The Accumulation of Angiostatin-like Fragments in Human Prostate Carcinoma[1]

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ABSTRACT

Purpose: **Angiostatin, a potent inhibitor of angiogenesis and, hence, the growth of tumor cell metastasis, is generated by a proteolytic enzyme from plasminogen. However, its localization and specific enzymes have yet to be ascertained in human tissue.**

Experimental Design: **To elucidate the generation and the localization of angiostatin in prostate carcinoma, we examined angiostatin generation in a panel of human prostate cancer cell lines and performed immunohistochemistry with the antibodies to angiostatin and prostate-specific antigen (PSA), a potent proteolytic enzyme of angiostatin in 55 cases of prostate carcinoma.**

Results: **We demonstrated that the lysates of human prostate carcinoma cell lines could generate angiostatin-like fragments from purified human plasminogen but could not generate angiostatin in the absence of exogenous plasminogen. The fragmented proteins were reacted with the monoclonal antibody specific for plasminogen lysine-binding site 1 (LBS-1). Immunohistochemically, the intracytoplasmic immunostaining of LBS-1 was positive in 87.3% (48 of 55) of prostate carcinoma cases, and the immunostaining of miniplasminogen was negative in all cases. There was a significant relationship between the positive immunostaining of LBS-1 and Gleason score ($P = 0.0007$). The intracytoplasmic immunostaining of PSA was positive in 37.0% (20 of 54) of prostate carcinoma cases, but there was no significant relationship between the expression of PSA and Gleason score, or between the positive immunostaining of LBS-1 and PSA.**

Conclusions: **These findings suggest that angiostatin is generated by prostate carcinoma cells and is accumulated within the cytoplasm. In addition, the generation of angiostatin-like fragments was correlated with tumor grade; however, PSA may not be the only enzyme for angiostatin generation in human prostate carcinoma.**

INTRODUCTION

Tumor growth and metastasis require new blood vessels and both are, thus, dependent on angiogenesis. Angiogenesis is regulated by a balance between angiogenic stimulators and angiogenesis inhibitors. Therefore, endogenous angiogenesis inhibitors are important for tumor development. O'Reilly *et al.* (1) discovered that angiostatin, which is a M_r 38,000 internal fragment of mouse plasminogen, inhibits angiogenesis of lung metastasis arising from murine Lewis lung carcinoma. It has been reported that angiostatin is generated from plasminogen by limited proteolysis-related enzymes, such as matrix metalloproteinases 2, 3, 7, 9, and 12 (2–5), plasmin (6, 7), cathepsin D (8), urokinase-type plasminogen activator (9) and tumor cell-derived plasmin thiolreductase (7, 10). It is well known that angiostatin-like fragments are produced through the digestion of human plasminogen under certain circumstances, often in patients with malignant disease (11, 12), and they are then secreted into the circulation. Plasminogen LBS-1,[3] which includes angiostatin, contains the kringle domains 1–3, and is inhibitory for new vessel formation in an *in vitro* angiogenesis model (13). Miniplasminogen, which does not contain angiostatin, is also produced through the digestion of plasminogen and comprises kringle 5 plus the light chain (14). The use of immunoblotting to determine the existence of fragments is a well-established procedure; however, the immunohistochemical method has not been investigated in human tissue, and little is known about the localization of angiostatin in human tissue.

Recently, Heidtmann *et al.* (15) reported that PSA is able to convert Lys-plasminogen to angiostatin-like fragments, containing kringles 1–4, *in vitro*. The purposes of the current study were to demonstrate the angiostatin generation from human plasminogen using a lysate of human prostate carcinoma cells *in vitro* and to clarify the immunohistochemical distribution of angiostatin in prostate carcinoma *in vivo*. Furthermore, we evaluated the correlation between angiostatin-positive immunostaining and PSA expression or clinicopathological factors. This is the first study to demonstrate the distribution of angiostatin-like fragments in human prostate carcinoma and to find the close relationship between the accumulation of angiostatin-like fragments and tumor grade.

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[3] The abbreviations used are: LBS-1, lysine-binding site 1; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR.

Table 1 Correlation between the immunostaining of LBS-1 or PSA and clinicopathological parameters[a]

	LBS-1			PSA			Coimmunostaining		
	Negative	Positive	*P*	Negative	Positive	*P*	Negative	Positive	*P*
Age									
<70 (n = 32)	3	29	0.435	18	13	0.412	19	12	0.392
>70 (n = 23)	4	19		16	7		17	6	
Clinical stage									
A or B (n = 40)	7	33	0.181	23	17	0.176	25	15	0.297
C or D (n = 12)	0	12		9	2		9	2	
Pathologic T category									
pT_1 or pT_2 (n = 33)	7	26	0.035[b]	18	14	0.158	20	12	0.375
pT_3 or pT_4 (n = 21)	0	21		16	5		16	5	
Serum PSA									
Not elevated (n = 25)	5	20	0.19	14	11	0.232	16	9	0.538
Elevated (n = 25)	1	24		18	6		18	6	

[a] Complete clinicopathological data were not available for five cases. Fisher's exact test was used to compute *P* values.
[b] Statistically significant.

