RAPID COMMUNICATION

Expression and action of the growth hormone releasing peptide ghrelin and its receptor in prostate cancer cell lines

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Abstract

This study has examined the expression of two new facets of the growth hormone axis, the growth hormone secretagogue receptor (GHS-R) and its recently identified putative natural ligand ghrelin, in prostate cancer cells. GHS-R 1a and 1b isoforms and ghrelin mRNA expression were detected by RT-PCR in the ALVA-41, LNCaP, DU145 and PC3 prostate cancer cell lines. A normal prostate cDNA library expressed GHS-R1a, but not the 1b isoform or ghrelin. Immunohistochemical staining for the GHS-R 1a isoform and ghrelin was positive in the four cell lines studied. PC3 cells showed increased cell proliferation *in vitro* in response to ghrelin to levels 33% above untreated controls, implying a potential tumour-promoting role for ghrelin in this tissue. This study is the first to demonstrate the co-expression of the GHS-R and ghrelin in prostate cancer cells. It is also the first study to provide evidence that a previously unrecognised autocrine/paracrine pathway involving ghrelin, that is capable of stimulating growth, exists in prostate cancer.

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Introduction

Prostatic carcinoma is the second most frequent cause of cancer mortality in males in the Western world, with aging identified as the number one risk factor. Insulin-like growth factor-I (IGF-I), the tissue biomediator of GH, has an autocrine/paracrine action in the prostate (Culig *et al.* 1996) and a positive association has been identified between circulating IGF-I levels and prostate cancer risk (Chan *et al.* 1998), although this link has been disputed (Cutting *et al.* 1999). Other components of the growth hormone (GH) axis may also have tumourigenic potential (Chopin & Herington 1999). Antagonistic analogues of GHRH inhibit the growth of androgen-independent human prostate cancer cell lines both *in vivo* and *in vitro* (Schally & Varga 1999) and prostate cancer cell lines express both GHRH and GHRH receptor (Chopin and Herington 2001).

The expression of GH receptor (GH-R) mRNA has been demonstrated in normal and cancerous human prostate tissue (Kolle *et al.* 1999, Ballesteros *et al.* 2000). GH and GH-R are expressed in prostate cancer cell lines at the mRNA and protein levels (Chopin *et al.*, in press), suggesting the presence of a locally active GH responsive system. Untergasser *et al.* (1999) have reported an increased *in vitro* proliferation rate of LNCaP prostate cancer cells in response to GH, and GH-R antagonists possess anti-neoplastic properties in numerous tumours (Friend *et al.* 1999). The naturally-occurring GH secretagogue receptor (GHS-R) was cloned in 1996 (Howard *et al.* 1996). The human functional GHS-R (type 1a) is a 366 amino acid peptide with seven transmembrane domains, and is a member of the heptahelical superfamily of G protein-coupled receptors (Camanni *et al.* 1998). The type 1b splice variant is predicted to encode a 289 amino acid protein lacking two of the C-terminal transmembrane domains and is believed to be non-functional (McKee *et al.* 1997). A recently identified endogenous ligand for the GHS-R, ghrelin, is a 28 amino acid peptide originally isolated from rat stomach tissue and subsequently from human stomach (Kojima *et al.* 1999). Ghrelin was found to stimulate pituitary GH release *in vitro* and *in vivo* with a potency and specificity comparable to GHRH (Kojima *et al.* 1999). With ghrelin and the GHS-R now recognised as new components of the GH axis, the aim of this study was to investigate their expression and potential role in proliferation in prostate cancer cell lines.

