Strategies to Accomplish Targeted Expression of Transgenes in Ovarian Cancer for Molecular Therapeutic Applications


ABSTRACT

Purpose: The purpose of the study was to determine the capability of the midkine (MK) and cyclooxygenase-2 (cox-2) gene promoter regions to function as tumor-specific promoters for use in targeted gene therapy of ovarian cancer. Experimental Design: Established and primary ovarian cancer and mesothelial cells were transduced by adenoviral vectors containing a reporter or thymidine kinase gene expressed under the control of the MK, cox-2, or cytomegalovirus (CMV) promoters. SCID or C57BL/6 mice were injected i.p. with these same vectors. In vitro reporter gene expression and cellular cytotoxicity was determined using luciferase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, respectively. Acute toxicity in vivo was assessed by histological evaluation of harvested tissues. Results: Consistent activation of the MK and cox-2 promoters was noted in all of the ovarian cancer cell lines in addition to primary ovarian cancer cells. In contrast, reduced reporter activity was reported in mesothelial cells transduced with adenoviruses containing the test promoters, which was especially apparent for the cox-2 promoter. Additionally, the cox-2 promoter exhibited significantly lower reporter gene levels in liver and peritoneum than the control promoter in in vivo experiments. Tumor-cell killing induced by Adcox-2 MTK was comparable to that observed with AdCMVTK. However, a clear differential toxicity pattern was observed in favor of animals treated with Adcox-2 MTK when compared with controls. Conclusions: These data clearly demonstrate that the transcriptional control afforded by the cox-2 promoter is tumor-specific and is able to mitigate associated toxicity in normal tissue while maintaining therapeutic efficacy in the context of an ovarian cancer molecular chemotherapeutic approach.

INTRODUCTION

A variety of anticancer molecular therapies are based upon selective transgene expression in target tumor cells. In this regard, molecular chemotherapy is predicated upon tumor-specific expression of toxin genes (1). In addition, viral agents may be designed to replicate selectively within the tumor cells whereby direct target cell oncolysis is achieved (2). In both of these contexts, the therapeutic index realized is a direct function of the effective exploitation of tumor-selective patterns of gene expression. One method to achieve this tumor selectivity is via the strategy of transcriptional targeting. In this approach, transcriptional regulatory units corresponding to tumor-specific or organ-specific genes are used for controlling expression of the heterologous transgene. For this purpose, a variety of such specific promoters (TSPs) have been used to restrict toxin genes such as HSV-TK (3-6) and cytosine deaminase (7-10). Indeed, an active human trial currently uses this strategy as a means to restrict toxin gene expression to osseous metastasis in carcinoma of the prostate (11). In addition, a subset of conditionally replicative adenoviral agents use TSPs as a mean to achieve tumor selective replication (2, 12, 13). In this strategy, the relevant TSP provides the basis of positively induced expression of key adenovirus early genes required for replication. The foregoing considerations clearly establish that the availability of selective promoters is mandated for the development of promising anticancer molecular therapeutics.

3 The abbreviations used are: TSP, tumor-specific promoter; TK, thymidine kinase; HSV-TK, herpes simplex TK; MK, midkine; cox-2, cyclooxygenase-2; ATCC, American Type Culture Collection; CMV, cytomegalovirus; pfu; plaque forming unit(s); EGF, epidermal growth factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GCV, ganciclovir.

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For cancer of the ovary, direct routing of therapeutic agents via the peritoneal cavity has mandated unique selectivity considerations. Specifically, the propensity for liver localization of i.p. delivered agents (14), with potential attendant hepatotoxicity (15–18), requires TSPs with noninducibility in the context of the liver, in addition to the requirement for positive inducibility in tumor targets (19). In addition, an expression profile predicating noninducibility within normal mesothelial cells would represent an additional useful capacity.

To this end, a variety of TSPs have been studied for utility in the context of molecular therapeutic approaches for ovarian cancer (20–25). Whereas limited application data have been provided in these studies, rigorous analysis of the functional candidate TSPs for ovarian cancer has not been endeavored. Previous studies (6, 26) from our laboratory using the promoter regions of the MK and cox-2 genes have shown that both genes exhibit very high tumor:liver expression ratios making both of these promoters very attractive candidates for future in vivo gene therapy applications. Therefore, we explored the utility of the MK and cox-2 promoters in an ovarian cancer gene therapy context. Our studies clearly demonstrate that these two promoters exhibit key capacities consistent with their exploitation for targeted molecular therapeutics in carcinoma of the ovary.

