Gonadotropin-releasing hormone receptor–targeted gene therapy of gynecologic cancers

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Abstract

The majority of ovarian, endometrial, and breast cancers express gonadotropin-releasing hormone (GnRH) receptors. Apart from reproductive organs (ovaries, fallopian tubes, and uterus) that are normally removed during surgical therapy of ovarian or endometrial cancer, pituitary gonadotrophs also express GnRH receptors. The signal transduction pathway in tumor cells is basically different from the classic GnRH receptor signal transduction, which is known to operate in the pituitary gonadotrophs and can therefore be considered tumor specific. Other organs and hematopoietic stem cells do not express GnRH receptors. We have recently shown specific activation of nucleus factor κB in ovarian, endometrial, and breast cancers after treatment with GnRH agonists. Based on this tumor-specific signaling pathway and the distribution pattern of GnRH receptors, we have developed and successfully tested a gene therapy concept by using a GnRH analogue as an inducer for the transcription of a therapeutic gene in cell culture and in nude mice. [Mol Cancer Ther 2005;4(2):225–31]

Introduction

More than 80% of ovarian and endometrial cancers and >50% of breast cancers express gonadotropin-releasing hormone receptors (GnRH-R; ref. 1). Apart from pituitary cells and reproductive organs (ovaries, fallopian tubes, and uterus) that are normally removed during surgical therapy of ovarian or endometrial cancer, other organs and hematopoietic stem cells do not express GnRH-R (2). The GnRH-R is therefore a promising target for a tumor-specific gene therapy.

In addition to its unique distribution pattern, the GnRH-R signal transduction pathway in tumor cells is different from the classic pathways via phospholipase C or protein kinase C, known from pituitary cells (1). In tumor cells, GnRH analogues rather interfere with the mitogenic signal transduction pathway of growth factor receptors and related oncogene products associated with tyrosine kinase activity (3). A comparable mechanism has been described in human prostate cancer cells (4). These findings indicate the existence of a tumor-specific signal transduction pathway for GnRH in GnRH-R-positive cancers.

We have recently shown activation of the nucleus factor κB (NFκB) in ovarian, endometrial and breast cancers after treatment with GnRH agonist triptorelin (5). This would make triptorelin an ideal candidate to induce the expression of a therapeutic gene fused to a NFκB-enhancer sequence in GnRH-R-positive tumors. In this study, we have evaluated this novel gene therapy concept based on tissue-specific GnRH-R expression pattern and its signaling pathway characteristic for these tumors.

Gene expression–directed prodrug therapy is a two step therapeutic approach for cancer gene therapy. In the first step, a therapeutic gene is delivered into the tumor and brought to expression. In a second step, a prodrug is given and is selectively activated in transfected cells by the expressed gene product. The first gene-directed enzyme prodrug therapy system described was the thymidine kinase gene of the herpes simplex virus thymidine kinase (HSV-TK) in combination with the prodrug ganciclovir (6). A large number of experiments have been done with this system, in different types of tumors and initial studies in animal models were very promising. This encouraged investigators to move into clinical trials where poor results have been obtained thus far. The current state of preclinical research and the results of the clinical trials as reviewed by Fillat et al. (7) show that in the last 15 years a large effort has been made with numerous different strategies to enhance HSV-TK/ganciclovir efficacy in cellular and in vivo models.

To show the proof of principle of our gene therapy concept, we decided to use HSV-TK/ganciclovir as it is a well known and well established therapeutic system.

Materials and Methods

Animals

Female athymic (nude) mice (CD1 nu/nu), 6 to 8 weeks old on arrival, were obtained from Charles River (Sulzfeld, Germany). The mice were housed in sterile cages in a temperature-controlled room with 12-hour light/12-hour dark schedule and were fed autoclaved chow and water ad libidum. All experiments were done according to the German ethical guidelines and the German laws for protection of animals.
Cell Lines and Culture Conditions

The human endometrial cancer cell lines Ishikawa, HEC-1A, and Hec-1B and the ovarian cancer cell lines EFO-21, OVCAR-3, and SK-OV-3 were obtained from American Type Culture Collection (Manassas, VA) or the sources detailed previously (8). For T3-1, a human GnRH-R-positive mouse gonadotrope cell line, was kindly provided by Dr. P.M. Mellon (Department of Reproductive Medicine, University of California, San Diego, CA). 3T3 is a GnRH-R-negative human fibroblast cell line obtained from American Type Culture Collection. The cell lines were maintained in MEM medium (Life Technologies, Berlin, Germany) supplemented with 2.2 g NaHCO₃/L (Biochrom, Berlin, Germany), 100 units/mL penicillin (Life Technologies), 100 μg/mL streptomycin (Life Technologies), 10% (v/v) FCS (Life Technologies), and 40 IU insulin/L (Hoechst, Frankfurt, Germany) at 37°C in humidified 5% CO₂. αT3-1 was maintained in RPMI 1640 (Life Technologies).

