2010

Optimization and toxocologic effects of cancer immuno-electrogene therapy using a tumor-targeted interleukin-12 gene construct

Scott Douglas Reed
Louisiana State University and Agricultural and Mechanical College, sreed@vetmed.lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations
Part of the Medicine and Health Sciences Commons

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/657

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
OPTIMIZATION AND TOXICOLOGIC EFFECTS OF CANCER IMMUNOELECTROGENE THERAPY USING A TUMOR-TARGETED INTERLEUKIN-12 GENE CONSTRUCT

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Science
through
The Department of Comparative Biomedical Sciences

by
Scott Douglas Reed
B.S., Virginia Tech, 1984
D.V.M., University of Florida, 1990
December 2010
DEDICATION

This dissertation is dedicated to my parents. Their academic prowess was far greater than mine, but their sacrifices to family and the advancement of their children prevented them from pursuing the academic path that I have been fortunate enough to follow. My mom, a nuclear chemist who was at the top of her high school class and one of the few women in science in her college classes, gave up her career to assure the successes of her sons and daughter. My dad, who was valedictorian of his high school class, dedicated himself to a career in government where he served as a chemist working for both the Food and Drug Administration and the Environmental Protection Agency. Despite a job that was often full of frustrations, he always was there to provide for our family and encouraged us to pursue careers in science and to be critical thinkers. Both parents have never stopped being there to support us and have continued to support my sister Ginny, who has Down syndrome, long into their retirement years.

Although this may seem trivial, to those who have known the unconditional love and companionship that only a dog can provide, my additional dedication to my dog Chi who has helped me through the worst times of my life will come as no surprise. Since he has no use for such a dedication, I will let him roll in something stinky in honor of our travels together.
ACKNOWLEDGEMENTS

Obviously this dissertation would not have been possible without the financial support and mentorship provided by Doctor Shulin Li. I am grateful for his help and guidance. Additional funding of this investigation was supported by the National Institutes of Health under a Ruth L. Kirschstein T-32 National Research Service Award; I am indebted to Doctor Thomas Klei, Associate Dean for Research and Academic Affairs, and Doctor Andrew Lackner, Director of the Tulane Regional Primate Center, for securing this funding. Additionally, there have been a number of people who have facilitated my research and who I owe great appreciation, starting with my committee members. My pathology mentors, Doctors Timothy Morgan and Nobuko Wakamatsu have taught me a great deal about pathology and have served as a great source of expertise and advice. Doctor Inder Sehgal has been nothing but pleasant, cooperative, and helpful with everything I needed from him, including serving as co-chair on my committee on short notice after Doctor Li accepted a position at the MD Anderson Cancer Center. The dean’s representative, Doctor Prosanta Chakrabarty has also been very flexible and pleasant throughout my interactions with him.

From the Li laboratory, Doctors Denada Dibra and Jeff Cutrera provided tumor cells and their valuable time demonstrating a variety of techniques; Summer Xia was also helpful in locating items and providing therapeutic plasmids. Histopathology technical expertise was provided by Cheryl Crowder, Hal Holloway, Kendra Schultz, and Sherry Ring – without their help, the bulk of my work could not have been completed. Del Phillips also deserves thanks for processing immunohistochemistry slides. Marylin Dietrick was very helpful in processing and analyzing flow cytometry data; she is a tremendous asset to LSU.
Last, but not least, the dedicated animal husbandry staff in the Department of Laboratory Animal Medicine always went above and beyond in caring for our experimental mice. I owe special thanks to Ms Cecelia Koon and Doctor Rhett Stout for their help and facilitating animal orders and assuring regulatory compliance. More importantly, the hundreds of mice that were sacrificed in order to help other species affected by cancer were essential for this work. Without the ability to do therapeutic testing using laboratory animals, many of the great advances in human and veterinary medicine would not be possible.
TABLE OF CONTENTS

DEDICATION .......................................................................................................................... ii
ACKNOWLEDGEMENTS........................................................................................................ iv
ABSTRACT .............................................................................................................................. vii

CHAPTER 1. INTRODUCTION................................................................................................. 1
REFERENCES.......................................................................................................................... 3

CHAPTER 2. ELECTROPORATION ADVANCES IN LARGE ANIMALS................................. 5
INTRODUCTION ...................................................................................................................... 6
THE MECHANISM FOR EP-MEDIATED DNA ENTRY INTO CELLS ....................................... 7
ACHIEVING HIGH-LEVEL AND LONG-TERM GENE EXPRESSION ............................... 9
ELECTRON AVALANCHE TRANSFECTION AND ELECTROSONOPORATION ................................................. 9
EP FORMULATIONS AND NOVEL EP PARAMETERS ......................................................... 11
APPLICATION OF EP IN LARGE ANIMALS ...................................................................... 15
APPLICATION OF EP FOR DNA VACCINES – OPTIMIZATION ................................. 18
APPLICATION OF EP FOR DNA VACCINES – MUSCLE VERSUS SKIN ....................... 19
APPLICATION OF EP FOR DNA VACCINES – ROLE OF ADJUVANTS ....................... 21
APPLICATION OF EP FOR “CELL VACCINES” ........................................................... 21
APPLICATION OF EP FOR DNA VACCINES – LARGE ANIMAL AND PRIMATE STUDIES ................................................................................................................................. 22
INNOVATIVE APPLICATIONS OF EP GENE THERAPY .................................................. 22
UNSOLVED CONCERNS PERTAINING TO EP .................................................................. 25
CONCLUSION ....................................................................................................................... 27
REFERENCES ....................................................................................................................... 27

CHAPTER 3. BLEOMYCIN/INTERLEUKIN-12 ELECTROCHEMOCONEUTERAPY FOR TREATING NATURALLY OCCURRING SPONTANEOUS NEOPLASMS IN DOGS ................................................................................................................................. 40
INTRODUCTION .................................................................................................................. 41
MATERIALS AND METHODS ............................................................................................ 42
Selection of Naturally Occurring Tumor-Bearing Dogs .................................................... 42
In Vitro IL12-Augmented Cytotoxicity Determination .................................................... 43
Imaging ............................................................................................................................... 43
Other Diagnostics ............................................................................................................. 44
Treatment ........................................................................................................................ 44
RESULTS ............................................................................................................................. 45
DISCUSSION ......................................................................................................................... 53
CONCLUSIONS ................................................................................................................... 55
REFERENCES ....................................................................................................................... 56
ABSTRACT

This dissertation includes a comprehensive current review of reversible electroporation (EP) and other related physical gene transfection techniques; an overview of results of electrochemogene therapy (ECGT) used to treat naturally occurring spontaneous neoplasms in dogs; and the results of comprehensive, pre-clinical toxicology testing of electrogene therapy (EGT) of a tumor-targeted version of interleukin-12 (IL-12) in mice.

Intralesional bleomycin (BLM) and feline interleukin-12 (fIL-12) DNA injection combined with trans-lesional EP resulted in complete cure of two recurrent oral squamous cell carcinomas and an acanthomatous ameloblastoma in a series of six cases of spontaneous neoplasia in pet dogs. The three remaining dogs, which had no other treatment options, had partial responses to ECGT. One of these dogs had mandibular melanoma with pulmonary and lymph node metastases; one dog had cubital histiocytic sarcoma with spleen metastases; and one had soft palate fibrosarcoma. Treatment of all six dogs was associated with minimal side effects, was easy to perform, was associated with repair of bone lysis in cured dogs; improved the quality of life for dogs with partial responses; and extended overall survival time.

For the purpose of meeting pre-clinical safety requirements for an Investigational New Drug filing, we assessed the safety of tumor-targeted interleukin-12 (ttIL-12) when administered by EGT in C3H/HeJ mice by identifying an initial safe dose for human dose escalation schemes, toxicity target organs, markers of toxicity, and toxicity reversibility. Dystrophic cardiac calcification in older, 5 µg ttIL-12-treated mice was the only serious toxicity. Based on these results and the lack of any effect on wound healing when combined with surgery, low-intensity EGT with ttIL-12 appears to be safe and well tolerated as both a single treatment modality and when combined with surgical tumor resection.
Although there have been tremendous advances in cancer treatment over the past few decades, it still remains a major killer; in 2007, the cancer mortality rate was second only to heart disease. In other words, cancer represented roughly a quarter of all deaths in the United States or 562,875 people\(^1\). The majority of these deaths continue to be a result of recurrent or metastatic disease; therefore treatments addressing prevention or treatment of cancer recurrence and metastasis are essential for making a significant impact in the war against cancer.

Surgery remains the primary treatment modality for many solid tumors and is often combined with or supplanted by radiation and/or chemotherapy. In some cases surgery alone may result in a clinical cure, but many times either the location of the tumor or pre-existing micrometastases preclude complete cure. Despite lack of clinical cure, one of the benefits of surgical removal of the primary tumor is that the majority of metastatic tumor cells enter into a growth phase either because of lack of growth factor inhibition by the primary tumor or because of inflammatory effects of the surgery. Entry into the growth phase is essential for efficacy of radiation or chemotherapy. Other small molecule inhibitors and monoclonal antibodies also perform best in the setting of cell proliferation. Cancer immunotherapy is somewhat unique in that it usually does not require actively proliferating cells, instead relying on tumor specific antigens or lack of MHC expression to recognize and kill tumor cells through the immunosurveillance activities of the immune system. This becomes even more important with the knowledge that many tumors have a small quiescent population of tumor stem cells which drive tumor recurrence and may be important in tumor metastasis. Targeting this population of tumor cells may be the key to curing many cancers.

Cancer immunotherapy has been used for a variety of tumors for several years now. Immunotherapeutic strategies include recombinant cytokine therapy, dendritic cell manipulation
through either ex vivo manipulation and re-introduction to other in vivo modifications, and use of a variety of non-specific immunostimulants. Although recombinant cytokine therapy has had a number of safety issues, cytokine gene therapy avoids these problems by targeting tumors allowing safe sustained (for a limited period of time) systemic levels of circulating cytokines. Our laboratory has been actively developing a number of these gene therapies, the most promising of which is Interleukin-12 (IL-12) electrogene therapy. We have also looked at co-stimulatory molecule gene therapy and a number of other cytokine genes, but at this point IL-12 appears to offer the most promise. We have found this therapy to be efficacious in mice and to be both safe and effective when combined with bleomycin electrochemotherapy or surgery in both mice and dogs.

Given that our chosen means of gene transfection is by electroporation, chapter two covers recent advances in large animal electroporation therapy. In chapter three, examples of clinical use of combination chemotherapy and cytokine gene therapy mediated by electroporation is discussed with demonstration of safety and efficacy in dogs with a variety of neoplasms. In chapter four, controlled experimental pre-clinical safety studies provide firm evidence of the safety of this treatment prior to escalation to human clinical trials. Finally, chapter five summarizes our findings and suggests further directions for this work along with potential applications.

REFERENCES


INTRODUCTION

Gene therapy has become an important potential treatment modality for a variety of disorders and has shown great promise for treating genetic deficiencies and mutations, as well as for providing secreted therapeutic proteins and immune modulators. Gene therapy has also become important in preventing disease by use of DNA vaccines. Furthermore, in production animals, gene therapy has been used to enhance reproductive efficiency and production gains.

A variety of means for introducing genes into mammalian cells have been used that can be broadly categorized as viral, chemical, and physical methods. There is a general consensus that viral vectors are the most efficient means of gene transfection, but significant safety concerns, such as the potential to illicit an immune response and/or cause cellular transformation limit virus use in a variety of settings. Chemical methods can be effective in vitro, but their use in vivo needs further improvement because their transfection efficiency is lower than for viral and physical methods in most of cases. Physical transfection avoids many of the undesirable effects of chemical and viral methods, can be used repetitively, is relatively simple and cost-effective, and has essential no limitation on the coding length of the gene to be introduced. Of the physical methods of gene transfection, electroporation (EP) is the most commonly used method in a variety of animal and human trials.

Historically, gene therapy in large animals (defined as species of animals other than rats, mice, and small rodents) has been pursued using viral and non-viral vectors carrying genes to be transfected into host cells. Since in vivo EP has emerged as one of the few powerful non-viral vector delivery methods for efficiently and effectively delivering plasmid DNA and nucleic acids in vivo, this review will be limited to EP-based gene therapy. For the purpose of this review, in vivo EP refers to reversible EP for the purpose of gene delivery, not irreversible EP where the
therapeutic intent is ablation of cells using EP technique alone. Several recent reviews examining the use of irreversible $^5$-$^11$ and reversible $^{12-15}$ EP are published elsewhere. The focuses of this review are novel aspects of EP as applied to large animals, improvement of EP delivery technique, and development of EP-based vaccines.

THE MECHANISM FOR EP-MEDIATED DNA ENTRY INTO CELLS

During EP, a series of square-wave electric pulses are used to drive naked DNA into cells. A stable, non-dividing population of muscle cells is transfected when long-term expression of a gene is desired, for example in supplementing clotting factors to treat hemophilia (though immunogenicity often neutralizes the circulated clotting factor).$^{16, 17}$ Alternatively, a tumor’s population of neoplastic cells, stromal cells, and attending inflammatory cells are transfected in tumor gene therapy, for example introducing interleukin 12 to trigger anti-tumor immunity.$^{18}$ Regardless of the target cells transfected, EP exposes tissue to a brief electric field which induces temporary and reversible breakdown of cell membranes and formation of pores. The electric field also takes advantage of the tendency of negatively-charged nucleic acid to migrate toward the positive pole in an electric field (an electrophoretic effect).

