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PRINCIPAL INVESTIGATOR: Shuang Huang, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

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Shuang Huang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, California 92037

E-Mail: shuang@scripps.edu

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13. ABSTRACT (Maximum 200 Words)
Angiogenesis is essential for the growth and metastasis of solid tumors including breast cancer. In vitro and in vivo experimental models clearly demonstrate that suppressing angiogenesis leads tumor suppression. The overall goal of this proposal is to develop an adenovirus-based gene therapy approach for suppressing angiogenesis. In first year of the funding period, we have successfully constructed a human endothelial cell-targeted adenovirus gene delivery vector. In second year of the funding period, we focused on efforts on constructing endothelial cell-targeted adenoviral vector containing therapeutic genes including soluble VEGF receptor (sFlk and sFlt) and dominant negative angiogenesis-essential signaling molecules including Raf-1 and PI-3K. All of these molecules were found to overexpressed in a substantial levels as determined by immunoblotting using specific antibodies. Transduction of sFlk and dominant negative Raf-1 individual significantly inhibited VEGF-induced human endothelial cell migration and in vitro angiogenesis. These results suggest that endothelial cell-targeted adenoviral vector can be used to deliver anti-angiogenesis agents to block angiogenesis. In our future studies, we will determine the efficacy of developed sFlk- and dominant negative Raf-1-containing adenoviral vectors to suppress breast tumor development in xenograph breast tumor models.

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Introduction

The survival, growth and metastasis of solid tumors including breast cancer depends on the formation of new blood vessels to provide tumors with nutrients and oxygen, a process called angiogenesis. In vitro and in vivo experimental models indicate that suppressing angiogenesis can also suppress solid tumors. However, the success of this approach largely depends on whether sufficient amounts of therapeutic agents can be delivered to tumor-associated endothelial cells without causing toxic effects to other tissues/cells. Our proposal is designed to develop an endothelial cell-targeted adenoviral vector and to use the targeted vector to express high levels of anticancer therapeutic genes in the sites of angiogenenic tumors specifically and efficiently.

Body

In the previous year, we have developed an endothelial cell-targeted adenoviral vector (adenovirus fiber was modified by inserting NGR peptide in its HI-loop). The goal of our research in the second year of this funding is to construct the endothelial cell-targeted adenoviral vector carrying anti-angiogenesis genes. To achieve this goal, we first subcloned genes encoding soluble VEGF receptor (sFlk and sFlt) or dominant negative angiogenesis-essential signaling molecules [MEK1(-) and PI-3K(-)] into adenoviral shuttle vector pShuttle-CMV. These plasmids were co-electroporated with fiber gene-deleted pAd.Easy-1 into recombinant-competent BJ5183 E.coli strain. The resulted colonies were analyzed for correct adenovirus recombination and the plasmids with correct recombination then individually transfected into NGR-fiber-expressing 293 cells. To determine the abilities of these adenoviral vectors to deliver anti-angiogenesis genes in human endothelial cells, human umbilical vascular endothelial cells (HUVECs) were infected with various amount of recombinant adenoviruses for 48 hr. The expression of sFlk and sFlt was determined by immunoblotting in the 2-day conditional media. Figure 1A clearly shows that enhanced levels of sFlk secretion with increased amount of Ad

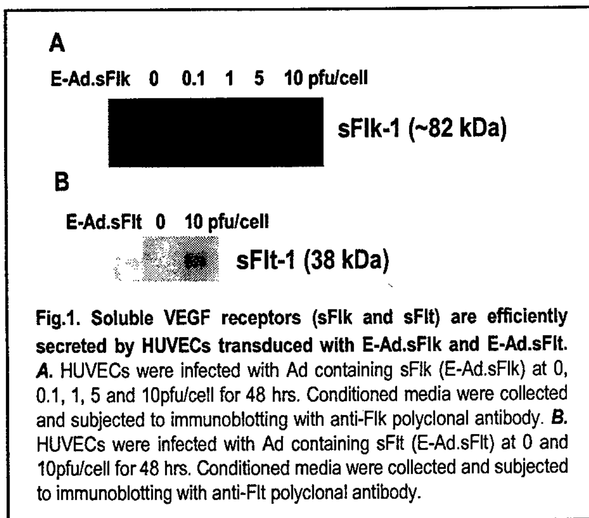

Fig.1. Soluble VEGF receptors (sFlk and sFlt) are efficiently secreted by HUVECs transduced with E-Ad.sFlk and E-Ad.sFlt. ***A.*** HUVECs were infected with Ad containing sFlk (E-Ad.sFlk) at 0, 0.1, 1, 5 and 10pfu/cell for 48 hrs. Conditioned media were collected and subjected to immunoblotting with anti-Flk polyclonal antibody. ***B.*** HUVECs were infected with Ad containing sFlt (E-Ad.sFlt) at 0 and 10pfu/cell for 48 hrs. Conditioned media were collected and subjected to immunoblotting with anti-Flt polyclonal antibody.

