INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

Assessing the Efficiency of *In Vivo* Electroporation as a Nonviral Gene Transfer Technique and Studying its Application in Antiangiogenic Cancer Gene Therapy and Cancer Immunotherapy

Antonio Maietta

Department of Pathology
McGill University, Montreal

August 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements of the degree of
Master of Science

Acknowledgements

I would like to thank my research supervisor, Dr. Prud'homme, for giving me the chance to experience the world of research and for the opportunity of working on such an interesting project.

I would also like to thank my advisor, Dr. Burnier, for his help, kindness, and advice.

Special thanks to the Royal Victoria Hospital Research Institute and the Department of Pathology for their financial support of this study.

Thanks to France Croze for helping me get started in the lab and for instilling in me the correct laboratory procedures.

Special thanks to Yi-Gang Chang who has spent innumerous hours working and learning together with me on this project. I would never have finished this project on time without your help. Your kindness and your friendship will never be forgotten.

Many thanks to the secretaries in our department for their help with purchasing supplies and for helping with the paperwork.

I would also like to thank Dr. Hawani and his wonderful technician, Sue, for all of their help with the flow cytometry work.

I must thank Antonio Morilla, with whom I had the pleasure of watching the Champions League games and watching his Barcelona get humiliated. Thanks for your friendship and for helping me keep my priorities straight.

I would like to thank the other members of Dr. Prud'homme's lab for their help and guidance.

I would like to thank my entire family who mean everything to me and without them I would never have made it this far.

Last, but definitely not least, I thank my exceptional girlfriend for her support and understanding and for making these last two years that much more enjoyable.

Abstract

Electroporation refers to a technique that makes use of electrical pulses to create transient and reversible pores into cellular membranes which allows the passage of drugs and macromolecules, such as plasmid DNA, into the cell's interior. It is a widely used laboratory procedure for the transfection of cultured mammalian cells, plant cells and bacteria. Only recently has *in vivo* electroporation been used for the transfer of genetic material into living tissue, such as muscle, hepatocytes, and tumors of experimental animals. Using a luciferase assay, we have shown that electroporation-mediated luciferase gene transfer into solid, subcutaneous tumors in mice resulted in a 10^3 fold increase in tumor luciferase activity compared to naked DNA injections. Using this technique, we succeeded in inhibiting the growth of LLC tumors in mice by delivering plasmid DNA encoding mouse Endostatin and mouse single-chain IL-12 directly into the tumor site. Lastly, *in vivo* electroporation was used to enhance DNA vaccination with the tumor associated antigen CEA and IL-12 gene adjuvant, which protected mice from a tumor challenge with LLC/CEA$^+$ tumor cells. Our results suggest that *in vivo* electroporation is an efficient nonviral, gene transfer vehicle that can be used in the fields of antiangiogenic cancer gene therapy as well as cancer immunotherapy.

Abrégé

L'électroporation est une technique qui utilise le courant électrique pour créer de façon transitoire et réversible des pores dans les membranes cellulaires et ainsi faciliter le passage de droques et de macromolécules telle du plasmide d'acide désoxyribonucléique (DNA) à l'intérieur des cellules. Cette procédure est très utilisée pour la transfection de cellules de mammifère en culture, de cellules de plants et de bactéries. Ce n'est que récemment que l'électroporation est utilisée *in vivo* pour transférer du matériel génétique à l'intérieur de tissus tels les muscles, le foie et des tumeurs expérimentales chez les animaux de laboratoire. En mesurant l'activité du gène reporteur de la luciférase nous avons montré que l'électroporation du plasmide contenant ce gène dans des tumeurs de cellules transplantées était 10^3 fois plus importante que dans les tumeurs injectées sans électroporation. En utilisant la technique d'électroporation, nous avons inhibé la croissance des tumeurs de la ligné cellulaire de poumons de Lewis (LLC) chez la souris par l'injection directement dans les tumeurs du plasmide contenant le gène de souris de l'endostatine ou d'une chaine simple de l'interleukine-12 (IL-12). Enfin, lorsque l'électroporation *in vivo* a été utilisé la protection de la vaccination contre des cellules tumorales LLC qui exprime le CEA de manière stable (LLC/CEA$^+$) par le plasmide de DNA encodant l'antigène embryonnaire cancérigène humain (CEA) et le gène de l'IL-12 a été augmenté. Nos résultats suggèrent que l'électroporation *in vivo* est une méthode non-viral efficace de transfert de gènes qui peut être utilisée comme thérapie anti-angiogénique et immunogénique contre le cancer.

Table of contents

List of Figures

List of Abbreviations

AAVs	Adeno-associated viruses
Ang	Angiopoietin
bFGF	basic Fibroblast growth factor
CEA	Carcinoembryonic antigen
DC	Dendritic cell
EC	Endothelial cell
ECT	Electrochemotherapy
EGT	Electro-gene therapy
Epo	Erythropoietin
HUVEC	Human umbilical vein endothelial cell line
IL-12	Interleukin-12
IP-10	Interferon-inducible protein-10
IRES	Internal ribosome entry site
IVEP	*In Vivo* electroporation
LLC	Lewis lung carcinoma cell line
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinase
NK	Natural killer cell
NO	Nitric oxide
PF4	Platelet factor 4
RenCa	Renal carcinoma cell line
SCID	Severe combined immunodeficiency disorder
TAA	Tumor-associated antigen
TNF-α	Tumor necrosis factor alpha
t-PA	tissue Plasminogen activator
TSP-1	Thrombospondin-1
uPa	Urokinase
VEGF	Vascular endothelial growth factor
vWF	von Willebrand factor

Introduction and Literature Review

Chapter 1: *In Vivo* Electroporation as a Nonviral Gene Transfer Technique

1.1 History and Applications of Human Gene Therapy

Somatic gene therapy can be defined as the introduction of DNA into somatic cells such that the protein encoded by this DNA can serve a therapeutic function in treating a given disease. The first approved clinical trials involving gene therapy were performed on two girls suffering from severe combined immunodeficiency disorder (SCID) (Blaese et al., 1995). SCID is an inherited, lethal, recessive disease caused by a mutation in the adenosine deaminase gene. Like cystic fibrosis and familial hypercholesterolemia, these diseases are classified as monogenic disorders and are excellent candidates for gene therapy (Prince, 1998). The rationale behind this type of treatment is to provide patients with the normal genes that are lacking and in essence curing them using genetic material as medication. Currently, more than 300 gene therapy clinical trials have been approved involving a wide range of diseases from cancer to cardiovascular diseases suggesting that this type of therapy is not restricted to monogenic disorders but can also be used in the treatment of complex disorders (Mountain, 2000). Furthermore, as we reach the completion of the Human Genome Project and the sequencing of the entire human genetic component, we are approaching an era where all of the genes involved in human disease will be identified and gene therapy will revolutionize the way medicine is practiced in this new century. However, despite all the excitement and the hundreds of clinical trials involving gene therapy, there have only

been few, if any true success stories to arise from the clinic. In fact, the death of Jesse Gelsinger, the only patient reported to have died from genetic therapy, reminds us that basic research into the methodology of gene transfer into human somatic cells must be carefully studied and optimized before we begin to treat human patients with this technique (Marshall, 1999).

1.2 Obstacles Preventing Successful Gene Therapy Clinical Applications

Although the idea of introducing DNA into human cells for therapeutic means appears straightforward enough, researchers quickly realized that transferring genes to human tissue would need to overcome several obstacles before gene therapy could become a routine procedure (Crystal, 1995). Ideally, the therapeutic gene should be targeted to a specific cell type. For instance, in the case of cystic fibrosis, which is caused by a lack of the CFTR gene, it is of extreme importance that the transgene is targeted specifically to the airways where it is normally expressed and not to other tissues where gene expression can have detrimental effects (Verma and Somia, 1997). Also in the above example, in order for gene replacement to play a therapeutic role expression of the transgene must be sustained over the course of the individual's lifetime. Besides duration of transgene expression, another important consideration in the successful application of gene therapy is regulation of transgene expression. Better understanding of the molecular mechanisms involved in gene transcription will enable us to place the therapeutic gene under the control of promoter/enhancer elements whose activity can be modulated. Another interesting approach is to place the transgene under the control of a drug-sensitive promoter such that administration of the prodrug results in transgene

expression (Ye et al., 1999). Gene transfer efficiency, which refers to the number of cells that can be transfected by a given approach, is another important consideration given that a certain number of cells must be transfected in order to achieve therapeutic levels of the gene product. Another major setback in the experience of human gene therapy has been the inability to produce an effective gene transfer vehicle which does not elicit a host immune response and potentially give rise to inflammation. Lastly, problems have been encountered in producing purified gene-transferring vehicles in large quantities and at high concentrations (Anderson, 1998).

1.3 The Three Different Approaches Used for Somatic Cell Gene Transfer

One of the first factors to consider in a gene therapy trial is how to physically target the tissue of interest with the therapeutic gene. Generally, there are three different categories of somatic cell gene therapy: *ex vivo*, *in situ*, and systemic *in vivo* (Anderson, 1998). The first one involves removing cells from the body, transfecting them in culture and then transplanting the engineered cells back into the host. An advantage of the *ex vivo* approach is that when retroviruses are used as the gene-delivery machinery, the transgene becomes incorporated into the cellular chromosomes and is expressed for the duration of the cell's lifetime. However, for certain cell types such as myoblasts, optimal cell transplantation procedures have yet to be defined (Mulligan, 1993). *In situ* gene transfer refers to the delivery of the transgene directly into the targeted tissue. In this case, the gene product is expected to have primarily local rather than systemic effects. An example of this type of approach would be transferring a gene encoding a cytotoxic protein or inflammatory cytokine directly into a tumor mass. Finally, injecting the gene

delivery vehicle directly into the bloodstream or such that the protein is released into the circulation, is referred to as a systemic *in vivo* approach (Desurmont et al., 2000).

1.4 Vehicles for Gene Transfer: Viral versus Nonviral Vectors

Devising effective vectors, or gene delivery vehicles, has been the greatest obstacle in human gene therapy. Presently, the field is divided between two camps: those using viral vectors and the nonviral enthusiasts. By far, viral vectors have been the preferred choice in clinical trials. The following is a brief discussion of the advantages and disadvantages with both methods.

Viral Vectors:

Viruses are obligatory, intracellular parasites specialized in transferring their genetic material into host cells making them excellent candidates for gene transferring vehicles. Three major types of viruses are used for human gene therapy: retroviruses, adenoviruses, and adeno-associated viruses (AAVs) (Kay et al., 1997).

Before being administered to humans, viruses are made replication-deficient by deleting the viral genes essential for virus replication and replacing them with appropriate expression cassettes containing the transgene. In order to obtain sufficient viral titers to transfect enough cells, viruses are produced in packaging cell lines which contain the genes necessary for virus replication. This automatically leads to two disadvantages associated with viral vectors: the first involves the problem of conveniently producing purified viral vectors at high enough concentrations and second is the potential for replication-competent virus production due to homologous recombination with the packaging cell line (Prince, 1998).

Retroviruses can only transfect dividing cells such that certain cell types, including muscle and neurons, cannot be targeted by retroviral vectors (Verma and Somia, 1997). Retroviruses are optimally used for *ex vivo* techniques where cultured cells are transfected and then transplanted into the host. The major advantage of using retroviral vectors is that the DNA is incorporated into the genome such that the transgene is expressed for the remainder of the cell's lifespan. This is considered beneficial for hereditary or chronic diseases where transgene expression must be maintained but can also be risky since the chronic expression of certain factors, such as cytokines, can be toxic to the patient. Furthermore, transgene incorporation into the host genome is a random event which can lead to drastic outcomes by inserting into a protooncogene or through deletion of a tumor suppressor gene (Crystal, 1995).

In humans, adenoviruses are responsible for such diseases as upper respiratory infections. Unlike retroviruses, adenoviruses can transduce non-dividing cells *in vitro* and *in vivo*, they can be produced at higher titers, and do not integrate into chromosomal DNA. Although *in vitro* and animal studies have shown adenoviruses to have very high gene transfer efficiency (Trapnell and Gorziglia, 1994), they have also revealed, probably, the greatest setback involving viral vectors. The immune system has evolved to help clear infections with such pathogens as viruses. Although adenoviral vectors are manufactured to express as little viral component as possible, leaky expression of viral proteins results in host immune responses which clear virus-infected cells and decrease transgene expression (Anderson, 1998). Evidence for this is provided by the fact that transgene expression is significantly increased in nude mice, or in mice which have been given immunosuppressive drugs (Dai et al., 1995). The immune response against

adenoviral vectors involves both the cell-mediated arm and the humoral component of the immune system. In the cellular responses, cytotoxic T lymphocytes play an important role in destroying virus-infected cells (Yang and Wilson, 1995). Inflammatory cytokines, such as tumor necrosis factor α (TNF-α), are also crucial in viral clearance, giving rise to potentially harmful inflammatory reactions in the host (Elkon et al., 1997). Furthermore, adenoviral vectors have been shown to elicit antibody production against viral proteins which prevents subsequent vector injections, limiting their use to cases where transgene expression is required for only short periods of time. Presently, researchers are attempting to manufacture "gutless" vectors which are devoid of all viral components and completely non-immunogenic, but the feasibility of producing such a vector remains uncertain (Yeh and Perricaudet, 1997).

AAVs are relatively new in the field of human gene therapy and AAV vectors have been used to achieve therapeutic levels of the factor IX clotting factor in immunocompetent mice (Herzog et al., 1997). Although these vectors appear to be less immunogenic than adenoviruses a large proportion of the adult human population have pre-existing AAV-specific antibodies which would inhibit repeat injections of this vector. Another major disadvantage with this vector is its very small genome, which limits the amount of foreign DNA it can carry (Mountain, 2000).

The obstacles involved in the use of viral vectors are formidable and pose serious health risks to the recipients. Viral vector-induced inflammation and immune responses are not only dangerous to the patients, but also limit the duration of transgene expression due to immune clearance of transfected cells and prevent the possibility of repeat administration of the vectors. The potential for insertional mutagenesis and the

production of replication-competent vectors can have catastrophic consequences. All these reasons justify the philosophy of the non-viral camp, which prefers to use safer gene delivery methods and devise ways of improving gene transfer efficiency.

Non-Viral Vectors:

In general, nonviral vectors result in relatively inefficient gene transfer but are safe and easy to produce. Naked DNA injections, cationic liposomes, gene gun delivery, and electroporation are some of the nonviral vectors used in gene therapy studies.

Although naked DNA does not transfect cells in culture, suprisingly it has been shown to transfect murine skeletal muscle cells *in vivo* (Wolff et. al. 1990). The mechanism through which naked DNA is taken up and expressed *in vivo* is not fully understood, but it appears that the two major obstacles to efficient gene transfer involve lysosomal degradation and nuclear rejection of foreign DNA (Coonrod et al., 1997). The use of naked DNA injections as a gene delivery system offers several advantages compared to viral vectors. Most importantly, this technique presents little or no safety hazards to recipients. Moreover, plasmid DNA can easily be prepared from bacteria in purified and enormous quantities. Unlike viral vectors, naked DNA can be administered repeatedly in order to obtain therapeutic levels of the transgene and there are no restrictions on the size of the DNA that can be transferred. However, viral vectors have been the preferred choice in clinical trials due to the fact that naked DNA injections result in low transfection rates (Doh et al., 1997). In spite of this, several studies have demonstrated clinical potential for naked DNA injections, especially in the field of DNA vaccination where intramuscular injections of naked DNA encoding viral or tumor antigens results in protective immunity in adult mice (Donnelly et al., 1997). One group

used intramuscular injection of a plasmid DNA encoding erythropoietin (Epo) to significantly increase serum Epo levels and obtain normal hematocrit levels in anemic adult mice (Tripathy et al., 1996). Also, cytokines or cytokine inhibitors have been administered using this technique to inhibit autoimmune diseases (Prud'homme and Piccirillo, 2000; and Chang and Prud'homme, 1999). Furthermore, one of the few reported human gene therapy success stories involves intramuscular injection of plasmid DNA encoding $VEGF_{165}$ to induce new blood vessel formation in patients with critical limb ischemia (Baumgartner et al., 1998; and Isner et al., 1996)). These findings suggest that nonviral methods could become the preferred strategy in future gene therapy trials.

In order to enhance transfection efficiency, several modifications of the naked DNA approach have been devised. The most popular one involves cationic liposomes which bind DNA tightly by electrostatic interactions and permit cellular entrance of the DNA through fusion of the liposome with the cellular membrane (Farhood et al., 1994). Alternatively, gold-coated DNA particles are propelled into the cytoplasm using a high-pressure helium stream in the gene gun approach (Mountain, 2000). Lastly, electroporation, which is the focus of the following section, makes use of voltage-induced, transient pores in the cell membrane which facilitates DNA uptake.

1.5 *In Vivo* Electroporation (IVEP): Principles and Methodology

Since Neumann et al. first showed that the herpes thymidine kinase (TK) gene can efficiently be transferred to cultured lyoma cells in the presence of electric impulses (Neumann et al., 1982), electroporation has become a routine laboratory technique for the transfection of cultured cells. Although the mechanism by which electric impulses

facilitates gene transfer is not fully understood, much has been learned studying artificial systems such as gene and dye uptake by unilamellar lipid vesicles in the presence of electrical pulses (Neumann et al., 1999). These studies have revealed that electric fields induce membrane pores and cell deformation, which then trigger downstream events such as pore enlargement and transport of small and large molecules across the electroporated membrane. The porous and permeable nature of the membrane is transient and reversible such that optimal electrical parameters provide an excellent degree of cellular permeability without severely damaging the cells. Also, it was shown that for efficient DNA uptake it is important that the DNA be present before pulsation. This suggested that the electric pulses not only induce cellular permeabilization, but also facilitate the migration of molecules from the periphery towards the cellular membrane. In fact, using analytical expressions it was shown that electrodiffusion of DNA is about ten times more effective than simple diffusion (Neumann et al., 1999). It was soon understood that these electrical phenomena studied in *in vitro* systems might be applied to living tissue. This eventually gave rise to the field of IVEP.

IVEP is a very simple procedure that lasts only a few seconds. All that is required is an electrical pulse generator and electrodes that are applied across the target tissue and transmit the electrical pulses. Electrodes can either be superficial or invasive. Superficial electrodes consist of parallel copper plates separated by an adjustable distance and placed on the surface of the skin. Invasive electrodes, or needle electrodes are surgically applied to target tissue such as liver, brain, and muscle. They exist as different numbered electrodes, such as two-needle or six-needle electrodes, which allow them to better surround the target tissue for a more even current distribution throughout the tissue.

The electrical parameters to be optimized for IVEP include pulse strength, pulse length and the number of pulses administered. Frequently, the number of pulses is fixed at eight. Pulse strength is the electric field generated and is reported as Volts per centimeters separating the electrodes. Pulse length is the duration of each pulse and is usually given as microseconds or milliseconds. The frequency is the time between each pulse and is normally fixed at one second. Depending on the application, there are generally two different types of conditions used: high voltage-short duration pulses versus low voltage –long duration pulses.

1.6 Electrochemotherapy (ECT): A Clinical Application of IVEP

Electrochemotherapy is a treatment that combines chemotherapy with IVEP by using high-voltage electric pulses to deliver impermeable drugs into tumors. By increasing the permeability of cytotoxic drugs, lower doses can be used, resulting in fewer side-effects and less systemic toxicity (Singh and Dwivedi, 1999).

Bleomycin is an extremely cytotoxic drug, but has had limited success as an anti-tumor agent due to the fact that it does not freely diffuse across cellular membranes into the cytosol which is its site of action. Initially, studies showed that electropermeabilization could be used to introduce bleomycin into cells and tissues resulting in cytotoxicity (Belehradek et al., 1994). Anti-tumor effects of this technique were studied in three different murine tumor models (Sersa et al., 1994). In this study, adult mice bearing solid subcutaneous tumors were given intravenous injections of bleomycin. Electric pulses were then administered across the tumor using two parallel plate electrodes placed at either end of the tumor. Tumor growth arrest occurred in all

mice treated with ECT, whereas no effect on tumor growth was observed in mice treated only with bleomycin or with electric pulses alone. ECT has also had exciting results in treating patients in various clinical trials. In one study, the anti-tumor effects of ECT using bleomycin on cutaneous and subcutaneous tumors of basal cell carcinoma, malignant melanoma, adenocarcinoma, and head and neck squamous cell carcinoma were studied (Mir et al., 1998). From 273 evaluable tumors from five different cancer centres, treatment resulted in objective responses in 85.3% of tumors with 56.4% of tumors showing a complete response. Bleomycin was administered either intravenously or directly into the tumors with both approaches being equally effective. Importantly, treatment was well tolerated with minimal adverse side-effects such as instantaneous contraction of the underlying muscles. In a similar study, 142 of 143 metastatic cutaneous nodules or primary tumors responded to intralesional bleomycin administration followed by short intense electrical pulses (Heller et al., 1998). 91% of those tumors showed complete responses. In addition to its impressive local effects on tumor nodules, ECT has been combined with immunotherapy to inhibit lung metastasis of subcutaneously transplanted Lewis lung carcinomas in mice (Orlowski et al., 1998). A final consideration must be made to the type of electrodes used for ECT. Typically, parallel plate electrodes have been used for administering direct current pulses to tumors in ECT trials. However, one study comparing different needle array electrodes to the parallel plate ones showed that the invasive type of electrodes resulted in better clinical responses in mice melanoma tumors (Gilbert et al., 1997). This is probably due to a more uniform distribution of electrical pulse across the tumor and suggests that better electrodes can be devised to improve ECT trials.

1.7 Electro-Gene Therapy (EGT)

Another exciting clinical application of IVEP is EGT. This technique is a very recent, nonviral means of gene transfer which previously accounted for approximately one percent of studies related to gene therapy. Comparing EGT to other nonviral gene transfer techniques, such as liposomes and gene gun, it has been reported that for localized target areas IVEP is the best method (Muramatsu et al., 1998). Most studies involving EGT have focused on transfection of muscle. It is generally accepted that low voltages of long duration provide best results for muscle transfection (Gehl and Mir, 1999). These results have also shown that intramuscular EGT results in a two to four-log enhancement of gene expression over naked DNA injections, interindividual variability is strongly decreased, gene expression is long-lasting and can be modulated by varying the amount of DNA injected and/or the electric-pulse parameters (Mir et al., 1999). Important clinical applications of this technique have been reported. Injecting as little as one microgram of plasmid DNA encoding Epo into mouse muscle followed by electroporation resulted in therapeutical serum Epo levels with a stable increase in hematocrit suggesting a safe and novel method of treating serum protein deficiencies (Rizzuto et al., 1999). Furthermore, the EGT of a hepatitis B surface antigen and a HIV gag antigen resulted in increased muscle expression and immune responses showing the potential of this technique for increased DNA vaccine delivery (Widera et al., 2000). Similar to ECT, EGT can also be used to transfer plasmid DNA into tumors whose gene product can inhibit tumor growth. The first such approach was used to transfer human monocyte chemoattractant protein-1 (MCP-1) cDNA into rat brain tumors (Nishi et al.,

1996). In this particular study, the brain tumor was first electroporated using invasive needle electrodes followed by intra-carotid artery injection of plasmid DNA encoding MCP-1 cDNA. This resulted in localized, increased MCP-1 protein expression in the brain with massive monocyte infiltration. Using parallel plate electrodes, another group reported transfecting 4% of subcutaneously transplanted murine melanoma tumors by intratumoral injections of plasmid DNA encoding B-galactosidase gene (Rols et al., 1997). More recently, several papers have reported using intra-tumoral EGT to significantly inhibit tumor growth. Using a suicide gene approach, transferring the herpes thymidine kinase gene into murine tumors followed by systemic ganciclovir injection resulted in up to 90% inhibition of tumor growth compared to control (Goto et al., 2000). Furthermore, EGT with plasmid DNA encoding the green fluorescent protein reporter gene produced numerous, bright, green signals within the tumors. Finally, in a rat model of hepatocellular carcinoma tumors failed to develop in mice injected intraperitoneally with antisense bcl-2 followed by IVEP of liver nodules using invasive electrodes (Baba et al., 2000). These results supported a previous study which showed that IVEP could be used to efficiently transfer reporter genes into hepatocellular carcinomas (Heller et al., 2000).

