Androgens Repress the Expression of the Angiogenesis Inhibitor Thrombospondin-1 in Normal and Neoplastic Prostate

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Abstract

In order to understand why the angiogenesis inhibitor thrombospondin-1 (TSP1) is often, although not always, associated with prostatic tumors, we have investigated its relationship with the testosterone and the vasculature on which both normal and tumorigenic prostatic epithelia depend. In vivo, androgen withdrawal led to increased TSP1 production and decreased vascularization in the normal rat prostate which was reversed by androgen replacement. Androgen repression of TSP1 production occurred at the transcriptional level and was dependent on the presence of the first intron of the TSP1 gene. In an experimental model of prostate tumorigenesis, TSP1, when delivered by admixed stromal fibroblasts, markedly delayed LNCaP tumor growth and limited tumor vascularization. However, prolonged exposure to TSP1 resulted in the growth of tumors secreting high levels of vascular endothelial growth factor in the bloodstream of tumor-bearing animals and tumor growth was no longer sensitive to TSP1 inhibitory effects. Clinical evidence also suggested that prostate carcinomas are able to adapt to escape the antiangiogenic effects of TSP1. In human androgen–dependent localized prostate carcinomas, TSP1 expression was inversely correlated with blood vessel density. Androgen deprivation in patients with hormone-responsive tumors led to increased TSP1 expression and vascular regression. In contrast, despite a sustained expression in the tumor bed, TSP1 was no longer associated with decreased vascularization in hormone-refractory prostate tumors. Overall, these results suggest that the high in situ TSP1 exposure triggered by androgen deprivation in patients with prostate cancer could lead to early tumor resistance. Such patients could benefit from a combination of androgen deprivation and antiangiogenic therapy in order to minimize the induction of such tumor escape.

Introduction

The structural and functional integrity of the prostate gland requires a constant supply of androgens. In rats, androgen ablation by castration results in a marked involution of the prostate that is preceded by a decrease in organ blood flow. Testosterone treatment of castrated animals stimulates rapid vascular expansion and subsequent regrowth of the gland (1, 2). In the rat prostate, this androgen-dependent regrowth is under the control of the vascular endothelium which, in turn, depends on the production of vascular endothelial growth factor (VEGF) elaborated by the prostate epithelium (3–5). In mice bearing androgen-dependent tumors, castration also leads to decreased VEGF expression by tumor cells and subsequent vascular regression, which precedes tumor growth inhibition (6, 7). Human androgen–responsive prostate carcinomas behave similarly in that on pharmacologic or surgical reduction of androgens (reviewed in ref. 8), tumors undergo clinical regression which is accompanied by suppression of VEGF production (9).

Yet, variations in VEGF alone do not seem to be sufficient to explain these androgen effects on prostate vascularization. In the rat prostate, involution following hormone ablation is sustained, although the down-regulation of VEGF is only transient (3). Similarly, although castration of animals effectively inhibits the growth of androgen-dependent Shionogi tumors, VEGF down-regulation is short lived and is followed by a second wave of increased VEGF production from the tumor cells (10). Such data suggest that modulations in the angiogenesis inhibitor levels may also be involved in the response of the normal and neoplastic prostate to androgen ablation. Androgen ablation was reported to increase inhibitors such as maspin (11) and pigment epithelium-derived factor (12). Here, we investigate the relationship between androgens and another inhibitor of angiogenesis found in the prostate, thrombospondin-1 (TSP1).

TSP1 was the first naturally occurring inhibitor of angiogenesis to be identified (13). TSP1, by binding to endothelial cell surface receptor CD36, induces endothelial cell apoptosis through a p59™, caspase 3, and p38 mitogen-activated protein kinase signaling pathway (14). Because of its antiangiogenic properties, TSP1 overexpression in tumor cells inhibits tumorigenesis in various mouse xenografts models, including breast and skin carcinomas, fibrosarcoma, melanoma, and glioblastoma (reviewed in refs. 15, 16). Similarly, the intratumoral administration of a TSP1 expression vector into androgen-independent DU-145 prostate cancer xenografts inhibits tumor growth in vivo through inhibition of tumor angiogenisis (17).

