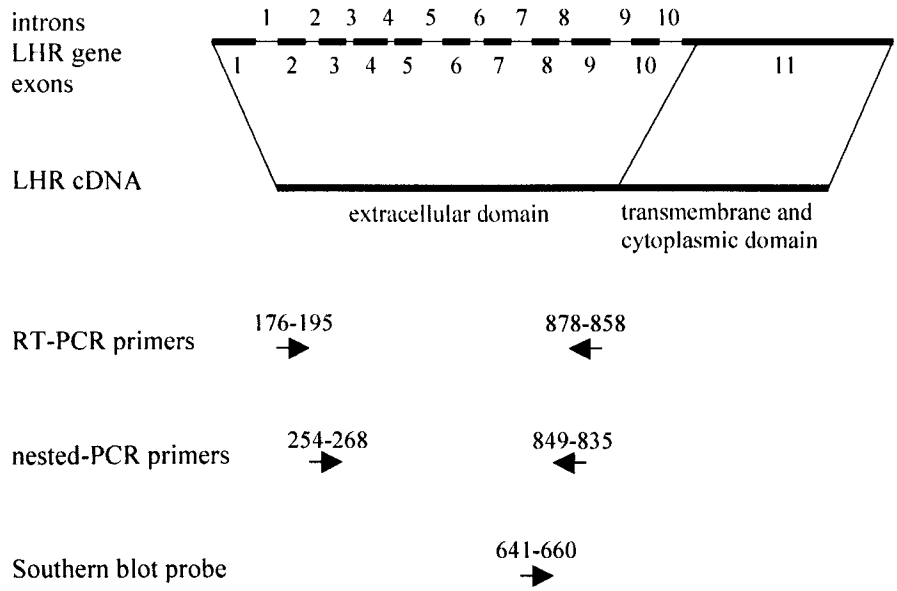

Figure 1. Schematic diagram of the LHR gene, LHR cDNA, and positions of the oligo primers and the oligo probes used. The positions of the primers and the probe in relation to cDNA are above arrows and respond to the scheme of LHR gene and cDNA. Exons 1–10 and the 5′-end of exon 11 encode the extracellular domain of the LHR; the rest of the exon 11 encodes the transmembrane and intracellular domains.

150 m*M* NaCl) that contained 0.1% Tween-20 (TBS-T buffer) overnight at 14°C. After incubation, the nitrocellulose membrane was washed three times with TBS-T, incubated with a 1:1000 dilution of LHR antibodies (anti-LHR 15-48, obtained from Dr. Patrick Roche, Mayo Clinic, Rochester, MN) for 1.5 hrs at room temperature, and washed again with TBS-T. Washed membranes were incubated with a 1:3000 dilution of biotinylated anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA) for 1 hr and washed three times with TBS. To develop color, TBS that contained 0.01% H_2O_2 and 0.04% 3,3′-diaminobenzidine was used. Molecular weights of bands were estimated using the Kodak 1D program (Eastman Kodak).

Statistical Analysis. Statistical analyses were conducted using analysis of variance followed by Tukey's multiple comparison tests in GraphPad Prism 2.0 (GraphPad Software, Inc., San Diego, CA). All data are expressed as the mean ± standard error (SE), and differences were considered to be statistically significant at $P < 0.05$.

Results

Hecate-CGβ Effective in *in vivo* Treatment. Hecate-CGβ and Hecate treatments began when the average tumor volume among the groups varied from 560 to 824 mm^3. The average number of tumors per rat in treatment groups was 1.9 ± 0.2. Eighty-eight percent of the Hecate-CGβ group had detectable tumors at the beginning of the experiment. The number of tumors per rat during treatment in control and Hecate-treated group increased 23%. The time course of tumor growth during treatment is shown in Figure 2. The first injection of Hecate-CGβ and Hecate significantly slowed the growth of mammary tumors ($P < 0.01$ and $P < 0.05$ vs. control, respectively). However, after the third injection, only Hecate-CGβ caused an important decrease of tumor growth, compared with the sham-treated control group ($P < 0.001$). A comparison of tumor volume before and after treatment revealed that the percentage of tumor volume increase was lowest and not statistically significant ($P > 0.05$) in the group treated with Hecate-CGβ (45.3 ± 27.6%) in comparison with Hecate-treated and sham-treated control groups (324.8 ± 78.1% and 309.9 ± 51.2%, respectively). Hecate-CGβ induced a significant decrease in tumor burden (9.5 ± 2.1 mg/g body wt; $P < 0.01$), compared with the sham-treated control animals (21.6 ± 2.9 mg/g body wt). A reduction in tumor burden was also observed in Hecate-treated females (17.6 ± 1.6 mg/g body wt vs. sham-treated controls; $P < 0.05$).

