Zinc α-2-Glycoprotein Is Expressed by Malignant Prostatic Epithelium and May Serve as a Potential Serum Marker for Prostate Cancer

Laura P. Hale, David T. Price, Luis M. Sanchez, Wendy Demark-Wahnefried, and John F. Madden

ABSTRACT

Zinc α-2-glycoprotein (ZAG) is a M, 41,000 glycoprotein secreted by a variety of normal epithelia. ZAG was recently shown to stimulate lipolysis in adipocytes, leading to the development of cachexia in animals with ZAG-producing tumors. To understand the possible contribution of ZAG to the development of cachexia in men with prostate cancer, ZAG production by normal and malignant prostate tissue was investigated using immunohistochemical assays. Anti-ZAG monoclonal antibodies reacted strongly with normal prostate epithelium but not with other components of prostate or seminal vesicles. The majority of prostate cancers tested (35 of 48; 73%) also reacted with anti-ZAG antibodies. High-grade tumors expressed significantly less ZAG than moderate-grade tumors (mean ZAG score 1.1 versus 1.9; P < 0.01). Men with ZAG-producing prostate carcinomas had elevated levels of serum ZAG relative to their normal age- and race-matched controls (1). It was found that a lipid-mobilizing factor isolated from the urine of human cancer patients with cachexia was identical to ZAG. Murine and human ZAG have an overall amino acid sequence identity of only 59% (7), but share up to 100% identity in specific regions hypothesized to be important in lipid metabolism (8). Thus, both human and murine ZAG stimulate lipolysis in both human and murine adipocytes, resulting in glycerol release and increased lipid utilization (6). Todorov et al. (9) quantitated ZAG production in vitro and cachexia induction in vivo using a panel of murine tumors including the MAC16 colon adenocarcinoma, M5 reticulum cell sarcoma, and B16 melanoma. The MAC16 tumor produced large quantities of ZAG and induced profound cachexia. The M5 tumor did not produce ZAG and failed to induce cachexia in vivo. The B16 tumor produced ~20% of the ZAG produced by MAC16 tumors and caused significant loss of carcass lipid, although profound cachexia had not occurred by 8 days after tumor implantation. Tumor-produced ZAG may thus contribute to the development of cancer cachexia. Whether ZAG has additional biological activities in addition to cachexia induction is currently unknown.

INTRODUCTION

ZAG is a secreted M, 41,000 protein first identified in human plasma in 1961 (1). It is named for its tendency to precipitate with zinc salts and for its electrophoretic mobility that is similar to plasma α2 globulins. Immunohistochemical studies have previously demonstrated immunoreactive ZAG protein within the cytoplasm of normal secretory epithelial cells, including those in breast, prostate, and liver, as well as in salivary, bronchial, gastrointestinal, and sweat glands (2). ZAG mRNA is expressed in a similar distribution, with placenta, ovary, and thyroid reportedly negative for ZAG mRNA (3). Consistent with its production by secretory epithelial cells, ZAG protein has been identified in most body fluids. The concentration of ZAG in normal human plasma or serum has been variously reported as between 25 and 140 μg/ml in different populations using various analytical techniques and may increase with age (4, 5).

The function of ZAG was unclear until recently, when Hirai et al. (6) found that a lipid-mobilizing factor isolated from the urine of human cancer patients with cachexia was identical to ZAG. Murine and human ZAG have an overall amino acid sequence identity of only 59% (7), but share up to 100% identity in specific regions hypothesized to be important in lipid metabolism (8). Thus, both human and murine ZAG stimulate lipolysis in both human and murine adipocytes, resulting in glycerol release and increased lipid utilization (6). Todorov et al. (9) quantitated ZAG production in vitro and cachexia induction in vivo using a panel of murine tumors including the MAC16 colon adenocarcinoma, M5 reticulum cell sarcoma, and B16 melanoma. The MAC16 tumor produced large quantities of ZAG and induced profound cachexia. The M5 tumor did not produce ZAG and failed to induce cachexia in vivo. The B16 tumor produced ~20% of the ZAG produced by MAC16 tumors and caused significant loss of carcass lipid, although profound cachexia had not occurred by 8 days after tumor implantation. Tumor-produced ZAG may thus contribute to the development of cancer cachexia. Whether ZAG has additional biological activities in addition to cachexia induction is currently unknown.

