Recombinant Human Uteroglobin Inhibits the in Vitro Invasiveness of Human Metastatic Prostate Tumor Cells and the Release of Arachidonic Acid Stimulated by Fibroblast-conditioned Medium

J. Leyton, M. J. Manyak, A. B. Mukherjee, L. Miele, G. Mantile, and S. R. Patierno

Departments of Pharmacology [J. L., S. R. P.] and Urology [M. J. M.], The George Washington University Medical Center, Washington, DC 20037, and Section on Developmental Genetics, Human Genetics Branch, National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892 [A. B. M., L. M., G. M.]

Abstract

Uteroglobin (UG) is a potent immunomodulatory and antiinflammatory secretary protein with high levels detected in human prostate tissue. We used three human prostate cancer cell lines (DU-145, PC3-M, and LNCaP) to test the hypothesis that UG may modulate invasiveness of prostatic carcinoma cells in the Boyden chamber assay for invasion through a reconstituted basement membrane preparation. Fibroblast-conditioned medium was used as the chemotactant. The most invasive cell line was DU-145, followed by PC3-M, whereas the androgen-dependent LNCaP cell line exhibited extremely low invasive potential. Pretreatment of DU-145 and PC3-M cells for 24 h with 0.01, 0.1, or 1.0 μM recombinant UG had no effect on basal invasiveness but inhibited fibroblast-conditioned medium-stimulated invasion in a dose-dependent manner, reaching up to 60.2 and 87.9% inhibition of DU-145 and PC3-M, respectively. UG had no effect on either cell-reconstituted basement membrane adhesion or simple chemotaxis in the absence of reconstituted basement membrane. UG also strongly inhibited the biphasic release of [14C]-labeled arachidonic acid from fibroblast-conditioned medium-stimulated DU-145 cells. These results suggest that UG may modulate prostate tumor cell invasiveness and that the mechanism may include inhibition of the arachidonic acid signal cascade.

Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths in American men with approximately 200,000 new cases expected to be diagnosed in 1994 (1). Although prostate cancer metastasis to regional lymph nodes occasionally follows an indolent course, within 5 years, 85% of patients with stage D1 disease progress, and 80% of patients with stage D2 disease die (2). Invasion is a prerequisite for migration of tumor cells in connective tissue stroma (3), and basement membranes form the major physical barriers to the migration process (4, 5). Tumor cell invasion can be measured in vitro (6) using recently developed assays that use RBM4 components to reproduce a barrier to invasion.

Signaling pathways that initiate tumor cell migration are among the least understood aspects of invasion and metastasis but are believed to result from specific lipid-receptor interactions. PLA2 is a key membrane signaling enzyme that modulates the level of available arachidonic acid, the substrate required for the production of eicosanoids (e.g., prostaglandins, leukotrienes, and thromboxanes). These proinflammatory mediators have been implicated as initiators of metastasis in primary neoplastic tissue (7). Inhibition of PLA2 has been suggested as a novel means to control chronic inflammation associated with tumor progression (8).

UG is a potent PLA2 inhibitor (9). Its synthesis and secretion are regulated by several steroids but is genetically distinct from the lipocortins. UG is a Mr 15,800, homodimeric secretory protein discovered in the gravid rabbit uterus and in several extrauterine tissues (10). UG is thought to play an immunomodulatory/antiinflammatory role in protection of the wet epithelia of organs that communicate with the external environment (10, 11). This protein has potent antichemotactic effects on neutrophils and monocytes (10). The human counterpart to rabbit UG was first found in nonciliated Clara cells in the distal bronchiolcelmway (12) and was originally designated CC10. The complementary DNA for human CC10 has been cloned and sequenced (13), and UG-like protein has been detected immunohistochemically in the human uterus (14), respiratory tract (15), and prostate gland (16). This study sought to determine the potential of UG as a modulator of prostate tumor cell invasiveness and the relationship of this activity to its antiinflammatory action.

Materials and Methods

Culture of Human Prostate Tumor Cell Lines. DU-145 (17) and the LNCaP (18) prostate epithelial tumor cell lines were obtained from American Type Culture Collection (Rockville, MD). PC3-M (19) cells were kindly provided by Dr. Isaiah Fidler (M. D. Anderson Cancer Center, Houston, TX). The cell lines were maintained in monolayer culture in α-minimal essential medium supplemented with glutamine, 10% fetal bovine serum, penicillin (100 units/ml, and streptomycin (100 μg/ml). All cell cultures were incubated at 37°C in 5% CO2/95% air, and the medium was replaced every second day.

