Expression and function of the ghrelin axis, including a novel preproghrelin isoform, in human breast cancer tissues and cell lines

P L Jeffery, R E Murray, A H Yeh, J F McNamara, R P Duncan, G D Francis, A C Herington and L K Chopin

Ghrelin Research Group, Hormone-dependent Cancer Programme, School of Life Sciences, Queensland University of Technology, PO Box 2434, Brisbane 4001, Australia

(Requests for offprints should be addressed to P Jeffery; Email: p.jeffery@student.qut.edu.au)

Abstract

While oestrogen, progesterone and growth factors, including growth hormone (GH), are clearly implicated in the pathogenesis of breast cancer, there is now evidence that the newly described ghrelin axis is also involved. The aims of this study were to investigate the expression of the ghrelin axis in breast cancer tissues and cell lines and to examine the effect of ghrelin on breast cancer cell proliferation *in vitro*. Ghrelin and its functional receptor, the growth hormone secretagogue receptor (GHSR) type 1a, were expressed in normal breast tissue and breast cancer specimens and cell lines. In contrast, the truncated GHSR type 1b isoform was exclusively expressed in breast carcinoma, suggesting that it has potential as a diagnostic marker. Ghrelin treatment significantly increases the proliferation of the MDA-MB-435 and MDA-MB-231 breast cancer cell lines *in vitro*. In addition, we have described the expression of a human preproghrelin isoform, exon 3-deleted preproghrelin, which encodes mature ghrelin plus a novel C-terminal peptide. Quantitative RT-PCR was used to demonstrate that this mRNA isoform is highly expressed in the MDA-MB-435 metastatic breast cancer cell line relative to the benign MCF-10A breast epithelial cell line. The unique C-terminal peptide of exon 3-deleted preproghrelin is expressed in the glandular epithelium of breast cancer tissues, with high-grade carcinoma exhibiting the strongest immunoreactivity. The data presented here suggest that components of the ghrelin axis may represent novel markers for breast cancer and potential therapeutic targets.

Endocrine-Related Cancer (2005) 12 839–850

Introduction

Breast cancer is the most commonly diagnosed malignancy in Western women (Jemal *et al.* 2004) and is a significant cause of mortality and morbidity. Ovarian hormones and their receptors are essential for normal mammaryogenesis and the initiation and progression of breast cancer (Anderson & Clarke 2004). Growth factors, however, also influence normal and pathological breast development (Laban *et al.* 2003), and there is emerging evidence that the endogenous growth hormone secretagogue, ghrelin, plays a role in breast cancer.

Mature ghrelin, a 28 amino acid peptide hormone, is post-translationally modified to give rise to a unique *n*-octanoylated peptide, which stimulates growth hormone (GH) release (Kojima *et al.* 1999). The cognate receptor for ghrelin, the growth hormone secretagogue receptor (GHSR), has two documented splice variants, producing the functional type 1a receptor and a truncated type 1b receptor (Howard *et al.* 1996). A non-acylated form of ghrelin (des-acyl ghrelin) does not activate the GHSR or induce GH release (Hosoda *et al.* 2000) but appears to have actions which are independent of the GHSR (Ariyasu *et al.* 2005). We have recently characterised an exon 4-deleted

preproghrelin mRNA isoform in mouse tissues, which is translated to produce a truncated proghrelin peptide possessing a unique carboxy terminus (Jeffery *et al.* 2005), and we have previously identified a homologous human isoform (exon 3-deleted preproghrelin) in prostate cancer cell lines (Jeffery *et al.* 2002, 2003).

Ghrelin is ubiquitously expressed in the body, suggesting that it possesses wide-ranging physiological functions, aside from stimulation of GH release. Its actions include a role in appetite regulation and obesity (Wren *et al.* 2001), while the evidence regarding its influence on adipogenesis is contradictory (Zhang *et al.* 2004a). Energy homeostasis is directly associated with reproductive function and the importance of ghrelin in a range of reproductive processes, including steroid hormone regulation and embryo development, has recently been demonstrated (Barreiro & Tena-Sempere 2004).