In human prostate cancer, results have been somewhat equivocal. Although some studies have reported a correlation between TSP1 and antiangiogenesis (18, 19), and between increased TSP1 expression in primary tumors and androgen deprivation in patients with prostate cancer (20), others have found no such association (21, 22). In an effort to more closely define the role of TSP1 in the normal and neoplastic prostate, we have combined...
molecular studies, rodent models, and a comprehensive clinical study to investigate the relationship between TSP1 expression, angiogenesis, and androgen dependency.

**Materials and Methods**

**Cell Culture, Transient Transfection and Cell-Based Reporter**

**TSP1 Gene Assay.** Human androgen–dependent LNCaP prostate cancer cells (gift from Dr. Chung, Emory University School of Medicine, Atlanta, GA) and cells explanted from mouse prostate glands were grown in DMEM medium (Invitrogen, Cergy Pontoise, France) supplemented with 5% (vol/vol) FCS, 5 mg/mL insulin (Sigma, Ile de l’Abeau, France), 136.5 mg/mL triodo-thyronine (Sigma), 5 mg/mL apo-transferrin (Sigma), 0.25 mg/mL biotin (Sigma), and 25 mg/mL adenine (Sigma). Human Saos-2 osteoblastic cells (American Type Culture Collection) were grown in McCoy’s medium (Invitrogen) supplemented with 15% (vol/vol) FCS. NT26 cells were derived from NIH3T3 mouse fibroblasts in which a tet-off expression system for TSP1 was introduced (23). NT26, TSP1-inducible mouse fibroblasts were grown in DMEM containing 10% FCS and 100 ng/mL of doxycycline, a tetracyclin analogue, to repress TSP1 expression. Human umbilical vascular endothelial cells were grown in RPMI supplemented with 10% FCS. The parental construct of the human TSP1 promoter (−2033, +750) was kindly provided by Dr. Paul Bornstein (University of Washington, Seattle, WA; ref. 24). Internal 5′-deletion fragments of the TSP1 promoter were cloned into the pGL2 luciferase vector (Clontech, Palo Alto, CA). These plasmids were then introduced into cells by transient transfection in Saos-2 cells overnight using the calcium phosphate precipitation method. After transfection, cells were incubated for 10 hours in DMEM medium containing 10% (vol/vol) charcoal-stripped FCS and then treated with 0.1 nmol/L of R1881 (NEN, Zaventem, Belgium), a synthetic analogue of testosterone, or the vehicle. Eighteen hours later, the luciferase activity in cell lysates was measured using the Luciferase Assay System as described by the manufacturer (Promega, Charbonnières, France) and normalized for protein content. For each TSP1 reporter construct, the luciferase activity was expressed as a percentage of the activity driven by the (−586, +750) hTSP1-luc construct in the absence of androgens. TSP1 in cell extracts were detected by immunoblotting as previously described (23), using anti-TSP1 antibody Ab-4 (Neomarkers, Union City, CA).

**TSP1-Inducible Cell Line.** The mouse fibroblastic NT26 TSP1-inducible cell line has been described elsewhere (23). Murine TSP1 inducible mouse fibroblasts were grown in DMEM containing 10% FCS and 100 ng/mL of doxycycline, a tetracyclin analogue, to repress TSP1 expression. Human umbilical vascular endothelial cells were grown in RPMI supplemented with 10% FCS. The parental construct of the human TSP1 promoter (−2033, +750) was kindly provided by Dr. Paul Bornstein (University of Washington, Seattle, WA; ref. 24). Internal 5′-deletion fragments of the TSP1 promoter were cloned into the pGL2 luciferase vector (Clontech, Palo Alto, CA). These plasmids were then introduced into cells by transient transfection in Saos-2 cells overnight using the calcium phosphate precipitation method. After transfection, cells were incubated for 10 hours in DMEM medium containing 10% (vol/vol) charcoal-stripped FCS and then treated with 0.1 nmol/L of R1881 (NEN, Zaventem, Belgium), a synthetic analogue of testosterone, or the vehicle. Eighteen hours later, the luciferase activity in cell lysates was measured using the Luciferase Assay System as described by the manufacturer (Promega, Charbonnières, France) and normalized for protein content. For each TSP1 reporter construct, the luciferase activity was expressed as a percentage of the activity driven by the (−586, +750) hTSP1-luc construct in the absence of androgens. TSP1 in cell extracts were detected by immunoblotting as previously described (23), using anti-TSP1 antibody Ab-4 (Neomarkers, Union City, CA).

