

CLINICAL STUDY

Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines

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Abstract

Background: Ghrelin, a natural growth hormone secretagogue (GHS), has been identified in prostate carcinoma cell lines.

Objectives: To investigate the presence of ghrelin and its receptors in human prostate tumours and in DU-145, PC-3 and LNCaP prostate carcinoma cell lines, and to assess the effects of ghrelin and its more abundant circulating form, des-octanoyl ghrelin, on cell proliferation.

Methods: Ghrelin and types 1a and 1b GHS receptor (GHS-R) were determined at the mRNA and protein levels by RT-PCR, *in situ* hybridization, immunohistochemistry and enzyme immunoassay in tissues, cell lines and culture medium. Ghrelin binding was determined by radioreceptor assay. The effects on cell proliferation were evaluated by growth curves.

Results: Ghrelin mRNA was found in prostatic carcinomas and benign hyperplasias, but immunohistochemistry was negative. GHS-R1a and 1b mRNAs were absent from carcinomas, but GHS-R1b mRNA was present in 50% of hyperplasias. Ghrelin peptide and mRNA were present in PC-3 cells exclusively, whereas GHS-R1a and 1b mRNAs were expressed in DU-145 cells only. Specific [¹²⁵I]Tyr⁴-ghrelin binding was detected in prostate tumour, DU-145 and PC-3 cell membranes and the binding was displaced by ghrelin, synthetic GHS and des-octanoyl ghrelin, which is devoid of GHS-R1a binding affinity and GH-releasing activity. Ghrelin and des-acyl ghrelin inhibited DU-145 cell proliferation, displayed a biphasic effect in PC-3 cells and were ineffective in LNCaP cells.

Conclusions: Specific GHS binding sites, other than GHS-R1a and 1b, are present in human prostatic neoplasms. Ghrelin, in addition to des-acyl ghrelin, exerts different effects on cell proliferation in prostate carcinoma cell lines.

European Journal of Endocrinology 150 173–184

Introduction

Ghrelin is a gastrointestinal hormone, identified in the rat and human stomach, that stimulates the release of growth hormone (GH), gut motility and food intake and causes positive energy balance (1–3). It is an acyl peptide consisting of 28 amino acids and esterified with octanoic acid on Ser³, although a form of 27 amino acids, des-Gln¹⁴-ghrelin (resulting from alternative splicing of the same gene) has also been isolated (4). Both forms exist in either acylated and, more abundantly, des-acylated forms (5), but the latter have been shown to be inactive in terms of GH-releasing activity *in vivo* (6, 7). Ghrelin expression has also been described in the pituitary gland, the arcuate nucleus of the hypothalamus, kidney, placenta, endocrine pancreas, testis (8–13) and in various endocrine neoplasms of pituitary, gastrointestinal and pancreatic origin (12, 14, 15).

The effects of ghrelin are mediated by specific receptors shared by non-natural compounds of the GH secretagogue (GHS) family, such as hexarelin and MK-0677 (16, 17). Two types of GHS receptor (GHS-R) have been cloned: the GHS-R type 1a and its inactive isoform, GHS-R1b, which are widely distributed in different regions of the brain, pituitary gland and other peripheral organs (18–20). GHS-R1a mediates the classical GH-releasing effect, in addition to some non-endocrine activities, whereas various uncloned GHS binding sites seem to be involved in mediating other non-endocrine actions, the significance and relevance of which are currently poorly understood (3, 21). The latter include inhibition of cell growth in neoplastic cells, as observed in thyroid, breast and lung tumours (22–25). Interestingly, the different binding pattern of natural and synthetic GHS ligands in some non-tumoural and neoplastic tissues or cell lines seemed to suggest

the possible existence of specific GHS-R subtypes different from the GHS-R1a (3, 23, 26, 27). The recent findings that breast cancer cell lines, H9c2 cardiomyocytes and guinea pig heart do not express GHS-R1a, but have high-affinity binding sites, common for ghrelin and des-acyl ghrelin, that are involved in mediating their antiproliferative, cardioprotective and negative cardiac inotropic effects, provide further support to the hypothesis that several receptors for ghrelin and GHS exist in normal and neoplastic tissues (23, 26, 27). Each receptor may then contribute independently to the wide array of biological activities induced by synthetic GHS, ghrelin and endogenous ghrelin-derived molecules (3, 28).

