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Award Number: DAMD17-98-1-8576

TITLE: PTEN, A Tumor Suppressor Gene for Prostate Cancer

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REPORT DATE: January 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2001	Final (1 Jul 98 - 31 Dec 00)

4. TITLE AND SUBTITLE
PTEN, A Tumor Suppressor Gene for Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8576

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The PTEN tumor suppressor gene encodes a lipid and protein phosphatase and is inactivated in a wide variety of human malignant neoplasms, including gliomas, melanomas, lymphomas and carcinomas of the endometrium, kidney, bladder, lung and prostate. Our work has conclusively demonstrated that loss of the PTEN gene is associated with increased rates of tumor progression in a mouse model of prostate cancer. A key observation is that loss of even a single allele of the PTEN gene can increase rates of tumor progression and this resolves a longstanding dilemma regarding the discordance between rates of LOH at the PTEN locus and observed rates of mutation of the gene. Our results indicate that at least one factor leading to increased tumor progression is increased tumor angiogenesis secondary to increased VEGF expression in tumors with loss of the PTEN gene. However, based on the current literature, alterations in apoptosis and proliferation are also likely to play an important role. The availability of numerous clonal cell lines and our bank of tissues from the PTEN $^{+/-}$ and WT$^{+/+}$ mice will be invaluable in dissecting out the many factors associated with PTEN inactivation that promote tumor progression in prostate cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
PTEN, tumor suppressor genes, prostate cancer, transgenic mice	25
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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PTEN, A TUMOR SUPPRESSOR GENE FOR PROSTATE CANCER

FINAL REPORT

INTRODUCTION

The PTEN tumor suppressor gene encodes a lipid and protein phosphatase and is inactivated in a wide variety of human malignant neoplasms, including gliomas, melanomas, lymphomas and carcinomas of the endometrium, kidney, bladder, lung and prostate. The tumor suppressor activity of PTEN is believed to be primarily due to its ability to dephosphorylate phosphatidylinositol (3,4,5) phosphate at the 3-position and in this manner negatively regulate the activity of the phosphotidylinositol 3-kinase pathway (1). A variety of biological effects have been attributed to loss of PTEN activity that are relevant to its role as a tumor suppressor gene, including enhanced cell proliferation, decreased apoptosis (1) and enhanced tumor angiogenesis (2). The PTEN tumor suppressor gene maps to human chromosome 10q23.3 and this region shows high rates of loss of heterozygosity (LOH) in a variety of human malignancies due loss of relatively large areas of one chromosome. It is generally believed that in the presence of such LOH the tumor suppressor gene present on the retained chromosome is inactivated by smaller deletions, resulting in homozygous deletion, or by mutation. However, for the PTEN gene, the rate of LOH at 10q23.3 is often much higher than the apparent rate of PTEN inactivation (3). For example, LOH at 10q23.3 has been detected in 15-49% of clinically localized human prostate cancers, while mutation or homozygous deletion of the PTEN gene is detected in less than 10% of these same cases. Similarly, in metastatic prostate cancer LOH at 10q23 is present in more than 50% of cases while PTEN inactivation is present in only a third of cases. Similar observations have been made in a variety of other

human malignancies. There are several possible explanations for this discordance. First, the retained allele may be inactivated by other mechanisms, such as methylation or promoter mutation. Methylation of the PTEN promoter has been reported in prostate cancer xenografts but to date there are no reports of methylation of the PTEN promoter primary or metastatic prostate cancer in vivo. A second potential explanation is that there is a second tumor suppressor gene located in the 10q23.3 region, closely linked to the PTEN gene. Finally, it is possible that loss of a single allele of PTEN (haploinsuffiency) is by itself sufficient to promote tumor progression. The goal of this proposal, as outlined in our revised Statement of Work (see Appendices) was to resolve this paradox and, if PTEN loss is critical in prostate cancer progression, to begin to understand the underlying biological mechanisms involved.

To differentiate between these three possibilities, we utilized the transgenic adenocarcinoma of mouse prostate (TRAMP) model. The TRAMP model was generated in the C57BL/6 inbred strain by microinjection of a construct harboring a minimal rat probasin regulatory element to direct expression of the SV40 early genes to prostatic epithelium. The earliest pathology is prostatic intraepithelial hyperplasia (PIN) and the mice can display well-differentiated adenocarcinoma as early as 12 weeks of age. Over the next 6 weeks period the TRAMP mice will commonly display moderately differentiated carcinoma and ultimately develop poorly differentiated carcinoma by the time they reach 24 to 30 weeks of age. Metastasis, both hematogenous and lymphatic, have been detected as early as 12 weeks of age and by the time the mice are 24-30 weeks of age the incidence of metastasis approaches 100%. Thus, the TRAMP model mimics many aspects of human prostate cancer and is an excellent model system.

BODY

Tasks 1 and 3. Survival analysis of PTEN X TRAMP mice and determination of status of PTEN gene in prostate cancer from these mice.

Survival analysis of wild-type and PTEN +/- TRAMP mice. To determine if loss of the PTEN tumor suppressor gene was associated with increased rates of tumor progression in TRAMP mice we bred female TRAMP mice with male mice heterozygous PTEN knockout male mice (PTEN $^{+/-}$) and analyzed the survival of the transgenic male progeny. Half of all progeny would be predicted to wild type at the PTEN locus, while half would have one inactivated allele. It has been reported previously that PTEN$^{+/-}$ mice develop hyperplastic and dysplastic lesions of the prostate but invasive adenocarcinomas are rare and occur at much later ages than the TRAMP carcinomas. Animals were followed until the reached criteria for euthanasia, including the presence of a large palpable tumor exceeding 10% of body weight, difficulty ambulating, huddled posture or an obviously moribund appearance. In some cases animals died unexpectedly prior to euthanasia. In all cases, necropsy was performed with gross and microscopic examination. The genotype of the animal was not determined until after necropsy in order to prevent bias in selection for euthanasia or in histopathological examination. Given that PTEN$^{+/-}$ animals develop a number of pathologies, including a lymphoproliferative disorder (4,5), a meaningful comparison of survival with wild type animals requires that animals dying or requiring euthanasia be analyzed by necropsy. A total of transgenic 70 animals have been comprehensively analyzed to date and included litters born over a seven month period. An additional 23 animals have been sacrificed and are still being analyzed and a further 30 mice are still under observation. We are currently preparing the data from the first 70

