Antagonists of growth hormone-releasing hormone inhibit proliferation induced by inflammation in prostatic epithelial cells

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Contributed by Andrew V. Schally, December 22, 2016 (sent for review November 29, 2016; reviewed by John T. Isaacs, Hari Koul, Natasha Kyprianou, and Jill A. Macoska)

The etiology of benign prostatic hyperplasia (BPH) is multifactorial, and chronic inflammation plays a pivotal role in its pathogenesis. Growth hormone-releasing hormone (GHRH) is a hypothalamic neuropeptide that has been shown to act as paracrine/autocrine factor in various malignancies including prostate cancer. GHRH and its receptors are expressed in experimental models of BPH, in which antagonists of GHRH suppressed the levels of proinflammatory cytokines and altered the expression of genes related to epithelial-to-mesenchymal transition (EMT). We investigated the effects of GHRH antagonist on prostatic enlargement induced by inflammation. Autoimmune prostatitis in Balb/C mice was induced by a homogenate of reproductive tissues of male rats. During the 8-wk induction of chronic prostatitis, we detected a progressive increase in prostatic volume reaching 92% at week 8 compared with control ($P < 0.001$). Daily treatment for 1 mo with GHRH antagonist MIA-690 caused a 30% reduction in prostate volume ($P < 0.05$). Conditioned medium derived from macrophages increased the average volume of spheres by 82.7% ($P < 0.001$) and elevated the expression of mRNA for *N-cadherin*, *Snail*, and *GHRH*. GHRH antagonist reduced the average volume of spheres stimulated by inflammation by 75.5% ($P < 0.05$), and TGF-β2 by 91.8% ($P < 0.01$). The proliferation of primary epithelial cells stimulated by IL-17A or TGF-β2 was also inhibited by 124.1% and 69.9%, respectively. GHRH stimulated the growth of BPH-1 and primary prostate spheres. This study provides evidence that GHRH plays important roles in prostatic inflammation and EMT and suggests the merit of further investigation to elucidate the effects of GHRH antagonists in prostatitis and BPH.

chronic prostatic inflammation | neuropeptide | prostatic hyperplasia | targeted therapy | experimental autoimmune prostatitis

Benign prostatic hyperplasia (BPH) is an age-dependent condition with a prevalence of 50–60% in men in their 60s (1). The term BPH refers to the enlargement of the prostate caused by expansion epithelial and stromal cells appearing primarily in the transition zone of the prostate (2). Patients with BPH frequently develop bladder outlet obstruction, leading to an increased resistance of the urethra, followed by the occurrence of various symptoms, collectively known as lower urinary tract symptoms (LUTSs) (3, 4). Current therapies include 5α-reductase inhibitors, which reduce dihydrotestosterone levels, and α1-adrenergic blockers, which lower the adrenergic tone (5, 6). These medical modalities have low efficacy and require continuous long-term administration. The number of patients with symptomatic BPH is expected to increase from 8.1 million in 2010 to 10.3 million in 2020 (7). Minimally invasive surgical techniques such as transurethral needle ablation and microwave thermotherapy are also used in severe cases (8). Although these techniques cause less complications than the invasive transurethral resection of the prostate, they still carry significant risks. Consequently, the development of an efficient, noninvasive medical therapy is urgently needed.

The pathogenesis of BPH is not completely understood, and it has been linked to many factors, including age-dependent changes in estrogen/testosterone ratio (9–11), age-related tissue remodeling, elevated levels of growth factors, hypoxia, and metabolic disturbances (12–15). Most recently, research has been more focused on the role of chronic inflammation as a central factor in the development of BPH (16). A majority of BPH specimens contain some degree of leukocyte infiltration (17–20). In addition, elevated levels of lymphocyte-derived cytokines, such as IL-2, IL-4, and IFN-γ, have been found in resected BPH tissue and appear to be involved in the stimulation of fibromuscular growth of the prostate (21). Similarly, cytokines released by infiltrated macrophages have also been demonstrated to stimulate stromal cell proliferation (22). Experimental animal models of bacterial prostatitis present a significant increase in epithelial proliferation induced by inflammation (23).

An established connection exists between inflammation and epithelial-to-mesenchymal transition (EMT), as demonstrated by the ability of inflammatory cytokines to regulate the expression of key genes of EMT in cancer and fibrosis (24). The occurrence of EMT has been confirmed in human BPH tissue and in an

Significance

The current therapeutic approaches to the treatment of benign prostatic hyperplasia (BPH) do not take into consideration that inflammation is an important factor in the pathogenesis of this disease. We previously demonstrated that growth hormone-releasing hormone (GHRH) antagonists reduce prostatic weights and decrease the level of inflammatory cytokines in a testosterone-induced BPH model. This study sheds light on the paracrine roles of GHRH in prostatic inflammation and demonstrates that GHRH stimulates the growth of BPH-1 and primary prostate epithelial spheres and that GHRH antagonists reduce prostate volume in an experimental model of prostatic inflammation.