Plasmids and Transfection

pNF-B-SEAP (Clontech, Palo Alto, CA) is designed to monitor the activation of NF-B and NF-B-mediated signal transduction pathways using the reporter gene secreted alkaline phosphatase (SEAP). pNF-B-SEAP contains four tandem copies of the κB4 enhancer fused to the HSV-TK promoter (Fig. 1). pTK-SEAP (Clontech) was used as a negative control to determine the background signals associated with the culture medium. The enhancerless pTK-SEAP contains HSV-TK promoter upstream of the SEAP coding sequence.

pNF-B-LacZ was constructed in our laboratory by insertion of LacZ gene (β-galactosidase) from pIND-LacZ (Invitrogen BV, Groningen, the Netherlands) into HindIII-XbaI site of pNF-B-SEAP after removal of the SEAP gene (Fig. 1A). pNF-B-TK was constructed in our laboratory by insertion of HSV-TK promoter into pNGVL1-TK (National Gene Vector Laboratory, University of Michigan, Ann Arbor, MI) into HindIII-XbaI site of pNF-B-SEAP after removal of the SEAP gene. pNF-B-TK contains four tandem copies of the κB4 enhancer fused to the HSV-TK promoter followed by the HSV-TK gene as therapeutic gene (Fig. 1A).

Transfections were done with Superfect liposome reagents (Qiagen, Hilden, Germany) according to manufacturer’s instructions.

GnRH Agonist Triptorelin-Induced NF-B Activation

Cells were grown to ~50% confluence on 30-mm plates. Transfections with pNF-B-SEAP (see above) were done using the Superfect liposome reagents and following the manufacturer’s instructions (Qiagen) and cells were cultured for 96 hours in the absence of FCS and phenol red with or without 100 nmol/L GnRH agonist triptorelin. Every 24 hours, 100 μL of the media were taken and analyzed for SEAP activity. Chemiluminescence detection of SEAP activity was done according the manufacturers instructions (Clontech) using a plate fluorometer (Berthold, Bad Wildbad, Germany).

GnRH Agonist Triptorelin-Induced NF-B Activation In vivo

Tumors were initiated by i.p. injection of 1 x 10⁷ cancer cells with Matrigel (Collaborative Biomedical Products, Bedford, MA). After 3 to 4 weeks, all animals had developed solid tumors of about 115 mm³ (HEC-1B) or 80 mm³ (OVCAR-3) and the treatment was initiated. Each group was composed of 10 animals. The in vivo experiments were done as follows: 50 μL pNF-B-LacZ (0.5 μg/μL) plus Superfect reagent (50 μL) were injected i.p. Half of each group were injected i.v. with triptorelin (300 nmol/20 g). After 24 hours, the mice were sacrificed. Tumor tissues were collected for β-galactosidase staining. All organs were removed, cleaned, and also stained for β-galactosidase activity. Stainings for β-galactosidase activity were done according to manufacturer’s (Promega, Madison, WI).
instructions for in situ staining of cells growing as monolayer. Tumor tissue and organs were washed with PBS and kept in fresh 0.25% glutaraldehyde (Sigma, St. Louis, MO)/PBS solution for 30 minutes before they were washed with PBS and stained over night at 37°C in staining solution. This solution contains in 30 mL PBS: 8 mg spermidine (Sigma), 60 μL of 1 mol/L MgCl₂, 2% NP40, 300 μL of 1% potassium deoxycholate, 150 μL of 0.5 mol/L K₃Fe(CN)₆, 3H₂O, 150 μL of 0.5 mol/L K₃Fe(CN)₆ (Sigma), and 15 mg 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Stratagene, La Jolla, CA).

GnRH Agonist Triptorelin-Induced Activation of HSV-TK In vitro

Cells were grown to ~70% confluence on six-well plates and transfected with pNF-B-TK (see above) or for control with pNGVL1-TK (see above) which contains a constitutively active HSV-TK. Transfections were done using the Superfect liposome reagents and following the manufacturer’s instructions (Qiagen). After 24 hours, the cells were treated with or without GnRH agonist triptorelin (100 nmol/L) followed by a treatment with or without ganciclovir (15 μmol/L). After 72 hours, viable cells were stained.

