ACQUIRED DISEASES

RESEARCH ARTICLE

Intravesical liposome-mediated interleukin-2 gene therapy in orthotopic murine bladder cancer model

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Using a novel orthotopic MBT-2 murine bladder tumor model, we evaluated the feasibility of intravesical gene therapy utilizing a cationic liposome, DMRIE/DOPE. Superficial bladder tumors were consistently established by intravesical instillation of 5×10^5 MBT-2 cells in syngeneic C3H female mice. In situ gene transfer to bladder tumors was accomplished via intravesical instillation of plasmid DNA/DMRIE/DOPE lipoplex. β -Galactosidase (β -gal) gene expression was preferentially evident in bladder tumors and was present for at least 7 days after a single 30 min in situ transfection. Murine interleukin-2 (IL-2) gene was used for treatment of 3-day-old pre-established bladder tumors. Forty percent of animals treated with IL-2 gene were completely

free of tumors by 60 days following the initial tumor implantation, while all control groups treated with β -gal gene died. Those animals initially cured of pre-established tumors were completely resistant to a subsequent tumor re-challenge and their splenocyte-derived cytotoxic T lymphocytes were shown to be specific to MBT-2 cells, indicating that immunological memory against MBT-2 tumors was elicited by the treatment. These results demonstrate the possibility of an effective clinical application of this in situ intravesical IL-2 gene delivery system to high-risk superficial bladder tumors, obviating a need for tumor procurement and ex vivo gene transfer. Gene Therapy (2000) 7, 844–851.

Keywords: bladder cancer; gene therapy; interleukin-2; intravesical; liposome

Introduction

Cancer immunotherapy continues to be a promising approach for the treatment of various cancers. Recently, a growing number of studies have shown that introduction of various cytokine genes such as IL-2,^{1,2} IL-4,³ IL-12,⁴ interferon- γ ⁵ or granulocyte-macrophage colony-stimulating factor (GM-CSF)⁶ into tumor cells can stimulate antitumor immune responses and in some cases, lead to the suppression of pre-established tumors in animal studies. Based on these studies, several clinical trials involving cytokine gene-modified, growth-inactivated, autologous tumor cells, so-called tumor vaccines, have already been initiated. Bladder cancer, known to respond to immunotherapeutic treatment with *Bacillus Calmette-Guérin* (BCG), is a potential target of this cytokine gene therapy strategy. Currently, intravesical BCG therapy is the major treatment of choice for recurrent high grade superficial bladder cancer.⁷ The mechanism of this BCG treatment, which is still not completely understood, is believed to act as an adjuvant and stimulates induction of antitumor cellular immune responses.⁸ However, this immunity is mostly a local event and does not induce a long-lasting systemic immunological memory against bladder cancer, leading to the later progression of the dis-

ease.⁹ In looking toward the development of effective tumor vaccines for the treatment of bladder cancer, we have assessed the ability of cytokine gene-modified bladder tumor cells to induce a state of immunity in animals against parental unmodified tumor cells. We have demonstrated that up to 50% of animals could be cured of pre-established bladder tumors by immunization with irradiated IL-2 gene-modified tumor cell preparations using retrovirus as the transfecting agent, which led to an induction of tumor-specific cytotoxic T lymphocytes (CTL).¹⁰ Moreover, animals cured of pre-established tumors were resistant to a subsequent tumor challenge, indicating tumor-specific immunological memory persisted in these cured animals. Recently, we have shown the effective use of cationic liposome-mediated plasmid DNA transfection instead of retroviral vector systems to modify not only established bladder cancer cell lines but also primary cultures of human bladder carcinoma.¹¹ Based on this achievement, IL-2 and B7, a T cell co-factor essential for T cell activation, genes were transfected to bladder tumor cells *ex vivo*. Seventy-five percent of tumor-bearing animals that were treated with the combined and programmed immunization utilizing both IL-2 and B7 gene-modified tumor cell preparations survived and rejected further tumor challenge.¹² Despite these previous results, limitations in the ability to harvest, transfect genes *ex vivo* and re-inject the autologous tumors on a patient-by-patient basis question the feasibility of this strategy for clinical application. *In vivo* direct gene transfer to the cancer cells could be a possible modality to