The treatment did not affect the body and organ weights of liver, spleen, adrenals, ovaries, oviducts, and uterus (data not shown). Kidneys weights were increased in the groups treated with Hecate-CGβ and Hecate, compared with the sham-treated control group (6.5 ± 0.1 and 6.5 ± 0.1 vs. 6.1 ± 0.1 mg/g body wt, respectively; $P < 0.05$).

Histopathologic Changes in Tumor Tissue on Treatment. Histological examination of mammary tumors revealed a large diversity of tumor morphology. Three types of tumors were found: adenocarcinoma, fibroadenoma, and adenoma. Mammary tumors showed moderate degrees of differentiation, usually with ductal and lobuloalveolar proliferation, often with evident secretory activity and many cystic structures. Mitotic figures (counted on nine slides in 1 cm^2 of tumor) were common in Hecate-treated and sham-treated control animals (16.3 ± 2.1 and 19.2 ± 2.5, respectively), but tumors in the Hecate-CGβ group showed a lower level of proliferation (8.1 ± 1.2 vs. 16.3 ± 2.1 and 19.2 ± 2.5; $P < 0.05$ and $P < 0.01$, respectively; Fig. 3).

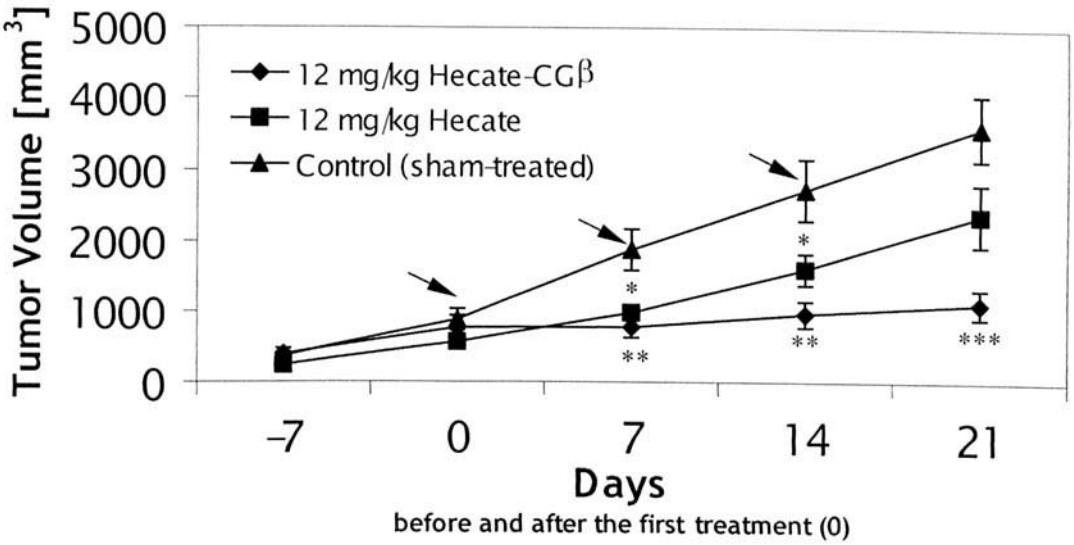

Figure 2. Tumor growth during the treatment with either sham or Hecate-CGβ or Hecate. Animals (10/group) were treated with either sham or Hecate-CGβ or Hecate once a week for 3 weeks by tail vein injections. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control sham-treated animals. Arrow, single injection of vehicle or 12 mg/kg body wt of Hecate or Hecate-CGβ conjugate.