In summary, IVEP appears to be an excellent alternative to viral vectors since any type of cell or tissue can be targeted, handling is easy and over in a matter of seconds, DNA can be administered repeatedly, no immunogenicity is expected and there are no restrictions on the amounts and sizes of DNA transferred.

Chapter 2: Anti-Angiogenic Cancer Gene Therapy

2.1 Angiogenesis in Health and Disease

Angiogenesis can be defined as the proliferation of new blood vessels from the preexisting parent vasculature. In human adults, endothelial cells are remarkably quiescent and angiogenesis, therefore, only occurs in specialized tissues under specific conditions. The female reproductive tissues are some of the few adult tissues to undergo angiogenesis, where it plays an important role in endometrial proliferation, corpus luteum formation, and pregnancy (Reynolds et al., 1992). Moreover, angiogenesis is crucial for wound and fracture repair and factors known to induce angiogenesis also play an important role in maintaining normal, established blood vessels as well as regulating such immune functions as lymphocyte trafficking and dendritic cell maturation (Ferrara, 1999; Carmeliet et al., 1999; and Barleon et al., 1996). It thus appears that angiogenesis is a finely regulated process such that it is turned "on" in specific conditions like wound repair and quickly turned "off" once the damage has been repaired. This has been referred to as the "angiogenic switch" which describes the existence of activators and inhibitors of angiogenesis and that when the inhibitors are in excess angiogenesis does not occur and only when the number of inhibitors decrease and activators are increased will angiogenesis take place (Hanahan and Folkman, 1996). In fact, the numbers of factors reported to have regulatory effects on the process of angiogenesis are many. Positive regulators of angiogenesis include growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), hypoxia, certain cytokines like IL-6, and such oncogenes as ras. Factors with anti-angiogenic activity

include platelet-derived factors such as platelet factor 4 (PF4) and thrombospondin-1 (TSP-1), cryptic angiostatic factors like endostatin, angiostatin, and PEX, IL-12, and certain tumor-suppressor genes, including p53. Certain diseases termed "angiogenic diseases" occur due to an imbalance in the factors involved in the "angiogenic switch (Folkman, 1995a). In the case of peptic ulcers and critical limb ischemia, the lack of angiogenesis results in disease and angiogenic activators such as bFGF and VEGF have been used to successfully treat these disorders (Folkman, 1995b). Contrarily, angiogenesis plays a causative role in other diseases such as diabetic retinopathy, rheumatoid arthritis, and tumor growth and metastasis. Therefore, a better understanding of the factors involved in the regulation of angiogenesis will eventually lead to its therapeutic control and treatment of a number of important diseases.

2.2 Angiogenesis: A Complex, Multi-Step Process

Angiogenesis is a complex, multi-step process involving cell-cell interactions, and the interaction of endothelial cells (EC) with extracellular matrix (ECM) components, soluble growth factors and proteases. The sequence of events involved in the angiogenic pathway include basement membrane degradation of the parent vessel, migration and proliferation of activated ECs, capillary sprout and lumen formation, and maturation of the new blood vessels involving basement membrane production and incorporation of peri-endothelial cells.

Normally ECs are remarkably quiescent with a turnover rate of approximately one thousand days (Folkman, 1995b). However, under given stimuli, such as hypoxia or inflammation, ECs become activated and angiogenesis occurs. The first step in the

angiogenic pathway is degradation of basement membrane. Invading macrophages or ECs themselves secrete matrix metalloproteinases (MMPs) which are crucial to the formation of new blood vessels (Stetler-Stevenson, 1999). MMPs play two very important roles in angiogenesis. Firstly, their proteolytic activity results in basement membrane degradation which facilitates EC migration. Secondly, the ECM acts as a reservoir for endothelial cell growth factors such as bFGF and VEGF which are not soluble proteins but rather sequestered by heparin (Schlessinger et al., 1995). MMPs play an important role in releasing these growth factors which can then act on ECs. Activated ECs then begin to penetrate the basement membrane and migrate towards the chemotactic factors resulting in the elongation and alignment of migratory ECs to form a capillary sprout. Importantly, activated ECs are structurally and metabolically different from quiescent cells. Not only do they become proteolytic, but they also express different cell surface molecules which are fundamental in the angiogenic pathway. One such surface marker is the $\alpha v\beta 3$ integrin which is specific for ECs undergoing angiogenesis and is involved in cell motility and growth (Eliceiri and Cheresh, 1999). In fact, this integrin inhibits EC apoptosis and has been used as a target for antiangiogenic therapies (Ruoslahti, 1997). Another feature of activated ECs is their hyperpermeability, due mainly to the action of VEGF, which is also known as vascular permeability factor and has been reported to be one of the most potent vascular permeabilizing agents known with a potency of about 50,000 times that of histamine (Dvorak et al., 1995). Hyperpermeability is not only a characteristic of activated ECs but plays an important function in angiogenesis. The endogenous ECM is not conducive to EC motility, but VEGF induces the EC release of matrix components which allows the formation of a

provisional ECM that is favorable for their migration (Dvorak et al., 1995). Finally, the new blood vessels undergo maturation by establishing normal intercellular junctions and ECs associate with perivascular cells such as pericytes which inhibit further EC proliferation. The balance between two groups of pro-angiogenic factors, VEGF and the angiopoietins (Ang), mediates this last step. Ang-1 acts via the Tie2 receptor to maintain and stabilize immature vessels by promoting the interaction of ECs with surrounding support cells, whereas Ang-2 has antagonistic effects on the same receptor. During the proliferative phase of angiogenesis, Ang-2 inhibits the association of perivascular cells which facilitates EC proliferation. In the absence of Ang-1, new vessels cannot mature and eventually regress (Holash et al., 1995; and Holash et al. 1995b). In fact, an anti-Ang-1 approach has been used to block angiogenesis and Ang-1 has been used therapeutically to protect against vascular leakage associated with VEGF overexpression in mice (Thurston et al., 2000).

In summary, angiogenesis is a process which involves numerous steps and is regulated by various factors. The better angiogenesis is understood at a molecular level, the more targets become available at blocking this phenomenon. And inhibiting angiogenesis is important when we consider the role it plays in various diseases, notably, tumor growth and metastasis.

2.3 Tumor Growth and Metastasis are Angiogenesis-Dependent

Angiogenesis is necessary, but not sufficient for tumor growth. Tumorigenesis is initiated by oncogenes and/or mutations in tumor suppressor genes which promote uncontrolled cellular proliferation and hyperplasia. In the early stages, tumors can divide

unimpeded in an avascular state until they reach a size of two to three millimetres in diameter (Folkman, 1986). Clinically, these asymptomatic lesions can be seen on the surface of the skin, on the cervix or in bladder. Studies on transgenic mice models of pancreatic islet cell carcinogenesis have clearly shown that an angiogenic switch must occur in premalignant tumors to progress to solid tumor formation and invasive carcinomas (Bergers et al., 1999; and Parangi et al., 1996). Prior to neovascularization, tumors are said to reach a saturation point where the proliferation rate of tumor cells is equal to their rate of apoptosis. This occurs when the metabolic requirements of the expanding tumor mass are no longer met by the endogenous blood supply. In this state of hypoxia, tumor cells, as well as inflammatory cells and connective tissue cells, secrete angiogenic factors such as VEGF and bFGF, which activate and attract ECs (Battegay, 1995). In turn, neovascularization augments tumor growth through two different mechanisms referred to as the perfusion effect and the paracrine effect (Folkman, 2000). The newly formed, hyperpermeable vessels literally bathe the tumor with nutrients and oxygen, while removing waste products through a perfusion effect. The latter mechanism involves the release of numerous mitogens, such as bFGF, by ECs which promote tumor growth. This two-way paracrine exchange of growth and survival factors, and the intricate relationship which exists between tumor cells and neighbouring ECs has been alluded to as the "two-compartment model of a tumor" (Folkman,1996a). In fact, it has been reported that up to forty percent of a tumor mass is composed of blood vessels.

Besides supplying the tumor with nutrients, angiogenesis also plays a crucial role in tumor metastasis by providing a primary route for tumor cells to enter the circulation and spread to distant organs (Zetter, 1998; and Folkman, 1996b). Strong evidence to support

the link between angiogenesis and tumor spread comes from the various studies showing that the vascularity of the primary tumor can be used as a prognostic marker for tumor aggressiveness and patient survival. Thus for many types of tumors, such as breast cancer, a highly vascular primary tumor is indicative of increased metastatic potential and decreased patient survival (Ellis and Fidler, 1995). Angiogenesis is involved not only at primary tumors, but also at distant metastases. When tumor cells from primary tumors are shed into the circulation they must extravasate at distant organ sites and adapt to the new microenvironment. These tumors can remain in a dormant state, as micrometastasis, for several years without causing any symptoms. Only once these tumors acquire an angiogenic phenotype does metastatic disease occur (Fidler and Ellis, 1994). Mechanisms explaining how micrometastases are activated have been proposed by studying a mice model of Lewis lung carcinoma (O'Reilly, 1997a). In this animal model, the removal of primary tumor results in neovascularization of dormant micrometastases and their subsequent growth. It has been shown that the primary tumor secretes an anti-angiogenic factor which is stable in the circulation and accumulates at sites of distant metastases suppressing their growth. Once the primary tumor is removed, the levels of the angiostatic factor decrease resulting in rapid growth of the distant tumors. Thus, it can be appreciated that the growth of distant tumors is regulated by a balance between angiogenic and angiostatic factors and that a decrease in the level of anti-angiogenic factor results in an angiogenic switch that is favorable for tumor growth. Therefore, angiogenesis is essential for growth of both the primary and secondary tumors. Hence, by blocking angiogenesis not only are you starving the primary tumor of essential nutrients but also preventing an escape route for malignant cells and keeping

micrometastases in a dormant state. This has given rise to a new approach for treating cancer, termed, antiangiogenic cancer therapy.

2.4 Antiangiogenic Cancer Therapy

The fundamental principle of this approach is the attempt to treat cancer, not by attacking tumor cells themselves, but rather the blood vessels which are necessary for tumor growth (Hanahan, 1998; and Ezzell, 1998). Theoretically, angiostatic therapy offers several advantages over conventional cancer therapy. Firstly, ECs, unlike tumor cells, represent a genetically stable population of cells eliminating the risk of drug resistance that is seen with regular chemotherapy. Evidence for this comes from young, hemangioma patients that are treated with an anti-angiogenic factor, interferon alfa-2a, without any occurrence of drug resistance and very little toxicity (Folkman, 1995b). Furthermore, studies in mice have shown that tumors treated with angiostatic factors regress completely. When treatment is halted tumor regrowth occurs. Readministration of the angiostatic factor results in further tumor regression and this treatment cycling can be repeated several times with the same outcome (Kerbel, 1997; and Boehm et al., 1997). Another advantage of angiostatic therapy is based on the fact that ECs undergoing angiogenesis are structurally and metabolically different from those in quiescent vessels. Therefore, treatment is more selective reducing the risks of potential side effects. Moreover, some researchers have taken advantage of the fact that activated ECs express specific cell surface markers, such as $\alpha v\beta 3$ integrins, to construct ligands for this integrin coupled with anticancer drugs to treat tumors in mice (Arap et al., 1998). New vessels are hyperpermeable and have abnormal architecture resulting in increased interstitial

pressure which acts as a barrier for chemotherapeutic agents to reach the tumor (Gasparini, 1999). By targeting ECs instead, antiangiogenic agents do not need to reach the tumor cells making the target more accessible. Similarly, antiangiogenic agents do not need to cross the blood-brain barrier. Finally, it is expected that antiangiogenic agents would be effective against most solid tumors, regardless of tumor cell type, since angiogenesis appears to be fundamental for tumor growth and metastasis (Taniguchi, 1998).

Many different strategies exist for inhibiting angiogenesis (Gastl et al., 1997; Brower, 1997; Keshet and Ben-Sasson, 1999;and Oehler and Bicknell, 2000). There exist endogenous angiostatic factors, such as endostatin and angiostatin, which can directly inhibit EC proliferation and migration. Administration of these molecules in mice tumor models has been successful in inhibiting tumor growth by preventing angiogenesis. Furthermore, tumors secrete angiogenic factors, including VEGF, which are necessary for tumor growth. Blocking the activity of these factors has also been useful in preventing tumor growth in animals. However, this approach is riskier since tumors have the ability to adapt to this sort of treatment by secreting different angiogenic molecules and can potentially lead to drug resistance. $\alpha v\beta 3$ integrins specific for activated ECs confer a necessary anti-apoptotic activity to endothelium undergoing angiogenesis. Antagonistic peptides, known as RGD peptides, can bind to this integrins and prevent angiogenesis (Bischoff, 1997). Activated ECs and tumor cells secrete MMPs which are involved in basement membrane degradation and EC migration. PEX, a C-terminal fragment of MMP-2 which binds to ECs and prevents MMP-2 activity, has been shown to block angiogenesis *in vivo* and inhibit tumor growth in mice (Brooks et al.,

1998). Finally there is evidence to suggest that antiangiogenic therapy in combination with classical cytotoxic therapy can potentiate the antitumor effects of either drug alone (Teicher et al., 1994).

Since antiangiogenic therapy is angiostatic in nature, therapy must be sustained to achieve long-term suppression of tumor growth. Moreover, although quiescent ECs differ from activated ones, angiogenesis is an important physiological response involved in wound repair and the female reproductive system. Furthermore, since one antiangiogenic agent, thalidomide, has already proven to have teratogenic effects and since the consequences of systemic antiangiogenic therapy have not been fully studied in humans, treatment should be kept local preventing any side effects which may be associated with systemic therapy. Theoretically, gene therapy can provide local, sustained expression of angiostatic factors.

2.5 Advantages of Angiostatic Cancer Gene Therapy

Combining the fields of angiogenesis research and gene therapy research has given rise to the exciting new field of antiangiogenic gene therapy (Kong and Crystal, 1998a; and Folkman, 1998). Several advantages are offered using a gene therapy approach over conventional drugs. Systemic administration of an anti-angiogenesis agent can result in promiscuous anti-angiogenesis and affect normal physiological functions. In fact, a study has already shown that systemic antiangiogenic treatment with AGM-1470 in nonpregnant mice inhibited endometrial maturation and corpus luteum formation (Klauber et al., 1997). Gene therapy would eliminate this risk by directing gene transfer to the tumor environment. Also, gene transfer would result in accumulation of the

antiangiogenic factor within the tumor without increasing systemic levels of the drug. Furthermore, since angiogenic and antiangiogenic factors have an effect on cells within the local environment and not necessarily on the cells expressing the factors, delivering and expressing the gene in cells in the tumor environment, without targeting a specific cell type, should be sufficient to have an antiangiogenic effect. Lastly, gene therapy would definitely be cheaper than daily injections with antiangiogenic factors and would solve the unresolved pharmacological problems with bolus injections.

Various animal studies have shown that gene therapy may prove to be an effective way to treat cancer by blocking angiogenesis in solid tumors (Kong and Crystal, 1998a). Many of these studies have targeted the activity of VEGF, which is one of the most specific and potent angiogenic factors involved in tumor angiogenesis. In a rat glioma model, retroviral transfer of a truncated VEGF receptor-2 (VEGFR-2) gene resulted in suppressed tumor angiogenesis and prolonged survival of rats with intracerebral tumors (Machein et al., 1999). The mutated VEGFR-2 works by sequestering available VEGF secreted by tumor cells and inhibiting its angiogenic activity. Another study using adenoviral transfer of soluble VEGFR-1 in a murine model of colon carcinoma showed that vector can be administered locally, either intravenously for liver metastases, intratracheally for lung metastasis, or intratumorally for subcutaneous tumors, to suppress tumor growth without the risk of systemic antiangiogenesis (Kong et al., 1998b). In fact, the use of soluble VEGF receptor gene therapy has been used to suppress the growth of a variety of mouse tumors including mammary, ovarian and lung carcinoma, as well as glioblastoma, suggesting a crucial role for VEGF in promoting tumor angiogenesis (Millauer et al., 1996; and Millauer et al., 1994). Antisense technology has also been

used to block VEGF activity and inhibit tumor growth. Growth of rat C6 glioma cells, stably transfected with antisense-VEGF cDNA, implanted subcutaneously was significantly inhibited and tumor vascularization was reduced (Saleh et al., 1996). Several antiangiogenic factors have also been used for gene transfer purposes to inhibit tumor angiogenesis. Transduction of established intracerebral gliomas by adenoviral vectors carrying soluble platelet factor-4 (sPF-4) cDNA, resulted in a decrease in tumor angiogenesis and increased animal survival (Tanaka et al., 1997). Angiostatin and Endostatin, two potent angiostatic factors, have also been used for antiangiogenic cancer gene therapy purposes in animals. One study showed that subcutaneous implantation of an angiostatin, stably transfected mouse fibrosarcoma cell showed a seventy-seven percent decrease in tumor growth compared to nontransfected cells and resulted in hypovascular tumors (Cao et al., 1998). Retroviruses were also used to transfer the angiostatin cDNA into rat RT2 glioma cells, resulting in small, pale tumor nodules when implanted into the subrenal capsule, intracranially, and subcutaneously, compared with the large, red, hypervascularized tumors which formed with the vector-transfected control tumor cells (Tanaka et al., 1998). Gene therapy trials using another potent antiangiogenic factor, Endostatin, have also been successful in preventing tumor growth in animals. Systemic administration of an adenovirus vector carrying endostatin cDNA was shown to significantly inhibit the growth of both JC breast carcinoma and Lewis lung carcinoma models in mice, and to prevent lung metastases in the Lewis lung model (Sauter et al., 2000). Also, subcutaneous implantation of endostatin-transfected mouse renal carcinoma cells (RenCa) and endostatin-transfected human colon carcinoma cells (SW620) resulted in hypovascular tumors that were up to ninety-one percent smaller than tumors from

nontransfected control cells and significantly decreased lung and liver metastases, respectively (Yoon et al., 1999). Similar results were obtained when a cell mixture of twenty-five percent transfected cells and seventy-five percent control cells were implanted, suggesting that gene delivery into a minority of tumor cells can be effective in blocking angiogenesis and preventing tumor growth and metastases. Although the great majority of these studies use viral vectors to transfer the angiostatic gene, the literature contains three reports of nonviral antiangiogenic gene therapy for cancer treatment. The sustained production of Endostatin following intramuscular injections of naked DNA formulated with a PINC polymer resulted in endostatin serum levels of seven nanograms per millilitre, which was enough to inhibit the growth of subcutaneously implanted mouse RenCa and Lewis lung carcinomas and to prevent the lung metastases of the RenCa tumors (Blezinger et al., 1999). Furthermore, intratumoral injections of liposomes complexed to plasmids encoding angiostatin and endostatin resulted in a thirty-six percent and forty-nine percent reduction, respectively, in a breast tumor cell line (MDA-MB-435) implanted in the mammary fat pads of nude mice (Chen et al., 1999). Moreover, intravenous injection of the endostatin-liposome complex reduced tumor growth in nude mice by nearly forty percent, providing further evidence for the efficacy of nonviral delivery of antiangiogenic genes. Lastly, systemic gene delivery of three different gene-liposome complexes, namely p53, GM-CSF, and angiostatin genes, were shown to be equally effective at inhibiting tumor metastasis and tumor angiogenesis in a murine model of invasive melanoma (Liu et al., 1999).

Chapter 3: Enhancing DNA Vaccination Against Tumor Associated Antigens (TAA) Through *In Vivo* Electroporation

3.1 Principles of Tumor Immunotherapy

Studies from laboratory animals and humans have provided convincing evidence to suggest that the immune system can recognize and destroy malignant cells. The isolation of tumor-specific T cells from cancer patients has led to the identification of a number of tumor-associated antigens (TAA) (Timmerman and Levy, 1999; Kawakami and Rosenberg, 1997; Boon and van der Bruggen, 1996; and Pardoll, 1994). New therapeutic approaches to cancer involve enhancing immune responses against these antigens. Before reviewing some of the current research in this field, the cells involved and the effector mechanisms used in cancer immunity will be discussed.

T Lymphocytes:

Most of the attention has focused on $CD8^+$ cytotoxic T lymphocytes (CTLs) for several reasons. Firstly, most tumors express MHC class I molecules but are MHC class II negative. Moreover, *in vitro* CTLs isolated from tumor-bearing mice are able to lyse tumor cells directly upon recognition of peptide-MHC class I complexes expressed by tumor cells. In fact, many of the TAAs have been identified by analyzing the antigen specificity of tumor-reactive CTLs isolated from cancer patients. However, given the crucial role of $CD4^+$ Th cells in regulating most antigen-specific immune responses, it is not surprising that $CD4^+$ T cells have also been shown to play an important role in the antitumor response. Recent studies have shown that $CD4^+$ T cells are involved in several distinct antitumor effector mechanisms (Toes et al., 1999). These effector mechanisms include priming of tumor-specific CTLs, either directly through the release of soluble

factors such as IL-2, or indirectly by activating dendritic cells (DCs) which present antigens to CTLs, and recruitment and activation of infiltrating macrophages and eosinophils involved in tumor clearance (Hung et al., 1998). Thus, both T cell subsets appear to play important roles in antitumor immunity and most T cell depletion studies have shown that both $CD4^+$ and $CD8^+$ T cells are required for systemic antitumor effects.

Natural Killer Cells and Macrophages:

Natural killer (NK) cells are a distinct group of lymphocytes that lyse tumor cells without prior sensitization and with no MHC requirements. NK cells have been shown to kill tumor cell lines *in vitro* and prevent metastasis in mice injected i.v. with malignant cells.

Activated macrophages mediate their antitumor effects via several effector mechanisms. The secretion of a number of cytotoxic substances, such as free oxygen radicals, or cytokines such as tumor necrosis factor (TNF) play a very important role in the tumoricidal effects of macrophages. Finally, phagocytosis is another mechanism by which macrophages can kill tumor cells.

Dendritic Cells:

Dendritc cells (DCs) are specialized antigen presenting cells (APCs) which play a pivotal role in the initiation of cell-mediated immunity (Banchereau and Steinman, 1998; and Hart, 1997). High level expression of MHC class I and class II molecules, as well as co-stimulatory molecules, makes DCs the ideal cell-type for presenting tumor protein antigens to T cells. Because of these characteristics, the use of tumor antigen-loaded DCs as vaccines for cancer immunotherapy have been extensively studied (Timmerman and Levy, 1999). Furthermore, initial clinical trials have shown that DCs pulsed with tumor

antigens could elicit specific anti-tumor immune responses in cancer patients (Hsu et al., 1996).