Within the cell membrane, pores form within 10 ns $^{19}$ and initially are less than 10 nm in diameter. $^{20, 21}$ While the duration of pore formation may be short, reconstitution of the cell membrane may be prolonged by decreasing temperature. However with longer duration electric field application, pore number increases and pores begin to coalesce. When large enough pores form, the damage becomes irreversible and cells die (irreversible EP). Fortunately, the size of pores normally is not a limiting factor, both small oligonucleotides and nucleotides larger than 150 kb, which is larger than the pores, have been shown to readily enter the cell during EP. This fact suggests that the mechanism of gene transfer into cells may simply be based on diffusion;$^{22}$ or, as other researchers have suggested, that electric-pulse-induced membrane instability causes
membrane bound vesicles containing DNA to form which are carried into the cell by endocytosis.\textsuperscript{23} Despite numerous theories, the mechanism of nucleic acid entry into cells remains open to conjecture.\textsuperscript{24} All models explaining nucleotide migration through the cell membrane must be based on several physical postulates including: the existence of long-lived electropores,\textsuperscript{25-27} a preliminary binding step at the cell surface due to membrane plasmid DNA interaction and then DNA diffusion through electropores,\textsuperscript{26} electrophoretic forces generated by the external field which push the plasmid DNA through the membrane.\textsuperscript{28,29} Interactions have been observed between DNA and model lipid bilayers which suggest that other mechanisms, including endocytosis, may also play a role in membrane-DNA interaction. In fact, DNA-induced endocytosis has been observed in a number of studies, in the absence of any electric field.\textsuperscript{30,31}

It is generally accepted that when the cell membrane is not permeabilized, electric field lines or vectors follow the outer profile of the cell, and DNA flows in the direction of the field around the cell to the anode. When the membrane is permeabilized, electric field lines enter the cell membrane and DNA is trapped in the region of the cell membrane opposite the cathode where it is effectively pushed up against the membrane by electrophoretic force.\textsuperscript{24} Interaction with the permeabilized membrane prevents DNA from flowing around the cell. Thus, Favard, \textit{et al.}, conclude that electrotansfection is a multistep process where negatively charged DNA migrates by electrophoresis towards the cell plasma membrane on the cathode side where it accumulates.\textsuperscript{24} When electric fields exceed a certain threshold, the plasma membrane is permeabilized allowing accumulated plasmid DNA to enter. This translocation of plasmid DNA from the plasma membrane to the cytosol and subsequent passage to the nuclear envelope takes minutes to hours. Intracellular movement also occurs by an as yet undetermined mechanism
which may involve simple diffusion, endocytosis, or electrophoretic movement. Upon entering the nucleus, gene transcription from plasmid DNA can take place.\textsuperscript{24}

ACHIEVING HIGH-LEVEL AND LONG-TERM GENE EXPRESSION

In most cases, the DNA that enters the nucleus is transiently transcribed and rarely integrated into the host cell genome. Although this means that the frequency of genome disruption is lower than techniques involving integration of the gene in the host genome,\textsuperscript{32} the optimal conditions for long-term levels of transgene expression without adverse side effects, remains a primary objective for researchers.\textsuperscript{33} Despite the fact that transient expression of the transgene remains one of the major shortcomings of nonviral DNA delivery, this shortcoming can partially be alleviated by using post-mitotic, stable cells, such as myocytes. EP-mediated gene therapy in continually dividing cells often yields declining transgene expression, probably due to degradation, since extrachromosomal DNA is known to persist in post-mitotic tissues. Several recent studies, however, have shown that the integrase from bacteriophage φC31 confers genomic integration of plasmid DNA and long-term expression in mammalian cells in a variety of contexts. Used together, EP-mediated transfection and φC31 integrase could be a powerful combination for long-term, nonviral gene therapy.\textsuperscript{34, 35}

ELECTRON AVALANCHE TRANSFECTION AND ELECTROSONOPORATION

Improvements in EP methods, equipment, and protocols are inevitable and there have been numerous alterations in EP technique and attempts to understand the mechanisms underlying EP in recent years. An interesting recent example of a technique based on EP that has been used for gene therapy in rabbit eyes, is EAT. In electrosonoporation, an electric field with high voltage amplitude is produced from microelectrodes adjacent to plasma bubbles or blebs (between the retina and choroid in this study). This forms a transient vapor cavity in the
plasma space which is ionized, allowing conductance from the electrode through the vapor cavity to the tissue. At the same time, the cavitation bubble generates a propagating acoustic wave that exposes the tissue to mechanical stress synchronized with an electric field. In initial studies on chorioallantoic membrane, electron avalanche transfection was >10,000-fold more efficient and produced less tissue damage than conventional EP. Efficient plasmid DNA transfer to the rabbit retina after subretinal DNA injection and trans-scleral EAT was also demonstrated in this study. Electroretinograms and histology showed no evidence of damage from the procedure.³⁶

EAT differs from conventional EP by using microelectrodes instead of large electrodes; by relying on ionization of the vapor cavity to deliver the electric field and mechanical stress; and by using short, biphasic electric pulses. Since arc production is considered detrimental in conventional EP, EAT delivers the electrical charge via an ionized vapor cavity which prevents arc generation, allowing use of much higher electric fields. Furthermore, short, biphasic pulses cause little or no muscle movement which is desirable for precision and patient comfort. Increased EP efficiency under the tensile stress created during EAT may occur because of increased lipid bilayer instability and resulting increased susceptibility to permeabilization.³⁶

Another modification of EP, electrosonoporation (ES) is similar to EAT in that EP is combined with a physical method of inducing pore formation through interaction with cavitation bubbles. Sonoporation (SP) uses ultrasound to temporarily permeabilize cell membranes allowing uptake of compounds from the extracellular environment; since membrane alteration is transient, the compound is left trapped inside the cell after ultrasound exposure. Ultrasound produces microscopic cavitation bubbles within the extracellular milieu; the cavitation bubbles implode producing a shockwave while on or near the surface of a cell membrane; and the tiny shockwave produces pores in the cell membrane allowing compounds to diffuse into the cell.³⁷
Steps in ES include preparation of a DNA-microbubble preparation, injection, and tissue ultrasound exposure.\textsuperscript{37}

Ultrasound offers good penetration through soft tissue, minimal damage to cells/tissues, and does not damage DNA; however, it is limited by breakdown of cell cytoskeleton which among other perturbations, alters DNA trafficking within cells.\textsuperscript{38}

Combining EP with SP has been used in multiple studies for gene transfer and has been found to be effective.\textsuperscript{39, 40} When used to transfec\t the luciferase reporter gene into muscle, ES was found to be twofold more effective than EP alone.\textsuperscript{40} The following table (table 1) has a brief comparison of some of the benefits and shortcomings associated with these techniques.

Regardless of how poration occurs, persistent, high-level gene expression remains a challenge. This is particularly true for secreted proteins because of their immunogenicity. Our results suggest that the secreted alkaline phosphatase (SEAP) reporter gene maintains long-term expression at a level of 5 ng/mL in blood, but expression is shut down rapidly after exceeding this level following $IM$ delivery of SEAP gene via EP (Figure 1). This observation is most likely due to development of an immune response, since a titer of anti-SEAP antibody was detected in the animals expressing a high level of SEAP. Other mechanisms such as death of the cells with high reporter gene expression may also contribute.

**EP FORMULATIONS AND NOVEL EP PARAMETERS**

Although EP is the determining factor that dictates gene transduction \textit{in vivo}, the carrier solution in which genes are delivered by EP can affect the efficacy and damaging effects of EP. Physiologic saline is most commonly used, and when combined with EP, expression of luciferase is enhanced by 10,000 fold over direct injection in muscle.\textsuperscript{41} In tumors, expression after EP of luciferase gene in saline was increased 1200 fold.\textsuperscript{42} Alternatively, concentrated
### Table 1. Summary Comparing Different Poration Gene Transfection Techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP</td>
<td>Equipment is readily available &amp; currently in clinical use</td>
<td>Mild transient discomfort at EP site &amp; mild local tissue damage</td>
<td>[8-11, 20, 28-29]</td>
</tr>
<tr>
<td></td>
<td>Short-term gene expression may be an advantage in some settings (cancer therapy)</td>
<td>Long-term gene expression remains a challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large amount of literature pertaining to optimization &amp; implementation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Proven in a variety of species with numerous transfection products</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Local tissue damage may offer advantages in terms of cytokine release</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibition of angiogenesis &amp; local tumor destruction</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mild transient discomfort at EP site &amp; mild local tissue damage</td>
<td></td>
</tr>
<tr>
<td>SP</td>
<td>Simple</td>
<td>Breakdown of cell cytoskeleton alters DNA trafficking</td>
<td>[33, 34]</td>
</tr>
<tr>
<td></td>
<td>Equipment readily available &amp; currently in clinical use</td>
<td>Long-term gene expression remains a challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimal damage to tissues and DNA</td>
<td>Limited species and genes tested</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lack of tissue damage may limit release of beneficial cytokines</td>
<td></td>
</tr>
<tr>
<td>EAT</td>
<td>Minimal tissue damage (applicable to sensitive neural tissue)</td>
<td>Specialized equipment</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td>High efficiency of transfection (greater than 10,000 fold more effective than EP)</td>
<td>Cumbrous for clinical use &amp; requires specialized training to implement</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimal patient discomfort</td>
<td>Long-term gene expression remains a challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High precision</td>
<td>Limited species and genes tested</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lack of tissue damage may limit release of beneficial cytokines</td>
<td></td>
</tr>
<tr>
<td>ES</td>
<td>More effective transfection than EP alone in one study</td>
<td>Combination equipment is not currently in clinical use</td>
<td>[35, 36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Long-term gene expression remains a challenge</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Limited species and genes tested</td>
<td></td>
</tr>
</tbody>
</table>
DNA formulation in phosphate-buffered saline (10 mg plasmid DNA per mL) yielded high levels of expression in skeletal muscle. Salt concentrations influence transfection efficiency by altering the ionic atmosphere, ionic strength, and conductivity of the DNA formulation. Lee, et al., demonstrated a general trend toward increasing efficiency of luciferase reporter gene expression with decreasing vehicle cationic strength. However, overall transfection efficiency was diminished by tissue damage because of hypo-osmotic stress and electrical injury induced by low conductivity. Ultimately these authors found that the optimal saline concentration was 71 mM for EP in skeletal muscle.

Ionic strength is not the only consideration when formulating a medium for EP of nucleic acids; a variety of polymers and adjuvants (in the case of vaccines) have also been added to EP formulations. Addition of a variety of polymers to DNA formulations have been shown to increase efficacy and decrease toxicity of EP. Poly-L-glutamate, polyacrylic acid, poly-L-aspartate, dextran sulfate, pectin, poloxamer 188, polyvinylpyrrolidone, and cationic liposomal formulations have been examined and found effective. Addition of 15-50 kDa poly-L-
glutamate is one example of the increased efficacy, without toxic effect, that may be provided by adding polymers to the DNA formulation; 6 mg/ml of ploy-L-glutamate has consistently improved EP efficiency by 4-12 fold. In the case of poly-L-glutamate, these effects are thought to be a result of its ability to decrease DNA clearance and increase DNA stability in muscle. Poloxamer 188 provides an example of a polymer which has been added to decrease EP damage. Perhaps most promising, are the cationic liposomal formulations that have been shown to increase transfection in a variety of mouse tumor systems. Addition of adjuvants to the formulation can increase vaccine efficacy and will be discussed in the vaccine application section of this review.

The actual EP parameters and conditions are as important as or more important than the nucleic acid formulation for effective electrotransfection. Aside from the desirability of having adaptive constant-current EP discussed previously, longer duration electric pulses with lower voltage have been shown to give the same EP effect as high voltage shorter duration pulses. Specifically, a pulse of 100 V/cm lasting 100 ms yields expression equivalent to 25 V/cm for 160 ms. Thus, to minimize tissue injury, lower voltages can be used to decrease heat build-up and resultant necrosis. In other studies, Satukauskas, et al., have shown that a train of long identical pulses, or combinations of pore-creating high-voltage, short-duration electric pulses and electrophoretic low-voltage, long-duration electric pulses are necessary for efficient gene transfection. More recently, André, et al., demonstrated high level gene expression in muscle by delivering a single 800 V/cm, 100 microsecond pulse, followed by four 80 V/cm, 100 millisecond pulses. Furthermore, when these investigators examined the effect of fast versus slow injection of transfection medium, they demonstrated that very fast injection of transfection medium into tissue (20 ul/2 sec) increases gene expression by 500-fold compared to the classic slow injection (20 ul/25 sec) technique.
APPLICATION OF EP IN LARGE ANIMALS

Until now, in vivo EP has been primarily conducted in murine models, but attempts and applications to large animals have gained momentum in recent years. Most applications in large animals use muscle as the target tissue. As alluded to previously, skeletal muscle is an ideal tissue for EP-mediated gene transfer. Muscle fibers are long-lived post-mitotic cells, and muscle is well vascularized, allowing efficient transport of gene products into the systemic circulation. Access to numerous muscle groups is also relatively easy in most species. Furthermore, gene expression in muscle after EP-mediated gene transfer has been reported to be as long as 9-19 months. Thus, skeletal muscle-targeted EP has been used for introduction of numerous genes to supplement production of critical secretory molecules in deficient hosts or augment levels of gene product already present.

Perhaps the largest amount of work using EP-mediated gene therapy in large animals has been conducted optimizing EP parameters in pigs. Bureau, et al. demonstrated efficient EP-mediated transduction of growth hormone releasing hormone (GHRH) gene using electric pulses of low field intensity. They also found that internal needle electrodes give a 25-fold increase in expression levels compared with caliper electrodes in skeletal muscle in swine, and demonstrated that by optimizing the EP method, favorable physiological changes, such as enhanced weight gain and improved body composition, could be obtained at extremely low plasmid doses in a large mammal. Furthermore, they found that the degree of permeabilization of the muscle cells is dependent on the electric field intensity, length of pulses, shape and type of electrodes. Somiari, et al. found that cell size was also an important parameter in determining degree of permeabilization. Use of needle electrodes in large mammals, such as pigs or humans, is necessary because of the increased resistance of the skin, the thickness of the subcutaneous fat
tissue, and the concern for tissue damage if the intensity of the electric field were to be proportionally increased using caliper or plate-type external electrodes. Brown, et al. further optimized muscle EP-mediated gene transfer by determining that using constant current pulses, between 0.4 and 0.6 A applied 80 seconds after injection of 0.5 mg plasmid DNA expressing secreted embryonic alkaline phosphatase reporter gene in a total volume of 2 mL produced the highest level of expression in semimembranosus muscle in pigs. Increased injection volumes and increasing lag time between injection and EP did not improve transfection efficiency.

Numerous other studies have applied EP-mediated gene transfer in pigs with excellent results. The bulk of applications thus far have been directed at regulating fat and muscle mass. Draghia-Akli, et al. note that EP-mediated gene transfer is particularly appropriate for modulating the intrinsic properties and mass of muscle and fat. Treatment conditions such as cachexia associated with chronic diseases, autoimmune diseases (e.g., myasthenia gravis), stimulation or suppression of appetite, and in vivo manipulation of glucose metabolism and fat deposition in patients with diabetes are some of the applications of EP-mediated gene therapy in muscle. Basic studies of muscle-specific transcription factors and their impact on development, also benefit from use of EP-mediated gene therapy. Additionally, it has recently been suggested that administration of the gene for leptin, a hormone predominantly produced by adipocytes and, functionally, a key regulator of body weight, may ameliorate obesity from a variety of causes.

Young pigs that underwent muscle EP with GHRH plasmid had significantly greater weight gain, significantly increased lean body mass, and decreased fat mass when compared with controls. Additionally, pigs undergoing EP with GHRH plasmid were leaner at end of study than controls, and had a proportional increase in all internal organs and higher bone density. Similarly, pregnant sows treated with GHRH gene IM EP had offspring with optimal health and growth characteristics and significantly reduced morbidity and mortality. Treated pigs also
expressed GHRH for at least one year, and beneficial effects on offspring occurred for three consecutive pregnancies.66

Similarly, a study of GHRH gene IM EP in thirty-two Holstein heifers yielded cows with improved immune function, health status, significantly increased body weights at 100 days of milk production, and improved body condition scores.67

Myogenic plasmid containing GHRH has also been delivered by muscle EP in severely debilitated dogs with naturally occurring tumors, and yielded significantly increased concentrations of IGF-1 and increased muscle mass.68 Similar to the previously mentioned work by Andre’, et al., work in dogs demonstrated that a combination of 1 high voltage pulse (600 V/cm, 100 μs), followed by 4 low voltage pulses (80 V/cm, 100 ms, 1 Hz) yielded the same transfection efficiency as the standard trains of low voltage pulses, and was able to yield detectable systemic expression of human interleukin-12. Only mild and transitory local side effects, without clinically detectable systemic side effects, were seen, indicating that electrotransfection is a feasible, effective, and safe method for muscle targeted gene therapy in dogs, which could have potential for clinical applications in small animal veterinary practice.69

In other applications of muscle EP in dogs, Fewell et al. were able to produce measurable levels of factor IX in treatment of hemophilia B and described a method for producing high transfection efficiency with high levels of systemic factor IX following a single administration;16,17 Draghia-Akli, et al. were able to demonstrate effects of GHRH in young, healthy Beagles;70 and Tone, et al. were able to demonstrate long-term gene expression in muscle EP in dogs.61

Electrotransfer of plasmid DNA into skeletal muscle has been successfully achieved in many different experimental animals including mice, rats and rabbits,41,71 cattle,67,72 goats,72 sheep,73 pigs,74,75 dogs,17,68 and monkeys.76 As in the dog studies previously mentioned, it has been shown that a better transfection efficiency can be achieved using combination of one high
voltage electric pulse followed by different numbers of low voltage electric pulses.\textsuperscript{56} It has been hypothesized that the high voltage pulse first causes permeabilization of cell membrane, followed by electrophoresis of DNA across destabilized cell membrane during the low voltage pulses.\textsuperscript{56, 62, 77} Large animal models and production use of EP-mediated gene therapy is growing significantly; applications of EP in general seem almost limitless, including the use of EP-mediated vaccine applications.