To estimate the possible side effects of Hecate-CGβ and Hecate treatment, we checked ovarian and oviduct morphology by histopathologic analyses. There were no significant differences in the number of atretic follicles in ovaries from the three treatment groups (Table 1). Follicular atresia was seen in three generations of follicles (24.1%–25.8% follicle degeneration). Mainly, well-defined corpora lutea were seen, but the number of them differed among the different treatment groups (Table 1). Treatment with Hecate-CGβ significantly affected the number of corpora lutea in the ovaries (12.1 ± 0.9 vs. 17.1 ± 1.6 for controls; $P < 0.001$). Similarly, the number of corpora lutea was reduced in ovaries obtained from Hecate-treated females, but to a lower degree in sham-treated control animals (13.5 ± 1.2 vs. 17.1 ± 1.6; $P < 0.05$).

Cross-sections of different organs (lungs, pancreas, liver, spleen, adrenals, and uterus) were studied to examine side effects or metastases. Kidneys in 50% of Hecate-CGβ–treated and 75% of Hecate-treated animals showed minor but evenly spread necrosis; in the control group, necrosis was seen in 13% of the kidneys studied (data not shown). However, such abnormalities were also seen in liver sections from all treatment groups. Adrenals in all three treatment groups showed evidence of cortical necrosis, usually in the zona fasiculata or zona reticularis; this ranged in severity from minor focal degeneration to extensive areas of necrosis (data not shown).

Hormone Levels in Treated Groups. Hecate and Hecate-CGβ treatment did not significantly affect estradiol, prolactin, LH, FSH, or TSH plasma levels, compared with the control (sham-treated tumor-bearing animals; Table 2). No significant differences were observed between the Hecate and Hecate-CGβ treatment groups (Table 2).

LHR Expression in the Mammary Gland Tumor Tissues. Because there are no data on the expression of LHR in the DES/DMBA-induced mammary gland rat tumor

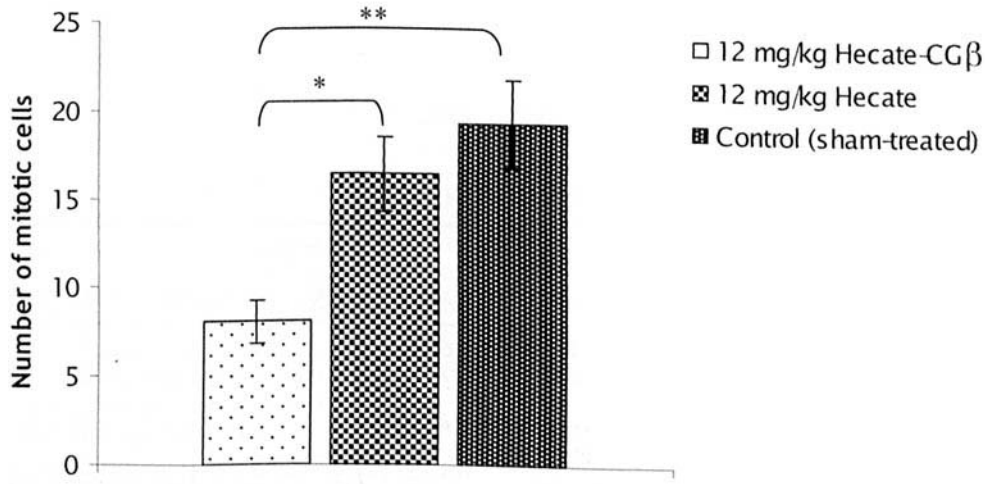

Figure 3. Level of cell proliferation in tumor samples obtained from Hecate-CGβ-, Hecate-, and sham-treated control animals. Mitotic figures were counted on 9 slides in 1 cm^2 of each tumor and are presented as a number of mitotic cells/1,000 nuclei examined. $^{*}P < 0.05$; $^{**}P < 0.01$.

Table 1. Number of Healthy and Atretic Follicles and Corpora Lutea in Both Ovaries from Females in Different Treatment Groups[a]

Treatment group	Corpora lutea	Diameter of follicles[b] <200 μm N	<200 μm A	200–400 μm N	200–400 μm A	>400 μm N	>400 μm A
Hecate-CGβ (12 mg/kg body wt; $n = 10$)	12.1 ± 0.9*	8.2 ± 0.6	0.8 ± 0.5	2.8 ± 0.4	2.4 ± 0.5	1.7 ± 0.2	1.2 ± 0.3
Hecate (12 mg/kg body wt; $n = 10$)	13.5 ± 1.2**	8.1 ± 1.3	0.7 ± 0.1	2.1 ± 0.4	2.2 ± 0.2	1.1 ± 0.2	0.8 ± 0.2
Control (tumor-bearing, sham-treated animals; $n = 10$)	17.1 ± 1.6***	8.2 ± 0.9	0.5 ± 0.2	3.1 ± 0.6	2.2 ± 0.5	1.2 ± 0.3	1.0 ± 0.3

