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14. ABSTRACT –SEE ATTACHED PAGE

The goal of this CDA was to investigate the regulation of Endothelial precursor cells by endothelial cell specific cytokines in mammary tumors. Initial studies anticipated a critical role of VEGF and PlGF, based on their ability to promote survival signaling in endothelial cells. Our investigations also pointed to another cytokine-receptor family–the Tie-2 signaling pathway in EPCs in breast cancer and in survival signaling through the Akt pathway (1, 2). Ongoing studies have pointed to Akt as a common downstream signaling pathway that may mediate proliferation and homing of EPCs to mammary tissue and mammary tumors. Transgenic models are proposed to regulate Akt signaling and mammary tumor promotion to investigate the relationship between EPCs and mammary tumor angiogenesis

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angiogenesis, Tie-2, angiopoietins, endothelial stem cells

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Introduction:

In the previous annual report, we described the progress made on this grant that led to two publications which were reported and submitted as appendicies (1,2). The second of these was in press last year so we have included the full reference as a reportable outcome in this report. We also pointed out the unexpected results presented in these publications, that Tie-2 was more indicative of breast cancer progression than VEGF receptors and other stem cell markers like ÂC133. Given those findings we described our plans to investigate Tie-2 signaling and function further, indicating a change in direction for the remainder of the grant. The reviewer's evaluation of our report did not recommend a change in the SOW, however, did recommend that we update the abstract, Subject Terms and Conclusions to more appropriately reflect the new direction. We were further instructed that even this final report should be written as an annual report and only focus on the results not previously reported rather than an overall summary. We have attempted to comply with these recommendations in this annual report.

Body: The review of last years report indicated the following:

CONTRACTUAL ISSUES: Information provided in this second Annual Summary supports the following estimate of progress relative to the approved SOW:

Task 1	Months 1-18	Complete
Task 2	Months 6-24	Extended, largely complete
Task 3	Months 12-36	Substitution in progress

The appended manuscripts show that the PI found unexpected results and appropriately chose to follow up rather than pursue the original approach. This reviewer concludes that information with potentially great clinical value was gathered and that no change in SOW is indicated.

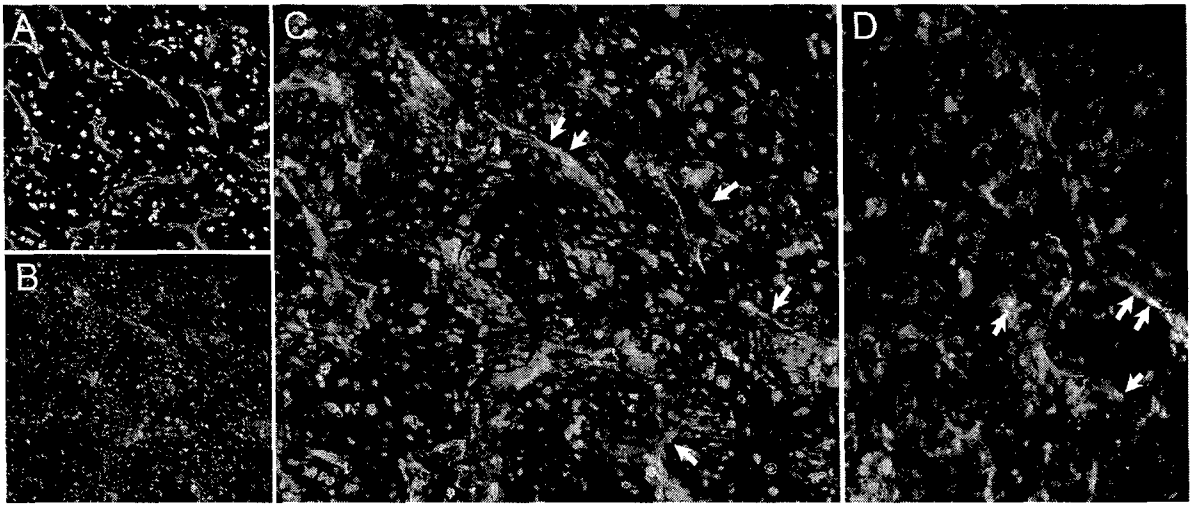
Thus we will address the uncompleted tasks:

Task 2 :

Investigation of tumors overexpressing VEGF indicated that the Akt signaling pathway was chronically activated in endothelial cells (Figure 1). Since our prior results reported in last year's report (2) had indicated that the Akt signaling pathway was a primary target of the Tie-2 receptor family signaling also, we have found that focusing on this pathway can bridge the originally proposed goals of investigating VEGF and the changed direction that we encountered when Tie-2 was identified as more indicative of breast cancer progression than VEGF receptors.