GnRH Agonist Triptorelin-Induced Activation of HSV-TK In vivo

Tumors were initiated by i.p. or s.c. injection of 1 × 10⁷ cancer cells with Matrigel (Collaborative Biomedical Products). After 3 to 4 weeks all animals had developed solid tumors of about 115 mm³ (HEC-1B) or 80 mm³ (OVCAR-3) and the treatment was initiated. Each group was composed of 10 animals. The in vivo experiments were done as follows: 50 μL pNF-B-TK (0.5 μg/μL) plus Superfect reagent (50 μL) were injected either i.p. or directly into the s.c. tumors. Transfection was repeated every fourth day. Half of each group were injected i.v. with triptorelin (15 nmol/g every fourth day). The pNF-B-TK-transfected mice were treated with ganciclovir (20 μg/g every fourth day). Tumor volumes were measured every 2 days. The mice were killed after 20 days.

Statistical Analysis

All data are expressed as the mean ± SE. The data from the time course experiments in nude mice were tested for significant differences by one-way ANOVA followed by Student-Newman-Keuls test for comparison of individual groups, after a Bartlett test had shown that variances were homogenous. Differences were considered statistically significant at a P < 0.05.

Results

Basic Mechanisms of the GnRH-R-Targeted Gene Therapy

A GnRH agonist (e.g., triptorelin) induces activation of NF-B in GnRH-R-positive cancer cells (Fig. 1B). Activated NF-B now couples to the κB DNA binding sites of the pNF-B-TK plasmid and induces expression of the therapeutic enzyme HSV-TK. Through this the prodrug ganciclovir is activated.

GnRH Agonist Triptorelin-Induced NF-B Activation In vitro

To examine GnRH agonist triptorelin-induced activation of NF-B, we transiently transfected GnRH-R-positive EFO-21 ovarian cancer cells with a reporter vector containing a κB cis-acting DNA sequence (response element) and the SEAP reporter gene or the pTK-SEAP vector as a negative control. SEAP activity was detected using a chemiluminescence assay. During culture of the transfected EFO-21 GnRH agonist triptorelin treatment resulted in a 5.4-fold (Fig. 2A) increase of NF-B-induced SEAP expression (P < 0.001). To show that our gene therapy concept based on signal transduction pathway of GnRH is specific for GnRH-R-positive tumors, we had to show that pNF-B-activation is observed neither in GnRH-R-negative cells nor in GnRH-R-positive nonmalignant cells, GnRH-R-negative SK-OV-3 ovarian cancer cells
were transfected with pNFβ-B-SEAP and treated with or without triptorelin. The SEAP-activity remained unchanged at a low level after triptorelin treatment (Fig. 2B). In pNFβ-B-SEAP transfected GnRH-R-negative 3T3 fibroblast cells NFβB activity remained unchanged at a low level after triptorelin treatment (Fig. 2C). The same experiment was done with the αT3-1 gonadotrope cells which express GnRH-R. In αT3-1 cells, NFβB-induced SEAP expression was not affected by triptorelin (Fig. 2D). These data indicate that GnRH agonist triptorelin-induced NFβB activation is restricted to cancer cells and requires the presence of GnRH-R (Fig. 2A).

**GnRH Agonist Triptorelin-Induced NFβB Activation In vitro**

To examine GnRH agonist triptorelin-induced activation of NFβB in vitro, mice bearing endometrial or ovarian tumors i.p. or s.c. were transiently transfected with a vector containing a kB4 response element and the LacZ reporter gene. Triptorelin-induced NFβB activity within the tumor is shown as β-galactosidase activity (Fig. 3). NFβB was induced by triptorelin in the tumor only (Fig. 4A). Apart from the reproductive organs ovary (Fig. 4B) and uterus (Fig. 4C) that are normally removed during surgical therapy, other organs (Fig. 4D–K) did not show triptorelin-induced NFβB activity in vivo. The blue staining on stomach (Fig. 4H) are tumor cells grown on the surface of the organ, not the organ itself.

**GnRH Agonist Triptorelin-Induced Activation of HSV-TK In vivo**

To examine GnRH agonist triptorelin-induced activation of NFβB and specific killing of GnRH-R-positive tumor cells in vivo, we used the therapeutic HSV-TK gene under the control of four copies of the kB enhancer fused to the HSV-TK promoter. Using HSV-TK as therapeutic gene, ganciclovir as prodrug, and GnRH agonist triptorelin as inductor we could show the specific killing of NFβB-TK transfected GnRH-R-positive cancer cells in vivo (Fig. 5, right). Without triptorelin treatment, the NFβB-TK transfected cells could not be killed by ganciclovir (Fig. 5, middle). Using a vector with a constitutively active HSV-TK gene (pNGVL1-TK) incubation with ganciclovir resulted in cell death with or without triptorelin treatment (Fig. 5, left). On GnRH-R-negative cells, not shown here, no killing effects were observed. Because of the different GnRH receptor signal transduction, no toxic effects were observed in GnRH-R-positive pituitary cells.

