AD ________________

Award Number: DAMD17-98-1-8094

TITLE: Endothelial Cell-Based Gene Therapy of Breast Cancer

PRINCIPAL INVESTIGATOR: John O. Ojeifo, M.D., Ph.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20057

REPORT DATE: August 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2002	Final (1 Aug 98 - 31 Jul 02)

4. TITLE AND SUBTITLE
Endothelial Cell-Based Gene Therapy of Breast Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8094

6. AUTHOR(S)
John O. Ojeifo, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20057

E-Mail: ojeifoj@georgetown.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

20021230 137

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)* Recurrence and metastatic dissemination of breast cancers account for a significant morbidity and mortality in women, and effective means of treating this subset of patients remain elusive. The reports that intravenously (IV)-administered, genetically modified endothelial cells (GMEC) can target and incorporate into sites of active angiogenesis suggest that this strategy may be useful for the treatment of metastatic breast cancer. We evaluated whether IV-injected, interleukin (IL)-2 or IL12 gene-modified murine microvascular endothelial cells (IL-2/GMEC) can target sites of metastatic breast cancer, and whether the expression of hIL-2 or mIL-12 transgene at the local tumor site can induce an anti-tumor immune response. Systemic administration of hIL-2/GMEC mediated significant reduction in the tumor burden of breast cancer and prolonged the survival of tumor-bearing mice. Immunocytochemical analysis of explanted tumors demonstrated presence of immune effectors (granulocytes, macrophages, CD4+ and CD8+ lymphocytes) within and around rhIL-2 positive tumors. Mice, which received only the hIL-2/GMEC without tumor cells, did not develop tumors and remained alive and well. Initial studies of tumor-bearing mice treated with IL-12/GMEC also show promising results. These findings suggest that systemic administration of GMEC is a potentially effective and safe strategy to target and treat metastatic breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, immunotherapy/immunology, endothelium, cytokine, angiogenesis, therapeutics	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

N/A Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

N/A Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

___ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

___ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Bio-safety in Microbiological and Biomedical Laboratories.

8/10/2002

PI - Signature Date

Table of Contents

Cover ....

SF 298 2

Foreword 3

Table of Contents 4

Introduction 5

Body 5

Key Research Accomplishments 11

Reportable Outcomes 12

Conclusions 13

References 13

Appendices 14

1. INTRODUCTION

Recurrence and metastatic dissemination of breast cancers account for a significant morbidity and mortality in women, and effective means of treating this subset of patients remain elusive. Previous reports from our laboratory have demonstrated that systemically-administered, *lacZ* or interleukin (IL)-2 gene modified endothelial cells (*lacZ*-GMEC or hIL-2-GMEC) can selectively migrate into, accumulate, and express the transgene at metastatic sites of breast cancer (1, 2), and that the expression of rhIL-2 at the local sites the metastases induced significant reduction in tumor burden in the mice (3). More recently, we reported that immune effector cells made up of neutrophils, macrophages, and both CD4+ and CD8+ lymphocytes infiltrate rhIL-2-positive breast cancer metastatic foci, suggesting that these immune cells may be involved in rhIL-2/GMEC-mediated regression of breast cancer metastases in mice (see our earlier reports for 1999, 2000 and 2001). However, because of the lack of specific, long-term systemic immunity in the mice, we investigated the therapeutic efficacy of IL-12 gene in mice with metastatic breast cancer. The product of this immune activating gene has been reported to induce complete tumor rejection and to confer long-term systemic immunity in animals with advanced tumors (4, 5). The objective of the present study was to determine the effect of intravenously (IV)-administered, murine IL-12 transgene-expressing endothelial cells (rmIL-12GMEC) on the growth of established breast cancer metastases in the lung and survival of the tumor-bearing mice.

2. BODY

2.1 Specific Aims and Statement of Work

The specific aims of this research are (1) To determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites; (2) To determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases will induce an anti-tumor immune response. The approved Statement of Work is as follows:-

Task 1: Months 1-24.