MATERIALS AND METHODS

Cell Lines. The six established human prostate carcinoma cell lines used in this study were PC-3, DU-145, LNCAP, PPC-1, ALVA 41, and ALVA 101 (16), provided by Dr. Bahk (Department of Urology, Medical College, Gyeongsang National University, Chinju, Korea). The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

Patients and Tumor Tissue. Prostatic tumor tissue was obtained from 55 patients with prostate carcinoma who had undergone radical prostatectomy. Before surgery, no other therapy had been given. Mean patient age was 68.4 years (range, 52–82 years). The histopathology and immunohistochemistry of all of the cases were reviewed by two of the authors (T. M. and M. T.). All of the primary carcinomas were classified according to the Gleason's grading system, which corresponds to tumor differentiation (17). Pathological Gleason score ranged from 5 to 10. In addition, in 11 cases the tumor had metastasized into the lymph nodes; however, distant metastasis to other organs was not observed in any cases. Formalin-fixed, paraffin-embedded materials were available for all of the patients. Preoperative serum PSA levels were determined by the Hybritech Tandem-R monoclonal RIA. Clinical and pathological stages were also compared with the results of immunohistochemistry (Table 1).

Antibodies. The monoclonal antibody to plasminogen (clone 4D2) including LBS-1 was purchased from Advanced Immuno Chemicals, Inc. (Long Beach, CA). We named this antibody "the monoclonal antibody to LBS-1," because we found that this antibody reacted with the epitope of the LBS-1 region (kringle domains 1–3), as described in the "Results" section. The polyclonal antibody for miniplasminogen was prepared from the antisera raised in rabbits by affinity chromatography on a Protein A-Sepharose column followed by a miniplasminogen-conjugated Sepharose 4B column (8). The monoclonal antibody to PSA was purchased from Dako (Tokyo, Japan).

Western Blot Analysis. To confirm the specificity of antibodies to LBS-1 and miniplasminogen, affinity-purified human plasminogen, LBS-1, the kringles 1–4, and miniplasminogen were applied on 12.5% SDS-PAGE. Human plasminogen was purified from the pooled human plasma, as previously reported (18). Fragments of the LBS-1, including the kringles 1–3 and miniplasminogen, were purified from human plasminogen as described previously (14). After electrophoresis, the proteins were transferred electrophoretically from the gel to a polyvinylene difluoride membrane (Millipore, Bedford, MA). The membrane was then blocked for 30 min in blocking buffer (4% skim milk in Tris-buffered saline) and probed with a 1:2000 dilution of a monoclonal antibody to LBS-1 or an affinity-purified polyclonal antibody to miniplasminogen. After being washed, the membrane was incubated for 1 h with horseradish peroxidase-conjugated rabbit antimouse IgG or goat antirabbit IgG as the secondary antibody and was developed using the chemiluminescence system (PerkinElmer Life Sciences, Boston, MA).

All of the six prostate carcinoma cell lines were cultured with 2 μM human plasminogen at 37°C for 18 h. After the culture, confluent cell monolayers were washed twice with PBS. Cell lysates were prepared by homogenization in a lysis buffer [50 mM Tris-HCl (pH7.4), 5 mM EDTA, 350 mM NaCl, 0.1% (v/v) NP40, and 50 mM NaF] and proteinase inhibitors. The protein concentration of each lysate was analyzed by SDS-PAGE under nonreducing conditions. Immunoblotting for LBS-1 was performed as described above. We also investigated PC-3 cells that were cultured with or without human plasminogen and with or without FCS to determine the influence of human plasminogen and FCS.

RT-PCR and Sequencing. Using RT-PCR, human plasminogen mRNA was assessed in PC-3 cells. Total RNA from PC-3 cell line was isolated using Trizol reagent (Life Technologies, Inc.). Reverse transcription was performed with 5 μg of total RNA in a total volume of 20 μl containing Superscript II reverse transcriptase (Life Technologies, Inc.). The PCR reagents, including 2.5 units of Taq DNA polymerase (Life Technologies, Inc.), were added to form a final volume of 50 μl. The PCR was performed in a GeneAmp PCR System 9600 (PerkinElmer, Foster City, CA). A 35-cycle amplification profile consisted of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Primer coding for the kringle 1-to-2 domain region of human plasminogen were as follows: forward, 5′-ACAGACCTAGATTCTCACCTGC-3′; and reverse, 5′-CTTCACACTCAAGAATGTCGC-3′ (nucleo-

