Independent Study Project Proposal
University of California, San Diego

Title of Project: Protein Kinase A gene therapy: Inhibition of angiogenesis and tumor metastases.

Abstract

Angiogenesis, the process of generating new blood vessels from pre-existing vessels, is highly regulated in normal physiologic states. The loss of regulatory control in angiogenesis is characteristic of many pathologic conditions including solid tumor cancers and their metastases.[3,10,11] Many advances have been made to develop techniques to inhibit angiogenesis and to thereby limit tumor growth. For example, recent studies demonstrated that activation of the enzyme protein kinase A (PKA) leads to the inhibition of endothelial cell migration as well as angiogenesis. This project aims to evaluate the effectiveness of the delivery of the activated form of PKA via gene therapy as a method to inhibit migration of endothelial cells *in vitro* and angiogenesis *in vivo*. In this project, a recombinant adenovirus containing the catalytic domain of PKA will be generated. Upon virus production, infected endothelial cells will be analyzed to confirm cAMP-mediated inhibition of migration on various ECM proteins, including fibronectin, vitronectin, and collagen. Furthermore, expression of the activated form of PKA *in vivo* through gene therapy will be evaluated for its potential to limit tumor angiogenesis and tumor growth. A recombinant adenovirus containing the regulatory type I domain of PKA will also be generated as a means to show that it is indeed the catalytic domain of PKA alone which carries the ability to suppress migration and angiogenesis.

Background

Angiogenesis is a process of neovascularization whereby new blood vessels are derived from pre-existing vessels.[11] This process involves endothelial cell proliferation, migration and maturation with simultaneous changes in the extracellular matrix (ECM).[5] Angiogenesis is a normal physiologic process under stringent regulation; the process is

controlled by a balance of positive and negative angiogenic regulators. The growth of new blood vessels is involved in specific physiologic processes: embryonic development, wound healing, the female reproductive cycle and inflammation. With the exception of cyclic angiogenesis that occurs during the female reproductive cycle, in mature organisms, factors that inhibit angiogenesis predominate and thus vascular endothelial cells usually remain quiescent.[3,11] Only upon stimulation by ischemic or hypoxic states in damaged or diseased tissues does angiogenesis proceed.[11,12]

However, angiogenesis also plays a key role in many pathologic conditions: solid tumor cancers and their metastases; ocular diseases including diabetic retinopathy and age-related macular degeneration; and inflammatory diseases such as psoriasis, rheumatoid arthritis, osteoarthritis and inflammatory bowel disease.[11] Tight regulatory control characteristic of neovascularization in normal physiologic states is lost in pathologic processes in which angiogenesis plays a role.[3,10]

Understanding the critical role of angiogenesis in tumor development and metastases has been groundbreaking and has led researchers towards the development of innovative approaches for the treatment of cancer. Early tumors begin as a few cell layers about 1mm in diameter. In order to survive and to grow beyond this size, solid tumors must establish their own blood vessels;[3,5,11] angiogenesis is initiated by growth factors and cytokines that are released from the tumor or from inflammatory cells that invade the tumor.[10,11] Such angiogenic factors include VEGF, basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF-α), and others.[11] The vascularization of tumors promotes not only their survival and growth, but also their metastases. Metastatic spread of cancer originates from primary tumor cells that enter the tumor's vasculature and subsequently are carried away to local and distant sites. Thus, using the density of microvessels within a tumor biopsy as a measurement of the extent of angiogenesis is a powerful prognostic tool for the identification of the tumor stage and its metastatic potential.[8,11]

Numerous studies have been done to understand and to dissect the molecular processes of angiogenesis in cancer growth and metastases. Many studies have already shown that a number of angiogenic growth factors, including bFGF, VEGF, IL-8 and TNF-α are significantly up-regulated in human tumors.[5,11] The role of these angiogenic

factors has been emphasized by many studies showing increased tumorigenicity when levels of these factors are elevated by transfection and reduced tumorigenicity when levels are decreased by specific antibodies or anti-sense techniques.[2,3,5,11] In fact, the very blood vessels feeding tumors show many differences from quiescent vessels; one difference being the up-regulation of receptors for angiogenic factors on angiogenic endothelial cells allowing such cells to proliferate up to 50 times more than quiescent vascular endothelial cells.[5]

