Initial Modulation of the Tumor Microenvironment Accounts for Thalidomide Activity in Prostate Cancer


Abstract  
Purpose: Disruption of stromal-epithelial interactions favoring prostate cancer progression may affect the phenotype of the disease. We did a preoperative study to test the hypothesis that thalidomide, an active agent in metastatic disease, is a modulator of the tumor microenvironment. Experimental Design: Eighteen men with high-risk prostate cancer were given thalidomide at doses escalated to 600 mg for 12 weeks, followed by radical prostatectomy. We constructed tissue microarrays from prostatectomy specimens from 15 treated patients and 15 matched untreated control subjects to assess effects of thalidomide on the tumor microenvironment. We compared the immunohistochemical expression of three groups of markers linked to angiogenesis, stromal-epithelial interactions, or the epithelial compartment. Levels of circulating basic fibroblast growth factor, interleukin-6, tumor necrosis factor-α, and vascular endothelial growth factor were also assessed. Results: Thalidomide was well tolerated and induced a median reduction in prostate-specific antigen of 41% without affecting testosterone. Tissue microarray analyses indicated modulation of vascular marker expression accompanied by a reduction in microvessel density in the treated group. Comparison of broader stromal-epithelial interaction markers between treated and control groups suggested a transition to a less aggressive phenotype as a result of thalidomide treatment. Hedgehog signaling was attenuated and the ratio of matrix metalloproteinases to E-cadherin shifted to favor E-cadherin. No differences were noted in proliferation or apoptosis in the epithelial compartment. Conclusions: These findings are the first clinical evidence to support the hypothesis that the reported thalidomide clinical efficacy is attributable to early modulation of the tumor microenvironment and suggest that stromal-targeting therapies will be effective against prostate cancer.

Therapeutically targeting the stromal-epithelial interaction implicated in invasion and metastasis has been proposed as a strategy to prolong the survival of patients with prostate cancer. We hypothesize that the assessment of the tumor microenvironment, following therapy with an agent that targets the stromal-epithelial interaction, will contribute to the prioritization of agents in the development of a rational treatment strategy. The “high-risk preoperative model” is the experimental platform to test the hypothesis in a clinically meaningful context. We clinically explored this paradigm by giving thalidomide preoperatively and assessed its effect on the tumor microenvironment.

Stromal-epithelial interactions have been identified as a potential driver of prostate cancer progression and metastasis (1). Drugs that disrupt the crosstalk between “host” stroma and tumor may reverse the lethal phenotype of the disease. Thalidomide may be one such drug; it is known to have antiangiogenic activity and is suspected of affecting the bone marrow microenvironment (2, 3). Moreover, in the context of prostate cancer, although in vitro studies have been discouraging, thalidomide has exhibited clinical efficacy in metastatic disease and may potentially offer a survival benefit in combination with docetaxel (4–6). Most investigators have attributed these observations to an initial effect on the tumor microenvironment, although alternative explanations have been proposed (7, 8).

We used the high-risk preoperative model to test whether thalidomide modulates the prostate cancer microenvironment in a manner that results in a less aggressive malignant phenotype. Candidate pathways of interest include those that regulate tumor-associated angiogenesis and pathways inherent in prostate development and associated with carcinogenesis (9–15).
Patients and Methods

Patients. Subjects in this prospective study of preoperative thalidomide were men with histologically confirmed prostatic adenocarcinoma with no evidence of regional or distant metastases; disease could be clinical stage T1c-T2c with Gleason score of ≥7 on initial biopsy or clinical stage T3. All subjects gave informed consent to participate in this phase II study, which is approved by the institutional review board of The University of Texas M.D. Anderson Cancer Center.

Thalidomide was given once daily in the evening at a starting dose of 200 mg/d. This dose was escalated by 200 mg/d weekly to a maximum of 600 mg/d if no grade 3-4 toxicity ensued. At 6 and 12 weeks, patients underwent digital rectal examination, transrectal sonography, and serum prostate-specific antigen (PSA) testing. Treatment was continued for a maximum of 12 weeks unless there was evidence of progression. PSA progression was defined as an increase in serum PSA of >25% over the baseline value. Progression of measurable intraprostatic lesions was defined as an increase of >25% in two dimensions. Radical prostatectomy followed treatment.

The statistical design of Thall et al. (16) was used, and a success probability of ≥0.20 was considered clinically promising. Clinical success was defined as stable disease at 6 weeks followed by a decline in serum PSA of ≥50% at 12 weeks.