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address these issues.^{13,14} Yet, in spite of its easy accessibility and the established role of intraluminally administered drugs for the treatment of bladder disease, only a few investigations have been reported for direct gene transfer to the bladder cancer.^{15–17}

The MBT-2 murine bladder cancer cell line, derived from a carcinogen-induced bladder tumor in C3H mice, is an appropriate tumor model for evaluating new therapeutic modalities of human bladder cancer. This poorly differentiated transitional cell carcinoma resembles both grossly and histologically its human counterpart. Treatments that have shown promise in this murine model have had similar therapeutic activities in human bladder cancer.^{18,19} As discussed previously, it is important that tumor cells outside their natural milieu behave quite differently than in their organ of origin.^{1,20} Based on this understanding, to reproduce the environment that exists in human bladder cancer, in our earlier studies, we used the orthotopic bladder tumor model, in which MBT-2 cells were directly injected into the bladder submucosa.^{1,10,12} In this study, we have modified our approach and established an easily reproducible intravesical seeding model that more closely represents the physiological features of human superficial bladder cancer. Using this novel MBT-2 superficial bladder tumor model, we have evaluated the feasibility of intravesical liposome-mediated IL-2 gene transfection in animals with pre-established superficial bladder tumors for the pre-clinical evaluation of a safe and effective *in vivo* gene delivery system.

Results

In vitro transfection of MBT-2 cells

To evaluate the *in vitro* optimal conditions for short-term liposome-mediated transfection, MBT-2 cells were transiently transfected with murine IL-2 plasmid, VR1110 (Figure 1)/DMRIE/DOPE lipoplex for 30 min. Among various plasmid DNA and cationic liposome combinations tested, the best murine IL-2 production was observed with the combination of 3 µg of VR1110 and 12 µg of DMRIE/DOPE for a 35-mm culture dish. Higher concentrations of plasmid DNA or cationic liposome did not improve murine IL-2 production level. Also 1 to 4 mass ratio (or 1 to 1 molar ratio) of plasmid DNA to DMRIE/DOPE was optimal in achieving maximal production level of the transduced gene of interest. The cell viability was not less than 95% with all the

VR1110/DMRIE/DOPE combinations tested. With VR1412 β-gal reporter plasmid transfection, the efficiency of gene transfection was determined and was calculated to be 36% by X-gal staining.

Utilizing these optimized conditions, murine IL-2 production level was 200 ng/10⁶ MBT-2 cells/24 h, which was undetectable in the supernatants of MBT-2 cells transfected with VR1019, the control parental plasmid.

In vitro morphology of IL-2 gene-modified MBT-2 cells was identical with the parental MBT-2 cells and their growth rate was the same. By staining with a monoclonal antibody, expression of the major histocompatibility complex (MHC) antigen was analyzed with a flow cytometer (data not shown). Parental MBT-2 cells express MHC class I antigen and IL-2 transfection up-regulated its expression in gene-modified MBT-2 cells. MHC class II antigen was not detected in either set of cells.

Establishment of intravesical MBT-2 tumor model

Since our goal in this experiment was to establish an intravesical transfection system against potentially invasive superficial bladder cancers, it was critical in animal studies to obtain a suitable superficial bladder tumor model which arises from bladder mucosa. We evaluated several methods of intravesical MBT-2 tumor implantation model including the utilization of cautery of the bladder wall.^{21,22} A more straightforward, precise physiological and reproducible bladder tumor model was created by simple intravesical instillation of highly tumorigenic MBT-2 cells into syngeneic C3H mice. As illustrated in Figure 2, the cauterization of the bladder mucosa before the instillation of tumor cells produced bladder tumors in all of the animals tested. However, unless laparotomy and direct visualization was performed, minor perforation of the bladder wall was frequently produced because of the inconsistent cauterization, which permitted tumor cells to be intraperitoneally disseminated. In our experiments, we observed that in the absence of cauterization, bladder confined tumors were still consistently obtained in a high incidence of more than 90%. These tumors were usually multi-focal but exclusively confined in the bladder. They then became palpable 17 days following the implantation. Just instilling the MBT-2 cells resulted in animal death by 5–6 weeks after tumor implantation. Most of the deaths appeared to be the

