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14. ABSTRACT Antiangiogenic therapy of cancers targets tumor blood vessels to deprive malignant cells of oxygen and nutrients. Therapy of human cancers has produced poorer results than therapy of mouse tumors, a disparity that may be explained by more extensive coverage of human tumor vessels (e.g. in breast cancers) by pericytes, which may be rendering 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 and are relatively refractory to antiangiogenic therapy compared to other mouse tumors. This project attempts to decrease pericyte coverage of vessels in these and other tumors by manipulating activity of endothelial cell (EC) Tie2 receptors and improve tumor response to antiangiogenic therapy. We created K1735 tumors and transgenic mice that inducibly express Tie2Ex, an inhibitor of EC Tie2 activation, under doxycycline regulation. Dox-nduced Tie2Ex expression in K1735 tumors reduces tumor vessel pericyte coverage and causes tumor EC death, vessel regression and tumor stasis. We created double transgenic mice that express Tie2Ex in mammary tissue under Dox.					
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Proposal Title: Targeting Tie2 to Increase Breast Cancer Responsiveness to Antiangiogenic Therapy

Introduction

Antiangiogenic therapy of cancers involves inhibiting tumor blood vessel development to deprive tumors of vital oxygen and nutrients. The potential benefits of antiangiogenic strategies have been dramatically shown in mouse tumor models. Results in human clinical trials, however, have been less striking. Recent trials have shown survival benefits, but tumor regression, which is often reported in murine tumors, is rarely seen in treated human cancers. 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 deficiency of pericytes surrounding vessels in commonly studied mouse tumors. Pericytes are periendothelial mesenchymal cells that surround capillaries. Their presence is associated with microvessel 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 MMTV-induced mammary carcinomas were resistant to rIL-12 antiangiogenic therapy, which was effective against every other mouse tumor model tested (Lee JC et al., *Canc. Res.* 62: 747-755. 2002).

The endothelial-specific receptor tyrosine kinase, Tie2, regulates microvessel pericyte coverage, as well as activating endothelial cell (EC) signal transduction pathways that promote their survival (e.g. the PI3 kinase-AKT signaling pathway). Studies proposed in this IDEA award examine whether inhibiting Tie2 activation diminishes pericyte coverage and apoptosis-resistance in tumor vessels in transplanted mouse tumors and MMTV mammary tumors and enhance their susceptibility to antiangiogenic therapy. Our strategy was to develop an inducible system for inhibiting Tie2 activation by expressing the extracellular domain of Tie2 (Tie2Ex) 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 for Tie2Ex expression was validated in this system, we would introduce it into MMTV-induced mammary carcinomas using transgenic manipulation of mice.

Body of Report

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

- a. Develop K1735 cell line that inducibly expresses Tie2Ex

The tetracycline (doxycycline)-inducible (“Tet-On”) system was chosen for regulating expression in tumors of Tie2Ex, the soluble, extracellular domain of the Tie2 receptor that acts as a decoy receptor for Tie2 ligands and inhibits Tie2 activation. We transfected K1735 tumor cells with a plasmid (CMV-rtTA, purchased from BD Bioscience) 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. Unexpectedly, expression of rtTA was poor in all transfected clones of K1735 cells we studied. Examination of the sequence of CMV-rtTA plasmid revealed the presence of a suboptimal translation initiation (Kozak consensus) site. Site-directed mutagenesis to create an optimal Kozak consensus sequence markedly improved rtTA expression. Additional analysis of the expression problem revealed that CMV was a relatively weak promoter/enhancer in K1735 cells. Changing to the eIF1 α promoter/enhancer (in pEF2 plasmid) resulted in much higher expression. These measures solved our problem of poor rtTA expression but delayed progress by 4-6 months. Eventually, we identified a single clone of transfected K1735 cells, K1735.m39, with robust rtTA expression and supertransfected it with a TRE-Tie2Ex plasmid to generate a clone of doubly transfected cells, K1735.Tie2Ex^{ind}, that secreted virtually no Tie2Ex in the absence of Dox in the culture medium and secreted abundant Tie2Ex when 1 μ M Dox was

present. K1735.Tie2Ex^{ind} cells secreted intermediate levels of Tie2Ex when lower concentrations of Dox (e.g. 0.1 μ M) was added and shut off Tie2Ex expression when Dox was removed. Control K1735.m39hygro^r cells did not express Tie2Ex with or without Dox.