[a] Ovaries were collected 1 week after last treatment with either sham or Hecate-CGβ or Hecate.
[b] A, atretic follicles; N, healthy follicles.
* vs. **$P < 0.001$, ** vs. ***$P < 0.05$.

model, we investigated the presence of LHR transcripts by RT–nested PCR and Southern blotting in these tumors. Possible mRNA fragments for LHR were detected in samples from 5 of 30 females. We amplified an expected 596-bp LHR fragment from few tumors, along with shorter (400 and 250 bp) and longer (900 bp) nucleotide sequences (Fig. 4A). The intensity of the signals obtained was much weaker in comparison to a positive control (Fig. 4C and D), with the exception of the 596-bp fragment (Fig. 4B, lane 2), which, however, was absent after the first-round amplification. Positive reactions with ovarian RNA samples were detected consistently in the first and second round of amplification and showed strong positive hybridization. RT–nested PCR amplification revealed additional 400 and 900 bp fragments in three animals (Fig. 4A; lane 1, 9 and 11) that, however, did not give hybridization signals (Fig. 4B). In contrast to parallel positive reactions, no signal was found in the control sections with kidney RNA, blank-only buffer without template, and in the absence of the reverse transcriptase. The possibility of multiplying genomic DNA was excluded by the fact that each mRNA fragment to be multiplied crossed several large intronic sequences. Contrary to the differences in receptor amplification, all tissues, including kidney, showed a similar amplification of a 315-bp β-actin nucleotide sequence.

Using Western immunoblotting, we further confirmed the expression of LHR protein in samples where mRNA for LHR was detected. The results showed that the tumors contain a 80-kDa receptor protein (Fig. 5), in contrast to positive controls (ovary), where an additional 130-kDa protein was detected. These proteins were not stained in samples where LHR transcripts were absent in RT–nested PCR. A lower intensity of blotting in tumor samples was observed, in comparison to the positive controls.

Discussion

One of the major goals in constructing the Hecate-CGβ conjugate was to reduce its toxic side effects (by the fusion of the CGβ fragment) and to increase the efficiency of Hecate in disrupting cancer cell membranes by delivering this peptide selectively to tumor cells expressing LHR. Numerous recent studies have demonstrated that rat and human breast tissues and breast cancer specimens express LHR (19–21); therefore, we decided to explore whether Hecate-CGβ will abolish, with high efficiency, DES/DMBA-induced mammary gland tumors in the rat. It was previously shown that LH/CGβ-lytic peptide conjugates selectively kill cancer cells through their LHR and that the cytotoxicity of the targeting drug can be increased *in vitro* and *in vivo* by prior treatment with FSH, dihydrotestosterone or 17β-estradiol, all of which are known to up-regulate LHR (28–31). Thus, our intent was to explore the possibility that Hecate-CGβ conjugate would be effective in the ablation of DES/DMBA-induced rat mammary gland tumor in rat.

Table 2. Means of Plasma Hormone Concentrations in Treatment Groups[a]

Treatment group	Hormone levels Estradiol (pg/ml)	Prolactin (ng/ml)	LH (ng/ml)	FSH (ng/ml)	TSH (ng/ml)
Hecate-CGβ (12 mg/kg body wt; $n = 10$)	90.1 ± 11.5	66.5 ± 4.2	0.7 ± 0.1	2.7 ± 0.6	23.4 ± 0.4
Hecate (12 mg/kg body wt; $n = 10$)	110.3 ± 20.7	77.4 ± 6.9	1.0 ± 0.2	2.2 ± 0.3	24.6 ± 1.1
Control (tumor-bearing, sham-treated animals; $n = 10$)	117.0 ± 21.7	83.0 ± 6.8	1.2 ± 0.2	3.2 ± 0.5	22.4 ± 1.0
Control (non–tumor-bearing, untreated animals; $n = 10$)	85.6 ± 12.0	77.9 ± 7.3	0.7 ± 0.2	2.9 ± 0.7	22.1 ± 0.5

[a] Plasma samples were obtained 1 week after last treatment with either sham or Hecate-CGβ or Hecate.