Determine (a) whether IV-injected, interleukin-2 gene-modified murine lung endothelial cells (IL-2/MLECs) can target sites of pulmonary metastases of breast cancer, and (b) how well IL-2/MLECs can express the IL-2 transgene at the metastatic sites.

a. Mouse lung endothelial cells (MLECs) will be isolated and enriched using FDG-FACS. The cells will be transduced with a retroviral vector containing human IL-2 gene.

b. Efficiency of IL-2/MLEC incorporation at different tumor sites:
   - Co-localization of IL-2/MLEC and tumor in animals: three experiments; 40 animals per experiment.

c. Determination of toxicity of IV IL-2/MLEC administration:
   - Acute toxicity following a single dose of 10^5 IL-2/MLEC administration
   - Cumulative toxicity following 3 IV injections of 10^5 IL-2/MLECs spaced 3-4 days apart.

Three experiments; 40 animals per experiment.

d. Optimization of IL-2/MLEC incorporation in tumor sites:
-Tumor-bearing animals will receive three IV injections of IL-2/MLECs closely (3-4 days) or widely (5-7) apart. Expression of IL-2 transgene at the metastatic sites determined by RNA PCR amplification of human IL-2 in discrete individual metastases. Four experiments; 40-50 animals per experiment will be performed.
-Comparison of the relationship between different administration schedules with the number of cells incorporated at sites of tumor metastases will be determined. Two experiments; 40 animals per experiment will be performed.

<u>Task 2:</u> **Months 24-36.**

Determine whether the expression of hIL-2 transgene at the local site of pulmonary metastases site will induce an anti-tumor immune response. Groups of experimental of experimental and control animals will be sacrificed weekly to monitor hIL-2 expression in the lungs, quantify metastastic foci, and to assess lung tumor response to IL-2/MLEC
treatment. One group of the experimental and control animals will be observed over time for survival. Survivors will receive additional MFP injection of 4T1 cells to determine their ability to reject tumor re-challenge.

2.2 Major Research Accomplishments

Overview
During the previous three years (1998-2001), we completed all the experiments outlined in the statement of work (SOW). In the past year, we generated murine microvascular endothelial cells expressing IL-12 transgene (IL-12/GMEC) and performed preliminary studies of the effect(s) of IV-injected, IL-12/GMEC on long-term survival of mice with lung metastases breast cancer.

MATERIALS AND METHODS

Animals
Female BALB/c mice, six to eight weeks of age, and purchased from the National Cancer Institute Animal Program (Frederick, MD) were used for this study. Protocols for husbandry and experimental manipulation were approved by the Georgetown University Animal Care and Use Committee. All mice were housed in a pathogen-free environment, and National Institutes of Health-Centers for Disease Control biosafety level 2 containment procedures were observed. This mouse strain was used these studies in order to ensure that the mice were syngeneic for both tumor cell lines and endothelial cells, thereby avoiding the immunological rejection of either tumor cell implants or the gene-modified endothelial cells.

Endothelial cell isolation
Mouse microvascular endothelial cells (MEC) were isolated from the lungs of BALB/c

mice using a multi step method as previously reported (6). Endothelial cells obtained retained differentiated endothelial cell makers, including cobblestone morphology and expression of acetylated-low density lipoprotein (Ac-LDL) receptor and von Willebrand Factor. Endothelial cells at passages 3 or 4 were used for subsequent studies.

Generation of endothelial cells expressing neomycin resistance and murine IL-12 trangenes

Endothelial cells expressing exogenous genes for neomycin phosphotransferace resistance (*NeoR*) and murine interleukin (mIL) 12 (rmIL-12/GMEC) were generated as previously reported (5). Dr. Michael T. Lotze generously provided a TFG-mIL-12-*neo* retroviral vector that was used in these studies. This vector expresses both p35 and p40 subunits of murine IL-12 as well as the *NeoR* genes from a polycistronic message utilizing internal ribosome entry site sequences (5). To generate the rmIL-12/GMEC, retroviral supernatant was first generated by transfecting TFG-mIL-12-*neo* proviral constructs into CRIP packaging cell line (5). The titer of the Crip TFG-mIL-12 producer cell line used in the subsequent experiments was 5 x 10^4 CFU/ml. To generate rhIL-2-GMEC, microvascular endothelial cells (MEC) were harvested from lungs of BALB/c mice and transduced with helper-free, culture supernatants of the Crip TFG-mIL-12 producer cell line. Twenty four hours prior to infection, MEC at passage 3 or 4 were seeded onto fibronectin-coated 100 mm plastic dishes at a 1:2-1:3 split ratio. Following overnight culture of the cells, the medium was replaced with 5 ml of amphotropic viral stock containing either the rmIL-2 gene (TFG-mIL-12-*Neo* construct) or the parent vector with only the neomycin resistance (*NeoR*) gene (MFG-*Neo*) and 8μg/ml polybrene to promote viral attachment to cells. The dishes were returned to the incubator and an additional 5 ml of fresh endothelial cell growth medium was added after two to four hours. Subsequently, the dishes were incubated overnight and then refed with complete growth medium. Colonies were isolated by selection in G418 100μg/ml, the minimum concentration of G418 that was required to kill all cultured endothelial cells (data not shown). After selection in G418, cell populations were expanded and characterized for rmIL-12 production as well as maintenance of endothelial cell phenotype. Recombinant mIL-12 protein secretion *in vitro* by the rmIL-2-GMEC was assayed from 24-hour culture supernatant of the cells.

