INTRAVESICAL TUMOR NECROSIS FACTOR-ALPHA GENE THERAPY MEDIATED BY A NOVEL LIPOSOME SYSTEM IN AN ORTHOTOPIC MURINE BLADDER CANCER MODEL

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SUMMARY

Purpose: To evaluate the safety and efficacy of intravesical instillation of a non-viral vector encoding TNF-α in an orthotopic bladder cancer model.

Materials and Methods: The murine TNF-α cDNA was cloned into vector pBud.CE4.1. A murine bladder cancer cell line MB49 was transfected by pBud-TNF-α using cationic liposome DOTAP plus methyl-beta-cyclodextrin solubilized cholesterol (MBC). TNF-α levels were determined by ELISA. Cell proliferation, cell cycle analysis and Annexin V staining were done to examine the effects of pBud-TNF-α. Flow cytometric analysis of MHC I, MHC II, ICAM I, B7-1 and Fas molecules were performed. *In vivo*, RT-PCR analysis of TNF-α expression in murine bladder was done. MB49 cells were implanted in 24 C57BL/6 mouse bladders. Two days after implantation, pBud-TNF-α was instilled in 12 mice with the rest getting the control vector pBud intravesically. On day 27, 5 days after the sixth instillation, all bladders were harvested, sectioned and examined. The infiltration of immune cells into bladder after TNF-α therapy was also investigated.

Result: MB49 cells produce 893.7±24.0pg/ml of TNF-α 48 hours after TNF-α transfection and their growth was inhibited. Cell cycle analysis and Annexin V staining showed MB49 cells were induced to apoptosis after transfection. MHC I, B7-1 and Fas expression were also enhanced significantly. *In vivo*, three mice died in the control group because of excessive bladder tumor burden while 1 died in the pBud-TNF-α treated group. Histological study showed that 9 of 12 mice in the control group had bladder tumor while only 3 of 12 in the pBud-TNF-α treated group demonstrated bladder cancer.

TNF-α mRNA was observed to increase after the first instillation and then return to basal level 1 month after the sixth instillation. CD3+ T lymphocytes and NK cells in bladder were enhanced after intravesical TNF-α transfection.

Conclusion: Intravesical instillation of pBud-TNF-α produces a significant anti-tumor effect in an orthotopic murine bladder cancer model. Cytokine gene therapy may be useful as an adjuvant therapy for bladder cancer.

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RELATED PUBLICATIONS AND CONFERENCE ABSTRACTS

Zang Z, Mahendran R, Wu Q, Yong T, Esuvaranathan K. Intravesical Liposome-mediated Tumor Necrosis Factor-alpha gene therapy in an orthotopic murine bladder cancer model. ***(Submitted to Gene Therapy)***

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ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
CIAP	Calf Intestinal Alkaline Phosphatase
CIS	Carcinoma *In Situ*
CMV	Cytomegalovirus
CTLs	Cytotoxic T Lymphocytes
DEPC	Diethyl Pyrocorbonate
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate)
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
G-CSF	Granulocyte Colony-Stimulating Factor
HSV-tk/GCV	Herpes Simplex Virus Thymidine Kinase/Ganciclovir
HSV	Herpes Simplex Virus
ICAM I	Intercellular Adhesion Molecule-I
MBC	Methyl-β-cyclodextrin Solubilized Cholesterol
MHC I	Major Histocompatibility Complex I
MHC II	Major Histocompatibility Complex II

MOI	Multiplicity Of Infection
MRI	Magnetic Resonance Imaging
ONPG	O-Nitropnenyl-ß-Galactopyranoside
PI	Propidium Iodide
PSA	Prostate Specific Antigen
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
TCC	Transitional Cell Carcinoma
TGF-β1	Transforming Growth Factor-beta1
TNF-α	Tumor Necrosis Factor-α
TURBt	Transurethral Resection of Bladder Tumor

CHAPTER ONE INTRODUCTION

1.1 BLADDER CANCER

1.1.1 Epidemiology of bladder cancer

Bladder cancer is the fourth most common malignancy in males and the eighth most common in women in western countries. It is also the second most common tumor and the second most common cause of mortality of all the genitourinary cancers in the United States. Each year, over 52,000 new cases are diagnosed with this disease and there are more than 12,000 deaths in United States (Lamm et al., 1995). In many Asia countries, bladder cancer is the most common urological cancer. It accounts for 52% of all the urological cancer in South Korea (Cheon et al., 2002). In Singapore, it lists ninth amongst the most common cancer in males (Chia et al., 1995). Noticeably, the incidence rate of bladder cancer had increased remarkably worldwide. In the USA, for example, the incidence of bladder cancer has increased by 36% in the last decade (Lamm et al., 2000).

There are many suspected risk factors of bladder cancer including: urinary tract infection (Kantor et al., 1984; La Vecchia et al., 1991), Schistosoma haematobium (Badawi et al., 1995), smoking (Mommsen et al., 1983; Fortuny et al., 1999), artificial sweeteners (Miller et al., 1977), hair dye (Yu et al., 2002; Gago-Dominguez et al., 2003), 2-naphthylamine and benzidine (Piolatto et al., 1991; Shinka et al., 1991).

1.1.2 Pathology of bladder cancer

More than 90% of bladder cancers are transitional cell carcinoma (TCC). Other types of bladder cancer such as squamous cell carcinomas (5%), adenocarcinoma (1%), primary lymphoma, sarcoma, rhabdomyosarcoma and leiomyosarcoma account for less than 10% of the total cases. Seventy to 80% of TCC appears as papillary tumors. Papillary tumors are often associated with recurrence, but seldom invade muscularis propria or metastasize. Non-papillary tumors account for 20-30% of all bladder tumors, with muscle invasion present in 90% of these patients. In TCC, the recommended grading system is the WHO classification:

Grade1 papillary TCC show an increased number of cell layers of superficial cells, there is reduced or absent cytoplasmic clearing, increased nuclear size, slight nuclear pleomorphism and slightly abnormal nuclear polarization, slight hyperchromatism, absent or rare mitoses and nuclear grooves are present.

Grade 2 papillary TCC show a variable number of cell layers, absent superficial cells, often absent cytoplasmic clearing, increased nuclear size, moderate nuclear pleomorphism, abnormal nuclear polarization, moderate hyperchromatism, mitotic figures and nuclear grooves are present.

Grade 3 papillary TCC show a variable number of cell layers, absent superficial cells, absent cytoplasmic clearing, greatly increased nuclear size, marked nuclear pleomorphism, absent nuclear polarization, marked hyperchromatism, prominent mitoses and absent nuclear grooves.

There are several staging systems that have been described for bladder cancer, one of the best known of which is AJCC/UICC (Table 1.1). Traditionally, Ta and T1 papillary urothelial carcinoma are called superficial cancer and T2 and above are termed as muscle invasive cancer.

Table1.1: Pathological staging of bladder cancer (AJCC/UICC) (Syrigos et al., 1999)

PATHOLOGICAL STAGING OF CARCINOMA OF THE URINARY BLADDER	
AJCC stage	Level of invasion
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ
T1	Tumor invades the lamina propria
T2	Tumor invades the muscularis propria (detrusor muscle)
T2a	Superficial muscularis propria
T2b	Deep muscularis propria
T3	Deep muscularis perivesical tissue
T3a	Microscopically
T3b	Macroscopically (extravesical mass)
T4	Invasion of adjacent structures
T4a	Prostate, uterus, vagina
T4b	Pelvic wall, abdominal wall
N1-N3	Lymph node metastasis
N1	Regional node < 2 cm
N2	Regional node 2-5 cm
N3	Regional node >5 cm
M	Distant metastasis

1.2 THE TREATMENT OF BLADDER CANCER

1.2.1 Surgical treatment and intravesical therapy of superficial TCC

Seventy to 80% of the patients with TCC present with low-grade, noninvasive tumors or superficial papillary tumor confined to the mucosa. The standard primary treatment for superficial bladder cancer is endoscopic transurethral resection of the bladder tumor (TURBt). However after surgical treatment, 70% of TCC will recur and about 30% of the recurrent tumors present with higher grade and/or with muscle invasion. The high recurrence rate and the unpredictability of the progression patterns have led to the widespread use of intravesical chemotherapy or immunotherapy.

The advantage of the intravesical route of administration are the high concentration of drug in contact with tumor-bearing mucosa or bladder epithelium at risk, and little or no systemic uptake of the drug. The commonly used intravesical agents in the treatment of bladder cancer include Bacillus Calmette-Guerin (BCG), thiotepa, mitomycin, doxorubicin, valrubicin, interferon, interleukin-2, keyhole limpet haemocyanin, bropirimine and levamisole.

1.2.2 BCG therapy

Among all these agents, BCG is favored as a first-line intravesical drug because of its well-documented effect on reducing tumor recurrences and tumor progression after

TURBt. A significant reduction in tumor recurrence is noted in most studies comparing BCG with TUR alone for superficial bladder cancer. Lamm reported that immunotherapy with BCG has resulted in complete tumor regression in one half of treated patients with papillary tumors and in more than 70% of those with carcinoma *in situ* (CIS) for 5 years or more (Lamm, 1992). In comparison, the existing studies of intravesical chemotherapy have failed to demonstrate significant reduction in long-term incidence of tumor recurrence. Moreover, intravesical BCG can also reduce the risk of tumor progression after transurethral resection to stage T2 disease or higher in patients with superficial bladder cancer. Sylvester et al followed up 4,863 patients for a median of 2.5 years and a maximum of 15 years. They found 260 of 2,658 patients on BCG (9.8%) had progression (T2 disease or higher) compared to 304 of 2,205 patients in the either resection alone or resection plus intravesical treatment other than BCG (13.8%), a reduction of 27% in the odds of progression on BCG (OR 0.73, $p = 0.001$) (Sylvester et al., 2002). However, compared with intravesical chemotherapy, intravesical BCG therapy appears to have more side-effects. Reported systemic side-effects include fever, flu-like symptoms, malaise, chills, pneumontitis, hepatitis, arthralgia, myalgia and rash. Local side-effects comprise BCG cystitis, dysuria, urinary frequency, hematuria, granulomatous prostatitis, epididyno-orchitis and urethral obstruction, etc.

The anti-tumor mechanism of intravesical BCG therapy in superficial bladder cancer has not been elucidated. But two premises seem certain (Martinez-Pineiro et al., 1997). First, it is necessary to bring living bacteria in contact with tumor cells. Consequently attachment, retention and internalization of BCG would take place. Thereafter, the

induction of immunological events follows and this may lead ultimately to tumor destruction (Ratliff et al., 1987). Second, T lymphocytes are required for BCG-mediated anti-tumor activity, evidenced by the phenomenon that depletion of total T cell, Th or Tc subsets in mice eliminated BCG-mediated anti-tumor activity (Ratliff, 1992). The infiltration of a broad range of immunological cells, including macrophage, T lymphocytes and natural killer cells, is observed after intravesical BCG treatment.

1.2.3 Treatment of invasive bladder cancer

For invasive TCC, squamous cell carcinomas and adenocarcinoma, radical cystectomy plus urinary diversion is recommended to optimize the 5-year survival rate. Radical cystectomy refers to the removal of the anterior pelvic organs. In males it includes the resection of the prostate, seminal vesicles, bladder with its peritoneum, and perivesical fat. In females, it includes the urethra, bladder, cervix, vaginal cuff, uterus, ovaries, and anterior pelvic peritoneum. Partial cystectomy is also used to treat solitary, primary invasive TCC. But the biggest disadvantage of partial cystectomy is the high rate of tumor recurrence (Sweeney et al., 1992). Radiation and chemotherapy have been studied as bladder-sparing treatments for muscle-invasive TCC. Invasive TCC is sensitive to cytotoxic chemotherapy, but the majority of patients does not achieve a complete response and eventually succumb to progression of chemo-resistant disease (Shipley et al., 1998). Invasive TCC is only partially responsive to radiation alone.

1.3 GENE THERAPY OF BLADDER CANCER

1.3.1 Introduction

Despite the efficacy of TURBt plus intravesical BCG immunotherapy, 30% of TCC patients will have tumor recurrence. As many as 30% of recurrent tumors will progress to higher grade or stage tumors, which can be potentially life-threatening (Soloway 1996; Nseyo et al., 1996; Saint et al., 2002). New treatment modalities must be developed to improve the overall treatment efficiency of TCC.

The basic concept of human gene therapy was developed more than 20 years ago. Gene therapy for cancer has become a more realistic approach because rapid advances in molecular genetic techniques have revealed the alteration of cellular oncogenes and tumor suppressor genes in cancer cell. Gene therapy is defined as the treatment of an acquired or inherited disease by direct transfer of genetic material and genetic modification of genes expression in somatic cells. It is an attractive new approach for the treatment of bladder cancer for several reasons. Firstly, the unique isolated environment and the accessibility of the bladder make it an optimal candidate for gene therapy. Secondly, patients who have undergone TURBt have a low tumor burden and like patients with carcinoma *in situ* (CIS), their cancer cells are accessible to either viral or non-viral agents, so they provide the best chance for a gene-based immunotherapy to succeed. Thirdly, the response of the tumor to treatment can be easily determined with cystoscopy and urine cytology.

1.3.2 Gene delivery vectors

1.3.2.1 Viral vectors

The therapeutic outcome for any form of gene therapy directly depends upon the availability of an efficient and safe delivery system. Currently, there are two major methods of delivering genetic material into target cells: non-viral and viral methods. Viral systems are the most commonly used gene transfer methods in gene therapy today. To construct a viral vector, a therapeutic gene is inserted into a modified viral genome, and a specific promoter such as the cytomegalovirus (CMV) promoter, is inserted into the viral genome to drive production of the therapeutic gene. Then the therapeutic gene is delivered into the target cells upon infection with the virus. Many kinds of viruses can be used to construct the viral vectors. The most widely used vector systems are adenovirus, retrovirus, herpes-simplex, vaccinia virus and adeno-associated virus. These viruses have different features in terms of the size of genes that can be carried, transfection efficiency, duration of expression and immunogenicity of the vector and transgene products (Table 1.2). In general, viral vectors are more efficient than non-viral vectors in terms of transfection efficiency. However, the risk of using live viruses in gene therapy protocols has always been of concern. Adenovirus, for example, was linked to the death of an18 year-old patient at the University of Pennsylvania (Lehrman, 1999). Duplicate sequences not engineered in the original form were discovered from this patient's organs after autopsy, revealing vector recombination (Smaglik, 1999).

Table 1.2: Viral vectors in gene therapy (Jian, 1998)

Characte-ristics	Retrovirus	Adenovirus	Herpes virus	Adeno-associat-ed virus	Vaccinia Virus
Size	8 kb	7-8 kb	30 Kb	4.5 kb	30kb
Integration	Yes	Occasional	No	Yes	No
Tissue specificity	Yes	Yes	Yes	No	No
Properties	Infect only dividing cells	Infect non-dividing cells	Neurotropic infects CNS cells	Integration in non-dividing cells	Wide host range
Delivery	*Ex vivo* or direct injection	*Ex vivo* or direct injection, aerosolization	*Ex vivo* or direct injection	Probably *ex vivo* only	Direct skin Scarification
Titer (cfu/ml)	10^6-10^9	10^8-10^{10}	10^6-10^8	10^6-10^8	10^7-10^9
Duration of expression	Good	Transient	Transient	Potentially good	Transient
Level of transgene expression	Moderate	High	Moderate	Moderate	High
Safety	Insertional mutagensis	Inflammatory response, insertional mutagenesis	Insertional mutagenesis	Insertional mutagenesis	Dangerous in immune suppressed patients

1.3.2.2 Non-viral vectors

Non-viral vector systems are important because they do not carry most of the risks implicit in the use of viral vectors. Techniques for non-viral gene transfer include physical and chemical methods (Table 1.3). Liposomes and electroporation are the most widely used non-viral approaches and may be suitable for gene therapy in localized bladder tumors (Harimoto et al., 1998).