Figure 1. Akt activation in tumor vessels. VEGF overexpressing tumor grown subcutaneously in Nu/Nu mice was double stained for TRITC-lectin (A, red) to

label blood vessels and phosphorylated Akt (B, green). Merged images are shown in (C), with arrows indicating endothelial cell nuclei positive for phosphorylated Akt. Phosphorylated Akt is also seen in tumor cells. A higher magnification of some blood vessels are shown in (D).



Task 3 (substitution): Explore the hypothesis that Akt signaling in endothelial cell precursors (EPCs)/stem cells is important for EPC contribution to angiogenesis of the mammary tumor.

One way we have investigated this is to mate the MMTV-PyT mammary tumor bearing mice to transgenic mice that overexpress activated Akt (myrAkt) only in endothelial cells and endothelial precursor cells. The basics of the myrAkt transgenic animals are described in a recent publication (3). To obtain these triple transgenic mice, we had to do some extensive breeding (as our desired genotype required 2 generations and only 1/32 offspring in the third generation were triple transgenic AND female). Our preliminary data suggests that Akt activation alone (which we have now shown would result from both VEGF expression or Tie-2 activation) does increase tumor size from 1.25 cubic centimeters at 12 weeks (n=4) to 1.75 cubic centimeters (n=6). The standard deviation for these groups was less than 0.05, and thus significant. These studies are continuing and additional animals are being used to increase the group size and hopefully the significance of the data. We find that expression of myrAkt in these mice leads to accumulation of cells sticking to walls of the vessels in the mammary gland which resemble stem cells (Figure 2) . They are mononuclear, have a large nuclear:cytoplasmic ratio, and were confused as leukemia cells by our EM pathologists. Although we have yet to prove they represent an expansion of the endothelial stem cell compartment, this is a leading hypothesis. Since they home more significantly to mammary glands rather than other tissues investigated we are investigating how this might relate to the contribution of endothelial precursor cells (EPCs) to breast cancer angiogenesis. One limitation in the tools available to study mouse EPCs were a lack of antibodies to mouse stem cell markers. We have made a polyclonal antibody to mouse AC133 that is efficient at staining fixed tissue and we will use this antibody to identify stem cells in situ. We are staining these unidentified cells present in the mammary gland vasculature in the myrAkt mice for EPC markers (AC133) and VEGF and Tie-2 receptors. We also plan to transplant bone marrow from these myrAkt animals into non-tumor bearing females and MMTV-

PyT females to see if they will home to the mammary gland after transplant. This would further indicate that these are bone-marrow derived cells. One potential explanation of their increased stickiness to the blood vessels is our observation that Akt expression in endothelial cells from these animals have increased expression of adhesion molecules such as VCAM and ICAM.

Taken together, although our initial studies on the proposed Aim indicated Tie-2 signaling may be most important for EPCs in breast cancer—we have found a common signaling pathway for VEGF receptor and Tie-2 receptor that may lead the way to EPC amplification and homing to the mammary gland. Although this is the final report for the IDEA award, my CDA award on this project has one more year and we will be continuing this line of investigation during that time.