At present, no data are available on the expression of ghrelin and GHS-R in human prostate carcinomas or in benign prostatic hyperplasias. However, a recent study reported the presence of ghrelin at both mRNA and peptide levels in various prostate carcinoma cell lines (29). This expression was accompanied by the presence of GHS-R1a. When the activity of ghrelin on cell proliferation was studied in one of the cell lines (PC-3), a mitogenic effect was found at low concentrations of the peptide (0.1–20 nmol/l). To clarify a possible autocrine/paracrine role of ghrelin in prostatic tumours, the effect of this hormone, and that of its endogenous des-acylated form, on the proliferation of cultured prostatic carcinoma cells should be validated by using a wide range of concentrations of ghrelin and by examining the expression of ghrelin and GHS-R on benign and malignant human prostatic neoplasms.

On the basis of the foregoing, the aims of the present study were first to investigate the expression of ghrelin in benign (hyperplastic) and malignant (carcinomatous) prostatic tissues and in one androgen-dependent (LNCaP) and two androgen-independent (DU-145 and PC-3) prostatic carcinoma cell lines. In addition, in the above cells and tissues, the presence of GHS-R was assessed either by RT-PCR or by binding studies. Finally, we studied the effects of ghrelin, des-octanoyl ghrelin and some synthetic peptidyl (hexarelin) and non-peptidyl (MK-0677) GHSs on the proliferation of DU-145, PC-3 and LNCaP human prostate carcinoma cell lines *in vitro*.

Materials and methods

Chemicals

Human ghrelin_(1–28), ghrelin (human) enzyme immunoassay kit and anti-human ghrelin polyclonal antibody were purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Hexarelin, MK-0677 and Tyr⁴-ghrelin_(1–28) were purchased from Neosystem SA (Strasbourg, France), and growth hormone-releasing hormone (GHRH)_(1–29) and somatostatin releasing-inhibiting factor (SRIF)_(1–14) were from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

[¹²⁵I]Tyr⁴-ghrelin (1800–2100 Ci/mmol) and [³H]thymidine (specific activity 2000 Ci/mmol) were purchased from Amersham Biosciences. Ghrelin was radioiodinated using a lactoperoxidase method and purified by reverse-phase high-performance liquid chromatography. Insulin-like growth factor (IGF)-I, penicillin, streptomycin, FCS, trypsin/EDTA solution and other tissue culture reagents were purchased from Sigma Aldrich Srl.

Tissue samples and cell lines

Ten surgical specimens of benign prostatic hyperplasia and 13 samples of prostatic carcinoma, including three neuroendocrine carcinomas, were obtained from the Department of Pathology. All patients gave their informed consent for the research use of their tissues and the study obtained approval from an independent local Ethics Committee. A small fragment of tissue adjacent to the portion taken for histopathological examination was immediately frozen at –80 °C and stored for 4–24 months until processed for RNA extraction, immunohistochemistry, *in situ* hybridization, membrane preparation and binding studies.

DU-145, PC-3 and LNCaP cell lines were purchased from the American Tissue Culture Collection (Rockville, MD, USA). Two of them (DU-145 and PC-3) were androgen-independent (30, 31), whereas LNCaP was an androgen-dependent cell line (32). DU-145 cells were routinely cultured in Iscove, and PC-3 and LNCaP were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin (5000 U/ml)/streptomycin (15 mg/ml) in a 5% CO₂ humidified atmosphere at 37 °C. These cultures were used for RNA extraction, immunohistochemistry, *in situ* hybridization and cell proliferation studies, and to obtain cell membranes for binding.