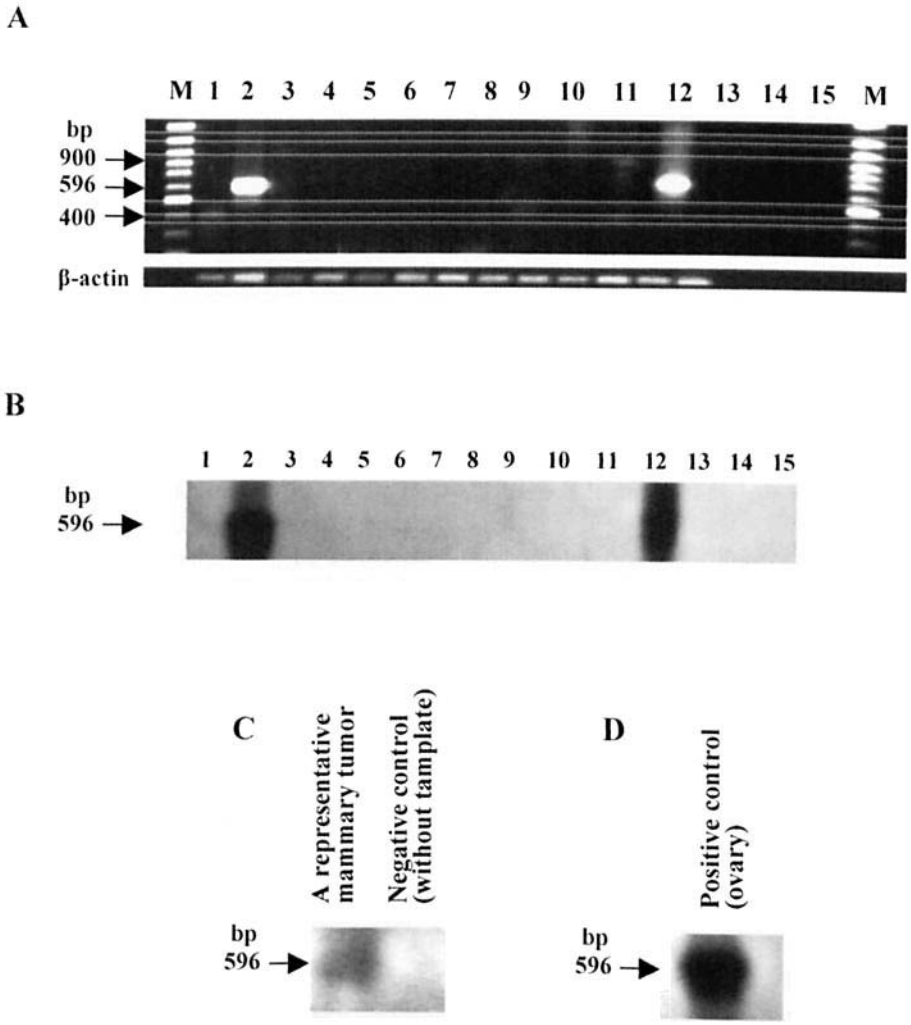

Figure 4. Representative RT–nested PCR (A) and Southern hybridization for LHR (B). A representative Southern blotting for mammary gland tumor with negative control without template (C) and positive control (ovary; D). Lanes 1–11 represent amplification of the LHR fragment from mammary tumors. Different lanes may represent different tumors of the same rat. Lanes 12–14 show control reactions with ovary RNA (12), kidney (13), blank-only buffer without template (14), and in the absence of the reverse transcriptase (15). A molecular-weight marker (M) was used (100-bp DNA Ladder Plus; Fermentas Inc., Hanover, MD). The exposure time was 7 days for the tumor samples (B and C) and overnight for ovary (D). The films were overexposed on purpose, to demonstrate the weak signals. Even 1 month of exposure did not reveal any other signals (data not shown).