Author contributions: P.P., A.V.S., and F.G.R. designed research; P.P., L.S., and K.K. performed research; A.V.S. contributed new reagents/analytic tools; P.P. and L.S. analyzed data; and P.P., A.V.S., and F.G.R. wrote the paper.

Reviewers: J.T.I., Johns Hopkins Oncology Center; H.K., Louisiana State University Health Sciences Center–Shreveport; N.K., University of Kentucky; and J.M., The University of Massachusetts, Boston.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620884114/-/DCSupplemental.

in vitro inflammation model (25–27). However, little is known about the exact molecular mechanism how the chronic prostatitis/EMT/BPH transition may occur.

The classical role of the hypothalamic neurohormone growth hormone (GH)-releasing hormone (GHRH) is to regulate the production and secretion of GH in the pituitary. Since its discovery, mitogenic effects of autocrine/paracrine GHRH have been demonstrated in various types of cancers, including prostatic, which express GHRH and GHRH receptors (GHRHRs) (28–33). An autostimulatory loop, composed of tumor-derived GHRH and its tumoral receptors, can be disrupted by specific GHRH antagonists, resulting in inhibition of tumor growth in experimental models. GHRH antagonists induce marked reduction in the growth of prostate cancer cells in in vivo cancer xenograft models and in vitro (34).

The involvement of GHRH signaling in the pathogenesis of BPH has been studied by our group in a testosterone-induced rat BPH model (35). The expression of protein for GHRH and GHRHRs was markedly elevated in this model, indicating the involvement of the GHRH autostimulatory loop in the pathogenesis of hormone-induced prostatic enlargement (36). In this experimental model, antagonists of GHRH, synthesized in our laboratories, demonstrated beneficial effects on prostate size, mitotic index, and the expression of various growth factors, inflammation-related genes, and key regulators of EMT (36–38). Anti-inflammatory effects for GHRH antagonists were also demonstrated in experimental models of cancer and ocular inflammation (39, 40).

In this study, we investigated the effects of the GHRH antagonists MIA-690 and JV-I-38 in experimental autoimmune prostatitis (EAP). We also report antiproliferative effects of MIA-690 in in vitro 3D models of inflammation and EMT.

Results

Immunohistochemical and Immunocytochemical Confirmation of the Expression of GHRH and GHRHR Proteins. As revealed by immunohistochemical analyses, GHRH and GHRHR are predominant in epithelial cells of the ventral prostates of mice (Fig. 1*A*). The expression of GHRH and GHRHR were also confirmed in human BPH-1 cells and primary prostate epithelial (PrEp) cells (Fig. 2*A*).

EAP Induces Prostatic Enlargement. Autoimmune prostatitis in Balb/C mice was induced by a previously established method (41). Mice were injected with two immunization injections of homogenate of prostate, seminal vesicle, and coagulating gland from rats that had been reported to induce a marked inflammation after 8 wk. Other models of experimental prostatitis showed an increase in the weights of the prostate induced by inflammation (42). Therefore, our first aim was to establish a