Ghrelin expression has been detected in many endocrine tumour types (Kanamoto *et al.* 2001, Korbonits *et al.* 2001, Papotti *et al.* 2001, Iwakura *et al.* 2002, Arnaldi *et al.* 2003, Corbetta *et al.* 2003, Volante *et al.* 2003, Tsolakis *et al.* 2004) and hormone-dependent cancers including testicular tumours (Gaytan *et al.* 2004). At the functional level, *n*-octanoylated ghrelin treatment increases the rate of proliferation in a number of cell lines, including human prostate cancer (Jeffery *et al.* 2002), HepG2 hepatoma (Murata *et al.* 2002) and pancreatic adenocarcinoma cancer cell lines (Duxbury *et al.* 2003), rodent pituitary tumour cells (Nanzer *et al.* 2004), as well as some normal cell lines and primary cell cultures (Pettersson *et al.* 2002, Andreis *et al.* 2003, Kim *et al.* 2004, Mazzocchi *et al.* 2004, Zhang *et al.* 2004b, Maccarinelli *et al.* 2005). There is evidence to suggest that a similar system could exist in female hormone-dependent cancers, such as breast cancer. While normal human breast tissue does not exhibit specific binding sites for synthetic growth hormone secretagogues (Papotti *et al.* 2000), binding activity has been described in neoplastic breast tissues and in breast cancer cell lines (Cassoni *et al.* 2001). However, GHSR 1a expression has not been demonstrated in these cell lines.

Ghrelin treatment resulted in an anti-proliferative response in these cell lines *in vitro*, although this occurred at relatively high (1000 nM) concentrations (Cassoni *et al.* 2001). This contrasts with our previous studies in prostate cancer cell lines, where proliferation was maximally stimulated at 10 nM ghrelin and inhibition of proliferation was not observed (Jeffery *et al.* 2002).

The present study aimed to investigate (a) the expression of the ghrelin axis, including the novel

exon 3-deleted preproghrelin isoform, in breast cancer cell lines and tissues and (b) the effect of ghrelin treatment on breast cancer cells *in vitro*, in order to determine if an autocrine/paracrine pathway exists to promote cell growth in breast cancer, as appears to be the case in prostate cancer (Jeffery *et al.* 2002).

Materials and methods

Cell culture

Oestrogen-dependent breast cancer cell lines T47D and MCF-7, the oestrogen-independent cell lines MDA-MB-231 and MDA-MB-435, and the transformed benign breast epithelial cell line, MCF-10A (Soule *et al.* 1990), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). While microarray studies showing that the MDA-MB-435 cell line clustered with melanoma cells (Ellison *et al.* 2002) had previously cast doubt on the derivation of this cell line, MDA-MB-435 cells are indeed a breast epithelial cell line and not derived from occult melanoma (Sellappan *et al.* 2004). All cell lines were cultivated in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 10% foetal calf serum (FCS; Invitrogen), 50 U/ml penicillin, 50 µg/ml streptomycin and 5 µg/ml gentamicin (Invitrogen) and grown in tissue culture flasks (Nagle Nunc International, Roskilde, Denmark) in a 5% CO₂ atmosphere at 37°C. Cells were free of *Mycoplasma* infection (ATCC Mycoplasma detection kit).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

On reaching 70% confluency, cultured cells were collected and total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions, and then treated with DNase (DNA free, Ambion, Austin, TX, USA). For reverse transcription, 0.5 µg/µl oligo dT18 primer was added to 5 µg total RNA and incubated for 10 min at 70°C. First strand buffer, to a final concentration of 50 mM TrisCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol (Invitrogen) and 300 µM of each dNTP (pH 7; Roche, Basel, Switzerland), was added and incubated with 10 units/µl SuperScript RT II (Invitrogen) at 43°C for 90 min. In order to test for genomic DNA contamination (critical for the valid detection of GHSR 1b, which contains intronic sequence) and to check the integrity of the cDNA, RT-PCR for β₂ microglobulin was performed (Table 1). Normal human breast and stomach RNAs were purchased from Clontech (Palo Alto, CA, USA). For real-time

Table 1 Primer sequences, expected amplicon sizes (bp) and annealing temperatures (Ta) for RT-PCR

RT-PCR primer pair	Primer sequence	Size (bp)	Ta (°C)
Full-length preproghrelin			
sense	5'GCCCACCTGTCTGCAACC3'	501	50
antisense	5'TGAACATTTATTGCGCTCCTG3'		
Exon 3-deleted preproghrelin			
sense	5'AACTGGAAGTCCGGAGGCCCC3'	151	62
antisense	5'GAACATTTATTGCGCTCCTG3'		
GHSR 1a			
sense	5'TCTTCCTTCCTGTCTTCTGTC3'	349	50
antisense	5'AGTCTGAACACTGCCACC3'		
GHSR 1a nested			
sense	5'ACCAGAACCACAAGCAAACC3'	192	50
antisense	5'TGATGGCAGCACTGAGGTAG3'		
GHSR 1b			
sense	5'TCTTCCTTCCTGTCTTCTGT3'	179	55
antisense	5'GATAGGACCCGCGAGAGAAA3'		
β ₂ microglobulin			
sense	5'TGAATTGCTATGTGTCTGGGT3'	248	50
antisense	5'CCTCCATGATGCTGCTTACAT3'		