Antibodies and B cells:

Although monoclonal antibodies specific for tumor antigens have been isolated, generally a strong humoral response against tumor antigens does not result in significant antitumor activity. *In vitro*, antibodies have been shown to kill solid tumor cells through several mechanisms. Antibody-coated tumor cells undergo opsonization and are phagocytosed by macrophages, with or without the presence of complement. Furthermore, antibody-coated tumor cells can be killed by antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of neutrophils, NK cells, or macrophages. The importance of these effector mechanisms *in vivo* is not fully understood. Antibodies have been used to treat tumors experimentally by coupling them with cytotoxic agents and targeting them to tumor epitopes. Finally, depending on the tumor model studied, B cell involvement in regulating tumor immunity may or may not be of importance.

3.2 Mechanisms Involved in Tumor Escape from Immune Destruction

Although tumor antigens exist and are targeted by specific immune effector mechanisms, cancer cells have devised several ways to elude antitumor immune responses and avoid destruction. The following is a brief discussion of the several mechanisms involved in tumor escape from immune surveillance.

Cancer cells are genetically and phenotypically unstable, and T cell responses against specific tumor antigens can lead to the selection of nonimmunogenic tumor cell variants in a similar way that microorganisms develop drug resistance. Also, tumor cells

are not professional APCs and presentation of tumor antigens by cancer cells to T cells may result in T cell anergy in a mechanism identical for maintaining peripheral tolerance to self-antigens. For instance, cancer cells may present antigens in the context of MHC molecules but lack the expression of co-stimulatory molecules necessary for proper antigen presentation. Moreover, tumors also show decreased expression of MHC molecules allowing them to escape host immunity. Finally, tumors can secrete their own immunosuppressive factors such as TGF-β, IL-10, or VEGF which results in ineffective tumor immunity. Therefore, one of the greatest challenges of cancer researchers and immunologists is to figure out ways to potentiate or amplify patients' antitumor immune responses. The next section will discuss one such attempt: boosting specific tumor immunity through the use of DNA vaccination against common specific TAAs.

3.3 DNA Vaccination Primes Antitumor T-Cell Responses

DNA vaccinations are very effective at priming MHC-restricted T- cell responses. Animal studies have shown that intramuscular injections of naked DNA encoding TAA elicit T cell responses that have the potential of rejecting tumor cells expressing the antigen (Kowalczyk and Ertl, 1999). Previous work from our lab made use of the human carcinoembryonic (CEA) antigen in mice to study different DNA vaccination strategies. Mice were immunized with CEA plasmid DNA and then challenged with a Lewis lung carcinoma cell line stably transfected with the CEA antigen. Tumor growth was retarded but eventually all mice developed solid, subcutaneous tumors. When a plasmid encoding the IL-12 gene was co-administered with the CEA plasmid, IL-12 acted as an adjuvant by enhancing the T cell-mediated immune response against the CEA antigen protecting up to

80% of the mice studied from developing tumors (Song et al., 2000). In a similar study, DNA vaccination with the TAA Neu induced protective immunity against the development of spontaneous mammary tumors in HER-2/neu transgenic mice as well as in mice challenged with tumor cells expressing the Neu antigen (Amici et al., 2000; and Chen et al., 1998).

In order for DNA vaccines to elicit potent, protective immune responses, efficient gene transfer is crucial. Thus, although DNA vaccines have been shown to induce protective immunity in small animals, the lower efficiency of naked DNA uptake by cells in larger animals, such as humans, may limit the use of this technique for human vaccination. Recently, it has been shown that electroporation can be used to increase DNA vaccine delivery and amplify protective immune responses (Widera et al., 2000). Using the weakly immunogenic hepatitis B surface antigen, this group showed that IVEP resulted in an earlier onset and a greater magnitude of anti-hepatitis B antibodies in mice. Also, the immunogenicity of an HIV gag DNA vaccine was increased as was seen by increased antibody titers, increased $CD8^+$ T cell responses, and a decrease in the amount of DNA required to induce an immune response. Furthermore, similar results were obtained using a combination HIV gag and env DNA vaccine in guinea pigs and rabbits. Thus, these results suggest that electroporation may be very useful in the field of cancer immunotherapy by amplifying the immune responses associated with DNA vaccination with TAA.

Chapter 4: Antiangiogenic and Immunomodulatory Factors Used for this Project

4.1 Soluble VEGF Receptor 1 (sFlt-1)

VEGF, also known as vascular permeability factor (VPF), is a potent angiogenic and vascular permeability factor. Alternative splicing of a single VEGF mRNA gives rise to five different isoforms, 121, 145, 165, 189, and 206, identified by the number of amino acids they contain. $VEGF_{165}$, the major and most potent isoform, is sequestered on heparan-sulfate proteoglycans of cell surfaces and in the extracellular matrix. The angiogenic actions of VEGF are mediated via two membrane-bound, tyrosine kinase receptors expressed almost exclusively on ECs. VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) both contain seven extracellular immunoglobulin domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. Upon VEGF binding, the receptors homo- or hetero-dimerize, and undergo autophosphorylation which begins a signal transduction cascade ultimately resulting in the production of proteases, the expression of specific integrins, and finally, in EC proliferation and migration (Neufeld et al., 1999).

VEGF plays a pivotal role in vasculogenesis, angiogenesis, and endothelial survival of the mature vasculature. Vasculogenesis occurs in the embryo and is defined as the formation of primitive blood vessels from mesenchymal tissue (Carmeliet, 2000; Risau, 1997; Beck, Jr., and D'Amore, 1997; and Risau, 1995). The most convincing evidence for the crucial role of VEGF in the development of the embryonic vasculature is provided by studies showing that embryonic mice lacking a single VEGF allele do not survive due to abnormal blood vessel formation (Carmeliet et al., 1996; and Ferrara et al., 1996). Furthermore, homozygous mouse mutants for the Flk-1 receptor fail to undergo

vasculogenesis and die *in utero*, between 8.5 and 9.5 days post-coitum, in the absence of any organized blood vessel formation (Shalaby et al., 1995). Interestingly, mice lacking the Flt-1 receptor are capable of forming ECs but organize them into abnormal vascular channels with an overgrowth of endothelial-like cells within the lumens resulting in embryonic death between post-coital days 8.5 and 9. (Fong et al., 1995). Thus, despite being structurally homologous the two VEGF receptors appear to have distinct biological properties. Biochemically, Flt-1 shows a ten fold greater affinity to VEGF but about a ten fold lower kinase activity than Flk-1. Mice expressing the Flt-1 receptor lacking the tyrosine kinase domain undergo normal embryonic development and angiogenesis suggesting that it may play an important role as a ligand-binding molecule, regulating the levels of VEGF available for angiogenesis (Hiratsuka et al, 1998). In support of this hypothesis, an endogenous soluble Flt-1 has been identified in pregnant mice whose levels are increased towards the end of pregnancy coinciding with a decrease in embryonic angiogenesis (He et al., 1999). A homologous soluble receptor has been isolated from a human umbilical vein endothelial cell (HUVEC) line suggesting that a similar mechanism of Flt-1- regulated angiogenesis may also be occurring in humans (Kendall and Thomas, 1993; and Kendall et al., 1996).

Deregulated expression of VEGF and its receptors appears to play a major role in various "angiogenic diseases", including cancer and diabetic retinopathy. Conclusive evidence exists demonstrating the correlation between increased serum VEGF levels and the incidence of tumor growth and metastasis in both mice and humans. A study examining VEGF serum levels in thirteen different types of mouse ascites tumors demonstrated that VEGF was significantly accumulated in the ascites fluids of all thirteen

tumors (Luo et al., 1998). In a human study of patients suffering from cancer of the breast, lung, prostate, esophagus, stomach, colon, rectum, brain, or head and neck or from melanoma, non-Hodgkin's lymphoma, mesothelioma, or angiosarcoma, both patients with local, regional and disseminated cancer had significantly increased serum VEGF levels compared with subjects without cancer (Salven et al., 1997). Also, patients undergoing cancer therapy were found to have lower serum VEGF levels than those without cancer therapy. One of the major causes of blindness in the Western World is ischemia-induced retinal neovascularization seen in diabetic patients (Henkind, 1978; Engerman, 1989; Merimee, 1990; Klein and Klein, 1997; Aiello et al., 1998; and Infeld and O'Shea, 1998). Examination of ocular fluid of patients with diabetic retinopathy showed significantly increased levels of VEGF suggesting that it plays a critical role in mediating the proliferation of blood vessels in the retina resulting in severe visual loss (Aiello et al., 1994). Moreover, in a mouse model of retinal neovascularization VEGF levels are increased following retinal hypoxia and remain elevated during the development of neovascularization suggesting that it plays a causative role in the pathological formation of new retinal vessels (Pierce et al., 1995). Lastly, rheumatoid arthritis is a chronic inflammatory disease characterized by lymphocytic infiltration of the joints. Angiogenesis plays an important role in recruiting and activating the inflammatory cells within the synovium. Through immunohistochemical staining, reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization analysis, VEGF levels have been shown to be significantly increased in the joints of arthritic patients (Nagashima et al., 1995).

Since VEGF plays such a crucial role in diseases caused by pathological angiogenesis, inhibiting the activity of this angiogenic factor can be used to treat such disorders. Several techniques have been used to block VEGF activity including neutralizing monoclonal antibodies, and antisense VEGF transcripts (Aiello, 1997; and Asano, 1999). Soluble or truncated VEGF receptors have proven to be extremely effective for blocking angiogenesis of solid tumors and inhibiting tumor growth (Lin et al., 1998; Millauer et al., 1996; and Goldman et al., 1998). The mutated receptors' ability to inhibit VEGF activity involves two different mechanisms. Firstly, soluble receptors act extracellularly by binding and quenching available VEGF molecules, preventing the interaction of VEGF with its receptors on the surface of ECs and its consequent angiogenic activities. Given that Flt-1 has a higher affinity for VEGF than the Flk-1 receptor, sFlt-1 should be more effective at sequestering available VEGF molecules. An *in vitro* study examining the ability of either soluble receptor from inhibiting endothelial cell proliferation and migration revealed that whereas sKDR, which is the human homolog of the mouse sFlk-1, could not prevent VEGF-mediated EC proliferation and only partially inhibit cell migration sFlt-1 almost completely blocked VEGF-induced cell proliferation and migration (Roeckl et al., 1998). The other mechanism involved in blocking VEGF activity is referred to as dominant-negative inhibition. This refers to the ability of soluble or membrane-bound receptors lacking kinase activity to bind VEGF and subsequently interact with full-length receptors found on the surface of EC. This association captures the normal, functional receptors on the cell surface, but prevents signal transduction which requires homo-or heterodimerization of two full-length monomeric receptor subunits. Lastly, a single intravitreal injection containing 200

nanograms of sFlt-1 or sFlk-1 inhibited retinal neovascularization in the mouse model of ischemia-induced retinal neovascularization in one hundred percent and ninety-five percent of animals, respectively (Aiello et al., 1995).

Thus, there exists abundant evidence suggesting the efficiency of soluble VEGF receptors to inhibit angiogenesis, making these molecules excellent candidates for their therapeutic use in preventing pathological angiogenesis.

4.2 Angiostatin

Angiostatin was first discovered in a murine model of Lewis lung carcinoma in which the primary tumor inhibited the growth of lung metastases (O'Reilly et al., 1994). Removal of the primary tumor resulted in the neovascularization and growth of lung micrometastases. It was discovered that the primary tumor was secreting an antiangiogenic factor which accumulated in the blood and urine of these mice and prevented metastatic growth by inhibiting angiogenesis at the secondary sites. This antiangiogenic factor was purified from urine of mice with primary Lewis lung tumors and was given the name Angiostatin. Molecular characterization and sequencing of this factor revealed that it was a thirty-eight kilodalton internal fragment of plasminogen containing the first four highly homologous, looped multiple disulfide-bonded domains, known as kringle domains. Proof that Angiostatin was indeed an internal fragment of plasminogen came from studies which showed that systemic administration of a recombinant molecule consisting of the first four kringle domains of human plasminogen successfully inhibited primary growth and metastasis of Lewis lung carcinomas in mice and inhibited EC proliferation *in vitro* (Sim et al., 1997).

Several mechanisms have been proposed to describe how Angiostatin is generated *in vivo*. In subcutaneous Lewis lung carcinomas, tumor cells secrete granulocyte-macrophage colony-stimulating factor which induces tumor-infiltrating macrophages to express the serine proteinase, metalloelastase (MME), involved in plasminogen cleavage into Angiostatin (Dong et al., 1997). Alternatively, human prostate carcinoma cells (PC-3) themselves secrete enzymatic activity that converts plasminogen to angiostatin (Gately et al., 1997). PC-3 cells secrete urokinase (uPa) which is involved in cleaving plasminogen into the plasmin intermediate. Plasmin, in the presence of free sulfhydryl donors (FSD) provided by the tumor cells, acts as both the substrate and enzyme for the generation of angiostatin. Angiostatin produced in a cell-free system by combining plasminogen with uPa and FSD has been used to inhibit angiogenesis *in vitro* and *in vivo* and to inhibit primary tumor growth and metastasis of Lewis lung carcinomas in mice. Furthermore, MMP-3, also known as stromelysin-1, and MMP-12 have been shown to cleave plasminogen *in vitro* to yield Angiostatin, implicating yet another class of proteinases in the production of this antiangiogenic factor (Lijnen et al., 1998; and Cornelius et al., 1998).

Although the mechanisms by which Angiostatin inhibits angiogenesis are not understood, several possibilities have been proposed. Angiostatin has been shown to bind the ATP synthase on the surface of HUVE cells (Moser et al., 1999). It has been proposed that ECs use the ATP synthase to produce an additional source of ATP which allows them to survive under the hypoxic conditions of a growing tumor. Angiostatin, by binding to this enzyme, may prevent ECs from producing needed ATP and result in decreased EC survival. Another recent study suggests that its antiangiogenic activity

relies on its ability to prevent the activation of plasminogen by tissue plasminogen activator (t-PA) (Stack et al., 1999). Vascular ECs secrete plasminogen activators, t-PA being the prominent one, which convert plasminogen into plasmin. Plasmin is an important protease that facilitates EC and tumor cell invasion through the ECM by digesting a number of molecules found in the BM and tumor stroma, such as laminin and fibronectin. t-PA can form a ternary complex with its substrate, plasminogen, and ECM components which enhances its catalytic activity. *In vitro*, Angiostatin can inhibit EC invasion by interacting with the ECM and disrupting its association with t-PA which prevents matrix enhancement of t-PA activation of plasminogen. Other evidence suggests that Angiostatin induces endothelial cell apoptosis and causes inappropriate activation of focal adhesion kinase, which is normally involved in crucial cellular responses, such as proliferation, migration, differentiation or death (Claesson-Welsh et al., 1998). Lastly, it has been reported that Angiostatin blocks angiogenesis *in vivo* by inhibiting endothelial cell proliferation by preventing the G_2/M transition induced by M-phase-promoting factors (Griscelli et al., 1998).

Systemic administration of human Angiostatin strongly inhibits the growth of primary human and murine tumors in mice (O'Reilly et al., 1996). However, given the pharmacological problems associated with systemic Angiostatin treatment, such as the need for high dosages, repeated injections and long-term administration of the protein, gene therapy is an attractive alternative for antiangiogenic therapy using Angiostatin (Cao, 1999).

4.3 Endostatin

Endostatin is one of the most potent angiogenesis inhibitors. It was discovered by looking for the production of novel inhibitors of endothelial cell proliferation by a murine hemangioendothelioma cell line, EOMA (O'Reilly et al., 1997b). Endostatin was the name given to the twenty kilodalton protein purified from the conditioned media of EOMA cells that specifically inhibited the proliferation of various endothelial cell lines. Like Angiostatin, Endostatin is a cleavage fragment of a larger precursor molecule that has no antiangiogenic activity. Microsequencing analysis of the twenty kilodalton protein revealed that it was identical to a C-terminal fragment of collagen XVIII. The ability of a recombinant twenty kilodalton protein produced from the C-terminal portion of mouse collagen XVIII to inhibit the proliferation of bovine capillary endothelial cells *in vitro* confirmed that Endostatin was indeed a cryptic fragment of collagen XVIII. Furthermore, systemic administration of recombinant Endostatin resulted in the complete regression of several different types of primary solid tumors, such as Lewis lung carcinoma, T241 fibrosarcoma, EOMA, hemangioendothelioma, and B16F10 melanoma, grown in mice to a size of at least one percent of total body weight. Similar to Angiostatin, Endostatin did not affect the proliferative index of the tumors. Immunohistochemical analysis revealed that the antitumor effect was due to an inhibition of tumor angiogenesis.

Several papers have reported plausible mechanisms for Endostatin activity. Recently, it has been demonstrated using three different apoptosis assays that Endostatin causes cow pulmonary artery endothelial cell apoptosis (Dhanabal et al., 1999a). Moreover, Endostatin treatment led to a significant reduction in Bcl-2, an anti-apoptotic

protein. These effects were specific to endothelial cells and did not occur with other cell types tested. Another study, using recombinant human Endostatin produced from a yeast expression system, showed that in addition to causing HUVE cell apoptosis, Endostatin treatment resulted in cell cycle arrest in the G_1 phase of ECs (Dhanabal et al, 1999b). Lastly, it has been shown that mouse Endostatin has a high affinity for heparin (Hohenester et al., 1998). Thus, Endostatin may also use an indirect approach at blocking angiogenesis by binding to the heparan sulfate proteoglycans involved in the signaling of such EC growth factors as VEGF and bFGF.

Despite Endostatin's impressive results in shrinking tumors in mice, human studies have not been as impressive. Although Endostatin and Angiostatin were seen as cancer cures following the initial results in mice, the fact is that these molecules have just recently become available as drugs for human applications (Ryan et al., 1999; and Rowe, 1999). Significant barriers must be overcome before pure and sufficient quantities of these proteins can be tested on various tumor models, assessed for toxicity, and eventually allowed into the clinic (Crystal, 1999). One feasible alternative is to use Endostatin gene therapy and give patients the task of manufacturing the drug themselves.

4.4 Interleukin-12 (IL-12)

IL-12 is unique from other cytokines because it is a heterodimer composed of two disulfide-linked chains, p35 and p40, encoded by separate genes. Active IL-12 requires coordinated expression of both genes. IL-12 plays a crucial role in regulating the early events of inflammatory responses and enhances the development of a Th1 response that favors cell-mediated immunity (Trinchieri, 1994; and Robertson and Ritz, 1996). Upon

T-cell interaction, antigen presenting cells, such as dendritic cells and B lymphocytes, produce IL-12 which causes T cell and natural killer (NK) cell proliferation and increased cytolytic activity and stimulates their secretion of IFN-γ. Numerous studies have shown that IL-12 administration causes regression of primary tumors and prevents metastasis of several tumor models in mice and has been tested in various clinical trials (Brunda et al., 1993; and Sun et al., 1998).

Anti-tumor effects of IL-12 are mediated by several effector mechanisms. One study examined the cellular and molecular mechanisms of IL-12- mediated anti-tumor activity in BALB/C mice bearing established subcutaneous RENCA or CT 26, murine colon adenocarcinoma, treated with daily one microgram intraperitoneal IL-12 injections (Tannenbaum et al., 1996). Cytokine and chemokine gene expression in tumors along with immunohistochemical analysis of tumors revealed that IL-12 responsive cells within the tumors, such as T cells and NK cells, secrete IFN-γ which induces tumor cells to synthesize the CXC chemokine interferon-inducible protein-10 (IP-10). Expression of IP-10 results in massive tumor infiltration of $CD8^+$ T cells and macrophages. The cytoloytic nature of infiltrating T cells, mediated by the effector molecules perforin and granzyme B, NK cells, and macrophage nitric oxide (NO) production are enhanced by IL-12 and result in tumor regression. These results support previous studies claiming that IFN-γ is a crucial mediator of the antitumor effects of IL-12 (Trinchieri, 1995; and Nastala et al., 1994). Furthermore, rabbit polyclonal antibodies specific for IP-10 resulted in inhibition of T cell infiltration into RENCA tumors and prevented IL-12 mediated tumor regression (Tannenbaum et al., 1998). This mechanism is now referred to as the "attraction-expansion" model and has been used by several researchers to treat

tumors in mice. The basic principle relies on using a combination of chemokines and cytokines such that the chemokine acts as a recruiting molecule, attracting immune cells to the tumor site, and cytokines are "activators" potentiating the cytolytic activity of the infiltrating cells (Paillard, 1999). In one study, intratumoral coinjection of two adenoviruses, one encoding IP-10 and the other IL-12, resulted in marked antitumor synergistic effect resulting in one hundred percent eradication of established CT26 tumors in mice (Narvaiza et al., 2000). Co-administration of the two vectors resulted in a potent tumor-specific CTL response with massive tumor infiltration of CD4 and CD8 T cells. NK cells were also shown to play a crucial role in the antitumor response. In a similar experiment, intratumoral coinjection of adenoviruses encoding IL-12 and lymphotactin, a chemokine that can cause local accumulation of $CD4^+$, $CD8^+$, and NK cells, results in tumor regression and protective immunity against solid, murine, breast adenocarcinomas stably transfected with polyoma middle T (PyMT) or Neu (8142) antigen (Emtage et al., 1999).

The fact that IL-12 also displays antitumor effects and antimetastatic effects in SCID mice demonstrates that additional, non-immune responses are involved in IL-12 mediated tumor regression. Several early studies demonstrated that IL-12 displayed *in vivo* antiangiogenic activity by inhibiting murine corneal vascularization induced by bFGF (Voest et al., 1995; and Kerbel and Hawley, 1995). Only later was it shown that the antiangiogenic activity was not due to IL-12 itself but by the downstream chemokine IP-10, and that neutralizing antibodies against IP-10 resulted in inhibition of IL-12 mediated antiangiogenic activity enhancing tumor growth and metastases of human lung tumors in SCID mice (Arenberg et al., 1996). Human Burkitt lymphoma cell lines give

rise to subcutaneous tumors in athymic mice. Inoculation of Epstein-Barr virus-immortalized normal human lymphoctes into these tumors results in elevated IP-10 levels and tumor regression. Constitutive IP-10 expression in mice Burkitt tumors inhibits the ability of the tumors to grow as subcutaneous tumors and results in visible necrosis. Histological examination of these tumors revealed that necrosis is due to vascular damage of tumor blood vessels, including intimal thickening and capillary damage (Sgadari et al., 1996). Further insight into the antiangiogenic activity of IL-12 has revealed the critical role of NK cells in vascular damage (Yao et al., 1999). In an *in vivo* model of bFGF-induced neovascularization, neutralization of NK cell function using specific asialo GM1 antibodies inhibited IL-12 mediated antiangiogenic activity. Burkitt lymphomas in nude mice, treated with local IL-12 injections, resulted in vascular damage with NK cell infiltration surrounding small vessels. Furthermore, NK cells isolated from nude mice and activated *in vitro* with IL-12 become strongly cytotoxic to syngeneic aortic endothelial cells. These results suggest that NK cells play an important role in IL-12 mediated antiangiogenic activity and that the endothelial cell cytotoxic nature of NK cells might be involved in IL-12 suppression of angiogenesis.

