A therapeutic model for advanced endometrial cancer: Systemic progestin in combination with local adenoviral-mediated progesterone receptor expression

Donghai Dai,1 Lina Albitar,1 Tan Nguyen,1 Laura L. Laidler,1 Meenakshi Singh,2 and Kimberly K. Leslie1

1Reproductive Molecular Biology Laboratory, Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico and 2Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado

Abstract
Cancer of the uterine endometrium is a frequent gynecologic malignant disease for which few therapeutic options are available for advanced disease. Progesterone is the normal female hormone that limits growth and proliferation of endometrial cancers; however, progesterone receptors are frequently down-regulated, leading to treatment failures. The current studies explored the effectiveness of adenoviral-mediated progesterone receptor gene transduction in combination with progestin therapy in mouse xenograft models. Pretreatment of cells with progesterone receptor–encoding adenovirus and progestin inhibited the development of s.c. tumors in athymic mice. In the i.p. xenograft model, replacement of both isoforms of progesterone receptor, PRA and PRB, in combination with progesterone treatment resulted in a significant 2.6-fold increase in overall survival time compared with control animals. These studies indicate that when progesterone receptor levels are maintained, progestin therapy is effective in limiting tumor growth. Future therapeutic regimens targeted at enhancing progesterone receptor expression have the potential to improve outcomes in women with endometrial cancer. [Mol Cancer Ther 2005;4(1):169–75]

Introduction
Carcinoma of the uterine endometrium is the fourth most common malignant disease in women and was responsible for the deaths of 6,800 American women in 2003 (1). Mortality is related to advanced disease, for which effective therapies are lacking; the median survival among patients with metastatic or recurrent endometrial cancer is < 1 year (2). Chemotherapeutic agents may achieve a transient response rate of 40% to 60% (2). A Gynecologic Oncology Group trial reports a slight improvement in median survival with a triple-agent regimen (3), but the long-term survival benefit is still limited. Likewise, the hormonal therapies employed to date have been similarly unsuccessful in producing lasting growth inhibitory effects, with published Gynecologic Oncology Group response rates of ~10% to ~33% (4–8). Yet again, the clinical benefit is only transient.

Growth of the uterine endometrium is controlled by estrogen and progesterone. Endometrial carcinogenesis is related to estrogen overexposure that is not modulated by the differentiating effects of progesterone (9), and the role of progesterone in the glandular epithelium of the endometrium is primarily to induce cellular differentiation and to antagonize estrogen-mediated cell proliferation (10). The biological functions of progesterone are mediated through progesterone receptors, which function as ligand-responsive transcription factors in the nucleus (11). It has been shown that progesterone, through progesterone receptor, activates the transcription of many genes (12–15). Signal transduction of progesterone may become more complex through its cross-talk with other signaling pathways, such as growth factors (16) and cytokines (17), and through direct interactions with protein kinases (18). Two isoforms of progesterone receptor, PRA and PRB, are expressed. Whereas each isoform controls a unique cadre of genes (12–15), they function together to enhance endometrial differentiation (19, 20). In humans, it is thought that the loss of the coordinated expression of the progesterone receptor isoforms, leading to abnormal ratios of PRA to PRB, may be an early event in endometrial carcinogenesis (21). This could indicate that expression of both receptors is important in the prevention of endometrial cancer as well as in the successful implementation of progestin therapy.

Expression of progesterone receptor has been positively correlated with response to progestin treatment and a good prognosis (22). The overall response rate has been reported to be 72% in patients with progesterone receptor–rich tumors but only 12% in patients with progesterone
receptor–poor lesions (23). Unfortunately, progestin treatment leads to depletion of progesterone receptor within the target tissue (24). Thus, persistent expression of functional PRA and PRB is likely to be required for successful progestin treatment.