Chemoinvasion Assay. The tumor cells in logaritmic growth phase were detached by brief exposure to 0.25% trypsin-0.25% EDTA and then centrifuged at 800 × g for 5 min. Cells were resuspended, and 1.5 × 10⁵ cells were seeded in 60-mm dishes in SF medium. The dishes were treated with 0.01, 0.1, or 1.0 μM uteroglobin for 24 h, and the cells were removed by gentle scraping with a rubber policeman. The human prostate tumor cell lines were tested for invasiveness according to the method of Albini et al. (6) using the blind-well Boyden chambers. The lower compartment of the Boyden chamber was filled with 220 μl of either FCM as the chemotactrant for analysis of stimulated invasion or serum-free culture medium for analysis of basal invasion. FCM was prepared by incubating proliferative cultures of 3T3 cell fibroblasts for 24 h in SF medium. The lower compartment was then overlaid with a 12-μm pore size polycarbonate membrane filter (Neuro Probe, Inc., Cabin John, MD) that had been precoated with 25 μg/50 μl RM (Matrigel; Collaborative Biomedical Products, Bedford, MA). Tumor cells were added to the upper compartment (3.0 × 10⁵ cell/well), and the chambers were incubated for 6 h at 37°C. Invasiveness was assayed in triplicate for each time point.

Quantification of Invasion. Cells that had invaded the RBM and had migrated through the filter were quantitated using the crystal violet staining technique adapted for use with the Boyden chamber assay (20). At the termination of the incubation period, the filters were removed from the
chambers and pinned to a wax plate with the invading cells, which had traversed the filter, facing upward. The cells on the pinned filters were stained for 10 min with 0.5% crystal violet in 25% methanol. The filters were rinsed four times in distilled water or until no additional stain leached from the filters. The lower surface of each filter in contact with the wax plate (originally the upper surface of the filter in the Boyden chamber) was scraped with a moist cotton swab (Q-tip) to remove nonmigrated cells, and then the filter was placed in a 24-well cluster plate and dried overnight. The crystal violet was extracted from the invading cells by adding 500 μl of 0.1 M sodium citrate in 50% ethanol for 10 min and reextracted with an additional 500 μl of 0.1 M sodium citrate in 50% ethanol. The samples were analyzed spectrophotometrically at 585 nm using a Spectronic GENESYS 5 (Milton Roy, Rochester, NY).

**Recombinant Human UG.** Recombinant human UG (also known as Clara cell M₁₀,₀₀₀₀ protein) was produced in Escherichia coli as described (21, 22). Briefly, a full-length complementary DNA coding for human UG originally cloned in pGEM4Z (Promega) was digested with PstI, and a 340-base pair fragment containing the entire coding region of mature Clara cell M₁₀,₀₀₀₀ protein plus 53 nucleotides of the pGEM4Z polylinker was excised. This fragment was purified by preparative low melting agarose gel electrophoresis and subcloned into the PstI site of pLD101 (22). The new plasmid, pGEL101, was used to transfet E. coli strain BL21 (DE3). Bacterial cultures containing pGEL 101 were induced with isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 0.45 mM. The recombinant protein was purified as described (21).

**Effect of UG on FCM-induced AA Release.** [14C]AA (specific activity, 58.0 mCi/mmol), obtained from Amersham (Arlington, Heights IL), was used to measure AA release stimulated by FCM from DU-145 prostate epithelial tumor cells in the presence or absence of 1 μg UG. Cells were labeled by preincubation with 1 μCi of the [14C]AA per 0.75 X 10⁶ cells in 2 ml α-minimal essential medium SF culture medium for 24 h at 37°C. Cells were washed three times with 20 ml 0.2% bovine serum albumin to remove free radioactivity. Following this, 2 ml of FCM alone or FCM containing UG (1 μg) were each added to three separate cell cultures and incubated at 37°C. At times 0, 5, 10, 20, and 30 min and 1, 2, 3, 4, and 5 h, 50-μl aliquots of incubation media were removed for time-course evaluation of release of [14C]AA, and radioactivity was measured in EcoLite Biodegradable scintillant (ICN, Irvine, CA). The amount of [14C]AA release stimulated by FCM was calculated by subtracting the small amount of [14C]AA released spontaneously in SF standard medium from the amount released from FCM and FCM/UG-treated cells.