The rmIL-12 + *NeoR*- and *NeoR* gene-expressing endothelial cells (rmIL-12-GMEC and *Neo*-GMEC, respectively) retained the endothelial cell phenotype, including cobblestone morphology, uptake of acetylated-low density lipoprotein (Ac-LDL), and expression of von Willebrand factor. No replication-competent helper virus was detected in the media of twenty-five passages in each of the gene-modified endothelial cells.

Quantitation of secreted rmIL-12 protein in endothelial cell culture media

Recombinant murine interleukin 12 protein secreted by cytokine gene-transduced and non-transduced endothelial cells were assayed in the culture media of the cells by enzyme-linked immunoabsorbance (ELISA) assay (Quantikine M murine IL-12 p70) (QuantikineTM R&D Systems, MN), following the manufacturer's instructions. The range of sensitivity of the ELISA assay was 2.5-500 pg/ml for IL-12. The bioactivity of IL-12 produced *in vitro* by IL-12 gene transduced MMEC was determined by the proliferative response of day 4 PHA-activated

lymphoblasts as previously described (5). IL-12 levels were expressed as nanograms/10^6/24 h. Recombinant mIL-12 secretion *in vitro* by the rmIL-12-GMEC ranged from 32-85 ng/10^6 cells/24h

Establishment of pulmonary metastases of mammary adenocarcinoma

The 4T1 tumor cell line (provided by Fred R. Miller, Michigan Cancer Foundation), was derived from a spontaneously-arising mammary tumor in a BALB/c mouse. The cells were harvested from dishes while in their exponential growth phase in culture with 0.1% trypsin/EDTA, washed once with culture medium and twice with PBS. They were counted electronically using a Coulter counter (Coulter Counter, Model ZB, Coulter Corporation, FL, USA) and suspended in PBS at a concentration of 10^6 cells/ml. More than 98% of the cells in suspension were singlets and more than 90% were viable based on their ability to exclude trypan blue. To establish pulmonary metastases of 4T1 tumors, the 4T1 cell line was first implanted in syngeneic BALB/c mice. The primary tumors were established by injecting 1 X 10^5 4T1 cells suspended in 0.1ml of phosphate buffered saline into the first right thoracic mammary fat pad (MFP) of the mice. Two weeks following the inoculation of 4T1 cells into the MFP, when many pulmonary metastases are 1- 2 mm in diameter, i.e., a point at which they begin to acquire a new blood supply (7), the primary tumors were completely excised, and mice were randomized into three groups. One group of mice was treated with three sequential tail vein injections of 1 x 10^5 hIL-12/GMEC in 0.1 ml PBS. The second group received three sequential tail vein injections of 10^5 *Neo*-GMEC in 0.1 ml PBS, and the third group was given three sequential tail vein injections of 0.1 ml PBS without GMEC. The recombinant mIL-12 secretion *in vitro* by the rmIL-12-GMEC ranged from 32-85 ng/10^6 cells/24h. Administration of the rmIL-12/GMEC was started on the third day following surgical excision of the primary tumor, and the injections were spaced 3 days apart. Three groups of non-tumor-bearing mice were also studied. These consisted of non-tumor-bearing mice given three sequential tail vein injections of 105 hIL-2-GMEC or *Neo*-GMEC, and non-tumor-bearing mice without GMEC. After GMEC administration, the animals were monitored for survival. Lungs of mice that died or sacrificed when they became ill were removed, processed and examined for number and size of pulmonary metastases. Otherwise, groups of mice were sacrificed at periodic intervals after the administration of the GMEC and various tissues, including individual lung metastasis were harvested and processed for rmIL-12 gene expression using DNA polymerase chain reaction (PCR) and reverse transcriptase (RT-PCR) techniques. Histology, and immunocytochemistry were also performed. The lungs and individual tumors were examined to follow the rate of growth or regression of the lung metastases, rmIL-12 gene expression, and the nature of cellular infiltrate of the tumors.