animals for publication and our results based on these animals are described below. Seven animals were excluded from this cohort due to the presence of other pathology (primarily lymphoproliferative disorder) and/or the absence of significant prostate pathology. Based on the necropsy findings animals had three basic findings that lead to death. In approximately 30% of cases, animals had local disease and required euthanasia for large primary tumors and/or bladder obstruction leading to obvious morbidity. In the remaining cases, animals had metastatic disease, often to multiple organs, including lung (28/63), abdominal lymph nodes (26/63), liver (22/63) and kidney (4/63). Kaplan-Meier analysis of the survival of the $PTEN^{+/-}$ TRAMP animals in comparison to WT $^{+/+}$ littermate controls revealed that $PTEN^{+/-}$ had a significantly decreased survival ($p<0.03$) compared to $WT^{+/+}$ littermate controls. The PTEN $^{+/-}$ animals died at a mean age of 25.4 +/- 1.0 weeks (SEM, n=35) as compared to a mean age of 32.1 +/-1.3 weeks (SEM, n=28) for $WT^{+/+}$ littermate controls. This difference is also highly statistically significant (P=0.0001, t test). Analysis of the pathology at necropsy revealed the $PTEN^{+/-}$ and $WT^{+/+}$ animals had quite similar pathological findings. Of note was the finding that in both cases approximately 30% of animals died of local disease rather than metastasis (32% for $PTEN^{+/-}$ vs 30% for $WT^{+/+}$). Thus, inactivation of the PTEN gene appears to be promoting aggressive tumor growth rather than metastasis per se. In addition, the pattern of metastatic spread to various organs was not altered in the $PTEN^{+/-}$ animals when compared to $WT^{+/+}$ controls. One possible explanation for decreased survival in the $PTEN^{+/-}$ animals was that they may be less healthy than wild type animals due to pathology related to loss of one PTEN allele and thus died more easily than control animals. However, we found that the mean size of the primary tumors in the $PTEN^{+/-}$

animals (4.3 +/- 1.0 g, SEM, n=34) was actually greater than WT controls (3.0 +/- .28 g, SEM, n=28) so this explanation appears to be unlikely.

Analysis of the PTEN gene and protein in prostate cancer from PTEN$^{+/-}$ and WT$^{+/+}$ mice.

In order to determine the basis for the decreased survival seen in the PTEN$^{+/-}$ we determined the status of the PTEN gene and protein in a subset of these tumors. This analysis was greatly facilitated by the fact that the large, poorly differentiated tumors that were the most common finding at necropsy were greater than 90% carcinoma by histopathology, in contrast to human prostate cancer specimens where such pure tumors are unusual in surgically resected human prostate cancers. We carried out Southern blot analysis of DNAs extracted from primary prostate cancers of 38 animals using a probe which distinguishes the knockout (KO) and wild-type (WT) alleles (5). No evidence of homozygous deletion was seen in the 19 WT tumors analyzed. Of the 19 PTEN$^{+/-}$ tumors analyzed, 13 had loss of the WT allele, resulting in complete loss of functional PTEN gene. The mouse PTEN gene has been mapped to 24 cM on chromosome 19, a region synteneic to the human chromosome 10q23 region. To determine the extent of loss on mouse chromosome 19 in tumors with loss of the WT allele, we analyzed a subset of tumors for LOH on chromosome 19 using PCR of polymorphic repeats. These repeats are known to differ between the chromosome 19 derived from the SV129 strain (carrying the KO allele) and that derived from the C57Bl/6 (carrying the WT allele) based on information from the Jackson Labs database. Analysis of the closely linked D19Mit119 polymorphic marker, which is located at 27.5 cM on chromosome 19, revealed loss of the band derived from the C57Bl/6 allele in all cases, consistent with the Southern blot analysis. Similar results were seen at D19Mit41 (41 cM) and D19Mit33 (53 cM). Thus,

in approximately two-thirds of primary prostate cancers from the $PTEN^{+/-}$ mice there is loss of the WT allele due to loss of large regions (24-53 cM) of the WT chromosome 19. Loss of the entire WT chromosome 19 cannot be excluded.

In 6 of the 19 prostate cancers from the $PTEN^{+/-}$ animals there was retention of the WT PTEN allele. By Southern blotting there was retention of both WT and KO alleles in four cases, while in two cases there was loss of the KO allele. Analysis of the closely linked D19Mit119 marker was concordant with the Southern blot results. To determine if the PTEN was inactivated by methylation or promoter mutations leading to loss of expression we analyzed the 5 of the tumors for which protein extracts were available by Western blotting for PTEN expression. In all cases, PTEN protein expression was still present. We also performed mutation analysis by RT-PCR and sequencing using RNAs derived from 3 tumors and found no evidence of point mutation. This RT-PCR analysis was facilitated by our previously published observation regarding lack of a PTEN pseudogene in mice (6). Thus it appears that in these cases the single WT allele expressed a WT PTEN protein. When the survival data for the PTEN animals was correlated with the status of the PTEN gene in the prostate cancer tissue we found that animals with complete loss of the WT PTEN gene had a mean survival of 22.3 +/- 1.8 weeks (SEM, n=13) while animals with a single copy of the WT PTEN gene had a mean survival of 25.3 +/- 1.3 (SEM, n=6). Comparison of the mean survival of animals with a single PTEN allele with the WT animals revealed a significantly shorter mean survival than the WT animals ($p<0.03$, t test). Thus the absence of one PTEN allele is associated with survival that is shorter than the wild-type animals and indicates that haploinsufficiency of the PTEN gene promotes tumor progression in prostate cancer.

Given the evidence that loss of one or both alleles of the PTEN gene is associated with accelerated tumor progression in TRAMP prostate cancers we sought evidence that the PTEN gene was altered in the tumors from the $WT^{+/+}$ animals in our cohort. Analysis of the D19Mit119 polymorphic repeat, which is closely linked to the PTEN gene, in the tumors from the $WT^{+/+}$ animals revealed that in the 19 animals for which DNA was available, 9 had lost one allele while 10 retained both alleles. Thus the $WT^{+/+}$ animals showed high rates of LOH in the PTEN region. Western blot analysis of five tumors from $WT^{+/+}$ animals (with and without PTEN LOH) revealed that all expressed PTEN protein. Mutation analysis of the PTEN gene by RT-PCR and sequencing revealed no mutations in the 3 tumors with LOH that we analyzed. Thus, LOH is common at the PTEN locus in the $WT^{+/+}$ animals but there is no evidence of complete PTEN inactivation. The mean survival of animals with somatic LOH at D19Mit119 was 29.3 weeks, while animals without LOH was 34.2 weeks. While this difference is not statistically significant ($p=0.13$, t test) it is consistent with our observation in the PTEN mice that loss of one allele promotes tumor progression. These findings are in complete concordance with our results using 40 tumors from TRAMP animals crossed into a DBA background that we have analyzed previously, in that 40% of such tumors showed LOH in the region of the PTEN gene but no evidence of mutation (by RT-PCR and sequencing) or homozygous deletion (by Southern blotting) was found.