**MATERIALS AND METHODS**

**Tissue Culture and Cell Lines.** The human ovarian carcinoma cell lines SKOV3.ip1 (a gift from Dr. Janet Price, M. D. Anderson Cancer Center, Houston, TX), OV-4 (generously provided by Dr. Timothy J. Eberlein, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA), and SW626 (HTB-78; ATCC, Manassas, VA) were grown in DMEM:Ham’s F-12, 50/50 mix. The human ovarian carcinoma cell line OVCAR-3 (HB-1947; ATCC) and gastric cancer cell line KATO-3 (JCRB, JCRB0611; Japanese Collection of Research Bioresources, Tokyo, Japan) were cultured in RPMI 1640. The PA-1 ovarian teratocarcinoma cell line (CRL-1572; ATCC) and gastric cancer cell line TAG-72 (28), was added to the cell suspension at a concentration of 10^7 cells, followed by incubation at 4°C for 1 h. The immunocomplexes formed by the antibody and the cells were recovered from the beads by brief trypsinization and cultured with RPMI complete medium. Cytological analysis confirmed that between 70 and 85% of the cells were ovarian carcinoma cells, depending on the patient sample (data not shown).

Mesothelial samples were obtained by the enzymatic disaggregation of peritoneal strips collected from female patients with no underlying malignant conditions undergoing intrabdominal surgery (29).

**Recombinant Adenoviruses.** Replication incompetent adenovirus vectors expressing firefly luciferase and HSV-TK, respectively, were constructed through homologous recombination in *Escherichia coli* using the AdEasy system (30). All of the vectors used in these experiments contained transgene cassettes placed in the E1-deleted region of an adenoviral vector backbone. Luciferase-expressing adenoviral vectors, Adcox2MLuc and Adcox2LLuc (26), were modified to carry the cox-2 promoter (−883/+59) (middle length) and the cox-2 promoter (−1432/+59) (large length) promoters (derived from the phPES2; a gift from Drs. Hiroyasu Inoue and Tadashi Tanabe, National Cardiovascular Center Research Institute, Japan; Refs. 31, 32), respectively, and AdMKLuc with midkine promoter (−2285/+27; Ref. 33) was also used. In separate experiments, both an HSV-TK expression vector, Adcox-2 MTK, containing the cox-2 promoter, and Ad-MKTK, containing the MK promoter (6), were used. AdMKLuc was used as a ubiquitous expression vector for luciferase and HSV-TK, AdCMVLuc and AdCMVTK, in which the expression of transgene was conferred by a CMV immediate early promoter, were used. Lastly, an *Escherichia coli* β-galactosidase expression vector AdCMV LacZ (generously provided by Albert B. Deisseroth, Yale University School of Medicine, New Haven, CT) was used. As a control for the in vivo luciferase expression assays, an adenovirus encoding the green fluorescent protein driven by the CMV promoter (AdCMVGFP; a
gift from Corey Goldman, Cleveland Clinic Foundation, Cleveland, OH) was used.

The viruses were propagated in the adenovirus-packaging cell line, 293, and purified by double CsCl density gradient centrifugation, followed by dialysis against PBS with 10% glycerol. The vectors were titrated by plaque assay and stored at −80°C until use.

In Vitro Analysis of Gene Expression. Twelve h after plating (5 × 10^4 cells/well in a 24-well plate), SKOV3.ip1, OVCAR-3, OV-4, PA-1, SW626, and purified, primary human ovarian cancer cells from six patients were infected with AdCMVLuc, AdMKLuc, Adcox2MLuc, Adcox2LLuc, or mock infected (control). Cells (5 × 10^4)/well were seeded in 24-well plates in triplicate for each virus and infected at 50 pfu/cell. A luciferase radioluminometric assay was performed after 48 h, and the readings (RLU) were normalized to the amount of protein as determined by a BCA assay. The experiment was performed in triplicate, and the mean percentage of expression of each promoter in relation to CMV is shown for every cell line.