RT-PCR for ghrelin and GHS-R1a and 1b

In 10 cases of prostatic benign hyperplasia and 10 cases of prostatic carcinoma, and in the three cell lines of prostate carcinoma studied, RT-PCR was performed to detect mRNAs for ghrelin and types 1a and 1b GHS-R. Total RNA extraction and complementary DNA transcription were performed as described elsewhere (33). The primers for ghrelin were synthesized according to the sequence reported by Gualillo *et al.* (11). The sequences were: 5'-TGAGCCCTGAACACCAGAGAG-3' for the forward primer and 5'-AAAGCCAGATGAGCGCTTCTA-3' for the reverse primer. Those for GHS-R1a and 1b were synthesized according to Korbonsits *et al.* (14) and used for RT-PCR under the same conditions as described by these authors. The following sequences were used: 5'-TCGTGGGTGCCTCGCT-3' as the forward primer for both GHS-R1a and GHS-R1b, 5'-CACCCTACAGCCAGCATTTTC-3' for the GHS-R1a reverse primer and 5'-GCTGAGACCCACCCAGCA-3' for

the GHS-R1b reverse primer. The reverse primers are specific for the two different C-terminal sequences of GHS-R1a and 1b respectively. In particular, the GHS-R1b reverse primer recognizes part of the 74 bp unspliced intron that is characteristic of GHS-R1b transcript. The expected sizes of the amplicons were 327 bp, 65 bp and 66 bp for ghrelin, GHS-R1a and GHS-R1b respectively. Amplification of β_2 -microglobulin served as a quality control for the RNA [see details in (12)]. Positive controls included normal gastric mucosa for ghrelin, and pituitary tissue for GHS-R1a and 1b amplifications. Negative controls were carried out by omitting cDNA from the PCR mixture and the reverse transcriptase enzyme during retrotranscription.

To test the specificity of the RT-PCR product further, Southern blot analysis was performed using the probe sequence (5'-TCCGGTTCAACGCCCCCTTTG-3') previously published by Korbonits *et al.* (14). Membranes were hybridized overnight at 42 °C with 25 pmol digoxigenin-labelled ghrelin and GHS-R oligonucleotide probes. The membranes were then washed with $2 \times \text{SSC}-0.1\%$ SDS for 5 min at 42 °C and $0.5 \times \text{SSC}-0.1\%$ SDS at 42 °C for 10 min. Digoxigenin-labelled specific hybridization was visualized using an immunological detection system (Boehringer Mannheim GmbH) using anti-digoxigenin antibodies conjugated with alkaline phosphatase. Detection was performed using the chemiluminescent substrate disodium 3-(4-methoxyphosphoryl)-1,2-dioxetane-3,2-(5-chloro) tricyclo (3.3.1.) decan; -4-yl) phenylphosphatase (Boehringer Mannheim GmbH), according to the manufacturer's instructions. All blots were exposed to X-ray films at room temperature for 1–5 h.

In situ hybridization for detection of ghrelin mRNA

Selected cases of benign prostatic hyperplasias (four cases) and of prostatic carcinomas (four cases) were studied by means of a non-radioactive *in situ* hybridization procedure in order to confirm the RT-PCR finding and to analyse the localization of ghrelin mRNA. Silane-coated slides were hybridized overnight with an equimolar mixture of two 45mer antisense probes corresponding to nucleotides 90–134 and 421–465 of the pre-pro-ghrelin sequence, at the final dilution of 33 nmol/l (1). Probes were digoxigenin-labelled with the Boehringer labelling kit, according to the manufacturer's instructions. Prehybridization treatments included a microwave passage (5 min at 800 W in citrate buffer pH 6.0) and proteinase K digestion (1 $\mu\text{g}/\text{ml}$) for 4 min. Hybridization products were revealed applying the Dako GenPoint kit (Dako A/S, Glostrup, Denmark) as described elsewhere (33), with the following minor modifications: a 1/5 dilution of tyramide was used and the sample was washed with hot PBS after the tyramide incubation (34). Positive controls were represented by sections of normal oxyntic

mucosa of the stomach. In order to test the specificity of the antisense probes and to exclude a possible tyramide-based background, negative controls were also run, such as hybridization with an unrelated probe, omission of the specific probe, and RNase digestion.