Table 1.3: Non-viral gene transfer methods (Peter et al., 2002)

Method	In vitro efficiency	In vivo efficiency	Stability
Chemical			
Liposomes	Medium	Low	Short
Calcium Phosphate co-precipitation	Medium	Impossible	Short-long
Physical			
Electroporation	Medium	Low-medium	Short-long
Microinjection	High	Impossible	Medium
Tissue-injection	-	Low	Short
Jet injection	High	Impossible	Medium
Gene gun	High	Medium	Medium

1.3.2.3 Liposome system

Liposome-based gene delivery is regarded as a promising gene transfer technique for gene therapy because of its safety, the lack of immnogenicity, unlimited size of DNA that can be delivered and relative ease in creating DNA-liposome complexes in large scale for use in the clinic. Generally, the liposomes can be grouped in two categories based on the mode of entrapment of DNA: positively charged or cationic liposome and negatively charged or anionic liposome or pH-sensitive Liposome (Table 1.4). Cationic liposomes are more commonly used due to their relatively high transfection efficiency in various types of cell and tissue. Both cationic and anionic liposomes may share the same mechanism of liposome-cell interaction which can be divided in three steps: internalization of liposome-DNA complex, delivery of DNA into the cytosol and entry of DNA into the nucleus (Behr et al., 1989; Pinnaduwage et al., 1989; Gustafsson et al., 1995; Rose et al., 1995).

Table 1.4: Two categories of liposome

Categories	Mode of Entrapment of DNA	Gene Transfer	Examples
Cationic Liposome	DNA remains outside to the positively charged liposomes due to the charge interactions.	Efficient for gene transfer because of their stronger adherence to negatively charged cell membrane.	Lipofectamine, DOTAP, DMRIE, DOTMA
Anionic Liposome	DNA is entrapped in the negatively charged liposomes.	Less efficient. Liposomes are destabilized in response to low pH in the endocytic vacuoles and release the content into cytoplasm.	DOSG, CHEMS

Liposome-DNA complex can enter the cell by local destabilization of membrane, fusion or endocytosis. Endocytosis is the major mechanism for pH-sensitive liposome. Usually the non-specific cellular binding of pH-sensitive liposome is low. This binding ability can be significantly enhanced if an appropriate ligand, such as an antibody, is attached to the liposome surface. In contrast, cationic-DNA liposomes can binds cells more strongly due to favorable charge interactions. This may lead to the higher transfection efficiency of cationic liposomes compared to ligand-free, pH-sensitive liposomes.

The second step is the delivery of DNA into the cytosol. For the liposome-DNA complex which enters the cell by local destabilization and fusion, DNA usually can be directly delivered to the cytosol. For those that enter by endocytosis, this leads to the formation of endosomes containing liposome-DNA complex. DNA can be released by the rupture of the endosome membrane. But unfortunately, many endosomes rapidly fuse with lysosomes and DNA is degraded subsequently.

The last step is the entry of DNA into the nucleus. Very little is known about how DNA moves from the cytosol into the nucleus. But Capecchi showed this process may be very inefficient, evidenced by the fact that the microinjection of DNA into cytosol induces much less gene expression than the injection of same amount of DNA into nucleus (Capecchi et al., 1980).

1.3.3 Gene therapy strategies for bladder cancer

The most common strategies in gene therapy of bladder cancer are immune inductive, corrective, cytotoxic gene therapy and anti-sense oncogene therapy.

1.3.3.1 Immune inductive gene therapy

Immune inductive strategies aim to enhance the host immune response by increasing the antigenicity of tumor cells or boosting host humoral and cellular immune response to any given tumor locally or systematically. There are mainly two treatment options designed to achieve the above aim: transferring co-stimulatory molecules for T-cell recognition/activation and transferring genes encoding for cytokines. B7, a co-stimulary molecule, has been tested in an attempt to enhance tumor antigenicity. B7 protein is usually expressed on macrophages and B cells and reacts with CD28 to activate T cells. Tumor cells typically do not express this molecule. Vaccination of B7-transfected tumor cells has been shown to generate an antitumor effect, eliminating established tumors (Fujii et al., 1996; Larchian et al., 2000). Gueguen et al obtained a panel of CTL clones

that can specifically lyse bladder tumor cells in a MHC class I-restricted fashion by stimulating blood lymphocytes with the B7-1 gene transfected bladder carcinoma cells. In bladder cancer, increased effort has been focused on modification of tumor and/or host immunogenicity by transferring cytokines genes, namely cytokine gene therapy. This will be discussed in detail in the section 4 of Introduction.

1.3.3.2 Corrective gene therapy

The goal of corrective gene therapy is to suppress the malignant phenotype of cancer cells or to restore the normal function of aberrant cells. The mutational loss of tumor suppressor genes and activation of oncogenes are important steps in malignant cell transformation. Under physiological conditions, both cell division enhancing oncogenes and cell division inhibiting tumor suppressor genes are in balance. Various mutations for both classes of genes have been reported for bladder cancer (Table 1.5).

Mutations in tumor suppressor genes can shift the balance towards cell division. Transfer of the wild type genes into the cells may restore the cell cycle and apoptosis control. P53 was selected as the first target for this therapeutic strategy to treat various malignancies because the expression of this gene is altered in more human cancers than any other known gene. Preclinical studies with adenovirus containing a wild-type p53 construct have shown that p53 transduction induces apoptosis and decreases cell proliferation in a number of cancer cell lines and *in vivo* as well (Nielsen et al., 1997; Spitz et al., 1996; Ohashi et al., 1999). In bladder cancer, the adenovirus-mediated transduction of wild-

type p53 resulted in dose-dependent growth inhibition of bladder cancer cells *in vitro* (Irie et al., 2001). Shirakawa et al reported Ad-CMV-p53 induced higher levels of p53 protein and mRNA in the drug-resistant bladder cancer cell lines than in the parental cell line and, consequently, higher levels of p21 and Bax mRNA, which resulted in higher percentages of G (1) cell-cycle arrest and apoptosis (Shirakawa et al., 2001).

Table 1.5: Common oncogene and tumor suppressor gene mutation in bladder cancer (Peter et al., 2002)

Gene	Physiology function	Result of mutation
Rb	Tumor Suppressor gene	Inactivation
p53	Tumor Suppressor gene	Inactivation
p16	Tumor Suppressor gene	Inactivation
p21	Tumor Suppressor gene	Inactivation
c-myc	Oncogene	Over activation
mdr-1	Oncogene	Over activation
c-erb-2	Oncogene	Over activation
Ras	Oncogene	Over activation

The transfer of retinoblastoma (Rb) is also a common target for gene therapy. The Rb tumor suppressor gene is inactivated in at least 25%-50% of bladder cancers. Xu et al. used adenovirus to transfect Rb-defective human bladder cancer cell lines 5637 and HT 1376 cells. The transfected cells demonstrated morphological changes as well as growth inhibition in a dose-and cell-type-dependent fashion (Xu et al., 1996).

1.3.3.3 Cytotoxic gene therapy

Cytotoxic gene therapy implies the selective destruction of cancer cell or cancer-bearing tissue. As the cells themselves generate the toxic product leading to their death, these

approaches are often called suicide gene therapy. Several suicide systems are under investigation in an attempt to demonstrate a drug-induced killing of the cancer. Herpes simplex virus thymidine kinase/ganciclovir (HSV-tk/GCV) is one of the best established of these systems. Ganciclovir (GCV) is an acyclic nucleoside analogue that is not normally metabolized by mammalian cell thymidine kinase. However, HSV-tk is able to monophosphorylate the relatively non-toxic prodrug GCV. The product is subsequently metabolized by endogenous mammalian kinases into ganciclovir triphosphate, which is a purine analog that competes with normal nucleotides and can inhibit DNA polymerase and thus lead to cell death (Matthews et al., 1988; Moolten et al., 1986; Samejima et al., 1995). In subcutaneous bladder cancer model, HSV-tk/GCV system has shown dramatic killing against cancer cell (Sutton et al., 1997; Freund et al., 2000). A potential problem for this system is the issue of tissue specificity. Ideally, the promoter used in HSV-tk/GCV system should either be from a cancer-specific gene or from a tissue specific gene.

Recently, attenuated, replication-competent herpes simplex virus (HSV) mutants such as G207 and NV1020 are attracting interest because of their ability to replicate within and kill tumor cells while remaining of low pathogenicity to normal tissue. G207 is genetically engineered oncolytic virus based on wild-type herpes simplex type-1. The key features of G207 include the deletion of both gamma (1) 34.5 genes and inactivation of ICP6 (ribonucleotide reductase) allows G207 to selectively replicate within tumor cells (Mineta et al., 1995; Yazaki et al., 1995). NV1020 is another attenuated recombinant herpes virus with deletions of the HSV joint region, with deletion of only one copy of the

gamma (1) 34.5 gene, and with the ICP6 gene intact (Delman et al., 2000; McAuliffe et al., 2000). Studies showed both these viruses were effective at infecting, replicating within, and achieving subsequent cell lysis for bladder cancer cells both *in vitro* and *in vivo* with a single intravesical instillation (Cozzi et al., 2001; Oyama et al., 2000). G207 and NV1020 have potential for intravesical treatment of human bladder cancer.

1.3.3.4 Anti-sense oncogene therapy

As the activation of oncogenes and mutational loss of tumor suppressor genes are important steps in malignant cell transformation, anti-sense oncogene therapy has been designed to inactivate oncogenes, reverse the malignant phenotype, inhibit tumor and decrease tumorigenicity. Several strategies have been used: including anti-sense oligonucleotides and anti-oncogene ribozymes. Anti-sense oligonulceotides specifically inhibit the activities of various oncogenes and proto-oncogenes, presumably by binding to mRNA and inducing translation arrest (Mizutani et al., 1994; Li et al., 1996). Anti-oncogene ribozymes are RNA molecules that exhibit specific catalytic activities. It can destroy RNA translation templates for "unwanted" gene products. Hammerhead ribozymes are one sub-group of ribozymes which can perform true enzymatic reactions and are named for their hammerhead-like structure (Sioud et al., 1999; Bi et al., 2001). Irie et al demonstrated intralesional injection of an adenovirus expressing an anti-H-ras hammerhead ribozyme resulted in significant antineoplastic effects in a dose-dependent fashion in a murine subcutaneous bladder cancer model (Irie et al., 1999).

1.4 CYTOKINE GENE THERAPY OF CANCER

1.4.1 Introduction

Cytokines have been shown to play an important role in the regulation of the host antitumor immune response and the direction of the maturation, activation and migration of the inflammatory cells. But the clinical application of cytokines is hampered because of the severe side effects associated with the systemic administration of cytokine. For TNF-α, for example, the maximal tolerated dose in human being (10 μg/kg) is about 40-fold less than the doses required to generate a significant anti-tumor response in mice (400μg/kg)(Asher et al., 1987). Additionally, their effectiveness is also decreased by the rapid elimination and short half-life when they are delivered systemically (Rosenberg et al., 1989; Lotze et al., 1985). Thus cytokine gene therapy is designed to circumvent these problems by transfecting the cytokine gene into tumor or carrier cells that will express the cytokine at the primary tumor site, mimicking paracrine cytokine release *in vivo* and enhancing the induction of tumor-specific immune response without many of the troublesome systemic side effects.

1.4.2 Modality of gene transfer for cytokine gene therapy

Depending on the approach of gene transfer, cytokine gene therapy can be divided into two general types: *ex vivo* transfection and *in vivo* tranfection (or *in situ* transfection). For *ex vivo* transfection, tumor cells are transfected with cytokine gene *ex vivo*, irradiated,

and injected to the host. The tumor cells transfected can be autologous or allogeneic. The use of autologous tumor cells requires that the patient's tumor be surgically removed. This strategy has the advantage that the patient's own tumor cells have the greatest chance to vaccinate against the spectrum of relevant tumor antigens both shared and unique to the individual. The strategy indeed has been shown to have the ability of inducing dramatic immune responses in experimental animals against parental unmodified tumor cells (Saito et al., 1994; Fearon et al., 1990).

But the limitations in the ability to harvest, transfect gene ex vivo and re-inject the autologous tumors on a patient-by-patient basis raise questions about the feasibility of this strategy for clinical application. In contrast, the use of allogeneic tumor cells (a single standardized transduced cells line) is much less labor-intensive and time-consuming. But its efficacy depends on whether the transduced cells share antigens with the patient's tumor. For *in vivo* transfection, the cytokine gene is directly transferred to the tumor cells *in vivo*. No *ex vivo* tumor cell tranfection and re-injection are required. Many studies have been carried out to evaluate the efficacy of this strategy (Saffran et al., 1998; Lee et al., 1994).

1.4.3 Target cells of gene transfer in cytokine gene therapy

Several types of cells have been used as targets for cytokine gene transfer of *ex vivo* gene transfection approach. For the most part, the cytokine gene therapy of cancer has involved the transfer of cytokine gene to tumor cells. Theoretically, this approach has advantages in that expression of the cytokine will be occurring in the same

microenvironment as expression of tumor antigens, which should facilitate a heightened immune response. A number of studies have shown that the direct cytokine gene transfer to tumor cells renders them vulnerable to immune attack, enhancing the ability of the tumor to initiate a protective immune response.

Recently, interest has also extended to transfection of other cell types, such as endothelium cells, dendritic cells, lymphocytes and fibroblast. Endothelial cells have been chosen as the targets for cytokine gene transfer because they function not only as a vascular framework for the intravascular delivery, but also as a source of cytokines that influence the growth and differentiation of neighboring vascular and parenchymal cells. The advantage of these cells is that they maintain their capacity to proliferate and offer a potential renewable and expandable source of therapeutic gene production at sites of tumor angiogenesis. Su et al injected highly metastatic human breast cancer cell line MDA-MB-435 with the genetically manipulated endothelial cells expressing IL-1α or IL-2 into the mammary pad of nude mice (Su et al., 1994). The results showed that cytokine-expressing endothelial cells not only inhibited the tumorigenesis of MDA-MB-435 cells, but also abrogated the formation of metastasis.

Dendritic cells (DC) are the most effective antigen presenting cells and are critical for the induction of primary, cell-mediated immune responses due to their ability to acquire antigen in the peripheral tissue and process, transport, and present it to naïve or memory-antigen-specific T cells in the secondary lymphoid organs (Steinman et al., 1991; Stingl et al., 1995). As the DC's effectiveness in presenting tumor antigen is mainly modulated

by cytokines, cytokine gene transferred DC may be able to stimulate potent and Ag-specific anti-tumor immune response. Nishioka et al showed intratumoral injection with IL-12 gene-modified bone marrow-DCs resulted in regression of day 7 established weakly immunogenic tumors (MCA205, B16, and D122) (Nishioka et al., 1999). Miller et al also found that intratumoral administration of adenoviral interleukin 7-transduced dendritic cells (DC-AdIL-7) resulted in complete tumor regression in two lung cancer models (Miller et al., 2000). Furthermore, all the DC-AdIL-7-treated mice completely rejected a secondary rechallenge, whereas the AdIL-7-treated mice had sustained antitumor effects in only 20-25% of the mice.

1.4.4 Cytokines used in gene therapy of bladder cancer

The direct modification of tumor cells using cytokine genes to increase host immunity has been studied intensively in experimental animals over the last decade. A number of cytokine genes have been demonstrated to have the ability of reducing tumorigenicity of cancer cells by stimulating localized inflammatory and/or immune response, including TNF-α, granulocyte-macrophage-colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-7, IL-12, IL-18, interferon gamma, etc. However, some cytokines also failed to show any properties in inducing immunity or deceasing tumorigenicity after they were transferred to tumor cells, such as IL-5 (Kruger-Krasagakes et al. 1993). Transforming growth factor-beta1 (TGF-β1) modified Meth A sarcoma cells are even much more tumorigenic than parental cells (Chang et al., 1993; Torre-Amione et al., 1990). This strategy has been tested in many cancer models and the clinical trials are under going in various cancer patients such as

melanoma, renal cell carcinoma, colon cancer, lung cancer, brain tumor and lymphoma. In spite of the unique anatomy structure of bladder and the established role of intravesical immunotherapy with BCG for the treatment of bladder cancer, only a few cytokine gene therapy studies have been reported in bladder cancer, including *ex vivo* cytokine gene transfer strategy (vaccination) (Table 1.6) and *in situ* cytokine gene transfer strategy (Table 1.7).

Table 1.6: Review of the studies on cytokine gene therapy of bladder cancer (*ex vivo* cytokine gene transfer strategy)

Cytokine& Reference	Strategy	Vector	Cell line/ Mouse strain	Animal model	Result
IL-2 (Larchian et al., 2000)	Vaccination using IL-2 followed by B7-1 gene modified tumor cell	Cationic Liposome (DMRIE /DOPE)	MBT2/ C3H	Ortho-topic	75% cure in IL-2/B7/B7 group.
IL-2 and IFN-γ (Connor et al., 1993)	Vaccination with IL-2 or IFN-γ modified cells	Retro-virus	MBT2/ C3H	Ortho-topic	60% cure in IL-2 group. The therapeutic effect of IFN-γ is minimal.
IL-12 (Yamanaka et al., 1999)	Parental cells mixed with MBT2/IL-12 cells were subcu-taneously injected.	Calcium phosphate	MBT2/ C3H	Subcu-taneous	100% rejection of parental tumor.

