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PRINCIPAL INVESTIGATOR: Howard A. Fine, M.D.

CONTRACTING ORGANIZATION: Dana Farber Cancer Institute
Boston, Massachusetts 02115

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I. INTRODUCTION
The growth of most solid tumors, including breast carcinoma, is highly dependent of the development of vascular structures to support the increasing need of the tumor for nutrients and oxygen, a process called angiogenesis. In adults, physiologic angiogenesis occurs only during limited times thus making angiogenesis a rather tumor-selective process, and therefore, a promising therapeutic target for cancer therapy. It has been recently demonstrated that endothelial progenitor cells (EPCs), or angioblasts, can be easily detected and isolated from the peripheral blood of adult mice and humans. It can be shown that following systemic injection into the circulation of an animal, EPCs can migrate to areas of angiogenesis. We, therefore, hypothesized that EPCs, first transduced in vitro with a prodrug activating or anti-angiogenic gene and then re-injected back into the host, could be utilized as a selective tumor targeting “vector” by migrating into, and delivering the therapeutic gene to areas of tumor-associated angiogenesis.

We initially proposed experiments designed to answer three basic questions:

1.) Can EPCs migrate to sites of tumor-mediated angiogenesis following in vitro expansion and re-injection into the systemic circulation;

2.) Can EPCs be transduced by retroviral and/or adenoviral vectors in vitro, and what are the optimal conditions for efficient transduction;

3.) Can EPCs transduced with prodrug activating/suicide or anti-angiogenic genes mediate a selective anti-tumor effect following systemic delivery.

Significant progress has been made toward answering many of these questions as summarized below:

II. BODY
Please find below our "Statement of Work" for the proposed experiments as outlined in our original proposal. As you will see, we have remained true to this outline plan and we are relatively on schedule toward completing our goals.

Task 1: To establish whether endothelial cells (EC) and/or endothelial progenitor cells (EPC) migrate to sites of tumor mediated angiogenesis (months 1-12).

- Establish human xenograft breast cancer model in immunodeficient animals:
We have established both a MDA-MB-435, and MCF-7 subcutaneous breast cancer as well as a murine TA3/st mammary peritoneal carcinomatosis model in immunodefficient animals. The models are now consistently predictable for tumor growth rate and time to animal death.

- **Determine whether retroviral transduced human umbilical vein endothelial cells (HUVE) will migrate to, and become incorporated into tumor microvasculature following systemic injection:** Although HUVEC cells could be easily transduced by retroviral vectors carrying marker genes, we could not document migration of HUVECs to sites of tumor angiogenesis following either systemic (tail vein) or local (intra-tumoral) administration despite what has been previously published (by a single group). Although we cannot be certain why this is so, it is our belief that the fully differentiated HUVECs do not possess the same chemotaxic properties that undifferentiated EPCs do.

- **Isolate adherent, Flk-1 positive mononuclear cells in whole blood from β-galactosidase (β-gal)-transgenic mice:**
- **Determine whether Flk-1 positive, adherent whole blood mononuclear cells (presumed EPCs)**

We and others have now demonstrated that FLK-1 positive cells from the whole blood of β-galactosidase transgenic animals can migrate to, and be incorporated into the growing vasculature of subcutaneous tumor implants.

**Task 2:** Determine whether EPCs can be transduced by retroviral (RV) and/or adenoviral (AV) vectors, and establish the optimal conditions for high efficiency transduction:

- **Isolate human EPCs from whole blood by use of anti-CD34 antibody and anti-Flk-1 antibody coated magnetic beads, and then selecting for doubly positive adherent cells.**

We obtained discarded alloquots of CD34+ cells from patients and volunteers treated with G-CSF. Immunophenotyping of these cells on day 0 revealed that 100% cells were CD34+ and approximately 50% were CD45+. We found that CD34/Flk-1 double positive cells are quit rare within the leukopacks with <0.1% of CD34+ cells expressing Flk-1. Additionally, other endothelial specific markers including E-selectin and Tie-2 were generally not expressed on these cells. Less specific markers such as CD31 and Flt-1 were found on approximately 90% and 10% of cells, respectively. We performed a series of experiments in an attempt to define the optimal growth conditions for the few EPCs within the CD34+ population. To date, our data demonstrates that CD34+ cells plated on fibronectin-coated cell culture plates, grown in RPMI1640 medium (GIBCO) containing 20% fetal bovine serum with a mixture of endothelial mitogenic growth factors (i.e. VEGF, bFGF) supplemented with GM-CSF yields the largest amount of EPCs. By day 7 under these culture conditions, approximately 30% of these cells were now Flk-1+, 45% were Flt+, 28% were Tie-2+, and 10% were E-selectin positive. The results of the immunophenotyping remained essentially unchanged for cells in culture for at least 3 weeks (the longest time period we investigated). The vast majority of adherent Flk-1+ cells had taken a spindle shape, although nearly 20% of the non-adherent cells grown under these selective conditions were also Flk-1, and Tie-2 positive.