It has been reported previously that introduction of progesterone receptor genes through adenoviral constructs results in the expression of progesterone receptor and restoration of progestin sensitivity in endometrial cancer cells grown in vitro (25, 26). Furthermore, cancer cell proliferation and invasion are inhibited, and apoptosis and differentiation are induced. These in vitro studies suggest that reestablishment of progesterone signaling could be an effective strategy to control endometrial cancer growth even for progestin-resistant tumors. These initial studies have now been expanded to include the athymic mouse xenograft model. We show that systemic progestin treatment in combination with adenovirus-mediated progesterone receptor gene expression significantly inhibits tumor growth and prolongs survival.

Materials and Methods

Cells and Reagents

A poorly differentiated human endometrial cancer cell line, Hec50co, has been characterized and maintained in our laboratory (25–27). Construction of adenovirus carrying progesterone receptor genes was described previously (25). Medroxyprogesterone acetate (MPA) was purchased from Pharmacia & Upjohn (Bridgewater, NJ). Six- to eight-week-old athymic Crl:NU/NU-nuBR mice were purchased from Charles River Laboratories (Wilmington, MA). The experimental protocol was approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Development of Subcutaneous Endometrial Tumors in Athymic Mice and Intratumoral Administration of Adenovirus

Hec50co cells were grown in cell culture medium (DMEM) with 10% fetal bovine serum until subconfluence and then harvested by trypsinization. Cells (3 × 10⁶) in 100 µL DMEM were injected s.c. into the flanks of athymic mice. The tumors were measured weekly, and tumor cross-sectional areas (mm²) were calculated by using the following formula: length (mm) × width (mm) × π / 4. Fifty microliters of adenovirus carrying PRB genes (AdPRB), 5 × 10¹⁰ green fluorescent units per milliliter, were injected directly into the tumors when diameter of the largest tumor reaches 10 mm.

Development of Intrapерitoneal Endometrial Cancer in Athymic Mice

Hec50co cells were grown to subconfluence and harvested with trypsin and EDTA. Cells (10 × 10⁶) in 300 µL medium were injected into the mouse peritoneal cavity under general anesthesia. Twenty mice were used in this study with a minimum of three mice sacrificed at 1, 3, 5, 7, 21, and 28 days after transplantation. The peritoneal cavity was opened and examined macroscopically. Peritoneal connective tissues, including omentum and mesentry, were collected and fixed in 10% phosphate-buffered formalin (Fisher Scientific, Fairlawn, NJ). These tissues were subsequently processed and embedded in paraffin blocks that were cut into 5 µm sections and stained with H&E.

Treatment of Peritoneal Endometrial Cancer

Three days after inoculation of human endometrial cancer cells into the mouse peritoneal cavity, 2 × 10⁶ green fluorescent units of adenovirus in 500 µL DMEM or vehicle alone was injected into the peritoneal cavity. One milligram of MPA or saline was injected i.m. weekly. Mice were euthanized when they were judged terminally ill according to criteria developed in advance in the animal use protocol. Decisions to euthanize animals were made by an employee of the animal care facility who was blinded to the treatment regimen under investigation.

Immunocytochemistry Staining for Progesterone Receptor and Ki-67 Expression in the Tumor Tissues

Progesterone Receptor Immunocytochemistry Staining.

The tissue was cut into 5 µm sections, mounted on slides, and baked at 60°C for 1 hour. After cooling, the slides were deparaffinized in xylene (3 × 5 minutes), 100% ethanol (2 × 5 minutes), 70% ethanol (1 × 5 minutes), and deionized water (2–5 minutes). Antigen retrieval was then done with the use of a decloaking chamber in which the slides were immersed in DAKO 1 × Target Retrieval Solution (DAKO, Carpinteria, CA) and boiled for 5 minutes at 120°C (20–25 p.s.i.). The slides were cooled in the buffer for 10 to 20 minutes and rinsed in three changes of deionized water. The slides were then loaded into the DAKO Cytomation autostainer after programming the following reagents and incubation times: buffer rinse (DAKO 1 × Wash Buffer); Dual Endogenous Enzyme Block (ready-to-use) for 5 minutes [DAKO Envision+ Dual Link (ready-to-use)]; buffer rinse [DAKO progesterone receptor (PgR 636) primary antibody, 1:600 in DAKO antibody diluent for 30 minutes; DAKO Universal Mouse Negative Control; buffer rinse; EDL Labeled Polymer (ready-to-use) for 30 minutes [DAKO Envision+ Dual Link]; buffer rinse; 3,3′-diaminobenzidine for 10 minutes (DAKO DAB+); water rinse; counterstained with hematoxylin for 5 minutes (DAKO Automation Hematoxylin); buffer rinse. The slides were dehydrated with 95% ethanol (2 × 1 minute), 100% ethanol (2 × 1 minute), and xylene (3 × 20 dips). Richard Allen mounting medium was applied and the slides were coverslipped.