ZAG accumulates in breast cyst fluids to 30- to 50-fold plasma concentration (10, 11) and is overexpressed in 40–50% of breast carcinomas (10, 12, 13). Serial analysis of gene expression (SAGE) and microarray analysis have confirmed the relative overexpression of ZAG in breast cancer relative to normal mammary epithelium (14). In breast carcinomas, ZAG...
expression was found to correlate with tumor differentiation and did not independently affect prognosis (13). ZAG has been reported to be present in normal prostate tissue (2) and also to constitute 30% of the protein present in seminal fluid (4).

In this study, the production of ZAG protein by normal and malignant human prostate tissues was examined. The levels of ZAG present in the serum of men with ZAG-producing prostate carcinomas were compared with those of normal age- and race-matched controls. To address the potential contributions of ZAG-producing tumors to serum ZAG concentrations, human ZAG levels were measured in sera from mice bearing syngeneic tumors engineered to produce human ZAG and in nude rats bearing orthotopic human prostate tumors. Taken together, these studies show that ZAG production by prostate cancer can lead to systemically elevated ZAG levels, which may be useful diagnostically. Because the level of ZAG in the serum of men with prostate cancer may potentially reach levels shown to induce cachexia in animal studies (6) when the tumor burdens become large, the effects of tumor ZAG production on cachexia-associated complications of advanced prostate cancer deserve additional investigation.

MATERIALS AND METHODS

Tissue and Serum Samples. Normal and malignant prostate tissues were identified through a computerized search of the surgical pathology archives of the Department of Pathology at the Duke University Medical Center (Durham, NC) and used as FFPE sections according to an Institutional Review Board-approved protocol for the use of archival tissues. To eliminate potential selection bias, all prostatectomy specimens obtained during a 3-month period in 1999 that had sufficient tumor available for examination were used in this study. This yielded 16 specimens with a combined Gleason sum of 5–6 (moderate grade), 13 specimens with a combined Gleason sum of 7 (borderline high grade), and 3 specimens with a combined Gleason sum of 8–9 (high grade). To obtain additional numbers of high-grade tumors for evaluation, all prostatectomy specimens with Gleason sums of 8–9 obtained in 1999 were added to the study (total, n = 19). Blocks that contained tumor as well as residual benign prostatic epithelium were selected for study. Nine additional cases of prostate tissue obtained by transurethral resection of the prostate with no evidence of malignancy were studied as controls. Clinical characteristics of patients from whom samples were obtained are summarized in Table 1. Matched frozen and FFPE samples of normal and prostate cancer tissues obtained anonymously as discarded tissue also were used as controls to verify appropriate antigen retrieval and to optimize immunohistochemical staining.

Table 1 Clinical characteristics of patients in case series for ZAG immunohistochemical staining

<table>
<thead>
<tr>
<th>Tumor grade</th>
<th>Age (yr)</th>
<th>Average Gleason sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>No tumor (n = 9)</td>
<td>71 ± 8 (61–84)</td>
<td>NA</td>
</tr>
<tr>
<td>Moderate (n = 16)</td>
<td>59 ± 6 (49–70)</td>
<td>5.6</td>
</tr>
<tr>
<td>Borderline high (n = 13)</td>
<td>65 ± 8 (49–78)</td>
<td>7.0</td>
</tr>
<tr>
<td>High (n = 19)</td>
<td>67 ± 8 (56–83)</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*Average ± SD (range).

a NA, not applicable.

Serum samples obtained as part of a previous hospital-based prostate cancer case-control investigation aimed at determining anthropometric and hormonal risk factors (Duke Institutional Review Board no. 668-93-5R2) were used to explore ZAG expression in both malignant prostatic tissue and sera. Methods for this study have been reported elsewhere (15, 16). In brief, both cases and controls for this study were weight-stable (<5% change in body weight within 1 year of study recruitment), had no current or past use of hormonal agents, no history of other cancers (with the exception of non-melanoma skin cancer), and were 50–70 years of age. Cases were ascertained within 3 months of diagnosis with early stage disease. Eligibility criteria for control patients required normal PSA values and negative digital rectal exams. Sera from this study had been stored at −70°C, and only aliquots from cases that were accrued before treatment were accessioned for the current study. Additionally, subjects who subsequently developed cancer (other than non-melanoma skin cancer) within 3 years of original participation were excluded from the current study. Selected serum aliquots were anonymized, coded, and analyzed for ZAG in a blinded fashion, with two race- and age-matched controls (n = 28) selected for every case (n = 14; see Table 2). FFPE tumor samples from each case patient were retrieved from the Duke Surgical Pathology Archives and assayed for ZAG by immunohistochemistry. Tumors with detectable ZAG immunoactivity (a score of ≥1) were scored as ZAG-positive.