Analysis of Gene Expression in Vivo. To evaluate the reporter gene expression with each promoter, 10^9 pfu of AdCMVLuc, AdMKLuc, Adcox2MLuc, Adcox2LLuc, or AdCMVGFP diluted to 1 ml with PBS or PBS alone were i.p. administrated to mice (three mice/group). After 72 h, the animals were sacrificed, and the peritoneal and retroperitoneal cavities (including the liver and kidney) were frozen and prepared for further study. Each sample (1 mg), having undergone three cycles of freezing and thawing in Cell Culture Lysis Buffer (Promega), was centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4°C. The supernatant was recovered and analyzed for luciferase activity and protein concentration. The same protocols were used in the in vitro assays.

In Vitro Analysis of the Cytocidal Effect of HSV-TK Expression Vectors in Combination with GCV. SKOV3.ip1 human ovarian carcinoma cells were seeded onto 96-well plates at a density of 10^4 cells/well. After 2 h of infection with AdCMVTk or Adcox2MTk at 1, 10, or 100 pfu/cell (and an additional 24 h of incubation with complete medium), 1, 10, or 100 μM of GCV was added. Five days later, the number of the surviving cells was analyzed by the MTS method using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega) and an automated E max spectrophotometric plate.
reader (Molecular Device Corp., Sunnyvale, CA) as described by the manufacturers. All of the experiments were carried out in triplicate.

The Analysis of in Vivo Toxicity. To analyze the toxicity after i.p. administration of the HSV-TK expression vector in combination with GCV, C57BL/6 mice received i.p. administrations of AdCMVTK or Adcox2MTK. The administration was performed at three different doses ($5 \times 10^8$, $5 \times 10^9$, and $5 \times 10^{10}$ pfu/mouse), with and without subsequent ganciclovir treatment. Control groups receiving only ganciclovir or PBS were also examined. The administration of ganciclovir was initiated 48 h after viral administration at 50 mg/kg, twice a day, for 7 days. Clinical data were recorded everyday for weight, activity, hair loss, and other major findings. On day 8, mice were euthanized and underwent necropsies. Key organs and tissues were fixed with 10% buffered formalin, paraffin-embedded, and sections were analyzed histopathologically following routine staining with H&E.

RESULTS
Activity of Promoters in Established and Primary Patient-derived Ovarian Cancer Cells. Initially, the activities of the MK and cox-2 promoters in an adenoviral context were analyzed in five established ovarian cancer cell lines using luciferase expression vectors. As a control of ubiquitous expression, an expression vector with the CMV promoter driving luciferase gene expression was used. The results are represented as the mean percentage of expression of each promoter relative to CMV promoter activity. In this analysis, the activity of the MK promoter was approximately 60% of the activity of the CMV promoter in the PA-1 cell line (Fig. 1). The MK promoter was moderately active in the other four cell lines but reduced by about 4-fold from that seen with the PA-1 cell line. The cox-2 M promoter was strongest in the SW626 cell line, at approximately 45% of the activity of the CMV promoter in the PA-1 cell line (Fig. 1). The MK promoter was moderately active in the other four cell lines but reduced by about 4-fold from that seen with the PA-1 cell line. The cox-2 M promoter activity decreased by about half in the SKOV3.ip1 and PA-1 cells lines when compared with the activity in SW626 cells. An even further decrease in activity was seen using the cox-2 M promoter in both the OV4 and OVCAR 3 cells. The cox-2 L promoter had measurable but weak activity in all of the five cell lines tested with the highest activity being in the SKOV3.ip1 cell line (5% of CMV). These results confirm that both the MK and cox-2 M promoters are active in many of the established ovarian cancer cell lines.

To further clarify the character of the promoters in a system with more direct relevance to human tumors, primary human...
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in the majority of primary human tumor cells.

Activity of Promoters in Primary Mesothelial Cells.
To analyze the therapeutic effect of these TSPs in the cells lining the peritoneum, the MK, cox-2 M, and cox-2 L promoters were analyzed in primary mesothelial cells. As shown in Fig. 3, all of the three promoters were relatively weak in comparison with the CMV promoter. The MK promoter was the most active with 2.5 to 7 times lower activity than the CMV promoter, however. On the contrary, both the cox-2 M and cox-2 L promoters showed extremely low activity in primary mesothelial cells in all of the three patients examined. This suggests that an advantageous promoter activity differential exists between normal mesothelial cells and malignant ovarian cells, which should have therapeutic potential.