1.5 CLINICAL TRIAL OF BLADDER CANCER GENE THERAPY IN THE NATIONAL INSTITUTE OF HEALTH

To date, only a few clinical trials have been carried out or are on going in NIH (Table 1.8). All of these trials use viral-vectors. No cytokine gene therapy clinical trial has been done although this strategy has an apparent theoretical advantage in bladder cancer.

Table 1.7: Review of the studies on cytokine gene therapy of bladder cancer (*in situ* cytokine gene transfer strategy)

Cytokine& Reference	Strategy	Vector	Cell line/ Mouse strain	Animal model	Result
IFN-β (Izawa et al., 2002)	Intratumoral injection with Ad-IFN-β	Adeno-virus	253JB/ BLAB	Subcu-taneous	Ad-mINF-β suppress established tumor significantly. No cure.
IFN-γ (Shiau et al., 2001)	Intratumoral injection with retro-IFN-γ supernatant	Retro-virus	MBT2/ C3H	Subcu-taneous	Ad-mINF-γ suppress established tumor. Significantly. No cure.
IL-2 (Horiguchi et al., 2000)	In situ gene transfer (intravesical instillation of lipoplex)	Cationic Liposome (DMRIE /DOPE)	MBT2/ C3H	Ortho-topic	40% cure in IL-2 group.
IL-12 (Chen et al., 1997)	Intratumoral injection with IL-12	Adeno-virus	MB49/C 57	Subcu-taneous	100% cure in IL-12 group.

Table 1.8: Clinical trial of bladder cancer gene therapy in NIH

Trial title	Institution	Status	Strategy	Vector	Reference
Phase I Study of Adenovirus p53 in Patients With Locally Advanced or Metastatic Bladder Cancer	University of Texas - MD Anderson Cancer Center	Closed	Corrective gene therapy	Adenovirus	Pagliaro et al., 2001
PV701 in Treating Patients With Advanced or Recurrent Peritoneal Cancer	Memorial Sloan-Kettering Cancer Center	Active	Cytotoxic gene therapy	PV701, a replication-competent strain of Newcastle disease virus	Not published yet

1.6 STUDY DESIGN

The high tumor recurrence and progression rate after TURBt plus intravesical BCG therapy highlights the necessity of exploring other therapeutic approaches. Cytokine gene therapy is an attractive strategy for bladder cancer because: (a) The unique and isolated environment of the bladder makes it an optimal candidate for cancer gene therapy. (b) Superficial bladder cancer patients who have undergone TURBt have a low tumor burden and like patients with carcinoma in situ (CIS), their cancer cells are accessible to intravesical administration of viral or non-viral agents. Theoretically, these patients are expected to especially benefit from gene therapy. (c) The lack of response to BCG immunotherapy has been correlated with a reduction in cytokine production after BCG induction. This could reflect that in these patients the reduced cytokine levels may contribute to the poorer response to BCG therapy. Thus direct cytokine gene transfer could be an effective way to boost the immune system in these patients. (d) A number of studies have shown that introduction of cytokine genes into tumor cells can stimulate antitumor immune responses and lead to significant tumor suppression in various cancer models. Bladder cancer, which is known to well respond to immunotherapy with BCG, is a potential target for cytokine gene therapy.

In recent years, several cytokine genes have been tested as candidates for cytokine gene therapy in bladder cancer such as interleukin-2, interferon-β and interleukin 12 with encouraging results. To date however, tumor necrosis factor-α (TNF-α) has not been evaluated by direct gene transfer in bladder cancer although TNF-α protein possess

potent multiple anti-tumor effects and the data in laboratory and clinical studies demonstrated its effectiveness in inhibiting bladder cancer.

The recent report of the induction of leukemia-like symptoms in children treated with retroviruses and the limited transfection of urothelial cells in the bladder by adenoviruses in spite of the presence of human coxsackie-adenovirus receptor (hCAR) highlight some of the problems associated with viral gene therapy. We therefore explored a novel non-viral transfection system comprising DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) plus methyl-beta-cyclodextrin solubilized cholesterol (MBC) which we have previously demonstrated to efficiently transfect urothelial cells both *in vitro* and *in vivo* using the β-galactosidase reporter gene (Lawrencia et al., 2001). The addition of MBC, a complex which may be capable of donating cholesterol to the cell membranes and affect the fluidity/ permeability of the cell membrane, to DOTAP can improve the transfection efficiency by 3.8 fold (Lawrencia et al., 2001).

The aim of this study is to evaluate the feasibility and efficacy of cytokine gene therapy using DOTAP plus MBC as gene deliver system and mouse TNF-α gene as therapeutic gene. To mimic clinical condition, an orthotopic murine model of bladder cancer was chosen and the intravesical approach of therapeutic gene applied.

CHAPTER TWO MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and biological reagents

Absolute Alcohol	(Merck Darmstadt, Germany)
Acetone	(BDH Lab Supplies, England)
Agarose	(Promega, Madison, WI)
Annexin-V-FLUOS	(Roche Diagnostics, Mannheim, Germany)
Aprotinin	(Sigma, St. Louis, MO)
ß-mercaptoetharol	(Sigma, St. Louis, MO)
BSA (100×)	(Promega, Madison, WI)
Buffer J (10×)	(Promega, Madison, WI)
Buffer K (10×)	(Promega, Madison, WI)
Calf Intestinal Alkaline Phosphatase (CIAP) (1 uint/µl)	(Promega, Madison, WI)
Cholesterol-Water Soluble	(Sigma, St. Louis, MO)
DNA Ladder (1 Kb)	(Promega, Madison, WI)
Deoxyribonucease I	(Invitrogen, Carlsbad, CA)
Diethyl Pyrocorbonate (DEPC)	(Sigma, St. Louis, MO)
DNA Polymerase I Large (Klenow) Fragment	(Promega, Madison, WI)
DNA Ligase	(Promega, Madison, WI)

Dnase Buffer	(Gibco BRL, Rockville, MD)
dNTP (100 mM)	(Promega, Madison, WI)
EcoR I (10 units/ul)	(Promega, Madison, WI)
Ethidium Bromide	(Sigma, St. Louis, MO)
Eosin	(Sigma, St. Louis, MO)
Ethylene Diamine-Tetra-acetic Acid (25 mM)	(Gibco BRL, Rockville, MD)
Fetal Bovine Serum (FBS)	(HyClone, Logan, UT)
Formaldehyde	(Sigma, St. Louis, MO)
Glutaraldehyde (25%)	(Sigma, St. Louis, MO)
Glycerol	(Merck Darmstadt, Germany)
Hepes Buffer Solution (1M)	(Gibco BRL, Grand Island, NY, USA)
Hematoxylin	(Sigma, St. Louis, MO)
Igepal CA 630	(Sigma, St. Louis, MO)
Ligase Buffer (10×)	(Promega, Madison, WI)
Isopropanol	(Sigma, St. Louis, MO)
LB Medium (Low Salt)	(NUMI, Sinagpore)
LB Medium (Normal Salt)	(NUMI, Sinagpore)
Load Dye (6×)	(Promega, Madison, WI)
L-glutamine (200mM)	(Sigma, St. Louis, MO)
Magnesium Chloride (25mM)	(Promega, Madison, WI)
Magnesium Chloride	(Merck Darmstadt, Germany)

Magnesium sulfate	(Merck Darmstadt, Germany)
Mineral Oil	(Sigma, St. Louis, MO)
Mounting Medium	(Surgipath, Canada)
N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP)	(Roche Diagnostics, Mannheim, Germany)
O-Nitropnenyl-ß-Galactopyranoside (ONPG)	(Sigma, St. Louis, MO)
pBud.CE4.1	(Invitrogen, Carlsbad, CA)
pCMVlacZ	(Clontech, Pala Altho, LA)
Penicillin-Streptomycin Solution	(Sigma, St. Louis, MO)
Penicillin	(Sigma, St. Louis, MO)
Pepstatin	(Sigma, St. Louis, MO)
Phenyl Methyl Sulforyl Fluoride	(Sigma, St. Louis, MO)
Phosphate Buffered Saline (×10)	(NUMI, Singapore)
Potassium Ferricynaide	(Sigma, St. Louis, MO)
Potassium Ferrocynaide	(Sigma, St. Louis, MO)
Potassium Chloride	(Merck Darmstadt, Germany)
Propidium Iodide (PI)	(Sigma, St. Louis, MO)
Taq DNA Polymerase (5U/ul)	(Promega, Madison, WI)
Thermophilic DNA Poly 10×Buffer	(Promega, Madison, WI)
Reverse Transcriptase	(New England Biolab, Beverly, MA)

Reverse Transcriptase Buffer	(New England Biolab, Beverly, MA)
Rnase A	(Sigma, St. Louis, MO)
RNAsin	(Promega, Madison, WI)
RPMI 1640	(NUMI, Sinagpore)
Sac I (10 units/ul)	(Promega, Madison, WI)
Sca I(10 units/ul)	(Promega, Madison, WI)
Sodium Acetate Buffer Solution (3M)	(Sigma, St. Louis, MO)
Sodium Azide	(Sigma, St. Louis, MO)
Sodium Carbonate	(Merck Darmstadt, Germany)
Sodium Chloride	(Merck Darmstadt, Germany)
Sodium Dihydrogen Phosphate Dihydrate	(Merck Darmstadt, Germany)
Sodium Fluoride	(Merck Darmstadt, Germany)
Sodium Hydrogen Phosphate Anhydrous GR	(Merck Darmstadt, Germany)
Sodium Hydroxide	(Merck Darmstadt, Germany)
Trypan Blue	(Sigma, St. Louis, MO)
TRIzol™	(Life Technologies, Inc. Gaithersburg, MD)
X-gal	(Bio-Rad, Herclues, CA)
Xylene	(Merck Darmstadt, Germany)
Zeomycin (100mg/ml)	(Invitrogen, Carlsbad, CA)

2.1.2 Commercial kits

ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit	(Applied Biosystems)
Endofree QIAGEN Plasmid Purification Kit	(QIAGEN)
Mouse TNF-α ELISA Kit	(Endogen, Pierce Biotechnology, Inc.Rockford, IL)
Micro BCA Protein Assay System	(PIERCE, Rockford, IL)
QIAquick Gel Extraction Kit	(QIAGEN)
Wizard Plus SV Minipreps DNA Purification System	(Promega, Madison, WI)

2.1.3 Antibodies

FITC anti-mouse CD3	(BD PharMingen,USA)
Hamster anti-mouse B7-1 mAb	(BD Pharmingen,USA)
Hamster anti-mouse ICAM I mAb	(BD Pharmingen,USA)
Hamster anti-mouse Fas mAb	(BD Pharmingen,USA)
Mouse anti-mouse H-2K^{b}/H-2D^{b} MHC I mAb	(BD PharMingen,USA)
Mouse anti-mouse I-A^{b} MHC II mAb	(BD Pharmingen,USA)
PE anti-mouse CD8a	(BD PharMingen,USA)
PE anti-mouse NK cells/2B4	(BD PharMingen,USA)
PE anti-mouse CD4	(BD PharMingen,USA)
Purified mouse IgG2a, κ isotype standard	(BD Pharmingen,USA)

Purified biotin polyclonal hamster IgG	(BD Pharmingen,USA)
Purified rat IgG2a, κ isotype standard	(BD Pharmingen,USA)
Rabbit anti-mouse Ig-FITC	(Dako, Danmark)
Rabbit anti-hamster IgG-FITC	(BD Pharmingen,USA)

2.1.4 Oligonucleotide primers

Mouse TNF-alpha Primer	(Research Biolabs, Singapore)
Mouse GAPDH primer	(NUMI, Singapore)
Mouse Fas Primer	(NUMI, Singapore)
Oligo dT	(NUMI, Sinagpore)
pBud CMV Forward Primer	(Invitrogen, Carlsbad, CA)
pBud CMV Reverse Primer	(Invitrogen, Carlsbad, CA)

2.1.5 Cell line and mouse strain

The murine transitional cell carcinoma cell line MB49 was obtained from Dr. Timothy Ratliff (University of Iowa, USA).

Female C57BL/6 mice (4-6 weeks) were purchased from the Laboratory Animal Center (LAC, Sembawang, Singapore). All animals were kept at the Animal holding Unit (National University of Singapore) and allowed to acclimatize for 1 week prior to the

study. All animals were kept in a photo-periodic room with artificial light. Animals were fed on standard laboratory chow and water was given *ad libitum*. The room temperature was maintained at 21°C with a relative humidity of about 50%. Mice ears were nicked for identifying.

2.2 METHOD

2.2.1 Cell culture

MB49 cells were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 50 U/ml penicillin and 0.05 mg/ml streptomycin at 37°C in a humidified chamber with 5% CO_2. The prepared medium was stored at 4°C. All the cell lines were grown in plastic culture flasks (Zellkultur-Produkte, Switezerland). For maintenance, cells were passaged every 4-6 days by diluting 1 ml cell suspension in 11 ml fresh medium. The cells were dislodged by scraping with a cell scraper (Nunc, Denmark).

2.2.2 *In vitro* transfection optimization using reporter gene pCMVlacZ

Briefly, $2x10^5$ MB49 cells were plated per well in 6-well tissue culture plates 24 hours before transfection. After three washings with blank RPMI 1640, 1 ml of blank RPMI 1640 medium was added to the cells. To prepare the transfection reagent, 20 μg of DOTAP and 40μg of methyl-β-cyclodextrin solubilized cholesterol(MBC) was diluted to

a final volume of 87 µl with 20mM Hepes buffer and different amounts (1µg, 2.5µg, 5µg, 7.5µg, 10µg) of pCMVLacZ were diluted to a final volume of 75 µl in 20mM Hepes buffer. The plasmid solution was added to the DOTAP/MBC mixture and incubated at room temperature for 15 minutes. Thereafter, this transfection reagent was added into the medium and incubated with MB49 cells for 2 hours at 37 °C. Then the medium was removed and the cells were washed, cultured for 48 hours in 3 ml of completed RPMI 1640. X-gal staining and ONPG assay was performed to access the transfection efficiency and galactosidase activity 48 hours after transfection.

X-gal staining

In brief, cells were washed 3 times with 1×PBS and fixed in 0.05% glutaraldehyde (GTA). The cells were then stained with X-gal solution (1mg/ml in 5mM potassium ferricynaide and 5mM potassium ferrocynaide, 2mM magnesium chloride, in Tris buffer (pH 8.5) for 4 hours. Transfection efficiency was determined by counting the number of blue colonies relative to the total number of cells in five quadrants at ×100 magnification. Transfections were performed in duplicates and repeated three times.

O-Nitropnenyl-ß-Galactopyranoside (ONPG) assay for transfection

After 48 hrs the cells were washed three times with 1×PBS, scraped and transferred to Eppendoff tubes. The cells were lysed with 150µl of lysis working solution. One ml lysis working solution consists of 1 ml lysis buffer, 10 µl Igepal CA 630, 0.2 µl Aprotinin(10 mg/µl), 10 µl PMSF(100mM) and 1 µl Pepstatin (1 mg/ml). Lysis buffer consists of 10 mM Tris-Hcl, 5mM EDTA, 150 mM Nacl and 50 mM NaF.

The protein content of the lysates was measured by the Micro BCA Protein assay system with bovine serum albumin as a standard. Fifty μg of cell protein lysates were assayed in a reaction mixture containing 4mg/ml of ONPG (dissolved in Z buffer) and incubated at 37°C for 1 hour. Z buffer consist of 60mM Na_2HPO4, 40mMNaH_2PO4, 10mM KCl, 1 mM MgSO4.7H2O, 50mM ß-mercaptoetharol and was adjusted to PH=7.0. Reactions were stopped by the addition of 500ul 1M Na_2CO_3 and the optical densities (OD) λ=420 were measured.

2.2.3 Construction and cloning of mouse TNF-α encoding plasmid

2.2.3.1 Preparation of insert fragment for cloning

The mouse TNF-alpha cDNA was digested from pCIneo-mTNF-α using EcoRI. Two μg of pCIneo-mTNF-α, 5 μl of universal buffer, 5 μl of BSA (10 x), 2 μl of EcoR I (24 units/ul) in a total volume of 50 μl was incubated at 37 °C for 1 hour.

2.2.3.2 Preparation of vector for cloning

This vector was chosen because it has two multiple cloning cites. Two μg of pBud.CE4.1 was incubated with 2 μl of Sca I(10 units/ul), 5 μl of buffer K, 5 μl of BSA (10 ×) in a total volume of 50 μl was incubated at 37 °C for 1 hour.