Immunohistochemical Studies. Immunohistochemical assays were optimized using matched samples of frozen and FFPE tissues to ensure that appropriate immunoreactivity was retained in FFPE tissues. Four-μm FFPE sections were stained using standard protocols, including the blocking of endogenous peroxidase activity (0.6% H2O2 in absolute methanol; 15 min), antigen retrieval with microwave citrate [10 mM sodium citrate (pH 6.0); 2 × 5 min; 600 W], and blocking with 10% horse serum in PBS. The slides were then sequentially incubated at 37°C with primary anti-ZAG monoclonal antibody 1H4 (11), biotinylated secondary antibody, and avidin-biotin-horseradish peroxidase complexes (VectaStainABC; Vector Laboratories, Burlingame, CA) with intervening PBS washes. Bound antibody was detected with 3, 3’-diaminobenzidine plus H2O2. The immunoreactivity of FFPE sections using this protocol was identical to that of frozen tissue, except that nuclear staining was occasionally seen focally in some FFPE tissues. As nuclear staining was never observed in frozen tissues or FFPE tissues treated with enzyme-based antigen retrieval, this occasional

Table 2 Clinical characteristics of patients in case-control series for serum ZAG measurements

<table>
<thead>
<tr>
<th>Race</th>
<th>Age</th>
<th>Body mass index</th>
<th>Average Gleason sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 white</td>
<td>64 ± 6 (52–70)</td>
<td>26.6 ± 3.1</td>
<td>6.3</td>
</tr>
<tr>
<td>1 black</td>
<td>(n = 14)</td>
<td>(20.7–33)</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>26 white</td>
<td>64 ± 5 (51–70)</td>
<td>27.8 ± 3.2</td>
</tr>
<tr>
<td>2 black</td>
<td>(n = 28)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average ± SD (range).

a NA, not applicable.
focal nuclear staining was clearly a fixation-dependent artifact of the microwave antigen retrieval process, and was ignored. The staining protocol was optimized such that serial sections stained with an equivalent concentration of isotype-matched control antibody showed a total lack of color development. The immunohistochemical reactivity of tumors was rated independently by two board-certified pathologists according to the following scale: 0, absence of reactivity in >50% of tumor cells; 1, faint but clearly detectable reactivity in >50% of tumor cells; 2, moderate reactivity in >50% of tumor cells; and 3, strong reactivity in >50% of tumor cells. The staining intensity of residual non-apocrine prostate epithelium in each section was assigned a score of 2 to allow normalization. Given that Gleason scores could be assessed at the time the ZAG-immunostained slides were reviewed, true blinding was not possible; however, the Gleason sum derived from examination of all slides obtained for each case was not available to the observers at the time the ZAG-immunostained sections were evaluated.

**Measurement of Serum ZAG.** Serum ZAG levels were determined by an antigen capture enzyme immunoassay, using anti-ZAG mAb IB5 (11) as the capture antibody. Bound ZAG was detected using biotinylated anti-ZAG mAb 1H4, streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch Labs, West Grove, PA), and 3,3',5,5'-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Standard curves were constructed using recombinant human ZAG, quantified by \( A_{280} \) of HPLC purified ZAG (1). Each serum sample was analyzed in quadruplicate for at least 2 independent dilutions and results were averaged. The sensitivity of the assay was 10 pg/ml.

**Generation of ZAG-producing Murine Cell Lines.** A full-length human ZAG cDNA including the endogenous secretory signal sequence was cloned from human liver using reverse transcription-PCR. The primers used corresponded to bp 3–21 and bp 938–920 (GenBank D90427). The construct sequence was verified by automated DNA sequencing using the Duke University Cancer Center DNA Sequencing Shared Resource and then inserted into the pCDNA3.1(--) Myc-His eukaryotic expression vector (Invitrogen) using restriction enzyme digestion and adapter ligation to ensure in-frame insertion relative to the myc and 6-His 3' epitope tags. Epitope-tagged human ZAG constructs were transfected into B16 murine melanoma cells and stable transfectants were obtained by G418 selection and then cloned by limited dilution. Selected clones expressed high levels of epitope-tagged human ZAG with the predicted molecular weight of \( M,46,000 \), as verified by antigen-capture ELISA and Western blot of the culture supernatant.

