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<td>In normal adults, the tissue vasculature is quiescent due to the predominance of angiogenesis inhibitors over that of inducers secreted into the tissue matrix. During tumor progression, this balance shifts to favor angiogenesis. Our goal is to characterize this transition in the prostate by 1) identifying the key angiogenic mediators, 2) investigating the clinical significance of mediator levels in prostatic fluid, and 3) designing anti-angiogenic therapies utilizing these mediators. We have found that normal prostate epithelial cells inhibit angiogenesis through secretion of thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor, with only low levels of inducers present. In the transition to a pro-angiogenic state, in BPH and PCAs, TSP-1 expression is downregulated or lost, and both BPH and PCAs, secrete increased levels of angiogenic inducers vascular endothelial growth factor (VEGF) and/or fibroblast growth factor-2 (FGF-2). Analysis of prostatic fluids from normal controls and prostatitis, BPH and PCa patients revealed no correlations between overall angiogenic activity or VEGF levels and patient group, and neither FGF-2 or TSP was detectable in these samples suggesting that angiogenic mediators are not a useful measure of disease in prostatic fluids. We have also initiated anti-angiogenic treatment studies in mouse models using TSP-1 and anti-VEGF approaches.</td>
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Annual Summary Report

Introduction

For progressive growth and metastasis, all tumors are dependent on angiogenesis, the growth of new blood vessels from the existing vasculature (1). Increased microvessel density has been correlated with advanced disease in prostate cancer (2-5), indicating an important role for angiogenesis in this disease. The design of rational anti-angiogenic therapies specifically for prostate cancer requires that we have a basic understanding of angiogenic regulation in the normal and in the diseased prostate. Many molecules can modulate angiogenesis; however, the majority of these molecules are multi-functional, and angiogenic function is tissue dependent (6-8). In the prostate, several known angiogenic inducers are expressed, such as vascular endothelial growth factor (VEGF); fibroblast growth factor-2 (FGF-2); interleukin-8 (IL-8); transforming growth factor-β (TGF-β); keratinocyte growth factor (KGF); and, hepatocyte growth factor (HGF) (9, 10). In addition, VEGF expression has been correlated with increasing tumor grade (11). However, to date, little is known regarding which molecules are the functional angiogenic mediators in the prostate. In our proposal, we set out to 1) identify such mediators in the prostate, 2) assess the clinical usefulness of measuring angiogenic mediator levels in prostatic fluid, and 3) test the efficacy of using the endogenous angiogenesis inhibitor in treating prostate cancer in animal models.

Description of Research Accomplishments and Training to Date

Identification of functionally relevant angiogenic mediators in the prostate: In our proposal we presented preliminary data that TSP-1, a potent angiogenesis inhibitor (12, 13), is the major angiogenesis inhibitor present in the normal prostate maintaining its angio-quiescent phenotype. This is based on work with secretions from short-term cultures of normal prostate epithelial cells using the microvascular endothelial cell migration assay, an in vitro angiogenesis assay (14), to measure angiogenic activity. TSP-1 secretion was verified by Western blot. We also observed that in contrast to normal cells, four PCa cell lines assayed (DU145, LNCaP, PC-3 and TSU-Pr1) induced angiogenesis, and TSP-I was not detectable in cell secretions (Figure 1). For each of these cell lines, the dose of conditioned media at which half the maximal angiogenic activity is present (ED50) was calculated from linear regression analysis of a dose-response curve. The ED50 for DU145, LNCaP, PC-3 and TSU-Pr1 is 4, 6, 10 and 12 µg/ml respectively.

To further characterize angiogenesis in the prostate, we cultured epithelial cells from three normal, four BPH, and two cancer specimens for immediate collection of conditioned media and cell lysates and have used these samples in our in vitro angiogenesis assays. We also collected samples directly from minced tissue of one normal and three BPH specimens. In addition, we are using three normal epithelial cell strains obtained from Clonetics (San Diego, CA; PrEC-18, -42, and -17) as well as the established PCa cell lines DU145, LNCaP, PC-3 and TSU-Pr1. In contrast to the PCa cell lines, BPH epithelial cells continue to express TSP-1 but at a diminished level compared to our short-term normal cultures (Figure 1B).
Figure 1. Expression of TSP-1 in Prostate Samples. Serum-free conditioned media was analyzed by Western blot using a TSP-1 antibody (A4.1, produced in our own laboratory; (13). A) Short-term cultures of normal epithelial cells and two normal epithelial cell strains obtained from Clonetics from 18 and 42 year old donors respectively. Lower band in panel A is a degradation product of TSP-1 (unpublished observation). B) PCA cell lines and short-term cultures of epithelial cells from BPH tissue (20 μg total protein loaded per lane; Lanes: 1, DU145; 2, LNCAP; 3, PC-3; 4, TSU-Pr1; 5, BPH3; and 6, BPH4). Purified TSP-1 protein served as a positive control.