Thereafter 2 μl of Calf Intestinal Alkaline Phosphatase (CIAP) (1 uint/μl), 10 μl of 10x CIAP buffer and 38 μl water were added to the above digested DNA to make up a total volume of 100 μl and incubated at 37 °C for 1 hour.

2.2.3.3 Gel electrophoresis for insert DNA and vector and gel extraction

Insert DNA fragment and vector were separated by electrophoresis through 0.7% of agarose gels that contained 0.5 μg/ml ethidium bromide. To extract and purify the insert DNA and vector, the DNA was excised from the agarose gel with a clean, sharp scalpel and weighed, then purified with QIAquick gel extraction kit. Briefly, 3 volumes of buffer QG were added to the 1 volume of gel slices and incubated at 50°C for 10 minutes. After checking the color of the mixture was yellow, 1 gel volume of isopropanol was added to the samples. To bind DNA, the samples were applied to the QIAquick column and centrifuged for 1minute. The columns were washed with 0.75 ml of buffer PE and centrifuged for 1 min. The flow-through was discarded and the columns were centrifuged for an additional 1minute at 13,000 rpm. Thereafter, 30 μl of buffer EB was added to the center of QIAquick columns and centrifuged for 1 minute at 13,000 rpm.

2.2.3.4 Filling in reaction, gel electrophoresis and gel extraction for insert DNA

To convert TNF-α from 3' overhang obtained from EcoR I digestion to blunt end, 5 μl of Klenow buffer (10×), 5 μl of BSA (10 ×), 1 μl of dNTP (2mM), 1 μl Klenow DNA polymerase and 8 μl sterile water were added to the above 30 μl purified insert DNA and incubated at room temperature for 20 minutes. The product was separated by electrophoresis through 0.7% of agarose gels that contained 0.5 μg/ml ethidium bromide and extracted using QIAquick gel extraction kit.

2.2.3.5 Ligation of vector and insert DNA

To obtain the optimal ratio of vector to insert DNA, a 1:3 molar ratio of pBud.CE4.1: TNF-α were incubated with 2 µl of T4 DNA ligase and 2 µl of 10 x ligase buffer at 4 °C overnight.

2.2.3.6 Preparation of competent cells

A single colony of *E.coli* (DH5α strain) was used to inoculate a starter culture consisting of 10ml LB medium. The culture was grown for 8 hours at 37°C with vigorous shaking at 200rpm. To set up the large culture, 2ml of the starter culture was used to inoculate 200ml of LB medium in a sterile 1litre flask. The culture was grown at 37°C with vigorous shaking to an OD_{600} around 0.45. The culture was then aliquoted into four 50ml centrifuge tubes and centrifuged at 3000x g (Beckman JA-14 rotor), 4°C for 10 minutes. The supernatant was poured off and each pellet was resuspended in 2ml of ice-cold 0.1M $CaCl_2$ solution and left overnight at 4°C. Cells were then mixed with 15% glycerol and aliquoted into 1m cryo tubes and stored at -70°C.

2.2.3.7 Transformation

After ligation, 100 µl aliquot of rapidly thawed competent cells was dispensed into each Eppendoff tube. Tubes were gently swirled to mix contents and stored on ice for 30 minutes. Tubes were then transferred to a 42 °C waterbath and shocked for 2 minutes. Thereafter 0.5 ml of LB medium (low salt) was added and the cells were placed in a 37°C waterbath for 30 minutes. Then the cells were pelleted at 14,000 for 2 minutes and the supernatant was removed. The transformed cells were resuspended in 30 µl LB medium

(low salt), then transferred to LB (low salt)/Zeomycin (25 μg/ml) containing agar plates and gently spread using a sterile bent glass rod. The plates were inverted and incubated at 37°C overnight.

2.2.3.8 Culture of colony and miniprep of plasmid

The colonies were randomly picked up and each of them was inoculated into 3 ml of LB medium (low salt) /Zeomycin (25 μg/ml) by a sterile yellow tip. The cultures were allowed to grow overnight at 37°C before they were pelleted (1.5 ml culture from each tube) for Minipreps using Wizard Plus SV Minipreps DNA Purification system.

Briefly, the pellets were resupended with 250 μl cell resuspension solution. Then 250 μl cell lysis solution was added to each sample and the samples then were inverted for 4 times to mix. After adding 10 μl alkaline protease solution, the samples were inversed for 4 times and incubated at room temperature for 5 minutes. Thereafter 350 μl neutralization solution was added into each the samples. The samples were centrifuged at top speed for 10 minutes at room temperature. The cleared lysates were then decanted into spin columns. After centrifugation of spin column, 750 μl of washing solution (ethanol added) were added to each column and then centrifuged. At last, 100 μl of nuclease-free water was added to the spin columns and the columns were transferred to sterile 1.5 microcentrifuge tubes for centrifugation to elute the plasmid DNA. The DNA was stored at -20 °C.

2.2.3.9 Positive colony screening and streaking

To confirm the presence of insert fragment mTNF-α cDNA and its orientation, 5 μl plasmid DNA obtained from Minipreps was digested by 0.5μl Sac I with 1 μl 10x buffer J, 1 μl BSA (10x) in a total volume of 10 μl at 37°C for 1 hour. Then the digested plasmid was separated by electrophoresis through 0.7% of agarose gels that contained 0.5 μg/ml ethidium bromide.

The cultures from positive colonies were streaked on LB (low salt)/Zeomycin (25 μg/ml) containing agar plates again. A single colony was picked up from each plate and cultured as described above. The miniprep was carried out, followed by Sac I screening again. The confirmed positive cultures were stocked at -80 °C in 15% of Glycerol.

2.2.3.10 Sequencing of insert fragment mTNF-α

The mTNF-α cDNA was confirmed by sequencing.

Quantitation of plasmid concentration: The concentration of pBud-TNF-α obtained for minipreps was determined by measuring the absorbance (260nm and 280nm) using spectrophotometer (Shimadzu, UV-1201, Japan). One OD at 260 nm equals to 50 μg/ml DNA.

Performing cycle seqencing: ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit was used to perform cycle sequencing. Five hundred ng pBud-TNF-α was mixed with 8 μl of terminator ready reaction mix, 3.2 pmol CMV Forward primer in a

total volume of 20 µl. The PCR reaction was consisted of 30 cycles of denaturation at 96°C for 30 seconds, primer annealing at 50°C for 30 seconds and DNA synthesis at 60°C for 30 seconds. Another reaction using CMV Reverse primer was run concurrently.

Purification of extension product: Eighty µl of ethanol/sodium acetate solution consisting of 3 µl of 3 M sodium acetate, PH 4.6, 62.5 µl of non-denatured 95% ethanol and 14.5 µl of deionized water was added to each PCR product and vortexed. The tubes were left at room temperature for 15 minutes to precipitate the extension products. The tubes were then centrifuged for 20 minutes at the maximum speed. The supernatant of each sample was carefully aspirated and discarded. Thereafter, 250 µl of 70% ethanol was added to each tube and mixed. The tubes were centrifuged for 5 minutes at the maximum speed. The supernatant was aspirated and the samples were put in a heat block at 75°C until they were dry.

Automated sequencing: The dry tubes containing samples were then sent to the sequencing facility at NUMI for automated sequencing.

2.2.3.11 Maxiprep of pBud-TNF-α for transfection

The Endofree QIAGEN plasmid purification kit was used for the isolation of pBud-TNF-α. A single colony was used to inoculate a starter culture consisting of 2-5ml LB medium containing 50µg/ml of ampicillin. The culture was grown for 8 hours at 37°C with vigorous shaking at 200rpm. To set up the large culture, the starter culture was diluted 1/500 to 1/1000 into 200ml of LB medium containing 25µg/ml of zeomycin and the

culture was grown overnight at 37°C with vigorous shaking at 200rpm. The cells were harvested by centrifugation (6000 x g, Beckman JA-14 rotor, 4°C, 10 minutes) and the bacterial pellet was resuspended in 10ml of Buffer 1 (50mM Tris-HCL (pH 8), 10mM EDTA and 100μg/ml Rnase A). Bacterial cells were lysed with 10 ml of Buffer 2 (200mM of NaOH and 1% (w/v) SDS). After gentle mixing, 10 ml of Buffer 3 (3M potassium acetate pH 5.5) was added and the contents were thoroughly mixed by inversion. The cell debris and chromosomal DNA were removed by filtering through a QIAfilter cartridge. To remove endotoxins 2.5ml of ER buffer was added to the filtered lysated and incubated on ice for 30 minutes. The lysate was then loaded onto a pre-equilibrated QIAGEN column by gravity flow. The salt and pH conditions of the lysate and the selectivity of the QIAGEN resin ensure that only plasmid DNA binds. The QIAGEN-tip is then washed with Buffer QC (1M NaCl, 50mM MOPS, pH 7 and 15% isopropanol) to remove any nucleic acid binding proteins. The plasmid DNA was eluted with a high salt buffer QN (1.6M NaCl, 50mM MOPS, pH 7 and 15% isopropanol). The eluted DNA was percipitated with 10.5ml of isopropanol and the nucleic acids was pelleted by centrifugation (14000 x g, Beckman JA-20 rotor, 4°C, 30 minutes). The pellet was washed with 70% endotoxin free ethanol to remove residual salt and isopropanol the nucleic acids were pelleted by centrifugation (14000 x g, Beckman JA-20 rotor, 4°C, 10 minutes). The purified DNA was briefly air-dried and redissolved in 200ul of endotoxin free TE buffer (10mM Tris-HCL pH 7.5, 1mM EDTA pH 8.0).

2.2.4 *In vitro* TNF-α transfection

Briefly, $2x10^5$ MB49 cells were plated /well on 6-well tissue culture plates 24 hours before transfection. Transfection was carried out as described before except that 2.5μg pBud-TNF-α was used.

2.2.5 *In vitro* TNF-α expression

The culture medium was collected 48 hours after transfection from MB49 cells transfected with pBud-TNF-α and centrifuged at 4000 rpm for 10 min at 4°C and kept at -80°C for later bioassay. The TNF-α expression level was measured using the mouse TNF-α ELISA kit according to the manufacturer's direction.

Briefly, the supernatants were thawed to room temperature before performing the ELISA. The provided TNF-α protein standard was reconstituted in blank RPMI 1640. Fifty μl of standards and samples were added in the 96-microtiter plate in duplicate. After 2 hours of incubation at room temperature, the unbound substances were washed away with wash buffer. Then 100 μl of prepared Streptavidin-HRP solution was added to the plate and incubated for 30 minutes at room temperature. Following five washings, 100 μl of TMB substrate solution was added to the wells to incubate for 30 minutes. The enzyme reaction was stopped and colorimetric results were read at 450 nm using a microplate reader with a reference wavelength of 550 nm for the correction of optical imperfections in the microtiter plate. The sample values were calculated from the standard curve using curve-

fitting software GraphPad Prism 3.0. The supernatant collected from untransfected cells and pBud transfected cells served as controls.

2.2.6 Cell counting after transfection

To test whether pBud-TNF-α has an anti-proliferative effect against bladder cancer cells, the cell number of pBud-TNF-α transfected cells were counted and compared with that of parent cells and pBud transfected cells 48 hours after transfection. Aliquots of cell suspension were diluted with equal volumes of 0.1% trypan blue in the saline. The cells were harvested and counted. The experiment was carried out in duplicate for three times.

2.2.7 *In vitro* killing of bladder cancer cell line with pBud-TNF-α

2.2.7.1 Cell cycle analysis

Propidium iodide (PI) staining was performed to detect late apoptotic and dead cells with disrupted plasma and nuclear membrane. Ten thousand events were acquired in each sample. Pre-G1 cells were regarded as apoptotic cells.

Two days after transfection, the cells were washed twice with PBS-glucose filtered sample buffer. The cells were removed by cell scraper. The cells were then resuspended in 1 ml of sample buffer for cell counting. $1x10^6$ cells were taken from each sample for further fixing. The cells were centrifuged, and the supernatant was removed. For fixing, 1

ml ice-cold ethanol was added to the cell pellet drop by drop while vortexing the cells. The cells were fixed in the ethanol overnight at 4°C.

The fixed cell sample was vortexed and centrifuged at 12,000 rpm for 2 min before the 70% ethanol was poured off. Cells were gently resuspended in residual ethanol. 0.5 ml PI staining solution composed of 25 µl PI stock (1mg/ml), 57 Kunitz units Rnase A and 0.5 ml sample buffer was added to each sample. Samples were incubated in the dark at room temperature for at least 30 minutes. Analysis was carried out within 24 hour on a flow cytometry (Beckman Coulter Epics Elite ESP, USA).

2.2.7.2 Annexin V staining

To detect early apoptotic cells that expressed extracellular phosphatidylserine after pBud-TNF-α transfection, Annexin-V-FLUOS staining was carried out. Briefly, the cell was washed with 1×PBS for three times and flushed with 1ml of 1×PBS. After centrifugation at 7000 rpm for 3 minutes at 4 °C, the cell pellet was resupended in 100 µl incubation buffer containing 10 mM Hepes/NaOH (PH4), 140 mM NaCl and 5 mM $CaCl_2$. Then 2µl Annexin-V-FLUOS was added into the cells and incubated at room temperature for 10-15 minutes. Thereafter, 0.3 ml incubation buffer was added to each tube. The cells were filtered through 40µm mesh and sent for fluorescence-activated cell sorter (FACS).

2.2.8 Flow cytometric analysis for surface immuno-related molecules

The effect on up-regulating immuno-related molecule was examined by flow cytometry 48 hours after pBud-TNF-α transfection, the cells were washed, scraped and then transfered to tubes. After centrifugation, the cells were divided into desired tubes and resuspended in 100 μl 1% BSA in PBS. The cells were stained by 2 μl of purified mouse anti-mouse H-2K^{b}/H-2D^{b} MHC I monoclonal antibody (mAb), mouse anti-mouse I-A^{b} MHC II mAb, hamster anti-mouse B7-1 mAb, hamster anti-mouse ICAM I mAb and hamster anti-mouse Fas mAb and corresponding isotype-matched nonspecific control antibodies (purified mouse IgG2a, κ isotype standard, purified biotin polyclonal hamster IgG, purified rat IgG2a, κ isotype standard) for 20 minutes at 4°C . After washing, the cells were then stained by 2 μl of Rabbit anti-mouse Ig-FITC or anti-hamster IgG-FITC for 20 minutes at 4°C. The cells were washed again and fixed in 0.1% formaldehyde overnight at 4°C and analyzed by flow cytometry. The untransfected and pBud transfected cells are stained in the same way as controls.

2.2.9 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis of TNF-α expression *in vivo*

TNF-α mRNA expression level was determined by RT-PCR. Total RNA was isolated from mouse bladders (with no tumor orthotopically implanted) 2 days after one intravesical instillation of pBud-TNF-α using TRIzol^{TM} according to the manufacturer's

instruction. Total RNA extracted from mouse bladder which was transfected with pBud was served as control.

RNA extraction: A mouse bladder was homogenized with 1 ml of TRIzol^{TM} using a homogenizer (Heidolph DIAX900, Germany). The homogenized samples were incubated for 5 minutes at room temperature. Then 0.2 ml of chloroform was added into the 1 ml of TRIzol and mixed for 15 seconds, incubated at room temperature for 3 minutes followed by centrifugation at 12,000 rpm for 14 minutes at 4°C. Following centrifugation RNA remained at the colorless upper aqueous phrase. RNA was removed and precipitated by mixing with 0.5 ml of isopropanol and centrifugation at 12,000 rpm for 10 minutes at 4 °C. RNA pellet was washed in 75% ethanol and centrifuged at 7,500 rpm for 5 minutes at 4°C. Thereafter RNA was resuspended in 25 µl of RNase-free DEPC (0.1%v/v) water.

RNA yield: RNA yield was estimated by measuring the absorbance (260nm and 280nm) using spectrophotometer (Shimadzu, UV-1201, Japan). One OD at 260 nm equals to 40 µg/ml RNA.

Dnase treatment: One µg total RNA from each sample was treated by incubating with 1 µl of Dnase, 1 µl of 10× Dnase buffer, 0.5 µl of RNAsin and DEPC water in a total volume of 10 ul for 15 min at room temperature. The reaction was stopped by adding 1 µl of 25 mM EDTA to the above solution and incubating at 65°C for 10 minutes.

Complementary DNA synthesis: One µl of Oligo dT (3 pmol/µl), 0.4 µl of reverse transcriptase, 2.5 µl of 10× reverse transcriptase buffer, 0.25 µl of RNAsin, 1 µl of 25 mM nucleotide were added to the above reaction solution. The volume was increased to 25 µl with DEPC water and tubes incubate for 1 hour at 37°C. After adding 225 µl of TE buffer to the above mixture, the cDNA was stored at -20°C until further use.