More recently, there has been a different target for the inhibition of angiogenesis: the integrin family of cell adhesion receptors. The invasion, migration and proliferation of endothelial cells and vascular smooth muscle cells during vascularization is regulated by integrins. Integrins bind to ECM proteins or cell-surface immunoglobulin family molecules. Not only do they facilitate cellular adhesion and migration on ECM proteins, integrins also regulate cellular entry into and withdrawal from the cell cycle. Coordination of integrins with their respective ECM protein ligands induces a cascade of intracellular signals. Likewise, prevention of integrin-ligand interactions suppresses cellular growth or induces apoptosis.[11]

Integrin $\alpha V\beta 3$ and integrin $\alpha 5\beta 1$ have shown to be powerful regulators of angiogenesis and have played integral roles in tumor survival and metastases.[6,7,9,10,11,12] Stimulated endothelial cells depend on $\alpha V\beta 3$ function for survival during a critical period of angiogenesis, as inhibition of $\alpha V\beta 3$-ligand interaction by antibody or peptide antagonists induces vascular cell apoptosis and inhibits angiogenesis.[11] Recent studies indicate that angiogenesis also depends in part, on the ligation of $\alpha 5\beta 1$ by the central cell-binding domain of fibronectin. Evidence also indicates that integrin $\alpha 5\beta 1$ indirectly potentiates integrin $\alpha V\beta 3$-mediated migration on a ECM protein, vitronectin.[6,7]

Moreover, it has now been concluded that $\alpha V\beta 3$-mediated endothelial cell migration and angiogenesis is highly regulated by PKA activity. This has led to an additional target for inhibition of angiogenesis involved in tumor growth and metastases. Experiments using agents that activate intracellular PKA, such as forskolin, dibutyryl cAMP and $\alpha 5\beta 1$ antagonists, have been shown to effectively suppress endothelial cell migration on vitronectin *in vitro* or angiogenesis *in vivo*. In addition, inhibition of PKA has pro-migratory or pro-angiogenic effects. Thus, expression of activated PKA through

gene therapy is now being evaluated as an effective approach towards inhibition of tumor angiogenesis.[7]

Today, with a better appreciation of the critical dependence of tumor progression on neovascularization, it is rational to hypothesize that suppression of this rate-limiting step could suppress the growth of a wide range of tumor types. Possible strategies include the neutralization of the endothelial cell growth factors produced by tumor cells or the inhibition of their interaction with endothelial cells; inhibitors of protease secretion or function which facilitate cellular migration through basement membrane proteolysis; peptide inhibitors, non-peptidic organic inhibitors and antibody inhibitors of integrins; and expression of activated PKA. [5,11] Gene transfer strategies specifically offer great promise in that they have the potential to provide sustained, high, local concentrations of anti-angiogenic mediators specifically targeted to organs with tumors, minimizing systemic side effects. In a low tumor burden state, anti-angiogenesis gene therapy strategies are predicted to be able to provide *trans* suppression of the growth of endothelial cells in the context of micrometastases.[1] Clinical trials with anti-angiogenesis agents are in their infancy, but agents acting at almost every step of angiogenesis are being tested and offer great promise towards future cancer therapy. [5]

Experimental Procedures

The latest approach to inhibit angiogenesis is through the expression of activated PKA in endothelial cells *in vivo* via gene therapy. Various techniques to deliver the activated PKA gene into endothelial cells have been hypothesized: introduction of naked DNA containing the activated PKA expression construct; delivery of the activated PKA expression construct using a liposome, which is DNA surrounded by a lipid coat; and delivery of the gene using a viral vector. Researchers in Dr. Varner's lab have already successfully expressed PKA in endothelial cells *in vivo* through introduction of naked DNA containing the activated PKA expression construct, thereby inhibiting angiogenesis. However, for clinical therapeutic purposes, viral vectors are likely to be the most effective and useful. Therefore, the main objective of this project will be to inhibit angiogenesis through the expression of activated PKA using adenoviral vectors to deliver the gene. Liposome-based delivery will be examined as an alternative technique.