**Capillary-Like Tube Formation Assay.** The surface of 24-well plates were coated with the basement membrane Matrigel (400 ng/mL) or in vivo in the water supply (100 μg/mL) of the animals. To prepare conditioned media, NT26 cells were plated in duplicate plates and grown for 3 days in medium containing doxycycline in one plate, and no additive in the other one, in the absence of other antibiotics. The number of cells were similar in the two conditions when media were collected.

**Capillary-Like Tube Formation Assay.** The surface of 24-well plates were coated with the basement membrane Matrigel (400 μl/well; 10 mg/mL). Endothelial cells (4 × 10⁴ cells/mL/well) were laid on Matrigel-coated plates in the presence of conditioned medium from NT26 cells that had been first normalized according to the number of fibroblastic cells then diluted 1:2 (vol/vol) in fresh RPMI medium. After a 16-hour incubation at 37°C in a 5% CO₂ incubator, the wells were photographed. The total length of the tube network on photographs was measured using a semiautomated analyzer (Videoplan, Kontron, Munich, Germany), and results were expressed as μm/cm².

**Animals.** Tumorigenicity assays were done on 6-week-old male athymic Swiss mice (IFFA CREDO, St Germain sur l’Arbresle, France) and castration assays were done on adult male Sprague-Dawley rats (weight 330 g; IFFA CREDO). Studies involving animals, including housing and care, method of euthanasia, and experimental protocols were conducted in accordance with a code of practice established by the local animal ethical committees in Lyon and Villejuif, France.

**Tumorigenicity Assay and VEGF Quantitation.** LNCaP cells were mixed with NT26 fibroblasts previously cultured in the presence of doxycycline. A mix of 2 × 10⁶ LNCaP cells and 3 × 10⁶ NT26 cells per site were injected subcutaneously in the hindquarters of male athymic mice in 50% (vol/vol) Matrigel. Animals were randomized into two groups: one group received doxycycline in the drinking supply, whereas the other group had no doxycycline. The sizes of the tumors were determined by external measurements of the tumors in two dimensions with a caliper. Volume (V) was estimated as V = L × P² × 0.52, where L is the widest diameter and P the smallest. When the mice were killed, tumors were collected, fixed in Bouin’s solution (Sigma) and paraffin-embedded for further immunohistochemical analysis. Blood was collected for measurement of VEGF in plasma using ELISA kit specific for human protein (R&D Systems, Lille, France) according to the manufacturer’s instructions.

**Castration Assay.** The castration of animals and the administration of testosterone were done according to the protocol described by Franck-Lissbrant et al. (4). Animals were castrated for 12, 24, 48, or 72 hours. Alternatively, 15 days after castration, animals received a subcutaneous pellet of long-acting testosterone (15 mg/pellet, Innovative Research of America, Sarasota, FL) for 1, 2, or 3 days. Intact animals were used as controls. At sacrifice, the ventral prostate was dissected, formalin-fixed and paraffin-embedded for further immunohistochemical analysis.

**Human Prostate Cancer Material.** Paraffin blocks of prostatectomy (radical or transurethreal) specimens were obtained from the Department of Pathology at Edouard Herriot Hospital, Lyon. Tumor tissues included localized prostate cancer (n = 60), and metastatic hormone-sensitive (n = 33), and hormone-refractory (n = 10) prostate cancers. The median patient age was 67 years. The median follow-up was 2.4 years and was therefore not long enough to perform a survival analysis.