Figure 1 Structures of CMV promoter-based plasmid DNA vectors used in this study. VR1110 and VR1412 plasmid vector encode either murine IL-2 or *E. coli* β-gal gene. CMV, cytomegalovirus: prom/enh., immediate-early promoter and enhancer; BGH, bovine growth hormone.

Figure 2 Effect of procedure of intravesical tumor implantation on the bladder tumor incidence and staging 21 days following the intravesical implantation of 5×10^5 MBT-2 cells. Each group consisted of 10 animals. Cautery, cauterization of bladder wall just before tumor cell instillation; lap, laparotomy.

result of urinary obstruction caused by locally extensive bladder tumors. In some of the animals in which cauterization was not used, peritoneal dissemination was sometimes observed late in the experiment when bladder tumors penetrated the whole layers of bladder wall. Remote metastatic lesions to other organs such as lung were rarely seen, which was commonly observed in the orthotopic submucosal tumor injection model.^{1,10}

Intravesical liposome-mediated gene delivery

Utilizing the β -gal reporter plasmid, we evaluated how efficiently a single short-term intravesical administration of plasmid DNA/cationic liposome could transfect the pre-established superficial bladder tumors. As shown in Figure 3a, gross observation of the β -gal transfected bladder revealed that MBT-2 tumors exclusively expressed β -gal, while surrounding bladder urothelium as well as normal bladder was not apparently stained by X-gal. With microscopic observation (Figure 3b,c), the stained cells were identified mainly at a surface layer of the pre-established bladder tumors 2 days after transfection and small numbers of the tumor cells within the parenchyma of the tumor still expressed β -gal as late as 7 days following the single 30 min *in situ* liposome-mediated transfection. With a similar experiment utilizing murine IL-2 plasmid instead of β -gal, we monitored urine IL-2 level after the intravesical transfection. The peak IL-2 level of 1300–1600 pg/ml was observed 1 day after the transfection and then steadily declined to an undetectable level by day 5.

Intravesical liposome-mediated IL-2 gene therapy in animals with pre-established MBT-2 tumors

Given the ability to transfect pre-established intravesical tumors preferentially, we conducted efficacy experiments utilizing murine IL-2 (VR1110) lipoplex (Figure 4). Mean survival for the IL-2 treatment group was 43.8 ± 4.8 days, while in the groups treated with parental blank (VR1019) and β -gal (VR1412) lipoplex the mean survival was 22.8 ± 2.2 days and 24.0 ± 2.3 days, respectively. Until day 60, this efficacy study demonstrated survival of four of 10 (40%) mice treated with IL-2 lipoplex, while none of the animals in either control group, parental blank and β -gal lipoplex treatment, survived. There was a statistically significant survival advantage in the IL-2 lipoplex treatment group when compared with both controls by log-rank (Mantel-Cox) analysis ($P = 0.0008$). Also, as shown in Figure 5, growth of the pre-established intravesical tumors in the animals treated with IL-2 lipoplex was significantly suppressed when compared with that of control groups on day 21 (Kruskal-Wallis test, $P = 0.0020$). No untoward side-effects of the treatment with the lipoplex were noted except that small bladder calculi were observed in a total of two of 30 animals treated.