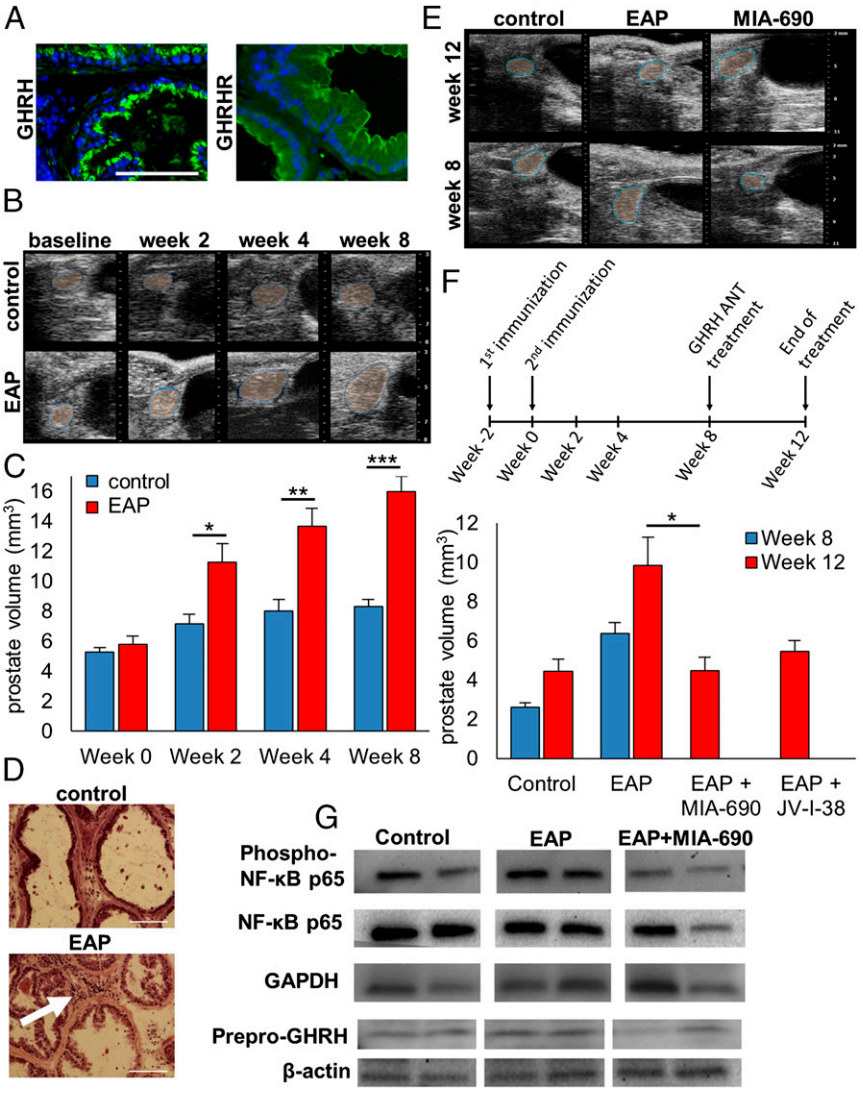

Fig. 1. GHRH antagonists MIA-690 and JV-I-38 reduce prostate enlargement in EAP. (*A*) GHRH and GHRHRs are localized mainly in the epithelial cells of the ventral prostate of Balb/C mice, as shown by immunohistochemistry. (Scale bar: 100 μm.) (*B*) Representative images showing volumetric changes in the ventral prostate detected by VEVO 1100 US imaging system. EAP was induced by two immunizations with a homogenate of rat male tissue injected 2 wk apart. Ultrasound images were recorded before the first injection (baseline) and 2 and 8 wk after the second injection. (C) Average volume of ventral prostates in EAP and control animals before and 2, 4, and 8 wk after the induction. (*D*) H&E staining shows leukocyte infiltrate in the ventral prostate of the EAP model (arrow) and no signs of inflammation in the control tissue. (Scale bar: 100 μm.) (*E* and *F*) GHRH antagonists reduce inflammation-induced enlargement of the ventral prostate in EAP. As depicted by the scheme, mice were treated with GHRH antagonists MIA-690 or JV-I-38 at 5 μg/d for 4 wk starting 8 wk after the induction of EAP and compared with vehicle-treated animals. Representative ultrasound images (*E*) and average volumes of the ventral prostates (*F*) are shown 8 and 12 wk after the induction of EAP. (*G*) Western blot analysis of the expression of phospho-NF-κβ p65, NF-κβ p65, and prepro-GHRH in ventral prostates from control, EAP, and EAP treated with MIA-690. GAPDH or β-actin were used as standard genes (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.01$ by Student's *t* test).