Tissue microarrays. A tissue microarray was constructed from radical prostatectomy specimens from 15 thalidomide-treated patients who underwent prostatectomy. A control tissue microarray was constructed from 15 prostatectomy specimens matched for pathologic stage and Gleason score at surgery. Areas representative of all histologic tumor patterns of the Gleason grades present were selected from the individual specimens. Each case was represented by a median of thirty 0.6-mm-diameter cores (range, 18-53). The treated microarray consisted of 453 cores, and the control of 523. Expression of 18 biomarkers (described below) was assessed by immunohistochemical staining. Antibodies and staining methods are shown in Supplementary Table S1. A DAKO autostainer (Carpinteria, CA) and standard 3,3-diaminobenzidine were used.

Biomarker analyses. Images of each biomarker in each core of the tissue microarray were acquired using a BLISS imaging system (Bacus

---

Table 1. Pathologic characteristics of radical prostatectomy specimens from thalidomide-treated patients

<table>
<thead>
<tr>
<th>Pathologic stage</th>
<th>No. patients (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organ-confined (pT2)</td>
<td>2</td>
</tr>
<tr>
<td>Extraprostatic extension (pT3a)</td>
<td>1</td>
</tr>
<tr>
<td>Seminal vesicle invasion (pT3b)</td>
<td>12</td>
</tr>
<tr>
<td>Positive lymph nodes (pT any, N1)</td>
<td>4</td>
</tr>
<tr>
<td>Positive surgical margins</td>
<td>9</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
</tr>
</tbody>
</table>

---

Fig. 1. Markers implicated in prostate cancer biology. Three groups of markers implicated in prostate cancer biology, especially with regard to disease progression, were tested: markers linked with endothelial cells [i.e., the vascular markers VEGF, IL-6, platelet-derived growth factor-A (PDGF-A), IL-8, bFGF, and the endothelial cell-specific marker CD31]; markers linked with broader stromal-epithelial cell interactions, such as members of the Shh pathway, the ligand Shh, the signaling intermediate Smoothened (Smo), the transcription factor gli2, MMP-9, MMP-2, E-cadherin, β-catenin, TGF-β, and TNF-α; and markers linked with epithelial compartment proliferation (Ki-67), apoptosis (active caspase-3), and survival (bcl2). For practical reasons, we address these markers according to their principal role in prostate cancer biology while acknowledging their overlapping properties.
Hierarchical clustering analysis revealed two main clusters of markers, depending on whether they were up-regulated or down-regulated in the treated group with regard to the untreated control group. Further analyses with standard t tests and a mixed model, allowing estimation of variability between and within individual samples, confirmed these results. A, image plot of the initial exploratory hierarchical clustering of the raw data. Involvement (extent of staining) for each marker (columns) and per sample (rows) is represented by color. Green to red, range of involvement from 0 to 3. B, image plot of relative involvement of only the differentially expressed markers between the treated group (upper half) and the control untreated group (lower half). Red, higher expression than the mean; green, lower; and black, no difference from the mean.
A four-point system was used for assessing involvement (percentage of tumor cells exhibiting detectable staining): 0, no staining; 1, up to 33% of cells stained; 2, 34% to 66% of cells stained; or 3, >67% of cells stained. The intensity of staining was scored as 0, low, or high. Subcellular localization of biomarker staining and the predominant histologic type of tumor in each core were noted.

Table 2. Differences in markers of angiogenesis and microvessel density in tumors from men not treated (control) or treated with thalidomide

<table>
<thead>
<tr>
<th>Angiogenesis</th>
<th>Control (mean)</th>
<th>Treated (mean)</th>
<th>SD between patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>2.24</td>
<td>1.63</td>
<td>0.52</td>
<td>0.0049</td>
</tr>
<tr>
<td>VEGF stroma</td>
<td>0.76</td>
<td>0.34</td>
<td>0.22</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.68</td>
<td>1.23</td>
<td>0.49</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-6 stroma</td>
<td>1.53</td>
<td>1.41</td>
<td>0.32</td>
<td>0.279</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>2.74</td>
<td>2.59</td>
<td>0.29</td>
<td>0.221</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.49</td>
<td>1.26</td>
<td>0.5</td>
<td>0.0008</td>
</tr>
<tr>
<td>bFGF</td>
<td>1.55</td>
<td>2.55</td>
<td>0.43</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Microvessel density</th>
<th>Control</th>
<th>Treated</th>
<th>SD between patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Median (min, max)</td>
<td>Mean</td>
<td>Median (min, max)</td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>32.6</td>
<td>30 (21, 54)</td>
<td>24.1</td>
<td>21 (11, 46)</td>
</tr>
</tbody>
</table>

NOTE: Values shown are “involvement” (extent of staining), expressed as a 0 to 3 scale for angiogenic markers and as absolute numbers of stains per core for microvessel density. P values refer to differences between control and treated groups according to univariate mixed model analyses (P < 0.05 for significance).