MBT-2 tumor re-challenge for cured animals by intravesical IL-2 gene therapy

In our previous studies, we have shown that animals cured of pre-established MBT-2 tumors by treatment with *ex vivo* IL-2 gene-modified tumor cell preparations were highly resistant to a subsequent re-challenge with parental MBT-2 cells.¹⁰ To evaluate this aspect in our current study, all the 60 day survivors received re-challenge of MBT-2 cells. All four animals initially cured by intravesical murine IL-2 gene therapy rejected the tumor once

Figure 3 β -Gal expression in pre-established bladder tumors following a single 30 min liposome-mediated intravesical VR1412, *E. coli* β -gal plasmid, transfection. Five days following the intravesical implantation of MBT-2 cells, 3 μ g VR1412/12 μ g DMR1E/DOPE lipoplex was instilled once into the bladder for 30 min. Two days (a,b) and 7 days (c) following this *in situ* transfection, bladders were removed and stained with X-gal. (a) Gross observation of bladder revealed preferential X-gal staining on intravesical tumor. Normal bladder transfected with the same β -gal lipoplex is shown on the left (bar corresponds to 1 mm). Ten (b) and 5 μ m (c) thick frozen sections were stained with X-gal. Arrows in (c) indicate the MBT-2 cells expressing β -gal in growing intravesical tumors. (Counter stained with hematoxylin and eosin. Original magnification $\times 100$. Bars correspond to 100 μ m).

again, while all six age-matched naive control animals died with bladder tumors (Figure 4). At the end-point of the experiment on day 120, the histological evaluation of all the surviving mice revealed no evidence of tumors or destruction of normal bladder architecture. For those surviving animals, CTL response against parental MBT-2 cells was evaluated. As shown in Figure 6, intravesical IL-2 gene therapy was capable of eliciting CTL response in a tumor-specific manner. EL4 cells used as control targets showed no specific lysis.

Figure 4 Treatment of tumor-bearing mice with *in situ* murine IL-2 gene transfection. Bladder tumors were established in C3H mice by intravesical implantation of 5×10^5 MBT-2 cells. Three micrograms of plasmid DNA/12 μ g DMRIE/DOPE lipoplex were instilled a total of six times with the schedule of once every other day from 3 days until 13 days after tumor implantation (small arrows). Experimental groups of animals were treated either by IL-2, β -gal or parental blank plasmid lipoplex. Each group consisted of 10 animals. * $P = 0.0008$ (log-rank analysis). Sixty day survivors were re-challenged with intravesical implantation of 5×10^5 MBT-2 cells (large arrow). A new set of six age-matched naive animals was used as a new control. (Δ) parental plasmid lipoplex (control); ($\circ$) β -gal lipoplex; ($\bullet$) IL-2 lipoplex; ($\blacktriangle$) new naive control.

Figure 5 Suppression of MBT-2 intravesical tumor growth. In the experiment shown in Figure 4, intravesical tumors were scored on a relative scale of 0 to 5 based on a daily observation. Mean tumor score of surviving animals of each three groups was plotted. There was a significant difference between IL-2 treatment group and the two control groups on day 21 ($P = 0.0020$, Kruskal-Wallis test). Tumor score: 0, no tumor; 0.5, palpable but not visible tumor; 1.0, small visible tumor; 2–4, increasingly larger tumors; and 5, abdomen full of tumor with erosion through skin. (Δ) parental plasmid lipoplex (control); ($\circ$) β -gal lipoplex; ($\bullet$) IL-2 lipoplex. Error bars indicate s.e.m.

Discussion

Cancer immunotherapy is based on the concept that tumor cells express tumor-specific antigens that can be recognized from self antigens and induce immune responses capable of eradicating tumor cells.²³ The goal of cancer immunotherapy, therefore, is to increase tumor-specific antigen recognition and enhance the antitumor responses of cancer patients. CD8⁺ CTL play an important role in tumor antigen recognition in the context of MHC class I molecules expressed on the surface

Figure 6 MBT-2 tumor-specific CTL responses in mice which survived from pre-established MBT-2 bladder tumor by the intravesical liposome-mediated *in situ* IL-2 gene therapy. Splenocytes from mice which survived from re-challenge in Figure 4 were harvested and a CTL assay was performed after 5 days of incubation. Splenocytes from naive mice were used as controls. Control target cells, EL4, showed insignificant lysis. This experiment was repeated three times with similar results.