Materials and methods

Cell Culture

ALVA41 cells were obtained from Dr P Leedman (Royal Perth Hospital, Perth, Australia) and DU145, LNCaP, and

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PC3 cells from the American Type Culture Collection (Rockville, MD). ALVA41 cells were cultured in RPMI 1640 medium (pH 7·4) (Life Technologies, Rockville, MD) with 5% foetal calf serum (FCS) (CSL Biosciences, Melbourne, Australia) and DU145, LNCaP and PC3 cells in RPMI 1640 plus 10% FCS. All cell lines were free from Mycoplasma and were cultured in 80 cm^2 cell culture flasks (Nagle Nunc International, Roskilde, Denmark) at 37 °C, 5% CO_2 with 50 units/ml penicillin G and 50 μl/ml streptomycin sulphate.

Reverse transcriptase PCR (RT-PCR)

On reaching 70% confluence, total RNA was extracted from cell pellets with Trizol (Life Technologies) according to the manufacturer's instructions. RNA was incubated in a 50 μl solution containing 20 nM $MgCl_2$, 2 mM DTT, 0·5 mg Dnase (Rnase free, Roche, Basel, Switzerland), 5·0 units of Rnase Inhibitor (Roche) and incubated for 30 min at 37 °C then heated at 90 °C for 5 min. Reverse transcription was achieved by the addition of 0·5 μg oligo dT_{18} primer, 5 μg total RNA at 70 °C for 10 min. This solution was incubated at 43 °C for 2 min in buffer (50 mM TrisCl pH 8·8, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT and 500 μl of each dNTP (pH 7; Roche). This was incubated with 200 units SuperScript RT II (Life Technologies) at 43 °C for 90 min, then 70 °C for 15 min. A normal human prostate cDNA library was obtained from Clontech, (Palo Alto, CA). PCR using β-actin primers 5′GTGGGGCGCCCCAGGCACCA3′; (sense) and 5′TTGGCCTTGGGGTTCAGGGG3′ (antisense) (annealing temp: 50 °C) confirmed the absence of genomic DNA contamination (330 bp product) in all cDNA samples. PCR was performed for the 1a GHS-R using 5′TCTTCCTTCCTGTCTTCTGTC3′ (sense) and 5′AGTCTGAACACTGCCACC3′ (antisense) primers (annealing temp: 50 °C). PCR for the 1b isoform was performed using the same sense primer as for the 1a isoform, and the antisense primer 5′TCAGAGAGAAG GGAGAAGG3′ (annealing temp: 58 °C). Ghrelin PCR was performed with 5′GAGGATGAACTGGAAGTC CG3′(sense) and 5′CATTTATTCGCCTCCTGAGC3′ (antisense) primers (annealing temp: 59 °C) and also with primers for the full length preproghrelin mRNA transcript – 5′GCCCACCTGTCTGCAACC3′ (sense) and 5′GAACATTTATTCGCCTCCTG3′ (antisense; annealing temp: 60 °C).

PCRs contained 10 × PCR Buffer, 100 μM dNTPs, 100 pM primers (Genset Pacific Oligos, Armidale, Australia), 2 μl cDNA or water (no template negative control) and 1 unit Red Hot Polymerase (Integrated Sciences, Melbourne, Australia). An additional 'minus RT' negative control was performed with the GHS-R 1b PCRs for all cell lines. Thermal cycling consisted of 5 min at 95 °C, 40 cycles of 30s, 95 °C, 30s at annealing temperature, 2 min at 72 °C, followed by 10 min at 72 °C on a PTC-200 Thermal cycler (MJ research, Watertown, Massachusetts).

Southern analysis and sequencing

RT-PCR products electrophoresed on a 2% gel were capillary blotted overnight in 20 × Standard Saline Citrate (3 M NaCl, 0·3 M sodium citrate pH 7) onto Hybond positively charged membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Internal oligonucleotide probes for GHS-R 1a 5′TGATGGCAGCACTGAGG TAG3′ and GHS-R 1b 5′GATAGGACCCGCGAGA GAAA3′ and ghrelin

5′TTGAACCGGACTTCCAGTTC3′ (Genset Pacific Oligos) were labelled using a DIG dUTP/dATP tailing kit (Roche) and quantified and hybridised according to the DIG user's manual (Roche). Membranes were exposed to X-ray film (Agfa-Gavaert, Morstel, Belgium) for up to 15 min and developed using the Curix 60 automatic processor (Agfa-Gavaert). RT-PCR products were purified from an agarose gel using the Concert Rapid Gel PCR purification kit (Life Technologies). Sequencing was performed at the Australian Genome Research Facility (University of Queensland, Brisbane, Australia) using the Applied Biosystems 377 DN automated DNA sequencer and ABI Big Dye Terminator reagents.