Polymerase chain reaction (PCR): The Full-length of TNF-α coding sequence was amplified from cDNA by using primer 5′ -ATGAGCACAGAAAGCATGATC-3′(sense) and 5′-TCACAGAGCAATGACTCCAAA-3′(antisense). As a control, a 237-bp fragment of murine GAPDH was amplified from the same RNA preparation by using primer 5′-TTCACCACCATGGAGAAGGC-3′ (sense) and 5′-GGAATGGACTGTGGTCATGA-3′ (antisense). PCR was performed by incubating 2.5-5 µl of RT-mixture with 0.5µM of 3′ and 5′ gene specific primer, 0.2 mM of dNTP, 1 × Mg free buffer and 1.5 mM MgCl2 in a final volume of 25 µl for 40 cycles using a thermal cycler (Hybaid Omni Gene System). The PCR reaction consisted of 35 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 55°C for 30 seconds and DNA synthesis at 72°C for 30 seconds. RT-PCR products were separated by electrophoresis through 1.2 % of agarose gels that contained 0.5 µg/ml ethidium bromide. Control PCR assays were performed in the absence of reverse transcriptase to confirm the absence of genomic DNA contamination in the total RNA sample. GAPDH was amplified for 30, 35, 40 cycles and analyzed to ensure the reaction was performed in the lineal stage.

To further understand the TNF-α mRNA expression in murine bladders after 1 and 6 transfections and 1 month after the 6th transfection with liposome mediated- TNF-α, the transfected bladders were harvested to extract mRNA and performed RT-PCR. All the mice were orthotopically implanted tumor into bladder 2 days before *in vivo* transfection to mimic the clinical application. Total RNA was isolated from 12 murine bladders using TRIzol according to the manufacturer's instructions. RT-PCR was done as mentioned above.

2.2.10 Orthotopic bladder cancer model

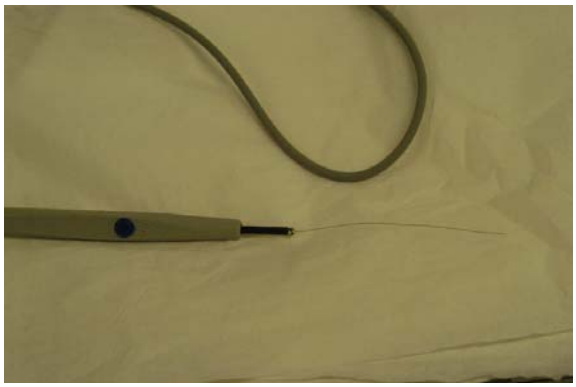
Female C57BL/6 mice (4-6 weeks) were purchased and used to produce an orthotopic bladder cancer model. Single cell suspensions were prepared from 50-60% confluence MB49 cells and adjusted to a concentration of 1×10^6 cells/ml in blank RPMI 1640 medium. The mice were anaesthetized and secured on a grounded plate with back fur shaved, and then catheterized with a 24-gauge plastic i.v. catheter through the urethra. A wire electrode was put into the bladder through the catheter. After a single transurethral cauterization at 0.2 watts to damage the bladder epithelium using monopolar coagulation function, (Aaron 800 high frequency desiccator, Aaron Medical Industries, INC. USA), 100ul of above mentioned cell suspension was immediately instilled into the bladder via the catheter and kept at least for one hour (Fig 2.1 A-G).

2.2.11 *In vivo* experiment with orthotopic bladder cancer model

Twelve mice were randomized into two groups, pBud-TNF-α group and pBud control group. Each group had six mice. The treatment was started on the day 3 after tumor implantation. Plasmid-liposome mixture was prepared using the same protocol as *in vitro*. After anesthesia, 100 μl of this mixture was instilled into each bladder. The instillations were conducted twice a week. The animals were inspected daily for the signs of toxicity caused by TNF-α, and the signs of excessive tumor burden. The mice were weighed twice every week. On day 27, 5 days after the sixth instillation, bladders were harvested and weighed. The kidneys and lungs were checked for the presence of metastasis or any other abnormalities. The abnormal organs were removed and fixed in 10% buffered formalin for the further H&E staining and histological study. This experiment was repeated once.

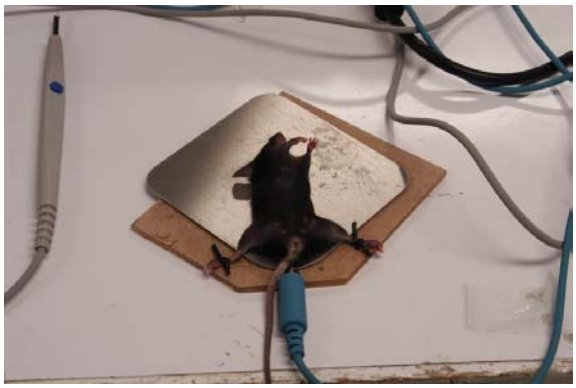
A

B



C

D



E

F

G

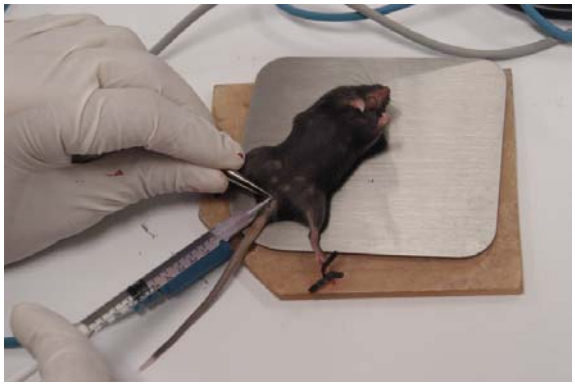


Figure 2.1 Materials and methods used in producing murine orthotopic bladder cancer model. (A): a female C57BL/6 mouse (4-6 weeks) used in this study, (B): a wire electrode to cauterize the mouse bladder, (C): Aaron 800 high frequency desiccator, (D): A mouse was anaesthetized and secured on a grounded plate with back fur shaved. (E): A mouse was catheterized with a 24-gauge plastic i.v. catheter through the urethra. (F): The wire electrode was put into the bladder through the catheter. (G): 100ul of MB49 cells (1×10^6 cells/ml) was instilled into the bladder after cauterization.

2.2.12 H&E staining

Following fixation in 10% buffered formalin, all tissue were paraffin-embedded, serially sectioned (5μM) and stained with hematoxylin and eosin. Briefly, the dry paraffin sections were stained in following procedure:

1. Pure xylene (5 minutes×2)

2.50%xylene&50%alcohol (2 minutes)

3.100% alcohol (5 minutes)

4.100% alcohol (5 minutes)

5.95% alcohol (5 minutes)

6.70% alcohol (5 minutes)

7. Tap water (2 minutes)

8. Hematoxylin (20-90 second)

9. Tap water (5 minutes)

10. Acid alcohol (5 dips)

11. Tap water (2 minutes)

12. Eosin (2-3 dips)

13. Tap water (2 minutes)

14. 70% alcohol (5 minutes)

15. 95% alcohol (5 minutes)

16. 100% alcohol (5 minutes)

17. Pure xylene (5 minutes×2)

The air-dry stained sections were then mounted by mounting medium. All the serial sections were examined carefully for the presence of the tumors.

2.2.13 Immune cells infiltration into bladder after TNF-α therapy

Four mice were orthotopically implanted bladder cancer and randomized into 2 groups. Two days after implantation, one group was transfected with pBud-TNF-α and another group was treated with pBud control vector. The intravesical instillation was carried out twice weekly. Two days after the third instillation, the mice were terminated and the bladders were harvested and rinsed with 1×PBS. The bladders were minced in 1 ml of blank RPMI 1640 and smashed with the plunge from 1ml syringe. Thereafter, the cells were filtered through a 40μm filter and centrifuged at 7K for 1 minute. The cell pellet was resuspended in 0.1% formaldehyde containing 10% of BSA and 0.01% azide and fixed for 1 hour. The fixed cells were then stained by 2μl of FITC anti-mouse CD3 and 2μl of PE anti-mouse CD4 or 2μl of FITC anti-mouse CD3 and 2μl of PE anti-mouse CD8a or 2μl of FITC anti-mouse CD3 and 2μl of PE anti-mouse NK cells/2B4 (BD PharMingen,USA) in a total volume of 100μl overnight. The stained cells were sorted by flow cytometry and the flow data was analyzed by WinMDI 3.0.

2.2.14 Statistical analysis

Data was analyzed using SPSS 11.0. Independent-samples T test was used to compare the means of two groups. ANOVA was used to compare the means of three groups.

CHARPTER THREE RESULTS

3.1 *In vitro* transfection optimization using reporter gene pCMVlacZ

Since the general practice in clinics is to keep intravesical drugs in the bladder for up to 2 hours after intravesical instillation, 2 hours of incubation time with MB49 cells after adding liposome-plasmid DNA lipoplex *in vitro* was used in this study. To optimize the plasmid DNA quantity used in this protocol, different amounts of pCMVlacZ gene were used to transfect MB49 cells. X-gal staining and ONPG assays were performed on the cells 48 hours after transfection.

X-gal staining showed that among various amount of plasmid DNA, namely 0.5μg, 2.5μg, 5μg, 7.5μg and 10μg of pCMVlacZ, 2.5μg gave the most cells with blue color, which is the indicator of transfection by reporter gene LacZ (Fig 3.1). Control cells which were transfected with 0μg of pCMVlacZ had no blue colonies. Similar results were obtained by using the reporter gene pBudCE.4.1/LacZ/CAT. Using 2.5μg of pCMVLacZ, the blue cells account for 30-40% of the total cell number after X-gal staining.

To confirm the optimal plasmid DNA quantity required for the transfection observed by X-gal staining, β-galactosidase activity was determined by the ONPG assay 48 hour after pCMVLacZ transfection. The results showed that the OD at 420nm of 2.5μg pCMVLacZ was much higher than that of other amounts of pCMVLacZ (Figure 3.2). Hence 2.5μg of plasmid DNA was used to transfect target cells in the subsequent experiments.

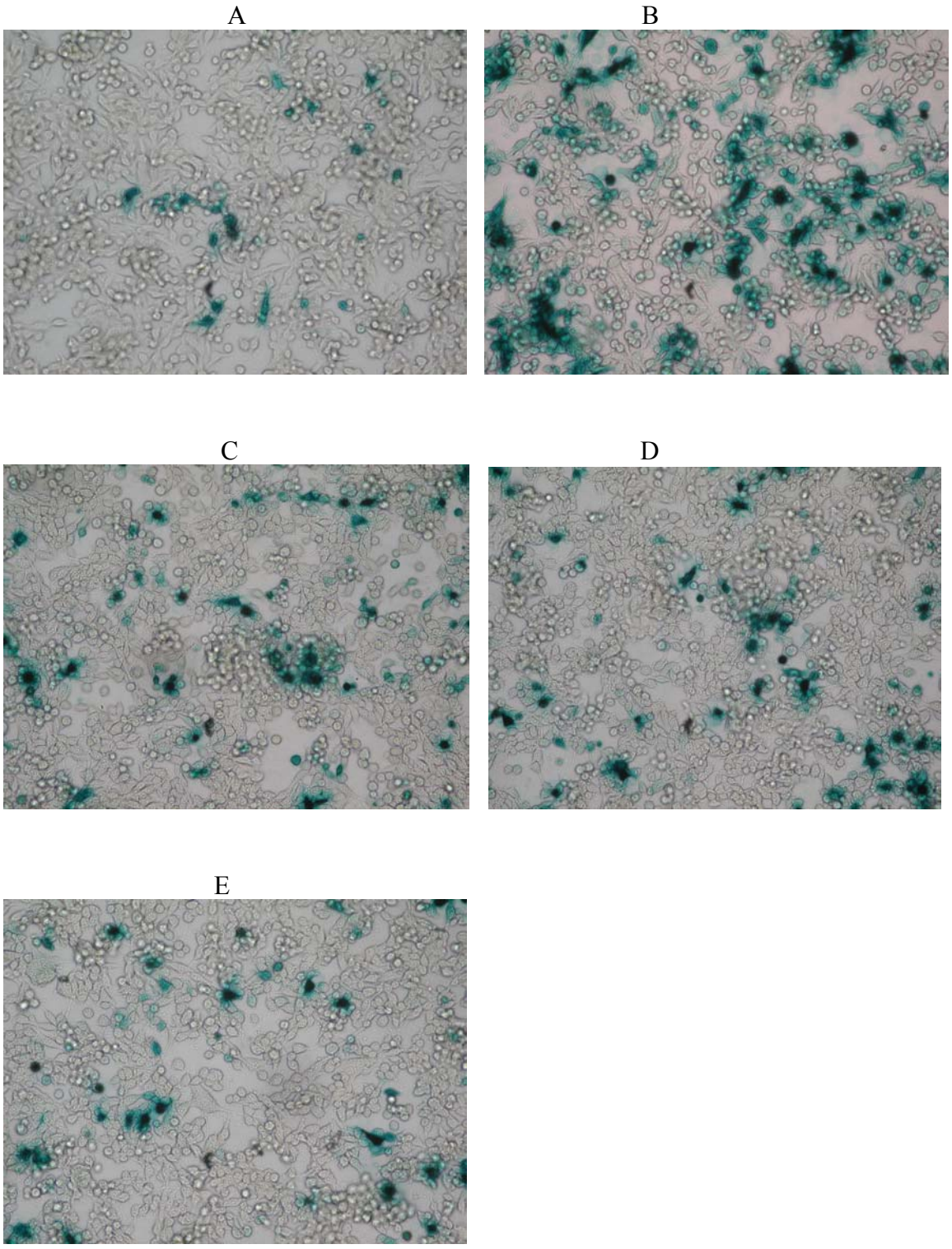


Figure 3.1 X-gal staining of MB49 cells 48 hours after transfection with different amounts of pCMVLacZ A: 0.5µg, B: 2.5µg, C: 5µg, D: 7.5µg, E: 10µg.

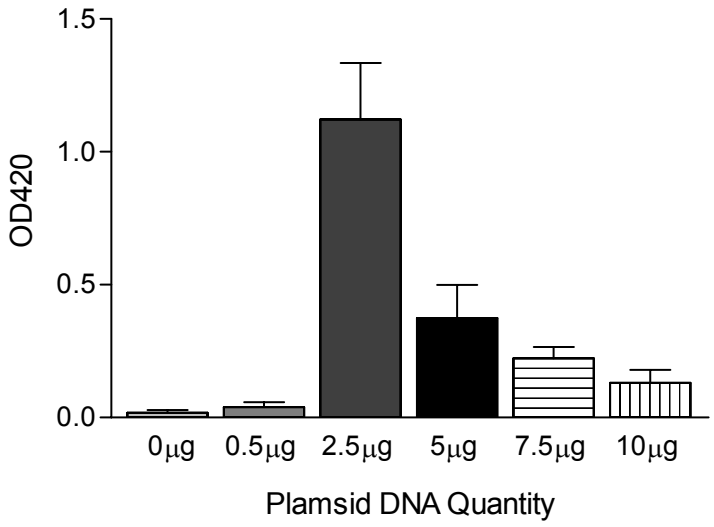


Figure 3.2: ONPG assay of MB49 cells which were transfected with different amounts of pCMVLacZ. MB49 cells were transfected with 0-10 μg of pCMVLacZ and harvested 48 hours after transfection. The ONPG assay was then carried out to determined galactosidase activity of the MB49 cells.

3. 2. Construction and cloning of mouse TNF-α encoding plasmid

The TNF-α cDNA was inserted into the multiple cloning site of pBudCE4.1 and its expression was driven by a pCMV promoter (Figure 3.3). The presence and orientation of TNF-α cDNA was checked by Sac I digestion and confirmed by automated sequencing (both forward and reverse). The alignment between the sequencing results and the TNF-α cDNA sequence in Genebank was done and shown to have 100% identity (Blast 1 and Blast 2).

