Loss of Uteroglobin Expression in Prostate Cancer: Relationship to Advancing Grade

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ABSTRACT
We have shown previously that the secretory protein uteroglobin (UG) is highly expressed in normal human prostate tissue but this expression is either lost or altered in human prostate cancer cell lines. Treatment of these cell lines with recombinant human UG inhibits their ability to invade human reconstituted basement membrane by up to 90%, implying that the loss of normal UG expression may be related to the invasive potential of prostate cancer. Because invasion represents a critical step in metastasis, the expression patterns of UG could provide a unique and relevant indicator of cancer progression. In this study, we present the immunohistochemical analyses of fresh frozen prostate tissues from surgical specimens taken from 50 patients.

Eight slides per patient were analyzed for UG staining. Slides from 26 patients showed evidence of prostate cancer, whereas slides from the remaining 24 patients showed only benign glands. The results demonstrate UG immunoreactivity in normal prostate, benign prostatic hyperplasia, and prostatic atrophy; low but clearly positive expression in prostatic intraepithelial neoplasia. This study sought to determine the immunohistochemical expression of UG in normal human prostate, BPH, PIN, and PC tissue and to assess the possibility that alterations in UG expression may be associated with prostate cancer.

INTRODUCTION
Adenocarcinoma of the prostate is the most commonly diagnosed form of cancer among men in the United States today. The number of cases diagnosed in 1996 exceeded 260,000, and that figure is expected to climb to around 334,500 in 1997 (1). It is projected that about 41,800 of these diagnosed cancers will result in death due to metastatic progression. Genetic alterations such as the loss of heterozygosity at several loci, including 6p, 7q, 8q, 10q, 11p, 13q, 16q, 17q, and 18q (2–8), the loss of putative metastasis suppressor genes such as KAI-1 (9) and CD44 (10), as well as changes in cell adhesion mechanisms such as E-cadherin/\(\alpha\)-catenin (11), are thought to contribute to this progression. However, despite the recent strides made in this area of study, it is generally accepted that the molecular alterations related to the malignant development of PC require better characterization.

One such alteration may involve a protein known as UG. UG is a low molecular weight, homodimeric secretory protein, also known as Clara cell 10 kDa (12) protein, which is found in abundance in human uterus, lung, and prostate (13–15). The UG locus has been localized to a single-copy gene on chromosome 11q12.3–13.1 (16). This chromosomal region is of interest in several hormonally regulated cancers (17) and has recently been identified as an additional site of loss of heterozygosity in PC (18). We have reported previously that UG expression is markedly decreased or absent in Several PC cell lines and that recombinant human UG modulates prostate tumor cell invasiveness, possibly by a mechanism related to the inhibition of the actions of PLA\(_2\) in the production of eicosanoids (19, 20).

This study sought to determine the immunohistochemical expression of UG in normal human prostate, BPH, PIN, and PC tissue and to assess the possibility that alterations in UG expression may be associated with prostate cancer.

MATERIALS AND METHODS
Tissue Source. Informed consent was obtained to study tissue from 30 patients who underwent radical prostatectomy for PC, cystoprostatectomy for transitional cell carcinoma, or open prostatectomy for BPH. Because a random sampling of cancer patients was desired, there was no inclusion or exclusion criteria before acquisition of these tissues. It is noteworthy, however, that none of these patients had prior endocrine treatment. Approximately two longitudinal specimens, of 1-cm each, extending from base to apex were excised parallel to the urethra and flash frozen in liquid N\(_2\) and stored at \(-70^\circ\)C. Lymphatic tissue containing PC was obtained from one laparoscopic pelvic lymph node dissection prior to a planned radical prostatectomy. Of 46 specimens resected for prostatic carcinoma, 26 contained carci-
nomata within the tissue samples obtained intraoperatively for UG determination. Tissue from the remaining 24 specimens contained benign prostatic tissue, prostatic atrophy, or fibromuscular tissue. The 26 cases with sampled carcinoma in the intraoperative sample had a similar histopathological tumor pattern to that present in the resected prostate. All 46 prostatectomy cases with preoperative diagnosis of PC contained carcinoma in the final surgical pathological review of the resected organ.