**Statistical Analysis.** Data between test group and controls in this study were evaluated using the standard repeated measures analysis of variance test. P < 0.01 was considered to be significant.

**Results**

**Quantitation of Tumor Cell Invasion in the Boyden Chamber.** The relationship between tumor cell invasion and absorbance was investigated with the DU-145 cell line. Table 1 shows the number of cells migrating to the lower face of the filters as a function of the number of cells seeded in the upper chamber. At cell seedings greater than 2 X 10⁵, approximately 22% of cells invaded the RBM and migrated through the filter in 6 h. Seeding higher numbers of cells did not increase invasiveness at 6 h (not shown). Using a Coulter Multisizer (Coulter, Hialeah, FL), we determined that 0.1 absorbance units corresponds to the amount of crystal violet stain extracted from 5000 cells.

<table>
<thead>
<tr>
<th>DU-145 prostate tumor cells were seeded into the top chamber. The number of cells migrating to the lower filter surface is expressed as a percentage of the total number of cells seeded in the top chamber. Values represent the mean ± SEM of triplicate determinations.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells seeded (X 10⁵)</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>300</td>
</tr>
</tbody>
</table>

* 0.1 absorbance unit corresponds to 5000 cells.

**Fig. 1.** Invasion of DU-145 (■), PC3-M (▲), and LNCaP (●) prostate tumor epithelial cells through RBM-coated filters in a Boyden chamber in response to FCM. Invading cells were quantitated by crystal violet staining, and the concentration of the extracted dye was measured at an absorbance of 585 nm. Points, means of triplicate cultures in a typical experiment; bars, SEM.

**Table 1** Relationship between cell invasion and absorbance (O.D.)

**Fig. 1** shows the relationship between FCM-induced tumor cell invasion per filter and absorbance (O.D.) of extracted crystal violet. Cells in the range of 1 X 10⁵, 2 X 10⁵, and 3 X 10⁵ were seeded in the top well and incubated for 6 h at 37°C. Migration to the underside of the filter increased linearly (DU-145, r = 0.95; PC3-M, r = 0.92; LNCaP, r = 0.99) with increasing numbers of cells seeded, reaching a maximum at 3 X 10⁶ cells per filter. The DU-145 cells showed the greatest migration, followed by PC3-M, whereas the LNCaP cell line was relatively less invasive.

**UG Effects on Tumor Cell Invasion.** Because of the relatively low invasive activity of the LNCaP cell line, we focused our experiments on the effect of UG on the DU-145 and PC3-M human prostate epithelial cells. Fig. 2 shows that DU-145 and PC3-M human prostate epithelial cell lines. Fig. 2 shows that DU-145 and PC3-M human prostate epithelial cell lines. DU-145 and PC3-M cells that were preincubated with 0.01 μg, 0.1 μg, and 1.0 μg concentrations of UG for 24 h prior to the invasion assay showed a dose-dependent inhibition in invasion in response to FCM (Fig. 2, column FCM/none); however, UG had no effect on basal invasiveness (Fig. 2, column SF-medium/UG). Inhibition was quantitatively determined by subtracting basal invasion from FCM-stimulated invasion in each treatment group and expressed as a percentage of the untreated control cells. Table 2 shows the average inhibition observed in three independent experiments, each performed in triplicate. The inhibition was found to be significant at the P < 0.05 level for the DU-145 cells treated with 1.0 μg UG and for the PC3-M cells treated with 0.1 and 1.0 μg UG; inhibition was significant at the P < 0.01 level for all combinations except PC3-M cells treated with 0.01 μg UG. As shown in Table 2, 1 μg UG inhibited invasiveness of PC3-M and DU-145 cells by 88 and 60%, respectively. Treatment of either DU-145 or PC3-M cells 24 h with 1 μM of either bovine serum albumin, myoglobin (a globulin protein of similar molecular weight to UG), or heat-inactivated UG had no effect on invasion (not shown).

To determine whether the antiinvasive activity of UG was related to effects on cell adhesion, DU-145 cells were seeded into culture dishes in the absence or presence of UG, and adherent cells were counted at 1, 3, and 6 h after seeding. To examine the potential effects of UG on
chemotaxis, DU-145 cells were seeded into Boyden chambers on membrane filters that were not coated with RBM. Migration in the absence and presence of FCM in the bottom chamber was measured. UG had no effect on either adhesion or chemotaxis (not shown).