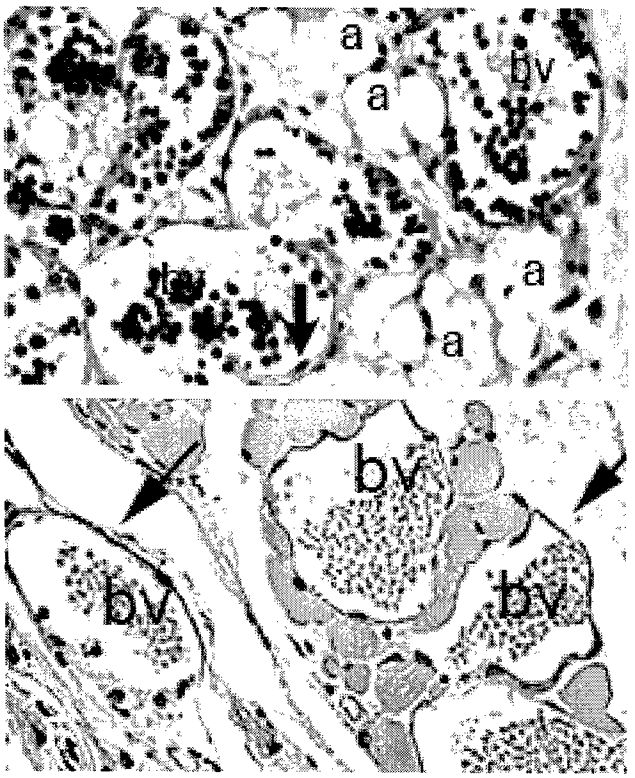


Figure 2 : Hematoxylin and Eosin Staining of mammary gland (top) and nearby skin (bottom) in transgenic mice expressing activated Akt in endothelial cells. Noteably the blood vessels become enlarged but the point here is that rather than a lumen full of erythrocytes (small red cells in lower panel), the mammary gland is full of sticky small round cells of unknown origin. We are investigating the possibility that these cells are stem cells or undifferentiated precursors. a=adipose bv=blood vessel

Key Research Accomplishments:

- Observation that VEGF expression in tumors leads to chronic activation of the Akt signaling pathway in tumor endothelial cells
- Creation of animals with constitutive activation of Akt in endothelial cells of mammary tumors
- Identification of 'putative' EPC amplification and homing to the mammary gland vasculature in non-tumor bearing mice with endothelial cell activation of Akt

Reportable Outcomes:

- Niu Q, Perruzzi C, Voskas D, Lawler J, Dumont D and Benjamin LE. Inhibition of Tie-2 signaling induces endothelial cell apoptosis, decreases Akt signaling, and induces endothelial cell expression of the endogenous anti-angiogenic molecule, thrombospondin-1. Cancer Biol. Ther. 2004, Apr, 3(4): 402-5.
- Production of mouse antibody to EPC stem cell marker AC133 that stains mouse EPCs in fixed tisssue

Conclusions

In conclusion we have found that Tie-2 is an important marker of EPCs in breast cancer and similar to VEGF is important for activation of the PI3Kinase/Akt signaling pathway in endothelial cells. Moreover, this pathway is chronically activated in endothelial cells of VEGF producing tumors. Using a model to induce Akt in endothelial cells regardless of which cytokine is present has presented a phenotype only in the mammary gland that appeared to our pathology colleagues to be a hematopoietic stem cell. We are pursuing this hypothesis and characterizing not only the results of of this Akt activation on mammary tumor progression but the identity of the mystery cells in the mammary gland vasculature.

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Research Paper

Inhibition of Tie-2 Signaling Induces endothelial Cell Apoptosis, Decreases Akt Signaling, and Induces Endothelial Cell Expression of the Endogenous Anti-Angiogenic Molecule, Thrombospondin-1

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KEY WORDS

AUTHOR: PLEASE PROVIDE KEY WORDS

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ABSTRACT

Small molecule inhibitors of endothelial cell specific tyrosine kinases are currently under investigation as potential means to block tumor angiogenesis. We have investigated the utility of blocking Tie-2 signaling in endothelial cells as a potential anti-angiogenic strategy. We have found that interruption of Tie-2 signaling either via RNAi or overexpression of a kinase-dead Tie-2 led to loss of endothelial cell viability, even in the presence of serum. Mechanistically, this is linked to a block in Akt signaling and increased thrombospondin expression. Thrombospondins are endogenous anti-angiogenic matricellular proteins known to regulate tumor growth and angiogenesis. We observed that both Tie-2 and subsequent PI3Kinase signaling regulates thrombospondin-1 expression. These data have lead to the model that Angiopoietin signaling through Tie-2 activates PI3Kinase/Akt, which represses thrombospondin expression. Thus, targeting Tie-2 with small molecules maybe efficacious as an anti-angiogenic therapy.