**APPLICATION OF EP FOR DNA VACCINES – OPTIMIZATION**

Vaccines (biological agents capable of triggering specific immunity against infectious diseases or cancer) can be delivered in a variety of ways, including EP. They can be categorized as inactivated/killed, attenuated/live, toxoid, component, and gene-based (DNA, RNA, oligonucleotides) vaccines that can be administered for a variety of purposes. These purposes were historically limited to infectious disease prevention, but now include tumor vaccine development and use in a variety of immune-mediated degenerative diseases. Of the different types of vaccine and their different targets, perhaps use of genetic vaccines as applied to both tumor vaccines and vaccination for infectious diseases shows the most promise when combined with EP.

Gene vaccines evolved from revolutions in molecular engineering and gene delivery, and their usage has become commonplace over the last few years. Gene vaccines use DNA to express immunogen and induce an immune response. Various gene delivery approaches are available to administer gene vaccines which, similar to other gene therapies, can be categorized as viral and non-viral. As stated previously, the use of viral vectors can be very effective in transfecting cells and inducing an immune response, but is limited by safety issues. Injection of naked DNA vaccine is safe, and in muscle yields long-term gene expression, but very little antigen response is produced.\textsuperscript{78} DNA injection followed by EP is much more effective, and
induces a similar level of immune response as protein immunization. Despite some early experimental successes, developing safe and effective DNA vaccines requires optimization of several variables before widespread EP-mediated gene vaccine administration becomes commonplace. Ultimately, the simplicity and effectiveness of genetic vaccination using EP may allow widespread use of gene vaccination in large animals and humans in the near future.

Optimization of gene construction can markedly enhance transfection efficiency and resulting immune responses when applied to DNA vaccines. To be an effective vector, plasmid DNA should contain a strong viral promoter and a strong polyadenylation transcription termination signal. Additionally, most vaccination vectors also contain an intron to increase expression. When the whole antigen is toxic or immunosuppressive, epitopes from the antigen may be utilized and can be expressed as mini-genes, which are inserted into unrelated but highly immunogenic sequences that successfully induce both cellular and humoral responses.

APPLICATION OF EP FOR DNA VACCINES – MUSCLE VERSUS SKIN

Optimization of EP parameters can also improve the outcome of genetic vaccine. As noted previously, to select optimal parameters for EP-mediated DNA delivery in vivo, specific needs for different tissues, vaccine formulations, and DNA dosages must be considered simultaneously. Also noted previously, muscle is the most commonly targeted tissue for EP-mediated gene delivery because of, among other reasons, the large quantity of tissue and its rich blood supply which allows systemic circulation of secreted proteins. For vaccination, EP-induced muscle cell damage may be beneficial because of the release of a variety of cytokines which may help initiate immune response by attracting antigen presenting cells (APCs) to the injection site. Muscle selection is also important; aside from accessibility, muscle should be chosen based on EP efficiency difference in different muscles. For example, in mice the anterior
tibialis muscle has been demonstrated to have the highest expression of muscles tested for secreted alkaline phosphatase.\textsuperscript{53} Unfortunately, even when all the aforementioned factors are optimized, \textit{IM} administration of gene vaccine may produce a less than optimal immune response in some circumstances.\textsuperscript{53}

Skin is a more traditional target tissue for vaccination because it is readily accessible and has a large population of unique antigen presenting cells. Keratinocytes are primarily responsible for transgene expression after intradermal (ID) administration.\textsuperscript{80} Expression of immunogen by keratinocytes can induce an immune response through interaction with bone marrow-derived dermal Langerhan’s cells and dermal dendritic cells.\textsuperscript{81} Similar to the findings of enhanced \textit{IM} expression of genes when delivered by EP in muscle, 100 to 1000-fold higher gene expression was induced after ID delivery of plasmid DNA when introduced by EP. Specifically, higher levels of prostate-specific antigen (PSA)-stimulated CD8\textsuperscript{+} T cells were induced after intradermal EP delivery of low-dose PSA DNA vaccine in a mouse model.\textsuperscript{82} Thus, skin continues to be a common target of EP-mediated vaccine use now and in the future.

Conversely, there is some evidence that in certain settings, IM EP does produce better immunization than ID EP of gene vaccines.\textsuperscript{71} The low levels of EP-mediated, gene vaccine-induced immunity in muscle alluded to before, may be a result of the lack of cytokines released by professional APCs. When expressed immunogen is secreted and taken up by large numbers of professional APCs, the APCs present antigen and cross-prime large numbers of cells.\textsuperscript{83} In contrast, ID administration exposes a much smaller number of APCs to transfected cells and development of immunity.
APPLICATION OF EP FOR DNA VACCINES – ROLE OF ADJUVANTS

As is the case for traditional vaccines, addition of adjuvant can significantly increase the magnitude and duration of vaccine-induced immune response in gene vaccines. Lipopolysaccharide (LPS), a component of gram negative bacterial cell walls and potent endotoxin, has been used to augment immune responses through toll-like receptor 4 (TLR4). Because granulocyte macrophage-colony stimulating factor (GM-CSF) has a potent effect on DC differentiation and maturation, and also on expression of MHC and co-stimulatory molecules, it has been utilized as immune adjuvant for vaccine against numerous infectious diseases and cancer. Oligonucleotides are also being investigated as adjuvant with promising initial results. Incorporating adjuvant into the gene construct has been demonstrated in an elegant example of enhanced anti-tumor vaccine efficacy using dendritic cells electrotransfected with mRNA containing the gene for tumor associated antigens (TAA) linked to mRNA encoding ubiquitin. The resulting ubiquitinated TAA product was effectively targeted to the proteasome, enhancing degradation of TAA which resulted in more efficient priming of TAA-specific CD8+ T-cells.

APPLICATION OF EP FOR “CELL VACCINES”

EP has also been used in the development of “cell vaccines”. For example, dendritic cells have been EP transfected with the gene for tumor-associated antigens ex vivo and reintroduced to patients to enhance their anti-tumor immune response. EP transfection of DCs with mRNA results in higher protein expression in DCs than DNA, and carries no risk of integration into host genome. RNA instability can be minimized by modifying the mRNA with a 3’-poly(A) tail and a 5’ 7-methylguanosine cap. A number of studies have been reported using this approach to various antigens such as melanoma, carcinoembryonic antigen, human
telomerase reverse transcriptase, and HER-2/neu antigen.\textsuperscript{96, 97} Using this approach for infectious disease vaccines has been much less common; nevertheless one recent publication reported improvement in hepatitis C prevention using mRNA-transfected DC-mediated vaccine.\textsuperscript{98}

**APPLICATION OF EP FOR DNA VACCINES – LARGE ANIMAL AND PRIMATE STUDIES**

Although active research using EP-mediated gene vaccines has grown exponentially over the past few years, most of the research to date has been in small animals. In general, studies in large animals have demonstrated less efficacy than in small animals, but the number of studies using large animals pales in comparison to those in mice. Effective EP-mediated gene vaccination in primates has been demonstrated however; Zhao and Xu looked at numerous combinations of EP parameters for vaccination against hepatitis B virus (HBV) and found a great variation in efficiency depending on the EP parameters selected.\textsuperscript{99} These authors also provided another example of using immune-modulating fusion genes (interleukin-2 and gamma interferon) as an adjuvant enhancing immune responses in EP-mediated gene vaccination.\textsuperscript{99} Thus EP-mediated gene vaccination shows great promise for achieving high gene expression, efficient humoral and cellular responses, and specific protection against antigens, including in more clinically relevant species such as the Rhesus macaques used in this study. Furthermore, EP-mediated gene vaccination has proven safe, stable, easy to manipulate, and relatively inexpensive.

**INNOVATIVE APPLICATIONS OF EP GENE THERAPY**

Investigations into treatment of type I diabetes mellitus (T1D) have also used muscle-targeted gene via EP delivery. T1D is due to a loss of immune tolerance to islet antigen and thus, there is intense interest in developing therapies that can re-establish tolerance. Tolerance is maintained by complex mechanisms that include inhibitory molecules and several types of
regulatory T cells (T\textsubscript{reg}). A major historical question is whether gene therapy can be employed to generate T\textsubscript{reg} cells. Recent studies indicate that gene transfer of immunoregulatory molecules can prevent T1D and other autoimmune diseases. In studies by Prud’homme, et al., \textit{in vivo} EP-mediated gene transfer was thought to have the potential to be used to perform DNA vaccination against islet cell antigens. When combined with appropriate immune ligands, this would result in the generation of T\textsubscript{reg} cells and protection against T1D. \textit{In vivo}, EP can also be applied for non-immune therapy of diabetes. It can be used to deliver protein drugs such as glucagon-like peptide 1 (GLP-1), leptin, or transforming growth factor beta (TGF-beta). These act in T1D or type II diabetes (T2D) by restoring glucose homeostasis, promoting islet cell survival and growth or improving wound healing and other complications of T1D.\textsuperscript{100}

Bone marrow cells, splenocyte and T cells generally are difficult to achieve a high level of gene delivery, regardless of gene delivery methods employed. Studies by Tervo, et al. found that both EP and nucleofection resulted in high-level transgene expression (up to 60% transgene-positive T cells) from both small and large green fluorescent protein reporter constructs in activated rabbit T cells with moderate cytotoxicity. Both non-viral gene delivery methods were vastly superior to retroviral, lentiviral, or adenoviral transduction approaches. These studies also established conventional EP as an efficient and inexpensive procedure to render primary rabbit T cells accessible to rapid functional \textit{ex vivo} analyses. Furthermore, the viability of electroporated rabbit T cells was remarkably high (47±7%); compared to analogous studies conducted in primary T cells from rats and mice.\textsuperscript{101}

In an interesting variant of \textit{in vivo} EP using transplantation of autologous hepatocytes that underwent EP \textit{in vitro}, hepatocytes were isolated from a surgically resected liver wedge, electroporated with an insulin expression plasmid \textit{ex vivo} and reimplanted intraparenchymally
under ultrasonic guidance into the liver in each of 10 streptozotocin-induced diabetic Yorkshire pigs. Based on positive results, authors concluded that autologous hepatocytes could be efficiently, simply and safely modified by EP of a plasmid DNA to express, process and secrete insulin. This strategy achieved significant and sustained therapeutic efficacy, and may have broader future applications for the treatment of other acquired and inherited diseases for which systemic reconstitution of a specific protein deficiency is desirable. Combining autologous hepatocytes with \textit{ex vivo} gene transfer has several advantages. Using this technique, hepatocytes are likely to be of higher quality and can be used fresh (instead of preserved). This also allows use of high voltage EP for transfecting primary somatic cells which otherwise might cause tissue necrosis \textit{in vivo}. Using autologous cells also overcomes the problem of donor scarcity and avoids the need for chronic immunosuppressive therapy.\textsuperscript{102}

Small double strand RNAs, involved in gene silencing or RNA interference, or closely related micro RNAs derived from endogenous hairpin precursors can bind to RNA-induced silencing complexes and either degrade messenger RNA, block translation, or otherwise suppress gene expression. EP may help overcome the fact that routine therapy or studies with siRNAs is complicated by the fact that these highly charged molecules do not easily enter cells.\textsuperscript{103}

In nonhuman primates, gene targeting can produce animal models for translational studies of human diseases. Gene targeting in fibroblasts followed by somatic cell nuclear transfer (SCNT) has been successful in numerous large animal species, including primates. In rhesus macaques gene targeting in a primary culture of adult rhesus macaque fibroblasts was accomplished by culture of adult male fibroblasts transfected by EP of S-phase synchronized cells with a construct containing a SV40 enhancer with human telomerase reverse transcriptase to overcome senescence and allow long term \textit{in vitro} manipulations.\textsuperscript{104} It is thought that these cell lines can be used for the production of null mutant rhesus macaque models of human genetic
disease using SCNT technology. Null mutant sheep, goats, pigs and cattle have been produced using an alternative approach: gene targeting in somatic cells followed by nuclear transfer to enucleated oocytes (SCNT; reproductive cloning) whose gene targeting efficiency could also potentially be improved using EP.

UN SOLVED CONCERNS PERTAINING TO EP

One concern in EP-mediated gene transfer in vivo is the amount of tissue damage produced secondary to heat generated. Draghia-Akli, et al. and others have suggested that constant current EP (instead of constant voltage) may reduce tissue damage and contribute to overall success. Unfortunately, exclusively focusing on using lowered voltage pulses in order to decrease cell death through necrosis/oncosis, may not prevent death through cell apoptosis which has been shown to take place even with low voltage EP. Although most of the adverse effects of EP have been characterized in muscle, mild damage has also been reported with ID EP, but this damage was resolved within one week of EP. Additional means of decreasing tissue damage include addition of polymers to the injected DNA formulation, alterations in ionic strength and composition, and augmentation of EP with other transfection techniques such as sonoporation.

Pain is another concern in cases of in vivo EP – especially if the technique is to be applied clinically to non-anesthetized patients. In humans, patients describe muscle contractions as being surprising, sometimes unpleasant, but not painful. Pain from EP is proportional to the absolute applied voltage, and one way of lowering the total voltage is by decreasing the gap between electrodes to 0.4 cm. During EP of cutaneous masses, muscle contractions can also be palliated by elevating or tenting the skin to be electroporated well above the underlying musculature. In certain settings, ex vivo EP may be practical, which would allow for the EP
procedure to be conducted on cells harvested from the patient for EP, and subsequently reintroduced in situ, completely eliminating the chance of EP-induced pain.\textsuperscript{102}

Vascular effects of EP have also been a concern for many investigators, but recent studies suggest that changes in afferent and efferent vessels during EP may be beneficial, particularly when applied to tumor gene therapy. High voltage pulses cause a brief reflex constriction of afferent arterioles in normal tissue, and in tumor tissue (which have more fragile and tortuous blood vessels) long-term hypoperfusion can occur after EP.\textsuperscript{120} These vascular effects may be beneficial in electrochemotherapy because higher concentrations of drug may remain trapped in the tumor due to lack of “wash out” at the time of EP. Similarly, in gene therapy, transient hypoperfusion has been shown to enhance gene expression.\textsuperscript{121-123}

One of the advantages of using EP and other non-viral vectors is that they are not hampered by vector immunogenicity if properly designed (by removal of CpG islands and residual bacterial sequences). If not properly designed, CpG-mediated nonspecific inflammatory effects (e.g. mediated through binding to TLR-9) can injure tissues and/or confuse the interpretation of immunological studies. Additionally, many viral promoters are turned off by inflammatory cytokines.\textsuperscript{124-126} Another approach to minimize immunogenicity is to delete most vector elements producing “minicircles” containing the expression cassette.\textsuperscript{127} Furthermore, if the gene being transfected is to be secreted, signal peptide sequences may also play an important role in functional expression. For example, studies involving nonhuman primates that received an erythropoietin encoding plasmid showed that changing the transgene leader sequence and optimizing the gene codon usage yielded higher levels of circulating transgene product and a more significant biological effect than the wild-type gene.\textsuperscript{76} Thus, by altering the plasmid or transfected DNA design and sequence, one can minimize the dose
necessary to attain physiological levels of the target hormone, enzyme, or peptide, and manipulate the expression of the newly produced transgene product.