**GnRH Agonist Triptorelin-Induced Activation of HSV-TK In vivo**

To show the proof-of-principle of our gene therapy concept in vivo, mice bearing endometrial or ovarian tumors i.p. or s.c. were transiently transfected with NFβB-TK (Fig. 1) and treated with GnRH agonist triptorelin and the prodrug ganciclovir. Transfection and treatment were repeated every fourth day. Every second day, the tumor volume (s.c. tumors) or development of ascitis indicated as increase of body weight (i.p. tumors) was measured. The increase of the tumor volume of the mice receiving gene therapy with s.c. endometrial or ovarian cancer was significantly lower ($P < 0.05$) than with the control animals (Fig. 6B). Development of ascitis indicated as increase of body weight of the mice receiving gene therapy with i.p. endometrial or ovarian cancer was significantly lower ($P < 0.01$) than that of control animals (Fig. 6A). Toxic side effects were not observed. To further exclude side effects, a long-time experiment using two animals without tumor was conducted. The mice were transiently transfected with NFβB-TK and treated with GnRH agonist triptorelin and the prodrug ganciclovir for 8 weeks. No effects were observed.

If the tumor growth had exceeded the limitations according the ethical guidelines the mouse had to be killed (t). One mouse in the gene therapy group bearing a s.c. endometrial tumor died 20 days after the beginning of the treatment (t).

**Discussion**

In this study, we show the proof of principle of a targeted HSV-TK suicide gene therapy which uses both tissue

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**Figure 3.** Transcription factor NFβB can be activated via GnRH-R and up-regulate target genes. Using the reporter gene LacZ, the concept of the gene therapy via GnRH-R-dependent tumor-specific signal transduction was successfully tested in nude mice bearing Hec-1A endometrial tumors (i.p.). Transfection with pNFβ-B-LacZ (i.p.). Treatment with GnRH agonist triptorelin (i.v.). X-Gal staining after tumor replacement. Experiments using OVCAR-3 human ovarian tumor cells gave identical results. Representative of three separate experiments. All experimental groups consisted of five animals.
specific expression of GnRH-R and tumor-specific signal transduction of this receptor.

TK is an enzyme that specifically functions in the salvage of thymidine for DNA synthesis. Although human TK is present in normal cells, the viral form can selectively activate antiviral agents such as ganciclovir which is thereafter toxic for mammalian cells. Selective expression of viral TK in cancer cells could permit effective treatment with ganciclovir. Application of drug sensitization with the HSV-TK/ganciclovir system in solid tumors was pioneered by Mootlen et al. and has been used in clinical trials of different tumors including cancers of the uterus, the ovary, and the breast (9–12). Although the mechanisms involved in tumor regression remain to be fully elucidated, it is likely that HSV-TK gene transfer to tumor cells results in the phosphorylation of ganciclovir to toxic nucleotide precursors, which in turn disrupt DNA synthesis in cells expressing the transgene, as well as not transfected neighboring tumor cells through cell-to-cell transfer of toxic metabolites (bystander effect; ref. 13). One of the unique features of the tumor destruction in the HSV-TK system is the observation that not all the tumor cells must contain the inserted gene to be killed by ganciclovir (9). Ural et al. (14) found that in situations in which only 10% of the cells were transfected, tumor cell proliferation decreased 70% compared with the control. This bystander effect is important in the gene therapy, because none of the thus far available gene transfer techniques is able to transfer a therapeutic construct into every single cell of a tumor. The bystander effect has been reported in gynecologic cancers as well (15–19). Recent studies from Kunishige et al. using lipofectin-mediated gene transfer of HSV-TK to a human cervical adenocarcinoma cell line and a human endometrial adenocarcinoma cell line showed sensitivity to ganciclovir and presented evidence of the bystander effect in both cell lines in vitro and in vivo (17). These investigators observed tumor reduction when mixtures of HSV-TK-negative cells with >20% HSV-TK-positive cells were injected into SCID mice (17).