INTRODUCTION

Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is a fundamental process required for normal embryonic development and for the development of pathological conditions such as cancer.[1-3] There are two major families of endothelial receptor tyrosine kinases that are critical for vascular development, the vascular endothelial growth factor (VEGF) receptors, VEGFR-1 and VEGFR-2, and Tie-1 and Tie-2 receptors.[4-8] The Tie-2 tyrosine kinase receptor is largely specific for the vascular endothelium and the ligands for this receptor is a family of proteins called Angiopoietins.[6,9] One current view of the functional properties of the angiopoietin/Tie-2 system is that in the quiescent state of the vasculature, Ang1, which is constitutively expressed in many tissues, acts on the endothelial cells (ECs) by activating Tie-2 , resulting in the maintenance of cell-cell contacts between ECs and pericytes, thereby stabilizing the blood vessel.[10-12] In addition, recent studies using inducible transgenics to overcome the earliest developmental phenotypes of the Tie-2 null animals clearly shows that Tie-2 function is critical for endothelial cell survival at later stages.[13] For neovascularization to take place in a mature tissue, destabilization of the blood vessels is thought to be induced by blocking Tie-2 signaling through binding of antagonistic Ang2.[14] The further fate of these vessels is dependant on the presence or absence of endothelial survival factors such as VEGF and PlGF.[15-17]

Investigators have used soluble Tie-2 ligand binding domains to block endogenous Tie-2 activation, angiogenesis and tumor growth in murine tumor models.[18] However, these approaches block ligand-binding rather than receptor function. Some literature on the Angiopoietins has suggested that there may be signaling and nonsignaling components to their functions (reviewed in ref. 12 and 19). One example of a nonsignaling function was reported by Carlson et al., who reported that angiopoietins may mediate integrin adhesion.[20] This raises the possibility that small molecule inhibitors of Tie-2 that block only the signaling function may not fully recapitulate approaches designed to block ligand binding. Reports of Tie-2 kinase inhibitors as anti-cancer therapeutics[21-23] suggest that loss of kinase activity may by a viable anti-angiogenic therapeutic. Given the possibility of nonspecific targets with such inhibitors, we sought to genetically validate the Tie-2 kinase as a target for anti-angiogenic therapy. We initiated a study to look at Tie-2 signaling that mediates endothelial cell survival, comparing both total loss of function and loss of kinase function approaches. These different approaches could have alternate phenotypes if nonsignaling functions were important to endothelial cell survival. We performed a series of experiments using both RNAi and overexpression of a kinase-dead mutant Tie-2 protein in primary dermal microvascular endothelial cells. We observed that loss of Tie-2 by either means induced apoptosis and thrombospondin-1 expression in endothelial cells.

Thrombospondin-1 is a matricellular protein that can induce endothelial cell apoptosis.[24] This function is in part due to activation of caspases.[25] These studies suggest that blocking Tie-2 signaling induces TSP-1 in endothelial cells where it may have profound autocrine effects on endothelial cell viability.

MATERIALS AND METHODS

Cell Culture. Primary human microvascular endothelial cells were isolated as previously described[26] and grown in EGM-2 MV medium (Cambrex, MD) on plates coated with 30 μg/ml Vitrogen 100 (Cohesion, CA). Passage 4–6 cells were used for studies.

pSuper Vector-Based RNAi System. pSuper vector was provided by Dr. Reuven Agami (Division of Molecular Carcinogenesis, the Netherlands Cancer Institute).[27] Human Tie2 RNAi oligos were designed and synthesized by Oligoengine Company (Oligoengine, WA). Four pairs of Tie2 oligonucleotides were synthesized. The sequences are:

T2.1 Forward
5'GATCCCCAGCTTCTATCGGACTCCCTTCAAGGAGGGAGT CGATAGAAGCTGTTTTTGGAAA

T2.1 Reverse
5'AGCTTTTCCAAAAACAGCTTCTATCGGACTCTCTCTTGA-AGGGAGTCCGATAGAAGCTGGGG

T2.2 Forward
5'GATCCCCGGTGCCATGGACTTGATCTTTCAAGAGAAGAA-CAAGTCCATGGCACCTTTTTGGAAA

T2.2 Reverse
5'AGCTTTTCCAAAAAGGTGCCAGACTTGATCTTCTCTTGA-AAGAACAAGTCCATGGCACCGGG

T2.3 Forward
5'GATCCCCGGCTTGTGAACTGCACACGTTCAAGAGACGT-GTGCAGTTCACAAGCCTTTTTGGAAA

T2.3 Reverse
5'AGCTTTTCCAAAAAGGCTTGTGAACTGCACACGTCTCTT-GAACGTGTGCAGTTCACAAGCCGGG

T2.4 Forward
5'GATCCCCGATGCGTCAACAAGCTTCCTTCAAGAGAGGAA-GCTTGTTGACGCATCTTTTTGGAAA

T2.4 Reverse
5'AGCTTTTCCAAAAAGATGCGTCAACAAGCTTCCTCTCTTG-AAGGAAGCTTGTTGACGCATCGGG

Table 1. **PRIMER SEQUENCES**

Gene	Primer sequence
Mouse Tie2	Forward 5'- TCCTGTGCTTGACTGGAATG-3'
	Reverse 5'- CGAATAGCCATCCACTATTGTCC-3'
Mouse TSP-1	Forward 5'- TAACGGTGTGTTTGACATCTT-3'
	Reverse 5'- TGGATAGATCTTGGCCCTTCA-3'
Mouse GAPDH	Forward 5'-GGCAAATTCAACGGCACAGT -3'
	Reverse 5'-AAGATGGTGATGGGCTTCCC -3'

All sequences were blasted to the genomic database to make sure they had no significant homology with other genes. Synthesized oligonucleotides were dissolved into water and mixed with annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Mg-acetate). After incubating at 95°C for 4 minutes and 70°C for 10 minutes, annealed oligonucleotides were slowly cooled to 4°C. Annealed oligonucleotides were incubated with T4 PNK at 37°C for 30 minutes to phosphorylate them and the enzyme was inactivated by incubation at 70°C for 10 minutes. The phosphorylated oligonucleotides were ligated into *Bgl*II, *Hind*III (NEB, MA) sites of the pSuper vector. The positive clones of Tie2-pSuper RNAi plasmid DNA were confirmed with *EcoRI-Hind*III digestion.

Nucleofector Transfection. Dermal microvascular endothelial cells were grown on flasks precoated with Vitrogen in EGM-2 MV complete medium. After 4 days, the cells were harvested with trypsin-EDTA and washed. For each 1 x 10⁶ cells, 100 μL Nucleofector (Amaxa Biosystems, Germany) with 2 μg DNA was used. The samples were transferred into Amaxa certified cuvettes (Amaxa Biosystems, Germany) and program S-05 was used. Endothelial cells transfected with RNAi or Tie-2 plasmids were then removed from the cuvette and plated into a precoated six-well tissue culture plate for 48 hours for further analysis.

Western Blots. Endothelial cell lysate were collected with RIPA buffer (Boston Bioproducts, MA) and protein concentrations were quantified with Bio-Rad DC Protein Assay Reagents (Bio-Rad, CA). 20 μg protein samples were run on 10% SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad, CA). The membranes were blocked with 5% nonfat milk and blotted with Anti-phospho Akt (Pharmingen, CA), Anti-PARP (Upstate Biotechnology, NY), Anti-Tie2 (Upstate Biotechnology, NY), Anti-human PTEN (Cascade Bioscience, MA), Anti-human TSP-1 monoclonal antibody MAI,[28] and anti-α-tublin (Sigma, MO) antibodies. Pierce detection reagents (Pierce Technology, IL) were used to visualize HRP-labeled secondary antibodies.