**Animal Studies.** 2 × 10^5 ZAG or vector-transfected B16 tumor cells were implanted s.c. in the flank in groups of five syngeneic female C57BL/6 mice. Serum was obtained and mice were weighed at 21 days, just before tumor-related death. The concentration of tumor-produced human ZAG in the serum was measured by antigen capture ELISA as described above. To address whether tumor-produced ZAG could be detected in the serum when tumors were grown orthotopically within the prostate, CWR22 androgen-dependent human prostate cancer cells suspended in Matrigel (Collaborative Research, Bedford, MA) at a concentration of 5 × 10^6 cells/100 \( \mu \)l were injected orthotopically into the ventral prostate of male nude rats 6 weeks of age (\( n = 9 \)). This orthotopic nude rat model facilitated accurate implantation and growth of a xenogeneic human ZAG-expressing tumor directly within the prostate. Sixty days after surgical implantation, animals were euthanized, and both serum and tumor were harvested and analyzed for expression of human ZAG. All animal experimentation was conducted under an Institutional Animal Care and Use Committee-approved protocol.

**Statistical Analysis.** To test the association between the ZAG score of immunostained prostate cancer samples and tumor grade, the Mantel-Haenszel correlation statistic with rank scores assigned to both variables was used. The association is described by giving means on ZAG scores by grade. To test whether the mean serum ZAG concentration of controls was different from that of cases, a difference score equal to the natural log of the serum ZAG concentration of the prostate cancer case minus the natural log of the serum ZAG concentration of the control was calculated and tested to determine whether the mean of the difference scores was equal to zero for each case/control match. Natural logs were used to successfully approximate normality. Repeated measures analysis using the MIXED procedure using SAS software (SAS Institute Inc., Cary, NC) was used to calculate the test statistic, because this procedure allowed the two difference scores for each case to serve as a correlated “cluster.” These matched data also were tested to determine whether the prostate cancer cases had higher serum ZAG concentrations significantly more often than their matched controls. This test was calculated using repeated measures logistic regression by the GENMOD procedure in SAS to account for the fact each case was matched to two controls.

**RESULTS**

**ZAG Is Expressed by Benign Prostate Epithelium, but not by Seminal Vesicles.** Normal benign epithelium was moderately to strongly reactive with anti-ZAG antibody in 9 of 9 normal human prostates tested. In addition, normal prostate acini present on sections that also contained prostate cancer were similarly moderately to strongly reactive with anti-ZAG antibody (48 of 48 cases; Fig. 1A). The immunohistochemical reactivity of the normal non-apocrine prostate epithelium in each section with anti-ZAG antibody was given a score of 2 to facilitate semiquantitative comparison of ZAG expression between different prostate cancers (see below). The overall immunoreactivity of normal prostate epithelium correlated with secretory activity and was highest in dilated apocrine-type glands containing copious luminal secretions (Fig. 1A, top). These highly reactive glands were assigned an immunohistochemical reactivity score of 3. The concretions present in normal acini also were highly reactive with ZAG mAb (Fig. 1B), indicating that ZAG protein is a prominent constituent of these concretions.

Because the concentration of ZAG in seminal fluid has been reported previously to be high (4), we sought to determine the cellular source of seminal fluid ZAG by immunohistochemical comparison of prostate and seminal vesicle tissues. We found no evidence of ZAG immunoreactivity in any of the 11 seminal vesicles studied (data not shown). The prostatic duct also was nonreactive with anti-ZAG antibody (data not shown). The high levels of ZAG immunoreactivity seen in normal prostate taken together with the total absence of ZAG immunoreac
Activity in seminal vesicle and associated ducts demonstrates that the ZAG described previously as present in seminal fluid (4) must be produced by the epithelium of the prostate itself.