Blast 1: Query:Insert(forward), subject: gi54844 Mouse mRNA for tumor necrosis factor

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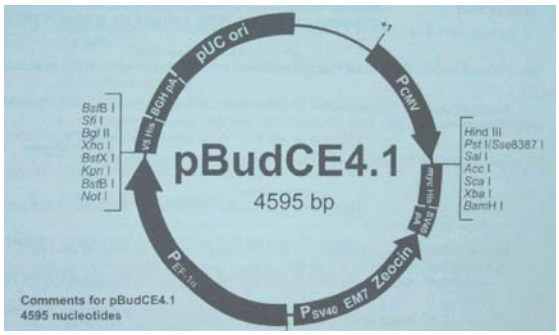


Figure 3.3: Map of the mammalian expression vector pBudCE4.1

3. 3 *In vitro* TNF-α transfection and expression

Endotoxin-free ultra-pure plasmid pBud-TNF-α was prepared for transfection after cloning. According to the optimal plasmid DNA quantity determined by LacZ gene, 2.5μg of pBud-TNF-α was used to transfect $2x10^5$ MB49 cells. About 893.7±24.0pg/ml of mouse TNF-α was detected by ELISA 48 hours after pBud-TNF-α transfection. TNF-α was undetectable in parent cells or pBud transfected MB49 cells. These results showed that transfection with pBud-TNF-α using this non-viral delivery system leads to a high level of TNF-α protein expression in MB49 cells.

3.4. Anti-proliferative activity after pBud-TNF-α transfection

Two days after pBud-TNF-α transfection, the cells were counted to assess its anti-proliferative activity. A significant inhibition of tumor proliferation was observed in pBud-TNF-α transfected cells (Fig. 3.4). The cell number at 48 hours after transfection was 55% ($p<0.05$) of that of parent cells or pBud transfected cells. This result suggests

that the TNF-α level expressed after transfection using our non-viral delivery system were high enough to suppress the bladder cancer cell growth *in vitro*.

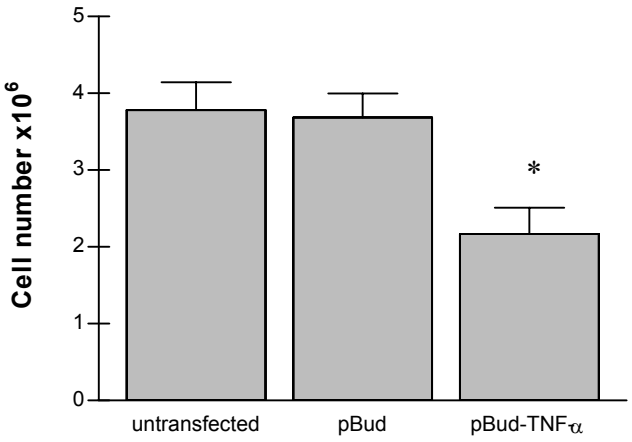


Figure 3.4: MB49 cell number of untransfected, pBud and pBud-TNF-α transfected cells 48 hour after transfection. * indicates $p<0.05$ compared with untransfected cell and pBud transfected cell.

3.5. *In vitro* killing of bladder cancer cell line with pBud-TNF-α

3.5.1 Cell cycle analysis

Cell cycle analysis of MB49 cells was performed 48 hours after transfection by PI staining (Fig 3.5). The percentage of cells in the G1 and G2 phases of the cell cycle in pBud-TNF-α transfected cells was lower than that of control cells. The sub-G1 cell population, which indicates the cells in late apoptosis (DNA fragmentation) was enhanced in TNF-α transfected cells in comparison with that of control cells (Table 3.1). This suggests that TNF-α transfection may directly induce bladder cancer cell apoptosis.

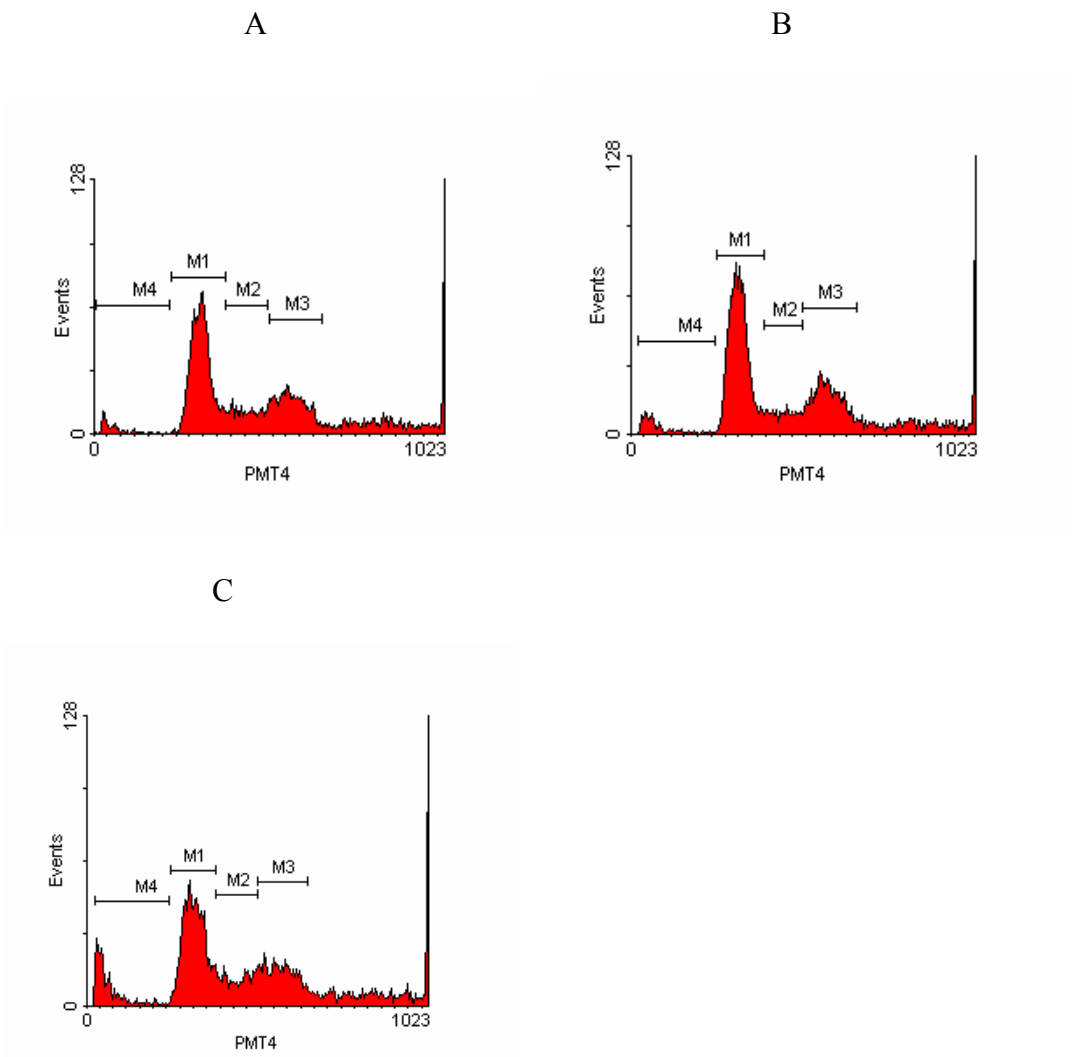


Figure 3.5 PI staining results of parental cells (A), pBud (B) and pBud-TNF-α (C) transfected MB49 cells 48 hours after transfection. M1, M2, M3, M4 denotes cells which were in G1, S, G2 and sub- G1 phase respectively.

Table 3.1: Cell cycle analysis of parental cells, pBud and pBud-TNF-α transfected cells

	Sub G1 phase	G1 Phase	S Phase	G2 Phase
Parental cells	2.11 ± 1.45%	38.76±4.38%	11.28±3.24%	21.15±1.04%
PBud	1.49 ± 0.74%	38.94±7.73%	11.42±1.54%	22.52±4.95%
pBud- TNF-α	8.63 ± 0.53%	31.52±1.94%	13.22±0.26%	19.42±0.57%

Data represent the Mean ± SD of three experiments done in duplicates

3.5.2 Annexin V staining

To confirm the effect of inducing bladder cancer cell apoptosis after pBud-TNF-α transfection, Annexin-V staining, a sensitive test for identifying pre-apoptotic cells, was done 48 hours after transfection. Approximately 32% of the MB49 cells were induced to a pre-apoptotic state after transfection (Figure 3.6).

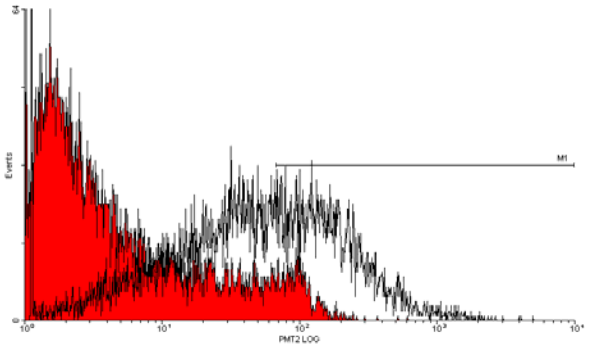


Figure 3.6 Annexin-V staining of pBud (filled histogram) and pBud-TNF-α (open histogram) transfected cells

Table 3.2 Annexin-V staining of parental cells, pBud and pBud-TNF-α transfected cells

Plasmid	Percentage of positive cells after Annexin-V staining
Parental cells	ND
Pbud	5.17 ± 0.08
pBudTNF-α	32.10 ± 7.52

Data represent the Mean ± SD of three experiments done in duplicates

3.6 Flow cytometric analysis for surface immuno-related molecules and Fas receptor

Forty-eight hours after pBud-TNF-α transfection, MHC class I, MHC-class II, B7.1, ICAM I and Fas antibody stained cells were analyzed by FACS. It showed that MHC class I, B7.1 and Fas molecule expression on MB49 cells were up-regulated after transfection compared with control cells (Table 3.3). There was no increase in MHC class II and ICAM I molecules on MB49 cells after treatment.

Table 3.3 The expression level of MHC I, B7.1 molecules and Fas antigen on MB49 cells 48 hours after pBud-TNF-α transfection

Plasmid	Percentage of positive cells		
	MHC I	B7.1	Fas
None	15.46 ± 9.61	5.83 ± 1.76	11.05 ± 7.91
pBud	13.04 ± 10.93	6.40 ± 2.19	16.43 ± 11.59
pBud-TNF-α	62.73 ± 11.83	34.73 ± 11.43	48.40 ± 18.95

Data represents the Mean ± SD from five experiments.

3.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis of TNF-α expression *in vivo*

Next, we checked the transfected gene expression level *in vivo* after *in situ* direct TNF-α gene transfer to mouse bladder by measuring the cytokine protein in the mouse urine. The mouse urine was collected after the first, third and sixth intravesical instillation of pBud-TNF-α. TNF-α protein level in urine was determined by ELISA. It showed negative

results in all urine samples from the transfected mice. We then switched to detect TNF-α expression *in vivo* by monitoring the mRNA level of TNF-α.

RT-PCR was performed using mRNA extracted from pBud-TNF-α and pBud transfected mouse bladders which have no tumor implanted. It showed TNF-α mRNA was readily detectable in pBud-TNF-α transfected mouse bladder 2 days after transfection. In contrast, it was undectable in pBud control vector transfected mouse bladder (Figure 3.7.1). This suggests that intravesical administration of the therapeutic gene with our novel liposome system is able to deliver the therapeutic gene to the target organ and get the gene expressed successfully. Using LacZ gene, we also demonstrated that this strategy is safe because the marker gene can only be found in bladder, but not in liver, kidney, heart, brain, lung or ovary.

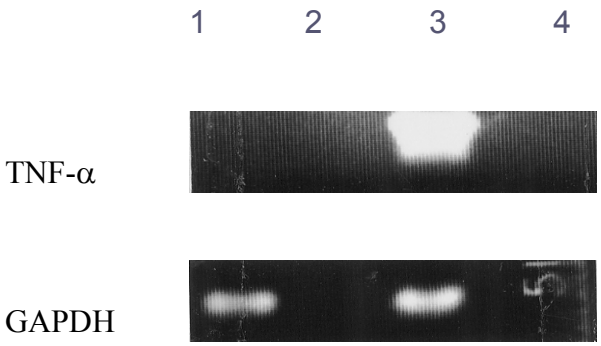


Figure 3.7.1 RT-PCR results after *in vivo* direct TNF-α gene transfer to mouse bladders (non-tumor implanted bladders). TNF-α mRNA expression level were determined by RT-PCR. GAPDH was amplified as control. lane 1&2: mRNA from control vector transfected mouse bladder, lane3&4: mRNA from TNF-a transfected mouse bladder, lane1&3: PCR with RT, lane 2&4: PCR without RT.

To monitor TNF-α mRNA expression in murine bladders after 1 and 6 transfections and 1 month after the 6th transfection, RNA was extracted from the transfected bladders and

RT-PCR was performed. All the mice had been orthotopically implanted with tumor cells 2 days before *in vivo* transfection to mimic the clinical application. The results showed that after 1 instillation with pBud-TNF-α, the TNF-α mRNA in bladders was enhanced significantly compared with that of the control vector transfected bladders (lane 4-6 compared to lane1-3, Fig 3.7.2). After 6 instillations, the TNF-α transcription was further increased and then returned to the level of control vector transfected bladders 1 month after the last transfection (lane 7-9 compared to lane10-12, Fig 3.7.2).

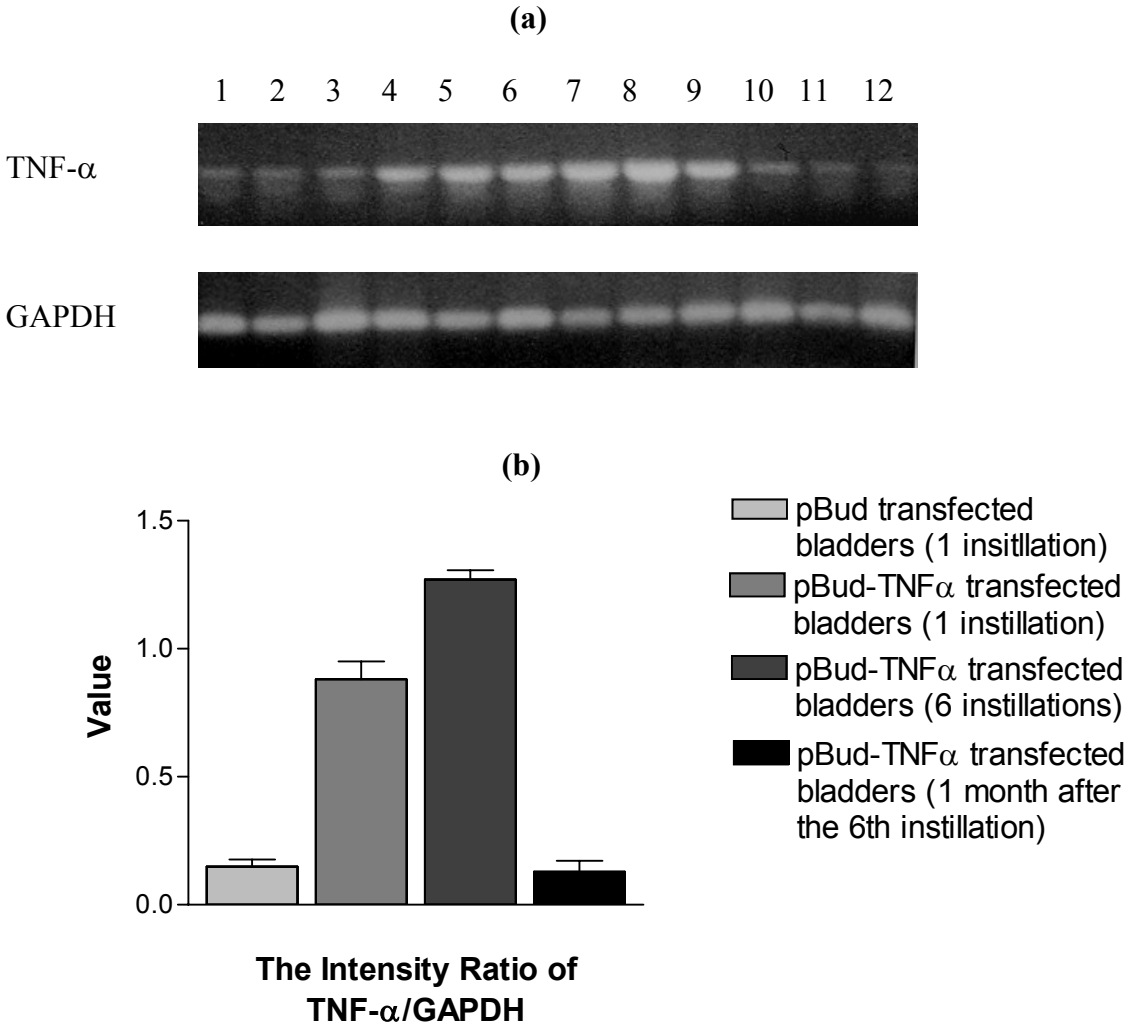
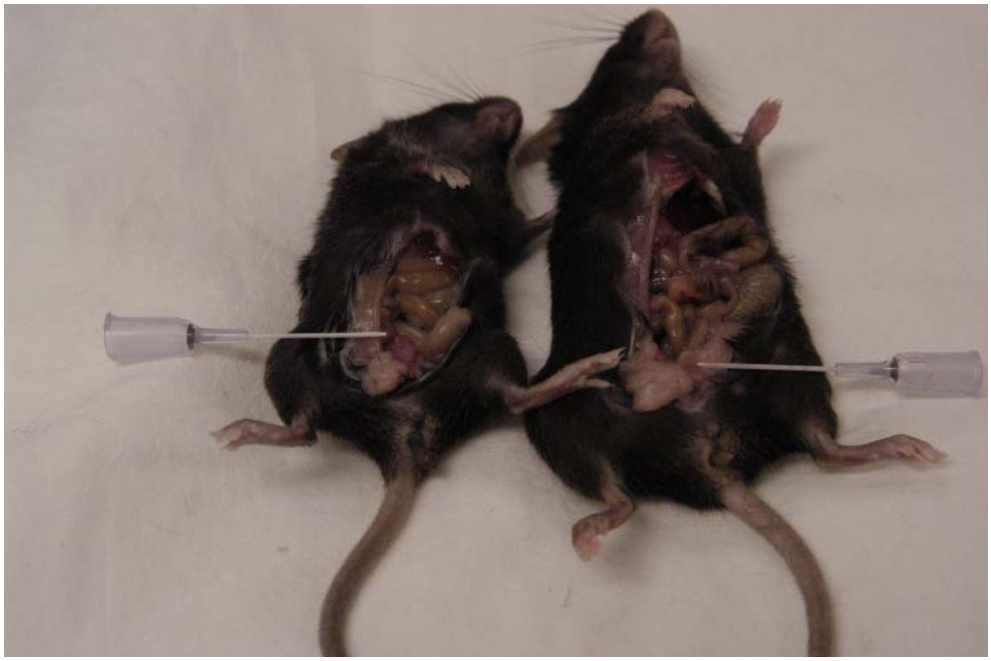