of the target tumor cells.²⁴ To date, many studies involving the treatment of tumor-bearing animals with non-immunogenic tumor cells engineered to secrete various cytokines have demonstrated the inhibition of pre-established tumors.^{1–6,10,25} In our previous experiments, *ex vivo* genetically modified IL-2 or GM-CSF secreting MBT-2 cell preparations were shown to induce effective immune responses against original MBT-2 tumors and cure up to 50% of animals with pre-established MBT-2 bladder tumors.^{1,10} Moreover, these antitumor immune responses of initially cured animals were persistent and highly effective against further MBT-2 tumor re-challenges. We successfully showed that this immuno-gene therapy leads to *in vivo* activation of tumor-specific CTL capable of lysing autologous tumors *in vitro* demonstrating exclusive specificity.¹⁰

Although previously used retroviral vectors provide genomic integration and a permanence of transduction, they require active cell division for efficient gene transfer.²⁶ In addition, the generation of retroviral vectors is complex and associated with safety issues for human trials that include the production of replication-competent recombinant virus.²⁷ Because of these limitations of the retroviral vector system as a transfection vehicle, we have successfully utilized liposome-mediated plasmid DNA transfer technique to primary cultures of human bladder carcinoma *ex vivo* as well as established bladder cancer cell lines, suggesting the clinical feasibility of this strategy.¹¹ In our current study, using an optimized 30 min transfection protocol, we demonstrated high levels of murine IL-2 production after the *in vitro* transfection of VR1110 plasmid DNA into MBT-2 cells. This level of expression is comparable with the adenovirus system and up to 100 times higher than that observed with the retrovirus transfection system.^{12,28–30}

Clinical trials of tumor vaccine strategy have been extremely limited due to the cumbersome *ex vivo* gene transfer procedures on a patient-by-patient basis. It also contains a potentially hazardous aspect to bring the tumor cells back into the patient, even if *ex vivo* gene-modified tumor cells are irradiated. From the clinical point of view, therapeutic targeting of superficial bladder cancers are appealing for *in situ* gene therapy because of their easy accessibility to urologists by the less invasive

maneuver of cystoscopy. Furthermore, the role of intravesical instillation of adjuvant chemotherapy (ie mitomycin C, doxorubicin) and immunotherapy (ie BCG) have already been established and validated to be effective, although limited, against tumor recurrence and extend the time before cystectomy is required. So far, these understandings have led some investigators to develop *in vivo* gene delivery system successfully utilizing adenovirus¹⁶ and vaccinia virus¹⁵ in animal bladder tumor models. Adenovirus-mediated approaches for *in vivo* transfection appeared to be highly efficient and since they are given intravesically, this approach may not encounter the host immune response against a virus.³¹ Also, as shown by Lee *et al*,¹⁵ vaccinia virus is capable of transfecting bladder tumors *in situ* and pre-immunity to vaccinia was found not to inhibit intravesical transfection. Yet the therapeutic use of these viruses as a gene delivery modality still entails safety issues. Since the *in vivo* gene delivery system may require multiple applications to generate a sufficient and optimal treatment force, the modalities of gene delivery should be extremely safe when considering human application. Liposome-mediated gene delivery *in vivo* has demonstrated safety in various animal studies and is being evaluated in a number of clinical trials.^{32–36} Successful gene transfer to the target tissues and the expression of the transduced gene of interest were achieved without any complications. When compared with retroviral transfection, multiple copies of the plasmid can be transduced per cell leading to higher production of the transduced gene of interest for a shorter period of time. Since the mechanism of transfection is not based on genomic integration, gene expression is transient, however, if gene expression persists long enough for significant immunomodulation, the transient nature of transfection becomes less important. The transient and less efficient nature of liposome-mediated transfection can be overcome by a multiple transfection protocol. These conclusions in our studies formed the basis for establishing an *in vivo* model of liposome-mediated bladder-directed gene transfer.