Clinical characteristics

Patients. Between December 2000 and March 2002, 18 patients (median age, 60 years; range, 43-71 years) were enrolled in the study. Ten (55%) patients had clinical stage T3...
disease, eight T2c, and 10 Gleason score of ≥8 at biopsy (Supplementary Table S2). Seven patients had baseline PSA of >20 ng/mL. Baseline PSA levels ranged from 3.0 to 190 ng/mL.

**Thalidomide and PSA level.** Reductions in serum PSA became quickly evident in all but one patient. At 6 weeks of treatment, PSA levels were a median 38% lower than at baseline (range, −12% to 49%). At 12 weeks, the median PSA reduction was 42% (range, −19% to 71%), and six patients (33%; 95% confidence interval, 16-56%) achieved a PSA reduction of ≥50%. Testosterone concentrations remained unaffected with medians of 308.85 ng/dL (range, 186.71-595) at baseline and 341.29 ng/dL (range, 208.88-923.97) at 12 weeks.

**Toxicity.** All patients were evaluable for toxicity. Dose was escalated to 600 mg daily in all patients. Seventeen (95%) completed treatment as scheduled. A total of 126 adverse events were reported and their incidence ranged from 5% to 61%. Toxicity consisted mostly of grade 1 somnolence, fatigue, neurotoxicity, and constipation. Two grade 3 neurotoxicity-related events and one grade 3 asthenia occurred. Twenty-one grade 2 events occurred, 10 of which were neurologic (Supplementary Table S3).

**Radical prostatectomy.** Fifteen of 18 patients underwent radical prostatectomy on completion of treatment. Surgery was aborted because of macroscopic lymph node infiltration in two cases and an aortic aneurysm in a third. Thalidomide treatment did not delay surgery. Median time to surgery from treatment completion was 5 days (range, 2-18 days). Prostatectomies were uneventful except for three involving difficulties in apical dissection, dissection from the rectum, or both. Median estimated blood loss was 575 mL (range, 200-1,300 mL). No
postoperative complications were reported and patients were hospitalized for a median of 3 days (range, 2-3 days).

**Pathology.** Pathologic features of radical prostatectomy specimens are listed (Table 1).

**Follow-up.** Eleven of 15 patients had undetectable PSA after surgery. No adjuvant treatment was given. At a median follow-up time of 37 months (range, 20-54 months), five patients still had undetectable PSA. To date, three patients have been diagnosed with metastatic disease (two in bone and one in lymph nodes) and one has died of the disease.

**Molecular effects**  
To efficiently investigate the molecular effects of thalidomide in prostate cancer, we compared the immunohistochemical expression of markers strongly implicated in prostate cancer progression with regard to angiogenesis, broader stromal-epithelial interactions, or markers of epithelial proliferation, survival, and apoptosis (Fig. 1) between the thalidomide-treated and control tissue microarrays, which were constructed from 15 prostatectomy specimens of thalidomide-treated patients and 15 untreated matched controls, respectively.

We scored expression of markers in the tumor epithelial compartment as well as the stroma near the tumor, where applicable. The main variable considered in the statistical analyses was mean involvement (i.e., proportion of tumor cells staining positively for a particular factor) used for initial hierarchical clustering (Fig. 2) and further statistical analysis; supplemental analysis was used to detect potential differences in intensity of expression (Supplementary Tables S4-S11).

**Markers of angiogenesis.** Expression of VEGF and IL-6, markers strongly implicated in prostate cancer angiogenesis (9), were lower in both the tumor epithelium and the stroma in samples from the thalidomide-treated group than in those from the control group (Table 2; Supplementary Fig. S1). The effect on VEGF expression was profound in both compartments. IL-6 was consistently expressed in the treated group but to a lesser extent than in the control. Expression of IL-8 and bFGF was higher in the treated group. IL-8 expression in the control was limited and predominantly cytoplasmic, which is consistent with other published reports (20); yet in the treated, it was primarily nuclear. Microvessel density was lower in the treated (mean $\pm$ SD, 24.1 $\pm$ 9.2; range, 11-46) than in the control (mean $\pm$ SD, 32.6 $\pm$ 9.8; range, 21-54; Table 2; Supplementary Fig. S2).

**Markers of broader stromal-epithelial interactions.** Comparison of the expression of markers of stromal-epithelial interaction between thalidomide-treated and control cases suggested a modulation of hedgehog signaling and the matrix metalloproteinase (MMP) to E-cadherin ratio by thalidomide. Results are summarized briefly below (Table 3).