Histological and immunocytochemical analysis of explanted tumor metastases

Tissue from the site of tumor inoculation as well as individual metastatic foci in the lungs were excised, fixed in 10% formal-saline and embeded in paraffin wax, sectioned at 4 μm., and stained with hematoxilin- and eosine. For immunocytochemistry, tissues were embeded in OCT compound (Miles Laboratory, Elkert, IN), snap-frozen in liquid nitrogen, and preserved at -80oC until sectioning. 5-μm cryostat sections were fixed in acetone and immunostained with purified rat mAb to: CD45 (MI/9.3.4 HL2 hybridoma, T200), CD8 (53.6.72 hybridoma, Lyt 2), CD4

(GK1.5 hybridoma, L3T4), anti-MAC-1 (MI/70.15.11.HL hybridoma), MAC-3 (M37/84.6.34 hybridoma:ATCC) and mouse granulocyte (RB6-8C5 hybridoma: Pharmigen, San Diego, CA). These were preincubated with rabbit serum to prevent nonspecific binding, and sequentially incubated with the optimal dilutions of the various mAb for 1 h, a rabbit anti-rat IgG (Organon-Teknica, Turnhout, Belgium) for 30 min, and a rat peroxidase antiperoxidase (Abbot Laboratories, North Chicago, IL) for 1 h. Each incubation step was followed by a 5-min Hanks balanced salt solution (HBSS) wash. The sections were finally incubated with 0.03% hydrogen peroxide (H_2O_2) and 0.06% 3.3' -diaminobenzidine (BDH Chemicals, Poole, United Kingdom) for 3 to 5 min. The slides were then washed for 5 min in running tap water, counterstained with hematoxilin for 1 min, and mounted in Canadian balsam. Endogenous peroxidase was inhibited by pretreatment with 0.01% v/v H_2O_2. Neutrophils did not show the inhibition-resistant endogenous peroxidase activity typical of eosinophils. Control slides were obtained by omitting the mAb to control the rabbit ant-rat Ig cross-reactivity, and using rat ant-human primary antibodies of the same IgG subclass. The percent of immunostained over total cells was determined at 400 x magnifications on five microscope fields with the help of a 1-mm square grid. Neutrophils were characterized by segmented or doughnut nuclei. Also, the presence of the typical oval granule with central rectangular crystalloid in the cytoplasm of the eosinophils allowed them to be unmistakably distinguished from neutrophils. Quantitative and semi-quantitative determination of the cellular infiltrate in the individual tumor specimen was performed. Hematoxilin- and eosine-stained sections from frozen tissue blocks were examined and the number of lymphocytes, granulocytes and macrophages per high-power fields scored.

RESULTS

Microvascular endothelial cell expression of mIL-12 transgene

The level of IL-12 secretion by various transduced cells is shown in Table 1. Cultured murine microvascular endothelial cells (MMEC) expressed recombinant mIL-12 gene in a stable fashion *in vitro*. All MMEC infected with TFG-mIL-12-*Neo* secreted significant amounts of rmIL-12 protein *in vitro*. MMEC transduced with the parent MFG-*neo* vector exhibited a pattern of mIL-12 expression that was similar to the parent (non-transduced) cells. Another interesting observation was that the rmIL-12 produced was both immunologically and biologically active, suggesting that splicing events between IRES domains did not occur, and that endothelial cells could produce and post-translationally process both subunits of IL-12. Furthermore, rmIL-12-expressing MMEC retained endothelial cell differentiated marker, including cobblestone morphology and expression of acetylated-low density lipoprotein (Ac-LDL) receptor and von Willebrand Factor, suggesting that endothelial cell expression of exogenous IL-12 gene may not effect the expression of their endogenous genes.

Table 1: Recombinant murine interleukin 12 (rmIL-12) production by parental and rmIL-12 gene-transduced mouse microvascular endothelial cells (MMEC)

Cell	Populations	IL-12 ($ng/10^6$ cells/24h)[a]
MMEC	Polyclonal	0
Neo-MMEC	Polyclonal	0
IL-12-MMEC	Polyclonal	40.4
IL-12-MMEC	Oligoclonal #	
	1	32.1
	2	44.1
	3	58.6
	4	74.2
	5	85.2

[a]The bioactivity of IL-12 produced in vitro by IL-12 gene transduced MMEC was determined by the proliferative response of day 4 PHA-activated lymphoblasts as previously described (5). IL-12 levels were expressed as nanograms/10^6/24 h.