- b. Determine if Tie2Ex reverses pericyte coverage of vessels in K1735 tumors.

K1735.Tie2Ex^{ind} cells injected into syngeneic C3H/HeN mice formed tumors. Dox (2.0 mg/ml) was placed in the drinking water of the mice when their tumors reached 6-8 mm diameter, and tumors were removed for analysis at various days post-Dox (progress up to this point was reported in June 2005). Western blot analysis of tumor lysate showed that K1735.Tie2Ex^{ind} tumors from mice given 2.0 mg/ml Dox in their drinking water had abundant Tie2Ex (starting 1-2 days after Dox treatment), while those from mice not given Dox had no detectable Tie2Ex (Figure 1A). Control tumors had no Tie2Ex whether or not their host was given Dox. Dox treatment virtually arrested growth of K1735.Tie2Ex^{ind} tumors but did not affect growth of control tumors. After 14 days of Dox, tumors were mostly necrotic. After 7 days of Dox, tumors had evident areas of necrosis mixed with areas of viable tumor (Figure 1B). Tumors removed after 1 or 2 days of Dox had increased endothelial cell apoptosis detected by TUNEL staining and small focal areas of early necrosis. Control tumors treated with Dox grew progressively and did not show these changes. From this, we concluded that high level Tie2Ex expression results in tumor endothelial cell death and vessel regression that produces tumor necrosis over time. In apparently healthy regions of K1735.Tie2Ex^{ind} tumors treated with Dox for 7 days, there was no significant change in the mean vessel density (19.3 vessels/hpf in –Dox tumors vs. 16.8 vessels/hpf in +Dox tumors, $p>0.05$), but pericyte coverage of vessels was decreased (30% of vessels in –Dox tumors vs. 14% of vessels in +Dox tumors, $p<0.01$). Thus, pericyte coverage of vessels was reduced by Tie2Ex expression in K1735 tumors. This justified proceeding with our plans to test the effect of Tie2Ex expression in MMTV-induced mammary carcinomas by creating transgenic mice (see Task 2). In terms of Task 1, analysis of Tie2Ex effects are ongoing. We are studying the effects of purified Tie2Ex protein injected into tumors that have not been genetically manipulated. Using immunohistological staining, we are also studying effects of Tie2Ex expression on vascular endothelial cell PI3K-AKT and Raf-MEK-ERK signaling.

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.
- b. Develop MMTV-infected female mice that will develop mammary tumors that express Tie2Ex under Dox induction.
- c. Determine if transgenically expressed Tie2Ex blocks or reverses pericyte coverage of vessels in MMTV-induced breast tumors.

Initiation of this Task 2 was delayed by about 8 months because of the delay in Task 1 and a widespread outbreak of Mouse Hepatitis Virus in the Penn vivarium. This prevented establishment of new lines of transgenic mice until the outbreak was contained and eliminated in late 2004. We excised and purified the TRE-Tie2Ex transcription unit from its plasmid, and the Transgenic Mouse Facility at Penn used this to generate C3H/HeN founder mice that carried the TRE-Tie2Ex transgene. Lines that stably transmitted the transgene were identified and crossed with MMTV-rtTA transgenic mice of the FVB strain (provided by Lewis Chodosh, University of Pennsylvania), which express rtTA in mammary and certain other tissues. Double transgenic (MMTV-rtTA⁺, TRE-Tie2Ex⁺) F1 progeny were recently obtained and screened. Female double transgenic mice showed high-level Dox-inducible Tie2Ex expression in mammary glands and certain other organs (e.g. salivary glands) (Figure 2). To generate mice with MMTV-induced tumors that carry the MMTV-rtTA and TRE-Tie2Ex transgenes, we crossed FVB MMTV-rtTA male transgenic mice with C3H/HeN TRE-Tie2Ex female transgenic mice that are infected with mouse mammary tumor virus. Since both transgenic lines are heterozygous and only female mice develop MMTV-induced tumors, we are expecting 1 in 8 pups will be double transgenic females. These will be infected with MMTV virus via milk transmission and should develop mammary tumors in which Tie2Ex expression is induced by Dox. These double transgenic female mice will have to age 5-8 months before tumors develop. Once tumors develop in these mice, fragments of these tumors will be transplanted into C3H/HeN x FVB F1 recipient mice to passage and expand these tumors for study. Because of delays in this project stemming from earlier problems, we need extra time to complete the proposed Tasks and have requested (and received) a no-cost extension of the grant.