In Vivo Promoter Activity in the Liver after i.p. Vector Delivery. The most used delivery route for therapeutic agents in ovarian cancer is i.p. administration. In animal experiments, a lethal adverse effect of suicide gene therapy was reported to be attributable to hepatic toxicity (15, 17). Thus, to analyze adverse effects using these TSPs, we analyzed the expression of the luciferase transgene with the candidate promoters in the peritoneum and the liver. 72 h after i.p. administration of the vectors. In the peritoneum (Fig. 4A), the transgene expression with the cox-2 M promoter was almost as low as that of negative controls (PBS or AdCMVGFP, approximately 20-fold lower than CMV). Luciferase expression as a result of MK and cox-2 L promoters was similarly low but approximately 2–4-fold higher than that of the cox-2 M promoter. In the liver (Fig. 4B), the same trend of MK promoter activity was observed; however, a much more profound decrease in activity was observed for the cox-2 M and cox-2 L promoters (over 300-fold lower activity than CMV). Taken together, with regard to the low transgene expression in the peritoneum and the liver in vivo, the cox-2 M promoter appears to be very promising as a transcriptional targeting motif in human ovarian carcinoma, which is capable of mitigating adverse affects.

Cytocidal Effect of the Cox-2 M Promoter-driven HSV-TK Expression in Ovarian Cancer Cell Lines. To verify that the cox-2 M promoter has strong enough activity to kill ovarian cancer cells, the cytocidal effect of the cox-2 M promoter driving HSV-TK expression, in combination with GCV, was analyzed in SKOV3.ip1 cells and compared with the CMV promoter-driven vector. Fig. 5 shows the data with fixed infectious units and variable doses of GCV. In this experiment, the cox-2 M promoter-driven vector has a significantly stronger cytocidal effect over the CMV-driven vector. These results indicate that the cox-2 M promoter is able to kill tumor cells using this suicide gene therapy strategy.

Mitigation of Peritoneal and Hepatic Inflammation in Adenoviral HSV-TK/GCV Suicide Gene Therapy Using the Cox-2 Promoter. As the cox-2 M promoter demonstrated a beneficial expression profile in the context of low activity in the peritoneum and liver but high enough activity to kill ovarian cancer cells in vitro, we examined the possibility of this promoter to mitigate peritonitis and hepatitis after TK/GCV gene therapy. Therefore, after i.p. administration of each vector and GCV treatment, the peritoneum and liver were extensively analyzed pathologically. In the liver, the group treated with AdCMVTK and GCV (Fig. 6A) showed increased lobular inflammatory activity indicating active hepatitis. Increased apoptosis was evident as measured by the presence of Councilman bodies. The inflammatory activity was diffuse but appeared to be greater in zone 3 (the area surrounding the central vein) as opposed to the periportal zone. Lymphoblast-like activated lymphocytes were also noted there. In the liver of the group with Adcox2MTK and GCV treatments (Fig. 6B), the histopathological findings indicated hepatitis was minimal or absent. Only rare collec-
tions of inflammatory cells in the parenchyma were noted that were equivalent to those seen in untreated control animals. In the peritoneum, the group treated with AdCMVTK and GCV treatments (Fig. 6C) showed evident peritonitis as characterized by a mixed inflammatory cell infiltrate sprinkled over the mesothelial-lined surface. In places, the mesothelium showed reactive changes to the inflammatory cell insult, and inflammatory cells could be seen in the submesothelial space. In contrast, in the group with Adcox2MTK and GCV treatments, no inflammatory response in the peritoneal lining was observed (Fig. 6D). These findings clearly indicate that the cox-2 promoter significantly mitigated hepatitis and peritonitis when compared with that seen after the treatment with conventional AdCMVTK and GCV.