Table 2 Primer sequences, expected amplicon sizes (bp) and annealing temperatures (Ta) for quantitative real-time RT-PCR

qRT-PCR primer pair	Primer Sequence	Size (bp)	Ta (°C)
Full-length preproghrelin			
sense	5' CCCTTTGATGTTGGAATCAAGCT 3'	106	60
antisense	5' GGGCCTCTTTGGCCTCTTC 3'		
Exon 3-deleted preproghrelin			
sense	5' CCAGCAGAGAAAGGAGTCAAGA 3'	126	60
antisense	5' GGCCTCCGACTTCCAGTT 3'		
18S rRNA			
sense	5' TTCGGAAGTGAAGCCATGAT 3'	151	60
antisense	5' CGAACCTCCGACTTTCGT 3'		

RT-PCR, reverse transcription was performed on RNA using the AMV first-strand cDNA synthesis kit (Invitrogen) as previously described (Jeffery *et al.* 2005).

RT-PCR was performed to investigate the expression of preproghrelin, exon 3-deleted preproghrelin and the type 1a and 1b transcripts of the GHSR in the MCF-7, MDA-MB-231, MDA-MB-435 and T47D breast cancer cell lines, and in the positive control tissue, human stomach. To detect preproghrelin mRNA transcripts, RT-PCR was performed using primer pairs specific for the full-length preproghrelin transcript and for exon 3-deleted preproghrelin (Table 1). For the detection of GHSR 1a, nested PCR was performed. The outer primers were used in the first reaction and 5 µl of this product was reamplified using the nested primers (Table 1). As GHSR 1b cDNA is identical to the genomicDNA, RNA was treated with DNase prior to reverse

transcription and a 'no reverse transcription' control was included. In all PCRs, a negative 'no template' control was also included. PCRs contained PCR buffer, 2.5 mM MgCl₂, 300 µM of each dNTP, 2 µl cDNA, 1 unit Red Hot Polymerase (Integrated Sciences, Melbourne, Australia) and relevant PCR primers (Proligo, Armidale, Australia) (Table 1). Thermal cycling, performed using a PTC-200 DNA engine (MJ Research, Watertown, MA, USA) consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, annealing temperature (Table 1) for 30 s and 72°C for 2 min and a final extension of 72°C for 10 min. PCR products were excised from the gel and purified (Concert Rapid Gel PCR purification kit, Invitrogen). Automatic sequencing was undertaken at the Australian Genome Research Facility (Brisbane, Australia) using Applied Biosystems Big Dye 3.1 (Applied Biosystems, Foster City, CA, USA).

Real-time quantitative RT-PCR

The Applied Biosystems (ABI) 7000 Prism Real Time PCR Sequence detection system was used to determine relative expression levels of full-length preproghrelin and exon 3-deleted preproghrelin, in the epithelial cell line MCF-10A and in the MCF-7, MDA-MB-231 and MDA-MB-435 breast cancer cell lines. All primers were designed using the Primer Express version 2.0 software (ABI), synthesised by Proligo (Table 2), and validated to ensure that the efficiencies of the target and reference genes were similar (User Bulletin #2, ABI Prism 7000 Sequence detection system). Each PCR contained 10 µl 2×SYBR[®] green master mix (ABI), 200 nM of each primer and 5 µl cDNA. All PCRs were performed in triplicate and normalised against the reference primer set (18S rRNA, Table 2). Data were analysed using the comparative C_T method (User Bulletin #2, ABI) and were represented as fold-change as compared with the calibrator cell line (MCF-10A).

Antibodies and tissue samples

Polyclonal anti-GHSR 1a, anti-GHSR 1b, anti-ghrelin and anti-exon 3-deleted preproghrelin antibodies were raised in rabbits (Institute for Medical and Veterinary Sciences, Adelaide, Australia) against peptide fragments synthesised by Mimotopes (Melbourne, Australia). Peptide fragments for GHSR 1a (RAWTESSINTC) (Feighner *et al.* 1998), GHSR 1b (GGSQRALRL-SLAGPILSLC), mature *n*-octanoylated ghrelin (GSSFLSPEHQVRVQQRKESKKPPAKLQPRC) and the unique C-terminal peptide derived from exon 3-deleted preproghrelin (RPQPTSDRPQALLTSLC) were conjugated to diphtheria toxin (Mimotopes) and antibodies raised separately in rabbits. Rabbit serum was affinity purified and antibodies were characterised by ELISA and Western analysis (Jeffery *et al.* 2002). The specificity of each antibody was also tested with stringent controls, including pre-absorption of each antibody with the peptide it was raised against.