This past summer, I worked to subclone the PKA gene into an adenovirus vector. Basically the procedure involved cloning of catalytic PKA cDNA and type I regulatory PKA cDNA into their respective adenoviral transfer vectors and co-transformation of these cDNA vectors with the adenoviral genome into competent bacterial cells. After selection of the recombinant adenoviral vectors (replication incompetent), they were screened by PCR, plasmid size and restriction enzyme analysis. Further work will be done to propagate and to amplify the positive recombinants in competent bacterial cells for the preparation of transfection quality DNA. The purified recombinant plasmids will then be transfected into stable viral packaging cells and plated for plaque formation. Next, plaques will be screened to determine if they are the expected recombinant viruses containing the PKA gene using various techniques: Western blot, immunoassay, PCR or Southern blot. Finally, a large-scale virus amplification will be performed followed by virus purification and titration.

After recombinant viruses containing either the PKA catalytic (PKA cat) or PKA type I regulatory (PKA RI) gene have been successfully constructed, the viruses will be used to express PKA both *in vitro* and *in vivo*. *In vitro*, the recombinant virus (1. empty virus = control; 2. PKA cat containing virus = experimental; 3. PKA RI containing virus = negative control) will be used to infect endothelial cells in culture and to observe its effect on cell migration. (Studies have already been performed where agents that activate PKA have suppressed endothelial cell migration).[7] To observe the effects of PKA on migration, migration assays will be performed as described. The underside of a membrane in a migration well will be coated with the ECM protein of interest. Endothelial cells (HUVEC) that were starved in serum-free media will be harvested. After adding 100,000 cells to the upper chamber of the migration well, the cells will be allowed to incubate at 37°C for 3-4 hours to allow the cells to migrate from the upper to the lower chamber. Cells will be removed from the upper chamber, and the membrane will be placed into an empty well for staining with 0.5% Crystal Violet in 50mM borate, pH 9.0, 2% ethanol. After washing, the cells that have migrated across the membrane will be counted.[7]

In ovo chick chorioallantoic membrane angiogenesis assays will be used to show that activated PKA gene expression limits angiogenesis. Angiogenesis assays will be

conducted as described. Chorioallantoic membranes (CAMs) of 10-day-old embryonated chicken eggs will be treated with filter discs saturated with growth factors that have been shown to be angiogenic. The recombinant virus containing no gene (empty virus as control), the PKA cat gene, or the PKA RI gene (negative control) will be applied onto the growth factor saturated filtered discs or injected intravascularly. CAMS will be harvested and evaluated in terms of mean number of blood vessel branch points to assess angiogenic potential. [7]

Most importantly, we would like to assess the expression of PKA via gene therapy and its potential to inhibit angiogenesis and thereby effectively limit tumor growth and metastases. To prove tumor growth inhibition by PKA expression, CAM tumor assays will be performed as outlined. [6] Basically, tumor cells will be placed on the surface of each CAM and cultured for 1 week. The resulting tumors will be excised and cut into 50 mg fragments. The fragments will be placed on additional CAMs and systematically injected intravenously with a recombinant PKA virus (1. empty virus = control; 2. PKA cat containing virus = experimental; 3. PKA RI containing virus = negative control). Forty-eight hours later, CAMs will be excised from the egg and the number of blood vessels entering the tumor will be counted. Subsequently, excised tumors will then be weighed to assess tumor growth or regression. [7]

Resources

I will be working directly with my faculty sponsor, Dr. Varner, and collaborating with a technician, Delphine Gros, to complete my research project. I have already completed some of the work described above as part of the NIH Summer Research Program. I will continue to visit Dr. Varner's weekly for the rest of this year, and I will maintain a working relationship with Dr. Varner and Delphine Gros to gather new data and to make a final conclusion.

Final Presentation

The final format of my independent study project will be a written report. It will include my original objectives and a presentation of my findings.

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