CONCLUSION

In summary, in vivo EP-mediated gene therapy is gaining ground as one of the most important means for non-viral gene therapy. Further understanding of the mechanisms of target cell DNA entry, intracellular DNA transport, and nuclear processing will allow further optimization of the technique through optimization of gene formulations and electrical pulse parameters. Furthermore, the ability to augment EP with tension forces (in EAT and ES), use it in an ex vivo setting, and incorporate integrase enzyme to allow genomic integration of transfected genes may broaden the appeal of this technique. At this point, there appear to be few limitations, and potential uses continue to grow as these limitations are overcome.

Because of the multitude of advantages in using EGT to attain gene expression, we have chosen this as our gene transfection method of choice for cancer immunogene therapy. In the next chapter, we discuss the effectiveness of using EP-mediated gene transfection of interleukin-12 when combined with EP-mediated chemotherapy for treatment of a variety of neoplasms in dogs.

REFERENCES


CHAPTER 3
BLEOMYCIN/INTERLEUKIN-12 ELECTROCHEMOGENE THERAPY FOR TREATING NATURALLY OCCURRING SPONTANEOUS NEOPLASMS IN DOGS*

*Reprinted by permission from Macmillan Publishers Ltd, 2010©
INTRODUCTION

As noted in chapter 2, electroporation (EP) is the application of a series of square-wave electrical pulses to increase cell permeability and allow movement of molecules into cells. Several recent reviews describe application of this technique to gene\textsuperscript{2,12-15} and drug therapy\textsuperscript{15,128-131}. Over the past fifteen years, EP has become a common technique for introducing large molecules into cells, both in vitro and in vivo.

IL-12 treatment can have a dramatic anti-tumor effect on a variety of neoplasms\textsuperscript{132-137}. Both innate resistance and adaptive immunity are affected by IL-12 mediated anti-tumor resistance. IFN-gamma and other cytokines induced by IL-12 either directly, or indirectly through other cytokine signaling, have both direct toxic effects on the tumor cells, and potent anti-angiogenic activity on tumor neovasculature. IL-12’s action in adaptive immunity relies on its ability to trigger or augment Th1 and CTL responses. Thus IL-12 has a potent adjuvant activity in cancer therapy and \textit{in vivo} electroporation delivery of genes or electrogenetherapy (EGT) has been described in numerous tumor-bearing murine models and in early human trials\textsuperscript{18,138}. In these studies, IL-12 EGT eradicates tumors and induces long-term anti-tumor memory, but is unable to eradicate large tumors\textsuperscript{138}.

BLM, an antitumor antibiotic, causes breaks in double-stranded DNA and releases free radicals which cause cellular oxidant damage\textsuperscript{139}. Delivery of BLM by local electrochemotherapy (ECT) is an ideal way to increase efficacy while reducing potential toxic side effects. Once BLM enters tumor cells during ECT, it becomes sequestered because of its large size and lack of transmembrane transporters. This sequestered site prevents non-target organ toxicity and allows specific targeting to the local EP site. In experimental animal models, delivery of BLM via intratumoral ECT consistently eradicates large tumors but often fails to extend survival time over standard therapy. EP delivery of chemotherapeutics has been described in animals for the
treatment of SCC in cats and a variety of cutaneous and subcutaneous tumors in cats, dogs, and horses. Bleomycin ECT has also been well tolerated in humans with between 9 and 100% complete response rates depending on the technique used. In fact, the European medical community has embraced ECT for many years, as evidenced by the results of the European Standard Operating Procedures of Electrochemotherapy (ESOPE) trials completed in 2003. These trials demonstrated that ECT was not only safe and effective, but also efficacious for local control of a variety of tumors. Two of the limitations of ECT are that it is currently only suitable for local treatment of superficial tumors, and it only targets the primary tumor.

Thus the combination of BLM, which is able to eradicate large primary tumors, and the gene for IL-12, which can induce anti-tumor immunity, seems ideally suited for treatment of most neoplasia which is accessible to local treatment. In fact, we and others have previously demonstrated that this combination can successfully eradicate and prevent recurrence of mammary cancer and melanoma in mouse tumor models. In addition to being efficacious, this treatment appears to be well-tolerated, has minimal side effects, preserves tissue architecture, and could be easily adapted to clinical use. However, this combination has not been tested in large animals bearing naturally occurring tumors, nor has it been used in humans at this point. In order to determine whether experimental results translate into positive clinical results, we used ECGT to treat six client-owned dogs with a variety of tumors.

MATERIALS AND METHODS

Selection of Naturally Occurring Tumor-Bearing Dogs. Privately owned dogs that were referred to the Cancer Treatment Unit of the Louisiana State University’s (LSU) Veterinary Teaching Hospital and Clinics were selected for the study from 2004 to 2008. Aside from having histologically confirmed neoplastic disease, inclusion criteria included the absence of overt heart, renal, or other life-threatening illness. Dogs were staged by obtaining a thorough
anamnesis, physical examination, complete blood cell count, serum biochemistry profile, urinalysis, electrocardiogram, thoracic radiographs (three view metastasis check), and abdominal ultrasound if indicated. Additionally, two of the dogs had computerized tomography (CT) scanning of the skull to gauge the degree of local tumor invasion and bone lysis.

**In Vitro IL-12-Augmented Cytotoxicity Determination.** A SCC cell line (SCCVII) was transfected with either two micrograms fIL-12 DNA or control DNA by in vitro EP, grown for 24 hours, and conditioned medium was collected. DNA was prepared using endotoxin-free preparation kits (Qiagen, Germantown, Maryland) using the standard protocol provided by the manufacture. Leukocytes were harvested from pre-treatment blood samples obtained from the second SCC-bearing dog. Leukocytes (5 x 10⁵) were then added to 1.5 mL of the conditioned medium and incubated for 18 hours at 37°C. Conditioned leukocytes were then mixed in a 50:1 ratio with fluorescent-labeled canine osteosarcoma target cells (D17) and incubated for 24 hours at 37°C. The number of remaining fluorescent cells (live cells) per microliter were then counted and compared to numbers remaining with control DNA.

**Imaging.** Except for abdominal ultrasound on the dog with histiocytic sarcoma, dogs were anesthetized for CT scans. An intravenous catheter was placed in the cephalic vein and maintenance fluids were delivered during anesthesia. Dogs were premedicated with various combinations of anxiolytic, analgesic, and parasympatholytic drugs, induced with propofol, intubated, and maintained on isoflurane in oxygen. Hemoglobin oxygen saturation, blood pressure, and electrocardiography were monitored during anesthesia.

For staging via thoracic radiographs, ventrodorsal, left lateral, and right lateral radiographs were obtained using digital radiography equipment (Siemens Medical Solutions USA, Incorporated, Malvern, Pennsylvania) and were evaluated by boarded veterinary
radiologists. For CT scans of the first SCC dog, a single slice spiral CT scanner (Picker PQ-5000, Picker Corporation – now GE Healthcare, Waukesha, Wisconsin) was used. For the second SCC dog, a 16-slice volume CT scanner (Lightspeed VCT, GE Healthcare, Waukesha, Wisconsin) was used. Abdominal ultrasound was also performed by a boarded veterinary radiologist and utilized a Logiq 9 ultrasound machine (GE Healthcare, Waukesha, Wisconsin).

**Other Diagnostics.** For clinicopathologic data, routine jugular phlebotomy and collection into EDTA blood and serum separator tubes were used to determine complete blood counts (CBCs) and serum chemistry analyses. Blood was run on an automated ADVIA 120 hematology analyzer with multispecies software (Bayer, Fernwald, Germany) and blood smear differential were confirmed manually. Serum was run on an Olympus AU640e automated chemistry analyzer (Olympus Incorporated, Center Valley, Pennsylvania). Urine was obtained by cystocentesis and routine urinalyses consisted of determining urine specific gravity, urine dipstick analytes, and sediment examination. In the case of the histiocytic sarcoma dog this was followed by routine urine culture and sensitivity.

For confirmation of tumor type, excision biopsies from the primary tumor were obtained surgically under anesthesia and were placed in 10% neutral buffered formalin. After being fixed, tissue was cut-in, paraffin embedded, sectioned at 3 µm, adhered to slides, stained with hematoxylin and eosin, cover-slipped, and evaluated by boarded veterinary pathologists.

**Treatment.** An intravenous catheter was placed in the cephalic vein and maintenance fluids were delivered during anesthesia. Dogs were premedicated with various combinations of anxiolytic, analgesic, and/or parasympatholytic drugs, induced with propofol, intubated, and maintained on isoflurane in oxygen. Hemoglobin oxygen saturation, blood pressure, and electrocardiography were monitored during anesthesia. Between 0.5 and 2.0 IU BLM (Bleo, Teva Parenteral Medicines, Incorporated, Irvine, California) and 150-400 µg fIL-12 DNA were
administered in 1-2 mL of 0.45% NaCl injected parenterally into the center of the mass, immediately followed by insertion of a hexagonal array of six needle electrodes (Inovio Biomedical Corporation, Blue Bell, Pennsylvania) surrounding the injection point (trans-lesionally) and electroporation. The only exception was the first case of SCC where simple caliper electrodes were used to deliver the pulses. Two 20 msec 400 V/cm electrical pulses were administered 100 msec apart using an ECM 830 electroporator (BTX, Genetronics – now Inovio Biomedical Corporation, Blue Bell, Pennsylvania). Additional treatments occurred ten or more days after the first. The number of treatments varied from one to three, and the study was approved by the Institutional Review Board of the LSU School of Veterinary Medicine.

Response to treatment was evaluated through recheck examinations, tumor measurement and gross characterization, communication with the pet’s owner, follow-up CBCs and serum biochemistry profiles, and in two cases, with follow-up CT scans. Complete response is defined as disappearance of all measurable tumors for 21 days and partial response was defined as more than 50% reduction in measurable tumor for 21 days. Lack of measurable tumor for greater than eight months was characterized as a cure for this study.

RESULTS

fIL-12 depicted in figure 2a provided by Dr Stephen Dunham (University of Nottingham, United Kingdom) was chosen due to the lack of canine IL-12 availability. There is 91% homology between the canine and feline IL-12 \(^{144}\) suggesting that the fIL12 should be functional on canine cells. To confirm this assumption, the biologic ability of fIL-12 to activate canine mononuclear cells was determined. Canine leukocytes activated by fIL-12 induced significantly more (p<0.05) canine target tumor cell death when compared to control DNA (figure 2b).
Figure 2: Analysis of fIL12 gene function against dog target tumor cells.  

**a.** The fIL-12 encoding gene in the pCI-neo vector (Promega Corporation, Madison, Wisconsin).  

**b.** Analysis of cytolytic activity of canine leukocytes activated by fIL12 conditioned medium. Significant (p<0.05) target cell death occurred when canine leukocytes were treated with fIL12. pCtrl and pFlIL12 represent conditioned medium obtained by transfecting cells with control DNA and fIL12 encoding plasmid DNA, respectively.

All dogs were treated with combinations of BLM and fIL-12 with a dosages extrapolated from those used in mice. The dose of BLM and Il-12 DNA was often divided between areas of the tumor receiving EP. For each cm² of tumor at its maximum cross sectional area (based on CT or caliper measurements), 0.5 IU of BLM and 150 μg of fIL-12 DNA were used.

All tumors were examined histologically to confirm histopathology diagnoses and extent of lesions and/or metastatic potential. Representative photomicrographs of available biopsy samples are depicted in figure 3. As noted previously, tumors treated represented a range of carcinomas and sarcomas, and all were diagnosed by boarded pathologists. Noteworthy is the fact that tumor emboli were noted within lymphatics in the biopsy from the first dog with SCC. Both the histiocytic sarcoma and the fibrosarcoma were considered grade 3 or high-grade soft-tissue sarcomas and carried a poor prognosis. The melanoma was also a high-grade given its high mitotic rate, poor differentiation, and diagnosis of lymph node and pulmonary metastases.
Figure 3: **Histologic analysis of dog tumor biopsies.** a. Section from the first dog treated with papillary squamous cell carcinoma. Bar = 500 µm b. Section from the dog with malignant melanoma (amelanotic). Bar = 100 µm c. Section from the cubital joint of dog with histiocytic sarcoma. Bar = 100 µm d. Section from dog with acanthomatos ameloblastoma. Bar = 200 µm. All sections are of hematoxylin and eosin stained formalin-fixed, paraffin-embedded biopsies.

Regardless of tumor type and anatomic location, all tumors had clinical response to ECGT. Furthermore, treatment in all cases was associated with minimal side effects. The most significant side effect was 48 hours of diarrhea for the dog with metastatic melanoma, but this dog had numerous other medical problems and demonstrated marked stress (aggression), making it difficult to directly link the diarrhea to the ECGT. Clinicopathologic data remained essentially unchanged after treatment for most dogs (Table 2). Only the dogs with histiocytic sarcoma and metastatic melanoma had clinicopathologically significant abnormalities. In the dog with histiocytic sarcoma, its bacterial cystitis resolved during treatment and there was no significant
progression of its cholestasis or hypercholesterolemia based on serum chemistries. In the dog with melanoma, alkaline phosphatase increased and was thought to represent increasing primary tumor bone involvement or cholestasis; this dog’s neutrophilic leukocytosis also progresses slightly and was thought to be related to increased necrosis and ulceration of the primary tumor. It is worthwhile noting that both these dogs had significant changes prior to treatment; therefore it is not thought that ECGT played any role in progression of pre-existing disease.