Breast cancer tissue sections were derived from excess surgical specimens from biopsies for pathological diagnosis and grading at Queensland Medical Laboratory (QML, Brisbane, Queensland, Australia), and were obtained in accordance with ethical guidelines (Queensland University of Technology ethics approval # 2820H). Normal breast, pituitary and stomach tissue sections were obtained from the Peterborough Hospital Tissue Bank (Peterborough, UK).

Immunohistochemistry on cell lines and tissues

Immunohistochemistry on breast cancer cell lines was performed as described previously (Jeffery *et al.* 2002). Briefly, cells were grown to 50–70% confluency in 96-well plates (Nagle Nunc), washed in PBS and fixed for 5 min in ice-cold 100% methanol. Cells were washed with 1% H₂O₂ in 50% methanol, followed by 0.3% Triton X in PBS and incubated with 1% bovine serum albumin (BSA) (Sigma-Aldrich Corp., St Louis, MO, USA). Purified ghrelin antibody was diluted 1/10, exon 3-deleted preproghrelin antibody was diluted 1/100, GHSR 1a antibody was diluted 1/50–1/100 and GHSR 1b antibody was diluted 1/50–1/100 in 0.01 M PBS with 1% BSA, and incubated with cells overnight. Immunohistochemistry was also performed on normal breast tissue (Peterborough Hospital Tissue Bank) and on 23 breast cancer tissue sections of clinical grades 1–3, as described above, using an Envision Plus DAB anti-rabbit immunostaining kit (Dako, Kyoto, Japan) according to the manufacturer's instructions. Human pituitary sections for GHSR 1a and GHSR 1b and human stomach for ghrelin (Peterborough Hospital Tissue Bank) were used as positive controls. For all immunohistochemical experiments, negative controls were performed by substituting immune sera with 1% BSA in 0.01 M PBS and also by pre-absorbing each antibody overnight with 1 mg/ml peptide to which the antibody was raised. Sections were counterstained with haematoxylin (Australian Chemical Reagents, Moorooka, Australia).

Cell proliferation assays (Wst-1 assays)

MDA-MB-231, MDA-MB-435, MCF-7 and MCF-10A cell lines were cultured in 96-well plates (Nagle Nunc) for 72 h at 37°C in the presence of *n*-octanoylated ghrelin peptide (Mimotopes) at concentrations ranging from 0 to 1000 nM. The culture medium contained 20 nM 4-(2-aminoethyl) benzene-sulphonyl fluoride hydrochloride (AEBSF, Sigma-Aldrich), a serine protease inhibitor that protects ghrelin from inactivation (Katugampola *et al.* 2001). Medium was changed daily, with controls receiving no treatment. At the completion of the 4-day protocol, cells were incubated for 2 h in Wst-1 reagent (Roche) at 37°C and cell proliferation was quantified by measuring photometric absorbance at 450 nm (BMG LABTECH, Offenburg, Germany). Each experiment provided 16 replicates and the experiment was repeated three times.

Statistics

Statistical analyses were undertaken using SPSS software (SPSS, Chicago, IL, USA). All numerical data are presented as means±standard error of the mean (S.E.M.). Statistical significance was determined using one-way analysis of variance (ANOVA) with Bonferoni post-hoc analysis, with a *P* value <0.05 considered to be statistically significant.

Results

Expression of the ghrelin axis in breast cancer (RT-PCR)

Expression of the ghrelin axis was investigated in the MDA-MB-231, MCF-7, MDA-MB-435 and T47D breast cancer cell lines with RT-PCR (Fig. 1). In each cell line, a cDNA product of the expected size for the full-length preproghrelin transcript (501 bp) was observed (Fig. 1A). The exon 3-deleted variant of the preproghrelin transcript was also amplified in all of the cell lines tested (392 bp; Fig. 1A). Using RT-PCR with primers specific for the exon 3-deleted preproghrelin transcript (Table 1), with the sense primer being designed to cross the unique exon 2/4 boundary, this variant was also demonstrated in all cell lines (Fig. 1B). Preproghrelin mRNA and the exon 3-deleted transcript were also detected by RT-PCR in the MCF-10A cell line and in normal breast cDNA (data not shown). mRNA for both GHSR isoforms (types 1a and 1b) was detected by RT-PCR in the MDA-MB-231, MCF-7, MDA-MB-435 and T47D cell lines (Fig. 1C