Tasks 2 and 4. Establishment and analysis of cultures from PTEN (+) and PTEN (-) TRAMP prostate cancers

Using tumors from the animals derived from our cross of TRAMP females with male $PTEN^{+/-}$ males we have established over 40 clonal cell cultures from primary and metastatic prostate cancers. We have analyzed PTEN expression in 29 of these cultures by Western blotting and have established that we have both PTEN expressing cell cultures and cultures with complete absence of PTEN expression. We are currently analyzing the biological consequences of loss of PTEN expression by examining these cell lines using a variety of biological and biochemical assays. Although many of these studies are ongoing, we have already generated several important findings. We have compared 13 PTEN (-) cultures derived from primary prostate cancers with 11 PTEN (+) cultures from similar primary tumors for production of vascular endothelial cell growth factor (VEGF). VEGF is generally believed to be a key regulator of tumor angiogenesis and we have previously shown that angiogenesis is increased in human prostate cancers in which PTEN has been homozygously deleted (2). Analysis of VEGF production by enzyme linked immunoabsorption assay has shown that the PTEN (-) cell cultures secrete 278 +/- 81 (SEM) pg/ml of VEGF while the PTEN (+) cultures secrete only 69.2 +/- 20 (SEM) pg/ml of VEGF. This difference is statistically significant ($p< 0.03$, t test). Thus a key mechanism for the increased angiogenesis seen in PTEN (-) prostate cancers in vivo may be increased VEGF expression by the prostate cancer cells. We are currently examining the basis for this increased expression of VEGF in PTEN (-) cells. We have also determined that there is increased expression of the anti-apoptotic protein Bcl-2 in PTEN (+) prostate cancer cells as compared PTEN (-) cells by Western blotting. Our

hypothesis is that in the absence of PTEN inactivation cells have acquired additional mechanisms to circumvent apoptosis i.e. increased expression of the apoptosis inhibitor Bcl-2. We are currently carrying out detailed studies of the regulation of apoptosis in our cell cultures in collaboration with other laboratories. We have also undertaken analysis of cell proliferation and of alteration in expression of cyclins and cyclin-dependent kinase inhibitors in these cells. The availability of these cell cultures provides a rich resource for such studies. At the present time we are not certain if these cultures are immortal (i.e. cell lines) but an important goal is determine if they are immortal, and if not try, and establish such immortal cell lines by nursing surviving cells through any replicative crisis.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that inactivation of the PTEN gene is associated with a significant increase in the rate of prostate cancer progression.
- Have shown that the increased rate of tumor progression due to PTEN activity is due to more aggressive tumor growth and not increased rates of metastasis.
- Demonstrated that haploinsufficiency of the PTEN gene is able to promote tumor progression in prostate cancer. This finding has broad implications for the analysis of the role of the PTEN gene in prostate cancer and other malignancies and indicates that PTEN is even more important than it is currently believed to be the case based on mutation analysis.
- Have established a large number of new, matched prostate cancer cell cultures that either express or completely lack PTEN protein for future studies of the biological role of PTEN in tumor progression.
- Have shown that human prostate cancers with homozygous deletion of the PTEN gene have increased rates of tumor angiogenesis and based on results from our cultured cells that this may be due to increased expression of VEGF.
- Demonstrated that mice lack a PTEN pseudogene (in contrast to humans) and that RT-PCR and sequencing is therefore a rapid and simple alternative to exon by exon analysis in mice.

REPORTABLE OUTCOMES

1) Publications and manuscripts.

- Giri, D. and Ittmann, M. 1999. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma. Human Path. 30:419-424.
- Kwabi-Addo, B., Thompson, T. and Ittmann, M. 2000. Absence of the PTEN/MMAC1 pseudogene in mice. DNA Cell Biol. 19: 301-305.
- Kwabi-Addo, B et al. 2001. Haploinsufficiency of the PTEN gene promotes prostate cancer progression. (in preparation for submission to Nature Medicine)

2) We have generated more than forty clonal mouse prostate cancer cell cultures from primary and metastatic prostate cancers, many of which have homozygous loss of the mouse PTEN gene. These are available for further study of the biological role of PTEN in prostate cancer progression.

3) We have developed a repository of frozen tissues, DNAs, RNAs, protein extracts and paraffin blocks from prostate cancers arising in our $PTEN^{+/-}$ and $WT^{+/+}$ mice. This is linked to a database describing survival and histopathology at necropsy. This resource will be invaluable for further studies of the biological role of the PTEN gene in prostate cancer.

CONCLUSIONS

Our work has conclusively demonstrated that loss of the PTEN gene is associated with increased rates of tumor progression in a mouse model of prostate cancer. A key observation is that loss of even a single allele of the PTEN gene can increase rates of tumor progression and this resolves a longstanding dilemma regarding the discordance between rates of LOH at the PTEN locus and observed rates of mutation. Our results indicate that at least one factor leading to increased tumor progression is increased tumor angiogenesis secondary to increased VEGF expression in tumors with loss of the PTEN gene. However, based on the current literature, alterations in apoptosis and proliferation are also likely to play an important role (1). The availability of numerous clonal cell lines and our bank of tissues from the PTEN $^{+/-}$ and WT$^{+/+}$ will be invaluable in dissecting out the many factors associated with the increased rate of tumor progression following PTEN inactivation in prostate cancer.

REFERENCES

1. Di Cristofano, A and Pandolfi, P.P. 2000 The multiple roles of PTEN in tumor suppression. Cell. 100:387-390.

2. Giri, D. and Ittmann, M. 1999. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma. Human Path. 30:419-424.

3. Ittmann, M. 1998. Chromosome 10 alterations in prostate adenocarcinoma (Review). Oncol. Reports 5: 1329-1335.

4. Di Cristofano, A., Kotsi, P., Peng, Y.F., Cordon-Cardo, C., Elkon, K.B. and Pandolfi, P.P. 1999 Impaired Fas response and autoimmunity in Pten+/- mice. Science. 285:2122-2125.

5. Podsypanina, K., Ellenson, L.H., Nemes, A., Gu J, Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E. and Parsons, R. 1999. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci 96:1563-1568.

6. Kwabi-Addo, B., Thompson, T. and Ittmann, M. 2000. Absence of the PTEN/MMAC1 pseudogene in mice. DNA Cell Biol. 19: 301-305.

APPENDICES

1) Publications and manuscripts.

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- Bernard Kwabi-Addo PhD
- Dipak Giri DVM, PhD
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Statement of Work (5/16/00, revised)

Overview of problem and experimental approach: Our analysis of numerous TRAMP prostate cancers has yielded a paradoxical observation. Although approximately 30% of the tumors show evidence of loss of heterozygosity (LOH) at the PTEN locus we have found no evidence of loss of PTEN protein expression, homozygous deletion of the PTEN gene or PTEN mutation. Two possible theories could explain this finding:

(1) Loss of one allele is sufficient to promote tumorigenesis (hemizygous sufficiency)
(2) A second tumor suppressor gene is present near the PTEN locus.