Ki-67 Immunocytochemistry Staining.

The tissue was cut into 4 to 5 µm sections, mounted on slides, and baked at 60°C for 1 hour. After cooling, the slides were deparaffinized in xylene (3 × 5 minutes) followed sequentially by dips in 100% ethanol (2 × 5 minutes), 95% ethanol (2 × 5 minutes), 70% ethanol (1 × 5 minutes), and deionized water (2–5 minutes). Antigen retrieval was done with the use of a decloaking chamber in which the slides were immersed in DAKO 1 × Target Retrieval Solution and boiled for 5 minutes at 120°C (20–25 p.s.i.). The slides were cooled in the buffer for 10 to 20 minutes.
and rinsed in three changes of deionized water. The slides were loaded into an immunohistochemistry autostainer (DAKO Cytomation) after programming the following reagents and incubation times: buffer rinse (DAKO 1 × Wash Buffer); Dual Enzyme-Endogenous Enzyme Block (ready-to-use) for 5 minutes [DAKO Envision+ Dual Link (ready-to-use)]; DAKO Ki-67 primary antibody, 1:800 in DAKO antibody diluent for 30 minutes; DAKO Universal Mouse Negative Control; buffer rinse; EDL Labeled Polymer (ready-to-use) for 30 minutes, from the DAKO Envision+ Dual Link; buffer rinse; 3,3′-diaminobenzidine for 10 minutes (DAKO DAB+); water rinse; counterstained with hematoxylin for 5 minutes (DAKO Automation Hematoxylin); buffer rinse. The slides were dehydrated with 95% ethanol (2 × 1 minutes), 100% ethanol (2 × 1 minutes), and xylene (3 × 20 dips). Richard Allen mounting medium was applied and the slides were coverslipped.

Statistical Analysis. When comparing two groups, unpaired t tests were used. When comparing three groups, one-way ANOVA was used.

Results

Development of Subcutaneous Endometrial Tumors and the Effect of Intratumoral Injection of Adenovirus in Mouse Xenografts

Subcutaneous endometrial tumors were created through injection of Hec50co cells into the flank subcutis. Changes in size were documented weekly as cross-sectional areas. Among the 10 mice receiving cancer cell inoculations, 9 of them developed visible tumors. The take rate was 90%. The average diameter of the tumors was 9.5 ± 3 mm in 3 weeks and 17 ± 2.7 mm in 5 weeks, respectively (Fig. 1). At 5 weeks, some animals with tumors having a diameter of >20 mm had to be euthanized, and further study was terminated because of size limits set by the animal protocol. The s.c. tumors were very solid with a well-delineated boundary. About 10% of tumors showed necrosis and internal bleeding within the tumor mass, particularly in tumors >15 mm. Tissue sections stained with H&E indicated that the tumors had circumscribed edges. Cancer cells formed sheets without glandular (endometrioid) or papillary serous morphology. They maintained a high-grade, poorly differentiated morphology similar to their progenitor cells.

To study the effect of progesterone on tumor development in vivo, Hec50co cells were infected separately with control virus (AdCon) or AdPRB with a multiplicity of infection of 10 and treated with 100 nmol/L progesterone for 24 hours before transplantation into athymic mice. Four mice that received Hec50co cells infected with AdCon developed s.c. tumors at a rate comparable with the growth curve described above. However, four mice injected with Hec50co cells infected with adenovirus carrying the PRB gene and treated with progesterone developed no visible tumors in 5 weeks. Thus, tumorigenicity was inhibited by progesterone in vivo when cells expressed PRB at the time of initial transplantation.