Prostate Carcinomas React with ZAG mAb. Thirty-five of 48 (73%) prostate cancers studied were reactive with anti-ZAG mAb (Table 3). The pattern of ZAG immunoreactivity in positive tumors varied from global cytoplasmic staining (Fig. 1C) to strong staining only on the luminal surface (Fig. 1D). In some tumors, there were local variations in the intensity of ZAG immunoreactivity, but usually with clear boundaries that suggested discrete tumor subpopulations. For example, one well-defined tumor nodule might be strongly positive with an adjacent tumor nodule only weakly positive (Fig. 1E) or even negative. As shown in Table 3, the intensity of immunostaining...
with anti-ZAG antibody also varied among tumors with similar Gleason scores. However, high-grade tumors were significantly more likely to be ZAG-negative or to have decreased ZAG immunostaining relative to moderate-grade tumors. The Mantel-Haenszel test of the association between ZAG and tumor grade gave a \( P \) of 0.01 for a mean ZAG score of 1.1 for high grade (Gleason sum, 8–9) versus 1.7 for borderline high (Gleason sum, 7) versus 1.9 for moderate grade (Gleason sum, 5–6) tumors.

Prostate tissues in which tumor cells demonstrated strong ZAG immunoreactivity also showed increased immunostaining of tumor-associated and benign stroma (Fig. 1C). These regions did not show increased background staining with isotype-matched control antibody. Therefore, increased immunostaining most likely represents detection of tumor-produced ZAG that has “spilled out” into the adjacent stroma. Unlike normal prostatic concretions (Fig. 1B), malignant crystalloids were nonreactive with ZAG mAb (data not shown).

**Serum ZAG Levels Increase in Patients with ZAG-positive Prostate Cancers.** To determine whether ZAG production in tumors was associated with an increased serum concentration of ZAG, serum ZAG concentrations were analyzed in a cohort of patients with documented prostate cancer (\( n = 14 \)) and age- and race-matched controls (\( n = 28 \)) using an antigen capture immunoassay. Eleven of 14 cancer patients had ZAG-positive tumors (ZAG score of \( \geq 1 \)) by immunohistochemistry. Two of three tumors with ZAG scores of 0 had small foci with faint ZAG staining but did not meet the 50% area requirement for ZAG positivity. Thus, 13 of 14 patients with prostate cancer had at least some ZAG production by cancer cells. Serum ZAG concentrations obtained for both patients and controls are shown in Fig. 2. The test of a mean difference in serum ZAG concentration between cases and controls gave a \( P \) of 0.10. The test of whether prostate cancer cases had higher serum ZAG concentrations significantly more often than the controls to which they were matched gave a \( P \) of 0.02; see text).

![Fig. 2](image)

**Serum ZAG levels are increased in patients with prostate cancer.** Serum ZAG was measured by antigen capture enzyme immunoassay as described in “Materials and Methods.” Prostate cancer patients had higher serum ZAG concentrations significantly more often than did the controls to which they were matched (\( P = 0.02 \); see text).

**Tumor-produced ZAG Contributes to Serum ZAG Levels in Murine Models.** To definitively test the hypothesis that tumor-produced ZAG contributes to an elevated concentration of circulating ZAG, it was necessary to generate a model system in which tumor-produced ZAG could be differentiated from ZAG produced by normal secretory epithelia. We therefore produced murine tumor cell lines expressing epitope-tagged recombinant human ZAG that could be specifically identified and distinguished from endogenous murine ZAG produced by normal secretory epithelium. To do this, we used antibodies that recognize either human ZAG or the epitope tag, but do not cross-react with murine ZAG. Human ZAG could be detected in the serum of mice bearing hZAG-transfected B16 tumors (\( 156 \pm 70 \) ng/ml; \( n = 5 \)), but not in the serum of mice bearing vector-transfected B16 tumors (\( n = 5 \)), when the tumor was implanted in a s.c. location. This level of increased ZAG production was sufficient to cause a mean weight loss of 15% in the group bearing hZAG-transfected tumors (ending weights: B16-vector, 20.6 ± 1.1 g; B16-ZAG 17.5 ± 0.8 g; \( P = 0.001 \)).

To show that human prostate carcinomas growing orthotopically within the prostate could similarly contribute to elevated serum ZAG levels, we implanted the ZAG-producing...
CWR22 human prostate carcinoma directly into the prostate of nude rats. As in the murine model described above, tumor-produced hZAG is readily distinguished from endogenous rat ZAG in this model using antibodies specific for hZAG that do not cross-react with rat ZAG. Rats with intraprostatic CWR22 tumors had 59 ± 24 ng/ml hZAG present in their serum (mean ± SD; n = 7), whereas two rats in whom tumors were implanted but failed to grow had undetectable serum levels of hZAG.