Fig 3.7.2. RT-PCR results after treatment using murine orthotopic bladder cancer model. (a) shows a representative mRNA expression of TNF-α after intravesical treatment in tumor implanted mouse bladders. lane 1-3: mRNA from control vector transfected mouse bladder (2 days after 1 instillation), lane 4-6: mRNA from TNF-α transfected mouse bladder (2 days after 1 instillation), lane 7-9: mRNA from TNF-α transfected mouse bladder (2 days after 6 instillations), lane 10-12: mRNA from TNF-α transfected mouse bladder (1 month after 6th instillation). (b) The ratio of TNF-α/ GAPDH measured by densitometry.

3.8 Murine orthotopic bladder cancer model

To reflect the biology of human bladder cancer as it presents in the clinics, a murine orthotopic bladder cancer model was developed in our lab. MB49 cells were intravesically instilled to the bladder of syngeneic mouse strain C57BL/6 after a single transurethral cautery at 0.2 watts to damage the bladder epithelium. A single tumor formed in bladder in 1 week (Figure 3.8). Histological study of the tumor showed it was transitional cell carcinoma (TCC) and the xenografts grew as superficial tumors with no evidence of invasion (Figure 3.9).

A



B

Figure3.8: Murine orthotopic bladder cancer model. A: The left mouse has tumor implanted in bladder while the right one is a control mouse with normal bladder. B: The left bladder is removed 1 week after tumor implantation and the right one is a normal control bladder.

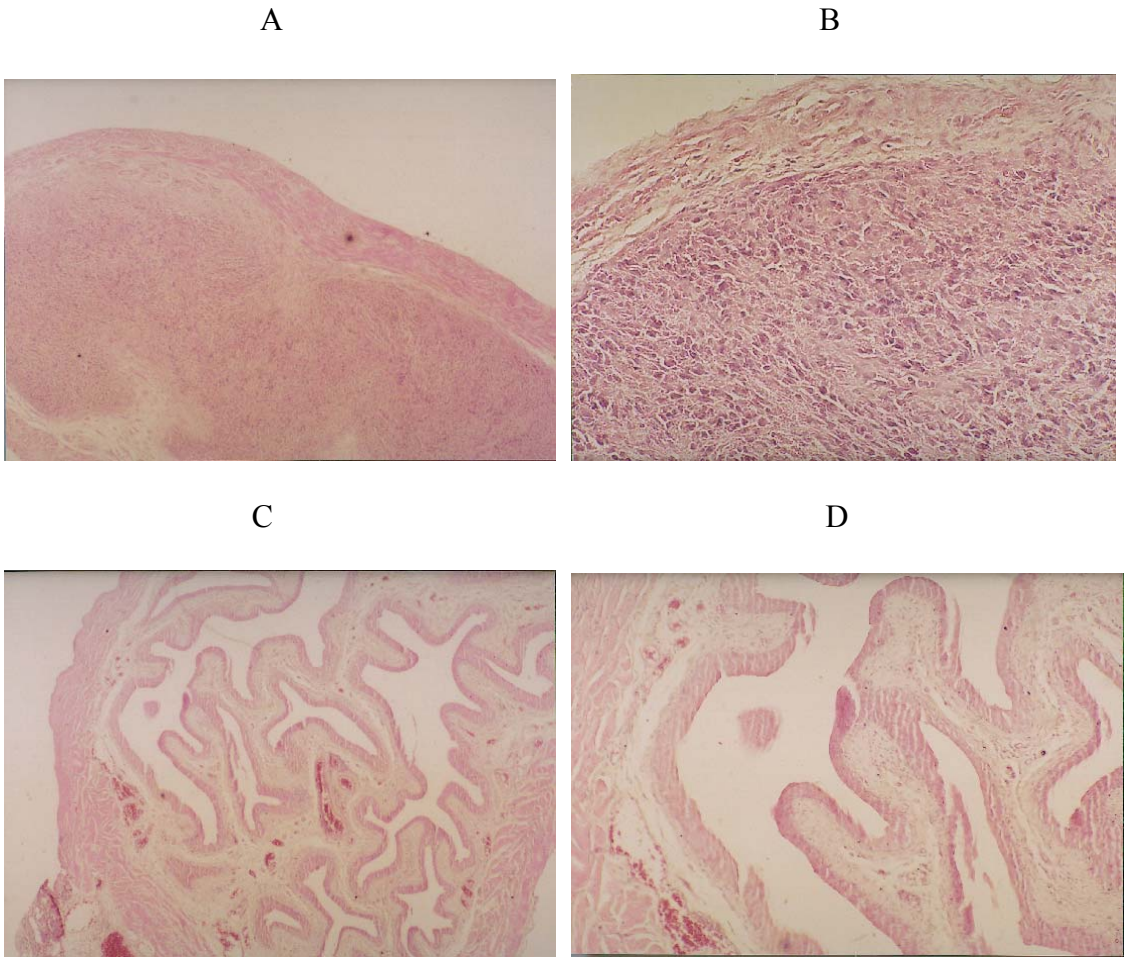


Figure 3.9: Histological study of mouse bladder (H&E staining). A&C: 4×10 magnification, B&D: 10×10 magnification) A&B: C57BL/6 mouse bladder which is instilled with MB49 cells after damaging the epithelium presents with a transitional cell carcinoma in bladder. The tumor does not invade muscle layer. C&D: The architecture of a normal C57BL/6 mouse bladder.

3.9 Tumor growth suppression of pBud-TNF-α *in vivo*

We then evaluated the effect of TNF-α gene on suppressing the growth of established bladder cancer *in vivo* using the orthotopic bladder cancer model mentioned above. The treatment was started 2 days after tumor implantation. Six intravesical instillations of liposome-mediated pBud-TNF-α were performed in total, i.e. twice weekly. All the mice (n=24) had severe hematuria 1-2 weeks after tumor implantation. Three mice died in the control group (n=12) while only 1 mouse died in pBud-TNF-α treated group (n=12). The remaining mice were terminated 5 days after the sixth instillation, namely day 27 after tumor implantation. Treatment with the TNF-α gene was well tolerated. No treatment-related death and adverse effects, such as diarrhea, rigor and weight loss were observed in the pBud-TNF-α group. The cured animals continually gained weight while some non-cured animals lost weight because of their tumor burden.

Necropsy showed that all the mice that died before the termination died from excessive bladder tumor burden. The average bladder weight of mice in the pBud-TNF-α treated group is 44.1% lower than that of the control group while the average body weight of mice in pBud-TNF-α group is 20.7 % higher than that of the control group. There was a significant difference in the relative organ weight (ROW) between the pBud-TNF-α treated group and control group ($p<0.05$) (Table 3.4). No metastasis was found in liver, heart, lung, intestine and bone. No hepatosplenomegaly was found in all the mice. Two mice had unilateral kidney enlargement and 2 mice had bilateral kidneys enlargement in the pBud control group while only 1 mouse in the pBud-TNF-α group had bilateral

kidney enlargement. All the abnormal kidneys were removed for the pathological study. All the bladders were removed and serially sectioned to exam the presence of tumors.

Table 3.4 Body and bladder weights of mice with orthotopic bladder cancer given intravesical TNF-α gene therapy.

Group (n=10)	Body Weight (g)	Bladder Weight	
		(g)	ROW
pBud	14.5± 0.9	0.059±0.0093	45.17±10.01
pBud-TNFα	17.5± 0.4	0.033±0.0050	18.91± 3.18

Data represents the mean ± SEM for bladder weight and ROW (relative organ weight). ROW= [organ weight (g) × 10^4]/Body weight (g). There is a significant difference in ROW between pBud-TNF-α and pBud group (p=0.03). (Two bladders in each group were randomly selected and distended by formalin before termination and therefore excluded from calculation)

Histological study showed that 9 of 12 mice in the control group had tumors in the bladder. In contrast, only 3 of 12 animals in the pBud-TNF-α treated group were demonstrated to have bladder cancer (Table 3.5& Fig 3.10). No renal metastasis was found in any of the mice. The enlargement of the kidney was caused by hydronephrosis. Four of 12 mice in the control group had hydronephrosis while only 1 of 12 mice in the pBud-TNF-α treated group had the same problem.

Table 3.5 Histological study of mouse bladder and kidney in intravesical TNF-α gene therapy.

Group (n=12)	No. with bladder tumor	No. with hydronephrosis
pBud	9/12 (75%)	4/12
pBud-TNF-α	3/12 (25%)	1/12

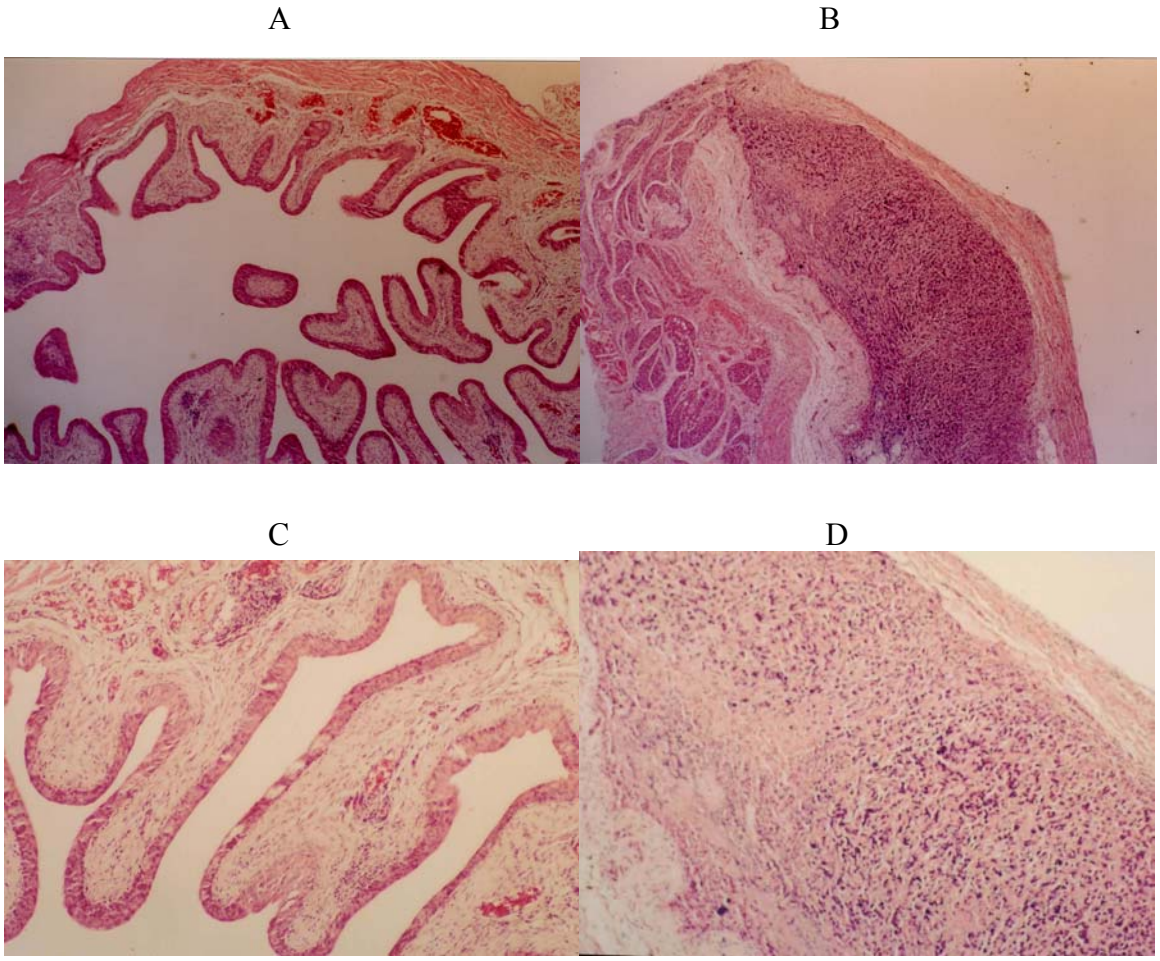


Figure 3.10: H&E staining of mouse bladder after treatment. (A&B: Magnification: 4×10, C&D: Magnification: 10×10) A&C: A tumor implanted bladder, after 6 instillation of pBud-TNF-α, shows normal epithelium. B&D: A tumor implanted bladder treated with pBud vector is filled with superficial tumor.

3.10 Immune cells infiltration into bladder after TNF-α therapy

To check whether direct TNF-α gene transfer to bladder can boost the host immune system by attracting the immune cells infiltration into the bladder, the mouse bladder was removed, minced and resuspended in RPMI 1640 2 days after the third instillation. The cells were stained with CD3, CD4, CD8 and NK monoclonal antibody and analyzed by flow cytometry. The results showed that the T lymphocytes and NK cells in bladder were enhanced after intravesical instillation of liposome mediated TNF-α.

Figure: 3.6 CD3, CD4, CD8 and NK positive cells in pBud-TNF-α and pBud transfected bladders.

Immune cell marker	pBud (n=2)	pBud-TNF-α (n=2)
CD3	30.63±1.63%	48.28±4.87%
CD4	20.65±1.53%	24.50±0.10%
CD3 CD4	16.98±1.19%	21.00±0.11%
CD8	20.24±4.46%	22.55±6.51%
CD3 CD8	15.56±2.21%	19.96±4.78%
NK	20.96±2.84%	26.77±2.82%
CD3 NK	16.74±2.44%	23.48±0.72%

CHARPER FOUR DISCUSSION

In this study, a novel cationic liposome mixture composed of DOTAP plus MBC was used to deliver the therapeutic gene. We demonstrated this non-viral system was efficient in delivering target genes to bladder cancer cells *in vitro* as well as bladder cancer tissues *in viv*. This is evidenced by the moderate amount of mouse TNF-α expressed in the supernatant and the detectable band in RT-PCR. Although TNF-α protein was undetectable in the urine after the first, third and sixth intravesical instillation of pBud-TNF-α, we speculate that several reasons may account for the results, such as the stability of TNF-α protein in urine, the amount of TNF-α protein which can be accumulated in the urine and the possibility of the denaturation of TNF-α protein in the salts or other compounds of the urine. The TNF-α titer obtained from murine bladder cancer cell MB49 cells using our transfection system is not as high as that of some other studies using adenovirus vector (Marr et al., 1998; Kianmanesh et al., 2001). However, some facts should be taken into account when comparing our results with these results.

