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13. ABSTRACT (Maximum 200 Words) Antiangiogenic therapy of cancers targets their blood vessels in an effort to deprive tumors of oxygen and nutrients. Results from human trials have been poorer than results from mouse testing. This disparity may be explained by more extensive coverage of vessels in human cancers, like breast cancer, by protective periendothelial mesenchymal cells (pericytes), rendering these vessels more therapy resistant. Mouse mammary tumor virus (MMTV)-induced mammary carcinomas reproduce the extensive pericyte coverage of tumor vessels seen in human breast cancers. This project will determine whether inhibiting activation of the endothelial cell Tie2 receptor reduces vessel pericyte coverage in MMTV-induced tumors and improves response to antiangiogenic agents. To do this, we are creating tumors with doxycycline-inducible expression of Tie2Ex, an inhibitor of Tie2 activation. Prior to doing this in mice that develop MMTV-induced mammary carcinomas, which will require transgenic introduction of regulatory components and the Tie2Ex gene, we are first developing and testing doxycycline-inducible Tie2Ex expression in transfectable K1735 tumors. Progress has been made towards the creation of these tumors and testing can soon begin on the effects of Tie2Ex expression on K1735 tumor vessel pericyte coverage and response to antiangiogenic therapy.				
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Proposal Title: Targeting Tie2 to Increase Breast Cancer Responsiveness to Antiangiogenic Therapy

Introduction

Antiangiogenic therapy of cancers involves targeting tumor blood vessels in an effort to deprive tumors of vital oxygen and nutrients. The potential benefits of antiangiogenic strategies have been dramatically shown in mouse tumor models. Results emerging from human clinical trials, however, have been less striking. A potential explanation for this disparity in treatment outcomes is that the vasculature of human tumors may be more resistant to antiangiogenic therapies. This may be due, at least in part, to extensive pericyte coverage of vessels in many common human cancers, such as breast cancers, compared to a relative paucity of pericytes surrounding vessels in commonly studied mouse tumors. Pericytes are periendothelial mesenchymal cells that surround capillaries, are thought to be a marker of vessel maturity and stability, and may confer resistance to certain antiangiogenic agents. Mouse mammary tumor virus (MMTV)-induced mammary carcinomas arising in C3H/HeN mice may more faithfully model human breast cancer vasculature inasmuch as vessels in these tumors have extensive pericyte coverage like in human breast cancers. Interestingly, we found these tumors were resistant to rIL-12 antiangiogenic therapy, which was effective against every other mouse tumor model tested. Evidence suggests that the endothelial-specific receptor tyrosine kinase, Tie2, regulates pericyte coverage of tumor vasculature in transplanted mouse tumor models. Studies in this IDEA award will examine whether inhibiting Tie2 activation diminishes tumor vessel pericyte coverage in transplanted mouse tumors and MMTV mammary tumors and enhance their susceptibility to antiangiogenic therapy. We will first develop an inducible system for inhibiting Tie2 activation using the extracellular domain of Tie2 as a decoy receptor in K1735 melanoma cells. These are easily transfectable tumor cells and produce tumors with well-characterized vasculature. Once the inducible system has been validated in this system, it will be brought into MMTV-induced mammary carcinomas using transgenic approaches.

Body

Task 1. Determine whether blocking Tie2 reverses pericyte coverage in K1735 tumors.

- a. Develop K1735 cell line that inducibly expresses Tie2Ex (Months 0-4).

We chose the tetracycline (doxycycline)-inducible ("Tet-On") system for regulating Tie2Ex expression in tumors. Tie2Ex is the soluble, extracellular domain of the Tie2 receptor, a decoy receptor for Tie2 ligands and an inhibitor of Tie2 activation. To achieve its regulatable expression in tumors, we had to engineer tumor cells to constitutively express the reverse tetracycline transactivator (rtTA), which activates transcription of genes under the control of a tetracycline-response element (TRE) only in the presence of doxycycline. Cells expressing rtTA must then be transfected with the Tie2Ex gene under TRE control. Properly engineered cells should not express Tie2Ex when there is no Dox present, should turn on Tie2Ex expression within 24 hours when Dox is added and should lose Tie2Ex expression when Dox is removed.

We obtained the CMV-rtTA(neo) and TRE(hygro) plasmid from BD Bioscience. When we transfected K1735 tumor cells with CMV-rtTA(neo), we obtained many G418-resistant clones, but even the best clones gave only very weak luciferase (luc) expression when they were transiently transfected with a TRE-luc plasmid and Dox was present in the cell culture media. Luminescence was generated by adding luciferin to the cells and detected using a Xenogen IVIS luminescence imaging system. We investigated the inadequate level of Dox-induced expression of genes under TRE control and noted two problems: (a) the CMV promoter in CMV-rtTA(neo) did not provide sufficient rtTA expression in K1735 melanoma cells, and (b) the rtTA clone provided by BD Bioscience has a pyrimidine in the -3 nucleotide position upstream of the ATG initiation codon. By Kozak consensus "rules", a purine in the -3 position is needed for efficient translation of rtTA. Accordingly, we performed PCR mutagenesis of the rtTA clone to substitute a purine for the pyrimidine in the -3 position and moved the altered rtTA (rtTA*) into the pEF2 expression vector, which uses the promiscuous and strong eIF1 α promoter/enhancer (which we know is very active in K1735 cells) to drive transcription of inserted genes.