Table 2: Summary of clinicopathologic abnormalities and side effects of treatment:

<table>
<thead>
<tr>
<th>Signalment</th>
<th>Pre-treatment clinicopathologic findings (followed by reference interval)</th>
<th>Post-treatment clinicopathologic abnormalities</th>
<th>Treatment Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 kg, 8-month-old, M Shih Tzu with maxillary SCC</td>
<td>NSF</td>
<td>NSF</td>
<td>Lethargy &amp; decreased appetite</td>
</tr>
<tr>
<td>4.5 kg, 6-month-old, F Jack Russell Terrier with mandibular SCC</td>
<td>ALP 488 (0-100) TB 1.1 (0-0.4) Chol 333 (150-240) UC: E.Coli</td>
<td>ALP 535 (0-100) TB 1.0 (0-0.4) Chol 335 (150-240)</td>
<td>None</td>
</tr>
<tr>
<td>40 kg, 9-year-old, FS Rottweiler with histiocytic sarcoma</td>
<td>NSF</td>
<td>ALP 269 (0-100) Alb 2.4 (2.6-4.2) TP 5.6 (5.8-7.5) Plat 1003 (220-600) WBC 16.2 (8-14.5) Neut 13.4 (3-11.5)</td>
<td>NSF</td>
</tr>
<tr>
<td>13 kg, 19-year-old, F Chow Chow cross w/ metastatic melanoma</td>
<td>NSF</td>
<td>ALP 399 (0-100) Plat 1008 (220-600) WBC 18.2 (8-14.5) Neut 15.6 (3-11.5)</td>
<td>Diarrhea 2 days</td>
</tr>
<tr>
<td>37 kg, 9.5-year-old, FS Golden Retriever w/ acanthomatous ameloblastoma</td>
<td>NSF</td>
<td>NSF</td>
<td>None</td>
</tr>
</tbody>
</table>

Alb = Albumin  ALP = Alkaline Phosphatase  Chol = Cholesterol  
F = Female  FS = Female Spayed  M = Male  
Neut = Neutrophils (x10^3/dl)  NSF = No significant findings  Plat = Platelets x10^3/dl  
TB = Total Bilirubin  TP = Total Protein  UC = Urine culture  
WBC = White blood count (x10^3/dl)

Of the six dogs treated, three had complete responses and three had partial responses. The first dog (with maxillary SCC) treated, had a complete response. This dog was the only dog to receive BLM/IL-12 ECGT using caliper electrodes as performed in tumor-bearing mice (17). Morphologically and on CT scan, this dog had clear bone lysis and a large tumor initially (figure 4a), whereas after the second treatment, visible tumor was eradicated. After six months, the
primary lesion had healed and much of the associated bony lysis had been repaired (figure 4b). Compared to surgery and radiation therapy, this dog’s treatment was without side effects and easily accomplished within a short period of time. This dog remains tumor free almost five years later (table 3).

The second dog treated also had a complete response. This dog was the first to receive ECGT via a six needle array electrode (as did all subsequent dogs). CT images taken initially for this dog with mandibular SCC demonstrate marked malocclusion and tumor invasion into adjacent bone (figure 5a). By the time the second CT images were taken 1.5 months after the last treatment, most of the bony lysis and much of the occlusal changes had resolved (figure 5b). Compared to surgery and radiation treatment, this dog experienced minimal side effects from ECGT. Aside for a single day of lethargy and decreased appetite, no side effects were noted. This dog remains tumor free almost three years later (table 3).

![Figure 4: Photographic and CT scan analysis of tumor volume change pre- and post-treatment.](image)

Figure 4: Photographic and CT scan analysis of tumor volume change pre- and post-treatment. **a.** Pre-treatment photograph in the dog with maxillary SCC. **b.** Photograph six months after treatment. **c.** Pre-treatment CT scan in the dog with maxillary SCC. **d.** CT scan six months after treatment. (Figure 3 is from figure 24.1 and 24.2, page 324 (Cutrera, Torrero et al. 2008) with kind permission of Springer Science and Business Media. ©2008 Humana Press. All rights reserved. No part of these figures may be reproduced, stored in a retrieval system, or transmitted in any form or by any means, electronic, photocopying, microfilming, recording or otherwise without written permission from the Publisher).
### Table 3: Clinical Summary (as of 7/2009):

<table>
<thead>
<tr>
<th>Signalment</th>
<th>Primary Tumor</th>
<th>Metastases</th>
<th>WHO Stage</th>
<th>Imaging</th>
<th>Treatments</th>
<th>Adjunct treatment</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 kg, 8-month-old, M Shih Tzu</td>
<td>2 cm diameter SCC of L rostral maxillary gingiva</td>
<td>Tumor noted in biopsy lymphatics</td>
<td>T2N0M0</td>
<td>CT consistent w/resolution</td>
<td>2 ECGT 10 day interval 2 ECGT 10 day interval</td>
<td>Previous surgery</td>
<td>Resolved, 56 months tumor-free</td>
</tr>
<tr>
<td>4.5 kg, 6-month-old, F Jack Russell Terrier</td>
<td>2 cm diameter SCC of the L rostral mandibular gingiva</td>
<td>None detected</td>
<td>T2N0M0</td>
<td>CT consistent w/resolution</td>
<td>2 ECGT 10 day interval 2 ECGT 10 day interval</td>
<td>Previous surgery</td>
<td>Resolved, 27 months tumor-free</td>
</tr>
<tr>
<td>40 kg, 9-year-old, FS Rottweiler</td>
<td>4-5 cm diameter histiocytic sarcoma of L cubital joint</td>
<td>Splenic masses</td>
<td>T3N0M1</td>
<td>U/S shows splenic metastasis</td>
<td>1 ECGT 10 day interval 2 ECGT 10 day interval</td>
<td>Tramadol</td>
<td>Primary tumor resolved, euthanized due to metastatic disease</td>
</tr>
<tr>
<td>13 kg, 19-year-old, F Chow Chow cross</td>
<td>6 cm x 4 cm diameter malignant melanoma of R mandibular gingiva</td>
<td>Submandibular lymph nodes &amp; pulmonary</td>
<td>T3N2bM1</td>
<td>Radiographs show pulmonary metastases</td>
<td>2 ECGT 10 day interval 2 ECGT 10 day interval</td>
<td>Piroxicam, tramadol</td>
<td>50% primary tumor reduction, euthanized due to poor quality of life</td>
</tr>
<tr>
<td>37 kg, 9.5-year-old, FS Golden Retriever</td>
<td>1.6 cm x 1 cm diameter mandibular acanthomatous ameloblastoma</td>
<td>None detected</td>
<td>T2N0M0</td>
<td>Radiographs within normal limits</td>
<td>3 ECGT 10 day intervals</td>
<td>Previous surgery</td>
<td>Resolved, 9 months tumor-free</td>
</tr>
</tbody>
</table>

**Figure 5.** Pre- and post-treatment CT scans delineating tumor and degree of bone lysis/repair for the dog with mandibular SCC. **a.** Pre-treatment CT demonstrating invasive mandibular SCC & displacement of incisor and canine teeth with bony lysis (circled). CT transverse sections are labelled sequentially from rostral to caudal (1-6) and refer to transverse sections through the tumor site (pre- and post-treatment images cover the same area). **b.** Post-treatment CT scan. The previous tumor site is circled.
The dog with acanthomatous ameloblastoma is our most recent success (figure 6a). After the initial diagnosis, this dog’s owners were offered either segmental mandibulectomy or radiation therapy. While both of these options offer a high probability of cure, they are associated with significant side effects. In the case of surgery, the dog would have had significant orthopedic pain, the chance of surgical complications, and would have had less desirable cosmetic and functional results. Radiation would have required frequent treatments, numerous periods of anesthesia, and the potential for radiation side effects. As can be seen from the photograph taken at the time of the last treatment (figure 6b), the tumor was completely eradicated with ECGT, avoiding many of the complications associated with surgery and radiation therapy. The area of gingival erosion depicted in figure 5b subsequently resolved completely leaving no residual tissue damage. Nine months later, this ten-year-old dog remains tumor free (table 3).

![Figure 6](image)

**Figure 6. Pre- and post-treatment photographs of completely eradicated acanthomatous ameloblastoma.** a. Photographs after the first ECGT treatment (arrows point to the tumor). b. After two more ECGT treatments (three total), tumor was completely eradicated (the gingival erosion subsequently resolved).

The remaining dogs, which were presented because they were thought to have no other treatment options (were not candidates for surgery, chemotherapy, or radiation therapy), had partial responses to ECGT. The dog with histiocytic sarcoma had marked enlargement of the left
cubital joint associated with the primary tumor and poorly-weight-bearing lameness (figure 7a). After treatment, the primary tumor was eradicated and lameness had improved markedly (figure 7b).

Figure 7. Pre- and post-treatment photographs of the histiocytic sarcoma. a. Photograph prior to treatment of the left cubitus (circled). b. Photograph sixty-nine days after a single ECGT treatment (previous tumor site circled).

Post-treatment nodules present were thought to represent scar tissue from the biopsy procedure, and the diameter of the joint was reduced by 1.5 cm. While not confirmed histologically, the size of the joint in comparison to the contralateral cubital joint was the same suggesting complete resolution of the primary tumor. Although the improvement in this dog’s ambulatory ability increased her quality of life, she ultimately was euthanized due to complications from splenic metastases.

Both the dog with metastatic melanoma and the dog with fibrosarcoma had an initial reduction in tumor size by greater than 50% after ECGT and therefore were also considered partial responders. In the case of the dog with melanoma, this sixteen-year-old dog had progressive neoplastic disease and numerous other medical problems which necessitated euthanasia. The dog with the fibrosarcoma also responded initially, but had rapid recurrence and owner’s declined further treatment.
The effect of treatment on metastatic disease was not determined. Both dogs with metastatic disease prior to treatment, ultimately were euthanized because of complications either associated with their metastases or with recurrence of the primary tumor. Generalizations regarding the type of tumors amenable to ECGT were also difficult given the small number of dogs treated. As is true with most neoplasia therapies, dogs with advanced metastatic neoplasia appear to be poor candidates for ECGT. At least two types of invasive oral neoplasms, SCC and acanthomatous ameloblastoma, responded well to treatment.

Table 3 summarizes the clinical findings and outcomes for five of the dogs treated. The sixth dog (not included in this table), a dog with fibrosarcoma of the soft palate, had initial tumor regression with rapid recrudescence after a single treatment and owners terminated their participation in this study.

**DISCUSSION**

As noted by several authors \(^{146}\), using client-owned dogs for clinical investigation of novel cancer therapies offers several advantages over mice and humans. Compared with humans, dogs have similar anatomy and physiology; dogs often share the same environment as humans; dogs naturally develop cancers sharing many characteristics with human cancers, the canine genome has been sequenced; and cancers in dogs grow over long periods of time yet their growth occurs over a short enough time period to be useful in evaluating new therapies. Dogs also share many of the intra-individual variations that humans do, including varied genetic constitution, varied immune responses, intra-tumoral heterogeneity, varied development of recurrent or resistant disease, and a varied degree of metastatic disease. Pet dogs also have the advantage of being cared for until ages commonly associated with the highest risk for cancer. For many of the naturally occurring cancers in dogs, histological appearance, tumor genetics, biological behavior,
and response to therapy are similar to their human counterparts. Furthermore, the lack of established “gold standard” veterinary cancer treatment protocols, allows dogs to be treated humanely with less proven treatment alternatives not possible in humans\(^{146}\).

From this series of treatments in dogs with spontaneous neoplasms and varied genetic makeup, environmental conditions, nutrition, and husbandry, we can conclude that the ECGT was well tolerated and had minimal side effects. Additionally, the treatments themselves can be easily accomplished with less than fifteen minutes of anesthesia. While not particularly painful, humans receiving EP describe uncomfortable muscle twitching; this discomfort combined with the need to access poorly accessible oral sites necessitates general anesthesia.

ECGT resulted in disease free intervals of greater than eight months in three tumors (considered a cure in this study); two of which had recurred after attempts at surgical resection prior to receiving this ECGT. Two of these tumors were SCCs which have previously been shown to respond to BLM ECT without gene therapy\(^{131}\); however, the period of follow-up was much less than the five cancer-free years that we report here. We were unable to evaluate whether these dogs would have been cured with BLM ECT alone, but theoretically, the augmentation with IL-12 gene therapy should help stimulate immune surveillance/memory and prevent recurrence as we found in mice (17). Acanthomatous ameloblastoma has not previously been reported to be ECT-sensitive; however, the lack of control subjects did not allow us to compare ECT to ECGT for this tumor either.

The dog with fibrosarcoma did not receive a full series of treatments. This dog’s partial response to therapy suggests that fibrosarcomas may be partially responsive to this treatment modality. In the two dogs with advanced metastatic neoplasia, ECGT enhanced the dogs’ quality of life prior to euthanasia; the effect of ECGT on metastatic disease was not determined. Both of
these dogs were not deemed to be candidates for surgery, radiation, or chemotherapy because of their advanced disease; thus ECGT offered a safe, relatively non-invasive, method of local tumor control without the side effects associated with conventional cancer therapies.

In humans, the rates of complete response after once-only treatments of single-agent, BLM ECT is reported to be between 9 and 100% depending on the technique used.\textsuperscript{141} Electrogene therapy of IL-12 has also reached phase one clinical trials in humans and has had similar positive results. In the first trial of gene transfer using electroporation in humans, the therapy was deemed, “safe, effective, reproducible, and titratable.”\textsuperscript{18} Moreover, IL-12 EGT without any other therapy gave complete regression of non-electroporated distant metastases in 10% of the subjects and produced disease stabilization or partial response in an additional 42%.\textsuperscript{18} Although impressive, if these therapies were combined, as in our study, the responses should improve significantly based on our findings in dogs and mice.\textsuperscript{138}

\section*{CONCLUSIONS}

Given the previous results achieved with experimentally induced tumors in mice\textsuperscript{138} and the encouraging responses achieved in three of the dogs in this study, ECGT offers a promising new approach to simple, relatively non-invasive tumor therapy with minimal side effects in dogs. Furthermore, ECGT may also be applicable to other species with readily available species-specific IL-12 DNA. These early applications support further research and treatment of a variety of neoplasms in dogs, and offer great promise for other species, including humans. For humans, additional pre-clinical studies assuring the safety of both EP and gene transfection with IL-12 are needed before applying for Investigational New Drug status. In the next chapter, safety studies looking at the toxicity of a tumor targeted version of the interleukin 12 gene were conducted to determine potential toxicity.
REFERENCES


CHAPTER 4
PRE-CLINICAL TOXICITY ASSESSMENT OF TUMOR-TARGETED INTERLEUKIN-12 LOW-INTENSITY ELECTROGENETHERAPY*

*Reprinted by permission from Macmillan Publishers Ltd, 2010©
INTRODUCTION

One of the major challenges associated with traditional surgical oncology, radiation, and chemotherapy is tumor recurrence and metastasis. One strategy to prevent tumor recurrence and metastasis is to induce or augment anti-tumor immunity. Interleukin 12 (IL-12) induces and maintains a powerful Th1-mediated anti-tumor immune response and has been used therapeutically in humans to treat neoplasia. The major effects of IL-12 are the stimulation of IFN-γ production by natural killer (NK) and T cells, which in turn stimulates additional IL-12 production \(^\text{147}\), and induction of anti-angiogenesis genes \(^\text{148}\). Another important role of IL-12 is to exhibit immunoregulatory functions in the generation of T helper 1 (Th1) and cytotoxic T lymphocytes (CTL) \(^\text{149}\). For these reasons, daily systemic administration of IL-12 recombinant protein (rIL-12) has been shown to generate a significant inhibitory effect on the metastatic tumor growth of B16F10 melanoma, established murine renal carcinoma (RENTCA), and CT26 tumors \(^\text{148, 150, 151}\). Unfortunately, rIL-12 has also been associated with significant adverse effects, including death \(^\text{152, 153}\). Investigation of rIL-12 toxicity suggests that cytokine-induced shock causing early deaths was mediated by NK cell production of IFNγ \(^\text{154}\). Much of this toxicity was attenuated by giving low, “desensitizing” doses of rIL-12 prior to treatment with the normal dose regimen; nevertheless, use of rIL-12 has now been supplanted by use of IL-12 gene therapy for cancer treatment.