This question is of some importance because it parallels similar observations in human prostate cancer (and several other human malignancies) in that the rate of LOH is substantially higher than the rate of complete PTEN inactivation by loss of protein expression and/or mutation. To answer this question we have setup a large experiment in which PTEN heterozygote mice are crossed with TRAMP mice. We currently have 120 TRAMP positive male mice from this cross. These male TRAMP transgene positive animals will be followed to the point at which 3-4 cm palpable tumors are present. These large tumors allow for simultaneous evaluation of PTEN protein expression by Western blotting, mutation detection by direct sequencing of cDNA and Southern blotting to determine the status of the normal and inactivated PTEN alleles. Eleven such tumors have been harvested to date. The cross results in 50% heterozygote PTEN knockout animals and 50% wild type animals, which will act as controls. If hypothesis (1) is correct we predict that heterozygote knockout animals will develop palpable tumors faster than controls, while if hypothesis (2) is correct no difference will be seen in the rate of tumor formation because the PTEN gene knockout is irrelevant for tumor formation. Furthermore, if hypothesis (1) is correct then examination of the tumors will show only a low rate of complete inactivation of PTEN (i.e. loss of the wild type allele) since loss of a single copy of the PTEN gene is sufficient to promote tumorigenesis. If hypothesis (2) is correct then we will see equal rates of loss of both the inactivated and wild-type alleles in the tumors since the PTEN gene is irrelevant to the LOH event. We will also quantitatively evaluate metastasis formation in these animals to determine whether metastasis may be promoted by loss of a single allele, since LOH at the PTEN locus is particularly common in metastatic prostate cancer.

As a critical adjunct to these experiments we will establish primary cultures and hopefully cell lines from as many primary tumors and large metastatic lesions as possible. Some of these cultures will be heterozygous for PTEN inactivation, some wild type and some will (perhaps) have complete PTEN inactivation. The availability of these cell lines from identical genetic backgrounds will allow us to examine the effect of PTEN hemi- or homozygous loss on numerous biological parameters that effect tumorigenesis in vivo including growth rate, invasion and motility, angiogenic activity, soft agar colony formation and apoptotic rate. To date all of our primary cultures of tumors have been successful and we are trying to establish permanent cell lines at the present time from all of these.

TASKS

Task 1. Follow PTEN heterozygote knockouts crossed with TRAMP mice for tumor development till 3-4 cm palpable tumors are present

A. Harvest fresh tumors (primary and large metastatic lesions), measure and weigh tumors, use portion for primary culture and snap freeze majority of remainder. One portion is submitted for paraffin–embedding as a part of the necropsy.
B. Perform complete necropsies on all animals to determine size and extent of primary tumors and metastasis in heterozygote knockouts and controls.
C. Analyze at least 100 animals in this fashion.
D. At end of experiment, unblind data regarding genotype of each animal and compare statistically.

Task 2. Establish primary cultures and cell lines from prostate cancers in mice analyzed in Task 1 above.

A. Establish primary cultures from primary and metastatic tumors harvested above from wild type and heterozygote mice for analysis.
B. Try to establish as many immortal cell lines from primary cultures above as possible.

Task 3. Analysis of prostate tumors in animals from Task 1 above

A. Determine PTEN status in all tumors by Southern blotting to determine whether there is loss is of the wild type and/or inactivated PTEN allele.
B. Perform Western blots on tumor extracts using anti-PTEN antibody
C. Perform mutation analysis using cDNAs from tumor RNAs.

Task 4. Characterization of primary cultures and cell lines from Task 2 above
A. Determine PTEN status of primary cultures and cell lines by Southern blotting, Western blotting and mutation analysis.
B. Characterize primary cultures and cell lines for morphology, proliferation rate (BrDU incorporation), apoptotic rate (TUNEL), motility and invasion (Boyden chamber assay), soft agar colony formation and angiogenic activity (HUVEC proliferation assay).
C. Correlate PTEN genotype and in vitro phenotype as determined above.

Inactivation of the PTEN Tumor Suppressor Gene is Associated With Increased Angiogenesis in Clinically Localized Prostate Carcinoma

DIPAK GIRI, PHD, AND MICHAEL ITTMANN, MD, PHD

The PTEN tumor suppressor gene encodes a dual-specificity protein phosphatase that may play a key role in modulating integrin-mediated signals. Inactivation of the PTEN gene has been detected in a small percentage of clinically localized prostate cancers but is common in metastatic disease. It has been shown in glioblastoma cell lines that loss of chromosome 10q, where the PTEN gene is located, is associated with increased angiogenic activity in the conditioned medium attributable to downregulation of thrombospondin-1, a negative regulator of angiogenesis. Therefore, we wished to determine whether inactivation of PTEN might be associated with increased angiogenesis in prostate cancers, because increased angiogenesis in localized cancers is associated with development of metastatic disease. Angiogenesis was assessed by counting microvessels in areas of maximal neovascularization after immunostaining with anti-factor VIII–related antigen antibodies in eight cases with proven homozygous deletion of the PTEN gene and 24 control cases. There was a statistically significant correlation between PTEN inactivation and increased microvessel counts. The microvessel density was higher at all Gleason scores in the cases with PTEN inactivation compared with control cases with the same score. To determine whether the increased angiogenesis in cases with PTEN inactivation was caused by downregulation of expression of the angiogenesis inhibitor thrombospondin-1, we analyzed a subset of the cases by immunostaining with anti-thrombospondin-1 antibody. Approximately 25% of cases showed decreased staining of prostate cancer cells, but there was no correlation with PTEN inactivation. Thus, PTEN inactivation is associated with increased angiogenesis, but the increased angiogenesis is not attributable to downregulation of thrombospondin-1 expression. HUM PATHOL 30:419-424. Copyright © 1999 by W.B. Saunders Company

Key words: **prostate carcinoma, PTEN, thrombospondin-1, angiogenesis.**

Abbreviation: **PSA, prostate-specific antigen.**

Tumor suppressor genes have been shown to be inactivated in most malignant neoplasms, particularly carcinomas. Recently a novel tumor suppressor gene known as PTEN[1] or MMAC-1[2] was cloned from the 10q23 region of chromosome 10. Mutation of this gene was subsequently shown to be the cause of Cowden disease,[3] a familial neoplastic syndrome characterized by a variety of benign hamartomatous tumors as well as a high frequency of breast and thyroid cancer in affected kindreds, proving that this gene is indeed a tumor suppressor gene in vivo. In addition, inactivation of the PTEN gene has been found in cell lines or primary tumors from a wide variety of malignant neoplasms, including prostate, breast, renal, bladder, and endometrial carcinomas, glioblastomas, melanomas, and sarcomas.[1,2,4-11]

The PTEN gene encodes a dual-specificity protein phosphatase[12] and also has extensive homology to tensin, a protein that interacts with actin filaments at focal adhesions. Focal adhesions are sites on the plasma membrane at which integrins aggregate and interact with a number of signal transduction proteins, including tyrosine kinases such as focal adhesion kinase as well as cytoskeletal proteins. In this manner, integrin-mediated signals are transmitted into the cell to control such integrin-mediated processes as cell migration, spreading, and growth. Recent studies by Tamura et al[13] have provided strong evidence that PTEN does inhibit the phosphorylation of focal adhesion kinase in response to integrin-mediated signals and plays a role in regulating the biological response to integrins.