**Immunohistochemical Analysis.** Paraffin-embedded serial tissue sections (5 μm thick) were deparaffinized, rehydrated, and immunostained using an automated immunostaining apparatus (NexES, Ventana, Strasbourg, France) with standardized duration and temperature of all the steps. For TSP1 and microvessel detection, immunostaining was done with rabbit polyclonal antibodies against TSP1 (Ab-8, Neomarkers), and von Willebrand factor (Dako, Trappes, France), respectively. Immunolabeling of transforming growth factor beta (TGFβ) was done using MAB1835 from R&D (formerly clone 1D11.16 from Genzyme), which recognizes only the active forms of TGFβ1, 2, and 3 as described in ref. 25. CD36 was immunodetected using FA6-152 monoclonal antibody (Immunotech, Marseille-Lumigny, France). Negative control experiments were done by omitting the primary antibody. The experimental procedures were carried out as described previously (23). For TSP1 immunostaining, tissue sections were considered as positive or negative according to the presence or absence of specific staining when compared with the staining obtained from a negative control. The intensity of the staining was scored arbitrarily as follows: strong (3+), moderate (2+), weak (+), and negative (−). Sections were evaluated independently by two examiners. For scoring of the microvessel density, highly vascularized regions of the tumors were selected at a low (10×) magnification and microvessels were counted in three nonoverlapping high power fields (HPF; 40× objective, area 0.283 mm² per field). For the castration assay, the lumen area of 40 immunostained vessels within each tissue section was measured using a semiautomated analyzer (Videoplan, Kontron), and results were expressed as the average area per vessel (μm²) for each immunostained section.

**Statistical Analysis.** For patients, data were analyzed using the χ² and Fisher’s exact tests. ANOVA followed by a Fisher’s protected least significant difference test was used to analyze all of the other experiments, and results were expressed as the mean ± SD. These analyses were done using the Stat-View 5.0 software. P < 0.05 was considered statistically significant.

**Results.**

**Expression of the Angiogenesis Inhibitor TSP1 in the Rat Ventral Prostate.** To determine whether androgens could regulate TSP1 expression, we analyzed the effects of castration
on TSP1 expression and vascularization in the rat prostate gland. In intact animals, a faint TSP1 expression (Fig. 1A) was mainly associated with numerous and large blood vessels found in the stroma of the ventral prostate (Fig. 1A, insert). Three days after castration, a moderate-to-strong staining for TSP1 was consistently observed in the stroma (Fig. 1B) and blood vessels were markedly smaller as compared with the size of prostatic ducts that remained almost unchanged (Fig. 1B, insert). Immunostaining for stromal TSP1 was even stronger 15 days after castration, and a striking increase in TSP1 immunoreactivity was also observed in the epithelium of prostatic ducts (Fig. 1C). At that time, the decrease in the size of blood vessels was associated with a drastic involution of prostatic ducts. In agreement with a previous study (4), morphometric analysis of prostatic tissues after castration revealed that the average lumen area of blood vessels gradually decreased compared with that observed in the prostatic tissue of intact animals, reaching a 75% decrease 15 days after castration (Fig. 1E). However, a 3-day treatment of the castrated rats with testosterone resulted in a rapid disappearance of TSP1 immunostaining in the stroma and in epithelial cells of prostatic ducts (Fig. 1D). Concomitantly, the size of blood vessels and prostatic ducts returned to normal (Fig. 1D, insert, and E). These results strongly suggested that androgens could repress TSP1 expression.

Evidence for the Presence of an Androgen Regulatory Element in the First Intron of the Human TSP1 Gene. We then first studied the effect of R1881 (a synthetic androgen receptor–specific agonist) on endogenous production of TSP1 by primary cultured cells grown from mouse ventral prostate explants (26). As shown in Fig. 2A, R1881 induced a marked...
reduction of TSP1 production in prostatic cells when compared with vehicle-treated cells.

We then analyzed whether androgens, by binding to their cognate androgen receptor, could repress the transcription of TSP1. The vast majority of cells from prostatic origin, such as the SV40-immortalized cell lines PNT1a and PNT1b or the tumorigenic PC3 or DU145 cells no longer express the androgen receptor. The androgen-dependent LNCaP cell line expresses the androgen receptor but TSP1 expression is repressed in this cell line by promoter methylation (26, 27). We therefore used the SaOS-2 osteoblastic cell line to examine the effect of androgens on TSP1 gene transcription because these cells express both TSP1 and a functional androgen receptor (28, 29). SaOS-2 cells were transiently transfected with a luciferase gene reporter under the control of a mouse primary prostate epithelial cells were grown for 3 days in hormone-stripped medium, then treated for 24 hours with 0.1 nmol/L R1881 (a testosterone analogue) or the vehicle. TSP1 and tubulin in cell extracts were immunodetected by Western blotting.