Task 3. Determine if Tie2Ex increases responsiveness of MMTV-induced breast tumors to antiangiogenic therapy.

Task 3 will proceed once Task 2 is accomplished and should be completed in the no-cost extension period.

Key Research Accomplishments

We created K1735.Tie2Ex^{ind} cells that engender tumors with robust Dox-inducible expression of Tie2Ex in vivo.

We determined that high-level Tie2Ex expression in tumors causes tumor vessel regression and tumor necrosis, suggesting that inhibiting Tie2 activity in tumor vessels is a potentially useful therapeutic approach.

We created TRE-Tie2Ex transgenic mice which, when crossed with MMTV-rtTA transgenic mice, yielded double transgenic progeny with robust Dox-inducible expression of Tie2Ex in mammary glands and certain other organs.

Reportable Outcomes (Publication bibliography)

Poster presentation (P19-9) at Era of Hope meeting, Philadelphia, PA (6/8/05 - 6/11/05).

No publications yet.

Personnel receiving pay: Jeff Tsai (graduate student)
 Stacey Jultine (research specialist)
 William Lee (principal investigator)

Conclusions

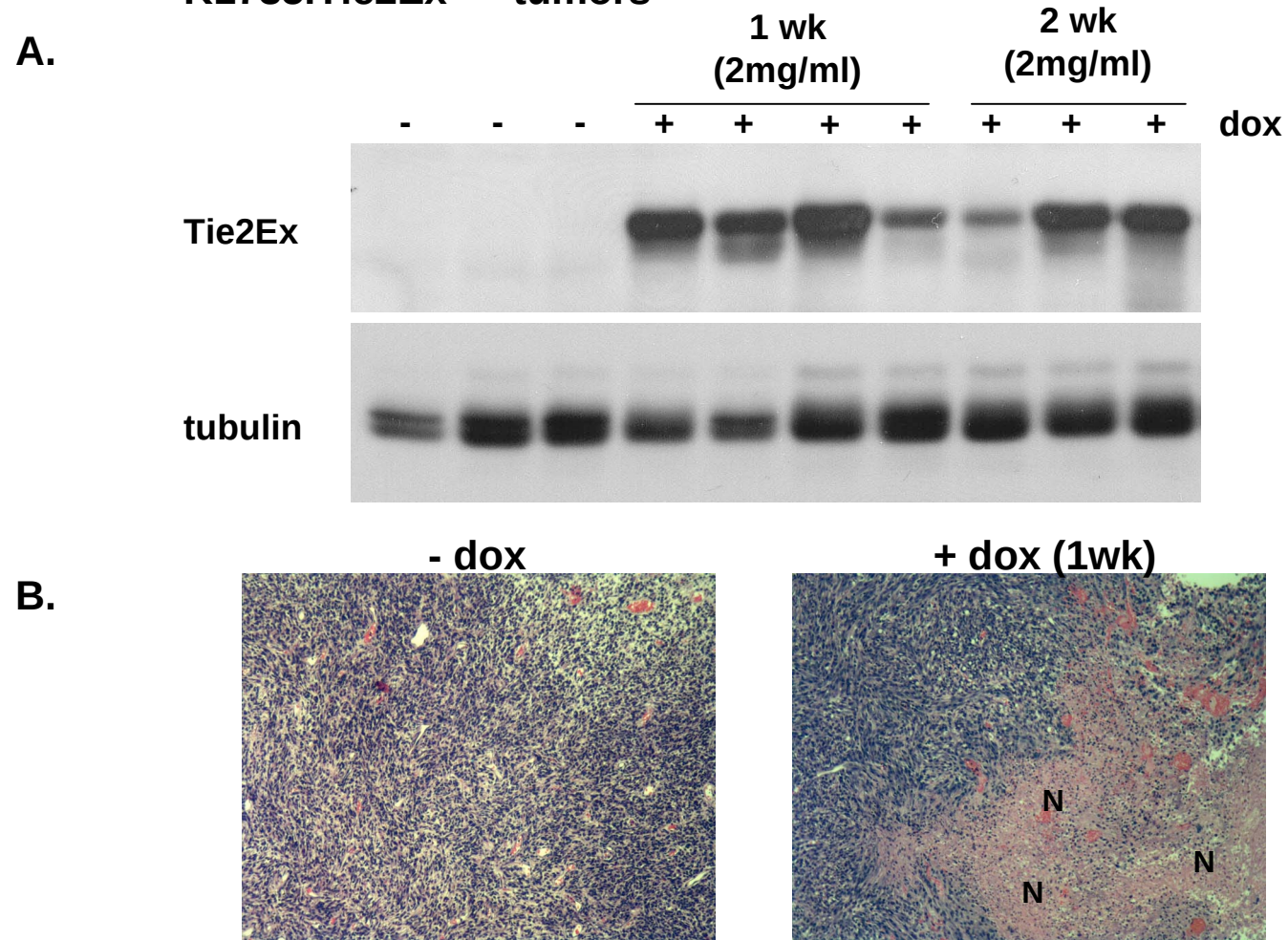
Expression of Tie2Ex in K1735 tumors induces vascular endothelial cell death, tumor vessel regression and tumor growth arrest.