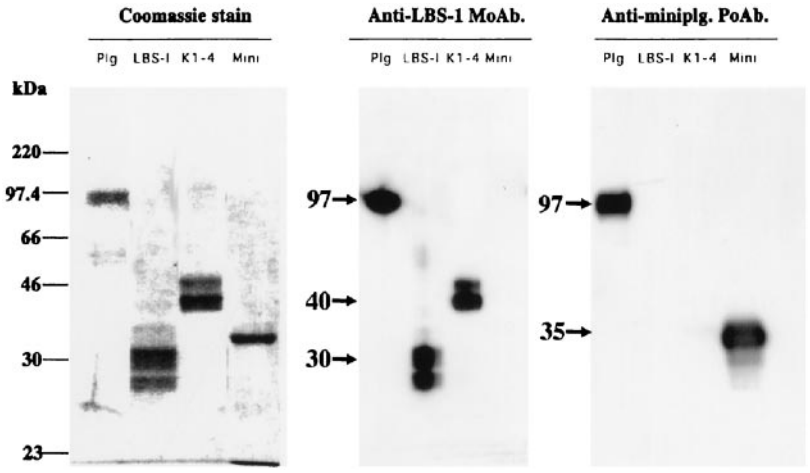

Fig. 1 Western blot analysis for determining the specificity of antibodies to LBS-1 and miniplasminogen under nonreducing conditions. The anti-LBS-1 monoclonal antibody reacted with bands the approximate sizes of which were M_r 97,000, 30,000, and 40,000. Each of these bands corresponds to that of plasminogen, LBS-1, and the kringles 1–4, respectively, when compared by Coomassie staining. The antiminiplasminogen polyclonal antibody reacted with bands the approximate sizes of which were M_r 97,000 and 35,000. Each of these bands corresponds to that of plasminogen and miniplasminogen, respectively, when compared by Coomassie staining. *Plg*, human plasminogen; *K1–4*, kringles 1–4; *Mini* and *miniplg.*, human miniplasminogen; *MoAb*, monoclonal antibody; *PoAb*, polyclonal antibody.

tides 447–596, 150 bp). Human β-actin primers were used as positive controls. Negative controls without RNA and without reverse transcriptase were also assessed. The sequence data were collected by ABI Prism 310 Collection Software and were analyzed by Sequencing Analysis and Sequence Navigator Software (Perkin-Elmer).

Immunohistochemical and Immunocytochemical Staining. Immunohistochemical staining for LBS-1, miniplasminogen, and PSA was performed using the streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). One paraffin-embedded block from each tumor was chosen for immunohistochemical studies. The normal liver tissue was used as a positive control. After blocking endogenous peroxidase, sections were pretreated at 100°C in a microwave oven. The sections were finally reacted in a 3,3′-diaminobenzidine, peroxytrichloride substrate solution, counterstained with hematoxylin.

We also performed immunocytochemical staining for LBS-1 and miniplasminogen in PC-3 cells incubated with 2 μM human plasminogen, according to the immunohistochemical method above. Negative control included testing the immunoractivity when an antibody was not present.

Evaluation of Staining. The degree of LBS-1 and miniplasminogen immunoreactivity was graded as follows: −, either no immunostaining, or else only minimal immunostaining was observed; +, either weak immunoreaction was diffusely observed or else strong reaction was recognized in more than 10% of the tumor cells; and ++, >50% tumor cells showed strong immunoreaction. In this study, we recognized cases graded as + or ++ to be positive cases. As previously reported (19), the extent and intensity of staining for PSA was evaluated subjectively, according to the following criteria: 0, no staining; 1, weak equivocal staining; 2, unequivocal moderate staining; and 3, strong staining. Only cells with an intensity of staining of >1 on a 0–3 scale (1 was equivocal) were considered to be positive.

Statistical Analysis. The correlation among each of the clinicopathological factors and the positive immunostaining of LBS-1 and PSA was evaluated using Fisher's exact test. The correlation between Gleason score and the positive immunostaining of LBS-1/PSA was assessed by Spearman's rank correlation. The statistical calculations and tests were performed using SAS software (version 6. 1. 2). Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Determining the Specificity of Antibodies for LBS-1 and Miniplasminogen by Western Blot Analysis

The anti-LBS-1 monoclonal antibody reacted with bands the approximate sizes of which were M_r 97,000, 30,000, and 40,000 (Fig. 1). Each of these bands corresponds to that of plasminogen, LBS-1, and the kringles 1–4, respectively, when compared by Coomassie Blue staining. The antiminiplasminogen polyclonal antibody reacted with bands the approximate sizes of which were M_r 97,000 and 35,000, but did not react with LBS-1 or the kringles 1–4. Each of these bands corresponds to those of plasminogen and miniplasminogen, respectively. We also investigated the possibility of the cross-reactions of these antibodies with apolipoprotein (a), thrombin, and urokinase-type plasminogen activator because of the high similarity between the kringle regions of plasminogen and these proteins; however, we found no cross-reactions with these proteins (data not shown).

Angiostatin Generation from Human Plasminogen by Prostate Carcinoma Cells

All of the six prostate carcinoma cell lines (PC-3, DU 145, ALVA 41, ALVA 101, LNCaP, and PPC-1) resulted in the generation of immunoreactive bands at M_r of ~35,000, 40,000, and 50,000 (Fig. 2, *left*). These bands were considered to be those of angiostatin-like fragments attributable to immunoreactivity with the monoclonal antibody LBS-1 and the fact that the size corresponded to the predicted mass of angiostatin-like fragments (6, 20). The levels of immunoreactivity of angiostatin-like fragments were variable in each of the cell lines. In the absence of exogenous plasminogen, there are no detectable angiostatin-like fragments in the lysates of PC-3 cells, irrespective of the absence or presence of FCS (Fig. 2, *right*). We also performed the RT-PCR and sequencing of human plasminogen