In using the normal prostate epithelial cell strains (PrEC) from Clonetics, we observed key differences between secretions from these strains and our short-term cultures of normal epithelial cells. In contrast to our short-term cultures that secrete high levels of TSP-1 and inhibit angiogenesis, PrEC strains secrete lower levels of TSP-1 (Figure 1A) and induce angiogenesis (Figure 2A). They also have elevated levels of growth factor secretions (VEGF and FGF-2) compared to the short-term cultures (Figure 2B).

Figure 2. Normal prostate epithelial cell strains are inductive and secrete higher levels of inducers than short-term cultures. A) Serum-free conditioned media collected from the epithelial cell strain, PrEC-42 was assayed in a microvascular endothelial cell migration assay alone and in the presence of neutralizing antibodies to a known inducer (αVEGF 20 μg/ml; αFGF-2, 10 μg/ml; αIL-8, αHGF and αKGF each at 40 μg/ml) or to the angiogenesis inhibitor, TSP-1 (αTSP-1, 40 μg/ml). BSA (0.1%) serves as a negative control for background migration levels, and VEGF (100 pg/ml) serves as a positive control. B) Levels of the angiogenic inducers FGF-2 and VEGF in conditioned media from short-term cultures and PrEC strains of prostatic epithelial cells were measured by ELISA (R&D Systems).

These data suggest a caveat in using these cell strains for angiogenic characterization. However, although TSP-1 is secreted at lower levels in these cells (see Figure 1A), it does contribute to the angiogenic phenotype because neutralizing antibodies to TSP-1 increases the activity (Figure 2A and data not shown), again supporting the role of TSP-1 in prostate angiogenesis. In addition, we have observed that the underlying inducers present in these cell strains are the same as in the short-term cultures as tested thus far, although some at higher levels than in our short-term cultures (see Figure 2B and data not shown). Therefore, the PrEC strains have proven useful for experimentation on the underlying functional angiogenic inducers present in the prostate which is a valuable resource considering the limited availability of truly normal prostate specimens (fresh tissue from 17-21 year old male donors).

Using neutralizing antibodies to a panel of known angiogenic inducers (VEGF, FGF-2, IL-8, HGF, KGF and TGF-β1), we identified IL-8 as a key angiogenic inducer in PrEC while HGF, KGF and TGF-β1 are not involved (Figure 2A and data not shown). We also have recent data that suggests there is synergy between FGF-2 and VEGF in these cells. When neutralizing
antibodies to both FGF-2 and VEGF are added to test samples, angiogenic activity is diminished significantly whereas with each alone, only a slight decrease is observed (Figure 2A and data not shown). In our BPH and PCa specimen and in the PCa cell lines, we have identified VEGF and/or FGF-2 as the primary inducers (data not shown). The PCa cell lines DU145 and LNCaP rely primarily on VEGF while PC-3 and TSU-Prl use primarily FGF-2. The BPH specimens also demonstrate some heterogeneity with some specimen using FGF-2 and others using both FGF-2 and VEGF to induce angiogenesis (data not shown). These samples were also tested with neutralizing antibodies to the panel of angiogenic inducers listed above, and inducers having minor contributions to the angiogenic phenotypes of these cells were identified; however, again, heterogeneity exists between the samples. In the DU145 and LNCaP PCa cell lines, HGF is involved; and, in the DU145 and PC-3 cell lines, IL-8 plays a minor role. In the TSU-Prl cell line, TGF-β1 contributes to the angiogenic phenotype. KGF was not found to be involved in any sample tested. However, any additional inducers present in BPH specimens have not yet been identified although IL-8 does not appear to be involved.

Levels of the major inducers FGF-2, VEGF and IL-8 were measured by ELISA. For FGF-2 and VEGF, levels are increased in three of the four PCa cell lines as compared to short-term cultures of normal epithelial cells, and BPH tissue had increased levels of VEGF (Figure 3). Levels of IL-8 were elevated in PCa cell lines DU145, PC-3 and TSU-Prl but not in LNCaP or in BPH specimen.