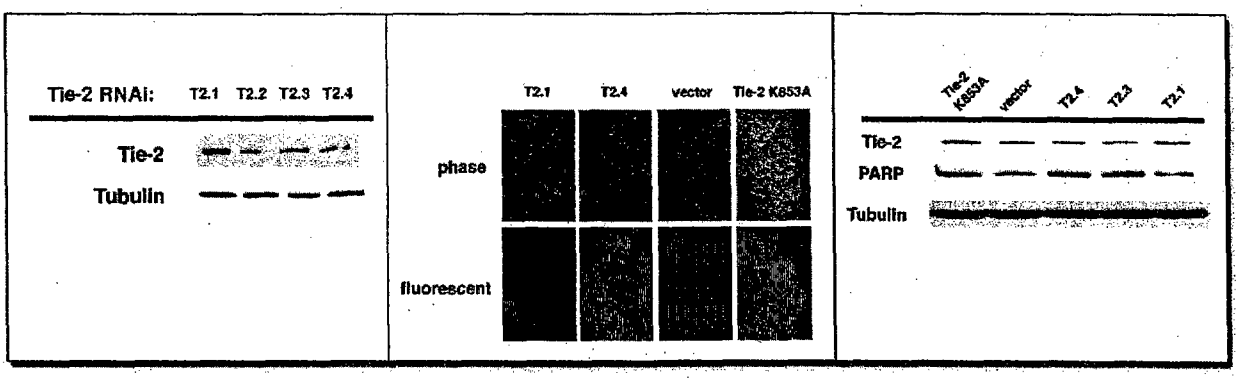

Figure 1. Loss of Tie-2 function induces endothelial cell apoptosis (A) Western blot analysis of Tie2 expression in human dermal microvascular endothelial cells transfected with Tie-2 RNAi's. (B) In Situ staining of activated caspases in endothelial cells transfected with Tie-2 RNAi and Kinase-dead Tie-2 (C) Western blot analysis of cleaved PARP in human dermal microvascular endothelial cells transfected with Tie-2 RNAi and Kinase-dead Tie-2.

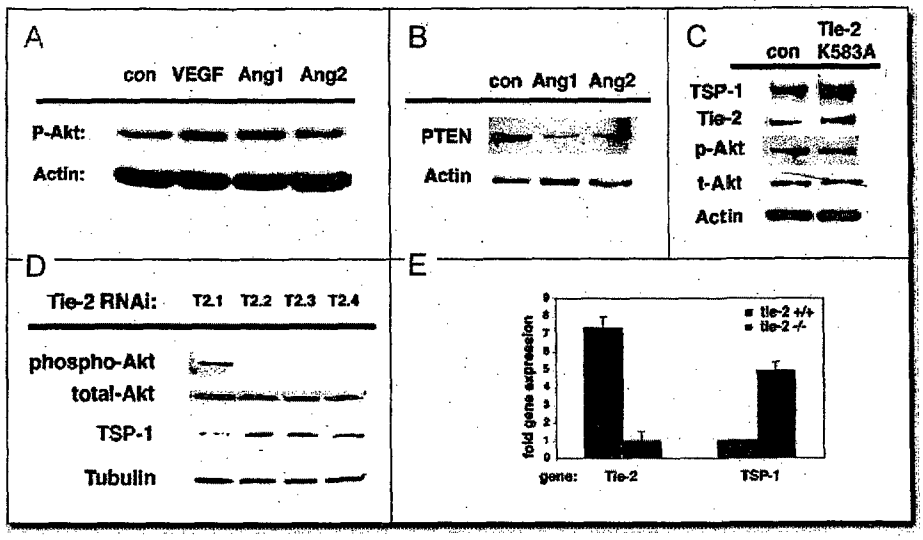

Figure 2. Signaling through Tie-2 mediates PI3Kinase regulation and thrombospondin expression. (A) 15 minute stimulation with VEGF-A and Ang 1 induced Akt phosphorylation in endothelial cells. (B) Overnight exposure to Ang1 and Ang2 decrease PTEN expression in endothelial cells. (C) Overexpression of kinase-dead Tie-2 leads to steady-state decreases in phosphorylated Akt, and increases in TSP-1. (D) Steady-state levels of phosphorylated-Akt are decreased when Tie-2 RNAi blocks Tie-2 expression. (E) Real time PCR analysis of TSP-1 and Tie2 RNA expression in Tie2 knockout and wildtype mice.