Systemic administration of cytokines at pharmacologic doses results in high concentrations of cytokines in the circulation and often in suboptimal levels in tissues at the site of tumors, therefore therapy is more likely to cause systemic toxicity and less likely to be efficacious in treating the primary tumor. In contrast, EP-mediated cytokine gene transfection allows localized expression of cytokine at targeted sites, avoiding deleterious side effects and
resembling the normal paracrine effect of cytokines. This localized effect can be enhanced more, if the gene is somehow targeted to specific tissue or the tumor itself.

Our laboratory has previously demonstrated and published the efficacy of intratumoral EP of the gene for IL-12\textsuperscript{155}. Others have also found that therapy using the gene for IL-12 is safe and effective in a variety of pre-clinical models along with in human phase 1 trials treating melanoma\textsuperscript{18}. However, in these safety trials B16 tumor-bearing mice were used, making it difficult to extrapolate to IL-12 cancer gene therapy in general (and making it difficult to rule out variables associated with tumor burden). Moreover, the electroporation field in those studies was a much higher voltage (1300 V/cm) and shorter duration (95\textmu sec) than the parameters used in this study\textsuperscript{156}. Our laboratory and others have found that lower voltage, longer duration pulses are associated with comparable transfection efficiency in a variety of mouse tumor models using standard reporter gene assays\textsuperscript{2, 54, 157}. Additionally, currently available commercial electroporators deliver low voltage over longer intervals.

Safety of EP has also been examined in numerous studies and numerous EP protocols have been found to be safe and effective for most commonly used tissues\textsuperscript{158-160}. There is some concern regarding use of EP in heart muscle, and patients with defibrillators or arrhythmias may not be good candidates for EP\textsuperscript{160}. To our knowledge, nobody has examined the toxic effects of EP over time when applied under clinical conditions in a systematic way, nor has anyone reported the safety and toxicity of a relatively low voltage, long duration electroporation protocol; therefore we sought to characterize EP changes at numerous acute and a chronic endpoints using a clinical electroporator to determine if there was severe or lasting toxicity.

To increase the efficacy of anti-tumor immune induction and further address the concern of safety, we have developed a plasmid DNA vector encoding a tumor-targeted peptide linked to the IL-12 gene, allowing the gene product to preferentially accumulate in tumors regardless of
the site of injection. Pre-clinical therapeutic studies using murine ttIL-12 encoding plasmid have been completed in four independent tumor models; we have found that accumulation of IL12 protein in tumors is safe and triggers a more aggressive anti-tumor immune response than systemic rIL-12 therapy (Cutrera, submitted manuscript). In this study, the safety and toxicity of ttIL-12 was thoroughly analyzed (according to FDA guidelines for Investigational New Drug (IND) approval) in order to determine whether this novel fusion gene can be safely used in humans under clinical circumstances.

MATERIALS & METHODS

GLP or GLP-like Protocols. Serum chemistries and complete blood counts (CBCs) were performed by Antech Diagnostics (Memphis TN), which is fully compliant with Good Laboratory Practice (GLP) Regulations as set forth in 21 CFR Part 58 as well as other regulatory requirements. All other procedures, including tissue archival, quality control, and data keeping procedures were conducted in GLP-like conditions (as stipulated by GLP regulations, but not certified by the Food and Drug Administration (FDA)).

Mice. All animal use studies had approval from the Louisiana State University Institutional Animal Care and Use Committee (Baton Rouge, LA). This committee follows USPHS Policy on Humane Care and Use of Laboratory Animals. Young adult male and female C3H/HeJ mice were obtained from a commercial vendor (Harlan Laboratories, Inc., Indianapolis, Indiana), were fed *ad libitum* a standard diet (Harlan Teklad irradiated mouse diet 7912, Madison, WI), and were housed in a temperature-controlled animal facility with a 12/12-hour light/dark cycle. Each group for the toxicity studies consisted of six male and six female mice; the age of mice at the time of the first treatment in this study was 8.5 weeks.

Cell Lines and Propagation. For tumor resection and wound healing studies, SCCVII murine squamous cell carcinoma cells which are syngeneic in C3H mice, originally obtained
from Dr Bert O’Malley (Baylor College of Medicine) were maintained as monolayers in Dulbecco’s Modified Eagle Medium. Tumors from study mice were harvested and confirmed to be spindyloid squamous cell carcinoma cells based on routine histopathology, cytokeratin immunohistochemistry, and transmission electron microscopy. Cells were harvested from flasks using trypsin-EDTA (0.05% trypsin-0.53 mmol/L EDTA; Mediatech), collected by centrifugation, and washed. Cells were counted and their concentration was adjusted to 2 x 10^5 cells/ml using sterile phosphate-buffered saline (PBS) prior to inoculation into mice.

**Tumor Inoculation and Monitoring.** Tumor cells were inoculated in syngeneic C3H mice by intradermal injection of SCCVII cells in the caudodorsal skin. 30 µl of approximately 2 x 10^5 SCCVII tumor cells in sterile PBS was delivered to each mouse. Tumors were monitored using a digital vernier caliper and volumes were calculated using the formula $\frac{4}{3}\pi(a^2 b)$ where “a” is the tumor long dimension (diameter) and “b” is the shortest measurement perpendicular to “a”.

**Anesthesia.** For all procedures requiring anesthesia, mice were anesthetized by chamber induction with 4% isoflurane in oxygen and were subsequently maintained by mask administration of isoflurane in oxygen to effect. During longer procedures, an infrared heat lamp was used for thermal support. Respiratory effort and rate along with anal tone and withdrawal reflexes were monitored throughout anesthesia.

**Tumor Resection Surgery.** When tumor areas averaged 30,000 mm³, mice were anesthetized, the area surrounding the tumor was clipped to remove hair, and the surgical site was prepared with three alternating 70% ethanol/2% chlorhexidine scrubs. The site was draped with a sterile drape and an elliptical incision was made with minimal tumor margins (approximately 1 mm – no effort was made to completely excise tumors beyond a minimal
margin) and the tumor was removed. The skin defect was closed with 2-3 cruciate sutures of 5-0 silk.

**Plasmid.** DNA encoding the tumor-targeting peptide (CHP) was inserted into the murine IL-12 gene construct immediately upstream to the p40 termination codon (Cutrera, submitted manuscript). This gene construct expressed CHP-IL-12, herein referred to ttIL-12, and was inserted into a pCLneo mammalian plasmid expression vector (Promega Corporation, Madison, Wisconsin).

**Electroporation.** Either skin overlying the dorsal lumbar epaxial musculature or quadriceps femoris muscle were electroporated using a fixed-voltage, four-needle array disposable electrode with a 4 mm gap delivering two 60 ms pulses of 46 V (200 V/cm) spaced approximately 190 ms apart, attached to a Medpulser™ (Inovio Incorporated, San Diego, California) clinical electroporator. For SC application (the first treatment), either 30 µL of 0.45% saline or ttIL-12 in 0.45% saline were injected subcutaneously; within 15-20 seconds the electrode was inserted transcutaneously straddling the injection site; and the pulses were delivered. For IM use, the same volume was injected bilaterally in the quadriceps muscles (the total volume was therefore 60 µL, and the total DNA was divided between the two limbs), followed by insertion of the electroporator needles deep into the musculature surrounding the injection site.

**Toxicity Study.** Groups of six female mice and six male mice divided equally between no treatment controls, 0.45% NaCl EP, 1 µg ttIL-12 DNA in 0.45% NaCl, and 5 µg ttIL-12 DNA in 0.45% NaCl were treated. The volumes injected were 30 µL in the case of SC treatment and 60 µL divided bilaterally into two 30 µL aliquots for IM treatment. Treatment consisted of initial SC EP followed ten days later by IM EP. Control and treatment groups were then sacrificed at acute time points (1, 3, and 7 days) and a 30 day chronic time point.
Dosages of ttIL-12 were a presumably toxic mega-dose of 5 ug IL12 DNA and a therapeutic
dose of 1 µg IL-12 DNA. The presumed toxic dose was chosen for determining the toxicity limit
but will not be translated to humans because it would equal an impractical 22.5 mg in a 90 kg
human (requiring a minimum injection of approximately 25 mL). The therapeutic dose chosen as
most likely to be translated into human trials is 1 µg for multiple administrations, which is equal
to 4.5 mg in a 90 kg human.

**Surgery Augmented with ttIL-12 EP Study.** To examine the effect of ttIL-12 EGT on
recovery from tumor resection surgery, groups of six male mice were treated with the
aforementioned protocol, but a massive dose of 20 µg of ttIL-12 was administered during each
episode of EGT.

**Physical Exam/Behavior Monitoring.** A brief physical exam including body weight,
respiratory rate and effort, mucus membrane character, cutaneous lesions, musculoskeletal
changes, and localized tissue damage was performed on each mouse the day after EP and every
seven days thereafter. Mice were also monitored for gait abnormalities and grooming behavior
along with any other detectable behavioral anomalies.

**CBC & Chemistry Analysis.** Mice were anesthetized and the brachial artery was
accessed through an incision created in the ventral axilla; the artery was severed and pooled
blood was collected with a sterile glass pipette. Approximately 400-600 µl of blood was
collected into a miniature EDTA tube (BD Microtainer™ Tubes, Becton, Dickenson and
Company) and mixed well. The remaining blood – usually approximately 800 µL – was
collected into a miniature plasma separator tube (BD Microtainer™, Becton, Dickenson and
Company) and thoroughly mixed. A blood smear was made from the EDTA blood sample and
the remaining blood and plasma were sent to a private GLP lab (Antech Diagnostics, Memphis,
Tennessee) for a CBC and select chemistry analysis.
**Euthanasia and Gross Necropsy.** Under isoflurane general anesthesia, mice were exsanguinated and death was assured through cervical dislocation. All mice received a complete necropsy. Any tissue which had gross lesions (in addition to those routinely collected) was saved and processed for histopathologic examination.

**Histopathology.** All major perfused organs as well as any tissues with lesions (2 sections of skin) were fixed in 10% neutral-buffered formalin. Routine tissues examined histologically on all mice were liver, spleen, kidneys, lungs, heart, thymus, tracheobronchial lymph nodes, brain, bone marrow (lumbar spine & femurs), and quadriceps musculature. After fixation the tissue was cut-in, embedded in paraffin, sectioned on a microtome, mounted on glass slides, and stained with hematoxylin and eosin. Cover-slipped slides were examined by a pathologist and lesions were recorded. All slides and paraffin blocks were subsequently archived.

**Statistical Analysis.** Groups in each phase of evaluation were compared to control mice using a two-tailed paired T-test with a confidence interval of 95%. Charts of group means include standard error bars unless indicated otherwise.

**RESULTS**

**Optimization of Medpulser™ Parameters.** Our ultimate goal is to effectively deliver ttIL-12 gene to tumors, tissues at the margins of tumors, or systemically via musculature using EP in order to prevent tumor recurrence after primary tumor ablation. To achieve this goal, it is important to have a set of EP parameters enhancing gene delivery in multiple tissues. To determine such a set of parameters, we compared each of the clinically used applicators provided by the manufacturer (Inovio, San Diego, California) of the electroporator used in this study. We found that using the Medpulser™ fixed-voltage applicator delivering 107 V/cm for two consecutive 60 msec pulses separated by a pause of approximately 190 msec allowed statistically
significant (p = 0.002 and p < 0.001) high levels of reporter gene and cytokine expression when delivered both SC and IM (Fig. 8A). This applicator delivers 46 V across a 4 mm gap, four-needle electrode array that is inserted transcutaneously.

**Cytokine Expression after ttIL-12 EGT.** In previous use of rIL-12, toxicity of IL-12 protein was seen with a large systemic acute dose of rIL-12. It is generally agreed that low constitutive IL-12 expression kinetics are produced by IL-12 EGT and one of the advantages of EGT is local expression without significant, potentially toxic systemic levels of cytokine. To determine whether clinical application of the optimized EP parameters with ttIL-12 yielded the safe expression kinetics, we determined plasma cytokine levels in all of the sacrificed mice. The previously determined parameters yielded efficient transfection of the gene for ttIL-12 in vivo using the Medpulser™ as indicated by detection of significant plasma cytokine levels only in IL-12 DNA-treated mice (Fig. 8B & 8C), yet we found no statistically significant elevation in systemic plasma cytokine levels. Mean levels of IL-12 p70 protein peaked at about 600 pg/mL in the 5 µg dose group on day three, and at approximately 60 µg/mL in the 1 µg therapeutic dose group on day seven. Levels of IFNγ also increased after treatment, with the mean peak value reaching approximately 120 pg/mL on day seven in the 5 µg treatment group, and approximately 360 pg/mL in the 1 µg treatment group.

Thus, EP parameters using the Medpulser™ electroporator and four-needle electrode array were effective in numerous reporter genes and for therapeutic ttIL-12. As expected, systemic levels of IL-12 were not significant enough to potentially cause systemic toxicity. Additionally, levels of IL-12 were greater in the higher dose group – particularly on day three. The results for IFNγ were similar, but higher levels were seen in the low dose IL-12 group compared to the high dose group at acute time points. It is possible that IFNγ production was higher in the lower dose ttIL-12 group because of greater attenuation of IFNγ response by the
high dose of ttIL-12, similar to the desensitizing dose of rIL-12 used to prevent IFNγ shock and fatality in rIL-12 therapy\textsuperscript{153}. Unfortunately, to achieve significance and make any definitive conclusions, a greater number of mice would be required.

**Figure 8.** Optimization of Medpulser \textsuperscript{TM} electroporation settings and Plasma IL-12 and IFNγ levels after ttIL-12 EGT. A, Comparison of luciferase gene expression after electroporation using different voltage applicators. B, There is no statistical difference (p > 0.05) in mean plasma murine IL-12p70 cytokine levels between groups, but trends are illustrated. C, Mean plasma murine IFNγ also were not statistically different between groups (p > 0.05), but did trend toward higher levels over time with return to normal. X-axis is the number of days after the IM EGT treatment. Error bars represent standard error of the mean.

**Background Lesions in C3H/HeJ Mice over Time.** Since all major perfused organs were evaluated histologically, we also noted lesions that appeared to have no relation to ttIL-12 EGT therapy. Two minor changes were noted over time in all groups (without increased severity or incidence in any particular treatment group). The incidence of bronchial-associated lymphoid tissue hyperplasia increased over time and was unassociated with treatment (presumably because of environmental inhalant antigen exposure). Additionally, the incidence of incisor malocclusion and overgrowth increased as mice aged. Lymphoid hyperplasia of lymph nodes, thymus and spleen were noted in all mice (both treatment and controls) throughout the study; and splenic extramedullary hematopoiesis, a common frequent finding in mice, was constant between groups and over time.

**Local Effects of Electroporation.** In order to determine the local effects of electroporation, muscle and skin were harvested from electroporation sites and examined microscopically. Focal to multifocal acute rhabdomyocyte necrosis with subsequent
pyogranulomatous to granulomatous myositis followed by myoregeneration and resolution were seen at site of needle electrode insertion in numerous mice in all of the groups that received IM EP (Fig. 9). The incidence of detectable muscle damage peaked at seven days for the ttIL-12 treated mice, and was maximal on day 1 for the 0.45% saline treated mice. These trends may reflect the more widespread tissue inflammation over time and chances of detecting a change, more than reflecting increasing severity; in fact, by day 30, no muscle lesions were detected except for a single small focus of muscle degeneration, myofiber loss, and mineralization in a mouse of the 5 µg ttIL-12 treatment group (in a mouse that also had marked dystrophic cardiac calcification (DCC)).