**Figure 3. Quantification of VEGF and FGF-2 levels in prostate samples.** Conditioned media samples were assayed by ELISA (R&D Systems) for levels of VEGF (A) and FGF-2 (B). Short-term epithelial cell cultures are labeled “Primary Epithelial” and BPH reflects combined data from 2-3 short-term cultures of BPH-tissue derived samples. Samples were quantified from a standard dose curve of purified protein.

Immunostaining for TSP-1 and VEGF has been performed on normal, BPH and PCa tissue. We have observed a decrease of TSP-1 staining in BPH and a decrease to loss of expression in PCa as compared to normal prostate tissue. In addition, VEGF staining is increased in BPH and PCa as compared to normal prostate tissue. Figure 4 shows examples of TSP-1 and VEGF staining in normal and PCa tissue. These data are consistent with our in vitro data identifying TSP-1 as the major endogenous inhibitor and VEGF as a key inducer secreted by BPH and PCa epithelial cells. Immunostaining for FGF-2 is currently in progress.

**Figure 4. Immunostaining for VEGF and TSP-1 in normal prostate and PCa tissue.** Paraffin-embedded archival prostate tissue was sectioned and stained using antibodies to either TSP-1 or VEGF and counterstained with H&E.

Unpublished data
Test of relationship between levels of angiogenic mediators in prostatic fluid secretions and prostate disease status: Prostatic fluid specimens from 9 PCa, 17 BPH, 14 prostatitis, and 3 normal patients were assayed for overall angiogenic activity and for levels of angiogenic mediators. All prostatic fluids assayed induced migration of microvascular endothelial cells. Levels of induction varied between individual samples but did not distinguish between patient groups (data not shown). None of the specimens contained detectable levels of TSP-1 by Western blot, suggesting that TSP-1 is not released into the lumen of the prostatic glands at detectable levels. Since VEGF and FGF-2 are the predominant inducers present in BPH and PCa specimens, we assessed the levels of these molecules in the prostatic fluid samples by ELISA (R&D Systems). No FGF-2 was detected in any of the samples assayed (data not shown). VEGF was detected in the samples at varying levels; however, again, no correlation existed between patient group and levels of VEGF. Thus, the measurement of these angiogenic mediator levels in prostatic fluids would not be a clinically useful parameter. If other key molecules are identified, these samples will be re-evaluated for the new molecules.

Determination of the efficacy of using the endogenous inhibitor in the prostate, TSP-1, to treat PCa in animal models: The initial experiment we proposed was to transfect PCa cell lines with a TSP-1 expression vector and test in vivo tumorigenicity by subcutaneous injection in nude mice. We have modified this experiment so that not only the efficacy of the endogenous inhibitor is tested, but also the efficacy of treatments targeting an endogenous inducer. Several groups have used anti-VEGF treatments against tumors of the DU145 cell line (15, 16). As stated above, we have found that VEGF is the dominant inducer in this cell line. However, PC-3 and TSU-Pr1 predominantly use FGF-2 in angiogenic induction; therefore, anti-VEGF therapies may not be effective against these cell lines. As prostate cancer is a notoriously heterogeneous disease, the heterogeneity observed in the dominant inducer used by the cell lines may also be reflected in human tumors. Therefore, we propose that replacing the endogenous inhibitor may be effective against a larger proportion of tumors. Conversely, using both treatments together may have a synergistic effect. Therefore, we plan to treat subcutaneous tumors of both the DU145 and TSU-Pr1 cell line in nude mice with DI-TSP alone, a peptide mimetic of TSP-1 which has demonstrated potent anti-angiogenic activity in other models (Bouck et al., unpublished data), a neutralizing anti-VEGF antibody alone, and both together. If our hypothesis is correct, the TSU-Pr1 tumors will not respond as well or at all to the anti-VEGF treatment as compared to the DU145 tumors, since, by in vitro studies, TSU-Pr1 induces angiogenesis by secretion of FGF-2 while DU145 relies primarily on VEGF. To ensure that the anti-VEGF antibody has anti-angiogenic activity, we tested it in a migration assay against purified human VEGF protein (Figure 5). It inhibited the migration of endothelial cells induced by VEGF, thus demonstrating its effectiveness.

Figure 5. Anti-VEGF antibody inhibits angiogenesis induced by human VEGF. Anti-VEGF antibody is tested against increasing doses of human VEGF protein in a microvascular endothelial cell migration assay. FGF-2 is used as a control for induction of migration and for specificity of antibody function. BSA (0.1%) serves as a negative control for background migration levels.
Currently, we have injected tumor cells into nude mice using both the DU145 and TSU-Pr1 cell lines to assess growth time and tumor characteristics. Immediately following this analysis, tumor cells will be injected for treatment studies using DI-TSP and the anti-VEGF antibody.