Our results showed that Hecate-CGβ, administrated once per week during a period of 3 consecutive weeks, efficiently inhibited the growth of DES/DMBA-induced rat mammary gland tumors, although we could document low levels of LHR in tumors in only 17% of treated rats. Tumor growth inhibition was also observed in the Hecate-treated group, although to a lesser extent. After first and second injections, the tumor growth progression was higher in the Hecate-treated group than in Hecate-CGβ–treated animals. Measurement of the tumor burden further proved that Hecate, like Hecate-CGβ, also inhibited tumor growth, but with lower efficiency. This analysis might be insufficient to complete evaluation of mammary gland tumor growth (43), given that we observed that the tumor proliferation level in Hecate-CGβ-treated animals was lowest, whereas, in animals treated with Hecate, it was similar to that of the control sham-treated group. Nevertheless, this Hecate activity was quite similar to the findings obtained by Gawronska *et al.* (30), who showed also significant cytotoxicity of Hecate, which was also comparable to Hecate-CGβ activity to OVCAR-3 xenografts. Moreover, recent findings showed that the lytic peptides are as much as 50 times more effective in killing cancer cells than nonmalignant cells (44). Additionally, in our experiment, treatment with Hecate-CGβ decreased tumor incidence during treatment from 23% in the Hecate and control groups to 12% in Hecate-CGβ–treated females. Because the treatment efficiency in decreasing the tumor growth was higher in Hecate-CGβ group than in Hecate group, we cannot rule out the possibility of an additional mechanism of action of Hecate-CGβ treatment that made this treatment more effective. Of interest, Hecate-CGβ showed remarkable ability to arrest the growth of DES/DMBA-induced mammary gland tumors in the rat, without any major side effects. However, the partial necrosis of the adrenal glands indicated in our experiment was in line with earlier

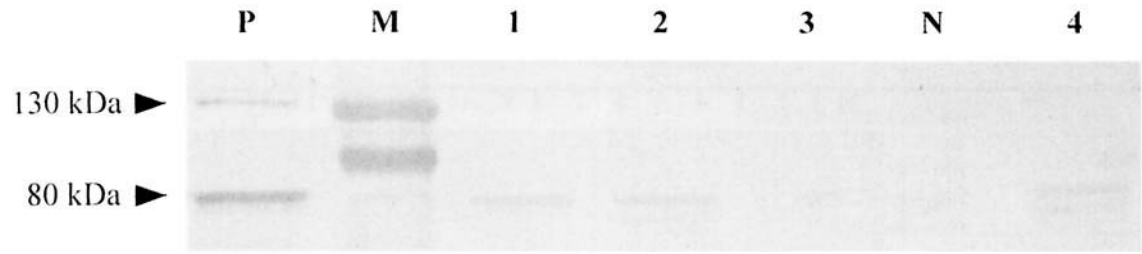

Figure 5. Western immunoblot for LHR in representative DES/DMBA-induced mammary gland tumor. Equal portions of each sample (35 μg protein) were separated on 10% SDS-PAGE. Specific rabbit anti-LHR antibodies and biotinylated anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA) were used for immunodetection. For 1–4 samples, P, positive control (ovary); M, molecular-weight marker (prestained SDS-PAGE Standard Broad Range, Bio-Rad Laboratories; 198, 115, 93, 49.8, 35.8, 29.2, 21.3 and 6.4 kDa); N, negative control (tumor without expression of mRNA for LHR).

observations, where similar abnormalities in adrenals of rats with DES/DMBA-induced mammary gland tumors were observed, possibly because of the transplacental action of these carcinogens (45).

We used RT–nested PCR to amplify a restricted region of the extracellular domain of the LHR. Our results showed, in 17% of females, that DES/DMBA-induced mammary gland tumors express the LHR gene. A similar fragment of mRNA was detected in mammary gland tumor samples and ovary. However, in contrast to the ovary, only weak expression was detected in tumors, and the product could be obtained only after second-round amplification. In addition, tumor tissues contained different forms of LHR transcripts that probably reflect differences in alternative splicing, transcriptional start sites or/and polyadenylation (46). The structure of LHR gene explains the likelihood that alternative splicing could be the reason for the various sizes of LHR mRNAs. The rat LHR gene is long, >60 kb, and contains 11 exons (47). There are 10 introns in the region coding for the extracellular domain of the receptor (47, 48), and this is the area where the majority of the polymorphism is likely to occur. A complicated alternative splicing phenomenon is typical for LHR expression (49–52), where the major mRNA species could be detected in range from 1.2 to 7.7 kb (53). RT-PCR analyses of LHR mRNA always indicate multiple cDNA species (52, 54, 55). Our results emphasized this contention—different mRNA transcripts were identified on RT–nested PCR with the extracellular primers.