Effects of IV-injected mIL-12/GMEC on mice with lung metastases of breast cancer

As has been observed in our previous studies (1, 3, and 2000 report), the lungs of untreated tumor-bearing mice and tumor-bearing mice treated with endothelial cells containing the empty vector (Neo-MMEC) had numerous tumor foci. All these mice died within four weeks of tumor inoculation. In contrast, only two of the ten tumor-bearing mice treated with the mIL-12/GMEC died within six weeks of tumor inoculation. The lungs of these mice contained tumor foci that were too numerous to count. As at the present time (nine weeks after tumor inoculation), all the remaining 8 tumor-bearing mice treated with the mIL-12/GMEC are still alive and appear to be well. Furthermore, we have not observed any tumor in the normal mice, which were given the same three IV injections of mIL-12/GMEC

Tumors that were recovered from the tumor bearing, mIL-12/GMEC-treated mice that died were all negative for rmIL-12, suggesting that these tumors may not have been targeted by the mIL-12/GMEC.

DISCUSSION

In our previous reports, we demonstrated ability of circulating hIL-2-GMEC to accumulate and express rhIL-2 at the local sites of breast cancer metastases (3, 8) or melanoma (1) *in vivo*. The presence of rhIL-2 protein at these sites was associated with a significant reduction of the established metastases. We have also shown that both macrovascular and microvascular endothelial cells expressing human IL-2 transgene can induce potent NK cell and CTL activation (9), and that the expression of recombinant hIL-2 gene at the local sites of lung metastases of breast cancer induced an increased infiltration of neutrophils, macrophages, and

both CD4+ and CD8+ lymphocytes. The presence of these cells and their distribution within and around the tumor mass suggest that they may be involved in the rhIL-2/GMEC-mediated inhibition of the growth of breast cancer *in vivo* (3, 8). This is supported by similar findings during IL-2-mediated regression of variety tumors *in vivo* (10-17), and the ability of IL-2 to stimulate and activate these immune effector cells (9, 17-19). Of interest, the administration of hIL-2-secreting GMEC did not produce any observable toxicity, such as body weight loss and lethargy, which is frequently reported in animals after systemic administration of IL-2 therapy (17). Furthermore, systemic hIL-2-GMEC did not produce any tumor in normal mice or increase tumor burden in tumor-bearing mice (1), suggesting that this strategy is potentially safe.

Although preliminary, our latest results suggest that IV-injected mIL-12/GMEC can prolong the survival of mice with advanced breast cancer.

Together, these data demonstrate that systemically administered endothelial cells expressing therapeutic transgene(s) can target foci of breast cancer metastases. Moreover, the genetically modified endothelial cells can express the transgenes at the tumor sites and induce an anti-tumor immune response. Thus, this strategy is a potentially safe and effective strategy to treat metastatic diseases. These findings provide evidence for endothelial cell-based gene therapy of advanced breast cancer and support for similar studies in a clinical trial.

2.3 Plans for the Future

We will continue our on-ongoing evaluation of the therapeutic efficacy of systemically administered IL-12/GMEC in mice with metastatic breast cancer and publish our findings in the near future. Subsequently, we plan to submit a proposal to the Department of Defense to study the effects of endothelial cells expressing a combination of immune activating and tumor suppressor transgenes on breast cancer metastases in mice. We also plan to determine the therapeutic efficacy and safety of systemically administered endothelial cells expressing a combination of human interleukin 2 and p53 transgenes in mice with metastatic breast cancer before undertaking a clinical trial of endothelial cell-based gene therapy of metastatic breast cancer.