Due to a long delay in availability of the TRAMP mice from Jackson Laboratories, we were not able to obtain the mice until July 24, 2000. However, at this time, we obtained two breeding pairs of TRAMP mice and immediately began breeding. Currently multiple liters are being genotyped by PCR to identify transgenic males for further breeding and experimentation. As soon as a sufficient number of mice are available, we will initiate the DI-TSP treatment study. In preparation for these studies, I have been assisting a co-worker with his mouse studies to learn proper animal handling, injection and tissue processing procedures, including tumor measurements and IHC for microvessel density measurements.

In summary, we have identified the key angiogenic mediators in the normal prostate, BPH and PCa tissue. TSP-1 is the endogenous inhibitor present in the normal prostate that is downregulated or lost during tumor progression. In addition, we have shown that in BPH and PCa the active inducers VEGF and/or FGF-2 are upregulated. Thus the transition from angi-inhibitory normal prostate tissue to angio-inductive BPH or PCa tissue involves both the down-regulation of the endogenous inhibitor and the upregulation of underlying inducers.

**Summary of Technical Training Received**

Through the course of these studies, I have become proficient in 1) cell cultures techniques, 2) in vitro angiogenesis assays and protein assays, and 3) using animal models. I have cultured established cell lines and strains and grown cells from explants of primary tissue. I have used these cells in proliferation and apoptosis assays and for collection of conditioned media and cell lysates that were used in Western blot analyses and for ELISAs. In addition, I have received extensive experience with the use of mouse models. I have learned basic handling and care procedures, subcutaneous tumor-cell injection and intraperitoneal injection procedures as well as autopsy and tissue preparation procedures.
Key research accomplishments

- Identification of TSP-1 as the major endogenous angiogenic inhibitor present in the normal prostate.

- Identification of the predominant angiogenic inducers, VEGF and FGF-2, present in BPH and PCa cells.

- Characterization of the alterations that shift normal anti-angiogenic prostate tissue to pro-angiogenic BPH or PCa tissue: 1) downregulation of TSP-1 and 2) upregulation of underlying inducers, predominantly FGF-2 and VEGF.

- Identification of additional angiogenic inducers, IL-8, HGF and TGF-β1, that contribute to the angiogenic induction by a subset of PCa cell lines and strains.

- Determination that measuring the levels of VEGF, FGF-2 or TSP-1 in prostatic fluids is not a clinically useful indicator of disease state.
Reportable Outcomes


- Manuscript in preparation for submission to Cancer Research within the coming month (draft of manuscript not included as not yet complete).

- Data obtained in these experiments were used as the preliminary data for submission of an Idea Development award submitted to the USAMRMC DOD Prostate Cancer Research Program by Dr. Sue Crawford, Dept. of Pathology, Northwestern University Medical School, whom I will be working with over the next year.
Conclusions

As stated above, we have identified the key angiogenic mediators in the normal prostate, BPH and PCa tissue. We have observed that the progression to a pro-angiogenic phenotype in BPH and PCa tissue occurs by both the down-regulation of the endogenous inhibitor, TSP-1, and the upregulation of underlying inducers, predominantly FGF-2 and VEGF. We have used these data to design anti-angiogenic treatment strategies to be tested in mouse models which are underway. These data have also led to several additional lines of research. From the IHC data, we observed alterations in the expression of angiogenic mediators in the stromal tissue. We hypothesized that these changes contribute to the overall angiogenic phenotype of the tissue. To determine the stromal contribution to the angiogenic phenotype, we have obtained a normal prostatic stromal cell strain from Clonetics (PrSC) and cultured stromal cells from a normal prostate tissue specimen, and we have initiated experiments to approach this question. Currently, our preliminary data supports our hypothesis, and we are pursuing the work.

In addition, from our data on TSP-1 in the prostate, we have begun investigating the mechanism of TSP-1 loss of expression in PCa. The chromosomal location of the TSP-1 gene (15q15) is not a known region of loss or mutation in prostate tumors, and some PCa cell lines do express the mRNA. Thus, loss of TSP-1 expression may be due to epigenetic factors, such as tissue hypoxia, or by suppression through another gene and/or protein dysregulated in PCa. Another question arising from our work with TSP-1 is if there is a relationship between TSP-1 and TGF-β1 in the prostate as TGF-β1 has been shown to be an important growth regulator in prostate tissue (17), and TSP-1 can activate TGF-β1 in vivo (18). However, the functional relationship of these molecules is known to be tissue specific (18), and their relationship in the prostate is currently uncharacterized.
References


PTEN may elicit its tumor suppressor function in part through regulation of angiogenic mediators.