Although IL-12 appears to be a promising anticancer drug, systemic administration of the cytokine can result in severe toxic effects and has already caused the death of two patients enrolled in clinical trials (Cohen, 1995). Several studies have provided evidence for the efficiency of local, intratumoral accumulation of IL-12 using gene therapy approaches. Replication-defective adenovirus carrying a bicistronic vector containing cDNAs for the p40 and p35 subunits of IL-12 was assessed for its antitumoral activity (Chen et al., 1997). Intratumoral injection of the vector resulted in elevated IL-12 and

IFN-γ levels over two to five days and levels returned to baseline by seven to fourteen days. These elevated IL-12 levels resulted in complete regression of subcutaneous murine MB49 bladder tumors in one hundred percent of animals studied, and cured mice showed protective immunity against rechallenge with the same cell line. An *ex vivo* approach was used to show the antitumor effects of IL-12 in advanced, terminally ill, melanoma patients (Sun et al., 1998). Melanoma cells were isolated from surgically removed metastases and were transfected with human IL-12 gene using ballistic gene transfer. Patients were then vaccinated with weekly, subcutaneous injections of the engineered cells for a period of six weeks and showed no signs of systemic toxicity other than a mild fever. Some of the patients developed delayed-type hypersensitivity (DTH) reactions against autologous melanoma cells and one patient showed a clinical response with regression of some cutaneous metastases over three months. Furthermore, biopsies of some of the metastases showed massive infiltration of $CD4^+$ and $CD8^+$ T lymphocytes suggesting that the vaccination protocol was successful in boosting antitumor immune response. Cationic liposomes complexed with a vector carrying the IL-12 gene have also been used to inhibit lung metastases in a mouse RENCA model (Blezinger et al., 1999). Intratracheal administration of the liposome vector resulted in elevated levels of biologically active IL-12 and IFN-γ in the bronchoalveolar lavage (BAL) fluid. These levels resulted in a significant reduction in the number of lung metastases twenty-four hours after subcutaneous challenge with RENCA cells, compared with control group. Moreover, intratracheal IL-12/lipid administration inhibited the growth of established lung metastasis and increased the survival of mice bearing RENCA tumors. A final consideration will be given to the amount of IL-12 needed at the tumor site in order to

achieve tumor regression. Using two different retroviral vectors carrying the IL-12 gene, one study has shown that the amount of IL-12 available at the tumor site is crucial for regression of solid, subcutaneous, C26 colon carcinomas in mice (Colombo et al., 1996). The first vector system resulted in the release of thirty to eighty picograms per millilitre of IL-12 at the tumor site, resulting in delayed tumor growth and scanty lymphocytic infiltration of the tumor in BALB/C mice. Switching to a retroviral system which released five nanograms per millilitre of IL-12 at the tumor site, resulted in complete tumor regression in one hundred percent of animals studied which was associated with massive tumor infiltration of $CD8^+$ T cells and NK cells. In order to achieve such elevated IL-12 levels through systemic administration of recombinant IL-12 protein, very high IL-12 concentrations would be required leading to severe, toxic effects. This study demonstrates that the amount of IL-12 required at the tumor site in order to achieve regression is quite high and that gene transfer techniques are most efficient at obtaining these levels.

Lastly, it should be noted that two different IL-12 genes were used in my project. pVR1255/bisIL-12 is an expression vector carrying the native mIL-12 gene in which both subunits, p35 and p40, are separated by an internal ribosome entry site (IRES) and are therefore expressed separately. A second pVR1255 expression vector, referred to as pVR1255/melIL-12, carries the mouse IL-12elasti gene which encodes a single chain IL-12. The single chain IL-12 makes use of a bovine linker motif that joins the two subunits together and allows both subunits to be transcribed together. A major disadvantage of the native bisIL-12 gene is the uncoordinated expression of p35 and p40 which gives rise to free p40 subunits that act as IL-12 antagonists by binding IL-12 receptors without any

signal transduction (Chen et al., 1997). Therefore, using melIL-12 prevents the production of free p40 subunits and ensures that every transcription event from a single promoter results in biologically active IL-12.

.

Chapter 5: Hypothesis and Objectives of this Study

The main objective of this study is to assess the efficiency of *in vivo* electroporation as a nonviral gene transfer technique. We hypothesize that electroporation can be used to efficiently transfer genes directly into the tumor environment. Once this is established, we intend to inhibit tumor growth in mice using *in vivo* electroporation to deliver genes encoding angiostatic factors and IL-12 directly into the tumor site. Lastly, electroporation-mediated intramuscular gene transfer will be used to enhance DNA vaccination with the CEA tumor antigen in the hopes of protecting mice against challenge with LLC tumor cells expressing CEA. Therefore the specific aims of this study are:

1. To assess the efficiency of *in vivo* electroporation in transferring genes into solid, subcutaneous tumors in mice using a luciferase assay.
2. To inhibit the growth of LLC tumors in mice through electroporation-mediated intratumor gene transfer of plasmid DNA encoding angiostatic factors, such as Endostatin, and the Th1 cytokine IL-12.
3. To protect mice against challenge with tumor cells expressing CEA through electroporation-mediated enhanced DNA vaccination with CEA and IL-12 adjuvant.

Chapter 6: Materials and Methods

6.1 Cloning of the Mouse Soluble VEGF Receptor-1 (sFLT-1)

6.1.1 RNA Isolation From Mouse Ten-Day Embryos (E10)

Mouse E10 embryos were used as our RNA source since sFLT-1 is highly expressed during mouse embryo organogenesis (E9.5-E12.5) (Peters et al., 1993). Pregnant CD1 mice in their tenth day of pregnancy were sacrificed by cervical dislocation and whole embryos were extracted. Total RNA isolation was performed using TRIzol® Reagent (Life Technologies), and was dissolved in 150μL of DEPC water at a concentration of 4.74μg/μL. The quality of the RNA was assessed on a 1% formaldehyde gel.

6.1.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR) Using sFLT-1 Specific Primers

RT-PCR of the isolated RNA was performed using the ThermoScript™ RT-PCR System (Life Technologies). Primers were constructed based on the FLT-1 sequence deposited in GenBank (accession number, LO7297) and were designed to amplify the entire 2.3 kilobases of the extracellular region of the native FLT-1 receptor (Finnerty et al., 1993). The sense primer contained 40 base pairs including four non-specific base pairs (the handle region), a Not I restriction enzyme site (5' GCGGCCGC) and 28 specific nucleotides (5' CGTCTTGCTCACCATGGTCAGCTGCTGG). The anti-sense primer also contained 40 base pairs including a three base pair handle region, an Eco RI restriction enzyme site (5' GAATTC), a stop codon (CTA), and 28 specific nucleotides (5' CAGGTTTGACTTGTCTGAGGTTCCTTGC). cDNA obtained from the RT

reaction was amplified using the above primers. Pfu DNA polymerase, and the following PCR conditions: initial denaturation at 94° for 45 seconds. 35 cycles of the following program. denature at 94° for 45 seconds, anneal at 60° for 45 seconds, and extend at 72° for 10 minutes. followed by a final 10 minute extension period. The PCR product was run on a 1% agarose gel and appeared as a single 2.3 kb fragment.

6.1.3 Cloning of the 2.3 kb Fragment Into the pVR1255 Expression Vector

The 2.3 kb fragment was cut from the agarose gel. purified. digested with EcoRI and Not I enzymes. and ligated to the pVR1255 vector which contains the human cytomegalovirus (CMV) immediate-early enhancer/promoter (IE-EP). the CMV intron A. and the minimal rabbit globin terminator sequence (RBG poly A). Following the ligation reaction. transformation of competent JM109 E. coli cells was performed. and the transformants were plated on Kan^{+} LB plates overnight. Out of ten colonies screened. a single clone was positive and was purified using the QIAfilter Endofree Plasmid Maxi Kit (Qiagen Inc.. Mississauga. Ontario). Plasmid DNA was resuspended in 100µL of endotoxin-free buffer at a concentration of 4.56µg/µL.

6.1.4 Sequencing of the 2.3 kb PCR Product

In order to sequence the 2.3 kb PCR product. the fragment was subcloned into the Bluescript sequencing vector using the restriction enzymes EcoRI and Not I. The new vector was sent to the Sheldon Biotechnology Centre where it was sequenced using standard primers. Sequencing results confirmed that the 2.3 kb fragment was indeed sFLT-1.

6.1.5 Western Blot of Conditioned Media From sFLT-1 Transiently Transfected COS-7 Cells

COS-7 cells were transiently transfected with sFLT-1/pVR1255 using Lipofectamine™ Reagent (Life Technologies) according to the manufacturer's instructions. 72 hours following transfection, conditioned media was collected and cells were harvested, and expression of sFLT-1 was analyzed through Western blotting. Samples were run on discontinuous SDS PAGE and transferred onto a PVDF membrane (BIO-Rad) using the MilliBlot-Graphite Electroblotter system. A 1:500 dilution of a mouse, monoclonal, anti-VEGF receptor-1 antibody (Sigma-Aldrich, Oakville, Ontario) was used to detect sFLT-1 expression.

6.2 Subcloning of pBLAST-mAngiostatin, pBLAST-mEndostatin, and pORF-melIL-12 into the pVR1255 Expression Vector

Vectors expressing the antiangiogenic genes mEndostatin, mAngiostatin, and full-length, single-chain IL-12 were purchased from InvivoGen (InvivoGen, San Diego, California). The genes were excised from the commercial vectors and inserted into our pVR1255 expression vector using the following technique. Genes were excised from the InvivoGen vectors using EcoRV and Nhe I restriction enzymes. Digestion products were run on a 1% agarose gel, the DNA bands were cut from the gel and the GeneClean Spin kit was used to purify the angiostatic cDNAs. pVR1255 plasmid DNA was linearized using EcoRV and Xba I enzymes, dephosphorylated, and ligated to the above purified cDNAs. Ligation products were used to transform competent E. coli JM109 cells using the ECM 830 Pulse Generator (Genetronics, Inc., San Diego, California) following the manufacturer's electroporation protocol.

6.2.1 Quantifying the Amounts of mEndostatin and melIL-12 in the Conditioned Media of Transiently Transfected COS-7 Cells

COS-7 cells were transiently transfected with the commercial mEndostatin and the pVR1255 vectors containing the mEndostatin and melIL-12 genes using the TransIT®-LT1 transfection system (Mirus Corporation, Madison, Wisconsin). The conditioned media was collected 24 hours following transfection. Commercial ELISA kits, OptEIA™ Mouse IL-12 (p70) Set (PharMingen Canada, Mississauga, Ontario) and ACCUCYTE® Murine Endostatin™ (CytImmune Sciences Inc., College Park, Maryland), were used to measure the levels of IL-12, and mEndostatin, respectively.

6.3 Plasmid DNA Preparation

Plasmid DNA was prepared by the alkaline lysis method using the QIAfilter Plasmid Giga kit (Qiagen Inc., Mississauga, Ontario) according to the manufacturer's instructions. Plasmid DNA was resuspended in sterile 0.85% NaCl and stored at -20°C. DNA purity was confirmed by electrophoresis with 1% agarose gel and spectrophotometric analysis revealed a 260/280 nm ratio greater than 1.60.

6.4 Preparation of Tumor Cell Suspensions

The murine Lewis lung carcinoma (LLC) cell line of C57BL/6 (H-2^b) origin and human carcinoembryonic antigen (CEA)- expressing stably transfected LLC (CEA$^+$/LLC) were cultured in DMEM (Life Technologies) containing 10% fetal bovine serum (FBS). Cells were washed twice with serum-free DMEM before trypsinization. DMEM-10 was used to inhibit trypsin activity. Cells were pelleted, resuspended in serum-free DMEM and counted using a hemacytometer. Cells were washed two more

times before being resuspended in serum-free DMEM at a final concentration of $4x10^6$ cells/mL

.

6.5 Tumor Cell Challenge

C57BL/6 mice were transplanted with $2x10^5$ LLC cells in a 50µL volume by subcutaneous injection in the left flank. Tumors were measured by caliper in two dimensions and the volumes were calculated using the formula ($width^2$ x length)/2.

6.6 Intramuscular and Intratumor Gene Transfer Using IVEP

Mice were anesthesized with a mixture containing two parts ketamine, and one part each of xylazine and saline. Areas were shaved to expose the injection sites. For i.m injections, rectus femoris muscles of both legs were injected with plasmid DNA preparations using disposable, sterile, plastic, insulin syringes and 29G1/2 needles (VWR Canlab, Mississauga, Ontario). The same syringes and needles were used for i.t injections. Immediately following DNA injections, conductive gel was applied over the exposed area to assure good contact between the electrodes and the surface of the skin. Electrical pulses were administered using BTX caliper electrodes model 384 (Genetronics, Inc., San Diego, California) attached to the ECM 830 Pulse Generator. The electrical parameters, pulse strength, pulse length, and the number of electrical pulses were set by turning the parameter control knob on the pulse generator.

6.7 Luciferase Assay

IVEP of 50 μg of pVR1255 plasmid DNA encoding the luciferase gene was injected into solid, subcutaneous LLC tumors. At different time points following gene transfer, tumors were excised and homogenized in luciferase cell culture lysis reagent (Promega, Madison, Wisconsin). Tumor homogenate was kept on ice for 30 minutes and then centrifuged at maximum speed for 15 minutes. Tumor lysate was collected and the total amount of protein within the lysate was quantified using the Bio-Rad DC protein assay reagent (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). Different protein concentrations were assayed for luciferase activity using the luciferase assay substrate (Promega, Madison, Wisconsin) and an automated luminometer.

6.8 Measuring melIL-12 and mEndostatin Amounts at the Tumor Site

Tumors were excised and homogenized in a PBS + 1% Triton X-100 solution. The homogenate was kept on ice for 30 minutes before centrifugation at maximum speed for 15 minutes. The tumor lysate was collected and stored at -80°C. melIL-12 and mEndostatin levels were measured using the aforementioned commercial ELISA kits.

6.9 Immunohistochemistry

Tumor tissue was fixed overnight in 4% formalin and paraffin-embedded. Sections were deparaffinized in 100% xylene at 60°C for 35 minutes and rehydrated in graded ethanol solutions. von Willebrand factor (vWF) staining were performed as described previously (Holmgren et al., 1995). Briefly, antigen was unmasked using 5μg/mL Proteinase K (Sigma, Saint Louis, Missouri) at 37°C for 15 minutes. Samples were then

treated with rabbit polyclonal antibodies against vWF (Dako Diagnostics Canada Inc., Mississauga, Ontario), or with non-immunized, rabbit immunoglobulin fraction (Dako Diagnostics Canada Inc., Mississauga, Ontario) as negative control. Sections were then incubated with biotinylated secondary antibody followed by an avidin-biotin peroxidase complex using the VECTASTAIN® *Elite* ABC Kit (Vector Laboratories Inc., Burlingame, California). Positive staining was visualized using DAB substrate (Vector).

6.10 Subcloning CEA cDNA from the pCI Expression Vector Into pVR1255.

Our lab has previously used a pCI vector encoding the human CEA gene for DNA vaccination purposes (Song et al., 2000). The CEA cDNA was excised using NheI and XbaI restriction enzymes. The NheI end was made into a blunt end using T4 DNA polymerase (Life Technologies) before being ligated to the pVR1255 vector linearized by digestion with EcoRV and XbaI enzymes. COS-7 cells were transiently transfected with the pVR1255/CEA plasmid and FACS analysis was used to confirm CEA expression.

6.11 Immunization Protocol

7 to 10-week old C57BL/6 mice were anesthesized and injected i.m followed by electroporation in the rectus femoris muscles of both legs with 50 μg of pVR1255/CEA vector mixed with 50 μg of blank pVR1255, 50 μg of pVR1255/melIL-12, or with 50 μg of pVR1255/bisIL-12 in a total volume of 50 μL of saline. Mice injected with 100 μg of blank pVR1255 vector served as control groups. Injections were administered once a week over a 5-week period. On the sixth week, mice were challenged with either LLC or CEA^+/LLC cells. In the former

case, once tumors reached a diameter just exceeding 2 millimetres 50 μg of pVR1255/CEA or 50 μg of blank pVR1255 as negative control was injected into the tumors followed by electroporation on every second day for a total of five injections. Tumor volumes were monitored 2-3 times weekly over a one month period.

Chapter 7: Results

7.1: Assessing Optimal Electroporation Parameters for Tumor EGT Using a Luciferase Assay

Tumor luciferase activity was measured on days 1,3, and 7 following luciferase EGT into solid LLC tumors using different electroporation parameters. Regardless of the parameters used, electroporation resulted in increased luciferase activity compared to plasmid DNA injections without electrical pulses at every time-point tested (Figure 1). Applying 8 square-wave pulses, each with a strength of 300V/cm, a duration of 20msec, and separated by 1 second, yielded the highest relative tumor luciferase activity resulting in 10^3 times greater activity than plasmid DNA injections alone on days 3 and 7. Furthermore, luciferase expression was sustained over a one week period using the above electrical parameters whereas luciferase activity dropped dramatically over the same time period using the other parameters. Lastly, although the results reported in Figure 1 were obtained using the LLC mouse tumor model, similar results were obtained with the murine P815 mastocytoma model (results not shown).

7.2 mEndostatin and melIL-12 Levels from Conditioned Media of Transiently Transfected COS-7 Cells

After subcloning mEndostatin and melIL-12 into the pVR1255 expression vector, COS-7 cells were transiently transfected with the new vectors and the amount of

mEndostatin and melIL-12 secreted into the media was quantified using commercial ELISA kits. Comparing the levels of mEndostatin in the conditioned media of cells transfected with pVR1255/mEndostatin with that of cells transfected with the commercial pBLAST/mEndostatin, our expression vector produced more than four times the amount of mEndostatin (600ng/ml vs. 140ng/ml) than Invivogen's vector. Conditioned media from cells transfected with pVR1255/melIL-12 contained 500-600 ng/ml of the cytokine.

7.3 Systemic Levels of mEndostatin and melIL-12 Following Intramuscular EGT

Adult CD-1 mice were injected i.m. with a single 50 μg injection of either pVR1255/mEndostatin or with pVR1255/melIL-12 with or without electroporation. Optimal electrical parameters for intramuscular EGT consisting of 8 pulses, each with a strength of 200V/cm and length of 20 msec. have already been established by others (Gehl and Mir, 1999) and were used for our purposes. Serum samples were collected on days 3, 7, and 14 following DNA injection and mEndostatin and melIL-12 levels were quantified using commercial ELISA kits. mEndostatin levels did not plateau over the two-week period, but rather increased steadily at each time point from 50 ng/ml at day 3, to 100 ng/ml at day 7, to a maximum value of 160ng/ml at day 14 (Figure 2). Electroporation did not increase the systemic levels of mEndostatin in this particular experiment. Finally, melIL-12 levels were below the ELISA kit's detection level at each time point.

7.4 Amounts of melIL-12 and mEndostatin Protein Located at the Tumor Site Following Intratumor EGT

C57Bl/6 mice bearing LLC tumors were treated intratumorally with 50 μg of pVR1255/melIL-12, pVR1255/mEndostatin, or blank vector followed with or without electroporation. Tumors were excised on days 1, 3, and 7 following DNA injections and melIL-12 and mEndostatin amounts were quantified with appropriate ELISA assays.

24 hrs. after DNA injections, there was no significant difference in melIL-12 amounts found at the tumor site between untreated tumors, mice treated with blank vector with pulse or mice treated with melIL-12 without electroporation. However, electroporation significantly increased the amount of melIL-12 levels to an average of 109.5 pg/mm^3 compared to 10.8 pg/mm^3 for mice receiving intratumor melIL-12 injections without pulse. On day 3 following DNA injections the difference between melIL-12 tumor amounts between electroporated and non-electroporated groups were even more striking. Whereas an average of 61.4 pg/mm^3 of melIL-12 was found at the tumor site of the electroporated group, only an average of 7.1 pg/mm^3 was found in mice treated with melIL-12 without electroporation. Finally, although the tumor concentration of melIL-12 was below the ELISA kit's detection level 7 days following melIL-12 DNA injection without pulse in three samples studied, the tumor amounts in the electroporated group remained elevated at the same time point at an average of 56.4 pg/mm^3.

Results obtained from measuring tumor mEndostatin amounts were not as promising. 24 hrs. following pVR1255/mEndostatin injections, protein amounts were below the detection limit for both electroporated and non-electroporated groups. Three days after DNA injections there was no significant difference between tumor amounts of mEndostatin between the two groups. On day 7, there was only a slight difference in

mEndostatin levels between the two groups with an average of 124 pg/mm^3 for the electroporated group and an average tumor mEndostatin amount of 91.5 pg/mm^3 for the non-electroporated group.

7.5 Systemic Antiangiogenic Cancer Gene Therapy through Intramuscular EGT of Three Different Angiostatic Vectors

The pVR1255 vectors mEndostatin, mAngiostatin, sFLT-1, and blank vector were used to treat C57 mice bearing LLC tumors. Mice were treated with 50 μg of plasmid DNA i.m. injections followed by electroporation one week before tumor transplant and once a week for three weeks upon tumor implantation. Results are shown in Figure 3. Significant differences between treated groups and mice receiving blank pVR1255 vector began to appear at day 9 following tumor transplantation. At that point, mice treated with Angiostatin had tumors which were 25% smaller than blank-treated mice (p=.03). By day 10, Endostatin treatment inhibited tumor growth by 21.3% (p=.03) and mice treated with Angiostatin resulted in tumors which were 24.8% smaller than the control group (p=.009). Best results were obtained on day 14 when Angiostatin treated tumors resulted in 30% inhibition of tumor growth (p=.006) and Endostatin treatment inhibited tumor growth by 22% (p=.03). On day 16, tumor growth was inhibited by 22.5% (p=.04), 21.2%, and 21.2% (p=.03) for the Endostatin, Angiostatin, and sFLT-1 treated groups, respectively. By day 20, the tumors began to grow very rapidly in all groups, massive ulcerations began forming in the center of some of the tumors, mice began dying due to tumor burden and thus, the experiment was terminated.

7.6 Intramuscular and Intratumor EGT With pVR1255/mEndostatin in Mice Bearing Solid Subcutaneous LLC Tumors

Treatment was begun once tumors reached a diameter between 4-5 mm. Mice received either 50 μg of blank pVR1255 i.m. injections, 50 μg of pVR1255/mEndostatin i.m. or i.t., or 50 μg of pVR1255/mEndostatin i.m. plus 50 μg of pVR1255/mEndostatin i.t., followed by electroporation. Treatments were administered once a week over a three-week period for a total of four DNA applications. Significant differences only began to appear 14 days after the first DNA injections (Figure 4). At that point, two of the mice in the i.t.-treated group bearing larger tumors had died off due to tumor burden, resulting in an average inhibition of tumor growth of 31% (p=.02) compared to blank vector-treated group. By day 18, with only six mice remaining in the i.t.-treated group and seven mice in the blank-treated group, the average difference in tumor size was 37.5% (p=.012). However, although the difference in tumor size between the i.m. + i.t.-treated group and the control group was only 26% (p=.03) all ten mice had survived to that point.