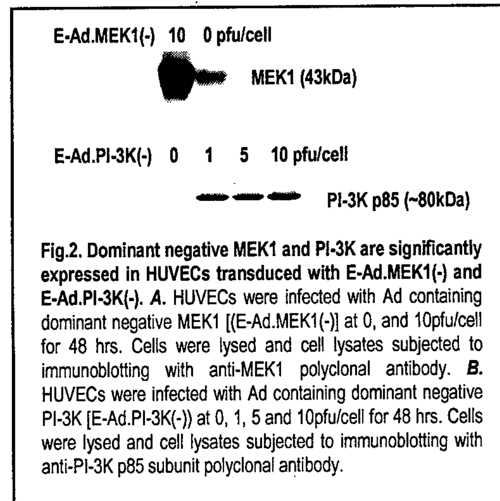

Fig.2. Dominant negative MEK1 and PI-3K are significantly expressed in HUVECs transduced with E-Ad.MEK1(-) and E-Ad.PI-3K(-). ***A.*** HUVECs were infected with Ad containing dominant negative MEK1 [(E-Ad.MEK1(-)] at 0, and 10pfu/cell for 48 hrs. Cells were lysed and cell lysates subjected to immunoblotting with anti-MEK1 polyclonal antibody. ***B.*** HUVECs were infected with Ad containing dominant negative PI-3K [E-Ad.PI-3K(-)) at 0, 1, 5 and 10pfu/cell for 48 hrs. Cells were lysed and cell lysates subjected to immunoblotting with anti-PI-3K p85 subunit polyclonal antibody.

(0.1-

10 pfu/cell). Figure 1B shows that sFlt was detectable in media with 10 pfu/cell Ad infection. The expression of dominant negative MEK1 and dominant negative PI-3K (the SH2 domain of the p85 subunit) was detected with 2-day Ad-transduced cell lysates. Overexpression of MEK1 and the SH2 domain of the p85 subunit could be readily observed in Ad-transduced HUVECs (Fig.2). These results demonstrate that our developed targeted adenoviral vector can be used to efficiently transduce anti-angiogenesis gene into human endothelial cells.

To determine the effect of adenovirus-delivered anti-angiogenesis genes in VEGF-induced human endothelial cell migration, HUVECs were infected with each individual adenoviral construct for 48 hrs and then subjected to migration assay using Transwell as we previously described. VEGF at 10ng/ml induced over 3-fold of cell migration above the basal cell migration (Fig.3). Expression of sFlk and dominant negative PI-3K almost completely blocked VEGF-induced cell migration (Fig.3). In contrast, expression of sFlt did not affect VEGF-induced cell migration and dominant negative MEK1 displayed only slight inhibition in VEGF-induced cell migration (Fig.3). These results suggest that both soluble VEGF receptor sFlk and dominant negative PI-3K can be used to inhibit VEGF-induced human endothelial cell migration.

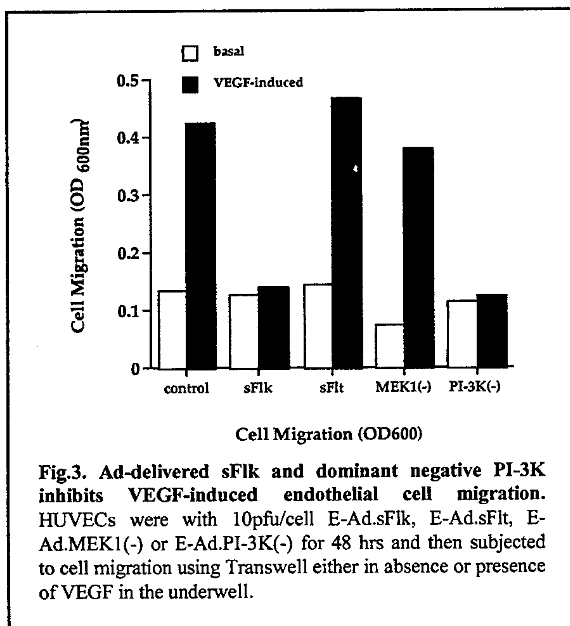

Fig.3. Ad-delivered sFlk and dominant negative PI-3K inhibits VEGF-induced endothelial cell migration. HUVECs were with 10pfu/cell E-Ad.sFlk, E-Ad.sFlt, E-Ad.MEK1(-) or E-Ad.PI-3K(-) for 48 hrs and then subjected to cell migration using Transwell either in absence or presence of VEGF in the underwell.