Immunohistochemistry for detection of ghrelin in tissue and cell lines

Sections from surgical specimens of benign prostatic hyperplasia and prostatic carcinoma were collected onto poly-L-lysine-coated slides; DU-145, PC-3 and LNCaP cells were grown on glass coverslips and fixed using methanol (5 min at -20°C) and acetone (5 s at -20°C), and stained for ghrelin using a standard immunoperoxidase procedure with streptavidin peroxidase (StrAvidin MultiLink kit, BioGenex Laboratories Inc., San Ramon, CA, USA). Slides were incubated with a 1/3000 dilution of an anti-human ghrelin antibody for 1 h at room temperature and the antigen was revealed as described previously (35). Anti-ghrelin antibody is a rabbit polyclonal antiserum that recognizes the C-terminal portion of both acylated and des-acylated ghrelin. After incubation with the primary antibody, the slides were incubated for 20 min with the secondary biotinylated antibody and then with a 1/50 dilution of the streptavidin–peroxidase conjugate for another 20 min. Finally, 3,3'-diaminobenzidine chromogen solution (LiquidDAB Substrate Pack, BioGenex Laboratories) was used to reveal the final reaction product and slides were counterstained in Mayer's haematoxylin (BioGenex Laboratories) for 30 s, dehydrated and mounted. Negative control reactions for ghrelin included omission of the primary antibody and preabsorption with a 100-fold excess of the antigen, as described elsewhere (15). The positive control for ghrelin was the endocrine cells of the oxyntic mucosa of the stomach.

Enzyme immunoassay for the detection of ghrelin in culture medium

In order to evaluate the secretion of ghrelin by prostate carcinoma cells, the peptide was assayed in the culture medium of cancer cell lines immunoreactive for ghrelin, by means of an enzyme immunoassay (EIA) technique. In a 75 ml flask, cells were grown until subconfluent. Medium was collected from the flasks and ghrelin was assayed in accordance with the instructions for the ghrelin (human) EIA kit. Control culture medium, in which human ghrelin was added at concentrations ranging from 10 to 100 pmol/l, was used as positive control.

Ghrelin binding assay

Ghrelin binding to membranes (30 000 g pellet) isolated from tumour tissues or cell line cultures was

carried out as described previously (17, 26, 27) using [125 I]Tyr⁴-ghrelin as a radioligand. Tyr⁴-ghrelin has been reported to have, *in vivo*, the same GH-releasing potency as native ghrelin and to be a reliable probe for labelling GHS-R in human tissues (6, 17). For the single-point binding assay, cell membranes [corresponding to 100 μ g membrane protein measured using the method of Lowry *et al.* (36)] were incubated in triplicate at 23 °C for 2 h under constant shaking with approximately 1 nmol/l [125 I]Tyr⁴-ghrelin in a final volume of 0.5 ml assay buffer. The composition of the assay buffer was as follows: 50 mmol/l Tris, 2.5 mmol/l EGTA, 0.002% bacitracin, 0.1% BSA, titrated to a final pH of 7.4 with HCl. Parallel incubations, in which 2.0 μ mol/l unlabelled ghrelin was also present, were used to determine non-specific binding, which was subtracted from total binding to yield specific binding values.

The binding reaction was terminated by the addition of ice-cold assay buffer, followed by filtration through Whatman GF/B filters. Filters were rinsed three times with assay buffer and the radioactivity bound to membranes was measured by a Packard auto- γ counter. Specific binding was expressed as a percentage of the total radioactivity added. Precautions were taken to minimize experimental variations in the binding of [125 I]Tyr⁴-ghrelin to tissue or cell membranes. Thus all binding studies related to one membrane preparation were carried out using the same batch of radiotracer. In some assays, receptor binding saturation studies were also conducted by incubating tissue membranes with increasing concentrations (from 0.03 to 3 nmol/l) of radioligand in the absence and in the presence of a fixed amount (2.0 μ mol/l) of unlabelled ghrelin. Saturation isotherms were transformed using the method of Scatchard (37) and the dissociation constant (K_d) and number of binding sites (B_{max}) were calculated with the Prism 3 program (GraphPad Software, San Diego, CA, USA). To establish binding site specificity, increasing concentrations of various unlabelled competitors were tested in competition assays with [125 I]Tyr⁴-ghrelin. The concentration of a competitor agent causing 50% inhibition of specific radioligand binding (IC_{50} value) was derived from the iterative curve-fitting analysis.