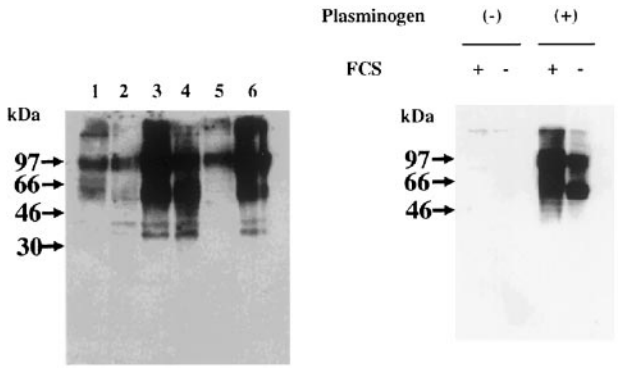

Fig. 2 *Left panel*, Western blot analysis of angiostatin-like fragments in prostate carcinoma cell lines with human plasminogen using the anti-LBS-1 monoclonal antibody under nonreducing conditions. The antibody reacts with protein bands corresponding to the molecular weight of plasminogen (*97 kDa*) and angiostatin-like fragments (*~35, 40,* and *50 kDa*) in the lysate of prostate carcinoma cells with human plasminogen. *Lane 1*, PC-3; *Lane 2*, DU 145; *Lane 3*, ALVA 41; *Lane 4*, ALVA 101; *Lane 5*, LNCAP; *Lane 6*, PPC-1. *Right panel*, Western blot analysis of PC-3 with either the absence or presence of human plasminogen using the anti-LBS-1 monoclonal antibody. The antibody reacts with protein bands corresponding to the plasminogen and angiostatin-like fragments in the lysate of PC-3 cells with human plasminogen. In the absence of exogenous plasminogen, there are no detectable immunoreactive bands, irrespective of the absence or the presence of FCS.

in PC-3 cells, but we found 14 base deletion and subsequent stop codon (data not shown).

Immunocytochemical and Immunohistochemical Staining

LBS-1. Immunocytochemically, PC-3 cells incubated with human plasminogen showed cytoplasmic immunoreactivity for LBS-1 (Fig. 3A). On the other hand, there was no any immunoreactivity for miniplasminogen, as well as negative controls (not shown in figure).

In the liver tissue, the staining pattern of LBS-1 was predominantly intracytoplasmic, demonstrating fine granules only at the hepatocytes around the central veins (not shown in figure). In the prostate tissue, normal and atrophic prostatic epithelium was always stained less intensely or was negative. On the other hand, prostate carcinoma cells were heterogeneously stained positively and the staining pattern was intracytoplasmic and homogeneous (Fig. 3*B*). Cases of Gleason primary pattern 4 and 5 adenocarcinoma tended to show strong staining in tumor cells rather than Gleason primary pattern 3 or lower-grade adenocarcinoma (Fig. 3*C*).

Miniplasminogen. In the liver tissue, the staining pattern of miniplasminogen was the same as that of LBS-1, and the positive cells corresponded with those of LBS-1 in serial sections (not shown in figure). In the prostate tissue, most prostate glands that included carcinoma cells were frequently negative; however, atrophic glands were strongly positive, and the staining pattern predominantly demonstrated fine granules (Fig. 3*D*). Intravascular serum was also strongly stained.

PSA. Most prostate glands that included carcinoma cells were frequently positive. The staining pattern of PSA was predominantly intracytoplasmic. As reported previously (21, 22), we also found that there was a heterogeneous distribution of PSA-stained cells in prostate carcinoma.

Correlation between the Positive Immunostaining of LBS-1/PSA and Clinicopathological Parameters. In the same areas within serial sections, there was strong immunoreactivity for LBS-1 and weak immunoreactivity for PSA in prostate adenocarcinoma (Fig. 3, *E* and *G*). There was no immunoreactivity for miniplasminogen in prostate adenocarcinoma and normal prostate glands (Fig. 3*F*).

The relationship between the positive immunostaining of LBS-1 or PSA and clinicopathological parameters is summarized in Table 1. A close relationship was found only between the positive immunostaining of LBS-1 and pathological T category ($P = 0.035$). In 48 (87.3%) of the 55 cases, the immunostaining of LBS-1 was positive in prostate carcinoma, and there was a significant correlation between the positive immunostaining of LBS-1 and Gleason score (Table 2; $P = 0.0007$; correlation coefficient, 0.4434). In 20 (37.0%) of the 54 cases, the immunostaining of PSA was positive in prostate carcinoma. Although we noted a trend of inverted correlation between PSA staining and the Gleason score, this relationship was not statistically significant (Table 3). The colocalization of LBS-1 and PSA was 33.3% (18 of 54), and there was no significant relationship between the positive immunostaining of LBS-1 and PSA in prostate carcinoma (Table 4).

DISCUSSION

O'Reilly *et al.* (1) reported that angiogenesis of lung metastasis arising from murine Lewis lung carcinoma was inhibited by angiostatin, a circulating angiogenesis inhibitor, which is a M_r 38,000 internal fragment of mouse plasminogen. Gately *et al.* (20) found angiostatin-generating activity in the media of prostate carcinoma cell lines. We also found angiostatin-generating activity in the lysate of human prostate carcinoma cells, as well as in the conditioned media (20).