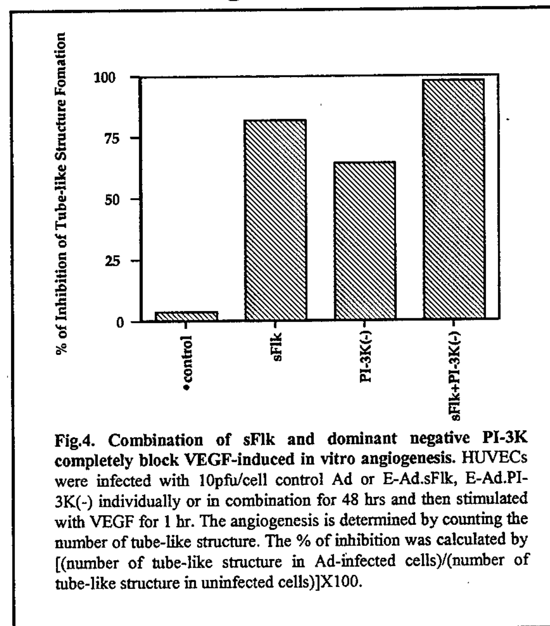

Fig.4. Combination of sFlk and dominant negative PI-3K completely block VEGF-induced in vitro angiogenesis. HUVECs were infected with 10pfu/cell control Ad or E-Ad.sFlk, E-Ad.PI-3K(-) individually or in combination for 48 hrs and then stimulated with VEGF for 1 hr. The angiogenesis is determined by counting the number of tube-like structure. The % of inhibition was calculated by [(number of tube-like structure in Ad-infected cells)/(number of tube-like structure in uninfected cells)]X100.

We next examined the ability of Ad-delivered sFlk and dominant negative PI-3K to inhibit VEGF-induced angiogenesis. HUVECs were infected with control Ad or Ad containing sFlk or dominant negative PI-3K individually or in combination for 48 hrs and then 10ng/ml VEGF added to HUVECs. The extent of angiogenesis in these cells was determined by examining their ability to form a tube-like structure. While tube-like structure was readily observed with VEGF-stimulated uninfected and control Ad-infected cells, cells infected with sFlk- or dominant negative PI-3K-containing Ad vector exhibited approximately 80% and 65% less tube-like structure (Fig.4). Cells expressing both sFlk and dominant negative PI-3K failed to form a tube-like structure. These results

suggest that VEGF-induced angiogenesis can be efficiently blocked by the combination of soluble VEGF receptor sFlk and dominant negative PI-3K.

Key Research Accomplishment

- We have constructed human endothelial cell-targeted Ad vectors containing sFlk and dominant negative PI-3K.
- We infected human endothelial cells with these two Ad vectors resulting in complete inhibition in VEGF-induced endothelial cell migration and angiogenesis.

Reportable Outcomes

One accepted and one submitted manuscript was partially supported by this grant:

Bian, D., Han, Q., Su, S., Mahanivong, C., Cheng, R.K., Pan, Z.K., Sun, P., and **Huang, S.** (2004). Lysophosphatidic acid stimulates ovarian cancer cell migration via a Ras-MEK kinase 1 pathway. *Cancer Res.*, in press.

Su, S., Han, Q., and **Huang, S.** (2004). Expression of urokinase receptor is regulated by interaction with urokinase plasminogen activator. Submitted.

Two other manuscripts currently in preparation are also partially supported by this grant.

Conclusions

We have generated endothelial cell-targeted Ad vectors containing sFlk and dominant negative PI-3K. In our experiments, we found that these vectors can suppress VEGF-induced human endothelial cell migration and angiogenesis. With continued proposed in vivo studies in the third year, we should be able to validate whether our approach represents an alternative therapeutic modality for breast cancer treatment.

References:

None

Appendices:

None