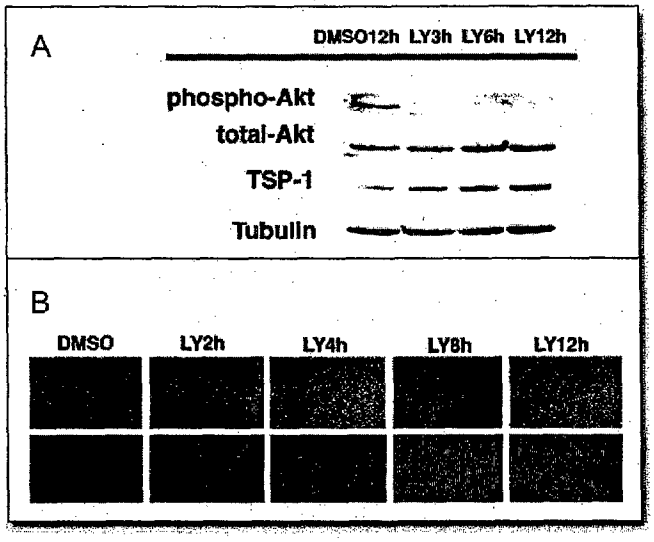

Figure 3. PI3 Kinase signaling mediates expression o f TSP-1 and caspase activation in primary endothelial cell culture. (A) Western blot analysis of TSP-1 and Akt expression and (B) activated caspases in human dermal microvascular endothelial cells incubated with LY 294002 at 25 μM from 2–12 hours.

Analysis of Caspase Activation. To determine whether human dermal microvascular endothelial cells were undergoing apoptosis after Tie2 RNAi transfection or incubation with LY 294002 (Calbiochem, CA) or a recombinant version of all three type' I reeats of TSP-1(8TSR), we incubated endothelial cells transfected with Tie2 RNAi or treated with LY 294002 at 25 μM or 3TSR at 100 μg/ml with CaspACE-FITC-VAD-FMK In Situ Marker (Promega, WI) at 10 μM for 20 minutes, which binds activated caspases.[29] Images were captured using the Lecia DC200 digital camera and imported into Adobe Photoshop 5.0.

Real time quantitative reverse transcription-polymerase chain reaction (QRT-PCR). Total RNA was isolated from freshly homogenized Tie2 knockout and Tie2 wildtype embro tissue as well as endothelial cells with RNeasy Mini Kit (Qiagen, CA) according to manufacturer's protocol. 2 μg of total RNA was used for reverse transcription with TaqMan Reverse Transcription Reagents Kit (Applied Biosystems, CA) according to the manufacturer's instructions. PCR primers were synthesized by Integrated DNA Technologies, Inc. (Integrated DNA Technologies, Inc., IA). Real time PCR reactions were conducted in 25 μl reaction volume containing 1 μl of cDNA and 12 μl 2 X Sybr Green Master Mix (Applied Biosystems, CA). PCR mixtures were preincubated at 50°C for 2 minutes, then 95°C for 10 minutes followed by 40 cycles of two-step incubations at 95°C for 15 seconds and 60°C for 1 minutes with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, CA). For each set of primers, specific standard curve was established to calculate the specific gene expression level normalized to GAPDH expression. Specific gene expression change was calculated by comparing the gene expressions normalized to GAPDH in different groups according to the manufacturer's instructions. Primer sequences are listed in Table 1.

RESULTS AND DISCUSSION

We compared endothelial cell viability after loss of total Tie-2 expression via RNAi to that of loss of kinase activity using overexpression of a kinase-dead Tie-2 protein.[8] Four RNAi sequences were designed and cloned into the pSUPER vector for transient expression following transfection. Three of the four sequences (T2.2-T2.4) were effective at reducing endogenous Tie-2 levels (Fig. 1A). The fourth RNAi (T2.1), which did not reduce Tie-2 expression, was used as a negative control in future experiments. Both blocking Tie-2 expression with RNAi and overexpression of a kinase-dead allele compromised endothelial cell viability in serum-containing media. To determine whether loss of viability was due to induction of apoptosis, we assayed for caspase activity and PARP cleavage. Figure 1B shows strong induction of caspases using a fluorescent Caspase substrate assay (CaspACE-FITC-VAD-FMK In Situ staining of activated caspases). Caspase induction was comparable between RNAi treated and Kinase impaired cultures. Similarly, both approaches induced PARP cleavage suggesting that indeed, even in serum-containing media, loss of Tie-2 signaling in endothelial cells was sufficient to cause apoptosis (Fig. 1C).