Inactivating alterations of the PTEN gene have been identified in prostate cancer cell lines and primary tumor specimens. Three of four prostate cancer cell lines analyzed inactivate both alleles of PTEN.[1] It should be noted that all of these cell lines were derived from metastatic prostate cancers. In collaboration with others,[4] we have recently evaluated 60 stage B prostate cancers both by polymerase chain reaction (PCR) analysis of polymorphic markers and quantitative Southern blotting. We found eight cases (13%) with homozygous deletions by quantitative Southern blotting, which were confirmed by the retention of heterozygosity at intragenic polymorphic markers.[14] A number of other groups have also analyzed primary human prostate cancer samples for inactivating lesions in the PTEN gene such as point mutations or homozygous deletions and found relatively low rates of PTEN inactivation in clinically localized cancers.[5-7] A higher rate of inactivation has been seen in the stage D cancers, with inactivating lesions seen in 13 of 39 such cases analyzed.[5,8] It is thus clear that PTEN is inactivated in a relatively small subset of clinically localized prostate cancers but in a much higher proportion of metastatic cancers. The reason for this strong association with tumor metastasis has yet to be established.

Angiogenesis plays a key role in tumor metastasis both by providing blood vessels that can be invaded so

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Supported by U.S. Army Medical Research Command DAMD17-98-1-8576 and Department of Veterans Affairs Merit Review funds.

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0046-8177/99/3004-0010$10.00/0

that tumor cells can enter the circulation and by promoting growth at metastatic sites. Weidner et al[15] have shown that increased tumor angiogenesis in primary tumor specimens correlates with metastasis in prostate cancer. It has been shown that introduction of chromosome 10q into glioma cell lines lacking chromosome 10 is associated with inhibition of angiogenic activity by conditioned medium from these cells because of induction thrombospondin-1 production.[16] Given that PTEN is strongly associated with metastatic disease, it is possible that PTEN loss may promote angiogenesis, directly or indirectly, and thus metastasis. We report here that clinically localized prostate cancers with homozygous deletions of the PTEN gene do indeed have significantly higher levels of angiogenesis than similar cancers without PTEN inactivation, but this alteration is not attributable to decreased thrombospondin-1 production by tumor cells in vivo.

MATERIALS AND METHODS

Tissue Acquisition and Analysis

All prostate cancer specimens were from radical prostatectomies performed for treatment of clinically localized prostate cancer. Tissues were received fresh and portions snap frozen in liquid nitrogen, DNA extracted,[17] and analyzed for homozygous deletion or mutation of the PTEN gene as described previously.[4] In each case, the tissue remaining after harvest of fresh tissue, including the entire prostatic capsule and both seminal vesicles, were subject to full pathological analysis of tumor stage and grade (Gleason score). No clinically or pathologically detectable metastasis was present in pelvic lymph nodes in any of the 60 cases. Prostate-specific antigen (PSA) recurrence was defined as a serum PSA greater than 0.4 ng/mL.

A total of 60 cases were analyzed, and eight of these had homozygous deletions of the PTEN gene by quantitative Southern blotting that were confirmed by retention of heterozygosity at intragenic polymorphic markers.[4] Of the 52 remaining cases, four had loss of heterozygosity in the 10q23 region without evidence of PTEN inactivation. These cases were excluded from analysis because of the difficulty in ruling out PTEN alterations. A total of 24 cases were randomly selected from the remaining 48 cases without PTEN inactivation based on the availability of corresponding paraffin blocks with adequate content of carcinoma.

Immunohistochemistry

Immunohistochemistry for factor VIII–related antigen was performed on 4-μm paraffin-embedded tissue sections of the 32 cases described. After deparaffinization, sections were treated with Autoblocker (R&D Systems) to inhibit endogenous peroxidase and avidin/biotin (Vector Laboratories, Burlingame, CA) to block endogenous biotin. The sections were incubated with 1 μg/mL rabbit anti–factor VIII polyclonal antibodies (Dako, Carpinteria, CA) at 4° for 12 hours at. After liberal washing with phosphate-buffered saline, pH 7.4, sections then were incubated with 7.5 μg/mL of biotinylated goat anti-rabbit secondary antibody (Vector Laboratories). Sections were then washed with phosphate-buffered saline containing 0.1% Tween 20 and incubated with avidin-biotin complex (Vectastain Elite, Vector Laboratories) for 15 minutes. The antigen-antibody reaction was shown using diaminobenzidine as substrate and the sections then counterstained with hematoxylin.

Immunohistochemistry for thrombospondin-1 was performed on frozen sections using anti-thrombospondin-1 antibody (Oncogene Research Products, Cambridge, MA), 200 ng/mL, at 4°C for 12 hours followed by 1 hour at 37°C. Immunoreactive thrombospondin-1 was detected following the protocol outlined for factor VIII using 7.5 μg/mL biotinylated horse anti-mouse secondary antibody (Vector Laboratories).

Vessel Counting

Analysis of microvessel density was based on the method described by Weidner et al.[15] Slides were scanned at 40× and 100× to determine the area with the highest level of neovascularization. Vessels were then counted in a total of five adjacent 200× fields in this area using a Nikon Labophot-2 microscope. The criteria of Weidner et al[15] were used to identify microvessels. In essence, any freestanding brown-stained endothelial cell or endothelial cell cluster was counted as a microvessel. The highest of the five fields was taken as the microvessel density and used to calculate the vessel density per square millimeter based on the area of the 200× field measured directly using an ocular micrometer. The PTEN status of the case was not known at the time of microvessel counting.