7.7 Inhibiting Tumor Growth by Combining mEndostatin and melIL-12 Intratumor EGT

Once LLC tumors reached a diameter between 2-3 mm, intratumor DNA injections were begun. Mice received either 50 μg of blank pVR1255, 50 μg of pVR1255/melIL-12, or 50 μg of pVR1255/melIL-12 plus 50 μg of pVR1255/mEndostatin followed by electroporation. A total of three DNA injections were given once a week for a two-week period (Figure 5). By 9 days after the first DNA injections, the tumors in the melIL-12 + mEndostatin group were 40% smaller (p=.025) than tumors in mice treated with blank

vector. Tumors remained remarkably small in this group up until day 14 where the average tumor size was 434.1 mm^3, which represented a 46.9% inhibition in tumor size ($p=.002$) compared to control group. By the same time point, tumors in the melIL-12 group alone had grown to an average size of 595 mm^3 resulting in 27.2% ($p=.03$) inhibition of tumor growth.

We repeated and modified the above experiment by including a group receiving only 50 μg of pVR1255/mEndostatin, increasing the dosage of blank vector to 100 μg, and beginning DNA injections when tumors had reached a size between 4-5 mm. In mice receiving mEndostatin alone or in combination with melIL-12, tumors were significantly smaller than tumors from control group on days 6, 9, and 13 (Figure 6). By day 9, tumors were 26.5% ($p< .05$) and 30% ($p< .05$) smaller than control groups for the mEndostatin and combination groups, respectively. On day 13, tumor growth was inhibited by 30% ($p< .05$) and 31% ($p< .05$), respectively, for the mEndostatin group and combination group. In this experiment, melIL-12 administration alone did not result in significant tumor growth inhibition at any time point. Furthermore, when tumors were injected with plasmid DNA without electroporation there was no difference in tumor sizes between any of the groups and tumors injected with saline followed by electroporation. Interestingly, tumors treated with blank vector and electroporation were significantly smaller than tumors injected with saline and electroporation at every time point, and were 28.5% smaller at day 13. Finally, at the end of the experiment immunohistochemical analysis of tumors in each group was performed by staining for the blood vessel marker vWF. In some of the treated groups, most notably those tumors treated with mEndostatin, positive staining of the blood vessels was a success allowing them to be easily identified and

counted. However, most of the tumors were highly necrotic and stained very poorly with vWF preventing us from comparing the number of blood vessels from the different treated groups.

In a final attempt to improve the antitumor effects of combining melIL-12 and mEndostatin plasmid DNA injections, the above experiment was repeated with the exception that DNA administration was initiated as soon as the tumors were big enough to inject into with an average diameter of approximately 2 mm. 14 days after the first series of DNA injections and after a total of four administrations there was a 43% ($p=.03$), 32%, and 19% reduction in tumor size compared to blank pVR1255-treated mice for the melIL-12, melIl-12 + mEndostatin, and mEndostatin groups respectively (Figure 7). Interestingly, one of the tumors from the melIL-12 group regressed completely after the second DNA injection when it measured 3 mm X 2 mm. The mouse has remained tumor-free for a period of at least 17 days. In addition, another tumor in the melIL-12 group remained the same size for a period of 21 days, measuring 3.5 mm X 3.5 mm. Eventually the tumor began growing normally and the mouse was sacrificed due to tumor burden. Lastly, another tumor in the mEndostatin group also regressed completely after growing to a size of 2.5 mm X 2.5 mm, and the mouse has remained tumor-free for a period of at least 17 days.

7.8 DNA Vaccination with CEA and bisIL-12 Protects Mice Against CEA$^+$/LLC Tumor Cell Challenge

One week after the last of five DNA injections, mice were challenged with CEA$^+$/LLC and tumor volume was measured bi-weekly for a period of 25 days. 100% of mice immunized with either blank vector alone, or with pVR1255/CEA + blank vector

developed tumors (Figure 8). However, 5 out of 8 mice (62.5%) immunized with pVR1255/CEA + pVR1255/bisIL-12 did not develop tumors and remained tumor-free over the course of the experiment.

In a final study, following the DNA immunization period mice were treated with normal LLC cells. Once the tumors reached a diameter of approximately 2 mm, pVR1255/CEA or blank pVR1255 (as negative control) intratumor injections followed by IVEP were performed in an attempt to transfect the tumor mass with CEA *in vivo*. However, as shown in figure 9, EGT of CEA did not inhibit tumor growth in the various immunized groups compared to immunized mice receiving i.t. injections of blank pVR1255 followed by electroporation.

Figure 1 Assessing optimal electroporation parameters for tumor EGT using a relative luciferase assay. 50 μg of pVR1255/luciferase DNA was injected into solid, subcutaneous LLC tumors followed by electroporation using different electrical parameters. Tumor luciferase activity was measured at different time points following DNA injections as described in Materials and Methods. Electrical pulses 300 V/cm with a duration of 20 msec resulted in highest luciferase activity. Each group represents the mean ± one standard deviation of ten mice.

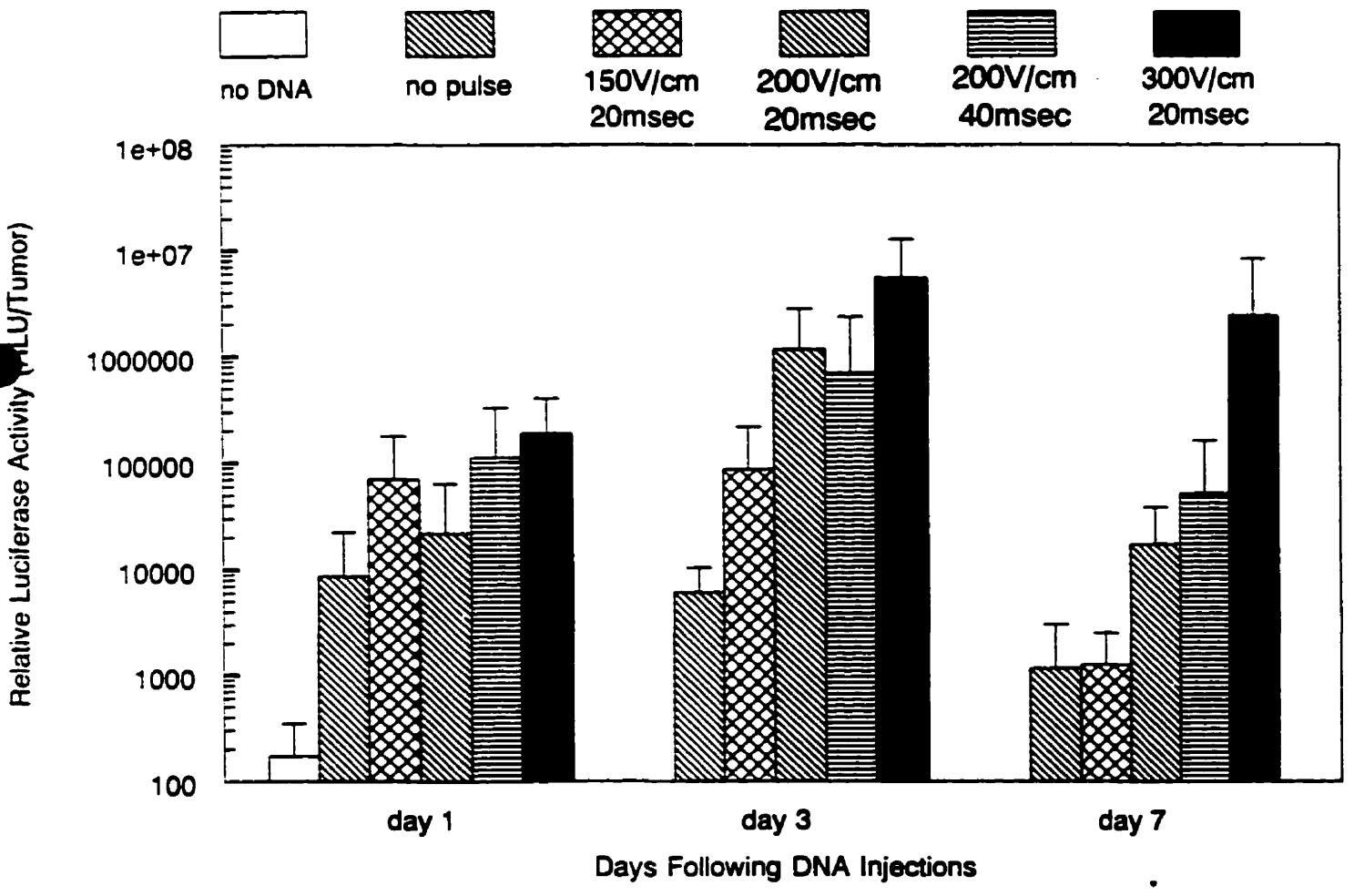

no DNA
no pulse
150V/cm 20msec
200V/cm 20msec
200V/cm 40msec
300V/cm 20msec
1e+08
1e+07
1000000
100000
10000
1000
100
Relative Luciferase Activity (RLU/Tumor)
day 1
day 3
day 7
Days Following DNA Injections

Figure 2 Systemic mEndostatin protein levels following intramuscular injection of pVR1255/mEndostatin with or without electroporation. Adult CD-1 mice were injected i.m. with 50 μg of pVR1255/mEndostatin with or without electroporation. Blood was collected at different time points following DNA injections and serum mEndostatin levels were measured. Each group represents the mean ± one standard deviation of 3-4 mice.

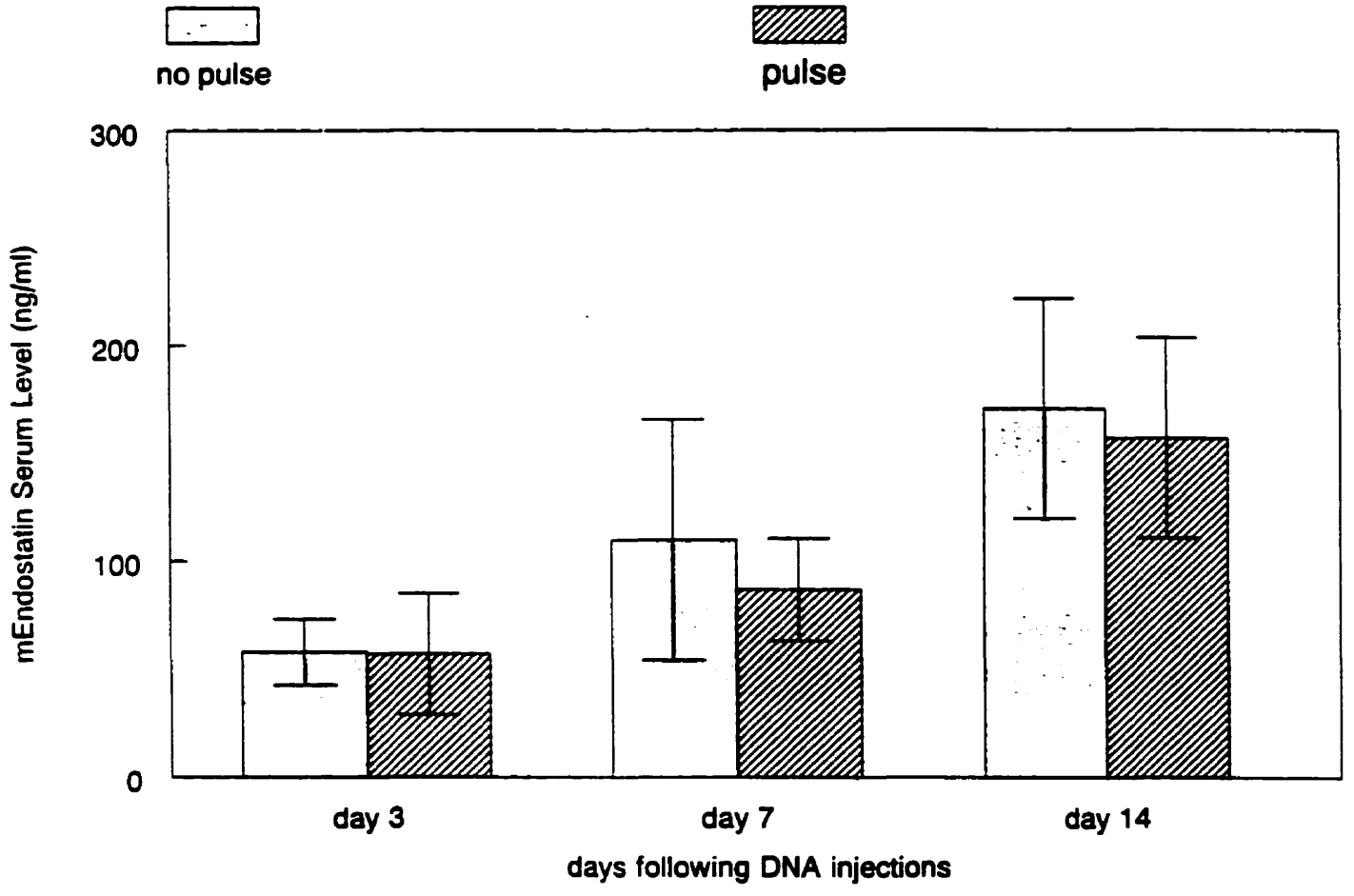

no pulse
pulse
300
200
100
0
mEndostatin Serum Level (ng/ml)
day 3
day 7
day 14
days following DNA injections

Figure 3 Systemic electroporation-mediated gene transfer of different angiostatic factors inhibits tumor growth. Mice were treated with 50 μg of plasmid DNA i.m injections followed by electroporation one week before subcutaneous tumor transplant and once a week for three weeks starting on the day of tumor transplantation. Significant differences in tumor size between treated groups and mice receiving blank pVR1255 vector began to appear at day 9 following tumor transplantation. Best results were obtained on day 14 when Angiostatin treated tumors resulted in 30% inhibition of tumor growth (p=.006) and Endostatin treatment inhibited tumor growth by 22% (p=.03). By day 16, tumor growth was inhibited by 22.5% (p=.04), 21.2%, and 21.2% (p=.03) for the Endostatin, Angiostatin, and sFLT-1 treated groups, respectively. Results are the mean ± one standard deviation of 10 mice per group and p values were obtained using an independent t-Test.

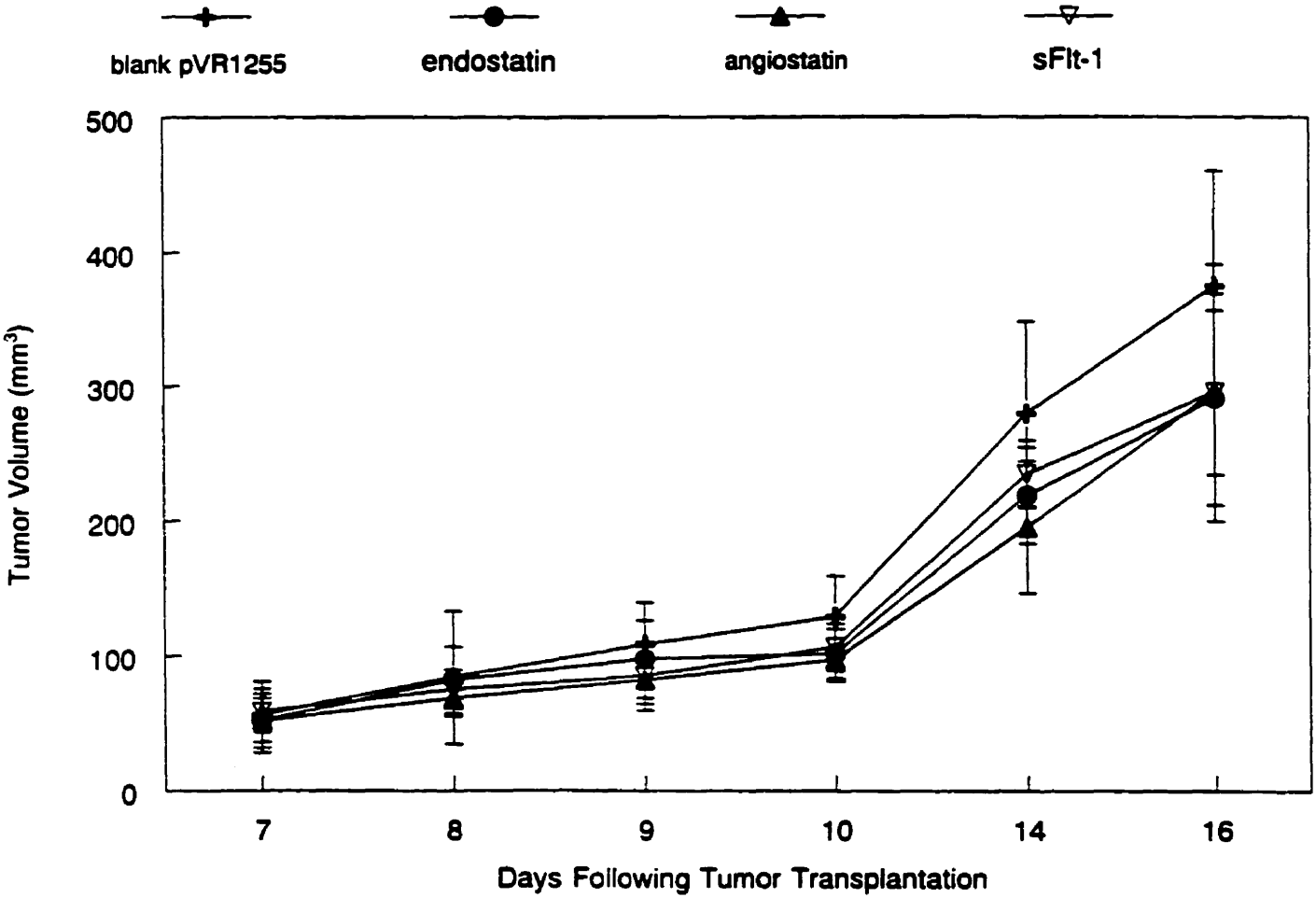

blank pVR1255
endostatin
angiostatin
sFlt-1
500
400
300
200
100
0
Tumor Volume (mm3)
7
8
9
10
14
16
Days Following Tumor Transplantation

Figure 4 Intramuscular and intratumor EGT with pVR1255/mEndostatin in mice bearing solid, subcutaneous, LLC tumors. Once tumors reached a diameter of 4-5 mm mice received either 50 μg of blank pVR1255 i.m. injections, 50 μg of pVR1255/mEndostatin i.m. or i.t., or 50 μg of pVR1255/mEndostatin i.m. plus 50 μg of pVR1255/mEndostatin i.t., followed by electroporation. Treatments were administered once a week over a three-week period. By day 18, with only 6 of 10 mice remaining in the i.t.-treated group and seven of ten in the blank-treated group, the average difference in tumor size was 37.5% (p=.012). Although the difference in tumor size between the i.m. + i.t.-treated group and the control group at the same time point was only 26% (p=.03), all ten mice had survived to that point.

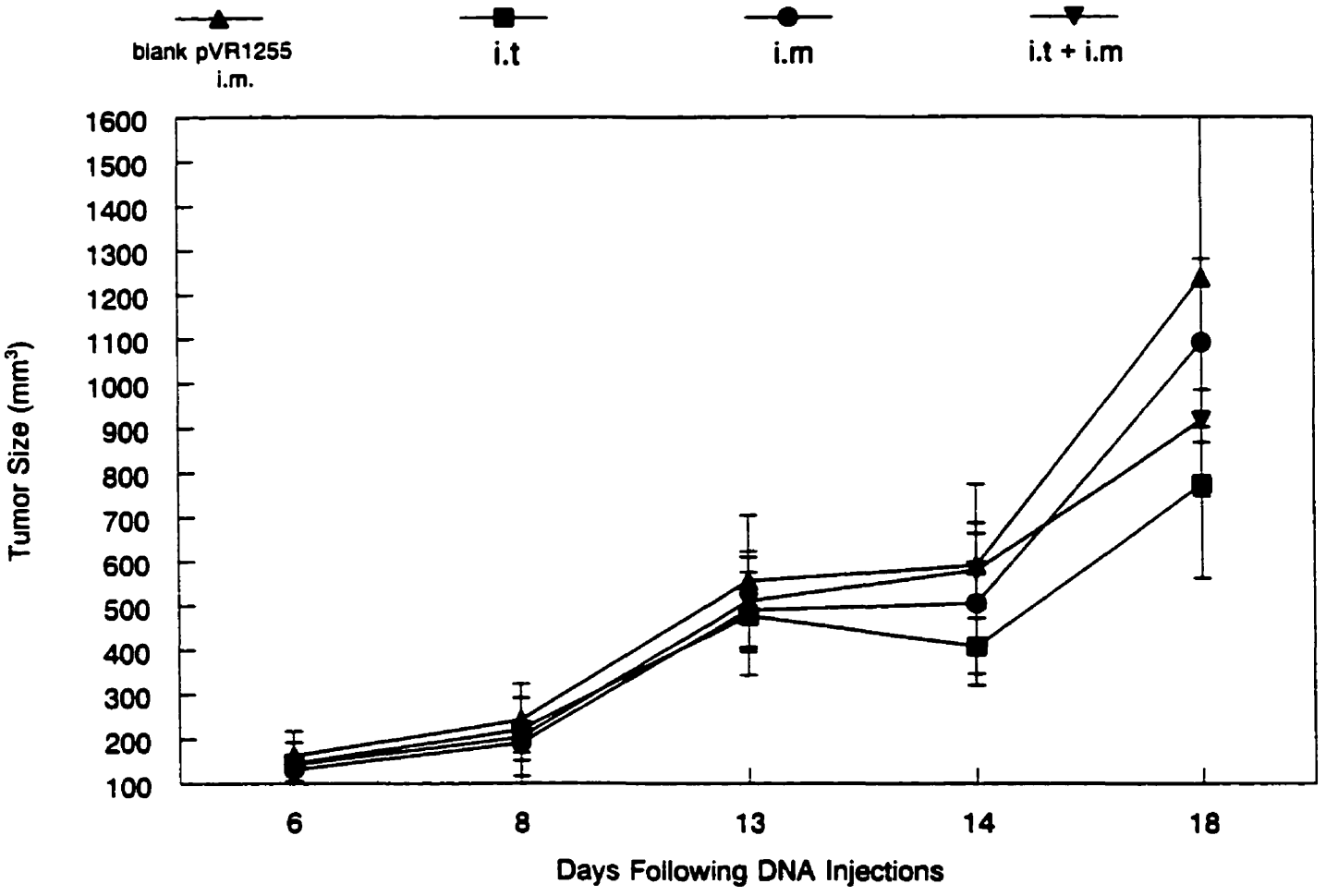

blank pVR1255
i.m.
i.t
i.m
i.t + i.m
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
Tumor Size (mm3)
6
8
13
14
18
Days Following DNA Injections

Figure 5 Inhibiting tumor growth through intratumor gene transfer of melIL-12 and mEndostatin combined using *in vivo* electroporation. Once LLC tumors reached a diameter between 2-3mm, mice received either 50 μg of blank pVR1255, 50 μg of pVR1255/melIL-12, or 50 μg of pVR1255/melIL-12 plus 50 μg of pVR1255/mEndostatin intratumor injections followed by electroporation. A total of three DNA injections were given over a two-week period. By 9 days after the first DNA injections, tumors in the melIL-12 + mEndostatin group were 40% smaller (p=.025) and 46.9% (p=.002) smaller than tumors in mice treated with blank vector by day 14. Results are the mean ± one standard deviation of ten mice per group and p values were obtained using an independent t-Test.