First, cytokine titer expressed by the transduced cells is highly cell-type dependent. Marr et al transfected human fibroblast MRC5 (ATCC CCL-171) cells and murine melanoma B16BL6 cells with the same human TNF-α encoding adenovirus vectors (AD-HCMV-TNF) under the same condition. The TNF-α titer from MRC5 was greater than 3000 ng/10^6 cells on day 6 after transfection, which was 1000 time higher than that from B16 cells on day 6 (Marr et al., 1998). A similar phenomenon was observed in other studies. Hu et al transfected a human bladder carcinoma cell line 5637 and prostate carcinoma

cell line LNCa.FGC with a tetracycline-regulatable recombinant adenovirus vector encoding TNF-α. The TNF-α produced by 5637 cells (6000 pg/10^6cell/24 hours) was more than 15 times higher than that obtained from LNCa.FGC cells (400 pg/10^6 cell/ 24hours) (Hu et al., 1997).

Secondly, it is expected that the TNF-α titer obtained using our non-viral gene deliver system would be lower than that obtained from adenovirus vectors because adenoviruses are well known to have very high transfection efficiencies in many cell lines. However, for a potent cytokine like TNF-α, too high a titer of protein product can also cause severe side-effects, even with local administration. Kianmanesh et al used adenovirus vector encoding TNF-α to transfect CT26 colon carcinoma. The titer of TNF-α in the supernatant was as high as 700-800 ng/ml 72 hours after transfection. Severe toxicities including significant weight loss and diarrhea (3/3) and treatment related death (1/3) were observed after intratumoral injection of this adenovirus vector into a subcutaneous model of colon cancer (Kianmanesh et al., 2001). Considering that man can only tolerate 2% of the dose/kilogram which is essential to obtain tumor regression in mice when TNF-α is admininstered systemically (Sidhu et al., 1993), special care should be taken to avoid too high titer of TNF-α which may enter into the circulation via local microvasculature even though it is applied locally. Moreover, lower concentrations of TNF-α do not necessarily mean lower inhibitory effects on tumor cells. Adenovirus mediated TNF-α transduced prostate carcinoma cells LNCaP.FGC secrete only moderate amounts of TNF-α (~400 pg/10^6 cell/ 24hours), at which level, however, the majority of the transduced LNCaP.FGC cells will die (Hu et al., 1997).

Thirdly, although viral vectors including adenovirus, retrovirus, and herpes-simplex virus are more commonly used in cancer gene therapy and may have higher transfection efficiency, these vectors also have their own limitations. Retroviruses may cause insertional mutagenesis because of random integration into the host genome. Recently, it was reported that a child participating in a gene therapy trial using retrovirus had developed leukaemia (Gore, 2003). Adenoviruses are strongly immunogenic. Furthermore, it has been shown that the expression of the human coxsackie and adenovirus receptor (hCAR), which is regarded essential for adenovirus to enter into target cells, was variable amongst different human bladder cancer cells (Li Y, et al., 1999). This raises the question of the feasibility of adenovirus-mediated gene therapy for bladder cancer. However, more recently, another study found that although immunohistochemistry results using a monoclonal antibody against hCAR showed homogeneous expression of the receptor throughout the full thickness of the urothelium, only the superficial layers were infected by the adenovirus while the monolayer cells derived from the same pieces of tissue and the same patients can be transfected by the adenovirus with much higher efficiency. Thus the authors argued that it is a physical barrier, most likely the multilayered structure of intact urothelium, rather than hCAR status that may be the main determinant of the transduction efficiency of the intact epithelium (Chester et al., 2003). In contrast, no receptors are required for cationic liposome-mediated gene delivery. Furthermore, liposome-mediated gene delivery *in vivo* has been demonstrated to be safe in various animal studies and is being evaluated in a number of clinical trials. 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 immuno-modulation, the transient nature of transfection becomes less important in bladder cancer. The ease of repeated intravesical instillation may compensate for the lower transfection efficiency of liposomes.

Mature tumor necrosis factor α (TNF-α) is a 17 kD polypeptide that binds to two cell surface receptors p55 and p75 (Asher et al., 1987). This proinflammatory cytokine has a variety of physiological effects including potent experimental antitumor activity. TNF-α was reported to exert its antitumor effects in three ways. The first is through direct cytotoxicity to tumor cells, by inducing apoptosis through engagement of the p55 TNF receptor (Fiers, 1991). The second mechanism is through the activation of immune cells like T cells, NK cells, macrophages as well as inducing cytokines like IL-6, GM-CSF, IL-8, etc (Tracey et al., 1994; Paleolog et al., 1994). The third mechanism is through its selective action on the neovasculature in the tumor microenvironment, leading to intravascular thrombosis within tumor and thereafter ischemic necrosis (Scheurich et al., 1987; Ranges et al., 1987).

TNF-α is believed to make an important contribution to the efficacy of BCG therapy of bladder cancer (Shin et al., 1995; Jackon et al., 1995; Balbay et al., 1994). Studies have shown that among the constituents of the urinary "cytokine soup" induced by intravesical

BCG, namely TNF-α, interferon-γ, interleukin-1 and interleukin-2 (Bohle et al., 1990; Prescott et al., 1990; Haaff et al., 1996), TNF-α and interferon-γ were the only cytokines which demonstrated significant anti-proliferative effects on the bladder cancer cells (Hawkyard et al., 1993). Furthermore, the data in laboratory and clinical studies also demonstrated its effectiveness in inhibiting bladder cancer. (Niell et al., 1994; Serretta et al., 1995; Lee et al., 1987; Kadhim et al., 1988; Sternberg et al., 1992; Glazier et al., 1995). The major problem which limits the clinical application of TNF-α in cancer treatments is the severe toxicity associated with systemic administration, such as sepsis-like syndrome, shock, hypotension, confusion, malaise, confusion, headache, rigors, fever, chills, diarrhea, lethargy, vomiting, hepatosplenomegaly, leukothrombopenia, anorexia, cachexia and weight loss (Chapman et al., 1987; Feinberg et al. 1988). Although intravesical immunotherapy of recombinant TNF-α shows good tolerance and little side-effect in patients, the response rate was inferior to BCG therapy. Part of the reason for this may be attributed to the limited retention time of TNF-α in bladder and the lack of the post-translation modification processes of recombinant TNF-α. Direct TNF-α gene transfer to bladder cancer cell may not only circumvent the problems encountered following systemic administration by providing high titers of cytokine in the vicinity of the tumor cell and lead to tumor suppression, but also solve the problems like rapid degradation and the short half life after administration of the recombinant TNF-α. Moreover, it has also been reported that endogenously TNF-α produced by the transfected tumor cells may promote the expression and secretion of further factors capable of synergizing with TNF-α to induce apoptosis at cytokine levels over 100-fold less than those required to achieve the same effect with exogenous added TNF-α (Gillio et

al., 1996). All these facts encouraged us to test the hypothesis that intravesical TNF-α gene therapy is effective to treat bladder cancer.

Our study showed that 75% of tumor implanted mice (9 of 12) which were treated with liposome mediated TNF-α gene were tumor-free while only 25% (3 of 12) of mice which were treated with pBud control vector had no tumors in the bladder. All the TNF-α treated mice were normal. No diarrhea and no treatment-related deaths were observed. The cured animals continually gained weight while non-cured animals lost weight because of their tumor burden. This results support the hypothesis that *in situ* cytokine gene transfer to bladder can achieve an anti-tumor effect with less risk of systemic effect. Kianmanesh et al showed that a single intratumoral injection of high dose (10^9 pfu) of TNF-α encoding adenovirus vector can completely suppress tumor growth in murine subcutaneous colon cancer model (3/3) and melanoma model (5/5). Unfortunately, this effect was also associated with severe toxicities including significant weight loss and diarrhea (3/3 in colon cancer, 3/5 in melanoma) and treatment related death (1/3 in colon cancer, 1/5 in melanoma). Two factors may account for the difference between this study and ours. Firstly, intratumoral injection which was applied in Kianmanesh's study may have resulted in systemic transfection of the TNF-α gene via the tumour vasculature, a problem that is rare with intravesical drug administration. Secondly, the large amount of adenoviruses vectors (10^9 pfu) used in this study and higher transfection efficiencies of adenoviruses may result in a much higher amount of TNF-α being produced *in vivo* with a concomitant increase in side-effects. Their study reported that 72 hours after transfection at a multiplicity of infection (MOI) of 70 there was >700ng/ml of TNF-α

produced *in vitro* which is 1000 fold more than that produced by our transfection system. This may also explain the reason that we did not achieve their cure rate of 100%. At a lower dose of 10^7 PFU adenovirus only partial suppression of the tumor (45% of suppression 2 weeks after vector administration) was observed. But there was no data as to how much TNF-α was produced by this dose of virus *in vitro*. In their study tumor reduction was solely dependant on an intact immune system.

In our animal study, 25% of the mice in the control group were tumor-free, indicating an implantation success rate of 75%. This rate is comparable with other orthotopic bladder cancer model studies using cauterization which showed a tumor incidence of 38-84% (Bonfil et al., 1997; Soloway et al., 1983; See et al., 1987). Unlike the bladder tumors induced by chemical carcinogens such as N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (Ibrahiem et al., 1983) and N-methyl-N-nitrosourea (NMU) (Elsasser-Beile et al., 2001) or created by applying corrosive chemicals like hydrochloric acid (HCL) and potassium hydroxide (KOH) followed by tumor cell instillation (Xiao et al., 1999; Chin et al., 1991), the bladder tumors formed using our technique are unifocal, superficial, papillary and have predictable location. These tumors resemble bladder cancers that occur in the clinics, both anatomically and histologically. Therefore this orthotopic tumor model is suitable for the evaluation of the treatment strategies of bladder cancer.

Our study showed at least two pathways might be involved in the anti-tumor effect of TNF-α gene transfection. The first is the direct effect of inducing apoptosis. This effect was shown by PI staining, which discriminates between hypodiploid DNA and diploid

DNA. It revealed the presence of an apoptotic peak 48 hours after pBud-TNF-α transfection. The effect was then confirmed by Annexin V staining, an indicator of the early stage of apoptosis. It demonstrated that as many as 35.4% of the MB49 cells were stained by Annexin V 48 hours after pBud-TNF-α transfection. Although PI staining showed only 6.3% pre-G1 cells, this may be because the 48 hour duration was not sufficient for MB49 cells to have DNA fragmentation under these circumstances. The direct apoptotic effect of TNF-α is consistent with another study which demonstrated that the transfer of the TNF-α gene into lymphoma T-cell can restore the physiologic cell death program lost by neoplastic cells (Gillio et al., 1996**).** Since it has been previously demonstrated that the expression of APO-1/Fas is up-regulated by exposure to either IFN-γ and/or TNF (Watanabe-Fukunaga R et al., 1992; Weller et al., 1994; Fellenberg et al., 1997), we then furthered our experiments by investigating the expression level of Fas receptor on the surface of MB49 cells after TNF-α gene transfection. This showed that APO-1/Fas receptor, a member of TNF/NGF receptor superfamily, was enhanced significantly after TNF-α transfection. As APO-1/Fas mediates apoptosis when it is cross-linked with Fas ligand on activated T cells and NK cells, we speculate that Fas/FasL may also play a role in the apoptotic effect *in vivo* after TNF-α transfection. Lee et al also demonstrated that the overexpressed Fas provides a functional target *in vivo* for T lymphocyte cells and results in the delay of *in vivo* tumor progression (Lee et al., 2000).

A more important mechanism is the stimulation of T-cell-mediated immunity to the tumor, which is critical to convert the immunological silence around the tumor cells. In

our study, we found a considerable increase in MHC I and B7-1 expression after TNF-α transfection. This event is crucial in activating tumor specific $CD8^+$ TCL considering that MHC I and co-stimulatory molecules such as B7-1 and B7-2 are the two essential signals in the activation and effector function of $CD8^+$ cytotoxic T lymphocytes (CTLs) and the responding T lymphocytes would be "anergized" or "tolerized" rather than activated if MHC I positive tumor cells presents tumor antigen in the absence of a co-stimulatory signal (George et al., 1989; Browning et al., 1992; Linsley et al., 1993; Townsend et al., 1993; Darnell et al., 1994;). Therefore, the up-regulation of these two molecules could significantly enhance the T cell mediated immune response which may be closely related to anti-tumor effect. As expected, MHC II expression was not found to be enhanced after TNF-α transfection. A similar result was reported elsewhere (Jackson et al., 1993). Nevertheless, Hawkyard et al showed that although TNF-α could not induce the expression of MHC II antigen from the cells which were initially MHC II-negative, it enhanced the expression on cells which already demonstrated a low level of the antigen (Hawkyard et al., 1993). In an analysis of 37 bladder tumors Nouri and Symes reported that about 54% were negative and the remaining had either strong or weak MHC class II expression (Nouri et al., 2000). Therefore, it is possible that when human bladder cancer which expresses low level of MHC II is transfected with pBud-TNF-α, MHC II expression may be enhanced, leading to the co-activation of $CD4^+$ tumor specific lymphocyte and a more potent anti-tumor immunity (Kern et al., 1986). The effect of the stimulation of T-cell-mediated immunity to the tumor after direct TNF-α gene transfer to tumor cells was further demonstrated by our *in vivo* study. It showed that $CD3^+$, $CD4^+$, $CD8^+$ and NK^+ cells in mouse bladders are significantly enhanced 2 days after the third

instillation of liposome mediated TNF-α gene. These results suggest that T lymphocytes and NK cells are also involved in the anti-tumor effects of TNF-α, providing the *in vivo* evidence of immune activation after *in situ* TNF-α gene transfection.

TNF-α has been reported to induce transformation of a normal rat urothelial cell line after 1 week of exposure at a concentration of 10ng/ml (Okamoto et al., 1997). This amount of TNF-α was at least 10-fold more than that produced by our transfection. Thus the likelihood that TNF-α could be detrimental in bladder cancer therapy is low. However, to confirm this, the long-term effect on mice of 6 intravesical instillations of TNF-α will need to be examined.

CHARPTER FIVE CONCLUSIONS AND FUTHER DIRECTIONS

In summary, our intravesical gene therapy using TNF-α as a therapeutic gene shows intravesical instillation of liposome mediated TNF-α gene can produce sufficient TNF-α protein in the vicinity of the bladder cancer cells to significantly inhibit their growth without any severe side-effects which have been observed in the approach of intratumoral injection of viral vector mediated TNF-α gene. It demonstrates that intravesical administration of therapeutic genes is a feasible and efficient *in situ* gene transfer approach for bladder cancer. Two mechanisms including direct apoptotic effect against bladder cancer cell and the activation of immune cells to tumor were shown to be involved in the antitumor activity of TNF-α gene therapy.

These results suggest that intravesical cytokine gene therapy may be useful as an adjuvant therapy for bladder cancer. Superficial bladder cancer patients who have undergone TURBt and patients with carcinoma-*in-situ* bladder cancer have low tumor burden but high risk of recurrence. They may benefit from this gene therapy strategy. It may be especially valuable for BCG-failures. Late stage invasive bladder cancer patients who have clinically unresectable tumors may also benefit from this strategy or through intratumoral injection of liposome mediated therapeutic cytokines.

However, as far as this study is concerned, some improvements can still be made to give a more accurate evaluation of cytokine gene therapy. For example, a technique which allows the confirmation of the presence of orthotopic bladder tumor before starting the

treatment and also permits the monitoring of the growth of the tumor during the treatment would give more convincing evidence. To achieve this aim, Magnetic Resonance Imaging (MRI) of the growth of the bladder tumor, green fluorescent protein (GFP) transfected bladder cancer cells and prostate specific antigen (PSA) transfected bladder cancer cells which can be implanted into the mouse bladder to simplify the tumor estimation are being studied.

Before a clinical trial can be initiated, more work should be done to refine our protocols. For instance, we may need to investigate whether six instillations are necessary to attain the optimal treatment effect or whether less would be as effective. Long-term observation after treatment may also be necessary to observe whether the cured mice would have recurrences or any side effects.

To augment the efficacy of the cytokine gene therapy, several other genes such as IL-2, GM-CSF, Interferon-γ are being screened. GM-CSF was chosen to be co-cloned into pBudCE4.1 with TNF-α to construct a mammalian expression vector which contained both mouse TNF-α gene and mouse GM-CSF gene (pBud-TNF-GM-CSF). Unfortunately, no synergistic effect was observed both *in vitro* and *in vivo* when plasmid pBud-TNF-GM-CSF was transfered into bladder cancer cells. Other strategies like the combination of cytokine gene therapy with corrective gene therapy (such as wild type P53 gene) may also be worth trying.

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