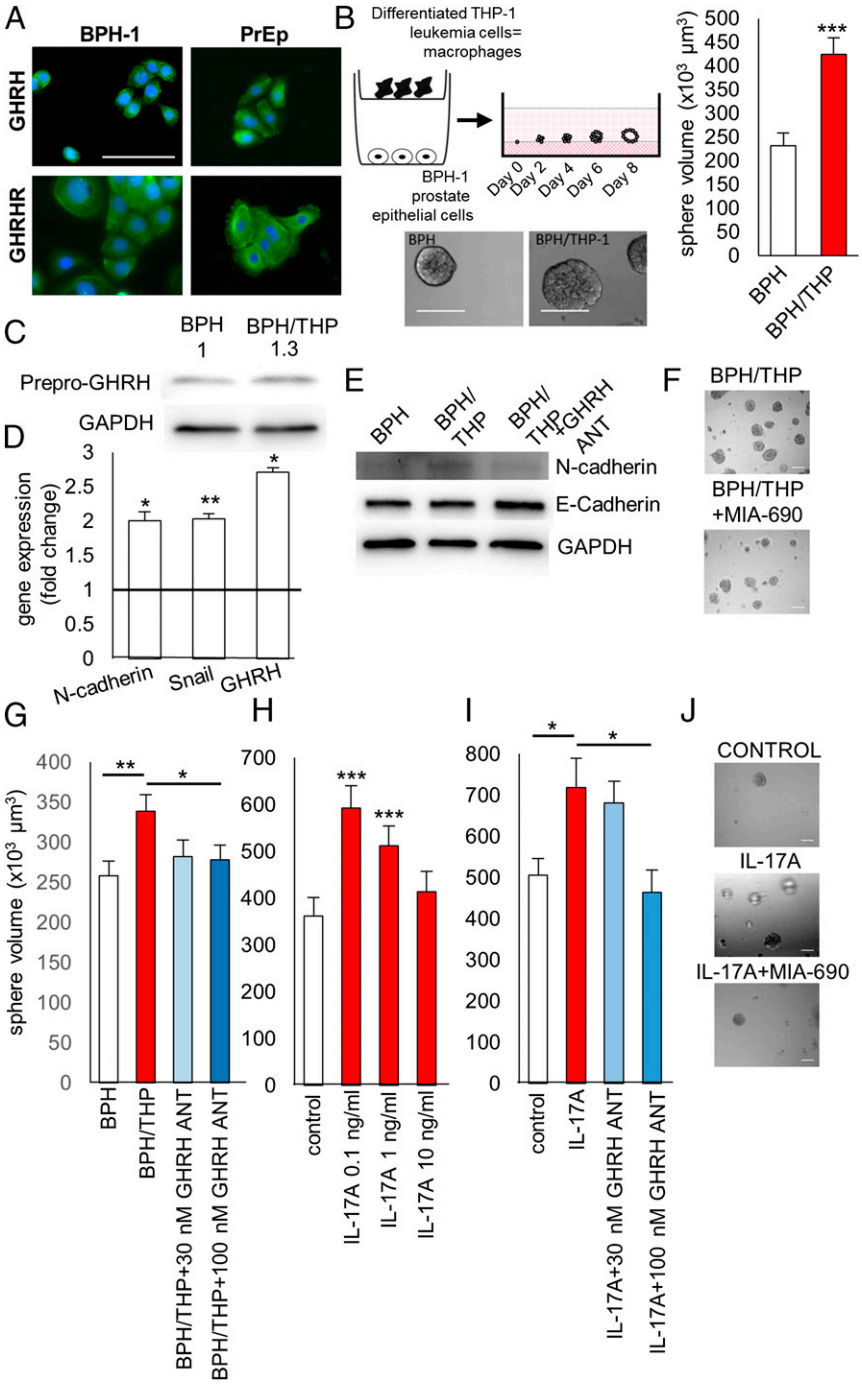

Fig. 2. GHRH antagonists inhibit proliferation and EMT induced by inflammation in 3D cell culture in BPH-1 cells and in PrEp cells. (*A*) GHRH and GHRHRs are expressed in BPH-1 cells and in PrEp cells as detected by immunocytochemistry. (Scale bar: 100 µm.) (*B*) In vitro model of inflammation-induced proliferation: BPH-1 cells were treated with conditioned medium derived from differentiated THP-1 macrophages that are cocultured with BPH-1 cells (BPH/THP) for 8 d. Control cells were incubated in medium derived from BPH-1 cells only (BPH). Representative images of BPH-1 spheres are shown. (Scale bar: 100 µm.) The average volume of spheres was determined from three parallel experiments. (*C*) Expression of protein for GHRH and (*D*) the expression of mRNA for *N-cadherin*, *Snail*, and *GHRH* are increased in cells treated with BPH/THP medium compared with control as determined by Western blot and quantitative PCR, respectively. (*E*) Representative images of Western blot analysis of the expression of N-cadherin, E-cadherin, and GAPDH in 3D cultures from BPH, BPH/THP, or BPH/THP with 100 nM treatment. (*F* and *G*) GHRH antagonist MIA-690 added at 30 nM or 100 nM concentration to BPH/THP medium suppresses the stimulatory action of macrophage-conditioned medium on the growth of BPH-1 spheres. (Scale bar: 100 µm.) (*H*) The effect of IL-17A on the growth of PrEp cells was tested at 0.1 ng/mL, 1 ng/mL, and 10 ng/mL. (*I* and *J*) GHRH antagonist MIA-690 inhibits growth of PrEp spheres stimulated by IL-17A. Cultures were treated with 0.1 ng/mL IL-17A alone or with MIA-690 at 30-nM or 100-nM concentrations. (Scale bar 100 µm.) $*P < 0.05$, $**P < 0.01$, and $***P < 0.01$ by Student's *t* test.