**DISCUSSION**

The results presented here confirm the previous observation that ZAG is produced by normal prostate epithelium. We extended this work further to show that the prostate is the major source of ZAG in seminal fluid, inasmuch as both seminal vesicle and prostatic ducts are negative for ZAG production by immunohistochemistry. In addition, we show that ZAG is produced by the majority (73%) of prostate cancers. ZAG production is associated with tumor differentiation status with decreased or absent ZAG production in more poorly differentiated (high Gleason grade) tumors. These results are similar to those seen in breast cancers, where the loss of ZAG production also was associated with lack of tumor differentiation (13). Our results differ from the 41–49% of prostatic adenocarcinomas reported previously to be ZAG-positive (Ref. 17; reviewed in Ref. 18). It is likely that differences in case selection criteria leading to inclusion of more poorly differentiated ZAG-negative tumors in the previous study contributed to these differences, because their requirement that at least 10% of the tumor consist of a non-gland-forming component (17) would bias selection toward cases with Gleason grade 4 or above. An increased sensitivity of our immunohistochemical assay relative to the previous study may also contribute to differences between these studies. Indeed, if the tumors with a ZAG score of 1 in our study are counted as negative, our overall percentages of ZAG-positive and ZAG-negative tumors agree very closely with those reported previously by Gagnon et al. (17).

Our studies show that, similar to what has previously been observed in PSA-secreting prostate cancers, tumor-produced ZAG contributes to an elevated serum ZAG level in mice bearing s.c. tumors and in both nude rats and humans with intraprostatic ZAG-secreting tumors. However, it also is possible that ZAG production by tumor-activated normal secretory epithelial cells within the prostate or elsewhere in the body may contribute to serum ZAG levels in prostate cancer patients. Determination of the relative contributions of tumor versus benign epithelia to the overall serum ZAG concentration in rodent models of prostate cancer must await the development of reagents that can specifically recognize rodent ZAG. It is likely that elevated serum ZAG also can result from pathological conditions other than prostate cancer, inasmuch as some of the human controls also had elevated serum ZAG that could not be related to the presence of prostate cancer (Fig. 2). The source of this increased serum ZAG is unknown.

The specificity of an elevated serum ZAG concentration as a marker for patients with prostate cancer is of considerable concern. However, many of the issues involved are not unique for ZAG, but have been addressed for PSA, a protein also produced by normal prostate epithelium as well as by many prostate cancers. Despite a lack of specificity of PSA for prostate cancer per se, serum PSA measurements have been proven useful to identify men at high risk for prostate cancer for whom additional diagnostic studies are warranted (19). Although it is possible that elevated serum ZAG levels may be observed in patients with benign prostatic hypertrophy alone, because the dilated normal glands common in benign prostatic hypertrophy are highly ZAG-immunoreactive, the normal dilated prostatic glands that are strongly immunohistochemically reactive with anti-ZAG mAbs retain their connectivity to the ejaculatory pathway unless they are disrupted by prostate cancer infiltration. Thus, the ZAG produced by these normal glands would not be expected to be available to the serum unless cancer is present. In contrast, many of the disorganized malignant glands do not connect to the ejaculatory system, and thus the ZAG produced by these glands may spill out into the stroma where it can be picked up by the lymph and contribute to systemic ZAG levels. Our observation that the stroma surrounding highly ZAG-immunoreactive normal glands in prostate typically fails to stain strongly with ZAG mAbs (Fig. 1A and 1D) compared with the moderate to strong anti-ZAG staining of stroma surrounding ZAG-positive cancers (Fig. 1C and data not shown) supports this interpretation. Other changes in the blood-prostate barrier attributable to the presence of malignancy also could contribute to the increased leakage of prostate-derived proteins, such as ZAG and PSA, into the serum. In addition, the absolute amount of ZAG secreted by tumors depends on both the rate of production by individual tumor cells and the overall tumor volume. Immunohistochemically strongly ZAG-positive tumors may contribute little to overall serum ZAG concentration if the overall tumor burden is low. It is clear that a determination of whether serum ZAG measurements will have sufficient specificity for clinical use as a serum marker of prostate cancer will require additional large-scale screening studies with long-term follow-up and must include sufficient numbers of patients with abnormal, but benign, prostate conditions.