Immunohistochemical Procedures. Two hundred serial sections of 5 μm each were taken from each tissue and placed on silanated slides (two sections per slide). Every tenth slide was stained with hematoxylin and diagnosed by a pathologist to determine which serial sections exhibited BPH, PIN, or cancer. This was to determine which slides contained both normal prostatic glands (as a positive control) as well as adjacent cancerous glands. The remaining slides were stored at −20°C until diagnosis was complete. Selected slides were immunostained as follows. Sections were fixed with 4% formaldehyde for 10 s and then blocked with a 1:100 dilution of mouse serum (SIGMA Immunostaining, St. Louis, MO) for 30 min at room temperature. Slides were then incubated overnight at 4°C with a 1:100 dilution of rabbit anti-human UG antibody, and corresponding serial sections used as a negative control were incubated with a 1:100 dilution of nonimmunized rabbit serum (BABCO Laboratories, Inc., Berkeley, CA).

The sections were washed three times for 10 min each in PBS and then incubated with a dilution of 1:1000 biotin-conjugated mouse anti-rabbit IgG (SIGMA Immunohistochemistry) for 30 min at room temperature. The sections were washed three times in PBS for 10 min, then incubated with streptavidin complex (DAKO, Carpinteria, CA) for 30 min, and washed again twice with PBS for 10 min. A 5-s immersion in chromogen (liquid DAB, DAKO) was followed by counter staining with hematoxylin.

Antibody Preparation. A peptide comprised of the 20 amino acid residues 37–56 of human UG was synthesized by Genemed (San Francisco, CA). This peptide was conjugated to keyhole limpet hemocyanin and used to raise antibody in rabbits (BERKLEY Antibody Company, Berkeley, CA). The specificity of this polyclonal serum to human UG was confirmed and titrated by ELISA and used in the immunostaining of prostate tissue specimens and performance of Western blot analysis.

Western Blot Analysis. Slices of tissue (~50 mg) adjacent to the areas of tissue used for immunohistochemistry were homogenized in protein lysis buffer [50 mM Tris Base, 350 mM NaCl, 0.1% volume NP40, 5 mM EDTA (pH 7.4), containing 2 mM PMSF, 20 μg/ml aprotinin, and 1 mM NaOVi] using an Ultratorrax homogenizer (Tekmar, Cincinnati, OH), three times for 30 s each. Cell debris was pelleted out by centrifugation at 8000 rpm for 20 min. The supernatant was then filtered through a 100-kDa molecular weight cut-off Centricon-100 spin filter (Amicon, Beverly, MA) by centrifugation for 1 h at 2300 rpm at 4°C. The lysates were then quantitated using a Bio-Rad protein assay (Bio-Rad, Hercules, CA), and 75 μg of the proteins below Mr 100,000 were heated for 3 min at 95°C in an equal volume of sample buffer (4% SDS, 100 mM Tris-HCl, 20% glycerol, 200 mM DTT, 0.2% w/v bromphenol blue, with 0.5% β-mercaptoethanol) and loaded on to a 15% denaturing SDS-polyacrylamide gel and subjected to electrophoresis for 40 min at 65 V. The proteins were transferred onto 0.2-μm polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) by electrotransf

RESULTS

Patient age in the 28 Caucasian and 22 African-American males ranged from 35 to 76 years with a median of 60.8 years. Forty-six patients had a preoperative histopathological diagnosis of prostate adenocarcinoma (26 Caucasians and 20 African-Americans), whereas 4 patients (2 Caucasians and 2 African-Americans) had no malignancy of the prostate and underwent surgery either for bladder carcinoma (1 Caucasian and 1 African-American) or for BPH (Table 1). These diagnoses were confirmed in histopathological examination of the surgical specimens. Of the 26 patients (15 Caucasians and 11 African-Americans) with carcinoma within the sections we examined, two had associated PIN. Two samples contained only fibromuscular tissue. The remaining 22 specimens tested contained only BPH or prostatic atrophy in the sample. However, the final surgical pathological diagnosis of these radical prostatectomies was adenocarcinoma with Gleason scores ranging from 4 to 10, with 16 patients determined to have moderately differentiated tumors (Gleason score from 4 to 6) and 30 patients with poorly differentiated tumors (Gleason score from 7 to 10). The latter included the patient with lymph node metastasis (Gleason 10).