Figure 2. Regulation of TSP1 expression by testosterone. A, mouse primary prostate epithelial cells were grown for 3 days in hormone-stripped medium, then treated for 24 hours with 0.1 nmol/L R1881 (a testosterone analogue) or the vehicle. TSP1 and tubulin in cell extracts were immunodetected by Western blotting. B, SaOS-2 cells, which express TSP1 and androgen receptor, were transiently transfected with reporter constructs placing the luciferase coding sequence (Luc) under the transcriptional control of different regions of the human TSP1 gene regulatory sequences. Dark lines, position of the first noncoding exon of TSP1. Restriction enzymes were used to clone internal deletion constructs. The position of a potential HRE, disrupted by site-directed mutagenesis in the last construct is indicated. For each construct, transfected cells were split in replicate wells and treated either with R1881 (black bars) or the vehicle (gray bars). The luciferase activity triggered by the (-548, +750) construct in the absence of R1881 was used as a control and set to 100%. Results, normalized for protein content, were then expressed as a percentage of the control. Values are the mean ± SD of four to eight separate experiments for each construct. * P < 0.05 for R1881-treated versus untreated cells.

TSP1 Delays the Onset of Tumorigenesis but Has no Effect on the Subsequent Growth Rate of LNCaP Xenografts. We used the LNCaP androgen–dependent prostatic cancer cell line which does not express TSP1. A mouse fibroblastic NT26 cell line, stably transfected with a tet-off regulatory expression vector for mouse TSP1 (23), was used to mimic in our tumorigenesis animal model the stroma reaction seen in human primary prostate carcinomas after androgen deprivation (see below). As shown by Western blotting, the synthesis and secretion of TSP1 by NT26 cells was repressed in the presence of doxycycline (Fig. 3A). In addition, the conditioned medium from NT26 cells drastically inhibited capillary-like tube formation only when TSP1 expression was induced by doxycycline withdrawal (Fig. 3B), demonstrating that TSP1 produced by NT26 cells was biologically active. In contrast, the growth of the LNCaP cells in the presence of the conditioned medium from NT26 cells was not modified by the expression of TSP1 (not shown). A mixture of LNCaP and NT26 cells was then injected subcutaneously into nude mice, and tumor growth was monitored during 9 weeks (Fig. 4A), at which time tumors were collected for immunohistochemical analysis. Tumors grown in doxycycline-fed animals, where TSP1 was repressed, were markedly larger than TSP1-expressing tumors grown in untreated animals (Fig. 4A). TSP1 expression induced an initial delay in tumor development of

Figure 3. Antiangiogenic effects of TSP1 in vitro. A, NT26-TSP1 inducible mouse fibroblasts were grown for 3 days in the presence or absence of doxycycline (dox). As judged by Western blotting, dox repressed TSP1 production in the conditioned medium from NT26 cells. B, endothelial cells were laid on Matrigel-coated plates in the presence of conditioned medium from NT26 cells treated (-TSP1) or not treated (+TSP1) with dox. The total length of the capillary-like tube network was measured using a semiautomatic analyzer.
about 10 days (Fig. 4A, insert) and, 65 days after tumor cell inoculation, tumor volumes in doxycycline-fed and untreated animals were 4.1 ± 1.0 and 1.7 ± 0.8 cm³, respectively (*P < 0.05). TSP1 secretion in tumors was detected by immunohistochemistry only in the absence of doxycycline (Fig. 4B). The tumor microvessel density in TSP1-expressing tumors (8.83 ± 1.46 vessels/HPF) was repressed by 37% as compared with untreated tumors (14.17 ± 3.38 vessels/HPF, *P < 0.05). TSP1 is a major activator of the latent forms of TGFβ and, using an antibody specific for the active forms of this cytokine, we also observed a marked TGFβ production in TSP1-expressing tumors (Fig. 4B). These data confirmed previous published work demonstrating the antiangiogenic and antitumor activities of TSP1 (16, 30). Nevertheless, despite an initial delay in tumor growth, the logarithmic linearization of the growth curves depicted in Fig. 4A (not shown) indicated that, once tumors started to grow, the tumor growth rates were similar in the presence or absence of stromal TSP1, suggesting that tumor cells had adapted to escape TSP1 inhibitory effects. To address this possibility, using ELISA, we measured the plasma levels of VEGF from human, LNCaP origin in tumor-bearing animals. We observed that this level was significantly increased in animals bearing TSP1-expressing tumors compared with that observed in doxycycline-fed animals (Fig. 4C). In addition, TGFβ did not modify the in vitro growth of parental LNCaP cells, whereas it did stimulate the growth of tumor cells explanted from TSP1-expressing tumors (doubling time: 30.1 ± 2.7 versus 39.9 ± 1.7 hours). To show that TSP1 no longer affected in vivo tumor growth, tumor cells that had grown in the presence of TSP1 were then re-injected together with fresh NT26 cells to nude mice that received or did not receive doxycycline. As shown in Fig. 4D, tumor growth was the same in doxycycline-fed and untreated animals. Tumor microvessel density was even increased (*P < 0.05) in TSP1-expressing tumors (14.20 ± 2.31 vessels/HPF) as compared with untreated tumors (10.50 ± 1.50 vessels/HPF). TSP1 expression was then studied in 103 prostate carcinomas from patients with localized or advanced disease, in order to examine the clinical relevance of our experimental findings.