As a result of the specificity of monoclonal antibody to LBS-1 and polyclonal antibody to miniplasminogen by Western blot analysis, we found that these antibodies react neither with endogenous proteins in tumor cells nor with other plasma proteins similar to plasminogen. Thus these antibodies were appropriate for identifying LBS-1 or miniplasminogen by immunohistochemistry. However, one should note that angiostatin cannot be directly identified by immunohistochemistry, because antibodies to each of LBS-1 and miniplasminogen also react with human plasminogen. In the present study, we demonstrated that, although plasminogen-producing hepatocytes showed positive immunoreactivity for both antibodies, prostate carcinoma revealed variable cytoplasmic immunoreactivity for LBS-1 and no immunoreactivity for miniplasminogen, in contrast to normal prostatic epithelium. Therefore, immunohistochemically, the immunoreactive product of LBS-1 can be interpreted as that of angiostatin-like fragments in prostate carcinoma, making it likely that angiostatin exists not only in the serum, but also in the cytoplasm of prostate carcinoma cells. Volm *et al.* (23) described the expression of angiostatin in non-small cell lung cancer, but the localization of angiostatin or morphological analysis was unknown. We were able to indirectly investigate the localization and the amounts of angiostatin *in vivo*.

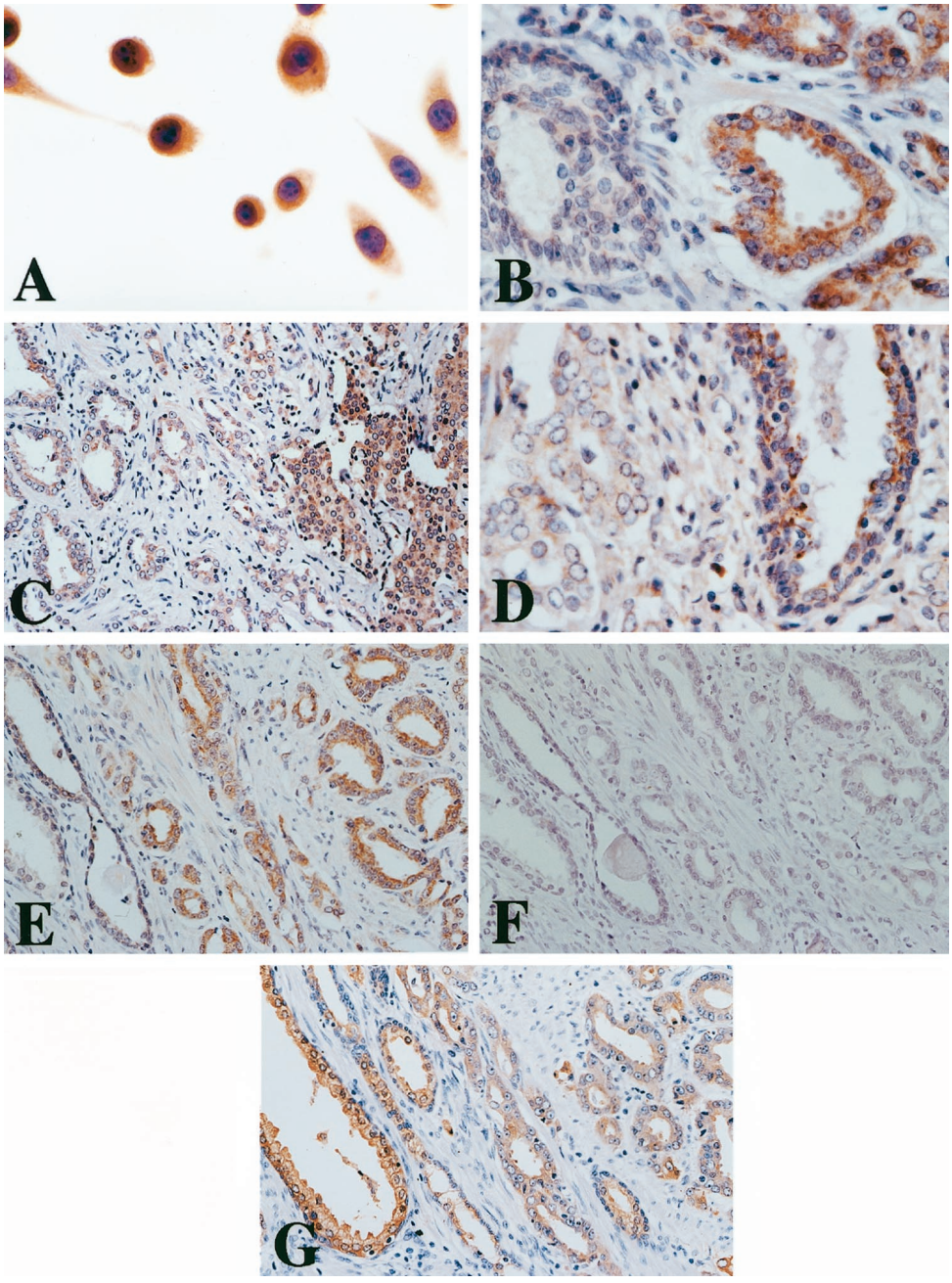

Fig. 3 *A*, immunocytochemical staining for LBS-1 in the cytoplasm of PC-3 cells. PC-3 cells incubated with human plasminogen showed cytoplasmic immunoreactivity for LBS- 1. ×400. *B*, immunohistochemical staining for LBS-1 in prostate tissue. Cancer cells (*right*) were stained positively in cytoplasm, compared with normal prostate glands (*left*). ×400. In *C*, there is a different intensity of LBS-1 staining between high-grade (*right*) and low-grade prostate carcinoma (*left*). ×200. *D*, immunohistochemical study for miniplasminogen in prostate tissue. Atrophic prostate glands show positive staining (*right*) but no reactivity in cancer cells (*left*). ×400. *E*, *F*, and *G*, immunohistochemical study for LBS-1 (*E*), miniplasminogen (*F*), and PSA (*G*) in prostate tissue of serial sections. In prostate carcinoma, strong immunoreactivity for LBS-1 can be seen (*E*, *right*), compared with that for miniplasminogen (*F*, *right*) and PSA (*G*, *right*). Strong immunoreactivity for PSA can be seen in normal prostate glands (*G*, *left*). ×200.

Some studies support the hypothesis that plasminogen exudes from blood capillaries, diffuses in the stroma, and binds to tumor cells (24, 25). Our study demonstrated that tumor cells contained angiostatin-like fragments in the cytoplasm; however, we found neither plasminogen nor angiostatin-like fragments on the surface of the tumor cells. Other studies have described the fact that tumor cells do not seem to express angiostatin molecules *per se* (2, 26). This speculation is supported by our results that PC-3 cells could not generate angiostatin in the absence of exogenous plasminogen and could not produce mRNA of human plasminogen. It is assumed that plasminogen binds to tumor cells and is converted into angiostatin probably by tumor-