We demonstrated the endothelial nature of these cells through their efficient formation of capillary tube-like structures in vitro, and their positive response in a chemoattractant migration assay to
VEGF and bFGF. Finally, we demonstrated endothelial-selective uptake of DiI-conjugated acetylated low density lipoprotein by the EPCs.

- **Transduce isolated EPCs with b-galactosidase expressing RV and AV vectors at various multiplicity of infection units (MOIs), and determine efficiency of transduction and the level of b-gal expression:**

  - **Determine the optimal conditions for efficient EPC transduction in vitro by varying the transduction conditions, including the extracellular matrix and the cytokines the EPCs are exposed to before, during, and after vector transduction:**

We have transduced our EPCs with E1/E3-deleted adenoviral vectors carrying both the beta-galactosidase and the green fluorescence protein gene. At MOIs of 50-100 we can efficiently transduce nearly 100% of all EPCs without significant vector-induced cytotoxicity. We have also demonstrated efficient Maloney-based retroviral transduction of the EPCs using both the MFG and LCNX vectors. We have evaluated both amphotropic and xenotropic murine envelopes, and although both allow for EPC transduction, we have found that the LCNX vector pseudotyped by the g-protein from the vesicular stomatitis virus (VSV) gives the highest efficiency EPC transduction. Finally, we have optimized retroviral transduction conditions which include the use of fresh (uncultured) CD34+ cells incubated for two days with a cytokine mixture including IL3, IL6, SCF, and VEGF. These pre-incubated CD34+ cells are then placed on fibronectin coated cell culture plates that have had retroviral vectors pre-loaded onto them. The cells are left in contact with the pre-loaded fibronectin for no more than 12 hours, and the procedure is repeated twice. This procedure results in >40% transduction efficiency of Flk-1 positive cells. We continue, however, to further optimize the protocol in an attempt to yield even higher levels of transduction efficiency. Within the last month, we have also demonstrated the ability of our in vitro retroviral transduced EPCs to migrate to sites of tumor-associated angiogenesis following systemic injection of the transduced cells.

Below is an outline of the work we plan to embark on over the next 12 months:

**Task 3: To evaluate whether EPCs transduced with prodrug activating (PDA)/suicide and/or anti-angiogenic genes mediate anti-tumor effects following systemic injection (months 12-36).**

  - **Human EPCs will be transduced by RV and AV vectors containing PDA genes (cytosine deaminase, cytochrome P4502B1, and deoxycytidine kinase), and in vitro cytotoxicity curves will be generated following exposure to the appropriate prodrug (months 24-36).**

  - **EPCs transduced by PDA-containing RV and AV vectors will be systemically administered to immunodeficient mice with established human breast cancer xenografts, and the anti-tumor effect of this strategy will be assessed following treatment of the animal with the appropriate prodrug. Optimization of the anti-tumor effect will also be performed by assessing the maximal amount of EPCs that can be safely injected, and evaluating the best timing of prodrug administration following EPC injections (months 24-36).**

  - **Construction and in vitro evaluation of the tetracycline repressible, platelet factor 4 or angiostatin (anti-angiogenic genes) expressing retroviral vectors (months 24-30).**
- In vivo evaluation of the antitumor activity of the EPCs transduced by the repressible anti-angiogenic vectors (months 30-36).
- Evaluation of the anti-tumor efficacy of combination therapy with EPCs transduced with PDA expressing vectors, followed by EPCs transduced by anti-angiogenic genes (months 30-36).

III. CONCLUSIONS:
We believe we have made significant progress toward our goal of utilizing endothelial cells as angiogenic-targeted genetic vectors. We have demonstrated our ability to isolate cells from both the bone marrow and peripheral blood of mice and humans that have the phenotypic and functional properties of endothelial progenitor cells. We have demonstrated our ability, and optimized the conditions to genetically manipulate these cells in vitro using both adenoviral and retroviral vectors. Finally, we have demonstrated the ability of these genetically manipulated vectors to migrate to sites of tumor-mediated angiogenesis and incorporate into tumor neovascularity. Thus, we have generated proof of principle that the strategy of endothelial progenitor cells as genetic vectors and angiogenesis-targeted gene therapy is feasible. We will spend the next several years attempting to optimizing the in vitro conditions of vector transduction and progenitor cell propagation. Finally, we will begin the process of evaluating the anti-tumor efficacy of various cytotoxic and cytostatic genes in the context of the endothelial progenitor cells. These studies will lay the framework for pilot studies of this strategy in humans.