Association of TSP1 Immunostaining with Clinico-Pathological Classification and Microvessel Density in Patients with Localized Prostate Cancer. In low-grade carcinomas (Gleason score < 6), TSP1 was consistently and strongly expressed in ductal epithelial cells, whereas very few blood vessels were present in the stroma. A moderate TSP1 staining was also observed in the stroma. In contrast, in high-grade carcinomas (Gleason score > 6), TSP1 immunoreactivity in ductal epithelial cells disappeared, concomitantly with the appearance of numerous blood vessels in the stroma. The analysis of 60 patients with localized prostate cancer indicated that there was an inverse relationship between TSP1 immunoreactivity and Gleason score, and between TSP1 immunoreactivity and microvessel density (Table 1). No significant association was observed between TSP1 and pathologic stage, tumor size, or PSA levels (not shown). There was no significant correlation between the microvessel density and the Gleason score, indicating that the histologic grade was not a confounding factor.
Association of TSP1 Immunostaining with Clinico-Pathological Classification and Microvessel Density in Patients with Prostate Cancer with Advanced Disease. All of the patients with advanced disease had a Gleason score > 6. In agreement with our results obtained with localized high-grade tumors, 22 out of 23 patients had a negative TSP1 score before androgen deprivation (Fig. 5A and Table 1). In addition, numerous blood vessels were present in the stroma (Fig. 5A, insert, and D). In sharp contrast, all of the patients undergoing androgen-ablation therapy had a significant increase in TSP1 expression (Fig. 5B and Table 1) with a concomitant decrease in the microvessel density (Fig. 5B, insert, and D). However, although TSP1 expression was substantially increased in hormone-refractory tumors (Fig. 5C and Table 1), the mean microvessel count significantly increased compared with that observed in hormone-responsive tumors following androgen deprivation (Fig. 5C, insert, and D). CD36-positive blood vessels were present in both hormone-responsive (Fig. 6A and B) and hormone-refractory tumors (Fig. 6C), suggesting that hormone-refractory tumors bypassed the antitumoral effect of TSP1 despite the presence of TSP-1 receptor CD36 on endothelial cells.