Our modification of the tumor implantation procedure allows reliable superficial intravesical seeding without artificial trauma as injury of the bladder. The model is convenient since simple intravesical implantation is always effective for tumor establishment in the bladder urothelium. In this model, metastatic disease was rarely seen compared with the submucosal tumor injection model. It can be speculated that at the time of submucosal tumor injection, there might be a chance for injected tumor cells to spread directly into blood stream, which resulted in hematological dissemination of tumor cells to other organs.

With this model system, we have shown that simple intravesical instillation of lipoplex is capable of delivering the gene of interest directly into growing bladder tumors. What has to be emphasized here is that intravesical instillation of the reporter gene yielded transfection only to bladder tumors, while surrounding normal bladder urothelium was not efficiently transfected. In recent reports by Engler *et al*,^{37,38} and Austin *et al*,³⁹ enhanced virus-mediated reporter gene uptake and expression were observed not only in cancerous urothelium but also in normal urothelium when bladders were co-administered or pretreated with ethanol, HCl or detergents to overcome the epithelial barrier. Based on these observations,

we speculate that the protective glycosaminoglycan (GAG) layer of the normal bladder urothelium may act as a physiological barrier to the lipoplex, while absence of the GAG complex allows bladder tumor transfection to occur. We also hypothesized that the difference of static electrical charges of the cell surface between tumor and normal urothelium, if any, might possibly affect the affinity of the instilled lipoplex. Currently, we are studying this aspect in our model system.

In the efficacy study, with intravesical administration of murine IL-2 lipoplex, 40% of animals were cured of pre-established bladder tumors, while none of the animals treated with the same dose of β -gal plasmid, which has the identical structure to the IL-2 plasmid other than the expression gene, and DMRIE/DOPE lipoplex survived. The results apparently indicated that our intravesical gene therapy was only effective when it resulted in attracting host immune response against parental tumor cells by local IL-2 secretion from *in vivo* transfected tumor cells themselves but not by the effects of proinflammatory cytokine production, if any, elicited by bacterial plasmid DNA transfection.⁴⁰ Moreover, it was consistent with our previous data that not only all the animals cured of pre-established tumors rejected the tumor re-challenge and survived for another 60 days, but also MBT-2 tumor-specific CTL activities were observed in these cured animals.

Our technique of liposome-mediated gene transfer was accomplished without any untoward side-effects except for a few cases of bladder calculi. Also, we did not find any undesirable consequences of IL-2 lipoplex administration including autoimmunity responses. On the contrary, production of IL-2 from the surrounding normal urothelium, although it is not likely to be high judging from the β -gal reporter gene experiment, is considered to be a favorable local immune milieu against bladder cancers.

Further studies of intravesical liposome-mediated gene therapy in the MBT-2 superficial bladder cancer model are currently being investigated. These studies will focus on the toxicological evaluation of intravesical gene therapy in order to accumulate data required for the approval of US Food and Drug Administration to conduct a clinical trial using the human grade, high purity human IL-2 encoding plasmid. Also, we are currently working with other novel liposome formulations to achieve better transfection efficiency in our bladder cancer model.

In summary, we have shown that superficial bladder tumors can be genetically modified using the technique of liposome-mediated *in situ* gene transfer via simple intravesical instillation. Intravesical introduction of exogenous genetic material is a potentially useful therapeutic modality not only for immuno-gene therapy but also various other gene therapy strategies against bladder diseases.

Materials and methods

Animals and cell lines

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center and The Cleveland Clinic Foundation. Eight-week-old female C3H/HeJ mice were purchased from The Jackson Laboratory (Bar

Harbor, ME, USA). The murine bladder carcinoma cell line, MBT-2 was originally obtained from Dr T Ratliff (The University of Iowa, Iowa City, IA, USA) from a transplantable N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide-induced bladder tumor in a C3H mouse. EL4 (murine thymoma cell, C57BL/6) was used as control for CTL assay. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 0.1 mM non-essential amino acids. All the cells used in this study were checked for the absence of Mycoplasma infection by a PCR-based assay at the Monoclonal Antibody Core Facility of Memorial Sloan-Kettering Cancer Center.