We generated clones of K1735 cells stably transfected with pEF2-rtTA*. Many of these had very

high level expression of luc following transient transfection of TRE-luc and growth in Dox. On the basis of level of luc expression, we selected 3 individual clones of K1735.rtTA* cells for subsequent analysis and transfection. To determine which was the best, we (a) stably transfected each of these clones with TRE-luc to determine which clone had the best operating characteristics and (b) injected 2×10^6 cells into syngeneic C3H/HeN mice to check for tumorigenicity (clones that had altered tumorigenic properties would not be very useful for the subsequent tumor studies we had planned). Clones of doubly transfected (pEF2-rtTA* + TRE-luc) cells were obtained by hygromycin selection and subsequently analyzed for Dox-regulable expression of luc. We looked for clones that provided (a) no luc expression in the absence of Dox, (b) rapid induction of high-level luc expression in the presence of 1 ug/ml Dox, (c) intermediate levels of luc expression in lower concentrations of Dox (e.g. 0.05-0.2 ug/ml), (d) sustained expression in the continued presence of Dox, and (e) rapid extinction of luc expression when Dox is removed from the medium. Several clones derived from K1735.rtTA* clone 39 fulfilled these criteria, so this clone has been chosen for transfection with TRE-Tie2Ex, and this transfection is underway.

In the meantime, to study the *in vivo* operating characteristics of our Dox-inducible system, we have injected into C3H/HeN mice cells from one clone of TRE-luc-transfected K1735.rtTA*(39) cells (K1735.rtTA*/luc cells). Once tumors arise, we will study induction and deinduction of luc expression *in vivo* by placing Dox in the drinking water and using the Xenogen IVIS system to image luc expression in mice following their injection with luciferin. The K1735.rtTA*(39) cells transfected with TRE-Tie2Ex will be screened by Northern blotting for Dox-inducible Tie2Ex expression. This should happen in the next two weeks as clones are emerging from hygromycin selection. Clones showing appropriate regulation will be ready for Task 1b.

- b. Determine if Tie2Ex reverses pericyte coverage of vessels in K1735 tumors (Months 5-8).

This will be underway shortly but was delayed by several months due to problems with the performance of plasmids we obtained from BD Bioscience, analysis of source of the problems, and finding solutions to the problems. Fortunately, we were able to identify the problems, manipulate the clones to achieve better expression, and operating characteristics. We had previously obtained preliminary data showing that constitutive, high level expression of Tie2Ex in K1735 tumors reduces tumor vessel pericyte coverage. We anticipate that inducible expression of Tie2Ex in these tumors will produce the same effect, except that now we should be able to dictate when and to what level the Tie2Ex is expressed.

Task 2. Determine whether Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors.

- a. Develop transgenic mice that inducibly express Tie2Ex (Months 5-16).
- b. Cross mice that inducibly express Tie2Ex with MMTV infected C3H mice (Months 17-24).
- c. Determine if transgenically expressed Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors (Months 24-36).

This Task should proceed quickly once we determine that the TRE-Tie2Ex provides inducible expression of Tie2Ex in cells expressing rtTA and that Tie2Ex reduces pericyte coverage in K1735 tumors. An investigator at the University of Pennsylvania, Dr. Lewis Chodosh, has mice that express rtTA in mammary glands due to a MMTV-rtTA transgene that he introduced into their germline. He has agreed to give us these mice in a collaborative study. We would create TRE-Tie2Ex transgenic mice and cross our strain with his MMTV-rtTA mice to obtain MMTV-rtTA x TRE-Tie2Ex double transgenics that should express Tie2Ex in mammary tissue and their tumors when Dox is placed in their drinking water. MMTV-induced mammary carcinomas arising in these mice should have Dox-inducible expression of Tie2Ex and allow us to study the effect of Tie2Ex on pericyte coverage of vessels in these tumors.

Task 3. Determine if Tie2Ex increases responsiveness of MMTV-induced breast tumors to antiangiogenic therapy (Months 24-36).

This Task will naturally proceed once Task 2 is accomplished.

Key Research Accomplishments

Creation of K1735.rtTA* cells with good expression of rtTA* providing absent basal (-Dox) expression of genes under the transcriptional control of TRE and excellent induced (+Dox) expression of genes under the transcriptional control of TRE.

Reportable Outcomes

None

Conclusions

Inducible expression of Tie2Ex should be attainable using the system chosen.

References

None

Appendices

None