Doll', Jennifer A., Campbell, Steve C., Bouck', Noel P., 'Robert H. Lurie
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The recently identified tumor suppressor gene PTEN encodes a dual specificity phosphatase whose loss activates PIP3 and thereby activates Akt which blocks apoptosis and promotes cell cycle progression. PTEN maps to chromosome 10q, a region that has previously been shown to suppress angiogenesis in glioblastoma cell lines by supporting the production of the anti-angiogenic protein thrombospondin-1 (Hsu et al. Ca.Res. 56:584-91, 1996). To determine if PTEN is the gene on chromosome 10q responsible for the angiogenic switch, both glioblastoma and prostate cancer cell lines were treated with wortmannin to mimic PTEN effects on PIP3 or were infected with retroviral constructs expressing wild type PTEN, and their secretions were analyzed for angiogenic mediators.

In the glioblastoma cell line U251, restoration of wild type PTEN decreased angiogenic activity as a result of an increase in thrombospondin-1 expression. There was no change in the production of stimulatory VEGF. Wortmannin treatment had similar effects on thrombospondin-1 suggesting that the PIP3 pathway is involved.

The prostate is similar to the brain in that thrombospondin-1 is the major natural inhibitor secreted by normal cells and loss of the PTEN tumor suppressor gene is associated with 50% of advanced tumors. Unlike glioblastomas which uniformly rely on VEGF as their major inducer of angiogenesis, the prostate lines fall into two classes: DU145 and LNCaP rely on VEGF while PC-3 and TSU-Prl use primarily bFGF. Prostate tumor lines also differ from glioblastomas in that when those lines mutant for PTEN (LNCaP and PC-3) were treated with wortmannin no change in TSP-1 expression was seen although a dramatic decrease in VEGF did occur. However in these lines introduction of wild type PTEN had no effect on VEGF secretion suggesting that if PTEN is affecting angiogenesis in these lines, it is not doing so via PIP3. These results suggest that in glioblastomas but not in prostate cells PTEN is capable of controlling angiogenesis and TSP-1 via PIP3 and emphasizes the remarkable tissue specificity of the regulation of key modulators of angiogenesis. (This work was done with support of NIH grants CA52750 CA64239 to N.P.B. and DOD grant DAMD17-99-1-9521 to J.A.D.)
IDENTIFICATION OF KEY ANGIONECIC MEDIATORS IN THE PROSTATE.
Jennifer A. Doll¹, Frank K. Reiher², Steve S. Campbell², Susan E. Crawford¹, ⁴ and Noel P. Bouck³, ⁴
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For growth and metastasis, all tumors require angiogenesis, the growth of new blood vessels from the existing vasculature. In most normal adult tissues, the vasculature is quiescent, an environment maintained in the tissue matrix by a predominance of angiogenesis inhibitors over inducers. In cancer tissue, the secreted levels of inducers and/or inhibitors are altered shifting the balance to favor induction. In prostate cancer (PCa), the changes in secreted angiogenic mediators have not been well characterized. In order to design rational anti-angiogenic treatment strategies, we have identified the key angiogenic mediators in benign prostatic hyperplasia (BPH) and PCa cells. Normal prostate epithelial cells secreted high levels thrombospondin-1 (TSP-1), a potent angiogenesis inhibitor, as observed by Western blot. In a functional angiogenesis assay of endothelial cell migration, normal prostate epithelial cell secretions inhibited migration, and TSP-1 neutralizing antibodies relieved this inhibition indicating that TSP-1 is the major active angiogenic inhibitor present in the normal prostate. TSP-1 secretion was down-regulated in cultured BPH epithelial cells and lost in PCa cell lines (DU145, LNCaP, PC-3 and TSU-Prl), and secretions from these cells induced migration. In addition to losing TSP-1, both BPH epithelial cells and PCa cell lines secreted elevated levels of vascular endothelial growth factor (VEGF; 4-20 fold) and/or fibroblast growth factor-2 (3-14 fold) which were the dominant angiogenic inducers. Other angiogenic inducers secreted by prostate cells played minor or no role in PCa-induced angiogenesis. Immunostaining revealed strong positivity for VEGF in PCa cells whereas minimal staining for TSP-1 was observed, consistent with our in vitro data. Similar patterns of VEGF and TSP-1 expression were observed in the stroma. Thus, it appears that angiogenesis in the prostate is influenced by both the epithelial and stromal cell secretions. (Supported by US Army grant #DAMD17-99-1-9521).

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