We next studied progesterone effects on established tumors using the same s.c. model. When the s.c. tumors grew to 10 mm in diameter, mice were simultaneously given AdPRB through intratumoral injection, and 1 mg MPA was given i.m. This study was prematurely terminated because in several cases tumor growth was greatly enhanced simply by the needle injection. Because tumors generated by Hec50co cells are solid and very firm, the viral solution apparently failed to stay inside the tumor, while at the same time the needle tract site became a route for further spread.

Development of Peritoneal Endometrial Cancer in Mice

A total of 20 mice were used to study the development of i.p. tumors after injection of endometrial cancer cells. By the end of the experiment, the presence of tumors or cancer cells in the i.p. cavity was confirmed in 19 of 20 mice (95% take rate). One day after i.p. injection of 10 × 10⁶ Hec50co cells, some of the cancer cells were able to attach to the peritoneal surface (Fig. 2A). However, many of the cells injected into the i.p. cavity were nonviable 24 hours after inoculation. Under microscopy, such cells exhibited disintegrated nuclei and extensive necrosis (Fig. 2A, arrows). It seems that only a small proportion of injected cells within close proximity to the serosa survive. These cells show large nuclei with prominent nucleoli and a limited amount of cytosol, resulting in an increased nuclear-to-cytoplasmic ratio (Fig. 2A, arrowheads). Other cells with decreased volume and clumped, pyknotic, and karyorrhectic nuclei were indicative of cells undergoing necrosis. Three days after grafting, tumor masses of limited size were formed around the serosa, consisting mainly of proliferating cancer cells (Fig. 2B). Focal areas of papillary subdifferentiation were now visible. Five days after grafting, a larger tumor mass was present with a more pronounced papillary appearance and clearly visible blood vessels (Fig. 2C). Seven days after grafting, the tumors were well formed and had the classic appearance of endometrial cancer of the serous papillary type (Fig. 2D). As illustrated in the figure, these tumor papillae were present on the surface of the peritoneum and displayed well-developed fibrovascular cores lined by pleomorphic tumor cells with classic hobnail nuclei and prominent nucleoli.

Three weeks after transplantation, multiple grossly visible tumors were present inside the peritoneal cavity, mostly located on the surface of the omentum, mesentery, and pelvic peritoneum (Fig. 2E). Tumor sizes ranged from 1 to 5 mm in diameter. At 4 weeks, watery ascites were commonly present when tumors reached the size of ≥10 mm in diameter (Fig. 2F). At the time of euthanasia, usually 4 to 6 weeks after inoculation in the control mice, the abdomen was usually distended and contained bloody ascitic fluid. Tumor explants were present throughout the peritoneal cavity and covered most organs. It is apparent that peritoneal tumors generated from poorly differentiated Hec50co endometrial cancer cells, used in these experiments, grow more aggressively than those formed by Ishikawa cells, as reported by others (28).
Progestin Treatment or Progesterone Receptor Expression Alone

Progestin treatment or progesterone receptor expression alone does not prolong the median survival of mice carrying peritoneal endometrial cancer. Eighteen mice were injected i.p. with $10^6$ Hec50co cells in 300 μL DMEM. They were divided into three groups of six mice each. The first group received DMEM i.p. and saline i.m. weekly. The second group received AdCon i.p. and MPA i.m. weekly. The third group was given AdPRB i.p. and saline i.m. Their median survival times were 6±1.1, 6.1±1.1, and 6.1±1.7 weeks, respectively (Fig. 3). There were no significant differences in tumor growth rates or survival among these three groups, indicating that progestin treatment in the absence of progesterone receptor or progesterone receptor expression alone in the absence of hormone therapy do not improve mouse survival.