Figure 1 Representative ethidium bromide-stained agarose gels of RT-PCR products from breast cancer cell lines. (A) RT-PCR demonstrating the mRNA expression of full-length preproghrelin (501 bp) in the MDA-MB-231, MCF-7, MDA-MB-435 and T47D cell lines. The lower band (392 bp) represents the exon 3-deleted mRNA variant. (B) Using primers and PCR conditions specific for the exon 3-deleted preproghrelin, PCR product was detected in the MDA-MB-231, MCF-7, MDA-MB-435 and T47D cell lines. (C) GHSR type 1a was also expressed in all cell lines tested, as was the truncated GHSR 1b isoform (D). All transcripts were amplified in the positive control tissue, human stomach. (E) β_2 microglobulin screening confirmed cDNA purity and quality.

and D), producing PCR products of expected sizes (192 and 179 bp respectively). All transcripts were detected in the stomach positive control and the RT-PCR for β_2 microglobulin was also positive (Fig. 1E). The identities of all transcripts were confirmed by cDNA sequencing.

Translation of exon 3-deleted preproghrelin mRNA (Fig. 2A) results in a truncated preproghrelin, which

Figure 2 Complete coding sequence of the human exon 3-deleted preproghrelin transcript. (A) Open reading frame of exon 3-deleted preproghrelin with the mature ghrelin peptide sequence underlined (accession # AY184207). The shaded type indicates nucleotide and amino acid sequence divergence from full-length preproghrelin and the dibasic motif is boxed. Numbers indicate nucleotide locations and amino acid locations. (B) The nucleotide and peptide sequence of the corresponding C-terminal region of full-length preproghrelin (accession # AB029434), displaying the difference between the two isoforms.

Figure 3 Real-time quantitative RT-PCR showing relative mRNA expression levels of (A) full-length and (B) exon 3-deleted preproghrelin in MCF-7, MDA-MB-231 and MDA-MB-435 cell lines. Full-length and exon 3-deleted preproghrelin mRNA levels in the MCF-10A cell line were normalised to 1. Data were expressed relative to the MCF-10A cell line mRNA levels and are represented as means $\pm$ S.E.M. ($n=3$ per cell line, assays performed in triplicate). ** $P<0.01$ relative to the calibrator cell line (MCF-10A).

possesses a unique C-terminal tail composed of 16 amino acids and a dibasic (RR) motif which is not present in the corresponding full-length preproghrelin sequence (Fig. 2B). Polyclonal antibodies were raised against the variant's unique peptide tail and used to determine protein expression in breast cancer cell lines and tissues.

Quantitation of full-length preproghrelin and the exon 3-deleted preproghrelin mRNA transcripts in breast cell lines by real time RT-PCR

Expression of full-length preproghrelin mRNA and the novel exon 3-deleted preproghrelin variant mRNA was quantitated in the MCF-10A, MCF-7, MDA-MB-231 and MDA-MB-435 cell lines by real-time quantitative RT-PCR analysis. For both transcripts, expression levels in the calibrator cell line (the benign breast epithelial cell line, MCF-10A) were set to 1 (Fig. 3). The relative expression level of full-length preproghrelin mRNA in the MCF-7 and the MDA-MB-231 cell lines was not significantly different to expression in the MCF-10A cell line ($P>0.05$). Although the MDA-MB-435 cell line displayed a 2.7-fold increase in full-length preproghrelin transcript levels over the MCF-10A cell line (Fig. 3A), this was not statistically significant ($P>0.05$).

The novel exon 3-deleted preproghrelin transcript was detected in all the breast cancer cell lines tested by real-time quantitative RT-PCR (Fig. 3B). There was no significant difference in the level of expression of the exon 3-deleted preproghrelin mRNA variant between the MCF-10A cell line and the MCF-7 cell line ($P>0.05$) (Fig. 3B); however, transcript was significantly elevated in the MDA-MB-231 cell line

compared with the MCF-10A cell line ($P=0.003$) (Fig. 3B), and expression of the novel mRNA isoform was 11.2-fold higher in the aggressive, metastatic MDA-MB-435 cell line than in the non-tumourigenic MCF-10A cell line ($P=0.001$) (Fig. 3B).