A total of 400 slides from the 50 specimens was evaluated, and UG expression in each slide was scored by three investigators (A. T. W., J. A. C., and A. S.). The degree of UG expression was graded by assigning a value of 0 to an absence of stain and assigning a value of 3+ to the most positive stain. Slides that stain with intensity between these two extremes were accorded intermediate values. Glands showing an absence of stain were uniformly marked as 0 by all three investigators. Intermediate scores were usually identical and never differed by more than a single value. Two of three scorers were blinded as to the source of the tissue. Immunohistochemical stain was also evaluated and scored according to distribution as focal or diffuse. In cases of suspected minimal disease, tissue sections were obtained from sites adjacent to the localized disease and did not always contain tumor. Of the 50 patient samples examined, we found we were able to analyze slides with cancer from 26. Two of these latter slides contained only cancer, with no evidence of any normal glands, and one contained cancer and PIN with no evidence of any benign glands. Two samples we examined contained only fibromuscular tissue, which lacks staining for UG. Therefore, we found 45 samples that contained benign...
Table 1  Patient data

<table>
<thead>
<tr>
<th>Detail</th>
<th>n = 50</th>
<th>No. of patients</th>
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<tbody>
<tr>
<td>Age</td>
<td>Range 35–76 yr</td>
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</tr>
<tr>
<td></td>
<td>Median 60.8 yr</td>
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</tr>
<tr>
<td>Race</td>
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</tr>
<tr>
<td></td>
<td>Caucasian</td>
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</tr>
<tr>
<td>Final pathological diagnosis</td>
<td>BPH</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Bladder carcinoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>PIN</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>PC Gleason’s grade 5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>PC Gleason’s grade 6</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PC Gleason’s grade 7</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>PC Gleason’s grade 8</td>
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</tr>
<tr>
<td></td>
<td>PC Gleason’s grade 9</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>Slide diagnoses</td>
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<tr>
<td></td>
<td>Prostatic atrophy</td>
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</tr>
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<td></td>
<td>PC Gleason’s grade 2</td>
<td>6</td>
</tr>
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<td></td>
<td>Fibromuscular tissue only</td>
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</table>

* The two patients with bladder carcinoma had a final prostatic diagnosis of BPH. PIN was associated with PC in all five cases; thus n = PC + BPH = 50.

b Patterns of glands and cells in the field of view at the time of slide analysis. The numbers here represent n (patients) of a total of 50 in which these pathologies were observed.


c See footnote 4 in text.

tissue, of which 42 contained benign glandular hyperplasia and three had prostatic glandular atrophy. Because of the difficulty of both finding and diagnosing PIN on frozen sections, the number of specimens in which PIN was detected remained low.

UG expression in benign tissue was predominantly diffuse and moderate in most cases. In 42 cases of histologically confirmed BPH, there was one that stained 3+, 25 that stained 2+, and 19 that stained 1+ for UG. In three cases of prostatic atrophy, two stained 2+ and one stained 1+. All were clearly positive (1+ or greater). The staining pattern was cytoplasmic and tended to be more pronounced at the luminal surface of the acinar cells (Fig. 1, A and B). Immunoreactivity was absent in sections of the prostate that lacked epithelial elements, such as fibromuscular tissue (Fig. 1A). The two cases of PIN examined for the expression of UG were both clearly positive and were scored as 1+ (Table 2, Fig. 1C).

Evaluation of slides containing PC contrasted sharply with normal, BPH, or PIN samples. Twenty of 26 specimens containing cancer did not stain for UG. Each of these cancerous glands was diagnosed as Gleason’s pattern4 of 3 or above (Fig. 1, C and D). This absence of stain was consistent among all cancerous glands of this Gleason’s pattern. Five samples contained cancerous glands that were graded with a Gleason’s pattern 1 or 2, and these were positive for UG staining. One sample contained several glands of Gleason’s pattern 3/4 as well as glands that were Gleason’s pattern 2. The Gleason’s pattern 3/4 lacked staining for UG (Fig. 1E, left), whereas the Gleason’s pattern 2 and the normal glands within the sample stained positive for UG (Fig. 1E, right). The prostate tumor cells in the metastatic lymph node likewise registered no signal (Fig. 1F).