Immunohistochemistry

Cells were grown to 70% confluence in 96 well plates (Nagle-Nunc), washed in phosphate buffered saline (PBS) and fixed for 5 min in 100% methanol. Immunodetection was performed using a Histostain-SP plus broad spectrum diaminobenzamine staining kit (Zymed, San Francisco, CA), according to the manufacturer's instructions. Polyclonal anti-GHS-R 1a antibodies were raised in rabbits (IMVS, Adelaide, SA) against the C-terminal peptide fragment (RAWTESSINTC) (Feighner *et al.* 1998) of the GHS-R 1a peptide conjugated to diptheria toxin (Mimotopes, Melbourne, Australia).

Polyclonal anti-ghrelin antibodies were raised against the mature human ghrelin peptide (GSSFLSPEHQRVQ QRKESKKPPAKLQPR; with the third residue *n*-octanoylated) (Mimotopes, IMVS) which was also conjugated to diptheria toxin. The titres of both antibodies were determined by ELISA. Sera were diluted 1:100 – 1:3200 in 1% bovine serum albumin in 0·01 M PBS, and cells were incubated at 4 °C for 24 h. Negative controls included the abolition of staining by pre-absorbing the relevant antibody with 1·0 mg/ml GHS-R 1a peptide fragment or ghrelin, or by the omission of primary antibody.

Western analysis

Western analysis was undertaken to confirm the expression of ghrelin peptide by the cells. Cell pellets of the four prostate cancer cell lines were lysed in buffer containing 1%

triton-X 100 and protease inhibitors (Roche). The homogenates were centrifuged and the supernatants containing total cell protein collected. 200 μg of protein was boiled for 2–3 min in 4X loading buffer (250 mM Tris–Cl pH 6·8, 2% SDS, 10% glycerol, 20 mM DTT, 0·01% bromophenol blue) and then electrophoresed, along with a Rainbow protein marker (Amersham) and 1 μg of synthetic ghrelin (Mimotopes) on a 10%SDS-PAGE gel. The protein was then transferred to a nitrocellulose membrane (Protran, Schleicher and Schuell, Germany) for 1 h in transfer buffer (10 mM $NaHCO_3$, 3 mM Na_2CO_3, methanol). The membrane was blocked overnight at 4 °C in 1X Tris Buffered Saline/0·05% Tween 20/1% BSA. This was followed by incubation of the membrane in primary anti-ghrelin antibody solution (1:500 dilution) at 4 °C overnight. After washing in TBS/Tween20, the membrane was incubated with an anti-rabbit secondary antibody (1:1000 dilution) (DAKO, Carpintera, CA) at room temperature for 1 h. After washing, a 1:10 dilution of Femto chemiluminescence solution (Pierce, Rockford, IL) was layered onto the membrane and incubated for 5 min. The membrane was then exposed to X-ray film overnight and then developed using the Curix 60 automatic processor (Agfa-Gavaert).

Cell proliferation assays

PC3 cells were seeded into 96-well plates at 10 000 cells/well. The cells were cultured for 3 days in the presence or absence of human mature *n*-octanoylated ghrelin (Mimotopes) at concentrations ranging from 0–20 nM. Ghrelin was dissolved in 1 XPBS (0·05% BSA) and added to RPMI 1640 medium containing 10% FCS. The negative controls received the same medium but without ghrelin. Medium was replaced every 24 h for both treated and untreated cells. After 72 h, the media were aspirated and the cells incubated in 0·5 mg 3-(4,5–dimethylthiazolyl-2yl)–2,5–diphenyl-tetrazolium (MTT)/serum free RPMI 1640 medium at 37 °C for 2 h. The MTT medium was then aspirated and the formazan salts solubilised in dimethyl sulfoxide (ICN, Costa Mesa, CA). A multi-well Biomek plate reader (Beckman, Brisbane, Australia) measured absorbances at 450 nm, with a reference wavelength of 650 nm.