We also monitored behavior and performed periodic physical examinations of the mice after EP and found that several mice were transiently lame in one or both pelvic limbs after IM EP. Mice that were lame were rechecked periodically and clinically detectable lameness resolved completely in all mice by seven days after IM EP. There was not relationship between the incidence or severity of lameness and the substance injected.

**Liver Toxicity from ttIL-12.** The biggest concern regarding adverse effects from IL-12 was hepatotoxicity; therefore several lobes of liver were examined histologically for ttIL-12 EGT lesions and a scoring system was used to document severity and incidence of lesions in different treatment groups (Fig. 10). There was a mild increase in the detection of liver lesions over time in the two ttIL-12 treatment groups, but none seen in either the control mice or the 0.45% saline treated mice (Fig. 10). Although none of the toxicity appeared serious, the most severe lesions, and the highest incidence of lesions, were seen on day seven (Fig. 10). Lesion incidence and severity were also greater in the 5 µg ttIL-12 treatment group than in the 1 µg ttIL-12 treatment group, but had essentially resolved by day 30.
To determine if there were any serologic indicators of liver toxicity and to see if these analytes correlated with histologic lesions, we compared mean levels of alanine transaminase (ALT) and alkaline phosphatase (ALP) activity in each group as well as levels of albumin (ALB) (Fig. 10). For alkaline phosphatase and albumin, there was no significant difference between treatment groups or over time. For ALT, there was a mild, but statistically insignificant (p.0.05), increase in treatment group deviation from controls up to day seven, which returned to normal by day 30. Levels never reached clinically relevant increases (2-3 fold higher than normal range), and levels of ALT in saline treatment groups were essentially as high as or higher than in the ttIL-12 treatment groups. Nevertheless, ALT levels did correlate with liver toxicity on an individual animal basis and is thought to be a good monitoring parameter for acute liver toxicity.

Figure 9. Muscle damage associated with EP. A, Photomicrographs illustrating the spectrum of muscle damage detected over time. On the left is quadriceps from an untreated mouse illustrating the normal muscle appearance. The next panel is from a mouse sacrificed one day after intramuscular EP demonstrating separation of the endomysium by fibrin and edema. The third panel is from a mouse three days post-EP demonstrating fragmentation, rounding, hyperesinophilic, and hyalinization of myofibers with a moderate multifocal to coalescing infiltrate of histiocytes and neutrophils. The fourth panel is from a mouse sacrificed seven days post-EP demonstrating resolving muscle damage with early regeneration and multifocal aggregates of histiocytes. The last panel is from a mouse sacrificed on day 30 post-EP illustrating normal musculature. Hematoxylin & eosin staining of paraffin embedded formalin-fixed tissue. 200x magnification, bar = 100 μm. B, Chart illustrating the percentage of mice with detectable quadriceps lesions.
Systemic Immune Effects of ttIL-12 EGT. In order to determine if there were any significant systemic effects of ttIL-12 EGT on the hematopoietic system and systemic immunity, CBCs and bone marrow histology were evaluated. In the case of WBC, there was a trend to decreasing total counts up to day seven which returned to normal by day 30 (Fig. 11). The trend was not significantly different from control mice in the 1 µg treatment group (p = 0.053 on day 3 and p = 0.109 on day 7), but was in the 5 µg treatment group (p = 0.017 on day three and p = 0.023 on day seven). The incidence of infectious disease was essentially non-existent (except possibly for mild BALT hyperplasia) in all groups except for the 5 µg ttIL-12 treatment group, which had two mice on day 30 that had severe, potentially life-threatening, infectious diseases. One mouse had a moderate to severe, multifocal to coalescing, pyogranulomatous cholangiohepatitis (Fig. 11B), and the other mouse had severe, bilateral, subacute to chronic, diffuse, pyogranulomatous pyelonephritis (Fig.11C). Of note is the fact that the second mouse also was one of the mice with DCC. No effects on bone marrow or other hematopoietic parameters were noted.

Dystrophic Cardiac Calcification. In histologically evaluating all other organs for potential ttIL-12 EGT toxicity, the only other major change seen in any of the mice was seen in the day 30, 5 µg ttIL-12 treatment group, which had 3 mice (25%) with moderate to severe DCC (Fig. 12). Of these mice, one died, and one had evidence of biventricular congestive heart failure (pulmonary edema with splenic and hepatic congestion) at the time of sacrifice.

As noted above, the only fatality in the 192 mice of this study was a female in the mega-dose 5µg chronic 30 day group that died on day 22 after the IM EGT treatment. The cause of death in this mouse was thought to be biventricular congestive heart failure secondary to DCC. Aside from severe myocardial mineralization (similar to Fig. 5B, but more severe), this mouse had hepatosplenomegaly due to congestion and mild hemosiderosis in the liver, spleen, and lungs. In the lungs, numerous alveolar macrophages contained either erythrocytes or
Figure 10. Incidence of mild liver toxicity in tIL-12 treated mice. A, Examples of liver histopathology for the liver toxicity scoring system used indicating that in all cases the toxicity was mild. The panel on the left illustrates non-affected liver. The second panel is consistent with a grade 1 score characterized by ≤ 1 lesion per 100 x field. The third panel is a grade 2 livers with 1-3 lesions per 100x field. The fourth panel is a grade 3 liver with 3-8 lesions per 100x field. Lesions consisted of small foci of hepatocellular necrosis with Kupffer cell hyperplasia with sporadic neutrophils & lymphocytes. Hematoxylin and eosin staining of paraffin-embedded formalin-fixed tissue. 100x magnification, bar = 200 µm. B, Incidence of liver lesions by lesion score and total group score (number at the top of the pie chart) for mice treated with 1 µg tIL-12. C, Incidence of liver lesions by lesion score and total group score (large bold number in the center) for mice treated with 5 µg tIL-12. D, Plasma levels of liver parameters by group and day. The 0.45% saline treatment group approached statistical significance for ALT on day 7 (p=0.51), but all other changes were not statistically significant (p>0.05).
Figure 11. Changes in total WBC in response to tTIL-12 over time. A, Chart depicting the change in total white blood cell count for each group over time. *Indicates significant change in total WBC. B, Photomicrograph from one of the mice in the 30 day 5 µg treatment group depicting a subacute moderate diffuse pericholangitis or portal hepatitis. C, Kidney photomicrograph from another mouse in the 30 day 5 µg treatment group depicting a severe, diffuse, subacute to chronic, pyogranulomatous pyelonephritis effacing the entire kidney. Hematoxylin and eosin staining of paraffin-embedded formalin-fixed tissue.
Figure 12. Photomicrographs comparing an untreated mouse heart to a heart with dystrophic cardiac calcification (DCC). A, Normal heart section with inset of the myocardium demonstrating no evidence of myocardial mineralization. B, Section and inset from a mouse demonstrating moderate, multifocal myocardial mineralization (intensely basophilic foci) throughout the ventricular free wall and interventricular septum characteristic of DCC. Hematoxylin and eosin staining of paraffin-embedded formalin-fixed tissue.

hemosiderin ("heart failure cells"), and the interstitial spaces surrounding major vessels were expanded. Definitive evidence of pulmonary alveolar edema was not seen, but the interval between death and post-mortem examination was estimated to be 12-24 hours. The female mouse with the heart depicted (Fig. 12B) was also in congestive heart failure at the time of sacrifice as evidenced by pulmonary edema along with hepatic and splenic congestion.

Effect of ttIL-12 EGT on Wound Healing. Because we anticipate combining ttIL-12 EGT with surgical resection of primary tumors, analysis of wound healing when ttIL-12 EGT was critical for assuring that the therapy did not interfere with surgical protocols. Therefore, we
grossly evaluated wound healing after EGT and tumor resection in squamous cell carcinoma tumor-bearing C3H mice and compared with wound healing in non-treatment controls. The last figure depicts healed tumor resection incisions seven days after surgery in mice treated with two treatments of 20 µg ttIL-12 prior to surgery (Fig. 13A & 13B). Wound healing was subjectively evaluated by observing the time to complete wound epithelialization (n =5 mice per group). As can be seen from the plot comparing wound epithelialization times (Fig. 13C), EGT treatment with massive doses of ttIL-12 produced no statistical difference (p=0.5415) in primary intention wound re-epithelialization.

![Figure 13](image)

**Figure 13.** Therapy with tumor-targeted interleukin 12 does not delay wound healing. Incision healing seven days after surgery in the untreated control group (A) and the ttIL-12 group (B). There was no significant difference in the time to complete epithelialization between the control and ttIL-12 EGT groups (p=0.54)
DISCUSSION

Several clinical trials are beginning or underway in humans using EP to deliver DNA in the form of vaccines or therapeutic genes; however, only one toxicology study was found in the literature using tumor-bearing mice to examine the safety of IL-12 gene EGT\(^{156}\). Furthermore, the aforementioned study used high voltage (1,300 V/cm), short duration (0.1 msec) EP pulses in contrast to the low voltage (200 V/cm), and long-duration (60 msec) pulses used in the current study. The electrode array used was also different than the one used in this study (a six-needle array versus the current four-needle array).

Comparing the different EP parameters, we did find effective transfection using the current parameters (Fig. 8), but it was important to also determine if these EP parameters caused any serious adverse effects. In addition to establishing the safety of the current set of EP parameters, it was also important to establish the safety of ttIL-12. Theoretically, a tumor-targeted gene should be both safer and more efficacious than a systemically-administered, non-targeted gene. As expected, therapeutic use of ttIL-12 EGT was found to be safe and well tolerated under conditions likely to be used for EGT clinically (Figs. 9, 10, & 11).

To our knowledge, this is also the first large scale study of IL-12 toxicity in non-tumor bearing mice, and the first examining the effects of relatively low voltage, long duration EP pulses. Tumor-bearing mice are not normal immunologically\(^{161-163}\), so extrapolation from studies in tumor-bearing mice is fraught with a number of variables which may affect toxicity and efficacy of immune therapy. Additionally, both transfection efficiencies and local EP effects vary widely depending on the EP system used.

Using these parameters, local muscle changes in response to EP were completely resolved by day 30. Interestingly, damage was more frequently detected on days one and three
in the group treated with carrier only – perhaps because of the hypotonic osmolarity of the half-strength saline compared to DNA solutions. A protective effect of ttIL-12, other than adding colloidal pressure, seems unlikely at these acute time points but cannot be ruled out. Although the damage was detectable, albeit transient, these changes should be viewed with the perspective that inserting the 4 mm wide, four electrode array into mouse quadriceps affected a much greater muscle mass than would be commonly used in humans – perhaps this would be analogous to penetrating human thighs with four equidistant railroad spikes spaced 25-50 cm apart and applying an electric field. In addition to the histologic muscle changes seen after EP, several mice were transiently lame in the pelvic limbs the day after EP, but subsequently returned to a normal gait and stance thereafter.

We chose C3H/HeJ mice in this study based on previously documented gross toxicity and our previous experience suggesting that they were more sensitive to IL-12 liver toxicity than other commonly used strains of inbred mice. Concerns about the well documented (16) defective TLR4 signaling in C3H/HeJ mice were not thought to be an issue because TLR4 signaling in mice appears to serve primarily as a lipopolysaccharide (LPS) response pathway. Thus, C3H/HeJ mice are immunologically normal, except for their inability to respond to LPS and to counter Gram negative infection. Liver and immune changes were detected in this study, but are not thought to be strain-specific.

Liver changes have been described before with rIL-12 therapy. Similar to our findings, mice in previous rIL-12 studies had foci of hepatocellular necrosis with aggregates of Kupffer cell hyperplasia. In this study we have also noted occasional neutrophil and lymphocytes interspersed within the aggregates of Kupffer cells in the acute treatment groups. Lesions in the mice in this study were mild to minimal and transient. The number of lesions
correlated with minimal elevations in ALT, but we found no increase in alkaline phosphatase (ALP) and no decrease in albumin (ALB) as was previously seen in rIL-12 toxicity \textsuperscript{164}. We chose not to determine levels of aspartate transaminase (AST) because this enzyme is also elevated in cases of muscle damage and is therefore not liver-specific. If liver toxicity were a concern, our results suggest that monitoring serum levels of ALT during therapy would be indicated.

Immune suppression and suppressed leukocyte counts have also been described with IL-12 therapy \textsuperscript{164}. In our study, both total white cell counts and individual absolute leukocyte counts were decreased after treatment with ttIL-12. Mean absolute leukocyte counts for neutrophils, lymphocytes, monocytes, and eosinophils never decreased significantly from control mice. Mean total WBC did decrease significantly (p<0.05) from control levels on days three and seven in mice treated with 5 µg of ttIL-12. Furthermore, in the 5 µg ttIL-12 group there were two mice that developed major infectious diseases; one mouse also had concurrent severe DCC which may have been a significant stressor predisposing the mouse to pyelonephritis (or pyelonephritis may have predisposed to DCC); the other mouse had moderate to severe cholangiohepatitis. Since no other mice in the study developed serious infectious disease, there is a possibility that mega-dose ttIL-12 may cause clinically relevant immune suppression. Conversely, at therapeutically relevant doses (1 µg), ttIL-12 EGT is unlikely to cause serious immunosuppression. Nevertheless, in addition to monitoring ALT, we suggest monitoring WBC during therapy – especially if combined with immunosuppressive chemotherapeutics.

DCC was the most serious problem potentially linked to ttIL-12 EGT. Although both of the females with clinical signs attributed to DCC (death and congestive heart failure) were well within the reported 81-89 day range for development of DCC in female C3H/HeJ mice \textsuperscript{166}, DCC
seen in the 100-day-old male mouse (which did not have signs of CHF) was well before the normal onset of 292-465 days reported previously. No other mice in the study developed detectable DCC suggesting that this 16% premature incidence in the mega-dose male mice may not have been significant. Nevertheless, until additional studies are done in mice that are not predisposed to DCC, mega-doses of ttIL-12 would be contraindicated in subjects with similar genetic predisposition.

DCC is common in many strains of inbred mice. In C3H mice, DCC is caused by a mutation in the \textit{ABCC6} gene leading to deficiency of its protein product, MRP6. MRP6 is expressed primarily in the liver and kidney but also exists in many other tissues throughout the body, and is in the family of ABC transporters. It has long been known that DCC can be triggered in susceptible mice by a variety of stimuli including: female sex – particularly in breeders, high fat diets, infectious agents, hormonal status, diet, and freeze-thaw injury. In this study, we found that ttIL-12 EGT also appears to hasten the onset of DCC in C3H/HeJ mice.

In summary, we can conclude that ttIL-12 EGT using low-voltage, long-duration pulses is safe and well-tolerated at therapeutically relevant dosages. Mild muscle changes seen after IM EP are unlikely to be a problem in humans given the small area electroporated. Transient acute decreases in total WBC may suggest possible mild immune suppression, so vigilant patient monitoring for infectious disease and serial CBCs would be prudent. However, no infectious diseases were seen in study mice at the clinically relevant dose of 1 µg ttIL-12. Likewise, DCC was not a problem in the 1 µg therapeutic dosage group. However, several mice (three of twelve, or 25%) were affected in the mega dose (5 µg) group at day 30, one of which presumably died of congestive heart failure secondary to DCC. Thus, until additional information is available,
people with \textit{ABCC6} gene defects or patients with Pseudoxanthoma elasticum (a heritable disorder of the connective tissue, caused by defective \textit{ABCC6} gene) should be excluded from treatment with mega-dose ttIL-12 EGT. Finally, no effect of ttIL-12 EGT on wound healing was seen – even at a dosage twenty times greater than those likely to be used clinically. Thus, given the safety of this treatment modality, and superiority to wild-type IL-12 therapy (Cutrera, submitted manuscript) we strongly recommend proceeding to phase I trials.