Discussion
The vasculature is thought to play a central role in the androgen-dependent growth of the prostate because endothelial cells respond to angiogenic factors like VEGF that are secreted by the prostate epithelium under testosterone stimulation (1–4, 31, 32). However, angiogenesis is almost always finely tuned by a balance between activators, and inhibitors. The lack of cell lines expressing the characteristic markers of normal prostatic epithelial cells has always been a major drawback to study the signal transduction pathways triggered by androgens in the normal prostate. More than 50 years of efforts from different laboratories led to only short-term primary cultures, not suitable for performing extensive molecular analysis. Therefore, in order to unravel the effects of androgens on the production of the angiogenesis inhibitor TSP1, and study its role in prostate pathophysiology, we combined molecular and in vivo experimental approaches, together with a comprehensive analysis of human primary prostate carcinomas at different stages of the disease. We first observed that castration induced the production of TSP1 by the normal prostate epithelium in vivo, whereas androgen replacement decreased it. Moreover, testosterone substantially repressed TSP1 production by primary prostate epithelial cells in culture. Similarly, androgens repress the expression and secretion of the antiangiogenic molecules, pigment epithelium-derived factor and maspin, in primary prostate epithelial and LNCaP cells, respectively (11, 12). The mechanism of androgen-induced repression for maspin in LNCaP cells is transcriptional but unknown for pigment epithelium-derived factor. TSP1 expression in LNCaP cells is constitutively repressed by methylation of the TSP1 gene promoter (26, 27), impeding the use of this human cell line to study the androgen regulation of TSP1. We therefore used the SaOs-2 osteoblastic cell line which expresses both the androgen receptor and TSP1 (28, 29). In these cells, androgens repressed TSP1 expression. This repression was transcriptional and required a regulatory region located in the first intron of the TSP1 gene. Although part of this region presented a sequence homology with a noncanonical hormone-regulatory element, disruption of this sequence did not affect testosterone-mediated repression of TSP1 transcription. It is therefore possible that the repression of TSP1 transcription by androgens occurs via the binding of androgen receptor to another noncanonical DNA element or depends on downstream effectors. Interestingly,

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*The intensity of the TSP-1 immunostaining was scored as follows: strong (3+), moderate (2+), weak (+), and negative (−).
†χ² test for the nominal clinico-pathological variables associated with TSP-1 expression.
androgens, via the HIF1α transcription factor, induce VEGF transcription (33), and androgen withdrawal destabilizes VEGF mRNA (7). VEGF and TSP1 are thus regulated in an opposite manner by hypoxia, p53 (34–36), and androgens (refs. 7, 33 and this study) in such a way as to position TSP1 as an effective negative counterpoint to stimulatory VEGF in the normal prostate. In other organs, such as breast, 17β-estradiol also up-regulates VEGF and represses TSP1 expression (37).

We next studied the role of TSP1 in tumorigenesis, using an expression system that allows the inducible production of TSP1 in the stroma of LNCaP tumor xenografts in nude mice. TSP1 inhibited both the vascularization and growth of LNCaP tumors, as it has been previously reported for DU-145 prostate tumors (17). Yet, on prolonged exposure to TSP1, LNCaP tumors came to grow at the same rate in the presence as in the absence of TSP1. In addition, transplantation experiments showed that they had become insensitive to inhibition by TSP1 and overproduced VEGF. Finally, transplantation experiments showed that TSP1-insensitive cells grew more effectively in the presence of TGFβ, a feature frequently observed during tumor progression (38, 39). Overall, these findings are in line with previous experimental works showing that fibrosarcoma and glioma cells are able to bypass TSP1 inhibition in vivo (23).

However, given that androgens repressed the TSP1 expression in the prostate (this study) and that human prostate cancers invariably relapse after hormone-ablation therapy (40), the key question was to know if a resistance to androgen deprivation may develop early in human primary prostate carcinomas as a result of high in situ exposure to stromal TSP-1.

To address this question, TSP1 expression was studied in 103 prostate carcinomas from patients with localized or advanced disease, before and after hormone-ablation therapy. As previously reported by others (20), in localized prostate tumors, there was an inverse relationship between TSP1 immunoreactivity and microvessel density as well as between TSP1 immunoreactivity and Gleason score. VEGF expression has been shown to be increased with increasing Gleason score (20). It is therefore most likely that the higher vascularization of high-grade carcinomas reflects an angiogenic switch in which the decreased TSP1 expression is associated with an increased VEGF expression. Interestingly, androgen deprivation induced a sustained reduction in blood vessel density as well as a substantial increase in TSP1 production by the neoplastic prostate epithelium, demonstrating that in human prostate carcinomas androgens also repress TSP1 expression. These findings were in complete agreement with previous works showing that androgen deprivation decreases VEGF expression and increases TSP1 expression in human prostate carcinomas (9, 10, 20). In addition, Chen et al. (41) recently showed that the androgen receptor antagonist, bicalutamide, induces the expression of TSP1 in prostate carcinoma xenografts in nude mice. The antiangiogenic effects of TSP1 are, at least in part, mediated by its interaction with its receptor CD36 on endothelial cells. CD36-positive blood vessels were observed in all the prostatic carcinomas studied, independently of the tumor grade and the hormonal status. Intriguingly, CD36 was also clearly expressed in prostate cancer cells and its expression pattern was similar to that of TSP1. For example, like TSP-1, CD36 was highly expressed in the cytoplasm of prostate cancer cells following androgen deprivation (Fig. 6B). These findings were reminiscent of our previous observations in primary breast tumors (42). CD36 is closely related to PAS-IV.
a differentiation-specific sialoglycoprotein secreted by breast epithelial cells during lactation which is also expressed in lactating adenomas. It is possible that prostate epithelial cells also secrete a PAS-IV/CD36-like molecule. Its up-regulation might correlate with the differentiation of prostate cancer cells following androgen deprivation. Results obtained here do not of course preclude the possibility that changes in the expression of other angiogenic (basic fibroblast growth factor, interleukin-8) and/or antiangiogenic (maspin, pigment epithelium-derived factor) factors may also occur during androgen deprivation (10–12, 19). They claim that TSP1 displays similar antiangiogenic properties in human prostate carcinoma xenografts in nude mice and primary androgen-responsive prostate tumors from patients.