Because the results obtained by Meduri *et al.* (19) suggested low or very low expression levels of LHR in patients with breast cancer, we could not rule out the possibility of very low expression of LHR in DES/DMBA-induced mammary gland tumors, which might not be detectable in all cases by RT–nested PCR (56), especially because the overall intensity of signal in tumors detected after amplification was much weaker than in the ovary. By Southern hybridization, we confirmed the expression of LHR only in samples where RNA products were detected by RT–nested PCR. The size of cDNA pieces agreed with those presented earlier after amplification, only with the exception of three cases, 400- and 900-bp cDNA fragments, where no hybridization was observed. Thus, besides the above mentioned mechanisms of LHR expression, we might have to take under consideration the crucial mutations in exon 8 of the LHR gene, which could eliminate bands complementary to the probe. Therefore, more exact and further characterization is necessary to prove the derivation of amplified LHR mRNA species.

Genomes of cancer are unstable, and this instability is manifested by extensive heterogeneity of cancer cells within each tumor (57). Such heterogeneity within human breast cancers during immunocytochemical studies of LHR has been previously reported (19). It has been shown that, in benign breast lesions, the LHR concentration is relatively higher than in breast cancer tissues, which might suggest that the LHR expression is down-regulated along with the tumor progression (19). Other experiments have implied that the loss of LHR mRNA isoforms, and therefore loss of the regulation of the cell-surface expression of mature LHR, may play a role in tumor pathogenesis (58). Our results also might suggest the extensive heterogeneity of the DES/DMBA-induced mammary gland tumors, as LHR expression in healthy rat mammary glands has already demonstrated (20).

Alternative splicing, as well as multiple gene mutations, may lead to crucial changes in protein function, changes in protein location, a deletion of protein activity, a modification of protein activity, novel protein activity, and changes in RNA stability or translational efficiency (59). Therefore, we used Western immunoblotting to reveal protein products of the LHR gene. Despite the presence of multiple receptor transcripts, analyzed tumor samples and ovary contained a single 80-kDa receptor protein. Furthermore, in ovary, besides the band of 80 kDa, a second band of 130 kDa was stained on the protein blot of LHR. The 80-kDa protein has previously been reported for the LHR (60); the 130-kDa protein observed in the ovary might represent a totally unreduced receptor-LH complex (61) or nonspecific binding. These results also correlate with earlier studies on lactating rat mammary glands, in which a 80-kDa LHR protein was identified, but the hormone binding form in mammary gland receptors have been suggested to exist as dimers (20). Similar to receptor transcripts, the amount of receptor protein in tumors was lower than in the ovary, which confirms the marginal expression of protein in DES/DMBA-induced mammary tumors.

Nevertheless, we observed the antiproliferative activity of Hecate-CGβ conjugate and Hecate in this study, but we can only speculate about the direct or indirect mechanisms of action in case of DES/DMBA-induced mammary tumors. A significant reduction in the number of corpora lutea and mitotic cells, and decreasing, but not significant, levels of estradiol, prolactin, and LH in Hecate-CGβ–treated group compared with Hecate-treated and sham-treated control rats might hint at the possibility of systemic effect of the treatment, although a local, ovarian effect cannot be excluded. On the other hand, lytic peptides are known to disrupt preferentially prokaryotic and cancer cell membranes maintaining large membrane potentials rather than healthy eukaryotic membranes (62, 63); thus, this known phenomenon could be involved also in the DES/DMBA-induced mammary tumors growth repression.

Taken together, our *in vivo* findings prove the fundamental hypothesis that Hecate-CGβ conjugate can be highly effective in decreasing the growth of DES/DMBA-induced mammary tumors. Further analysis of the *in vivo* effects of the action of Hecate-CGβ on mammary tumors possessing very low levels of LHR or lacking LHR and the molecular mechanisms underlying this direct or indirect effect of Hecate-CGβ need to be further studied. Although we are unable to show the mechanisms of the action of the Hecate-CGβ conjugate, we are able to establish the principle that the Hecate-CGβ conjugate has the potential for *in vivo* treatment of rat mammary gland tumors.

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