3. Key Research Accomplishments

We have:

a) Isolated pure population of lung endothelial cells from BALB/c mice. The cells have been transduced with a retroviral vector containing human IL-2 gene and high expressing clones have been isolated and fully characterized.
b) Determined the efficiency of hIL-2/MLEC incorporation into sites of breast cancer metastasis.
c) Optimized hIL-2/MLEC incorporation into sites of pulmonary metastasis of breast cancer.
d) Determined acute and cumulative toxicity of IV-administered hIL-2/MLECs.
e) Established that the expression of hIL-2 transgene at the local site of pulmonary metastases

site can induce an anti-tumor immune response *in vivo.*

f) Determined the level the nature, level, and duration of anti-tumor immune response that is induced at the local tumor site following IV administration of hIL-2/MLEC.

g) Determined the cellular mechanisms involved in IL-2/GMEC-mediated regression of breast cancer metastases in mice.

h) Generated interleukin (IL) 12 transgene-expressing endothelial cells and performed preliminary studies of their therapeutic utility in a mouse mammary adenocarcinoma model.

i) Published six abstracts and one manuscript of some of our study results. Another manuscript is under revision for publication in *Cancer Research.*

j) Obtained an NIH-RO1 grant award based on our findings to further develop and refine our systemic gene therapy strategy.

4. Reportable Outcomes

A) Manuscripts:

1. Ojeifo JO, Lee HR, Vezza P, Su N, Zwiebel JA (2001). Endothelial cell-based systemic gene therapy of metastatic melanoma. Cancer Gene Therapy. 8(9):636-648.
2. Ojeifo JO, Notario V, Herscowtiz HB, Zwiebel JA. Interleukin-2 gene-modified endothelial cell therapy of metastaic breast cancer in a murine model (Under revision)

B) Abstracts:

1. Hill J, Wu AG, Ojeifo JO, Miao Y, Herscowitz H, Meehan KR (1998) Immunologic effects of IL-2 post transplant in breast cancer. Proceedings of ASH, vol. 17:365b. Abstr. #4574.
2. Ojeifo JO, Vezza P, Kallakhury B, Lippman ME (1998). Interleukin-2 gene-modified endothelial cell treatment of metastatic breast cancer in mice. Proceedings of Gene Regulation and Cancer, AACR's special conference, Hot Springs, Virginia, October 1998 (abstr. # B-14).
3. Hannum RS, Ojeifo JO, Zwiebel JA, McLeskey SW (1999). RNA from endothelial cells isolated from xenograft tumors produced by control MCF-7 cells or MCF-7 cells transfected with angiogenic factors consistently contains transcripts for isoforms of FGF receptors 1 and 2 but not VEGF **but** not VEGF receptors. Proceedings of AACR, vol. 40:455 (abst. # 3007).
4. Ojeifo, JO, Wu AG, Miao Y, Herscowtiz HB, Meehan KR (1999). Docetaxel mobilization of hematopoietic stem cell in mice: Kinetics, dose titration, and toxicity profile. Proceedings of AACR, vol. 40: 721 (abst. # 4764).
5. Ojeifo JO, Herscowitz HB and Zwiebel JA. (2000). Interleukin 2 gene-modified endothelial cell targeting of breast cancer metastasis in mice. Proceedings of the Department of Defense Breast Cancer Research Program Meeting. Era of Hope. vol. II: 650.
6. Ojeifo JO, Herscowitz HB and Zwiebel JA. (2002). An endothelial cell-based gene therapy approach for treatment of breast cancer metastases. Proceedings of the Department of Defense Breast Cancer Research Program Meeting. Era of Hope. Orlando, Florida, September 25-28, 2002.

C) Grant awards:

Agency: National Cancer Institute, NIH.
Grant number: R01 CA093495-01
Grant title: Combined Gene Therapy for Metastatic Breast Cancer.
PI: John O. Ojeifo
Duration: 01/01/02 – 12/31/06

5. Conclusions

These results suggest that systemic administration of genetically modified endothelial cells expressing cytokine genes is a potentially effective and safe strategy to target and treat metastases of breast cancer. The approach may be particularly useful for targeting recombinant therapeutic molecules to sites of macrometastases throughout the body.