RESULTS

Comparison of Clinically Localized Prostate Cancers With and Without PTEN Inactivation

A comparison of the pathological stage and grade of cases with homozygous deletion of PTEN (PTEN [−]) and cases without PTEN deletion (PTEN [+]) is shown in Table 1. Most of both PTEN (−) and PTEN (+) tumors had microscopic evidence of extracapsular extension (T3a or T3b). A higher proportion of PTEN (+) cases were organ confined (T2), but there was also a higher proportion of cases with seminal vesicle invasion in this group, so that overall the pathological stage was comparable between the two groups. None of the observed differences in stage were statistically significant. The Gleason sum of the PTEN (−) cases was higher than that of the PTEN (+) cases (6.9 *v* 6.1), and this difference approached statistical significance

TABLE 1. Comparison of PTEN (−) and PTEN (+) Prostate Cancers

	PTEN (−)	PTEN (+)
Stage (TNM)		
T2a	0	1 (4)
T2b	0	1 (4)
T2c	1 (13)	5 (21)
T3a	3 (37)	11 (46)
T3b	4 (50)	4 (17)
T3c	0	2 (8)
Gleason score	6.9 ± 0.8	6.1 ± 1.0
PSA (+) 2 yrs	2/5 (40)	5/16 (31)

NOTE. Stage is pathological stage. The percentage in each category is indicated in parentheses. PSA (+) is defined as a serum PSA >0.4 ng/mL with at least 2 years follow-up. The percentage with PSA recurrence is indicated in parentheses.

($P = .07$, t-test). Thus, the cases with homozygous deletion of PTEN are slightly more poorly differentiated, but the pathological stage is similar to cases with retention of PTEN. Only limited clinical follow-up is available on these cases because the follow-up period in most cases is less than 2 years, but, as can be seen in Table 1, the cases with PTEN inactivation are somewhat more likely to have had a PSA recurrence at 2 years.

Validation of the Determination of Microvessel Density

Microvessel density was determined after anti–factor VIII–related antibody immunohistochemistry by the method of Weidner et al,[15] who found a statistically significant correlation of microvessel density determined by their technique and metastasis. We chose to use anti–factor VIII–related antigen rather than other vascular markers so that we could directly compare our results with those of Weidner et al,[15] who found a strong association of microvessel counts, as determined by their technique, and metastasis. Examples of prostate cancers with high and low microvessel counts are shown in Figure 1. To validate our method, we therefore compared our results with those obtained by Weidner et al. The cases analyzed by these authors contained a group of patients with metastasis at the time of radical prostatectomy or within 4 years of follow-up and a second group without evidence of metastasis in this period. Given the absence of metastatic disease in the pelvic lymph nodes of all of our patients and the fact that less than 20% of such patients would be expected to develop metastatic disease at 4 years,[18] we compared the mean microvessel density in our cases with that of Weidner's cases without metastasis after 4 years' follow-up. The mean microvessel count per square millimeter was 48 for our cases and 53 for the Weidner's metastasis-negative group, a difference that is not statistically significant. We also observed a correlation between microvessel density and Gleason sum, similar to that observed by Weidner (Fig 2). Thus we are confident that our application of the microvessel counting technique of Weidner et al is accurate and reproducible.

Microvessel Density in Prostate Cancers With Homozygous Deletion or Retention of PTEN

The mean microvessel density in cases with homozygous deletion of PTEN was 63.3 vessels per square millimeter (± 5.5; SEM, $n = 8$), whereas those cases with retention of PTEN had a mean of 40.2 vessels per square millimeter (± 5.5; SEM; $n = 24$). This difference is statistically significant ($P < .05$, t-test). Given that the microvessel number increases with increasing Gleason score and that the mean Gleason score was greater for the PTEN (−) cases, it is possible that the observed difference is a function of the poorer differentiation of the PTEN (−) cases. However, as can be seen in Figure 3, the mean microvessel count was higher in PTEN (−) cases, irrespective of Gleason score, compared with PTEN (+) cases with the same Gleason score. Thus, PTEN inactivation is associated with increased microvessel counts independent of the differentiation of the tumors.

Thrombospondin-1 Expression in Prostate Cancers With and Without PTEN Deletion

To determine whether the increased microvessel density in the PTEN (−) cancers was caused by decreased expression of the angiogenesis inhibitor thrombospondin-1 in these cases, we evaluated the expression of thrombospondin-1 by immunohistochemistry using 17 cases for which frozen tissue containing cancer was available for frozen sections. We chose to use frozen sections because we were unable to obtain strong, reproducible immunostaining in paraffin-embedded tissue even with antigen retrieval. The normal prostatic epithelium showed weak to moderate, thrombospondin-1 immunostaining of the luminal epithelial cells

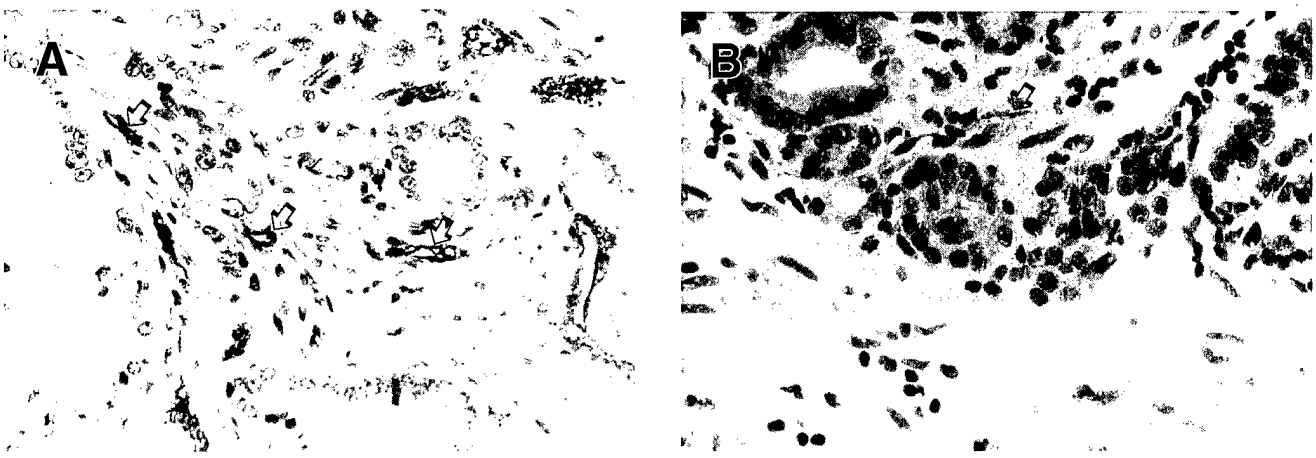

FIGURE 1. Antifactor VIII antibody immunostaining of prostate carcinomas. Immunohistochemistry with anti-factor VIII-related antigen antibody was performed on paraffin-embedded sections of prostate carcinomas as described in Materials and Methods. (A) Prostate carcinoma with numerous microvessels, some of which are indicated by arrows. (B) Prostate carcinoma with low microvessel count. A single stained cell is indicated by the arrow. (Original magnification ×200. Only a portion of the 200× field is present in the photomicrograph.)