In the setting of ovarian cancer, the HSV-TK has been used for gene therapy. HSV-TK-based gene therapy protocols have relied on the fact that 90% of ovarian cancers are confined to the peritoneum, even at relapse. This closed compartment permits uncomplicated i.p. delivery of concentrated liposomal and viral vectors for effective transduction of plasmids containing HSV-TK. Efficacy of this approach is further magnified by the bystander effect (20). Incorporation of the viral enzyme by 10% of tumor cells yields a 70% cell kill in some studies with xenografts, obviating the need to target 100% of the

Figure 4. Due to the tissue specific expression and signaling of GnRH-R, GnRH agonist triptorelin-induced activation of NFκB is limited to the reproductive tissues and their carcinomas. Hec-1A endometrium carcinoma grown i.p. (A), ovary (B), uterus (C), brain (D), lung (E), heart (F), kidney (G), stomach (H), intestine (I), liver (J). Transfection with pNFκ-B-LacZ (i.p.) with (left) or without (right) treatment with GnRH agonist triptorelin (i.v.). X-Gal staining after tumor/organ replacement. Experiments using OVCAR-3 human ovarian tumor cells gave identical results. Representative of three separate experiments. All experimental groups consisted of five animals.

Figure 5. Selective killing of GnRH-R-positive EFO-21 ovarian cancer cells in vitro. Using the gene therapy vector pNFκ-B-TK containing HSV-TK as therapeutic gene under the control of four copies of the xB enhancer fused to the HSV-TK promoter, the prodrug ganciclovir and GnRH agonist triptorelin as inductor the specific killing of GnRH-R-positive cancer cells could be shown (right). Without triptorelin treatment, the cells could not be killed by ganciclovir (middle). Using a vector with a constitutive active HSV-TK (pNGVL1-TK), treatment with ganciclovir resulted in cell death with or without triptorelin treatment (left). Representative of three separate experiments. Experiments using GnRH-R-positive endometrial cancer cell lines Ishikawa, HEC-1A, and Hec-1B and GnRH-R-positive ovarian cancer cell line OVCAR-3 gave identical results.
tumor cells (20). Several trials have been completed (21–23). In phase I reports, 38% of patients with recurrent disease have experienced stabilization of tumor growth (23). Toxicities have been tolerable. However, if such a gene therapy could be made tumor specific, this would increase effectiveness and reduce side effects. In our study, we could show that GnRH agonist-induced activation of NFκB via NFκB enhancer sequences was restricted to GnRH-R-positive human cancer cell lines in vitro. Using the reporter gene LacZ, the concept of gene therapy via GnRH-R-dependent tumor-specific signal transduction pathway was successfully tested in nude mice bearing xenografts of human ovarian or endometrial cancers. Only the GnRH-R-positive tumors as well as the reproductive organs that are normally removed during surgical therapy showed GnRH-induced activation of NFκB. All other organs did not show any activation of NFκB. Using HSV-TK as therapeutic gene, ganciclovir as prodrug and GnRH agonist triptorelin as inducer we could show specific killing of GnRH-R-positive cancer cells in vitro. No effects were observed on GnRH-R-negative cells. Because of the facts that the GnRH signal transduction in pituitary gonadotrophs is different to that acting in reproductive tumors and in breast cancer cells (1) and in addition that GnRH do not induce NFκB activation in pituitary gonadotrophs, the therapeutic gene could not be activated in pituitary gonadotrophs and therefore no toxic effects could as well be observed in the GnRH-R-positive pituitary gonadotrophs in vitro. To further exclude side effects in vivo a long time experiment (8 weeks) using two animals without tumor was conducted. No effects were observed.

The proof-of-principle could be shown in vivo in nude mice bearing endometrial or ovarian cancers s.c. and i.p. The tumor volume of the mice receiving gene therapy was significantly lower as compared with control animals. Toxic side effects were not observed.

Until now, for practical reasons, we have only used liposomal transfections, which might be adequate for i.p. tumors, such as ovarian cancer, where tumor spread limited to the peritoneal surface is common. Therefore, our nude mouse model with i.p. xenografts of human ovarian cancers was adequate for the evaluation of our gene therapy concept for ovarian cancer. If successfully optimized, this strategy could be directly taken to clinical development for the treatment of ovarian cancer. Because modern liposomal techniques are not toxic and have nearly the same efficacy as viral systems, the use of a nonviral method should be preferred in the therapy of ovarian cancer. Using i.p. xenografts of human endometrial cancer cells, this model was used to test the principal applicability of our approach for the gene therapy of this cancer. As this tumor mainly form metastases outside the peritoneal cavity, the development of a viral transfection strategy will be necessary.

In summary, the proof-of-principle of the GnRH-R-targeted gene therapy could be shown in vitro and in vivo on tumor-bearing nude mice. The tumor volume in mice receiving gene therapy was significantly lower compared with control animals. Toxic side effects were not observed.

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References


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