Western analysis was performed on prostate tissue from six of the patients in this study presenting with cancers of Gleason’s grade 8 or 9. The UG protein runs at around Mr, 10,000 on an SDS polyacrylamide gel (21). This protein was present in normal tissue lysates but absent in the lysates taken from cancerous tissue (Fig. 2).

DISCUSSION

We have previously reported our discovery of abundant expression of UG in the epithelial cells of the human prostate gland (15). In that preliminary description, there was no analysis of the comparative expression of uteroglobin in tissue sections exhibiting different pathological conditions. This study examines UG expression in fresh frozen cellular fields of BPH, PIN, and prostate cancer. We report that UG was absent from prostatic stromal tissue but was clearly expressed in normal glandular epithelial cells, in glandular areas exhibiting BPH and prostatic atrophy, in acinar glands identified as PIN, and in cancerous glands of Gleason’s pattern 2 or lower. In contrast, UG expression in glands of Gleason’s pattern 3 or higher was absent. In addition, in Western analysis of six patient samples of Gleason’s grade 8 or higher, the expression of the UG protein is undetectable as compared to normal tissue. This observation links the loss of UG expression with the pathological pattern of prostatic neoplasia.

The loss of normal UG expression in fresh frozen tissue samples of PC shown here corroborates our observations of human prostatic tumor cell lines derived from metastases (19, 20). The cell lines DU145, TSUpr1, and PC-3 were analyzed for UG mRNA expression by Northern blotting and compared with normal tissue. Normal prostatic cells expressed an abundant 600-bp mRNA transcript, whereas UG expression in the cell lines DU145 and PC-3 was absent. In TSUpr1 cells, an overexpressed but aberrantly processed (>10-kilobase pair) transcript was detected (20).

The absence of UG expression in prostatic tumor cells is also consistent with reports on the expression of the Clara Cell 10 kDa protein (CC10) gene, which is believed to be UG (21), in lung tissue. Broers et al. (14) and Linnoila et al. (22) showed that CC10 mRNA was abundantly expressed in normal bronchial tissue but was detectable in only 1 of 19 non-small cell lung carcinoma specimens. A large percentage of non-small cell lung carcinoma (primarily adenocarcinomas) exhibit ultrastructural and morphological characteristics indicative of peripheral airway cell origin (22). Because CC10 was a strong positive marker for normal peripheral airway cells, it is likely that the low percentage of CC10-positive tumors was due to the loss of CC10 expression during tumorigenesis. Sandmoller et al. (23) also supported this observation by fusing the 5’ flanking region

4 The Gleason’s number referred to here is that of the particular glands within the field of view at the time of slide diagnosis and refers to the pattern of the individual glands as opposed to the overall Gleason’s grade of the patient.
Fig. 1  Expression of UG in human prostate tissue. In A, UG expression in benign glandular hyperplasia is abundant and is found along the luminal surface of the acinar cells. Fibromuscular elements lack staining for UG (×40). In B, UG expression is also abundant in normal prostatic glands undergoing atrophy (×200). In C, UG expression is present in PIN (gland on the left), although it is somewhat diminished from BPH (center). In glands that are PC of Gleason’s pattern ≥3 (right), UG expression is absent (×100). D. contrast between positive UG staining in benign tissue (left) and the lack of UG staining in PC glands of Gleason’s pattern ≥3 (right, arrow; ×100). In E, in a single patient sample, regions of Gleason’s pattern ≤2 stained positive for UG (right), whereas glands on the same tissue section that were Gleason’s pattern ≥3 (left) were negative for UG expression (×200). In the patients studied, UG staining was consistently positive in sections of tissue that were of Gleason’s pattern ≤2 and consistently negative in those that were Gleason’s pattern ≥3. In F, UG expression is absent in prostate tumor cells that have metastasized to the regional lymph nodes (×100).

of the UG gene to SV40 T antigen to target SV-40-induced tumorigenesis to tissues that normally express abundant endogenous UG such as lung, salivary gland, stomach, and prostate. Endogenous production of UG in SV-40 T antigen-transformed cells was clearly detectable during the early stages of tumorigenesis but was undetectable in later stages. This suggests that the UG gene may be transcriptionally silenced as a tumor progresses past its earliest stages.