**Hedgehog signaling.** We assessed the expression of three main components of the sonic hedgehog (Shh) pathway: gli2, Smo (Smo), and the Shh ligand (21). Thalidomide treatment attenuated hedgehog signaling. The transcription factor gli2, the main downstream effector of the Shh pathway (12), was consistently expressed in the control in both the nucleus and cytoplasm of tumor cells; in contrast, gli2 expression was significantly lower in thalidomide-treated and localization was predominantly cytoplasmic (Supplementary Table S6). The Shh ligand and the transmembrane protein Smo are upstream components of the pathway responsible for the level of Shh activation (12). Expression of the ligand was the same in both groups, but expression of Smo, considered the determining factor of aberrant activation of hedgehog signaling in prostate cancer (13, 15), was much higher in the control (Fig. 3; Table 3).

**MMP/E-cadherin ratio.** Expression of MMP-2 and MMP-9 was significantly lower in the thalidomide-treated group than in the control group. E-cadherin, a marker of cellular adhesion, was consistently higher in the treated group (Table 3). A three-way scatter plot (Fig. 4) by discriminant analysis of the relative expression of MMP-9, MMP-2, and E-cadherin, which may predict prostate cancer phenotype more accurately than conventional variables of disease stage and tumor grade (10, 11, 22), distinguished between thalidomide-treated and control samples with 93% accuracy.

**Other markers of stromal-epithelial interactions.** TNF-α expression was higher in the thalidomide-treated group. No significant differences were detected in β-catenin or transforming growth factor (TGF)-β expression (Table 3).

**Markers of the epithelial compartment.** The apoptotic index of tumors, assessed with an antibody to active caspase-3, was low in both groups, although slightly higher in the treated (mean $\pm$ SD, 1.9 $\pm$ 2.6%; range, 0-3%) than in the control (mean $\pm$ SD, 0.64 $\pm$ 0.75%; range, 0-3%; $P = 0.064$; Table 4).

No statistically significant difference was found between treated and control tumors with regard to proliferative index; mean Ki-67 expression in the treated was 6.0% ($\pm$ 8.8; range,
Interestingly, baseline plasma VEGF levels were lower in patients whose PSA dropped <50% than in the others (P = 0.016) compared with those with a PSA drop of ≥50%. Analysis was done by Wilcoxon signed rank test for paired data. P < 0.05 was considered significant.

The results of our “proof-of-principle” study support the hypothesis that the inhibitory effects of thalidomide on prostate cancer progression suggested from phase II studies (4, 5) are consistent with an effect on the tumor microenvironment that precedes the effect on the epithelial compartment. Ascribing a single biological effect of thalidomide responsible for our observations is beyond the scope of this study, given the multiplicity of actions that have been assigned to the drug and the elusiveness of its mechanism of action.

We found that angiogenic signaling was profoundly affected by thalidomide treatment, both in the epithelium and the stroma. Guided by the significant decrease in microvesSEL density after 12 weeks of thalidomide treatment, we conclude that reduction of VEGF and IL-6 expression in the tumor microenvironment accounts for the antiangiogenic activity of thalidomide in prostate cancer. The observed increase in bFGF and IL-8 resembles a stress-related response that could be compensating for microvessel density decrease, as suggested in other contexts (23).

Our observations about hedgehog signaling and the MMP/E-cadherin ratio provide the first translational evidence to support the idea that thalidomide effects in the tumor microenvironment are not restricted to antiangiogenesis. Shh signaling governs epithelial-mesenchymal crosstalk during organogenesis, including prostate development, and tissue repair (14, 24, 25). Hedgehog signaling reciprocates with pathways and molecules acknowledged as crucial in this crosstalk, including members of the TGF-β superfamily, MMPs, epidermal growth factor, vascular markers, and the wnt pathway (26–29). The implication of hedgehog signaling in the pathogenesis of various malignancies such as prostate cancer is in keeping with the belief that carcinogenesis results from perturbations of the homeostatic equilibrium controlled by the microenvironment (11).

Activation of the hedgehog pathway by overexpression of the transcription factor gli has been shown to confer invasiveness and increased metastatic potential to prostate tumor cells, probably by down-regulating E-cadherin (12). Our work provides evidence of active hedgehog signaling in high-grade, locally advanced prostate cancer that is attenuated after treatment with thalidomide. The strikingly lower expression of Smo after thalidomide treatment most probably accounts for this effect. This central signaling intermediate has been designated the key determinant of aberrant hedgehog activation in advanced prostate cancer (12, 14, 21).