References

1. Ojeifo JO, Lee HR, Rezza P. Su N, Zwiebel, JA. (2001). Endothelial cell-based systemic gene therapy of metastatic melanoma. *Cancer Gene Therapy 8(9)*: 636-648.
2. Ojeifo JO, Herscowitz HB, Zwiebel JA. Interleukin-2 gene-modified endothelial cell targeting of breast cancer metastases mice. Proceedings of the U.S. Department of Defense (DOD) Breast Cancer Research Program (BCRP) Era of Hope meeting at Hilton Atlanta and Towers, Atlanta, Georgia, June, 2000. (abstr. # AA-15).
3. Ojeifo JO, Vezza P, Kallakhury B, Lippman ME. Interleukin-2 gene-modified endothelial cell treatment of metastatic breast cancer in mice. Proceedings of Gene Regulation and Cancer, American Association For Cancer Research's special conference, Hot Springs, Virginia, October 1999 (abstr. # B-14).
4. Tahara H, Zitvogel L, Storkus WJ, Zeh III HJ, Robbins PD, Lotze MT (1995). Effective Eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J Immunol* 154:6466-6474.
5. Forni G., Cavallo F., Consalvo M., Allione A., Dellabona, P., Casorati G.,and Giovarelli, M. Molecular approaches to cancer immunotherapy. Cytokines and Molecular Therapy 1:225-246, 1995.
6. Ojeifo JO, Su N, Ryan US., Verma, U.N., Mazumder, A., and Zwiebel, J.A. Towards endothelial cell-directed cancer immunotherapy: *In vitro* expression of human recombinant cytokine genes by human and mouse primary endothelial cells. *Cytokines and Molecular Therapy* 2: 89-101, 1996.
7. Barnhill RL, Piepkorn MW, Cochran AJ, et al. Tumor vascularity, proliferation, and apoptosis in human melanoma micrometastases and macrometastases. *Arch Dermatology* 1998; 134: 991-994 and 1027-1028.
8. Ning, Su; Ojeifo JO, MacPherson, A., and Zwiebel, JA. (1994).Breast cancer gene therapy: transgenic immunotherapy. Breast Cancer Research and Treatment 31:349-356.
9. Ojeifo JO, Su N, Ryan US, Verma, UN, Mazumder A, and Zwiebel, J.A. Towards endothelial cell-directed cancer immunotherapy: *In vitro* expression of human recombinant cytokine genes by human and mouse primary endothelial cells. *Cytokines and Molecular Therapy* 2: 89-101, 1996.

10. Forni G, Giovarelli M, and Satoni A. Lymphokine-activated tumor inhibition in vivo1: The local administration of interleukin 2 triggers nonreactive lymphocytes from tumor bearing mice to inhibit tumor growth. J Immunol. 139; 3933-3940, 1985.
11. Forni G, Giovarelli M, Satoni A, Modesti A, and Forni M.. (1987). Interleukin-2 activated tumor inhibition in vivo depends on the systemic involvement of host immunoreactivity. *J. Immunol.*138: 4033
12. Pickaver AH, Ratcliffe NA, Williams AE, and Smith H.(1972). Cytotoxic effects of peritoneal neutrophils on syngeneic rat tumor. *Nature New Biol.* 235: 187-.
13. Fady C, Reisser D, and Martin F. (1990). Non-activated rat neutrophils kill syngeneic colon tumor cells by the release of low molecular weight factor. *Immunobiology* 18:1-
14. Midorikawa Y, Yamashita T, and Sendo F. (1990). Modulation of the immune response to transplantation tumors in rats by selective depletion of neutrophils in vivo using a monoclonal antibody. *Cancer Res.* 50:6243.
15. Colombo MP, Ferrari G, Stoppacciaro A, Parenza M, Rodolfo M, Mavilio F, and Parmiani D. (1991).Granulocyte-colony-stimulating factor gene transfer suppresses tumorigenecity of a murine adenocarcinima in vivo. *J. Exp. Med.* 173:889-..
16. Musiani P, De Campors E, Valittutti S, Castellino F, Calearo C, Cortesuina G, Giovarelli M, Jemma C, De Stefani A, and Forni G. (1989). Effect of low doses of interleukin 2 injected perilymphatically and peritumorarily in patients with advanced primary head and neck squamous cell carcinoma. *J. Biol Resp. Modif.* 8: 571-.
17 Rosenberg, S.A (1997): Principles of Cancer Management: Biologic Therapy. In DeVita VT.Jr., Hellman S, Rosenberg SA (eds): Cancer: Principles & Practice of Oncology, Fifth Edition, J.B. Lippincott-Raven Publishers, Philadelphia.pp 349-375.
18. Salvadori S, Gansbacher B, Pizzimenti AM, et al. (1994).Abnormal signal transduction by T cells of mice with parental tumors is not seen in mice bearing IL-2-secreting tumors. *J. Immunol* 153: 5176-5182.
19. Porgador A, Feldman M, Eisenbach L. (1994). Immunotherapy of tumor metastases via gene therapy. *Nat Immun.* 13: 113-130.

Appendices: None