Inducible expression of Tie2Ex in mammary carcinomas should be achieved using the system chosen.

References and Appendices

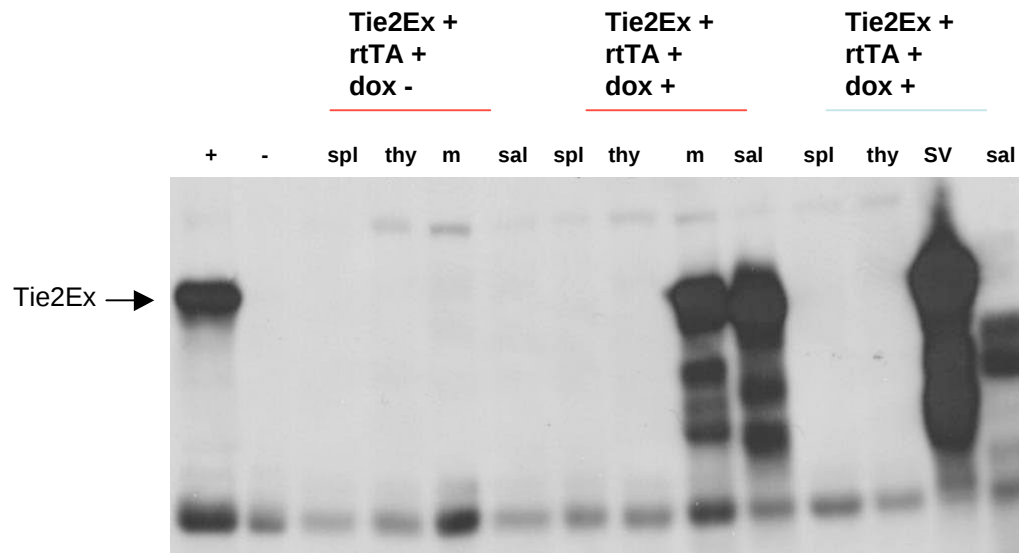
Presentation P19-9 at Era of Hope meeting, Philadelphia, PA (6/8/05 - 6/11/05).

Figure 1. Inducible Tie2Ex expression and effects of expression in K1735.Tie2Ex^{ind} tumors



Legend (A) Anti-Tie2 antibody western blot of K1735.Tie2Ex^{ind} tumor lysate taken from untreated mice or mice given Dox in their drinking water for 1 or 2 weeks; the blot was stripped and probed with anti-tubulin antibody to determine Lane loading. (B) H&E-stained tumor sections from an untreated mouse or a mouse given Dox for 1 week (N = necrosis).

Figure 2. Inducible expression of Tie2Ex in mammary glands of MMTV-rtTA x TRE-Tie2Ex double transgenic mice



Legend Organs were removed from MMTV-rtTA x TRE-Tie2Ex double transgenic mice treated (dox+) or not treated(dox-) with doxycycline (2mg/ml) in their drinking water for 2 days. Spleen (spl), thymus (thy), mammary glands (m), salivary glands (sal) and seminal vesicles (SV) were examined for Tie2Ex expression by SDS-PAGE/western blotting with anti-Tie2 antibody. Positive (+) and negative (-) Tie2Ex control samples were lysates fromTie2Ex-transfected and parental K1735 cells, respectively. Note dox-induced expression of Tie2Ex in mammary and salivary glands and in seminal vesicles in males.