Table 2 Correlation between the immunostaining of LBS-1 and Gleason score in 55 prostate carcinomas[a]

	Gleason score						Total
	5	6	7	8	9	10	(%)
LBS-1 immunostaining							
Negative	5	2	0	0	0	0	7 (12.7)
Positive	6	16	14	6	5	1	48 (87.3)

[a] P, 0.0007; correlation coefficient, 0.4434. Spearman's rank correlation was used to compute *P*s.

Table 3 Correlation between the expression of PSA and Gleason score in 54 prostate carcinomas[a]

	Gleason score						Total (%)
	5	6	7	8	9	10	
PSA expression							
Negative	9	7	8	4	5	1	34 (63.0)
Positive	2	11	5	2	0	0	20 (37.0)

[a] P, 0.4482; correlation coefficient, −0.1054. Spearman's rank correlation was used to compute *P*s.

Table 4 Correlation between the immunostaining of LBS-1 and PSA in 54 prostate carcinomas[a]

	PSA expression		
	Negative (%)	Positive (%)	Total (%)
LBS-1 immunostaining			
Negative	5 (9.3)	2 (3.7)	7 (13.0)
Positive	29 (53.7)	18 (33.3)	47 (87.0)

[a] $P = 0.962$; Fisher's exact test was used to compute *P*s.

associated proteinases of plasminogen, and finally, angiostatin may be translocated and accumulated in the cytoplasm by endocytosis or some other unknown mechanism.

We also found an increased frequency of LBS-1 staining as the tumor grade increased. Several studies have reported microvessel density correlated with tumor grade and progression in prostate carcinoma (27, 28), in contrast with the studies that lacked correlation (29, 30). We examined the microvessel density in prostate cancer but failed to find any relationships between microvessel density and immunoreactivity of angiostatin (data not shown). We need to examine the mechanism of angiostatin accumulation and the biological activity of the accumulated angiostatin in future studies.

PSA is a family of kallikrein-like serine proteases and is produced exclusively by prostate epithelium (31). Gately *et al.* (20) demonstrated that PC-3 cells express plasminogen-angiostatin-converting enzymes in serum-free conditioned medium and that the enzymatic activity requires a serine proteinase; however, there was no mention of PSA in their study. Heidtmann *et al.* (15) found that PSA is able to convert Lys-plasminogen to angiostatin-like fragments, containing kringles 1–4, and that the purified angiostatin-like fragments inhibited proliferation and tubular formation of human umbilical vein endothelial cells. Fortier *et al.* (32) reported that PSA may function in tumors as an endogenous antiangiogenic protein. Therefore, we should evaluate the effect of PSA on tumor angiogenesis in human prostate carcinoma.

Some reports have demonstrated that there was a tendency toward lower amounts of PSA and its mRNA in prostate carcinoma than in BPH by Northern blot analysis (33), *in situ* hybridization (33, 34), and immunohistochemistry (35–39). We also demonstrated that PSA immunostaining showed heterogeneous staining in prostate carcinoma, and the expression of PSA had no relevance to the positive immunostaining of LBS-1. There can be no doubt that there is generation of angiostatin-like fragments from plasminogen, at least in part, by PSA *in vitro* (15). However, the present study demonstrated that the colocalization of LBS-1 and PSA was seen in only 33.3% (18 of 54) of all patients, whereas the positive immunostaining of LBS-1 alone was seen in 53.7% (29 of 54).