Results

Single RT-PCR products of the expected size for GHS-R 1a (349 bp), GHS-R 1b (209 bp) and ghrelin (264 bp) were generated from cDNA derived from the ALVA41, DU145, LNCaP and PC3 prostate cancer cell lines (Fig. 1). Southern analysis of these products detected signals corresponding to the expected size (data not shown) and their identity was confirmed by automated sequencing with all products 100% homologous to published sequences. Normal prostate cDNA library expressed the GHS-R 1a mRNA isoform but not the GHS-R 1b isoform or ghrelin. In addition to the β-actin screen (data not shown), RNA was Dnase-treated prior to reverse transcription to ensure that GHS-R 1b RT-PCR products were derived from cDNA and not genomic DNA, as the 3′ end of the GHS-R 1b mRNA transcript contains a short intronic sequence (Howard *et al.* 1996).

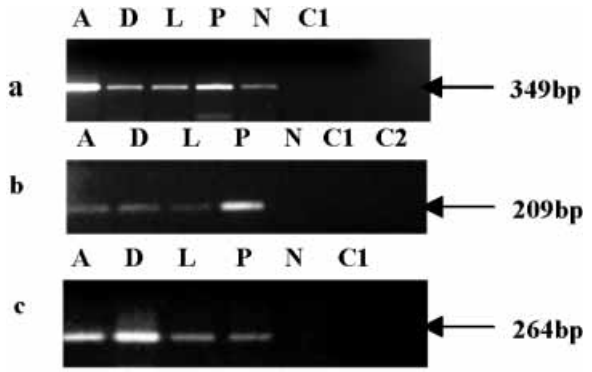

Figure 1 (a) GHS-R 1a and (b) GHS-R 1b and (c) ghrelin RT-PCR products amplified from the ALVA41 (A) DU145 (D) LNCaP (L) and PC3 (P) prostate cancer cell lines and normal prostate cDNA library (N). C1 = no template negative control, C2 = GHS-R 1b-representative PCR performed on ALVA41 RNA preparation prior to reverse transcription.

RT-PCR using primers for the full length preproghrelin sequence also identified a novel transcript with a complete exon 3 deletion (data not shown).

Positive immunohistochemical staining for GHS-R 1a (Fig. 2a-d) and ghrelin (Fig. 2f-i) was present in the cytoplasm of all the prostate cancer cell lines tested, providing evidence that these cells synthesise the GHS-R and ghrelin proteins. Primary antibody-free negative controls (Fig. 2e) and pre-absorption controls (data not shown) failed to stain for either antibody.

Western analysis of cell lysates detected a single band at 3 KDa in each of the four prostate cancer cell lines (Fig. 3). These bands were exactly the same size as a concurrently run sample of synthetic, *n*-octanoylated mature (28 amino acid) ghrelin peptide (Mimotopes). No other bands were observed, indicating that the anti-ghrelin antibody used in the immunohistochemical studies detects only mature ghrelin in these prostate cancer cell lines.

Incubation of PC3 cells with ghrelin over a 3 day period resulted in increased cell proliferation as compared with untreated controls (Fig. 4). This dose-related increase peaked at 5 nM ghrelin where viable cell numbers increased to 33% above untreated controls.