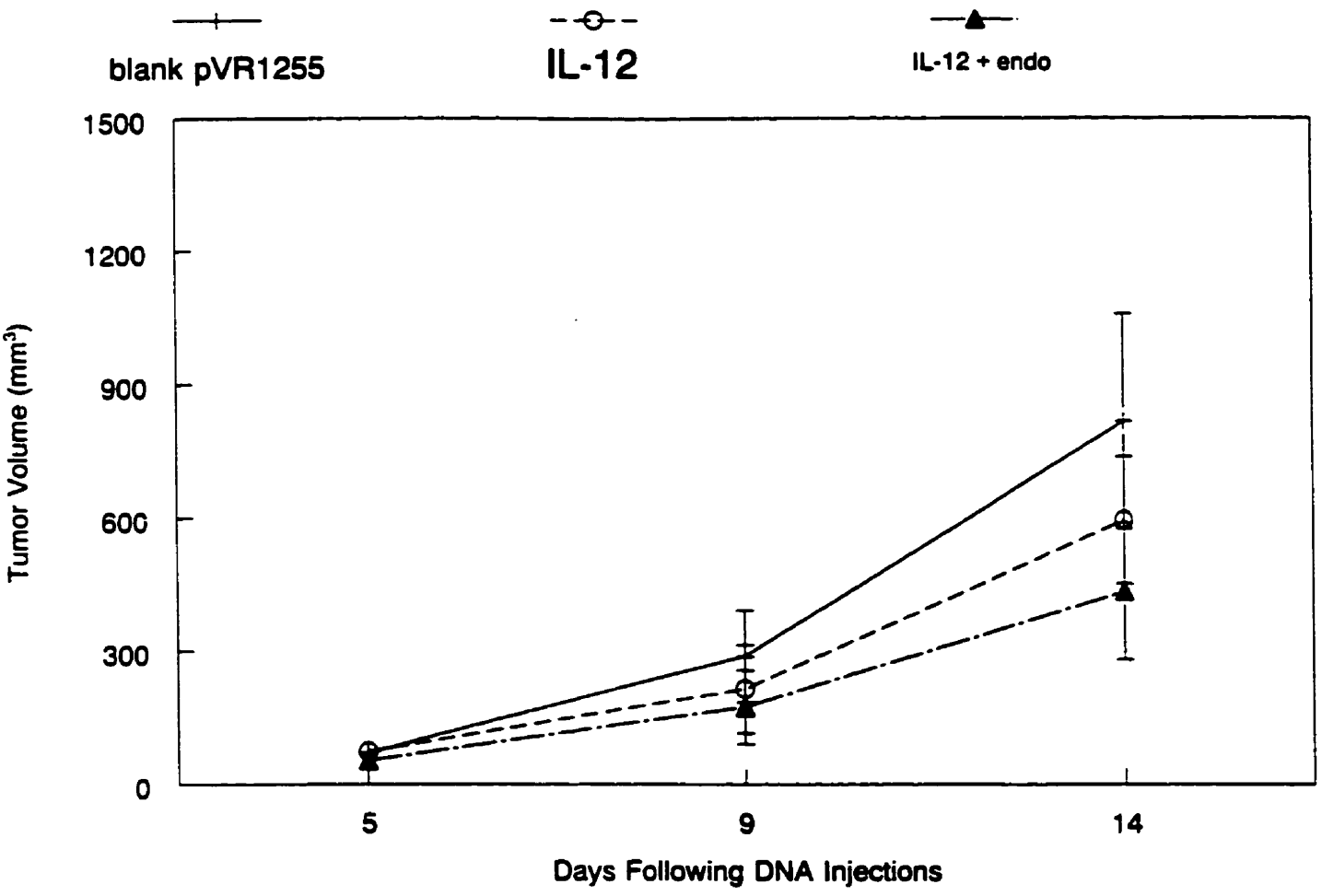

blank pVR1255
IL-12
IL-12 + endo
1500
1200
900
600
300
0
Tumor Volume (mm3)
5
9
14
Days Following DNA Injections

Figure 6 Comparing the antitumour effects of mEndostatin and melIL-12 intratumor gene transfer into fairly large tumors, either alone or in combination, through *in vivo* electroporation. Intratumor DNA injections were begun when tumors reached a diameter between 4-5 mm. Tumors were treated with 50 μg of pVR1255/mEndostatin, 50 μg of pVR1255/melIL-12, 50 μg of pVR1255/mEndostatin plus 50 μg of melIL-12, or 100 μg of blank pVR1255 vector as control group. A total of three injections were administered over a two-week period. In mice receiving mEndostatin alone or in combination with melIL-12, tumors were significantly smaller than tumors from control group on days 6, 9, and 13 following the first DNA injection. melIL-12 administration alone did not result in significant tumor growth inhibition at any time point. Results are the mean ± one standard deviation of 8 mice per group.

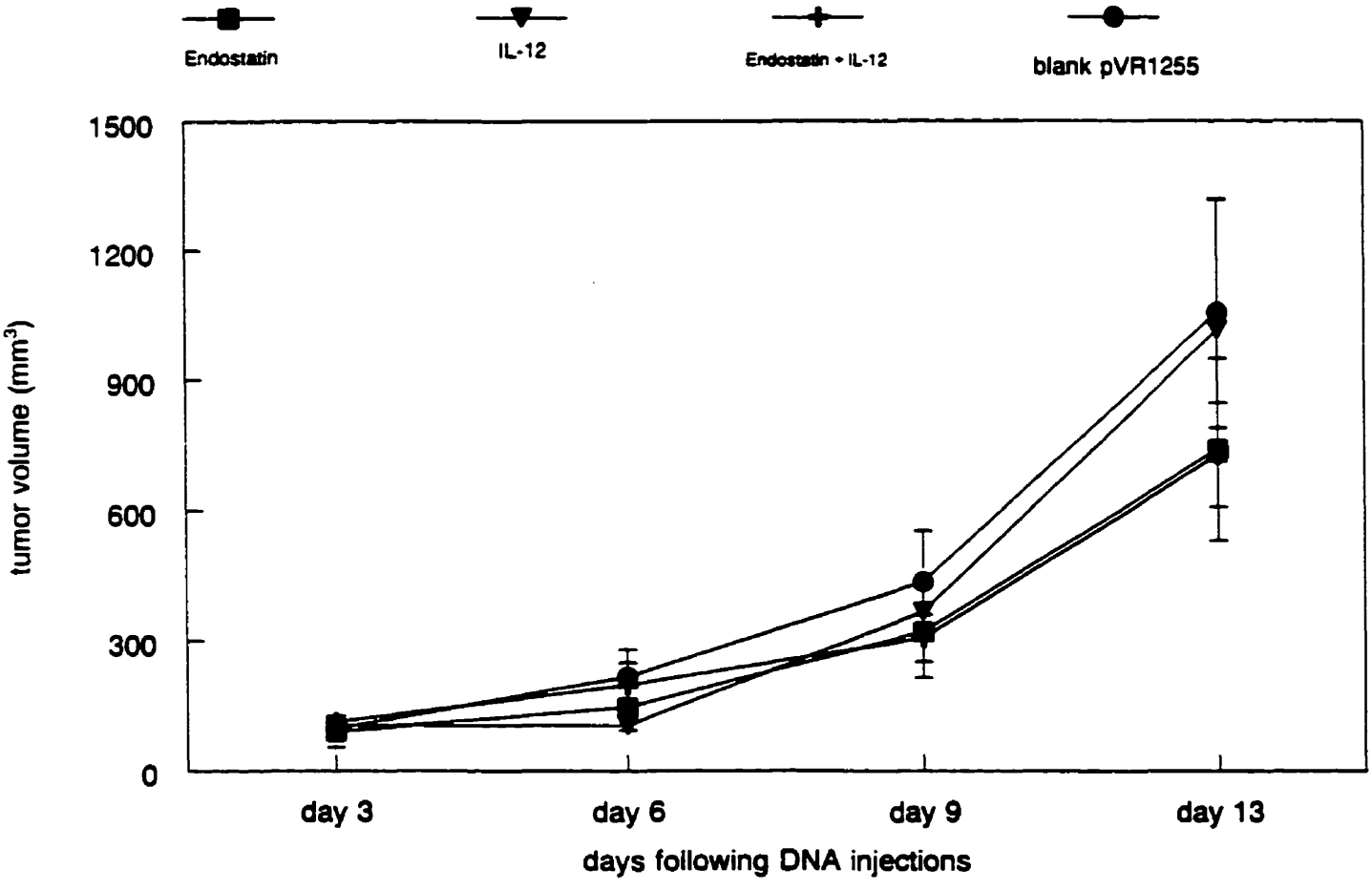

Endostatin
IL-12
Endostatin + IL-12
blank pVR1255
1500
1200
900
600
300
0
tumor volume (mm3)
day 3
day 6
day 9
day 13
days following DNA injections

Figure 7 melIL-12 intratumor gene transfer into smaller tumors results in significant tumor growth inhibition. Mice were treated as described in Figure 6 except that DNA injections were initiated when tumors reached a diameter of approximately 2 mm. 14 days after the first series of DNA injections and after a total of four administrations there was a 43% (p=.03), 32%, and 19% reduction in tumor size compared to blank pVR1255-treated mice for the melIL-12, melIL-12 + mEndostatin, and mEndostatin groups, respectively. Complete tumor regression occurred in one mouse in the melIL-12 group and one mouse in the mEndostatin group after having grown to a diameter of about 3 mm. Results are the mean $\pm$ one standard deviation of 8 mice per group and p values were obtained using an independent t-Test.

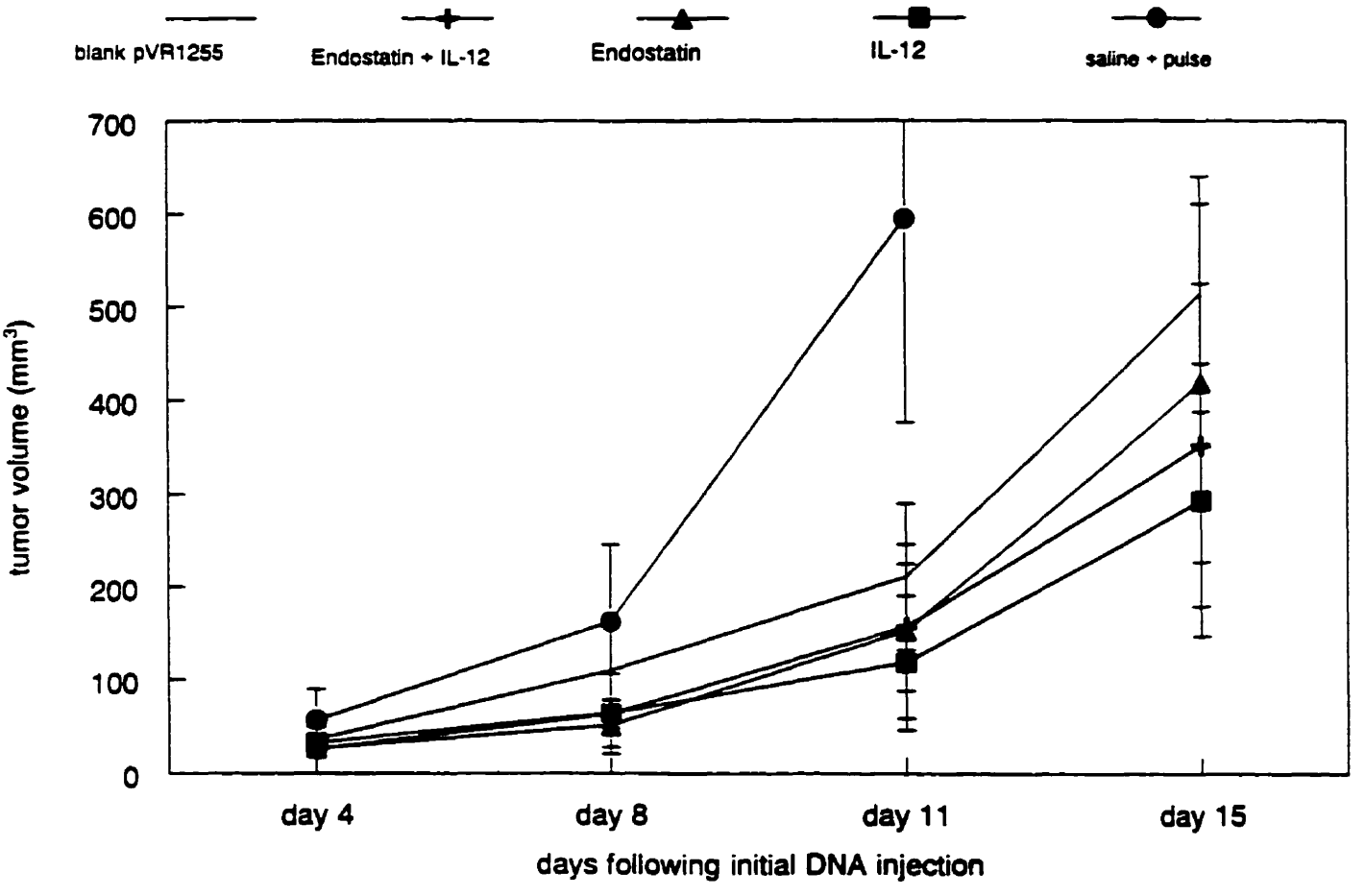

blank pVR1255
Endostatin + IL-12
Endostatin
IL-12
saline + pulse
700
600
500
400
300
200
100
0
tumor volume (mm3)
day 4
day 8
day 11
day 15
days following initial DNA injection

Figure 8 Using electroporation-mediated DNA vaccination with CEA and bisIL-12 adjuvant to protect mice against LLC/CEA$^+$ tumor cell challenge. Mice were immunized with pVR1255/CEA + pVR1255/bisIL-12, with pVR1255/CEA + blank pVR1255, or with only blank pVR1255 as described in Materials and Methods and then challenged with LLC/CEA$^+$ tumor cells. Only mice immunized with both CEA and bisIL-12 were protected from tumor challenge where 5 of 8 mice did not develop tumors and remained tumor-free over the course of the experiment.

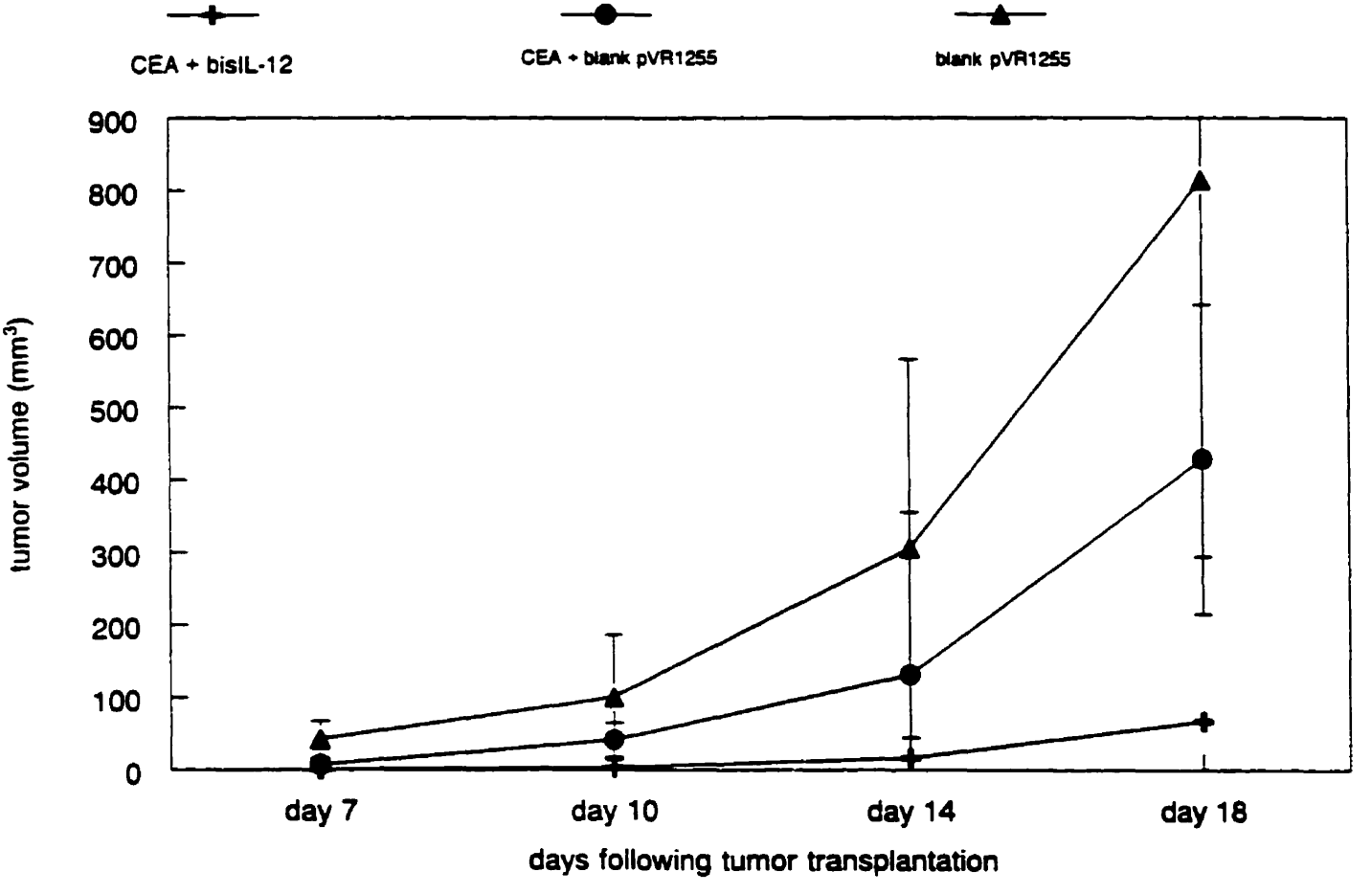
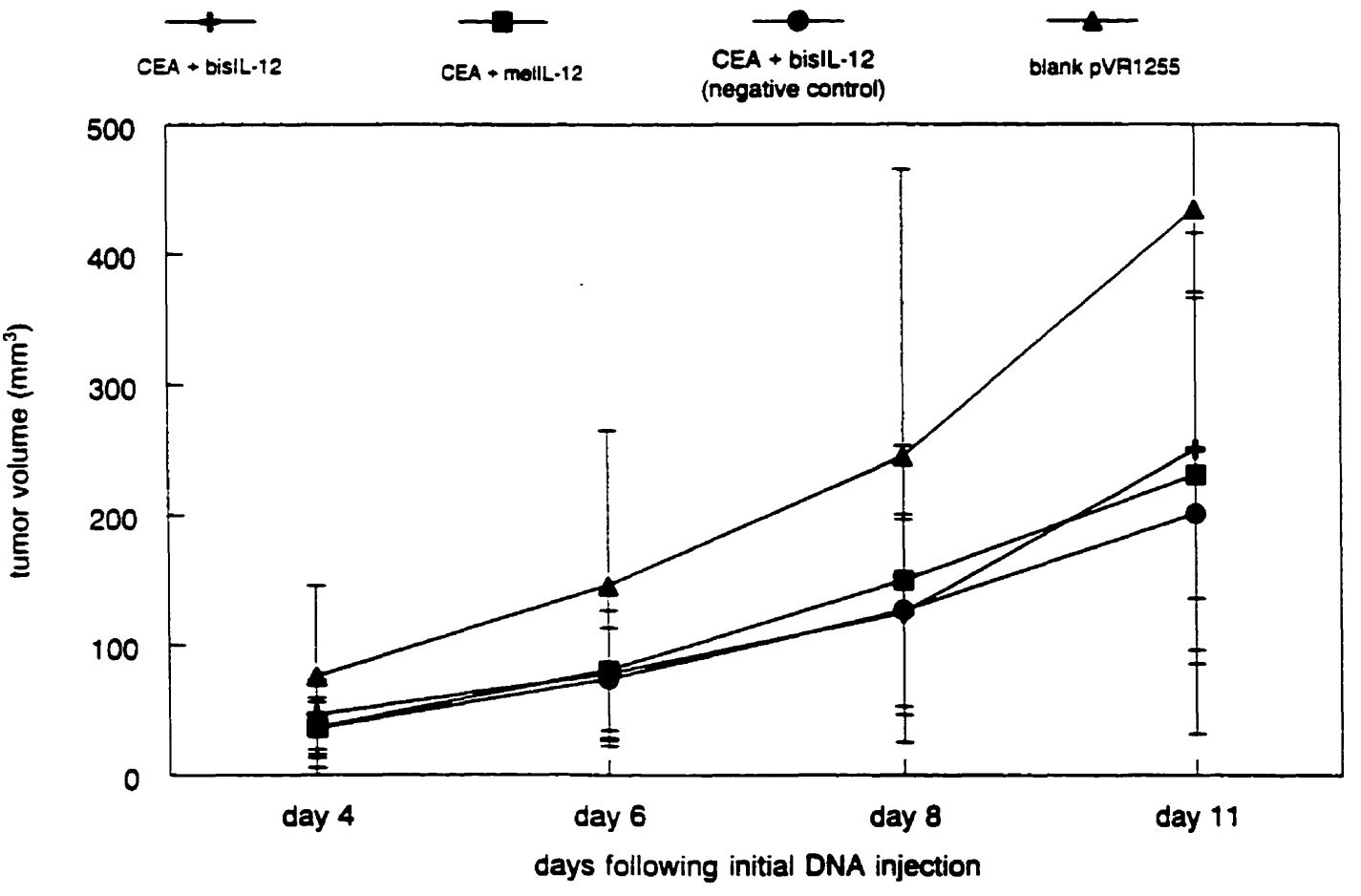

CEA + bisIL-12
CEA + blank pVR1255
blank pVR1255
900
800
700
600
500
400
300
200
100
0
tumor volume (mm3)
day 7
day 10
day 14
day 18
days following tumor transplantation

Figure 9 Assessing the antitumour effects of electroporation-mediated intratumor gene transfer of CEA into solid, subcutaneous LLC tumors following DNA vaccination with CEA and IL-12 adjuvant. Mice were immunized with pVR1255/CEA + pVR1255/bisIL-12, with pVR1255/CEA + pVR1255/melIL-12 or with blank PVR1255 vector alone as depicted above the graph of figure 9. Following the DNA vaccination protocol, mice were challenged with LLC tumor cells. When tumors reached a diameter of approximately 2 mm, 50 μg of pVR1255/CEA or 50 μg of blank pVR1255 vector (labelled as negative control) were injected into the tumors followed by electroporation. DNA injections were repeated every second day for a total of three injections. There was no significant difference in tumor growth between tumors treated with blank pVR1255 vector and all other groups.