The serum ZAG concentration that we measured in normal men 51 to 70 years of age is higher than the 25–140 μg/ml reported previously using different analytical techniques and other patient populations (4, 5). Approximately 25 mg of ZAG can be recovered per liter of serum using an affinity column made with anti-ZAG mAb 3C5, which appears to extract most of the ZAG (data not shown); however, the efficiency of ZAG elution was not tested. The higher absolute values that we measured with our antigen capture ELISA assay could occur because of differences in the affinity of the anti-ZAG mAbs used in our ELISA assay for recombinant human ZAG versus the human serum ZAG used in the studies reported previously as well as due to errors in absolute quantitation of the recombinant human ZAG standard used or to increases in serum ZAG concentrations in older individuals. Comparison of our recombinant human ZAG preparation versus ZAG purified from human serum as described previously (11) showed that color development in our ELISA assay was ~5% less for recombinant versus serum-derived human ZAG (data not shown). This suggests that
differences in mAb affinity may account for some of the increase in ZAG that we observed in our patient serum samples. Differences in the amount of full-length ZAG present in these two standard types may also contribute to different measured values for serum ZAG, because the antigen capture format requires both epitopes to be present for signal generation. The relative locations of the epitopes for the anti-ZAG mAbs 1B5 and 1H4 used in the antigen capture ELISA assay are currently unknown. Western blot of the recombinant human ZAG preparation used in this study showed a single broad band (data not shown) that may mask minor degradation. Errors in quantitation of the standards used would provide different absolute values for ZAG concentrations measured in patient serum, but any differences in ZAG concentrations between control and prostate cancer populations would still be valid. These studies highlight the importance of establishing normal ranges for each type of assay and standard preparation used. Although these technical considerations may potentially have influenced the absolute value of our ZAG measurements slightly, we feel that it is also likely that serum ZAG concentration increases with age. The studies published previously showed an increase in mean ZAG concentration from fetal to postnatal life (5). mRNA for ZAG has been reported to be induced by corticosteroids (20), and age-related changes in the homeostatic control of the hypothalamic-pituitary-adrenal axis result in increased plasma corticosteroid levels in older adults (21, 22). However, determining the contribution of age-related increases in corticosteroid concentration to our higher measured ZAG concentrations will require additional study.

Our studies definitively demonstrate that ZAG production by tumors can contribute to the elevation of systemic ZAG concentrations and result in marked weight loss in mice. Hirai et al. (6) found that induction of a lipolytic response leading to cachexia in mice required the administration of sufficient purified hZAG to raise the serum ZAG concentration by ~50% (e.g., an elevation of ~50 μg/ml). Despite the small elevation of total serum ZAG (~150 ng/ml) that we measured at the time our mice bearing hZAG-transfected tumors were killed, weight loss in these mice was evident within 1 week of tumor implantation and was sustained throughout the 3 weeks of tumor growth. The lower-than-expected serum levels of hZAG 3 weeks after tumor implantation may reflect down-regulation of the cytomegalovirus immediate early promoter used in our ZAG construct with time, a well-documented occurrence in murine in vivo models (23). Testing the possibility that tumors also may induce secretion of endogenous ZAG by normal prostatic or other secretory epithelia, thus affecting total (tumor-produced + endogenous) serum ZAG, will await the development of reagents recognizing rodent ZAG.

Taken together, our human and animal studies suggest that ZAG is a potential serum marker of prostate cancer that may be elevated early in tumor development. Whether measurement of ZAG in addition to free or conjugated PSA may allow increased sensitivity and discrimination of patients with prostate cancer versus benign prostate conditions in screening studies remains to be studied. Also unexplored are potential associations between ZAG levels and the development of clinically significant prostate cancer. It is known however that the levels of circulating ZAG are a function of both tumor ZAG production and tumor burden. ZAG also has been reported to be induced by androgens (20), but no information is available as to how androgen deprivation treatments for prostate cancer affect ZAG levels. However, serum ZAG is sufficiently elevated in a subset of men with prostate cancer to have the potential for physiological effects. Given that men with advanced prostate cancer often develop cachexia, there is a need to explore whether ZAG expression may portend risk and progressive disease, or whether ZAG secretion and/or functional effects can be modified with the potential to prevent cachexia-associated complications in patients with advanced prostate cancer.

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