detection method that enables the in situ detection of the growth of the prostate during the development of chronic prostatitis. Ventral prostates of mice were imaged by ultrasound by using a VEVO 1100 imaging system (FujiFilm VisualSonics), and volumes were calculated as described in *Materials and Methods*. Average volumes were not significantly different before the immunization (5.29 mm^3 in control vs. 5.81 mm^3 in EAP; Fig. 1 *B* and *C*). Two weeks after induction, EAP prostates were enlarged by 57% to 11.25 mm^3 vs. 7.17 mm^3 in the control group ($P < 0.05$). At week 4, prostates in the EAP group were 70% larger than controls (13.65 mm^3 vs. 8.01 mm^3; $P < 0.01$) and further increased to a final measurement of 92% at week 8 (15.99 mm^3 vs. 8.33 mm^3; $P < 0.001$). Prostatic inflammation was further confirmed by the transcriptional up-regulation of genes involved in inflammatory response such as bone morphogenic protein 3 (*BMP3*), *Ctf2*, growth differentiation factor 10 (*Gdf10*), *Mstn*, *IFN-γ*, *IL-10*, *IL-13*, and *IL-7* ($P < 0.05$; Table S1). Inflammation was also confirmed histologically by the presence of leukocyte infiltrates 12 wk after the induction of inflammation by visual assessment of H&E staining of frozen sections of the ventral prostates (Fig. 1*D*).

GHRH Antagonists Reduce Prostatic Enlargement Induced by EAP. Prostatic inflammation was induced by immunization with tissue of male rats, and animals were randomized into three groups (Fig. 1 *E* and *F*). Mice were treated with the GHRH antagonists MIA-690 or JV-I-38 at 5 µg/d for 4 wk, whereas control animals received vehicle treatment. The volumes of ventral prostates of

control animals increased from 2.60 mm^3 to 4.46 mm^3, and there was also a further increase in the growth of EAP mice from 6.37 mm^3 to 9.86 mm^3 (Fig. 1*F*). Treatment with MIA-690 reduced the average volume of the ventral prostates in EAP mice by 30% to 4.48 mm^3 ($P < 0.05$). JV-I-38 also decreased the average volume of the prostate, but this effect was not statistically significant (reduction of 14.3% to 5.46 mm^3). In addition, MIA-690 reduced the phosphorylation of NF-κB and the elevation in the protein levels of prepro-GHRH induced by inflammation (Fig. 1*G*).

The Expression of *GHRH* Is Induced in an in Vitro Model of Inflammation. An in vitro model of inflammation was generated according to a method published by Lu et al. (25). The average spherical volume increased by 82.7% in cultures treated with conditioned medium derived from BPH-1 and human acute monocytic leukemia cell lines (BPH/THP) compared with control (232,063.7 μm^3 vs. 423,966.9 μm^3; $P < 0.001$; Fig. 2*B*). The expression of mRNA for the mesenchymal markers *N-cadherin* and *Snail* was increased by the conditioned medium by one-fold ($P < 0.05$) and 1.03-fold ($P < 0.01$), respectively. The expression of mRNA and protein for *GHRH* was also up-regulated by 1.7-fold ($P < 0.05$) and 30%, respectively (Fig. 2 *C* and *D*).

GHRH Antagonists Inhibit Proliferation and EMT Induced by Inflammation. The average volume of BPH-1 spheres that were stimulated by conditioned medium was reduced by GHRH antagonist MIA-690 at 30 nM and 100 nM by 56,332.9 μm^3 and 60,658.2 μm^3 (inhibition of 70.1% and 75.5%), respectively [not significant (NS) and $P < 0.05$, respectively; Fig. 2 *F* and *G*]. The protein levels for N-cadherin were also suppressed by 100 nM MIA-690 in cultures treated with BPH/THP medium (Fig. 2*E*).

GHRH Antagonists Reduce the Proliferation of Primary Epithelial Cells Stimulated by IL-17A. IL-17A was tested at 0.1 ng/mL, 1 ng/mL, and 10 ng/mL to investigate its effect on the proliferation of PrEp cells grown in 3D culture and to establish an additional in vitro model of inflammation. The average sphere volume was significantly increased in cultures treated with 0.1 ng/mL and 1 ng/mL IL-17A (increase of 230,129.2 μm^3 and 150,157.2 μm^3 compared with control, respectively; $P < 0.001$), whereas, at the concentration of 10 ng/mL, IL-17A did not cause a significant change (Fig. 2*H*). The GHRH antagonist MIA-690 reduced the average volume of spheres by 191,458.2 μm^3 (inhibition of 124.1%) at 100 nM concentration, but had no significant effect at 30 nM concentration (Fig. 2 *I* and *J*).