Systemic MPA Treatment in Combination with Local Adenovirus-Mediated PRB Expression

Systemic MPA treatment in combination with local adenovirus-mediated PRB expression prolongs the survival of mice with peritoneal endometrial cancer. To investigate the effect of the combination of PRB gene transduction and administration of progestin as therapy for peritoneal endometrial cancer, mice were inoculated i.p. with $10^6$ Hec50co cells in 300 μL DMEM 3 days before treatment. The control group, which consisted of eight mice, received AdCon i.p. and 1 mg MPA i.m. Fifteen mice in the treatment group were given AdPRB and MPA. Treatment continued weekly until the animals became terminally ill and had to be euthanized. Their median survival times were 6±1.3 weeks for control animals and 9.4±2 weeks for animals receiving AdPRB and MPA (Fig. 4). A statistically significant difference in survival rates for the two groups ($P<0.01$, Student’s $t$ test) indicates that progestin inhibits tumor progression and prolongs animal survival in the presence of PRB.

MPA Treatment in the Presence of Both PRA and PRB

MPA treatment prolongs the survival of mice with peritoneal endometrial cancer in the presence of both PRA and PRB. In this study, we tested the hypothesis that expression of both PRA and PRB would prolong survival and provide additional benefit over PRB alone. Seven mice were injected with a mixture of equal amounts of adenovirus carrying PRA genes (AdPRA) and PRB (1×10^9 green fluorescent units each) in 500 μL DMEM and treated with 1 mg MPA i.m. The control group of four mice received only AdCon and MPA. The animals receiving MPA plus AdPRA and AdPRB had a median survival of 13.7±1.8 weeks, whereas the control animals (AdCon plus MPA) had a median survival time of 5.1±0.6 weeks (Fig. 5). The difference between these groups was significant ($P<0.01$, Student’s $t$ test). Comparing Figs. 4 and 5, it is apparent that the presence of both PRA and PRB provides a substantial benefit to animal survival compared with PRB alone, and it is likely that the expression of both progesterone receptor isoforms significantly enhances the effectiveness of progestin therapy.

Immunocytochemical Analysis

Immunocytochemical analysis documents the induction of progesterone receptor expression in the tumor cells transduced with adenovirus-encoding progesterone receptor and the reduced proliferation rate of these cells with the use of the marker Ki-67. Like many poorly differentiated endometrial tumors, Hec50co cells fail to express...
endogenous progesterone receptor (25, 29). Similarly, the tumors generated from Hec50co cells in mice xenografts are progesterone receptor negative as shown in Fig. 2G. However, when animals were treated with a mixed adenovirus containing PRA and PRB constructs, progesterone receptor expression was identified in some malignant cells at the periphery of the tumors (Fig. 2H). However, despite the successful transduction of some cells, others remained progesterone receptor negative. Ki-67 is a marker for active cell proliferation. With the use of this antigen, it was estimated that 80% of the tumor cells from control mice compared with 50% of the tumor cells from mice treated with progesterone receptor and progestin were actively proliferating.

**Discussion**

Endometrial cancer is the most common gynecologic malignant disease and is generally considered to have a favorable prognosis. The majority of the nearly 40,000 cases every year are treated successfully with surgery. However, in cases where surgery fails to cure, other treatment modalities are generally not effective. Chemotherapy and radiation are options for advanced disease, and hormonal therapy with progestin is another possibility. Because of its minimal side effects, progestin is often used to treat patients with metastatic or recurrent endometrial cancer (30). The efficacy of progestin treatment is positively correlated to receptor status and is more effective in tumors that express progesterone receptor. However, even in those favorable cases, tumors eventually develop progestin resistance because of the loss of progesterone receptor when progestin treatment is given in a continuous manner (31). From a molecular standpoint, it is well known that progesterone down-regulates its own receptor by a mechanism that targets progesterone receptor for destruction in the proteasome (32). We and others have shown that poorly differentiated endometrial cancers lose progesterone receptor expression (22–24, 29). Hec50co cells themselves, from which the in vivo tumors arise in this model, are representative of this receptor-depleted form of advanced endometrial cancer. As such, they are excellent models to study the effects of reintroducing progesterone receptor expression. Interestingly, these data indicate that, even in tumors in which progesterone receptor have been down-regulated, reestablishing progesterone receptor expression and signaling can have therapeutic benefit. Newer therapeutic trials, such as Gynecologic Oncology Group Study 119 and 153, have shown that addition of tamoxifen, acting as an estrogen surrogate to induce progesterone receptor, in combination with MPA provides a better response rate than progestin alone, but there is no substantial benefit in progression-free survival and overall survival (7, 8). Treatment failures are likely due to the growth of tumor cell subpopulations that are progesterone receptor negative (33). Thus, maintenance of progesterone receptor expression is a critical challenge in developing an effective progestin treatment protocol.