CEA + bisIL-12
CEA + melIL-12
CEA + bisIL-12
(negative control)
blank pVR1255
500
400
300
200
100
0
tumor volume (mm3)
day 4
day 6
day 8
day 11
days following initial DNA injection

Chapter 8: Discussion

We have used a luciferase assay to assess the intratumor gene transfer efficiency of *in vivo* electroporation using parallel plate electrodes. Two very important advantages of this assay are its sensitivity and the quantitative nature of the results. Data from two different tumor models, LLC tumors and P815 mastocytomas, clearly demonstrated that electroporation is an effective nonviral strategy for delivering genes into tumors *in vivo*. In both studies, eight electrical pulses each with a strength of 300 V/cm and duration of 20 msec resulted in the greatest luciferase activity. Using these optimal electrical parameters, relative luciferase activity was increased 10^3 times, on the third day following DNA injections, compared to tumors having received only DNA injections without electroporation. Other papers have also reported electroporation-mediated intratumor gene transfer but most have shown this using qualitative methods such as the expression of reporter genes like green fluorescent protein (Goto et al., 2000) or β-galactosidase expression (Rols et al., 1998). In the former case, the authors concluded that electroporation resulted in numerous, scattered bright green fluorescent signals throughout the tumor whereas β-gal staining resulted in positive signals in 4% of the tumor cells. Due to the quantitative nature of the luciferase assay, our results provide a clearer indication of the intratumor gene transfer efficiency of electroporation. It must be noted that one other study has used a luciferase assay to assess the efficiency of this nonviral gene transfer technique (Heller et al., 2000), but since they used an absolute assay, measuring the amount of luciferase protein produced within rat hepatocellular carcinomas *in vivo*, and we used a relative assay which measured luciferase activity it is impossible to compare our results. Moreover, using parallel plate electrodes to administer the electrical pulses we have determined that the optimal parameters consist of low voltage pulses of 300

V/cm of long duration (20 msec). This is in contrast to another study which showed that pulses of 800 V/cm and length of 4 msec resulted in most efficient gene transfer into solid, subcutaneous mouse melanomas using the same type of electrodes (Rols et al., 1998). Although we obtained similar results using two very different tumor models, namely LLC tumors and P815 mastocytomas, their results seem to suggest that optimal electrical parameters will vary depending on the tumor model studied. Also, when we used a voltage of 800 V/cm damage to the tumor was evident immediately after pulsation in the form of darkening of the tumor surface. In addition, another group using a six-needle array electrode reported efficient gene transfer with voltages as low as 66 V/cm (Goto et al., 2000). Since we only began obtaining enhanced gene transfer at a voltage of 150 V/cm using our parallel plate electrodes, the invasive needle array electrodes may be even more effective at permeabilizing solid tumors for gene transfer. Finally, our results showed that luciferase expression plateaus three days following gene transfer and then begins to decline. This confirms one of the major disadvantages of nonviral vectors, which is the very transient expression of nonintegrated foreign DNA. However, one of the greatest advantage of our technique is the ease of handling and the ability to readminister DNA injections as soon as expression begins to drop.

Intramuscular delivery of the endostatin gene using electroporation resulted in approximately a 20% inhibition in the growth of solid, subcutaneous LLC tumors. In contrast, a recent paper has shown that intramuscular administration of the endostatin gene complexed to a PVP polymer used to condense the DNA particles resulted in up to 40% growth inhibition of primary LLC tumors and significantly reduced the number of lung metastases (Blezinger et al., 1999). Looking at the serum levels of Endostatin following intramuscular DNA injections they reported that Endostatin

levels peaked at day 7 at 8 ng/ml. Using electroporation we obtained drastically different results. In our study, Endsotatin expression was sustained and had not yet peaked by day 14 following DNA injections where Endostatin levels reached a value of approximately 160 ng/ml. In fact, similar results were obtained following naked DNA injections without electroporation. This suggests that the pVR1255 vector is especially adept at transfecting muscle *in vivo* and confirms the findings from another study which showed that intramuscular injection of as little as 10 μg of a similar vector containing the erythropoietin gene was sufficient to significantly increase hematocrit levels in immunocompetent adult mice (Tripathy et al., 1996). Thus, although we obtained serum Endostatin levels almost twenty times greater than what was reported in that paper, inhibition of tumor growth, using the same LLC tumor model, was less pronounced in our study. This leads to the assumption that our strain of LLC tumor cells were more aggressive or less dependent on angiogenesis and that the same treatment modality on a less malignant tumor model may have yielded more encouraging results. Our initial studies with local, intratumor transfer of the endostatin gene resulted in increased tumor growth inhibition, attaining a growth inhibition of nearly 40%.

Co-transfer of the mEndostatin and melIL-12 genes into tumors resulted in enhanced antitumor effects. However, the tumor growth inhibiting activity of melIL-12 was clearly dependent on the size of the tumors when the DNA was administered. Intratumor transfer of the melIL-12 gene showed potent antitumor effects when delivered to tumors with a diameter just exceeding 2 mm, resulting in greater than 40% growth inhibition compared to mice treated with blank pVR1255 vector and resulted in the complete regression of a tumor which had grown to a volume of 10 mm^3. However, when administered to mice bearing tumors with an average volume

of 55 mm^3. melIL-12 had no antitumor activity compared to control group. Thus beyond a certain size, the immunostimulatory activity of melIL-12 is insufficient to slow the growth of the rapidly dividing tumor cells. A similar finding was reported in an earlier study which showed that the ability of IL-12 to eradicate tumors was inversely proportional to the tumor burden (Chen et al., 1997). In contrast, mEndostatin gene transfer into larger tumors retained its antitumor effects, inhibiting tumor growth by 30% compared to blank-treated mice. This was not surprising considering that earlier studies have shown that systemic treatment with recombinant Endostatin protein resulted in tumor eradication in mice carrying tumors 1% their body weight (O'Reilly et al., 1997).

Another interesting finding was that intratumor gene transfer of blank pVR1255 showed tumor growth inhibiting activity. In fact, tumors treated with blank vector followed by electroporation showed a 30% decrease in tumor growth compared to the same tumors treated with saline and electroporation. This is in accordance with the various studies which have demonstrated that naked DNA alone can provoke immune responses (Krieg et al., 2000; Barry et al., 1999; Leclerc et al., 1997; and Klinman et al., 1996). It is well established that bacterial plasmid DNA contains CpG dinucleotides which stimulate a Th1 response resulting in the secretion of such cytokines as IL-12 and IFN-γ. Thus, it is likely that intratumor administration of naked pVR1255 vector results in a Th1-type immune response which can impact tumor growth. Interestingly, this phenomenon did not occur when naked DNA was injected without electroporation providing further evidence for the efficiency of electrical pulses in promoting the uptake of foreign DNA by solid tumors.

Lastly, *in vivo* electroporation was used in a DNA vaccination study to protect mice against tumor challenge. Previous work in our lab had established that a DNA

vaccination protocol consisting of five weekly injections of 50 μg of pCMV-CEA vector plus 100 μg of pVR1255/bisIL-12 DNA protected mice from tumor challenge with CEA^+/LLC tumor cells. Recently, a study has shown that electroporation can increase DNA vaccine delivery and immunogenicity resulting in an enhanced Th1 response against a HIV gag antigen (Widera et al., 2000). In the previous study performed in our lab, mice were protected from tumor challenge only if bisIL-12 was administered together with the CEA tumor antigen. In our study, we hypothesized that by using electroporation we could increase the transfection efficiency to a point where protection could occur without bisIL-12 adjuvant. Furthermore, the CEA cDNA was transferred from the pCI vector into the pVR1255 vector which is thought to yield a higher transfection rate into living muscle tissue. However, our findings were consistent with the previous study, so that only mice receiving both CEA and bisIL-12 were protected from tumor challenge. An important difference was that in our study mice received only 50 μg of bisIL-12 compared to 100 μg in the previous study. Furthermore, a previous study has shown that following DNA vaccination with the hepatitis B surface antigen, growing P815 mastocytoma tumors can be rejected in mice through repeated intratumor injections of the viral antigen complexed with liposomes (Bohm et al., 1997). We attempted a similar approach by immunizing mice with CEA DNA vaccines followed by intratumor electroporation-mediated CEA gene transfer into growing LLC tumors. However, no antitumor effects were obtained using this approach compared to tumors treated with blank pVR1255 vector. A likely explanation for this is that the LLC tumor model is a more aggressive tumor than the P815 model and that further studies using our approach on a less aggressive tumor system should be pursued.

In conclusion, we have shown IVEP to be a safe, convenient, and efficient alternative to viral vectors in the field of gene therapy. Our approach resulted in excellent gene transfer efficiency into murine, solid, subcutaneous tumors. Although the duration of gene expression was shown to be very transient, the ease of DNA readministration without eliciting a host immune response is a favorable aspect of this technique that can be exploited to maintain elevated gene expression levels. With the development of new electrodes designed to better concentrate the electrical pulses to the target tissues and to facilitate electroporation of internal organs, any tissue type could be targeted with this technique. Moreover, the simplicity of this approach makes it possible to administer any combination of genes encoding tumoricidal factors directly into the tumor environment. Furthermore, electroporation may enhance gene delivery to a point where DNA vaccination would finally become a routine procedure used to immunize humans against viruses and possibly even malignant cells. The potential applications of IVEP makes it absolutely necessary to pursue basic research into the mechanisms by which electrical pulses create permeation sites into living tissue in the hope that one day electroporation may be routinely used to transfer genes into human somatic tissue.

References

Aiello, LP., Gardner, TW., King, GL., Blankenship, G., Cavallerano, JD., Ferris III, FL., and Klein, R. (1998). Diabetic retinopathy. Diabetes Care **21(1),** 143-56.

Aiello, LP. (1997). Vascular endothelial growth factor: 20th-century mechanisms, 21st-century therapies. Investigative Ophthalmology and Visual Science **38(9),** 1647-52.

Aiello, LP., Pierce, EA., Foley, ED., Takagi, H., Chen, H., Riddle, L., Ferrara, N., King, GL., and Smith, LEH. (1995). Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proceedings of the National Academy of Sciences of the USA **92,** 10457-10461.

Aiello, LP., Avery, RL., Arrigg, PG., Keyt, BA., Jampel, HD., Shah, ST., Pasquale, LR., Thieme, H., Iwamoto, MA., Park, JE., Nguyen, HV., Aiello, LM., Ferrara, N., and King, GL. (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. The New England Journal of Medicine **331,** 1480-7.

Amici, A., Smorlesi, A., Noce, G., Santoni, G., Cappelletti, P., Capparuccia, L., Coppari, R., Lucciarini, R., Petrelli, C., and Provinciali, M. (2000). DNA vaccination with full-length or truncated Neu induces protective immunity against the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Gene Therapy **7,** 703-706.

Anderson, WF. (1998). Human gene therapy. Nature **392,** 25-30.

Arap, W., Pasqualini, R., and Ruoslahti, E. (1998). Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science **279,** 377-80.

Arenberg, DA., Kunkel, SL., Polverini, PJ, Morris, SB., Burdick, MD., Glass, MC., Taub, DT., Iannettoni, MD., Whyte, TI, and Strieter, RM (1996). Interferon-γ inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. Journal of Experimental Medicine **184,** 981-92.

Asano, M., Yukita, A., and Suzuki, H. (1999). Wide spectrum of antitumor activity of a neutralizing monoclonal antibody to human vascular endothelial growth factor. Japanese Journal of Cancer Research **90,** 93-100.

Baba, M., Iishi, H., and Tatsuta, M. (2000). *In vivo* electrophoretic transfer of Bcl-2 antisense oligonucleotide inhibits the development of hepatocellular carcinoma in rats. International Journal of Cancer **85,** 260-66.

Banchereau, J., and Steinman, RM. (1998). Dendritic cells and the control of immunity. Nature **392,** 245-52.

Barleon, B., Sozzani, S., Zhou, D., Weich, HA., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood **87,** 3336-3343.

Barry, ME., Pinto-Gonzalez, D., Orson, FM., McKenzie, GJ., Petry, GR., and Barry, MA. (1999). Role of endogenous endonucleases and tissue site in transfection and CpG-mediated immune activation after naked DNA injection. Human Gene Therapy **10,** 2461-80.

Battegay, EJ. (1995). Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. Journal of Molecular Medicine **73,** 333-46.

Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., Isner, JM. (1998). Constitutive expression of phVEGF$_{165}$ after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation **97(12),** 1114-23.

Beck Jr., L., and D'Amore, PA. (1997). Vascular development: cellular and molecular regulation. The FASEB Journal **11,** 365-73.

Belehradek, J.Jr., Orlowski, S., Ramirez, LH., Pron, G., Poddevin, B., and Mir, LM. (1994). Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin. Biochimica et Biophysica Acta **1190,** 155-163.

Bergers, G., Javaherian, K., Lo, KM., Folkman, J., and Hanahan, D. (1999). Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science **284,** 808-12.

Bischoff, J. (1997). Cell adhesion and angiogenesis. Journal of Clinical Investigation **99(3),** 373-76.

Blaese, RM., Culver, KW., Miller, AD., Carter, CS., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, JJ., Rosenberg, SA., Klein, H., Berger, M., Mullen, CA., Ramsey, WJ., Muul, L., Morgan, RA., and Anderson, WF. (1995). T-lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years. Science **270,** 475-80.

Blezinger, P., Freimark, BD., Matar, M., Wilson, E., Singhal, A., Min, W., Nordstrom, JL., and Pericle, F. (1999). Intratracheal administration of Interleukin 12 plasmid-cationic lipid complexes inhibits murine lung metastases. Human Gene Therapy **10,** 723-31.

Blezinger, P., Wang, J., Gondo, M., Quezada, A., Mehrens, D., French, M., Singhal, A., Sullivan, S., Rolland, A., Ralston, R., and Min, W. (1999). Systemic inhibition of tumor growth and tumor metastases by intramuscular administration of the endostatin gene. Nature Biotechnology **17,** 343-48.

Boehm, T., Folkman, J., Browder, T., and O'Reilly, MS. (1997). Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature **390,** 404-407.

Bohm, W., Schirmbeck, R., and Reimann, J. (1997). Targeting an anti-viral cytotoxic T cell response to a growing tumor facilitates its rejection. Cancer Immunology and Immunotherapy **44,** 230-38.

Boon, T., and van der Bruggen, P. (1996). Human tumor antigens recognized by T lymphocytes. Journal of Experimental Medicine **183,** 725-29.

Brooks, PC., Silletti, S., von Schalscha, TL., Friedlander, M., and Cheresh, DA. (1998). Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. Cell **92,** 391-400.

Brower, V., (1999). Tumor angiogenesis- new drugs on the block. Nature Biotechnology **17,** 963-68.

Brunda, MJ., Luistro, L., Warrier, RR., Wright, RB., Hubbard, BR., Murphy, M., Wolf, SF., and Gately, MK. (1993). Antitumor and antimetastatic activity of interleukin 12 against murine tumors. Journal of Experimental Medicine **178(4),** 1223-30.

Cao, Y. (1999). Therapeutic potentials of angiostatin in the treatment of cancer. Haematologica **84,** 643-50.

Cao, Y., O'Reilly, MS., Marshall, B., Flynn, E., Ji, RW., and Folkman, J. (1998). Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. Journal of Clinical Investigation **101,** 1055-63.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nature Medicine **6(3),** 389-95.

Carmeliet, P. Lampugnani, MG., Moons,L., Breviario, F., Compernolle, V., Bono, F., Balconi, G., Spagnuolo, R., Oosthuyse, B., Dewerchin, M., Zanetti, A., Angellilo, A., Mattot, V., Nuyens, D., Lutgens, E., Clotman, F., de Ruiter, MC., de Groot, AG., Poelmann, R., Lupu, F., Herbert, JM., Collen, D., and Dejana, E. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell **98,** 147-157.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature **380,** 435-39.

Chang, Y., and Prud'homme, GJ. (1999). Intramuscular administration of expression plasmids encoding interferon-gamma receptor/IgG1 or IL-4/IgG1 chimeric proteins protects from autoimmunity. Journal of Gene Medicine **1(6),** 415-23.

Chen, L., Chen, D., Block, E., O'Donnell, M., Kufe, DW., and Clinton, SK. (1997). Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. The Journal of Immunology **159,** 351-59.

Chen, Y., Hu, D., Eling, DJ., Robbins, J., and Kipps, TJ. (1998). DNA vaccines encoding full-length or truncated Neu induce protective immunity against Neu-expressing mammary tumors. Cancer Research **58,** 1965-71.

Chen, QR., Kumar, D., Stass, SA., and Mixson, AJ. (1999). Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. Cancer Research **59,** 3308-12.

Claesson-Welsh, L., Welsh, M., Ito, N., Anand-Apte, B., Soker, S., Zetter, B., O'Reilly, M., and Folkman, J. (1998). Angiostatin induces endothelial cell apoptosis and activation of focal adhesion kinase independently of the integrin-binding motif RGD. Proceedings of the National Academy of Sciences of the USA **95,** 5579-5583.

Cohen, J (1995). IL-12 deaths: explanation and a puzzle. Science **270,** 908.

Colombo, MP., Vagliani, M., Spreafico, F., Parenza, M., Chiodoni, C., Melani, C., and Stoppacciaro, A. (1996). Amount of interleukin 12 available at the tumor site is critical for tumor regression. Cancer Research **56,** 2531-34.

Coonrod, A., Li, FQ., and Horwitz, M. (1997). On the mechanism of DNA transfection: efficient gene transfer without viruses. Gene Therapy **4,** 1313-1321.

Cornelius, LA., Nehring, LC., Harding, E., Bolanowski, M., Welgus, HG., Kobayashi, DK., Pierce, RA., and Shapiro, SD. (1998). Matrix metalloproteinases generate Angiostatin: effects on neovascularization. The Journal of Immunology **161,** 6845-52.

Crystal, RG. (1999). The body as a manufacturer of endostatin. Nature Biotechnology **17,** 336-37.

Crystal, RG. (1995). Transfer of genes to humans: early lessons and obstacles to success. Science **270,** 404-10.

Dai, Y., Schwarz, EM., Gu, D., Zhang, W., Saretnick, N., and Verma, IM (1995). Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. Proceedings of the National Academy of the USA **92,** 1401-1405.

Desurmont, C., Caillaud, JM., Emmanuel, F., Benoit, P., Fruchart, JC., Castro, G., Branellec, D., Heard, JM., and Duverger, N. (2000). Complete atherosclerosis regression after human apoE gene transfer in apoE-deficient/nude mice. Arteriosclerosis, Thrombosis, and Vascular Biology **20,** 435-442.

Dhanabal, M., Ramchandran, R., Waterman, MJF., Lu, H., Knebelmann, B., Segal, M., and Sukhatme, VP. (1999). Endostatin induces endothelial cell apoptosis. Journal of Biological Chemistry **274(17),** 11721-11726.

Dhanabal, M., Volk, R., Ramchandran, R., Simons, M., and Sukhatme, VP. (1999). Cloning, expression, and *in vitro* activity of human Endostatin. Biochemical and Biophysical Research Communications **258,** 345-52.

Doh, SG., Vahlsing, HL., Hartikka, J., Liang, X., and Manthorpe, M. (1997). Spatial-temporal patterns of gene expression in mouse skeletal muscle after injection of lacZ plasmid DNA. Gene therapy **4,** 648-663.

Dong, Z., Kumar, R., Yang, X., and Fidler, IJ. (1997). Macrophage-derived metalloelastase is responsible for the generation of Angiostatin in Lewis lung carcinoma. Cell **88,** 801-10.

Donnelly, J., Ulmer, JB., Shiver, JW., and Liu, MA. (1997). DNA vaccines. Annual Review of Immunology **15,** 617-648.

Dvorak, HF., Brown, LF., Detmar, M., and Dvorak, AM. (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. American Journal of Pathology **146(5),** 1029-39.

Eliceiri, BP., and Cheresh, DA. (1999). The role of αv integrins during angiogenesis: insights into potential mechanisms of action and clinical development. The Journal of Clinical Investigation **103(9),** 1227-30.

Elkon, KB., Liu, CC., Gall, JG., Trevejo, J., Marino, MW., Abrahamsen, KA., Song, X., Zhou, JL., Old, LJ., Crystal, RG., and Pedersen, EF. (1997). Proceedings of the National Academy of the USA **94,** 9814-9819.

Ellis, LM., and Fidler, IJ (1995). Angiogenesis and breast cancer metastasis. Lancet **346,** 388-90.

Emtage, PCR., Wan, Y., Hitt, M., Graham, FL., Muller, WJ., Zlotnik, A., and Gauldie, J. (1999). Adenoviral vectors expressing lymphotactin and interleukin 2 or lymphotactin and interleukin 12 synergize to facilitate tumor regression in murine breast cancer models. Human Gene Therapy **10,** 697-709.

Engerman, RL. (1989). Pathogenesis of diabetic retinopathy. Diabetes **38,** 1203-1206.

Ezzell, C. (1998). Starving tumors of their lifehood. Scientific American **279(4),** 33-34.

Farhood, H., Gao, X., Son, K., Yang, YY., Lazo, JS., and Huang, L. (1994). Cationic liposomes for direct gene transfer in therapy of cancer and other diseases. Annals of the New York Academy of Sciences **716,** 23-34.

Ferrara, N. (1999). Molecular and biological properties of vascular endothelial growth factor. Journal of Molecular Medicine **77(7),** 527-543.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, KS., Powell-Braxton, L., Hillan, KJ., and Moore, MW. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature **380,** 439-42.

Fidler, IJ., and Ellis, LM. (1994). The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell **79,** 185-188.

Finnerty, H., Kelleher, K., Morris, GE., Bean, K., Merberg, DM., Kriz, R., Morris, JC., Sookdeo, H., Turner, KJ., and Wood, CR. (1993). Molecular cloning of murine FLT and FLT4. Oncogene **8,** 2293-2298.

Folkman, J. (2000). Incipient angiogenesis. Journal of the National Cancer Institute **92(2),** 94-95.

Folkman, J. (1998). Antiangiogenic gene therapy. Proceedings of the National Academy of Sciences of the USA **95,** 9064-66.

Folkman, J. (1996a). Tumor angiogenesis and tissue factor. Nature Medicine **2(2),** 167-168.

Folkman, J (1996b). Fighting cancer by attacking its blood supply. Scientific American **275(3),** 150-4.

Folkman, J. (1995a). Angiogenesis in cancer, vascular, rheumatoid and other disease. Nature Medicine **1(1),** 27-30.

Folkman, J. (1995b). Clinical applications of research on angiogenesis. The New England Journal of Medicine **333(26),** 1757-63.

Folkman, J. (1986). How is blood vessel growth regulated in normal and neoplastic tissue?- G.H.A. Clowes memorial award lecture. Cancer Research **46,** 467-473.

Fong, GH., Rossant, J., Gertsenstein, M., and Breitman, ML. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature **376,** 66-70.

Gasparini, G. (1999). The rationale and future potential of angiogenesis inhibitors in neoplasia. Drugs **58(1),** 17-38.

Gastl, G., Hermann, T., Steurer, M., Zmija, J., Gunsilius, E., Unger, C., and Kraft, A. (1997). Angiogenesis as a target for tumor treatment. Oncology **54,** 177-184.

Gately, S., Twardowski, P., Stack, MS., Cundiff, DL., Grella, D., Castellino, FJ., Enghild, J., Kwaan, HC., Lee, F., Kramer, RA., Volpert, O., Bouck, N., and Soff, GA. (1997). The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proceedings of the National Academy of Sciences of the USA **94,** 10868-10872.

Gehl, J., and Mir LM. (1999). Determination of optimal parameters for *in vivo* gene transfer by electroporation, using a rapid *in vivo* test for cell permeabilization. Biochemical and Biophysical Research Communications **261,** 377-380.