\textbf{ADDITIONAL OBSERVATIONS}

In addition to the data published herein, Appendix 1 includes data demonstrating that there was no difference in weight gain between groups, and the incidence of lameness following IM electroporation (consistently higher in the 0.45\% saline control group).

\textbf{REFERENCES}


2. Reed SD, Li S. Electroporation Advances in Large Animals. \textit{Curr Gene Ther} 2009.


92


93


CHAPTER 5
CONCLUDING REMARKS
Cancer continues to remain a major cause of morbidity and mortality throughout the world. Conventional surgery, chemotherapy, and radiation therapy have failed to result in a significant impact on the rates of death from cancer; therefore, there is a real need for better therapies. Immune therapy in general and cytokine gene therapy specifically, offers several advantages over conventional treatment modalities. In addition to being able to effectively target tumor metastases and recurring primary tumors, we have found that cancer electroimmunogene therapy is cost effective, safe, and easy to perform.

If treatment in humans shows as much promise as seen in our pre-clinical trials, and is as safe as seen in the studies described herein, this therapeutic may offer a significant impact in the war against cancer. As summarized in this dissertation, there have been significant advances in the use of electroporation resulting in several clinically-approved electroporators. We have already proved utility of this technique when combined with electrochemotherapy in treating locally aggressive oral tumors in dogs in addition to our extensive previous data in mice. Perhaps most importantly in terms of being able to advance to human clinical trials, we have shown that this technique is safe.
Mean Body Weights for Each Group: There was no significant difference in body weights between treatment groups. (Mean +/- Standard Deviation in grams)

<table>
<thead>
<tr>
<th>GROUP*</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 10**</th>
<th>Day 11</th>
<th>Day 19</th>
<th>Day 26</th>
<th>Day 33</th>
<th>Day 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL₁</td>
<td>25+/-3.1</td>
<td>25.2+/-2.9</td>
<td>25.1+/-3.2</td>
<td>25.1+/-3.1</td>
<td>25.4+/-3.2</td>
<td>25.4+/-3.2</td>
<td>25.4+/-3.2</td>
<td>25.4+/-3.2</td>
<td>25.4+/-3.2</td>
</tr>
<tr>
<td>SALINE₁</td>
<td>24.9+/-2.1</td>
<td>25.1+/-2.2</td>
<td>25.2+/-2.5</td>
<td>25.1+/-2.8</td>
<td>25.4+/-3</td>
<td>25.4+/-3</td>
<td>25.4+/-3</td>
<td>25.4+/-3</td>
<td>25.4+/-3</td>
</tr>
<tr>
<td>1 µg₁</td>
<td>25.2+/-3.1</td>
<td>25.3+/-3</td>
<td>25.2+/-3</td>
<td>25.4+/-3.1</td>
<td>25.3+/-3.1</td>
<td>25.3+/-3.1</td>
<td>25.3+/-3.1</td>
<td>25.3+/-3.1</td>
<td>25.3+/-3.1</td>
</tr>
<tr>
<td>5 µg₁</td>
<td>24.2+/-2.3</td>
<td>24.3+/-2.3</td>
<td>24.6+/-2.3</td>
<td>25.3+/-2.6</td>
<td>25.2+/-2.4</td>
<td>25.2+/-2.4</td>
<td>25.2+/-2.4</td>
<td>25.2+/-2.4</td>
<td>25.2+/-2.4</td>
</tr>
<tr>
<td>CONTROL₃</td>
<td>23.9+/-2.9</td>
<td>24+/-2.5</td>
<td>24.3+/-2.5</td>
<td>25.2+/-2.6</td>
<td>24.6+/-2.1</td>
<td>24.4+/-2.6</td>
<td>24.4+/-2.6</td>
<td>24.4+/-2.6</td>
<td>24.4+/-2.6</td>
</tr>
<tr>
<td>SALINE₃</td>
<td>24.4+/-3.6</td>
<td>24.8+/-3.6</td>
<td>24.8+/-3.5</td>
<td>25.4+/-3.6</td>
<td>25.3+/-3.6</td>
<td>25.4+/-3.6</td>
<td>25.4+/-3.6</td>
<td>25.4+/-3.6</td>
<td>25.4+/-3.6</td>
</tr>
<tr>
<td>1 µg₃</td>
<td>25+/-3.4</td>
<td>25.6+/-3.3</td>
<td>25.6+/-3.3</td>
<td>25.8+/-3.5</td>
<td>25.7+/-3.5</td>
<td>25.6+/-3.8</td>
<td>25.6+/-3.8</td>
<td>25.6+/-3.8</td>
<td>25.6+/-3.8</td>
</tr>
<tr>
<td>5 µg₃</td>
<td>25+/-2.9</td>
<td>25.3+/-3.1</td>
<td>25.4+/-3.3</td>
<td>25.8+/-3</td>
<td>25.7+/-3.4</td>
<td>26+/-3.3</td>
<td>26+/-3.3</td>
<td>26+/-3.3</td>
<td>26+/-3.3</td>
</tr>
<tr>
<td>CONTROL₇</td>
<td>23.5+/-3</td>
<td>23.9+/-3.1</td>
<td>24.1+/-2.9</td>
<td>24.4+/-2.8</td>
<td>24.9+/-2.8</td>
<td>24.7+/-3</td>
<td>24.7+/-3</td>
<td>24.7+/-3</td>
<td>24.7+/-3</td>
</tr>
<tr>
<td>SALINE₇</td>
<td>23.4+/-1.9</td>
<td>24.3+/-2.1</td>
<td>24.7+/-2.1</td>
<td>24.8+/-2</td>
<td>25.1+/-2.2</td>
<td>25.6+/-2.7</td>
<td>25.6+/-2.7</td>
<td>25.6+/-2.7</td>
<td>25.6+/-2.7</td>
</tr>
<tr>
<td>1 µg₇</td>
<td>22.9+/-2.7</td>
<td>23.6+/-2.6</td>
<td>24+/-2.6</td>
<td>24.4+/-2.7</td>
<td>24.5+/-2.9</td>
<td>25+/-3</td>
<td>25+/-3</td>
<td>25+/-3</td>
<td>25+/-3</td>
</tr>
<tr>
<td>5 µg₇</td>
<td>23.1+/-2.3</td>
<td>23.8+/-2.3</td>
<td>24.1+/-2.5</td>
<td>24.2+/-2.3</td>
<td>24.9+/-2.3</td>
<td>25.7+/-2.2</td>
<td>25.7+/-2.1</td>
<td>25.7+/-2.1</td>
<td>25.7+/-2.1</td>
</tr>
<tr>
<td>CONTROL₃₀</td>
<td>22.8+/-2.2</td>
<td>23.7+/-2.1</td>
<td>23.6+/-1.9</td>
<td>22.3+/-1.9</td>
<td>24+/-2.1</td>
<td>24.6+/-2.1</td>
<td>25.5+/-2</td>
<td>26.2+/-1.8</td>
<td>27+/-2</td>
</tr>
<tr>
<td>SALINE₃₀</td>
<td>23.1+/-2.1</td>
<td>23.9+/-2.1</td>
<td>23.8+/-1.9</td>
<td>23.3+/-2.3</td>
<td>24.5+/-2</td>
<td>25.3+/-2</td>
<td>26.3+/-1.9</td>
<td>26.7+/-1.7</td>
<td>27.4+/-1.7</td>
</tr>
<tr>
<td>1 µg₃₀</td>
<td>23.8+/-3.1</td>
<td>24.1+/-2.8</td>
<td>23.6+/-3.2</td>
<td>23+/-3.7</td>
<td>23.9+/-3.5</td>
<td>24.4+/-3.3</td>
<td>24.7+/-3</td>
<td>25.1+/-3</td>
<td>25.4+/-2.9</td>
</tr>
<tr>
<td>5 µg₃₀</td>
<td>23.9+/-2.7</td>
<td>24.4+/-2.7</td>
<td>23.7+/-3.4</td>
<td>23.3+/-3.9</td>
<td>24.2+/-3.4</td>
<td>24.6+/-3</td>
<td>24.8+/-2.7</td>
<td>25.3+/-2.5</td>
<td>25.9+/-2.1</td>
</tr>
</tbody>
</table>

*The Subscript following the group identity is number of days between treatment and euthanasia.

**Day of intramuscular electroporation.
**Percentage of mice with detectable lameness after electroporation:** All groups had some members with detectable lameness. The only consistent trend was a greater incidence of lameness in the saline control group.

<table>
<thead>
<tr>
<th>GROUP*</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 10**</th>
<th>Day 11</th>
<th>Day 19</th>
<th>Day 26</th>
<th>Day 33</th>
<th>Day 40</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALINE1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SALINE2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µg2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 µg2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CONTROL7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SALINE7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1 µg7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5 µg7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>CONTROL30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SALINE30</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>50</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 µg30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 µg30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The Subscript following the group identity is number of days between treatment and euthanasia.

**Day of intramuscular electroporation.
APPENDIX 2
LETTERS OF PERMISSION
Grant of Permission

Dear Dr. Reed:

Thank you for your interest in our copyrighted material, and for requesting permission for its use.

Permission is granted for the following subject to the conditions outlined below:

"Electroporation advances in large animals" CGT 9:316-326

To be used in the following manner:

1. Bentham Science Publishers grants you the right to reproduce the material indicated above on a one-time, non-exclusive basis, solely for the purpose described. Permission must be requested separately for any future or additional use.

2. For an article, the copyright notice must be printed on the first page of article or book chapter. For figures, photographs, covers, or tables, the notice may appear with the material, in a footnote, or in the reference list.

Thank you for your patience while your request was being processed. If you wish to contact us further, please use the address below.

Sincerely,

AMBREEN IRSHAD

Permissions & Rights Manager

Bentham Science Publishers Ltd

Email: permission@bentham.org

URL: www.bentham.org
This is a License Agreement between Scott D Reed ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

License Number 2522031084901
License date Oct 04, 2010
Licensed content publisher Nature Publishing Group
Licensed content publication Cancer Gene Therapy
Licensed content title Bleomycin/interleukin-12 electrochemogenetherapy for treating naturally occurring spontaneous neoplasms in dogs
Licensed content author S D Reed, A Fulmer, J Buckholz, B Zhang, J Cutrera, K Shiomitsu, S Li
Volume number
Issue number
Pages
Year of publication 2010
Portion used Full paper
Requestor type Student
Type of Use Thesis / Dissertation
Billing Type Invoice
Company Scott D Reed
Billing Address 4343 Hyacinth Avenue
Baton Rouge, LA 70808
United States
Customer reference info
Total 0.00 USD
Terms and Conditions
Terms and Conditions for Permissions
Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
2. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

3. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

4. The credit line should read: Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication) For AOP papers, the credit line should read: Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

5. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows: Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

6. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows: Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Hello,
I request permission to use the recently accepted Cancer Gene Therapy manuscript #
CGT-10-0077R in my PhD dissertation

Scott

AKA: Scott D Reed, BS, DVM, DABVP

Reply

Dear Dr. Reed,

Thank you for your Nature permissions request. You are free to use your manuscript for your dissertation. Here is a link for information on the author policy for the Gene Therapy journal:

Author Guidelines: http://www.nature.com/gt/for_authors.html.

Kind regards,

Charlotte Kinnah
Permissions Assistant
nature publishing group
The Macmillan Building
4-6 Crinan Street
London N1 9XW
e:permissions@nature.com
p: + 44 207 014 4129
f: + 44 207 843 4998
VITA

Scott Douglas Reed is the son of Margaret McCracken Reed and Donald Vincent Reed and was born in Jeannette, Pennsylvania, a small city just southeast of Pittsburgh. He has a younger brother, Steve, and a younger sister, Ginny. His elementary school years were spent in Maryland, Ohio, and Virginia. James Madison High in Vienna, Virginia, a Fairfax County suburb of Washington, D.C., is where he completed high school.

After high school, Scott attended Virginia Tech where he received his bachelor’s degree in biochemistry. Upon graduation from Virginia Tech, he accepted a graduate position in molecular and cellular biology at the University of Florida, where he conducted research involving the cloning of chloroplast-targeting sequences for introduction of the glutamate dehydrogenase gene, a rate limiting step in nitrogen assimilation for plants. After a frustrating year of unsuccessful research, Scott elected to pursue his lifelong dream of becoming a veterinarian. For the following year he worked as a chemist for Doctor David A Williams (current Department Head of Veterinary Clinical Sciences at the University of Illinois) and completed prerequisites for veterinary school. He was subsequently accepted to the University of Florida’s College of Veterinary Medicine where he received numerous awards (Phi Zeta research award, Iams scholarship award, and Hill’s nutrition award) and his Doctor of Veterinary Medicine in 1990. During veterinary school he also met and married his wife, Doctor Frances Minnich Reed, and developed his love for pathology after inspiring mentorship by an excellent group of pathologists at the University of Florida (UF) including Doctor Guy Palmer (Director of School for Global Health at Washington State University) and Doctor Claus Buergelt (UF professor emeritus). After a year spent in private companion animal practice in Gainesville, Florida, Scott pursued his passion for pathology by accepting a position as a pathology resident
at North Carolina State University. Unfortunately because of financial difficulties and family problems, Scott was forced to return to private practice.

While in practice, Scott worked for many years as an emergency clinician, and eventually transitioned to daytime practice, becoming a successful companion animal practitioner. In practice, he received numerous awards and recognition in addition to attaining Diplomate status from the American Board of Veterinary Practitioners in canine and feline medicine, a distinction held by less than 1% of private practice veterinarians. Although many aspects of daytime family practice were enjoyable, soon the monotony of practice, physical problems with his hands, and increasing management responsibilities took their toll. Building his own practice and struggling through the early years of practice ownership provided the final incentive for Scott to give pathology another chance. Fortunately, he was provided an opportunity to pursue a combined pathology residency and doctorate at Louisiana State University. He completed the bulk of his pathology residency and most course work in 2008 under the direction of several excellent pathologists. He then joined the laboratory of Doctor Shulin Li (currently a Professor at MD Anderson Cancer Center), a prominent researcher in electroporation-mediated cancer immunogene therapy. Doctor Li not only provided a terrific project which allowed Scott to apply skills gained as a pathologist and a veterinarian, but also went out of his way to assure adequate funding and support, and has been very tolerant of Scott’s questions and critical thinking. As noted in the acknowledgements, this dissertation would not have been possible without Dr Li’s funding and direction.

Scott has published a number of peer reviewed papers in clinical veterinary medicine and surgery, pathology, and in electroporation-mediated gene therapy. Currently he is author on over twenty papers, the majority of which are first authorships. He was inducted into Phi Zeta, the
veterinary honor society, in 2008, and was recently awarded a 2010 CL Davis Foundation Student Scholarship Award in 2010.