We have studied the in vitro and in vivo effects of reintroducing progesterone receptor into endometrial tumors that have lost it. Previous work with cell models clearly shows that progestin therapy in the presence of high levels of progesterone receptor strongly inhibits
tumorigenesis (25, 26). The present studies now expand these findings and show that the principle of progesterone receptor reintroduction into tumor cells as a therapeutic avenue also applies to the mouse xenograft model.

Using the s.c. tumor model, we show that expression of progesterone receptor through gene transduction in combination with progesterone treatment before implantation into mice strongly inhibits tumor development. We also developed an i.p. tumor mouse model that more clearly recapitulates the spread of advanced endometrial cancer. Endometrial cancer is generally curable when the tumor is limited to the uterine corpus. However, with tumor extension into the peritoneal cavity, as occurs in 16% of cases, the prognosis is very poor (30). Among these, more than half of the cases show disease that is still limited to the pelvis with involvement of the pelvic peritoneum and adnexa (30). These patients are excellent candidates for treatment regimens aimed at the peritoneal cavity.

Using the i.p. model and a gene therapy approach whereby progesterone receptor is introduced via adenoviral infection into the peritoneal cavity, our data show that animal survival is prolonged significantly with progesterone receptor and MPA treatment. The best results are obtained when both progesterone receptor isoforms A and B are introduced together (compare Fig. 4 with Fig. 5). These data confirm the findings of Arnett-Mansfield et al. (21), who showed that endometrial carcinogenesis is related to loss of one or both progesterone receptor isoforms or an abnormal ratio of PRA to PRB. These data indicate that progesterone receptor expression in endometrial tumors can be obtained by a gene therapy approach. When a sufficient number of tumor cells are transduced, progestin treatment is effective in prolonging survival. The high expression of progesterone receptor in transduced tumor cells is driven by the viral vector promoter and seems to overcome the effects of ligand-induced down-regulation of progesterone receptor, which limits the effectiveness of progestin treatment in tumors expressing lower levels of receptors. The depth of adenovirus infection is a major limitation for gene therapy in endometrial cancer treatment. As we indicate, this therapy is an adjuvant method targeting scattered cancer cells, which could be used after a successful debulking surgery. However, in the work presented here, penetration of adenovirus is reasonably good. We detected progesterone receptor gene expression distant from the tumor surface (Fig. 2). Others have reported that adenovirus can reach the center of a 0.5 cm³ tumor (34). Penetration depends on the tissue type, and investigations have shown that 90% of hepatocytes within the liver express the transgene after adenoviral infection (35). However, in the same study, only 10% to 20% of cells within lung tissue expressed transgene. Because we have shown a clear growth and survival advantage in this animal study, we propose that a gene therapy strategy could be promising for scattered residual cancer cells or implants in the peritoneal cavity. We further propose that the effectiveness of progestin therapy for endometrial cancer can be significantly enhanced by the use of multiple strategies, including the future use of progesterone receptor gene therapy and the choice of a hormonal regimen (such as an estrogen surrogate in combination with sequential progestin) that maintains maximal progesterone receptor expression and activity.

References
Molecular Cancer Therapeutics

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