Gilbert, RA., Jaroszeski, MJ., and Heller,R. (1997). Novel electrode designs for electrochemotherapy. Biochimica et Biophysica Acta **1334,** 9-14.

Goldman, CK., Kendall, RL., Cabrera, G., Soroceanu, L., Heike, Y., Gillespie, GY., Siegal, GP., Mao, X., Bett, AJ., Huckle, WR., Thomas, KA., and Curiel, DT. (1998). Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. Proceedings of the National Academy of Sciences of the USA **95,** 8795-8800.

Goto, T., Nishi, T., Tamura, T., Dev, SB., Takeshima, H., Kochi, M., Yoshizato, K., Kuratsu, JI., Sakata, T., Hofmann, GA., and Ushio, Y. (2000). Highly efficient electro-gene therapy of solid tumor by using an expression plasmid for the herpes simplex virus thymidine kinase gene. Proceedings of the National Academy of the USA **97(1),** 354-359.

Griscelli, F., Li, H., Bennaceur-Griscelli, A., Soria, J., Opolon, P., Soria, C., Perricaudet, M., Yeh, P., and Lu, H. (1998). Angiostatin gene transfer: Inhibition of tumor growth *in vivo* by blockage of endothelial cell proliferation associated with a mitosis arrest. Proceedings of the National Academy of the USA **95,** 6367-6372

Hanahan, D. (1998). A flanking attack on cancer. Nature Medicine **4(1),** 13-14.

Hanahan, D., and Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell **86,** 353-64.

Hart, DN. (1997). Dendritic cells: unique leukocyte populations which control the primary immune response. Blood **90,** 3245-87.

Heller, L., Jaroszeski, MJ., Coppola, D., Pottinger, C., Gilbert, R., and Heller R. (2000). Electrically mediated plasmid DNA delivery to hepatocellular carcinoma *in vivo*. Gene Therapy **7**, 826-829.

Heller, R., Jaroszeski, MJ., Reintgen, DS., Puleo, CA., DeConti, RC., Gilbert, RA., and Glass, LF. (1998). Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. Cancer **83(1)**, 148-157.

Henkind, P. (1978). Ocular neovascularization: The Krill memorial lecture. American Journal of Ophthalmology **85(3)**, 287-301.

Herzog, RW., Hagstrom, JN., Kung, SH., Tai, SJ, Wilson, JM., Fisher, KJ., and High, KA. (1997). Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. Proceedings of the National Academy of the USA **94**, 5804-5809.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T., and Shibuya, M. (1998). Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. Proceedings of the National Academy of Sciences of the USA **95(16)**, 9349-54.

Hohenester, E., Sasaki, T., Olsen, BR., and Timpl, R. (1998). Crystal structure of the angiogenesis inhibitor endostatin at 1.5 A resolution. The EMBO Journal **17(6)**, 1656-64.

Holash, J., Maisonpierre, PC., Compton, D., Boland, P., Alexander, CR., Zagzag, D., Yancopoulos, GD., Wiegand, SJ. (1999a). Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science **284**, 1994-98.

Holash, J., Wiegand, SJ., and Yancopoulos, GD. (1999b). New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. Oncogene **18(38)**, 5356-62.

Holmgren, L., O'Reilly, MS., and Folkman, J. (1995). Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nature Medicine **1(2)**, 149-53.

Hsu, FJ., Benike, C., Fagnoni, F., Liles, TM., Czerwinski, D., Taidi, B., Engleman, EG., and Levy, R. (1996). Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. Nature Medicine **2(1)**, 52-58.

Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D, and Levitsky, H. (1998). The central role of $CD4^+$ T cells in the antitumor immune response. Journal of Experimental Medicine **188(12)**, 2357-2368.

Infeld, DA. and O'Shea, JG. (1998). Diabetic retinopathy. Postgraduate Medical Journal **74**, 129-33.

Isner, JM., Pieczek, A., Schainfeld, R., Blair, R., Haley, L., Asahara, T., Rosenfield, K., Razvi, S., Walsh, K., and Symes, JF. (1996). Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. The Lancet **348(9024),** 370-74.

Kawakami, Y., and Rosenberg, SA. (1997). Human tumor antigens recognized by T-cells. Immunology Research **16,** 313-39.

Kay, MA., Liu, D., and Hoogerbrugge, PM. (1997). Gene therapy. Proceedings of the National Academy of Sciences of the USA **94,** 12744-12746.

Kendall, RL., Wang, G., and Thomas, KA. (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochemical and Biophysical Research Communications **226,** 324-28.

Kendall, RL., and Thomas, KA. (1993). Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proceedings of the National Academy of Sciences **90,** 10705-10709.

Kerbel, SK. (1997). A cancer therapy resistant to resistance. Nature **390,** 335-36.

Kerbel, R., and Hawley, R. (1995). Interleukin 12: newest member of the antiangiogenesis club. Journal of the National Cancer Institute **87,** 557-59.

Keshet, E., and Ben-Sasson, SA. (1999). Anticancer drug targets: approaching angiogenesis. The Journal of Clinical Investigation **104(11),** 1497-1501.

Klauber, N., Rohan, RM., Flynn, E., and D'Amato RJ. (1997). Critical components of the female reproductive pathway are suppressed by the angiogenesis inhibitor AGM-1470. Nature Medicine **3,** 443-6.

Klein, R., and Klein, BEK. (1997). Diabetic eye disease. Lancet **350,** 197-204.

Klinman, DM., Yi, AK., Beaucage, SL., Conover, J., and Krieg, AM. (1996). CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proceedings of the National Academy of Sciences of the USA **93,** 2879-83.

Kong, HL., and Crystal, RG. (1998a). Gene therapy strategies for tumor antiangiogenesis. Journal of the National Cancer Institute **90(4),** 273-286.

Kong, HL., Hecht, D., Song, W., Kovesdi, I., Hackett, NR., Yayon, A., and Crystal, RG. (1998). Regional suppression of tumor growth by *in vivo* transfer of a cDNA encoding a

secreted form of the extracellular domain of the *flt*-1 vascular endothelial growth factor receptor. Human Gene Therapy **9,** 823-33.

Kowalczyk, DW., and Ertl, HCJ. (1999). Immune responses to DNA vaccines. Cellular and Molecular Life Sciences **55,** 751-70.

Krieg, AM., Hartmann, G., and Yi, AK. (2000). Mechanisms of action of CpG DNA. Current Topics in Microbiology and Immunology **247,** 1-21.

Leclerc, C., Deriaud, E., Rojas, M., and Whalen, RG. (1997). The preferential induction of a Th1 immune response by DNA-based immunization is mediated by the immunostimulatory effect of plasmid DNA. Cellular Immunology **179,** 97-106.

Lijnen, HR., Ugwu, F., Bini, A., and Collen, D. (1998). Generation of an Angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). Biochemistry **37,** 4699-4702.

Lin, P., Sankar, S., Shan, S., Dewhirst, MW., Polverini, PJ., Quinn, TQ., and Peters, KG. (1998). Inhibition of tumor growth by targeting tumor endothelium using a soluble vascular endothelial growth factor receptor. Cell Growth and Differentiation **9,** 49-58.

Liu, Y., Thor, A., Shtivelman, E., Cao, Y., Tu, G., Heath, TD., and Debs, RJ., (1999). Systemic gene delivery expands the repertoire of effective antiangiogenic agents. The Journal of Biological Chemistry **274(19),** 13338-13344.

Luo, JC., Yamaguchi, S., Shinkai, A., Shitara, K., and Shibuya, M. (1998). Significant expression of vascular endothelial growth factor/vascular permeability factor in mouse ascites tumors. Cancer Research **58,** 2652-60.

Machein, MR., Risau, W., and Plate, K. (1999). Antiangiogenic gene therapy in a rat glioma model using a dominant-negative vascular endothelial growth factor receptor 2. Human Gene Therapy **10,** 1117-28.

Marshall, E. (1999). Clinical trials- Gene therapy death prompts review of adenovirus vector. Science **286(5448),** 2244-2245.

Merimee, TJ. (1990). Diabetic retinopathy: a synthesis of perspectives. The New England Journal of Medicine **322(14),** 978-983.

Millauer, B., Longhi, MP., Plate, KH., Shawver, LK., Risau, W., Ulrich, A., and Strawn, LM. (1996). Dominant-negative inhibition of Flk-1 suppresses the growth of many tumor types *in vivo*. Cancer Research **56,** 1615-20.

Millauer, B., Shawver, LK., Plate, KH., Risau, W., and Ulrich, A. (1994). Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. Nature **367,** 576-79.

Mir LM, Bureau, MF., Gehl, J., Rangara, R., Rouy, D., Caillaud, JM., Delaere, P., Branellec, D., Schwartz, B., and Scherman, D. (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proceedings of the National Academy of the USA **96,** 4262-4267.

Mir, LM., Glass, LF., Sersa, G., Teissie, J., Domenge, C., Miklavcic, D., Jaroszeski, MJ., Orlowski, S., Reintgen, DS., Rudolf, Z., Belehradek, M., Gilbert, R., Rols, MP., Belehradek, J.Jr., Bachaud, JM., DeConti, R., Stabuc, B., Cemazar, M., Coninx, P., and Heller, R. (1998). Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. British Journal of Cancer **77(12),** 2336-2342.

Moser, TL., Stack, MS., Asplin, I., Enghild, JJ., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, HW., and Pizzo, SV. (1999). Angiostatin binds ATP synthase on the surface of human endothelial cells. Proceedings of the National Academy of Sciences of the USA **96,** 2811-16.

Mountain, A. (2000). Gene therapy: the first decade. Trends in Biotechnology **18,** 119-28.

Mulligan, RC. (1993). The basic science of gene therapy. Science **260,** 926-32.

Muramatsu, T., Nakamura, A., and Park, HM. (1998). *In vivo* electroporation: a powerful and convenient means of nonviral gene transfer to tissues of living animals. Bioorganic and Medicinal Chemistry Letters **1,** 55-62.

Nagashima, M., Yoshino, S., Ishiwata, T., and Asano, G. (1995). Role of vascular endothelial growth factor in angiogenesis of rheumatoid arthritis. The Journal of Rheumatology **22,** 1624-30.

Narvaiza, I., Mazzolini, G., Barajas, M., Duarte, M., Zaratiegui, M., Qian, C., Melero, I., and Prieto, J. (2000). Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-γ-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. The Journal of Immunology **164,** 3112-22.

Nastala, CL., Edington, HD., McKinney, TG., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W., and Lotze, M. (1994). IL-12 administration induces tumor regression in association with IFN-γ production. Journal of Immunology **153(4),** 1697-706.

Neufeld, G., Cohen, T., Gengrinovitch, S., and Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. The FASEB Journal **13,** 9-22.

Neumann, E., Kakorin, S., and Toensing, K. (1999). Fundamentals of electroporative delivery of drugs and genes. Bioelectrochemistry and Bioenergetics **48,** 3-16.

Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, PH., (1982). Gene transfer into mouse lyoma cells by electroporation in high electric fields. The EMBO Journal **1(7),** 841-845.

Nishi, T., Yoshizato, K., Yamashiro, S., Takeshima, H., Sato, K., Hamada, K., Kitamura, I., Yoshimura, T., Saya, H., Kuratsu, JI., and Ushio, Y. (1996). High-efficiency *in vivo* gene transfer using intraarterial plasmid DNA injection following *in vivo* electroporation. Cancer Research **56,** 1050-1055.

Oehler, MK., and Bicknell, R. (2000). The promise of anti-angiogenic cancer therapy. British Journal of Cancer **82(4),** 749-52.

O'Reilly, MS. (1997a). Angiostatin: an endogenous inhibitor of angiogenesis and tumor growth. EXS **79,** 273-94.

O'Reilly, MS., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, WS., Flynn, E., Birkhead, JR., Olsen, BR., and Folkman, J. (1997). Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell **88,** 277-85.

O'Reilly, MS., Holmgren, L., Chen, C., and Folkman, J. (1996). Angiostatin induces and sustains dormancy of human primary tumors in mice. Nature Medicine **2,** 689-92.

O'Reilly, MS., Holmgren, L., Shing, Y., Chen, C., Rosenthal, RA., Moses, M., Lane, WS., Cao, Y., Sage, EH., and Folkman, J. (1994). Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell **79,** 315-28.

Orlowski, S., An, D., Belehradek, J.Jr., and Mir, LM. (1998). Antimetastatic effects of electrochemotherapy and of histoincompatible interleukin-2-secreting cells in the murine Lewis lung tumor. Anti-Cancer Drugs **9,** 551-556.

Paillard, F. (1999). Cytokine and chemokine: a stimulating couple. Human Gene Therapy **10,** 695-96.

Parangi, S., O'Reilly, M., Christofori,G., Holmgren, L., Grosfeld, J., Folkman, J., and Hanahan, D. (1996). Antiangiogenic therapy of transgenic mice impairs *de novo* tumor growth. Proceedings of the National Academy of Sciences of the USA **93,** 2002-7.

Pardoll, DM. (1994). Tumor antigens: A new look for the 1990s. Nature **369,** 357.

Peters, KG., De Vries, C., and Williams, LT. (1993). Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth. Proceedings of the National Academy of Sciences of the USA **90,** 8915-8919.

Pierce, EA., Avery, RL., Foley, ED., Aiello, LP., and Smith, LEH. (1995). Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization. Proceedings of the National Academy of Sciences of the USA **92,** 905-909.

Prince, HM. (1998). Gene transfer: a review of methods and applications. Pathology **30,** 335-347.

Prud'homme, GJ., Piccirillo, CA. (2000). The inhibitory effects of transforming growth factor-beta-1 (TGF-β1) in autoimmune diseases. Journal of Autoimmunity **14(1),** 23-42.

Reynolds, LP., Killilea, SD., and Redmer, DA. (1992). Angiogenesis in the female reproductive system. The FASEB Journal **6,** 886-892.
Risau, W. (1997). Mechanisms of angiogenesis. Nature **386,** 671-74.

Risau, W. (1995). Differentiation of endothelium. The FASEB Journal **9,** 926-33.

Rizzuto, G., Cappelletti, M., Maione, D., Savino, R., Lazzaro, D., Costa, P., Mathiesen, I., Cortese, R., Ciliberto, G., Laufer, R., La Monica, N., and Fattori, E. (1999). Efficient and regulated erythropoietin production by naked DNA injection and muscle electroporation. Proceedings of the National Academy of Sciences of the USA **96,** 6417-6422.

Robertson, MJ., and Ritz, J. (1996). Interleukin 12: basic biology and potential applications in cancer treatment. The Oncologist **1,** 88-97.

Roeckl, W., Hecht, D., Sztajer, H., Waltenberger, J., Yayon, A., and Weich, HA. (1998). Differential binding characteristics and cellular inhibition by soluble VEGF receptors 1 and 2. Experimental Cell Research **241,** 161-70.

Rols, MP., Delteil, C., Golzio, M., Dumond, P., Cros, S., and Teissie, J. (1997). *In vivo* electrically mediated protein and gene transfer in murine melanoma. Nature Biotechnology **16,** 168-171.

Rowe, PM. (1999). What is all the hullabaloo about endostatin? The Lancet **353,** 732.

Ruoslahti, E. (1997). Integrins as signaling molecules and targets for tumor therapy. Kidney International **51,** 1413-17.

Ryan, DP., Penson, RT., Ahmed, S., Chabner, BA., and Lynch Jr., TJ. (1999). Reality testing in cancer treatment: the phase I trial of Endostatin. The Oncologist **4(6),** 501-508.

Saleh, M., Stacker, SA., and Wilks, AF. (1996). Inhibition of growth of C6 glioma cells *in vivo* by expression of antisense vascular endothelial growth factor sequence. Cancer Research **56,** 393-401.

Salven, P., Maenpaa, H., Orpana, A., Alitalo, K., and Joensuu, H. (1997). Serum vascular endothelial growth factor is often elevated in disseminated cancer. Clinical Cancer Research **3,** 647-51.

Sauter, BV., Martinet, O., Zhang, WJ., Mandeli, J., and Woo, SLC. (2000). Adenovirus-mediated gene transfer of endostatin *in vivo* results in high level of transgene expression and inhibition of tumor growth and metastases. Proceedings of the National Academy of Science of the USA **97(9),** 4802-07.

Schlessinger, J., Lax, I., and Lemmon, M. (1995). Regulation of growth factor activation by proteoglycans: what is the role of the low affinity receptors? Cell **83,** 357-360.

Sersa, G., Cemazar, M., Miklavcic, D., and Mir, LM. (1994). Electrochemotherapy: variable anti-tumor effect on different tumor models. Bioelectrochemistry and Bioenergetics **35,** 23-27.

Sgadari, C., Angiolillo, AL., Cherney, BW., Pike, SE., Farber, JM., Koniaris, LG., Vanguri, P., Burd, PR., Sheikh, N., Gupta, G., Teruya-Feldstein, J., and Tosato, G. (1996). Interferon-inducible protein-10 identified as a mediator of tumor necrosis *in vivo*. Proceedings of the National Academy of Sciences of the USA **93,** 13791-13796.

Shalaby, F., Rossant, J., Yamaguchi, TP., Gertsenstein, M., Wu, XF., Breitman, ML., and Schuh, AC. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature **376,** 62-66.

Sim, BKL., O'Reilly, MS., Liang, H., Fortier, AH., He, W., Madsen, JW., Lapcevich, R., and Nacy, CA. (1997). A recombinant human angiostatin protein inhibits experimental primary and metastatic cancer. Cancer Research **57,** 1329-34.

Singh, BN., and Dwivedi, C. (1999). Antitumor drug delivery by tissue electroporation. Anti-Cancer Drugs **10,** 139-146.

Song, K., Chang, Y., and Prud'homme, GJ (2000). Regulation of T-helper-1 versus T-helper-2 activity and enhancement of tumor immunity by combined DNA-based vaccination and nonviral cytokine gene transfer. Gene Therapy **7,** 481-92.

Stack, MS., Gately, S., Bafetti, LM., Enghild, JJ., and Soff, GA (1999). Angiostatin inhibits endothelial and melanoma cellular invasion by blocking matrix-enhanced plasminogen activation. Journal of Biochemistry **340,** 77-84.

Stetler-Stevenson, WG. (1999). Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. The Journal of Clinical Investigation **103(9),** 1237-1241.

Sun, Y., Jurgovsky, K., Moller, P., Alijagic, S., Dorbic, T., Georgieva, J., Wittig, B., and Schadendorf, D. (1998). Vaccination with IL-12 gene-modified autologous melanoma cells: preclinical results and a first clinical phase I study. Gene Therapy **5,** 481-90.

Tanaka, T., Cao, Y., Folkman, J., and Fine, HA. (1998). Viral vector-targeted antiangiogenic gene therapy utilizing an angiostatin complementary DNA. Cancer Research **58,** 3362-69.

Tanaka, T., Manome,Y., Wen, P., Kufe, DW., and Fine, HA. (1997). Viral vector-mediated transduction of a modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth. Nature Medicine **3(4),** 437-42.

Taniguchi, T., Rigg, A., and Lemoine, NR (1998). Targeting angiogenesis: genetic intervention which strikes at the weak link of tumorigenesis. Gene Therapy **5,** 1011-13.

Tannenbaum, CS., Tubbs, R., Armstrong, D., Finke, JH., Bukowski, RM., and Hamilton, TA. (1998). The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. The Journal of Immunology **161,** 927-32.

Tannenbaum, CS., Wicker, N., Armstrong, D., Tubbs, R., Finke, J., Bukowski, RM., and Hamilton, TA. (1996). Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. The Journal of Immunology **156,** 693-99.

Teicher, BA., Holden, SA., Ara, G., Sotomayor, EA., and Dong, HZ. (1994). Potentiation of cytotoxic cancer therapies by TNP-470 alone and with other antiangiogenic agents. International Journal of Cancer **57,** 1-6.

Thurston, G., Rudge, JS., Ioffe, E., Zhou, H., Ross, L., Croll, SD., Glazer, N., Holash, J., McDonald, DM., and Yancopoulos, GD. (2000). Angiopoietin-1 protects the adult vasculature against plasma leakage. Nature Medicine **6(4),** 460-463.

Timmerman, JM., and Levy, R. (1999). Dendritic cell vaccines for cancer immunotherapy. Annual Review of Medicine **50,** 507-29.

Toes, REM., Ossendorp, F., Offringa, R., and Melief, CJM. (1999). CD4 T cells and their role in antitumor immune responses. The Journal of Experimental Medicine **189(5),** 753-56.

Trapnell, BC., and Gorziglia, M. (1994). Gene therapy using adenoviral vectors. Current Opinions in Biotechnology **5,** 617-25.

Trinchieri, G. (1995). Interleukin-12 and interferon γ: do they always go together? American Journal of Pathology **147(6),** 1534-38.

Trinchieri, G. (1994). Interleukin-12: a cytokine produced by antigen-presented cells with immunoregulatory functions in the generation of T-helper cells type I and cytotoxic lymphocytes. Blood **84,** 4008-4027.

Tripathy, SK., Svensson, EC., Black, HB., Goldwasser, E., Margalith, M., Hobart, PM., and Leiden, JM. (1996). Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. Proceedings of the National Academy of the USA **93,** 10876-10880.

Verma, IM. and Somia, N. (1997). Gene therapy- promises, problems and prospects. Nature **389,** 239-42.

Voest, EE., Kenyon, BM., O'Reilly, MS., Truitt, G., D'Amato, RJ., and Folkman, J. (1995). Inhibition of angiogenesis *in vivo* by interleukin 12. Journal of the National Cancer Institute **87,** 581-86.

Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, SW., Chen, M., Leung, L., Otten, GR., Thudium, K., Selby, MJ., and Ulmer, JB. (2000). Increased DNA vaccine delivery and immunogenicity by electroporation *in vivo.* The Journal of Immunology **164,** 4635-4640.

Wolff, JA., Malone, RW., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, PL. (1990). Direct gene transfer into mouse muscle *in vivo.* Science **247,** 1465-1468.

Yang, Y., and Wilson JM. (1995). Clearance of adenovirus-infected hepatocytes by MHC class I-restricted $CD4^+$ CTLs *in vivo.* The Journal of Immunology **155,** 2564-2570.

Yao, L., Sgadari, C., Furuke, K., Bloom, ET., Teruya-Feldstein, J., and Tosato, G. (1999). Contribution of natural killer cells to inhibition of angiogenesis by interleukin-12. Blood **93(5),** 1612-21.

Ye, X., Rivera, VM., Zoltick, P., Cerasoli Jr., F., Schnell, MA., Gao, G., Hughes, JV., Gilman, M., and Wilson, JM. (1999). Regulated delivery of therapeutic proteins after *in vivo* somatic cell gene transfer. Science **283,** 88-91.

Yeh, P., and Perricaudet, M. (1997). Advances in adenoviral vectors: from genetic engineering to their biology. The FASEB Journal **11,** 615-623.

Yoon, SS., Eto, H., Lin, C, Nakamura, H., Pawlik, TM., Song, SU., and Tanabe, KK. (1999). Mouse endostatin inhibits the formation of lung and liver metastases. Cancer Research **59,** 6251-56.

Zetter, BR. (1998). Angiogenesis and tumor metastasis. Annual Review of Medicine **49,** 407-24.