UG Effects on FCM-induced AA Release. DU-145 prostate epithelial cells were prelabeled with [14C]AA as described above and examined for FCM-stimulated release of radioactivity in the presence and absence of 1 μM UG (Fig. 3). FCM induced a biphasic increase in the rate of release of [14C]AA from the cells, peaking at 20 min, followed by reuptake of radioactivity at 60 min and a subsequent sustained release over the 5-h observation period. UG (1 μM) reduced FCM-stimulated release of [14C]AA by 77% at 20 min in the first phase and 86% at 5 h in the second phase.

Discussion

The results of this study showed that recombinant human UG can significantly inhibit prostate tumor cell invasiveness as well as FCM-stimulated release of AA. In light of the discovery of UG-like protein in human prostate tissue (16), this study provides evidence that UG may have an important functional role to play in controlling the early events of tumor invasiveness in human prostatic carcinoma. Although the exact concentration of UG-like protein in the intact human prostate has not been measured, the prostate exhibits UG-like immunoreactivity similar to uterine tissue, which has UG levels ranging from several μM to over a mM in concentration. Thus, the antiinvasive activity of UG at submicromolar concentrations is highly physiologically relevant.

In vivo prostate tumor cells respond primarily to stromal cell secretory proteins for their growth and proliferation. In this study, we modeled prostate tumor cell extracapsular invasiveness using RBM, which selects for cells that have attached to the RBM, degraded the RBM enzymatically, and finally migrated through the filter towards FCM used as a chemoattractant. These events are consistent with the important in vivo steps in the metastatic spread of tumor cells through the basement membrane (5).

The inhibition of invasiveness by UG was not due to inhibition of cell-RBM adhesion and also is not due to nonspecific toxic effects, since treatment with UG (1.0 μM for 24 h) had no effect on colony formation or plating efficiency (not shown). This is further supported by the lack of inhibition of basal invasiveness by UG. The finding that

![Graph](image-url)
Uteroglobin inhibits prostate tumor invasiveness

UG decreased responsiveness of prostatic tumor epithelial cells to chemoattractants but did not affect basal invasiveness suggests that UG interferes with a specific invasion-associated inducible signal mechanism. Although UG has antichemotactic activity on macrophages, it did not interfere with the migration of DU-145 cells in the absence of the RBM barrier. This indicates that UG probably interferes with processes specifically involved in invasion through RBM.

Activation of PLA₂ and AA release and metabolism are recognized as important mechanisms for second messenger production in animal cells. Earlier reports have established the potent antichemotactic activity of UG (23) as well as UG-active site peptides (24). UG noncompetitively inhibits low molecular weight PL₂ activity in vitro (23), which regulates the intracellular release of secondary messengers like AA. We have now shown that UG strongly inhibits the release of AA from prostate tumor cells. Given that the prostate gland is prone to inflammatory disease, a role for an endogenous antinflammatory protein like UG becomes apparent. Additionally, a role for AA in tumor growth and metastasis has been suggested by reports of a positive correlation between the amount of prostaglandin production and tumor invasiveness in the lung (25). Bennett et al. (26) have reported that malignant human breast tumors contain more "PGE-like material" than do benign tumors or normal breast tissue. The highest levels of PGE-like material were associated with those tumors demonstrating histological evidence of invasiveness (7).

It is, therefore, tempting to speculate that the AA released from these prostate tumor cells participates in an autocrine loop that facilitates cell invasiveness. Indeed, a potential biochemical pathway linking AA with regulation of action of GAP and p21ras has been described (27). Activation of the p21ras has in turn been associated with acquisition of the metastatic phenotype (28) and activation of PL₂ (29). If AA stimulates prostate tumor invasiveness, then it is possible that UG would inhibit invasiveness by interfering with the signal pathway either by direct interaction with PL₂ or by other, as yet uncharacterized, mechanisms involved in receptors or downstream second messengers. Experiments are under way to examine these possibilities.

Acknowledgments

We thank Dr. John LaRosa for his continued support of this project.

References

Fibroblast-conditioned Medium
Release of Arachidonic Acid Stimulated by Invasiveness of Human Metastatic Prostate Tumor Cells and the in Vitro
Recombinant Human Uteroglobin Inhibits the Invasiveness of Human Metastatic Prostate Tumor Cells and the
Release of Arachidonic Acid Stimulated by Fibroblast-conditioned Medium

J. Leyton, M. J. Manyak, A. B. Mukherjee, et al.