These findings suggest the possibility that the angiostatin generation results not only from PSA, but also from the other proteinases *in vivo*. Additional investigations are necessary to determine any additional relationship between angiostatin generation and other proteinases.

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REFERENCES

1. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.

2. O'Reilly, M. S., Wiederschain, D., Stetler-Stevenson, W. G., Folkman, J., and Moses, M. A. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. J. Biol. Chem., *274:* 29568–29571, 1999.

3. Lijnen, H. R., Uguwu, F., Bini, A., and Collen, D. Generation of an angiostatin-like fragment from plasminogen by stromelysin-1 (MMP-3). Biochemistry, *37:* 4699–4702, 1998.

4. Patterson, B. C., and Sang, Q. A. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J. Biol. Chem., *272:* 28823–28825, 1997.

5. Dong, Z., Kumar, R., Yang, X., and Fidler, I. J. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell, *88:* 801–810, 1997.

6. Gately, S., Twardowski, P., Stack, M. S., Cundiff, D. L., Grella, D., Castellino, F. J., Enghild, J., Kwaan, H. C., Lee, F., Kramer, R. A., Volpert, O., Bouck, N., and Soff, G. A. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc. Natl. Acad. Sci. USA, *94:* 10868–10872, 1997.

7. Stathakis, P., Fitzgerald, M., Matthias, L. J., Chesterman, C. N., and Hogg, P. J. Generation of angiostatin by reduction and proteolysis of plasmin. J. Biol. Chem., *272:* 20641–20645, 1999.

8. Morikawa, W., Yamamoto, K., Ishikawa, S., Takemoto, S., Ono, M., Fukushi, J., Naito, S., Nozaki, C., Iwanaga, S., and Kuwano, M. Angiostatin generation by cathepsin D secreted by human prostate carcinoma cells. J. Biol. Chem., *275:* 38912–38920, 2000.

9. O'Mahony, C. A., Seidel, A., Albo, D., Chang, H., Tuszynski, G. P., and Berger, D. H. Angiostatin generation by human pancreatic cancer. J. Surg. Res., *77:* 55–58, 1998.

10. Stathakis, P., Lay, A. J., Fitzgerald, M., Schlieker, C., Matthias, L. J., and Hogg, P. J. Angiostatin formation involves disulfide bond reduction and proteolysis in kringle 5 of plasmin. J. Biol. Chem., *274:* 8910–8916, 1999.

11. Sten-Linder, M., Linder, C., Strander, H., Munck-Wikland, E., Wersäll, P., Linder, S., and Wiman, B. Angiostatin fragments in urine from patients with malignant disease. Anticancer Res., *19:* 3409–3414, 1999.

12. Gorrin Rivas, M. J., Arii, S., Furutani, M., Harada, T., Mizumoto, M., Nishiyama, H., Fujita, J., and Imamura, M. Expression of human macrophage metalloelastase gene in hepatocellular carcinoma: correlation with angiostatin generation and its clinical significance. Hepatology, *28:* 986–993, 1998.

13. Barendsz-Janson, A. F., Griffioen, A. W., Muller, A. D., van Dam-Mieras, M. C., and Hillen, H. F. *In vitro* tumor angiogenesis assays: plasminogen lysine binding site 1 inhibits *in vitro* tumor-induced angiogenesis. J. Vasc. Res., *35:* 109–114, 1998.

14. Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E., and Magnusson, S. The primary structure of human plasminogen: isolation of two lysine-binding fragments and one "mini-"plasminogen (MW, 38,000) by elastase-catalyzed-specific limited proteolysis. Prog. Chem. Fibrinolysis Thrombolysis, *3:* 191–209, 1978.

15. Heidtmann, H-H., Nettelbeck, D. M., Mingles, A., Jäger, R., Weelker, H. G., and Kontermann, R. E. Generation of angiostatin-like fragments from plasminogen by prostate-specific antigen. Br. J. Cancer, *81:* 1269–1273, 1999.

16. Plymate, S. R., Loop, S. M., Hoop, R. C., Wiren, K. M., Ostenson, R., Hryb, D. J., and Rosner, W. Effects of sex hormone binding globulin (SHBG) on human prostatic carcinoma. J. Steroid Biochem. Mol. Biol., *40:* 833–839, 1991.

17. Gleason, D. F. Histologic grading of prostate cancer: a perspective. Hum. Pathol., *23:* 273–279, 1992.

18. Deutsch, D. G., and Mertz, E. T. Plasminogen: purification from human plasma by affinity chromatography. Science (Wash. DC), *170:* 1095–1096, 1970.

19. Bostwick, D. G., Pacelli, A., Blute, M., Roche, P., and Murphy, G. P. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma. Cancer (Phila.), *82:* 2256–2261, 1998.