Moreover, our data shows for the first time, that despite a sustained expression in the tumor bed, TSP1 was no longer associated with a decreased vascularization in hormone-refractory prostate tumors. CD36-positive blood vessels were present in both hormone-responsive and hormone-refractory tumors, suggesting that hormone-refractory tumors are able to bypass the antitumoral effect of TSP-1 despite the presence of endothelial cell TSP-1 receptor CD36. These results are also in agreement with recent findings showing no difference in CD36 expression between benign and malignant human prostate tissues (22). As observed in our tumorigenesis model, TSP1 fostered the increased expression of VEGF and TSP1-activated TGFβ-stimulated tumor growth. In addition, TGFβ up-regulates VEGF transcription via a Smad pathway and a SP1 site in the VEGF promoter (43, 44). The homology of TSP1’s behavior between experimental and clinical prostate tumor specimens strongly suggests that a resistance also developed in primary prostate tumors as a result of high in situ exposure to TSP1, leading to increased VEGF production and/or TGFβ-dependent tumor growth. Although such an observation does not apparently argue for antiangiogenic therapy, it must be pointed out that selection for drug resistance inevitably occurs as a consequence of tumor regression following effective therapy (40). In this respect, there may be no cells that are sufficiently resistant to tolerate a combination of very effective therapies. This contention is supported by the fact that impairing the synthesis of VEGF severely limits the resistance of fibrosarcoma cells to TSP-1 antiangiogenic activity (45). In line with this, we believe that patients with hormone-responsive prostate cancer whose in situ TSP1 expression in primary tumors is high because of androgen deprivation could benefit from early antiangiogenic treatment, such as anti-VEGF therapy, in order to minimize the induction of such tumor escape.

Acknowledgments
Received 7/19/2004; revised 10/13/2004; accepted 11/2/2004.
Grant support: Grants from the NIH and Medical Research (Institut National de la Sante et de la Recherche Medicale; P. Clezardin), the Association pour la Recherche sur le Cancer (F. Cabon and P. Clezardin), the Association pour la Recherche sur les Tumeurs de la Prostate (M. Colombel and F. Cabon), and the Groupement des Entreprises Francaises dans la Latte contre le Cancer (F. Cabon). P. Fournier is a recipient of a fellowship from the Association pour la Recherche sur le Cancer.

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We thank Drs. Noël Bouck for critical reviewing of the manuscript and helpful advice, P. Bornstein (University of Washington, Seattle) for providing hTSP-luciferase constructs, Prof. L.W. Chung (Emory University School of Medicine, Atlanta) for the generous gift of LNCaP cells, and Céline Bourillon for her excellent technical assistance.

Figure 6. Immunodetection of CD36 in metastatic human prostatic carcinomas. A, androgen-sensitive carcinomas before hormone-suppressive treatment. CD36 was strongly expressed in blood vessels (black arrows) and, to a much lower extent, in tumor cells; B, androgen-sensitive carcinomas following androgen deprivation. In addition to blood vessels (black arrows), CD36 was strongly expressed in tumor cells (white arrows); C, hormone-refractory carcinomas. CD36 was strongly expressed in blood vessels (arrows). A faint staining was also observed in tumor cells (magnification ×200).
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