GHRH Antagonists Inhibit Proliferation and EMT Induced by TGF-β2. The increase in the expression of N-cadherin observed in BPH-1 cells stimulated with conditioned medium is dependent on TGF-β activity (25). To establish an EMT-induced model of proliferation, we tested the effect of TGF-β1 and TGF-β2 at 0.1 ng/mL and at 1 ng/mL concentrations in 3D cultures of BPH-1 cells. TGF-β1 did not affect the proliferation of BPH-1 cells. In contrast, TGF-β2 at 0.1 ng/mL increased average sphere volume by 29,210.9 μm^3 (NS) and 69,816.78 μm^3 at 1 ng/mL concentration ($P < 0.05$; Fig. S1*A*). Protein levels of GHRH were also increased by 1 ng/mL TGF-β2 (Fig. S1*D*). The GHRH antagonist MIA-690 at 100 nM reduced the average volume of spheres of BPH-1 cells stimulated by 1 ng/mL TGF-β2 (91.8% inhibition of TGF-β2 stimulatory activity; Fig. S1 *B* and *C*). The expression of protein for N-cadherin was also suppressed by MIA-690 in cells stimulated by TGF-β2, whereas the expression of E-cadherin was not presented with consistent changes (Fig. S1*E*). The stimulatory action of TGF-β2 was diminished by stable transfection of shRNA constructs that target the expression of GHRHR and its splice variant (SV1) as shown on the representative Western blot (Fig. S1*F*). The volume of BPH-1 spheres expressing scrambled control shRNA was increased by 31.4% upon stimulation with 1 ng/mL TGF-β2 (183,995.5 μm^3 vs. 241,736.0 μm^3 with stimulation; $P < 0.001$). In contrast, treatment with 1 ng/mL TGF-β2 decreased the volume of spheres composed of shRNAGHRHR1 expressing cells by 24.3% (144,882.4 μm^3 vs. 109,625.5 μm^3 with stimulation; NS). The stable transfection of another construct, shRNAGHRHR2, also decreased the stimulatory action of TGFβ-2 by 16.7% (159,700.5 μm^3 vs. 186,341.1 μm^3 with stimulation; NS). The effects of TGF-β1 and TGFβ-2 were also tested on the proliferation of PrEp cells in 3D cultures. At 0.1 ng/mL, TGF-β1 and TGF-β2 increased the average volume of spheres by 106,594.3 μm^3 ($P < 0.05$) and 150,387.0 μm ($P < 0.05$), respectively, whereas TGF-β1 at 1 ng/mL had an inhibitory effect (Fig. S1*G*). The stimulatory effect of TGF-β2 was significantly inhibited by GHRH antagonist MIA-690 at 30-nM and 100-nM concentrations (reduction of 73,999.8 μm^3/69.9% and 67,678.8 μm^3/63.9%, respectively; $P < 0.01$ for both; Fig. S1*H*).

GHRH Stimulates the Proliferation and Expression of N-Cadherin in Prostate Epithelial Cells. GHRH(1-29)NH_2 at low concentrations of 0.01 nM and 0.1 nM increased the volume of BPH-1 spheres by 86,712.0 μm^3 ($P < 0.05$) and 84,448.5 μm^3 ($P < 0.01$), respectively (Fig. S1*I*). GHRH(1-29)NH_2 at 0.1 nM concentration also increased the volume of PrEp spheres by 250,084.2 μm^3 ($P < 0.001$; Fig. S1*J*). GHRH(1-29)NH_2 at 0.1 nM increased the expression of N-cadherin protein but had no effect on the protein levels of E-cadherin (Fig. S1*K*).

Discussion

The clinical management of BPH shifted from surgical to medical care in the 1990s, decreasing risks related to surgery, but this converted the disease into a chronic condition, increasing the economic burden on the US healthcare system (43). Currently available treatments, 5-α reductase inhibitors and α-blockers, have been selected based on their beneficial action in alleviating symptoms, but these therapeutic medical modalities have low efficiency (43). The development of new therapies is challenging because the root cause of the condition is still unresolved. BPH is an age-related disease that is most likely caused by multiple factors (14). There is a growing body of evidence implying that inflammation is a causal factor in the pathogenesis of BPH, including a recently identified positive correlation between the presence/degree of inflammation and prostatic volume/weight (16, 44).

The proinflammatory environment may stimulate prostatic growth by multiple ways depending on the origin of inflammation (autoimmune vs. bacterial prostatitis) and the duration of the inflammatory episode (acute vs. chronic). Inflammation and EMT have been linked in the context of carcinogenesis and metastatic potential (45), but little is known about their interplay in benign cells. The first evidence of the involvement of EMT in BPH was provided by Alonso-Magdalena et al., who reported the down-regulation of CK8 and E-cadherin and up-regulation of pSMAD, Snail, and Slug in multilayered BPH ducts (27). We used an in vitro model of inflammation that contains various factors secreted by macrophages originally reported by Lu et al. (25). Lu et al. reported that, in this system, cells undergo EMT characterized as up-regulation in the expression of N-cadherin and Snail with a down-regulation of E-cadherin. The authors identified this phenomenon as an enrichment of mesenchymal cells linked with the concept that BPH is primarily a proliferative stromal disease (25). In our study, we were unable to show the down-regulation of E-cadherin, but this might be the result of the small number of cells that underwent EMT. Interestingly, immortalized human mammary cells similarly undergo EMT upon TGF-β1 treatment, but they also gain stem cell properties (46). This is consistent with earlier findings reported by Wang et al. that showed an increased number of epithelial progenitor cells in the prostate in a bacterially induced mouse model of inflammation (47). In our study, the increase of N-cadherin–positive cells might indicate the appearance of stem-like cells that fuels the expansion of epithelial cells. Our finding that antagonists of GHRH disrupt the increased

proliferative activity and decrease the expression of N-cadherin in prostatic epithelial cells triggered by the inflammatory environment was not previously described and is of great interest.