DISCUSSION

A major theoretical rationale for endeavoring molecular therapeutics of cancer is to achieve an improved therapeutic index. In adenoviral vector-based strategies, which represent one of the most widely used vector systems because of its high transduction efficiency in a variety of cells, targeted expression of the transgene has been studied both transductionally and transcriptionally. Transductional targeting is accomplished by modification of the native viral tropism (34). Transcriptional targeting, which is the focus of this study, is achieved by the utilization of TSPs to direct tumor-specific transgene expression. A number of promoters have been analyzed in gene therapy studies for transcriptional control in ovarian cancer. In an adenoviral vector context, the human H9251 folate receptor gene promoter, P1, has been shown to direct specific reporter gene expression of ovarian cancer cell lines (22). The MUC1 promoter has been used to drive expression of the proapoptotic bax gene that specifically kills ovarian cancer cell lines in a murine ovarian cancer model (23). In addition, the L-plastin gene promoter restricts reporter gene expression to ovarian cancer cells while sparing the surrounding mesothelium in vivo (24). Despite the apparent specificity of these vectors, analysis of the liver toxicity has not been addressed, which is of utmost importance for the human realization of these vectors. Therefore, the focus of this study was to use transcriptional targeting via three promoters (MK, cox-2 M, and cox-2 L) that have been reported previously (6, 26) to be beneficial for the mitigation of transgene expression in the liver, especially with regard to therapeutic index after i.p. Ad vector administration.

In our analysis using established cell lines, the activity of the MK and cox-2 M promoters in an Ad construct were relatively high, although weaker than the CMV promoter, which is known to be one of the strongest promoters. To further clarify their function in a system that closely recapitulates the in vivo situation, experiments were done using these promoters with...
primary ovarian cancer cells from six patients. The results were consistent, in all but one patient, with the results obtained in established cell lines. This is an important fact considering that often the information derived from cell lines has been known to over-represent efficacy when compared with in vivo patient experiments. Furthermore, these six patients suffered from a number of different ovarian carcinoma morphological subtypes, yet the promoter activities are similar, making this gene therapy approach very attractive for future clinical use.

Key to a successful therapeutic index is the importance of minimal effects on surrounding non-neoplastic tissue, peritoneal lining and liver cells being the most important foci. When the promoter activities were analyzed in human primary mesothelial cells, both cox-2 M and cox-2 L promoters showed almost baseline activity, whereas the MK promoter demonstrated activity. The activities of these promoters were next analyzed in vivo. In the mesothelial cells of the peritoneal lining, the cox-2 M promoter showed almost no activity, whereas MK and cox-2 L promoters were slightly more active. In the liver, the cox-2 M promoter showed the same low activity, but the activity of the MK and cox-2 L promoters increased to unacceptable levels. Taken together, the cox-2 M promoter, which showed high activity in ovarian cancer cells and the lowest activity in peritoneum and liver, is the most promising candidate promoter for ovarian cancer gene therapy clinical trials.

When the cytocidal effect of the cox-2 M promoter driving HSV-TK expression was analyzed in a HSV-TK GCV gene therapy context, a strong cytocidal effect was observed that was comparable with the CMV promoter-driven vector in SKOV3.ip1 cells. Others (35, 36) have reported that changing the promoter from CMV to Rous sarcoma virus did not weaken the cytocidal effect of the HSV-TK expression vector as the reduction maintains the activity in a range above the threshold. Thus, this might explain the strong efficacy of Adcox-2 M TK noted despite the weaker activity of this promoter as compared with the CMV promoter.

In the context of an application of this promising promoter in patient-based trials, we tested the toxicity of cox-2 M promoter-driven TK expression (Ad cox-2 M TK) in combination with GCV treatment in mice. In contrast to the CMV promoter-driven activity, which showed concomitant extensive peritonitis and hepatitis after GCV administration, Adcox-2 M TK did not show significant peritonitis or hepato-
titis. These morphological findings indicate that the activity profile of the cox-2 M promoter is targeted to an extent that nullifies undesired adverse events. The mitigation of in vivo toxicity with adenoviral gene therapy has been reported previously by ourselves and others (6, 17, 26). However, all of these analyses have been done after i.v. injection aimed at blocking toxicity after systemic adenoviral gene therapy. The expression profile after i.p. injection is reported to be different from that observed via i.v. systemic administration (14). In summary, our results clearly indicate that cox-2 M promoter-based transcriptional targeting of adenovirus can be used for tumor-specific transgene delivery while successfully mitigating adverse effects after i.p. administration. Thus, this system may contribute to the development of safer adenoviral gene therapy with toxin- or converting enzyme-expressing vectors for use in an ovarian cancer gene therapy in the near future.

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