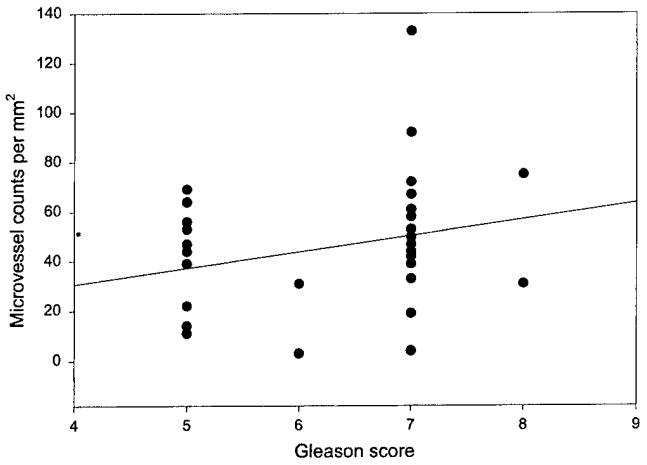

FIGURE 2. Correlation of microvessel count with Gleason score. Microvessel counts were determined as described in Materials and Methods after immunostaining with anti-factor VIII antibody. The line indicates the best fit by linear regression of the microvessel count as a function of Gleason score.

and basal cells that was somewhat variable among different acini within the same section. Endothelial cells stained strongly, as has been reported previously,[19] providing an internal control and occasional fibroblastic cells were also stained. Thirteen cases showed cytoplasmic immunostaining for thrombospondin-1 in 10% to 95% of the prostate cancer cells (Fig 4A). Four cases showed no staining except for rare, individual cells (Fig 4B). All four PTEN (−) cases analyzed had positive staining for thrombospondin-1, as did five of nine PTEN (+) cases. Thus, although a subset of prostate cancers appear to have decreased thrombospondin-1 production, there was no evidence that loss of PTEN led to decreased thrombospondin-1. It should be noted that no correlation between thrombospondin-1 production and microvessel density was observed in our cases (data not shown).

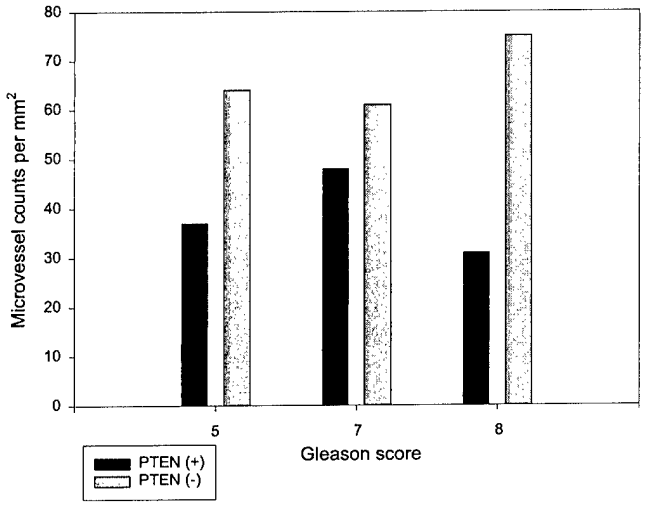

FIGURE 3. Microvessel counts for PTEN (−) and PTEN (+) prostate cancers with equivalent Gleason score. Microvessel counts were determined after immunostaining with anti-factor VIII antibody. The mean microvessel count of PTEN (−) and PTEN (+) cases at the indicated Gleason score is shown. Two PTEN (+) cases with a Gleason score of 6 are not shown because no PTEN (−) cases with that score were available.

DISCUSSION

Inactivation of the PTEN tumor suppressor gene has been shown to occur in 5% to 15% of clinically localized prostate cancers as well as approximately 30% of metastatic prostate cancer samples.[4-8] We report here that the subgroup of clinically localized prostate cancers with homozygous deletion of the PTEN gene have significantly higher levels of tumor angiogenesis, as assessed by microvessel counting, than those cases with retention of the PTEN gene. Such increased angiogenesis is correlated with the eventual development of metastatic disease in prostate cancer[15] as well as in many other forms of cancer and is consistent with the known association of PTEN inactivation with metastasis in prostate cancer. A similar association of increased microvessel density, as determined after immunostaining with either anti-CD31[20] or anti-CD34[21] antibodies, with higher rates of recurrence supports the original observation of Weidner et al[15] using factor VIII immunostaining. Increased angiogenesis may be associated with tumor metastasis for two reasons. First, the increased number of blood vessels provide more sites for invasion by tumor cells, and these newly formed vessels may be susceptible to invasion. Second, the increased angiogenesis observed in the primary site may reflect the underlying ability of the tumor to induce angiogenesis in metastatic deposits, and this ability may be crucial for the tumor to be able to implant and grow in these metastatic sites.

Although our cases with PTEN inactivation had a somewhat higher rate of PSA recurrence at 2 years than the cases with retention of the PTEN gene, compatible with more aggressive behavior, the small number of cases and the short follow-up do not allow us to make a firm conclusion regarding the impact of PTEN inactivation on clinical outcome in patients undergoing radical prostatectomy for localized disease. To accurately determine whether PTEN inactivation in clinically localized prostate cancers is correlated with the eventual development of metastasis, a much longer follow-up will be necessary (5 to 10 years), and more cases will need to be analyzed. However, the higher microvessel counts in our PTEN(−) cases suggest that they will have higher rates of metastasis in the future.[15,20,21] It should be noted that this report may underestimate the difference between cases with PTEN inactivation and those with PTEN retention, because of the possibility that some of the cases that we have assigned to the PTEN (+) group may actually have inactivation of PTEN by mechanisms we did not analyze in our tumors, such as transcriptional repression, mutations of the promoter region of both PTEN alleles, or small homozygous deletions outside the region analyzed by our Southern blot probes. The presence of such cases would lead to underestimation of the differences between PTEN (−) and PTEN (+) groups. The availability of an antibody that gives strong specific staining of the PTEN protein