The significance of the loss of UG expression in tumor cells is presently unknown. During development and in the adult genitourinary tract, its expression is a function of hormone receptor expression and hormonal status. Therefore, it is possi-
Staining of the 50 patient samples for UG can be summarized as follows. Of 50 samples, 2 contained only fibromuscular tissue, which does not express UG, and therefore were excluded from this table. Two samples contained cancer (Gleason's pattern \( \geq 3 \)) but no benign tissue, and one more sample contained cancer (Gleason's pattern \( \geq 3 \)) and PIN but no benign tissue. Thus, the 23 remaining cancers (17 Gleason's pattern \( \geq 3 \) and 6 Gleason's pattern \( \geq 2 \)) form a subset of 45 samples that contain benign tissue. Cancers were divided into those of Gleason's pattern \( \geq 2 \), which show positivity for UG, and those of Gleason's pattern \( \geq 3 \), which lack staining for UG. Immunoreactivity is ranked as in the previous table, where absence of staining is marked as negative, or 0, low positivity is +, moderate positivity is ++, and strong positivity is +++.

<table>
<thead>
<tr>
<th>Staining for UG expression</th>
<th>Negative (0)</th>
<th>Low (+)</th>
<th>Moderate (++)</th>
<th>Strong (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>19</td>
<td>25</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PIN</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer (( \geq 2 ))</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer (( \geq 3 ))</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the existence of UG has been known for almost 30 years (24), its physiological function and mechanism of action remains unknown. We have found that recombinant UG inhibited fibroblast conditioned medium-stimulated tumor cell invasion but did not affect simple cell motility. Therefore, it is possible that the ability of UG to interfere with cell motility may be cell type specific or may itself be altered as a function of tumorigenicity. It has also been shown that UG noncompetitively inhibits the activity of PLA\(_2\), in vitro (25, 26), which may account for the potential anti-inflammatory activity of UG, but it is not known whether this inhibition occurs in vivo. We have shown that treatment of prostate tumor cell lines with recombinant UG inhibits the biphasic release of arachidonic acid that was also induced by fibroblast conditioned medium (19). This may be due to inhibition of cell-membrane associated PLA\(_2\) activity, but presently the relationship between the inhibition of arachidonic acid release and the inhibition of invasion is merely correlative. It remains to be seen whether either intracellular or extracellular metabolites of arachidonic acid are the proximate activators of invasive motility in these cells, or whether, alternatively, UG acts through a specific receptor and signaling mechanism to regulate genes governing invasive motility. An alternate mechanism is suggested by the observation by Zhang et al. (27) that UG binds to fibronectin. Thus, it is possible that UG binding to fibronectin may also modulate the role of fibronectin in cell-matrix attachment and invasive motility.

With an aging population, PC will continue to have an impact on health care well into the next century. Over 50% of patients undergoing radical prostatectomy have locally advanced disease, and 25% of these will eventually exhibit disease progression. Clearly, there is a need to develop prognostic markers that will help predict individual outcome and thereby direct appropriate therapy. If UG proves to be an autocrine/paracrine inhibitor of localized cellular invasiveness, then the degree of loss of UG expression in biopsy specimens may be predictive of increased potential for metastasis. In contrast, positive expression of UG, such as we observed in PIN, may indicate an earlier stage in the continuum from neoplastic change to wide dissemination. Differential UG expression by PIN may distinguish those cases with a predilection for invasion from those with a less aggressive course. This hypothesis is presently being tested by expanding this study to include archived specimens, where PIN can be readily selected from among paraffin sections used for pathological diagnosis.

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