In the past few years, essential roles for fibrosis consequent to inflammation and aging in the development of LUTSs have been suggested (48). Fibrosis is defined as the progressive buildup of fibrotic connective tissue involving an increased number of myofibroblasts emerging through the transdifferentiation of epithelial cells and fibroblasts (49). The up-regulation of mesenchymal markers in BPH-1 cells triggered by the inflammatory environment in our study may mark the appearance of the mesenchymal phenotype and represent the very early stages of tissue fibrosis. Aging may contribute to the preservation of the fibrotic tissue by triggering changes in cell homeostasis and resistance to apoptosis (50). Further studies of this phenomenon would be of interest to determine how repeated activation of inflammatory pathways induces proliferation and/or mesenchymal transition in prostate epithelial cells.

In an effort to model inflammation in PrEp cells, we tested the effect of IL-17A at different concentrations on the growth of spheres. IL-17A is secreted by a subclass of T helper cells (T_H17), linked to autoimmune prostatitis, and stimulates the expression of other cytokines and chemokines such as TNF-α, IL-6, and IL-8 (51, 52). An earlier study could not demonstrate stimulatory activity on the proliferation of prostatic epithelial cells at the concentration of 20 ng/mL (53). We were able to verify the stimulatory effects of IL-17A added at low concentrations; moreover, we showed that this effect was also inhibited by GHRH antagonists.

The up-regulation of N-cadherin induced by the inflammatory environment inspired our further tests with TGF-β peptides, which are the primary regulators of EMT but are known to inhibit the proliferation of noncancerous epithelial cells in various contexts (54). In our system, we used long-term administration of low concentrations that are rarely used in other studies, and we found that TGF-β2 in BPH-1 and PrEp cells, and TGF-β1 in PrEp cells, stimulate proliferative activity. Moreover, GHRH seems to be a key modulator of this effect given the following: (*i*) the expression of GHRH is stimulated by TGF-β2, (*ii*) GHRH antagonists decrease the volume of spheres induced by TGF-β2, and (*iii*) the effect of TGF-β2 on the growth of BPH-1 spheres is disrupted when the expression of GHRHRs is silenced by the stable transfection of shRNA. Our finding that the down-regulation of GHRH signaling counteracts the proliferative effects of TGF-β2 is particularly intriguing but requires further investigation to reveal the exact interaction between these pathways. Untreated cells in which GHRH receptors are silenced to a greater extent with shRNAGHRH1 form spheres with smaller volume. Therefore, it is a challenge to estimate the distinct impact of GHRHR signaling on the effects of TGF-β2; it is also not possible to identify if this interaction is direct or indirect. Nevertheless, these important findings stimulate further investigations of the interplay between GHRH and TGF-β2 in healthy and inflamed cells.

In our previous studies that used a testosterone-induced BPH model in rats, we found that GHRHRs in the prostate are up-regulated after treatment with testosterone, whereas treatment with GHRH antagonists decreased prostate weights and reduced the protein levels of IL-1β, NF-κβ/p65, and cyclooxygenase-2 (36). In the current study, we used a model of autoimmune prostatitis that has been previously shown to develop progressive and chronic inflammation (41). We demonstrated the up-regulation of pro- and anti-inflammatory genes 8 wk after the immunization by PCR array. These genes have been associated with the induction of proliferation (*IL-7*, *IFN-γ*) (21), interference with steroidogenesis (*IL-13*) (55), and inhibition of inflammation (*IL-10*) (56) or have currently unknown function in the prostate (*cardiotrophin-2*). We also detected an increase in the expression of members of the transforming growth factor-β superfamily such as *myostatin*, *GDF-10*, and *BMP-3*. We established an ultrasound method that enables the in situ detection of volumetric growth of the ventral prostate during the progression of prostatitis. Enlarged prostates were shrunk by a 1-mo treatment with GHRH antagonists, which could be the consequence of multiple actions of the blockade of GHRH in vivo. First, GHRH antagonists exert their effects by down-regulating the activity of the GHRH/GH/IGF1 axis (33). IGF1, in fact, has been shown to direct the effects of IL-1 during experimental prostatic hyperplasia (57). Second, GHRH antagonists potentially act on local receptors in the prostate. This is supported by our finding that the expression of GHRH is up-regulated in vivo and in the in vitro inflammation model, as well as by treatment with TGF-β2. This paracrine GHRH is most likely a growth factor based on our results demonstrating that low concentrations of exogenous GHRH stimulated the growth of prostatic spheres. In addition, GHRH also increased the expression of N-cadherin, which implies that GHRH may be a key regulator of EMT and/or stimulate the expansion of progenitor cells.