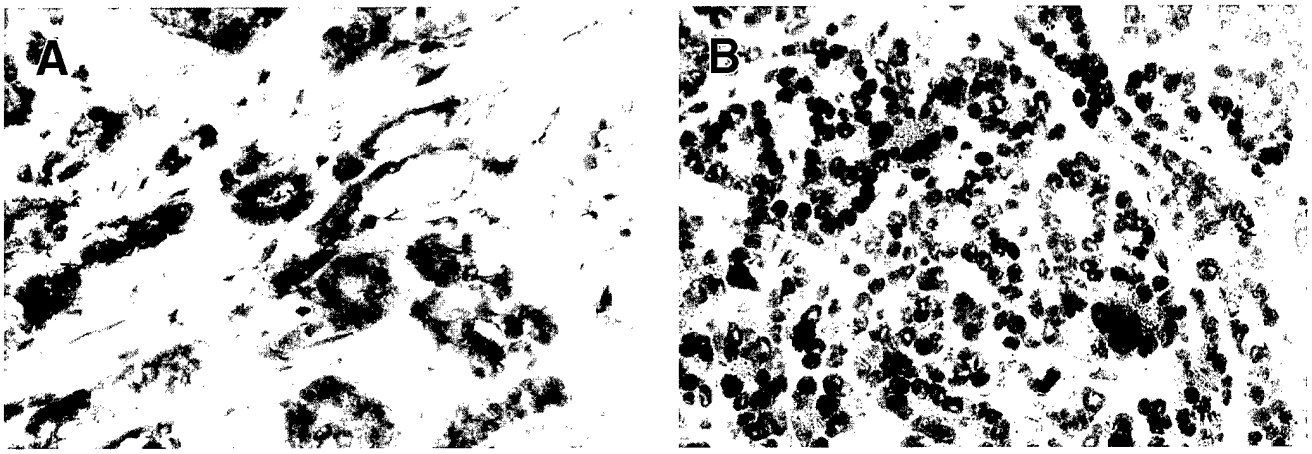

FIGURE 4. Immunohistochemistry of prostate cancers with anti-thrombospondin-1 antibody. Immunohistochemistry with anti-thrombospondin-1 antibody was performed as described in Materials and Methods. (A) PTEN (−) case with positive staining of cancer cells for thrombospondin-1. (B) PTEN (+) case with no thrombospondin-1 staining. (Original magnification ×200.)

in tissue sections, particularly paraffin-embedded sections, would be extremely useful in detecting such inactivation and in assessing the prognostic significance of PTEN inactivation in prostate cancer specimens, but such an antibody is not available.

The mechanism by which PTEN inactivation leads to increased angiogenesis in localized prostate cancers is unclear. Recent studies have shown that PTEN inhibits integrin-mediated cell motility,[13] consistent with its postulated role as a negative regulator of integrin-mediated signal transduction at focal adhesion complexes. An increase in cell motility and invasion after PTEN inactivation might indirectly increase angiogenesis by release of angiogenic substances from surrounding tissues or other indirect mechanisms. Alternatively, PTEN inactivation may lead to increased production of angiogenic factors or decreased production of angiogenesis inhibitors by the prostate cancer cells themselves. It has been shown that introduction of chromosome 10q into glioblastoma cell lines lacking chromosome 10 leads to decreased angiogenic activity in conditioned medium from these cells because of increased production of the angiogenesis inhibitor thrombospondin-1. Given that the PTEN gene is located on 10q, it is possible that it may be mediating this effect. However, in prostate cancer we found no correlation between loss of PTEN and decreased expression of thrombospondin-1. We also found no correlation with loss of thrombospondin-1 expression and microvessel density, so it appears unlikely that thrombospondin-1 is a major modulator of angiogenesis in clinically localized prostate cancer. However, many factors can inhibit angiogenesis, such as thrombospondin-2, as well as angiogenesis inducers such as vascular endothelial growth factor. Thus the manner in which loss of PTEN expression leads to increased angiogenesis may be complex, but elucidation of this process will be important in understanding the role of PTEN inactivation in the biology of prostate cancer.

Acknowledgment. The authors appreciate the assistance of Shea Scott and Dr Robert Genta in cutting of paraffin sections.

REFERENCES

1. Li J, Yen C, Liaw D, et al: PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science 274:1943-1947, 1997

2. Steck P, Pershouse M, Jasser S, et al: Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nature Genet 15:356-362, 1997

3. Liaw D, Marsh D, Li J, et al: Germline mutations of the PTEN gene in Cowden disease, and inherited breast-thyroid cancer syndrome. Nature Genet 16:64-67, 1997

4. Wang S, Parsons R, Ittmann M: Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas. Clin Cancer Res 4:811-815, 1998

5. Cairns P, Okami K, Halachmi N, et al: Frequent inactivation on PTEN/MMAC1 in primary prostate cancer. Cancer Res 57:4997-5000, 1997

6. Feilotter H, Nagai M, Boag A, et al: Analysis of PTEN and the 10q23 region in primary prostate carcinomas. Oncogene 16:1743-1748, 1998

7. Pesche S, Latil A, Muzeau F, et al: PTEN/MMAC/TEP1 involvement in primary prostate cancers. Oncogene 16:2879-2883, 1998

8. Suzucki H, Freije D, Nusskern D, et al: Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res 58:204-209, 1998

9. Teng D, Hu R, Lin H, et al: MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res 57:5221-5225, 1997

10. Tashiro H, Blazes M, Wu R, et al: Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 57:3935-3940, 1997

11. Wang S, Puc J, Li J, et al: Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 57:4183-4186, 1997

12. Myers M, Stolarov J, Eng C, et al: P-TEN, the tumor suppressor from chromosome 10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A 94:9052-9057, 1997

13. Tamura M, Gu J, Matsumoto K, et al: Inhibition of cell migration, spreading and focal adhesions by tumor suppressor gene PTEN. Science 280:1614-1617, 1998

14. Cairns P, Tokino K, Eby Y, et al: Homozygous deletions of 9p21 in primary human bladder tumors detected by comparative multiplex polymerase chain reaction. Cancer Res 54:1422-1424, 1994

15. Weidner N, Carroll P, Flax J, et al: Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 143:401-409, 1993

16. Hsu S, Volpert O, Steck P, et al: Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. Cancer Res 56:5684-5691, 1996

17. Ittmann M, Wieczorek R, Heller P, et al: Alterations in the p53 and MDM-2 genes are infrequent in clinically localized Stage B prostate adenocarcinomas. Am J Pathol 145:287-293, 1994

18. Wheeler T, Dillioglugil O, Kattan M, et al: Clinical and pathological significance of the level and extent of capsular invasion in clinical stage T1-2 prostate cancer. HUM PATHOL 29:856-862, 1998

19. Campbell S, Volpert O, Ivanovich M, et al: Molecular mediators of angiogenesis in bladder cancer. Cancer Res 58:1298-1304, 1998

20. Siberman M, Partin A, Veltri R, et al: Tumor angiogenesis correlates with progression after radical prostatectomy but not with pathological stage in Gleason sum 5 to 7 adenocarcinoma of the prostate. Cancer 79:772-778, 1997

21. Bettencourt M, Bauer J, Sesterhan I, et al: CD34 immunohistochemical assessment of angiogenesis as a prognostic marker for prostate cancer recurrence after radical prostatectomy. J Urol 160:459-465, 1998