Immunohistochemistry on breast histological sections

Ghrelin immunoreactivity was observed at very low levels in the normal breast epithelium, with moderately higher levels of staining in all of the 23 breast cancer samples stained within the same immunohistochemistry experiment (Fig. 4A,B,C). Immunohistochemistry, using the anti-exon 3-deleted preproghrelin antibody specific to the C-terminal tail of the isoform, revealed weak staining of the epithelial cells of normal breast glands (Fig. 4D). A greater intensity of specific staining for this isoform was observed in grade 1 carcinoma (Fig. 4E) and grade 2 carcinoma (Fig. 4F) when compared with normal breast epithelium (Fig. 4D). The highest level of exon 3-deleted preproghrelin immunoreactive staining, indicating high protein expression of the unique isoform, was detected in grade 3 breast carcinoma specimens (Fig. 4G). Up-regulated expression of the isoform was observed in all breast cancer tissues examined. Cytoplasmic GHSR 1a immunoreactivity was observed in normal breast tissue (data not shown) and in the glandular epithelium of grades 1, 2 and 3 breast cancers (Fig. 4I,J,K). The GHSR type 1b isoform was highly expressed in the epithelium of all breast cancer tissues examined, as demonstrated by intense immunostaining in a representative sample (Fig. 4L), whilst normal breast tissues failed to show specific immunoreactivity,

Figure 4 Representative immunohistochemical localisation of the ghrelin/GHSR axis in normal breast and breast cancer tissues. (A) Ghrelin immunoreactivity was present in normal breast where there were low levels of staining. (B,C) Higher levels of immunoreactivity were seen in low-grade carcinoma samples stained at the same time. (D) Low levels of immunoreactivity for the exon 3-deleted preproghrelin C-terminal peptide were demonstrated in normal breast tissue and (E) grade 1 carcinoma, whilst higher levels were seen in (F) grade 2 carcinoma and (G) high-grade (grade 3) breast cancer. (H) Representative negative control for GHSR 1a where the primary antiserum was omitted. (I) GHSR 1a staining was present in breast cancer grade 1, (J) grade 2, and (K) grade 3 breast cancer specimens. (L) GHSR 1b immunostaining was strong in breast cancer tissues; however, there was no specific staining for GHSR 1b in (M) normal breast tissue. Three independent tissue samples were stained for each antibody and tumour type, in at least three experiments each. Scale bars represent 100 μ m in A, D, H, I and M and 50 μ m in B, C, E, F, G and J-L.

Figure 5 Effect of *n*-octanoylated ghrelin on proliferation of MDA-MB-231 and MDA-MB-435 breast cancer cells *in vitro*. (A) Wst-1 proliferation assay performed on the MDA-MB-231 breast cancer cell line showing an increase in cell growth after treatment with increasing concentrations of ghrelin for 72 h. Proliferation, represented as percentage absorbance above control, is significantly increased with 10 and 100 nM ghrelin treatments ($P < 0.05$). (B) The MDA-MB-435 cell line proliferated faster in the presence of 0.1 nM ghrelin ($P < 0.05$), and in the presence of 1, 10 and 100 nM ghrelin ($P < 0.01$), than in controls. All data represent the mean $\pm$ S.E.M. of 3 experiments ($n = 16$ per treatment). * $P < 0.05$, ** $P < 0.01$ relative to controls.

suggesting that the GHSR type 1b isoform is not translated in normal breast tissue (Fig. 4M). The stromal tissue in the breast did not exhibit specific staining and negative controls also failed to show immunoreactivity for all antibodies used in this study (see Fig. 4H). Immunohistochemistry also demonstrated protein expression of the ghrelin/GHSR axis in breast cancer cell lines (data not shown).

Wst-1 proliferation assays

Cell proliferation assays were performed in the MDA-MB-231, MDA-MB-435, MCF-7 and MCF-10A breast cancer cell lines with the addition of a range of concentrations of ghrelin (0–1000 nM) for 72 h. Growth rate of the MDA-MB-231 cells was significantly increased by approximately 15% when treated with 10 nM *n*-octanoylated ghrelin ($P = 0.04$) and 17% when treated with 100 nM ghrelin ($P = 0.04$) when compared with controls receiving no ghrelin treatment (Fig. 5A). Ghrelin treatment also increased the rate of cell proliferation in the MDA-MB-435 cell line at 0.1 nM ($P = 0.04$), 1 nM ($P = 0.003$) and 10 nM ($P < 0.001$), with the greatest response seen at 100 nM ghrelin treatment (36% above control, $P < 0.001$) (Fig. 5B). Cell proliferation was not affected by ghrelin treatment (0–1000 nM) in the MCF-10A or MCF-7 cell lines (data not shown).