20. Gately, S., Twardowski, P., Stack, M. S., Patrick, M., Boggio, L., Cundiff, D. L., Schnaper, H. W., Madison, L., Volpert, O., Bouck, N., Enghild, J., Kwaan, H. C., and Soff, G. A. Human prostate carcinoma cells express enzymatic activity that converts human plasminogen to the angiogenesis inhibitor, angiostatin. Cancer Res., *56:* 4887–4890, 1996.

21. Aihara, M., Lebovitz, R. M., Wheeler, T. M., Kinner, B. M., Ohori, M., and Scardino, P. T. Prostate specific antigen and Gleason grade: an immunohistochemical study of prostate cancer. J. Urol., *151:* 1558–1564, 1994.

22. Sakai, H., Yogi, Y., Minami, Y., Yushita, Y., Kanetake, H., and Saito, Y. Prostate specific antigen and prostatic acid phosphatase immunoreactivity as prognostic indicators of advanced prostatic carcinoma. J. Urol., *149:* 1020–1023, 1993.

23. Volm, M., Mattern, J., and Koomägi, R. Angiostatin expression in non-small cell lung cancer. Clin. Cancer Res., *6:* 3236–3240, 2000.

24. Brutin, P., Chavanel, G., and Andre, J. The plasmin system in human colonic tumors: an immunofluorescence study. Int. J. Cancer, *35:* 307–314, 1985.

25. Clavel, C., Chavanel, G., and Birembaut, P. Detection of the plasmin system in human mammary pathology using immunofluorescence. Cancer Res., *46:* 5743–5747, 1986.

26. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, R. W., and Folkman, J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. J. Clin. Investig., *101:* 1055–1063, 1998.

27. Lissbrant, I. F., Stattin, P., Damber, J. E., and Bergh, A. Vascular density is a predictor of cancer-specific survival in prostatic carcinoma. Prostate, *33:* 38–45, 1997.

28. Bettencourt, M. C., Bauer, J. J., Sesterhenn, I. A., Connelly, R. R., and Moul, J. W. CD34 immunohistochemical assessment of angiogenesis as a prognostic marker for prostate cancer recurrence after radical prostatectomy. J. Urol., *160:* 459–465, 1998.

29. Gettman, M. T., Bergstralh, E. J., Blute, M., Zincke, H., and Bostwick, D. G. Prediction of patient outcome in pathologic stage T2 adenocarcinoma of the prostate: lack of significance for microvessel density analysis. Urology, *51:* 79–85, 1998.

30. Rubin, M. A., Buyyounouski, M., Bagiella, E., Sharir, S., Neugut, A., Benson, M., Taille, A., Katz, A. E., Olsson, C. A., and Ennis, R. D. Microvessel density in prostate cancer: lack of correlation with tumor grade, pathologic stage, and clinical outcome. Urology, *53:* 542–547, 1999.

31. Watt, K. W., Lee, P. J., M'Timkulu, T., Chan, W. P., and Loor, R. Human prostate-specific antigen: structural and functional similarity with serine proteases. Proc. Natl. Acad. Sci. USA, *83:* 3166–3170, 1986.

32. Fortier, A. H., Nelson, B. J., Grella, D. K., and Holaday, J. W. Antiangiogenic activity of prostate-specific antigen. J. Natl. Cancer Inst., *91:* 1635–1640, 1999.

33. Hakalahti, L., Vihko, P., Henttu, P., Antio-Harmainen, H., Soini, Y., and Vihko, R. Evaluation of PAP and PSA gene expression in prostatic hyperplasia and prostatic carcinoma using Northern-blot analysis, *in situ* hybridization and immunohistochemical staining with monoclonal and bispecific antibodies. Int. J. Cancer, *55:* 590–597, 1993.

34. Qiu, S., Young, C. Y. F., Bilhartz, D. L., Prescott, J. L., Farrow, G. M., He, W. W., and Tindall, D. J. *In situ* hybridization of prostate-specific antigen mRNA in human prostate. J. Urol., *144:* 1550–1556, 1990.

35. Keillor, J. S., and Aterman, K. The response of poorly differentiated prostatic tumors to staining for prostate specific antigen and prostatic acid phosphatase: a comparative study. J. Urol., *137:* 894–896, 1987.

36. Ellis, D., Leffers, S., Davies, J. S., and Ng, A. B. Multiple immunoperoxidase markers in benign hyperplasia and adenocarcinoma of the prostate. Am. J. Clin. Pathol., *81:* 279–284, 1984.

37. Epstein, J. I., and Eggleston, J. C. Immunohistochemical localization of prostate-specific acid phosphatase and prostate-specific antigen in stage A2 adenocarcinoma of the prostate: prognostic implications. Hum. Pathol., *15:* 853–859, 1984.

38. Sinha, A. A., Wilson, M. J., and Gleason, D. F. Immunoelectron microscopic localization of prostatic-specific antigen in human prostate by the protein A-gold complex. Cancer (Phila.), *60:* 1288–1293, 1987.

39. Purnell, D. M., Heatfield, B. M., and Trump, B. F. Immunocytochemical evaluation of human prostatic carcinomas for carcinoembryonic antigen, non-specific cross-reacting antigen. β-chorionic gonadotrophin, and prostate-specific antigen. Cancer Res., *44:* 285–292, 1984.

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