Discussion

This is the first study to report the expression of ghrelin mRNA, the type 1a and 1b GHS-R mRNA isoforms and ghrelin and GHS-R protein in human prostate cancer cell lines. In addition, we have demonstrated the expression of GHS-R 1a mRNA expression in a normal prostate cDNA library. This study also provides functional evidence that ghrelin may have an autocrine/paracrine role in

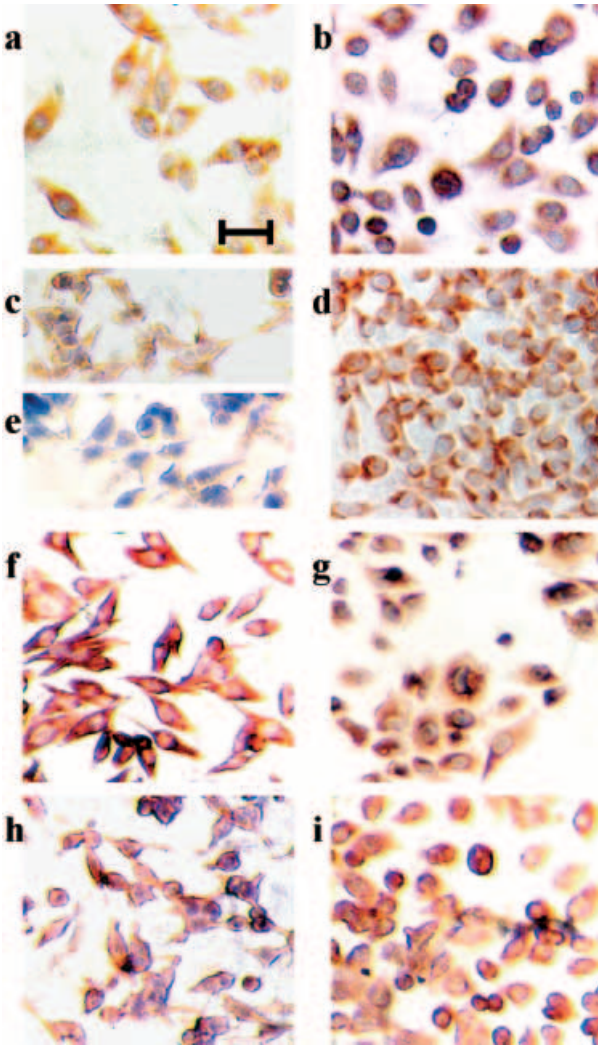

Figure 2 Immunohistochemistry performed on ALVA41 (a,f), DU145 (b,g), LNCaP (c,h) and PC3 (d,i) cells using GHS-R 1a antibodies (a-d) and ghrelin antibodies (f-i). Positive staining is indicated by the brown cytoplasmic staining. All cell nuclei are non-immunoreactive. Representative ghrelin negative control (e) demonstrates lack of immunoreactivity in LNCaP cells. Scale bar=5 µm for each photomicrograph.

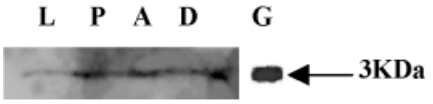

Figure 3 Western immunoblot of cell lysates from LNCaP (L), PC3 (P), ALVA41 (A) and DU145 (D) prostate cancer cell lines (24 h exposure to X-ray film) using anti-human ghrelin antibody showing bands of approximately 3 KDa, identical to *n*-octanoylated ghrelin (1 µg, lane G) which was exposed for a shorter time (5 min).

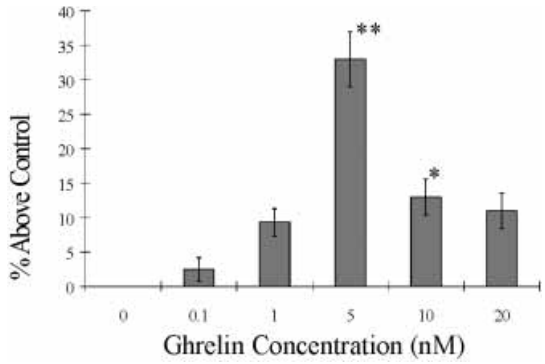

Figure 4 Effect of ghrelin on PC3 cell proliferation, as determined using the MTT dye method. Absorbance readings for each treatment were converted to percentages above control ± S.E.M., indicated by error bars. * denotes P values<0·01, ** P<0·001 (one-way ANOVA with Tukey's post hoc comparisons). Data represent one of 3 identical experiments, each n=16.