Intravesical implantation of MBT-2 cells

Confluent MBT-2 cells were trypsinized to a single cell suspension and greater than 95% cell viability was determined by trypan blue exclusion. The cells were re-suspended in phosphate-buffered saline (PBS) before inoculation. Animals were anesthetized with 50 mg/kg of intraperitoneal sodium pentobarbital injection and secured on a grounded plate with the back skin shaved. A 24 G Teflon-coated catheter was introduced into the bladder through the urethra. In the groups in which bladder cautery was performed, a surgical steel wire electrode was put through the catheter, which was protruded 0.5 mm outside the catheter into the bladder lumen, and was activated with an electrosurgical unit, SSE3B (Valleylab, Boulder, CO, USA) for 2 s at the 10–20 watts coagulation setting, such that the bladder wall was cauterized to produce a 2 mm spot lesion. Following the cautery, the electrode was removed leaving the indwelling catheter in place and 5×10^5 MBT-2 cells in 50 μ l of PBS were then instilled into the bladder. The catheter with the injection syringe was held in place for 15 min. The procedures were compared with or without a 1.0-cm suprapubic laparotomy to observe bladder manipulation directly. Twenty-one days following the implantation of MBT-2 cells, mice were killed and intravesical tumors were histologically evaluated. All the procedures were well tolerated and post-operative mortality was less than 5%.

Assessment of tumors

Mice were checked every day and the objective findings (ie general status, palpable mass, gross hematuria and ascites) were recorded. The mice which died before the end-point were necropsied immediately and the cause of the death was determined. If mice died before the end-point of the experiment without any histological evidence of cancer, they were excluded from the experiments. The mice which survived until the end-point underwent laparotomy to check the tumors and metastases. All bladders and tumors were removed and histological evaluation was conducted. The bladder tumors observed were classified into three categories: (1) metastatic: the tumor penetrated the bladder wall and extended outside the bladder. We usually observed peritoneal disseminations and lymph node metastases rather than hematological metastases in this stage; (2) invasive: the tumor did not penetrate the bladder wall but extended into the bladder muscle layers. Bladders were nearly replaced by the tumors in most of these cases; (3) superficial: the tumor was confined in the bladder and histologically remained within the bladder submucosa.

Liposome and plasmids

Lipid reagent and plasmid DNA expression vectors used in our experiments were generously provided by Vical (San Diego, CA, USA). Cationic liposome 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE)/dioleoylphosphatidylethanolamine (DOPE) was composed of the positively charged lipid DMRIE in combination with the neutral lipid DOPE in a 1:1 molar ratio. Details regarding general structures of the novel plasmid DNA vectors used in this study have been described previously.^{14,41} These plasmids commonly contained the human cytomegalovirus immediate-early promoter and enhancer plus intron A with a transcription terminator from the bovine growth hormone gene and kanamycin resistance gene for selection. VR1019 is the parental plasmid DNA expression vector. Murine IL-2 or *Escherichia coli* β -gal gene were cloned into VR1019 plasmid and designated as VR1110 and VR1412, respectively (Figure 1).