The work of others has shown that Ang1 activates PI3Kinasee and Akt phoshorylation via Tie-2 (18-20). We reproduced this result in our system and saw increased Akt phosphorylation following 25 ng/mL VEGF-A and 50ng/mL Ang1 but not 50ng/mL Ang2 stimulation (Fig. 2a). However, overnight exposure to both Ang1 and Ang2 reduced PTEN expression, suggesting that there may be both transient links to Akt signaling and more sustained effects via

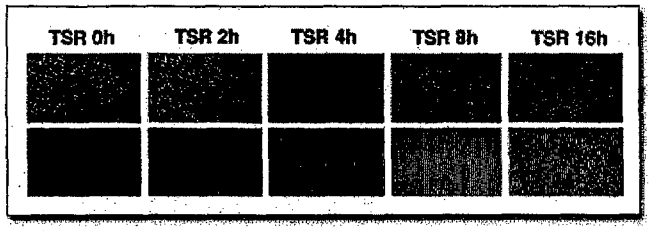

Figure 4. 8-16 hours of treatment with recombinant type I repeats from TSP-1 induce caspase activation. In Situ staining of activated caspases in human dermal microvascular endothelial cells incubated with 3 TSRs at 100 μg/ml show similar result on endothelial cell caspase activation as LY 294002 but with slightly delayed kinetics.

PTEN (Fig. 2b). When we blocked Tie-2 signaling with the kinase-dead mutant, we saw that basal levels of Akt phosphorylation were reduced in serum-containing media (Fig. 2c). In addition, we observed increased expression of TSP-1. We observed similar results following RNAi transfection (Fig. 2d). Additional support for the hypothesis that TSP-1 is regulated by Tie-2 signaling was obtained from the Tie-2 null mice. We compared *tsp-1* mRNA levels in Tie-2 null and wildtype embryos using real time quantitative RT-PCR and found that *tsp-1* levels were increased in null embryos (Fig. 2e). Genotyping of the embryos was further confirmed by RT-PCR of *tie-2*.

While we saw both effects on Akt phosphorylation and TSP-1 expression following loss of Tie-2 function, we did not know whether these two observations were mechanistically linked. To test this, we used the LY 294002 inhibitor to block PI3 Kinase activity in endothelial cell culture for 3,6 and 12 hours. We observed loss of Akt signaling by 3 hours and a gradual increase in TSP-1 levels from 3-12 hours (Fig. 3). We also observed induction of caspases after 8 hours of LY treatment. This suggests that sustained inhibition of PI3Kinase activity will both induce apoptosis and TSP-1 expression in endothelial cells.

Previous investigators have shown that the type I repeats of TSP-1 are capable of anti-angiogenic activity via apoptosis.[30] In our caspase induction assay, we observed that recombinant type I repeats of TSP-1 (designated 3TSR), recapitulated the activation of caspases between 8-12 hours, similar to the effect of Tie-2 blockade and with slightly delayed kinetics compared to the LY treatment (Fig. 4). Together, these data support the hypothesis that kinase inhibition of Tie-2 may be effective at reducing endothelial cell viability, in part by blocking PI3Kinase activity which subsequently induces TSP-1 autocrine signaling. Both loss of PI3Kinase activity and induction of TSP-1 can cause endothelial cell apoptosis.

While PI3Kinase regulates cell survival via several downstream pathways, these findings suggest that inhibition of Tie-2 signaling, even in full media with serum, abrogates this pathway and that at least one downstream mediator of the ensuing apoptosis is autocrine TSP-1 expression. Thrombospondins are negative regulators of angiogenesis that can induces apoptosis (27,39) and TSP-1 expression is correlated to a good prognosis in human tumors.[31-37] According to the hypothesis that angiogenesis is modulated by a balance in pro- and anti- angiogenic molecules, the Tie-2 inhibitors may inhibit tumor growth by shifting the balance of gene expression in favor of anti-angiogenic molecules.

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