In summary, we demonstrated that GHRH antagonists reduce prostatic enlargement, proliferation of prostatic epithelial cells, and expression of N-cadherin induced by inflammation. This effect is exerted through at least in part by prostatic GHRHRs. GHRH antagonists also disrupt the effects of TGF-β on cell proliferation and EMT. Our findings also indicate that GHRH acts as a local growth factor that is induced by the inflammatory environment. Our current and previous findings strongly indicate that GHRH antagonists may be clinically useful in the treatment of chronic prostatitis and BPH.

Materials and Methods

Peptides and Reagents. The GHRH antagonists MIA-690 and JV-I-38 were synthesized in our laboratory by solid-phase methods and purified by reversed-phase high-performance liquid chromatography as described previously (58). The chemical structure of MIA-690 is [(PhAc-Ada)^0-Tyr1, D-Arg^2, Cpa^6, Ala^8, Har^9, Fpa5^{10}, His^{11}, Orn^{12}, Abu^{15}, His^{20}, Orn^{21}, Nle^{27}, D-Arg^{28}, Har^{29}]hGH-RH(1–29)NH_2. The synthesis and structure of JV-I-38 was previously published (59). TGF-β1, TGF-β2, and IL-17A were purchased from Cell Signaling Technology.

Animals and EAP. Eight-week-old BALB/c mice, purchased from Harlan Laboratories, were housed in a climate-controlled environment with a 12-h light/dark cycle and fed standard laboratory diet with water ad libitum. Animal care was in accordance with institutional guidelines and complied with National Institutes of Health policy. EAP was induced as previously described (41). Tissues of male rats (prostate, seminal vesicle, and coagulating gland) were homogenized in PBS solution with protease inhibitors, and homogenate was centrifuged at 10,000 × *g* for 30 min. Supernatant was collected and emulsified with an appropriate volume of complete Freund's adjuvant by using opposed glass syringes. Mice were immunized with emulsion containing 1 mg protein in 150 μL volume administered twice, 2 wk apart, by s.c. injections into the flank. Control animals received no injections.

Study Design. Animals were randomized 8 wk after the second injection of immunization and were treated with MIA-690 or JV-I-38 at a dose of 5 μg/d for 4 wk. Control animals received 0.1% DMSO in 10% (vol/vol) aqueous propylene glycol solution. Mice were killed under anesthesia after 4 wk of treatment. Ventral prostates were collected and snap-frozen, preserved in optimal cutting temperature compound (OCT) or immersed in buffered 10% (vol/vol) formalin (pH 7.4), and embedded in paraffin for histological analysis.

Monolayer and 3D Culture of Cells. The human prostate epithelial BPH-1 cell line was donated by Simon Hayward (Vanderbilt University Medical Center, Nashville, TN) and maintained in RPMI-1640 medium (Life Technologies) supplemented with 5% (vol/vol) FBS. THP-1 cells (human acute monocytic leukemia cell) were obtained from American Type Culture Collection and were maintained in RPMI-1640 with 10% (vol/vol) FBS. PrEp cells were purchased from Millipore and were cultured in EpiGRO Basal Medium (Millipore). Cells were kept at 37 °C in a humidified atmosphere containing 5% CO_2. The 3D cultures were generated as described previously (25). Briefly, 45 μL of Matrigel was added to wells in an eight-well chamber slide and placed in a 37 °C incubator for 1 h. BPH-1 and PrEp cells were plated at 2,000 cells per well in medium containing 2% (vol/vol) Matrigel. Treatments (conditioned medium, TGF-β1, TGF-β2, or IL-17A) were added the next day for 8 d.

Images were acquired on a Nikon Eclipse Ti fluorescence microscope at 10× magnification, and the diameters of spheres were measured by using ImageJ software (National Institutes of Health). The average diameter of cells was calculated from triplicates. Additional information is provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported in part by the Urology Care Foundation Research Scholar Award Program and the American Urological Association Southeastern Section (to P.P.), Medical Research Service of the Veterans Affairs Department (A.V.S.), Departments of Pathology and Medicine, Division of Hematology/Oncology, and Sylvester Comprehensive Cancer Center of the Miller Medical School, University of Miami (A.V.S.), and South Florida Veterans Affairs Foundation for Research and Education (A.V.S.). A.V.S. is listed as coinventor on patents for GHRH antagonists, which have been assigned to the University of Miami and the Veterans Affairs Department; however, this study was purely experimental.

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