In vitro transfection

For *in vitro* transfection, MBT-2 cells were plated at a density of 3×10^5 cells in 35-mm culture dishes and allowed to incubate for 24 h at 37°C. DMRIE/DOPE was provided as a dried powder formulation and was re-suspended in sterile water to a concentration of 1 mg/ml (0.747 mM). Various amounts of DMRIE/DOPE (0, 4, 8, 12, 20 and 40 μ g) and plasmid DNA VR1110 (0, 1, 2, 3, 5, 10 and 20 μ g) were separately diluted in serum-free medium to a total volume of 500 μ l and then mixed rapidly together in polystyrene tubes. This 1.0 ml solution was allowed to form plasmid DNA/DMRIE/DOPE lipoplex at room temperature for 30 min. Ten minutes before the transfection, culture medium was gently aspirated and adherent MBT-2 cells were rinsed once with serum-free medium. The VR1110 lipoplex in a volume of 1.0 ml was then added to the cellular monolayers. After 30 min of incubation at 37°C, the medium containing the lipoplex was aspirated and replaced with 2.0 ml fresh serum-free medium. Twenty-four hours later, supernatants were collected and tested in triplicate for murine IL-2 enzyme-linked immunosorbent assay determination (BioSource International, Camarillo, CA, USA). Cell counts of each dish were performed at this point and the results were reported as ng IL-2/ 10^6 cells/24 h. As a control, MBT-2 cells were transfected with the parental plasmid VR1019.

Intravesical reporter gene transfection

The lipoplex was prepared in the same manner as described above except that the final volume of the solution was prepared as 0.1 ml. Five days following the intravesical implantation of MBT-2 cells, 3 μ g of β -gal reporter plasmid, VR1412, and 12 μ g of DMRIE/DOPE lipoplex was instilled transurethrally for 30 min in a total volume of 0.1 ml of saline. Two and 7 days following this single *in situ* transfection, animals were killed. Whole bladders were dissected and harvested into fixative (2% formaldehyde, 0.2% glutaraldehyde) for 1 h. The bladders were opened longitudinally and rinsed overnight with PBS, and the whole bladder was then stained overnight in 5-bromo-4-chloro-indolyl- β -D-galactopyranoside (X-gal) solution (2 mg/ml X-gal, 5 mM potassium ferri-cyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, 0.02% NP-40). Alternatively, bladders were snap frozen in liquid nitrogen, sectioned by cryostat and

fixed in 1% glutaraldehyde for 10 min followed by overnight incubation at 37°C with X-gal solution.

Intravesical IL-2 gene therapy model

Three days following the intravesical implantation of 5×10^5 MBT-2 cells, *in situ* murine IL-2 gene transfection was initiated, which was followed by five instillations. The treatment was given every other day until day 13. Three micrograms of plasmid DNA and 12 µg of DMRIE/DOPE lipoplex in a volume of 0.1 ml were instilled for 30 min per treatment. Experimental groups of animals were treated with either VR1019, VR1412 or VR1110 lipoplex. Each group consisted of 10 animals. All animals were monitored daily and tumors were evaluated at the time of death of each animal. Sixty days following the MBT-2 cell inoculation, all of the surviving animals which remained free of disease were re-challenged with intravesical implantation of 5×10^5 MBT-2 cells. As an untreated control, a new set of six age-matched naive mice was also implanted with the same inoculum of MBT-2 cells. These re-challenged survivors as well as new control animals did not receive any further treatment. Statistical comparisons of Kaplan–Meier survival data for this study were performed with the log-rank analysis (Mantel–Cox test) and differences in tumor score were determined with the Kruskal–Wallis test.

CTL analysis

Splenocytes were harvested and depleted of red blood cells with ammonium chloride Tris buffer. Fifteen million splenocytes were cultured with 7.5×10^5 irradiated stimulator cells (MBT-2 cells irradiated at 75 Gy) in 5 ml of RPMI 1640 medium with 10% FBS, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 mg/ml streptomycin and 50 µM β-mercaptoethanol per well in a six-well tissue culture plate. Cells were cultured for 5 days at 37°C and 5% CO₂.

The cell-mediated cytotoxicity was measured by Cyto-Tox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA). The target cells and serial dilutions of effector cells at varying effector:target ratios were added in 200 µl of RPMI 1640 medium with 10% FBS in 96-well U-bottom plates. The plates were centrifuged at 250 g for 4 min and incubated at 37°C and 5% CO₂ for 4 h. Fifty microliters of the supernatant were harvested and lactate dehydrogenase release was measured by the reagent provided by the assay system.

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