Discussion

The data presented here suggest that ghrelin plays a role in breast cancer by stimulating cell proliferation,

possibly via an autocrine/paracrine mechanism. This study is the first to report the expression of a new exon 3-deleted preproghrelin variant in the breast that is more highly expressed in breast cancer compared with normal breast tissues. We also report, for the first time, that GHSR 1b is differentially expressed in normal breast tissue compared with breast cancer specimens. We have demonstrated the expression of ghrelin, exon 3-deleted preproghrelin, and GHSR 1a and 1b in the MCF-7, MDA-MB-231, MDA-MB-435 and T47D breast cancer cell lines, in the nontumorigenic MCF-10A cell line and in a range of breast cancer tissues.

The potential tumour-promoting role of ghrelin is supported by our finding that ghrelin increases proliferation in breast cancer cell lines. Evidence provided here demonstrates that the addition of exogenous *n*-octanoylated ghrelin significantly stimulates proliferation in the highly metastatic MDA-MB-435 breast cancer cell line by up to 36% over controls and up to 17% above control in the oestrogen-independent MDA-MB-231 breast cancer cell line. An autocrine/paracrine role for ghrelin in a wide-range of cancers has previously been proposed (Jeffery *et al.* 2003). Acylated ghrelin has been shown to stimulate proliferation of cancer cell lines including prostate cancer (Jeffery *et al.* 2002), HepG2 hepatoma cells (Murata *et al.* 2002) and rat GH3 pituitary somatotroph tumour cells (Nanzer *et al.* 2004). Proliferation was also attenuated by up to 40% in the human erythroleukaemic HEL cell line after endogenously produced ghrelin was apparently neutralised with an anti-ghrelin antibody (De Vriese *et al.* 2005). This cell line secretes

substantial amounts of active ghrelin into culture medium (De Vriese *et al.* 2005), as does the TT thyroid carcinoma cell line (Kanamoto *et al.* 2001). Ghrelin may also have other roles in cancer, as ghrelin treatment increases the proliferative, migratory and invasive potential of pancreatic cancer cells. This potentially occurs via an autocrine/paracrine pathway, as these cells co-express ghrelin and the GHSR (Duxbury *et al.* 2003).

In contrast to our study, Cassoni and co-workers reported that treating MCF-7, MDA-MB-231 and T47D breast cancer cell lines with 1 μ M ghrelin significantly inhibited cell proliferation (Cassoni *et al.* 2001). We have demonstrated that the proliferative response to ghrelin increases at ghrelin concentrations of 10 and 100 nM in the MDA-MB-231 cell line, and 0.1–100 nM in the MDA-MB-435 cell line, which encompasses normal circulating ghrelin levels ($\sim$ 0.1–1 nM; Kojima *et al.* 1999, Pemberton *et al.* 2003, Groschl *et al.* 2004). As the octanoylated form of ghrelin is highly unstable in normal assay conditions, AESBF can be used to prevent the rapid deacylation of ghrelin (Katugampola *et al.* 2001). There is no evidence that ghrelin was protected in the Cassoni studies and, as a result, des-acyl ghrelin may be produced causing a negative impact on cell growth, as has been observed in the des-acyl overexpressing mouse (Ariyasu *et al.* 2005).

The mechanism underlying ghrelin-induced proliferation in breast cancer is unknown, although it is likely to act by stimulating the mitogen-activated protein kinase pathway, as demonstrated in our own studies in the prostate (Yeh *et al.* 2005) and in similar studies in HepG2 cells (Murata *et al.* 2002), cardiomyocytes (Baldanzi *et al.* 2002), adrenal cells (Andreis *et al.* 2003, Mazzocchi *et al.* 2004), 3T3-L1 adipocytes (Kim *et al.* 2004) and rodent GH3 cells (Nanzer *et al.* 2004). Alternatively, ghrelin could act by stimulating GH secretion from the breast epithelium. GH appears to contribute to breast carcinogenesis by a variety of means, including the autocrine stimulation of cell proliferation and survival (Kaulsay *et al.* 2001), and therefore targeting autocrine/paracrine GH pathways could benefit breast cancer patients (Waters & Conway-Campbell 2004). Enhancement of endogenous GH secretion may, at least partly, explain the proliferative effect of ghrelin on breast cancer cells *in vitro* that we have demonstrated here.