The MMP/E-cadherin ratio has been proposed as an independent predictor of prostate cancer aggressiveness (10, 11). Logically, this ratio should represent a biologically meaningful readout, as consistent E-cadherin expression indicates increased cell-to-cell adhesion whereas lower MMP expression has been linked to decreased invasiveness. In this light, the discrepancy in MMP/E-cadherin ratios between the control and thalidomide-treated groups should speak to a transition to a less aggressive disease phenotype in the treated group.

Survival and proliferation of the epithelial compartment were not significantly affected after 12 weeks of thalidomide treatment. Although a more prolonged treatment period may

### Table 4. Differences in proliferation and apoptosis in the epithelial compartment in tumors from men not treated (control) or treated with thalidomide

<table>
<thead>
<tr>
<th>Epithelial compartment</th>
<th>Control</th>
<th>Treated</th>
<th>SD between patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 (%)</td>
<td>Mean</td>
<td>Median (min, max)</td>
<td>Mean</td>
<td>Median (min, max)</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>5.0 (0, 23)</td>
<td>6.0</td>
<td>3.0 (0, 33.5)</td>
</tr>
<tr>
<td>Active caspase-3 (%)</td>
<td>0.64</td>
<td>0 (0, 3)</td>
<td>1.9</td>
<td>0 (0, 3)</td>
</tr>
</tbody>
</table>

NOTE: Values shown are “involvement” (extent of staining), expressed as percentage of tumor cells positive for proliferation. P values refer to differences between control and treated groups according to univariate mixed model analyses (P < 0.05 for significance).

<1-33.5%; median, 3.0%) as compared with 8.9% (± 7.1; range, <1-23.5%; median, 5.0%) in the control (P = 0.333; Table 3). No difference was detected in bcl2 expression (Supplementary Table S4) and androgen receptor expression and localization (Supplementary Fig. S5).

### Circulating factors

Plasma levels of TNF-α, measured in 16 thalidomide-treated patients, were higher after treatment (Supplementary Table S12). Serum bFGF and IL-6 measured in 14 patients remained unchanged. Viewed in terms of PSA drop, bFGF was slightly reduced in patients whose PSA dropped ≥50% (P = 0.09). Interestingly, baseline plasma VEGF levels were lower in patients with a PSA drop of ≥50% than in the others (P = 0.055) and did not change after treatment, contrary to those with a PSA drop of <50% (Fig. 5).
well reveal epithelium growth arrest, it could limit or obscure observations of the initial biological effects.

The reason for the decrease in PSA levels that we observed is unclear. We may speculate a correlation between this finding and the observed angiogenic effect. Furthermore, we cannot totally exclude the possibility that the decline in serum PSA was attributed to direct modulation of the epithelial compartment although no detectable modulations of cell death or androgen receptor expression were observed.

The preoperative high-risk model we used ensures that these findings are relevant to the lethal variety of prostate cancer and provide a potential molecular level explanation for the reported efficacy of thalidomide in metastatic disease (4, 5).

In conclusion, our observations suggest that thalidomide affects the tumor microenvironment in a manner that may transform the tumor phenotype to less invasive. The components of the microenvironment that seem to be modulated by thalidomide at the time of analysis include both angiogenesis and the broader stromal-epithelial cell interaction, as reflected by changes in Shh signaling and MMP/E-cadherin ratio. The observations we report suggest that targeting the microenvironment as a component of a rational cotargeting strategy may enhance the efficacy of more traditional epithelial targeting strategies in prostate cancer.

Acknowledgments

We thank Melissa Brown, Pauline Dieringer, Cynthia Carter, Erin Horne, Sherie Hodges, and other staff members from the University of Texas M. D. Anderson Cancer Center Genitourinary Medical Oncology Clinic for their excellent care of patients; Cindy Soto and Ina Prokhorov for expert technical assistance with pathologic specimens from the Department of Pathology, M. D. Anderson; Sabina Magedson and Ahn G. Hoang from the Department of Molecular Pathology, M. D. Anderson, for tissue microarray construction and immunohistochemical staining; Christine F. Wogan from the Department of Scientific Publications, M. D. Anderson, for editorial assistance; and Brooke J. Reynolds and Kim-Ahn Vu from the Department of Pathology Research, M. D. Anderson, for assistance in the preparation of illustrations and manuscript.

References


Initial Modulation of the Tumor Microenvironment Accounts for Thalidomide Activity in Prostate Cancer

Eleni Efstathiou, Patricia Troncoso, Sijin Wen, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/4/1224

Cited articles
This article cites 26 articles, 9 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/4/1224.full#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/13/4/1224.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.