stimulating prostate cancer cell proliferation. GHS-R expression has been shown previously in the pituitary and hypothalamus (Howard *et al.* 1996), in neuroendocrine tumours (de Keyzer *et al.* 1997) and *in vitro* in rat pituitary tumour cells (Adams *et al.* 1998), but not in prostate cancer. GHS-R overexpression may be associated with tumourigenesis in some tissues, as GHS-R mRNA expression is 200 fold higher in somatotroph tumours than in normal pituitary tissue (Skinner *et al.* 1998). The type 1a and 1b GHS-R isoforms are co-expressed in central nervous system tumours (Korbonits *et al.* 1999) and in the prostate cancer cell lines studied here. A normal prostate cDNA library did not express GHS-R 1b transcripts and this may represent a difference between the normal and cancerous state. Although no function has yet been ascribed to the type 1b GHS-R, given the conservation of the intron-derived coding sequence between human and swine type 1b cDNAs, the 1b isoform may actually have functional significance in some tissues (Van der Ploeg 1998). The role of the 1b receptor in prostate cancer needs to be further elucidated, and its differential expression may represent a novel marker for prostate cancer.

Ghrelin mRNA expression has been demonstrated in rodent brain and some rodent peripheral tissues and in human stomach (Kojima *et al.* 1999). To our knowledge, no previous studies have examined the expression or function of ghrelin in prostate cancer. In this study, ghrelin mRNA expression was demonstrated by RT-PCR in the prostate cancer cell lines, but not in a normal prostate cDNA library. This could reflect a very low abundance of ghrelin mRNA in normal tissue and a relative over-expression of ghrelin in prostate cancer. Interestingly, an mRNA variant of preproghrelin, with a complete exon 3 deletion, was amplified in all of the prostate cancer cell lines. The physiological relevance of this variant is currently unknown, however, other ghrelin variants have been described. Sequence variations in the ghrelin gene may play a role in the etiology of obesity in humans (Ukkola *et al.* 2001), and a testis-specific and developmentally-induced ghrelin gene-derived transcript (GGDT) has been reported (Tanaka *et al.* 2001).

Ghrelin significantly increases the proliferation of prostate cancer cells *in vitro*. This finding implies that ghrelin activates the GHS-R 1a receptor to induce proliferation, either by the autocrine action of secreted prostatic GH (Chopin *et al.*, in press) or by some other more direct signalling mechanism. The bell-shaped proliferation response curve demonstrated by the PC3 cells in response to ghrelin treatment could be a result of down-regulation of GHS-R expression by prostatic GH, as GHS-Rs in the rat hypothalamus are down-regulated by GH (Bennet *et al.* 1997).

The discovery that ghrelin induces a proliferative response in PC3 cells is the first demonstration of such a role for ghrelin in this cell type. This finding is pertinent given that the GHRH-GH-IGF axis is a promising target for anti-tumour therapies for GH responsive cancers. Blockade of the GHS-R receptor and/or the inhibition of ghrelin activity could provide future targets for the development of cancer therapies. The use of GHS treatment in the aged, where there is a decline in GH secretion, has been proposed (Fuh and Bach 1998). In light of the present study, caution is indicated regarding the use of GHSs and ghrelin in medical and non-medical settings, particularly in the aging male.

In conclusion, this is the first study to demonstrate the co-expression of ghrelin and the GHS-R in prostate cancer cells and the first to provide evidence that a previously unrecognised prostatic autocrine pathway involving ghrelin is capable of stimulating growth of prostate cancer cells *in vitro*.

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