GHSR binding sites have been demonstrated previously in the MCF-7, MDA-MB-231 and T47D breast cancer cell lines, although the same study failed to demonstrate specific expression of GHSR type 1a mRNA using RT-PCR (Cassoni *et al.* 2001). In the

present study, however, nested RT-PCR amplified both known isoforms of the GHSR in these cell lines. Cytoplasmic protein expression of the functional receptor was also detected in breast cancer tissues of varying pathological grades by immunohistochemistry. The presence of both ghrelin and GHSR 1a in breast cancer tissues strengthens the proposition that ghrelin stimulates cell proliferation in the breast via an autocrine/paracrine mechanism.

The immunohistochemical and PCR data presented here demonstrate that GHSR 1b is highly expressed in breast cancer tissue with negligible expression in normal breast specimens. This is the first report of differential expression of the GHSR type 1b isoform in the breast and this finding suggests that GHSR 1b could represent a new marker for breast cancer. Similarly, GHSR 1b mRNA copy number is reported to be significantly higher in somatotroph adenomas compared with normal pituitary tissue (Korbonits *et al.* 2001), and mRNA for the GHSR type 1b isoform was not detectable in normal prostate cDNA, but abundantly expressed in prostate cancer cell lines (Jeffery *et al.* 2002). To date, there has been no function ascribed to this isoform of the receptor. In cells overexpressing the type 1b isoform, the GH secretagogue, MK-0677, failed to bind GHSR 1b or activate intracellular signalling through calcium release, as occurs with GHSR 1a (Howard *et al.* 1996). To our knowledge, binding and signalling studies have not been reported for the natural ligand, ghrelin.

Ghrelin is synthesised as a 117 amino acid prohormone, preproghrelin. The human ghrelin gene consists of 4 coding exons and a very recently identified non-coding exon located upstream of the coding region which corresponds to non-coding sequence in the mouse ghrelin gene (Nakai *et al.* 2004). However, we have based our nomenclature of the human preproghrelin variant on the coding exons only. The 28 amino acid mature ghrelin peptide is translated from the coding exon 1 and part of exon 2 (Kojima *et al.* 1999). The splicing out of exon 3, therefore, does not interrupt the coding region of mature ghrelin itself, but alters the C-terminus of proghrelin by the introduction of a frameshift and an early stop codon. Thus, the C-terminus of exon 3-deleted preproghrelin consists of a unique peptide sequence of 16 amino acids and begins with a dibasic motif (RR), which may allow cleavage of the tail (Fig. 2). We have recently reported the expression profile of an homologous murine preproghrelin variant (Jeffery *et al.* 2005), suggesting that this conserved peptide may serve a functional role within the ghrelin axis. Using real-time RT-PCR, the present study demonstrates the overexpression of

the exon 3-deleted isoform of preproghrelin in the MDA-MB-231 and MDA-MB-435 cell lines, which are derived from aggressive breast tumours, when compared with the benign MCF-10A cell line. Up-regulated expression of the exon 3-deleted mRNA variant may be associated with increased malignant potential in breast cancer cells, as occurs with other mRNA transcripts borne of cancer-specific alternative splicing (Venables 2004). Correspondingly, protein expression of the unique exon 3-deleted preproghrelin was up-regulated in breast cancer specimens when compared with normal tissue. This indicates that elevated expression of the novel peptide may play a functional role in breast pathogenesis. A recent study identified immunoreactivity for C-terminal ghrelin peptides derived from the last 66 amino acids of native proghrelin in human serum and it is speculated that these C-ghrelin peptides may have relevance in cardiovascular disease (Pemberton *et al.* 2003). Further studies are warranted to determine if the unique C-terminal peptide of exon 3-deleted preproghrelin is cleaved from the remaining proghrelin peptide and if it circulates in human plasma in substantial quantities.

In summary, this study has described the expression of the ghrelin axis, including a novel exon 3-deleted preproghrelin isoform, in breast cancer specimens and cell lines and reports functional evidence that the proliferative rate of breast cancer cell lines increases in response to ghrelin treatment. This suggests that blockade of the ghrelin axis may have some therapeutic benefit. Ghrelin, the novel exon-3 deleted preproghrelin isoform and the GHSR type 1b isoform also display potential as diagnostic/prognostic markers for breast pathogenesis.

Funding

This work was supported by grants from the National Health and Medical Research Council of Australia and by the Queensland University of Technology. No conflict of interest prejudices the impartiality of this manuscript.

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