Cell proliferation studies

Cell proliferation was evaluated either by the incorporation of [3 H]thymidine into DNA or by counting the number of cells after appropriate incubation with different compounds.

Studies of [3 H]thymidine incorporation were performed as described previously (22–24). Briefly, starved DU-145 prostate cancer cells (2×10^5 cells/ml) were incubated at 37 °C, with or without 20 ng/ml IGF-I in the absence or the presence of different concentrations (from 0.1 nmol/l to 1 μ mol/l) of ghrelin, des-octanoyl

ghrelin, hexarelin, MK-0677 and GHRH. After 20 h, [3 H]thymidine 1 μ Ci/well was added and the incubation was continued for further 4 h, when the cells were harvested onto glass-fibre filter strips. Incorporation of [3 H]thymidine was measured in a scintillation counter.

In the experiments involving a study of cell numbers, DU-145, PC-3 and LNCaP cells were seeded in triplicate in 48-multiwell plates ($2-4 \times 10^3$ cells/well) in standard culture medium and allowed to become attached for 24 h. At 8 h after plating, cells were synchronized, by being left for 36 h in 0.5% FCS. They were then grown in a standard culture medium for 48 h in the absence or the presence of different concentrations of ghrelin or des-octanoyl ghrelin (10 pmol/l to 1 μ mol/l). After 48 h of treatment, cells were fixed in 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol and solubilized in 10% acetic acid. Cell growth was evaluated by measuring absorbance at 590 nm in a microplate reader (Multiskan Bichromatic, Thermo-Labsystems Oy, Helsinki, Finland). A calibration curve was set up with known numbers of cells and a linear correlation between absorbance and cell counts was established up to 1×10^5 cells. For time-course studies, DU-145 and PC-3 cells were treated as in the previous experiment, except that 0.5×10^3 cells were seeded per well and cell growth was followed in the absence or the presence of low (10 pmol/l) or high (1 μ mol/l) concentrations of ghrelin or des-octanoyl ghrelin at more than one time point (24, 48, 72 and 96 h after adding the hormone), with a change of medium every 48 h.

Statistical analysis

Values are expressed as median and range unless otherwise noted. In competition binding experiments and in cell proliferation studies, data are given as mean \pm S.E.M. The number of cases or experiments is indicated as *n*. Significant differences between groups were assessed by Kruskal–Wallis or Duncan's multiple range test. $P < 0.05$ was chosen as the level of significance.

Results

Expression of ghrelin mRNA and peptide

All cases of either benign prostatic hyperplasia or prostatic carcinomas were positive for expression of ghrelin mRNA, as studied by RT-PCR. This is depicted in Fig. 1, in which two representative example are given for both conditions: lanes 4 and 5, and 6 and 7 respectively. PC-3 cells (lane 3) were also positive, whereas DU-145 (lane 1) and LNCaP (lane 2) were negative. Analysis of mRNA by means of *in situ* hybridization confirmed the RT-PCR positivity for ghrelin mRNA in both benign prostatic hyperplasia (Fig. 2A) and prostatic

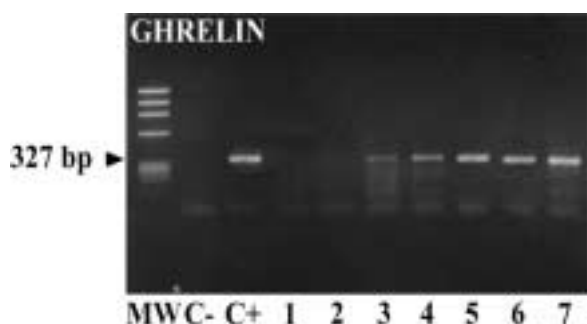


Figure 1 Detection by RT-PCR of mRNA for ghrelin in human prostate tumours and DU-145, PC-3 and LNCaP prostate cancer cell lines. The 327 bp band in lanes 1, 2 and 3 corresponds to ghrelin mRNA expressed by DU-145, LNCaP and PC-3 prostate cancer cell lines; lanes 4–5, benign prostatic hyperplasias (two selected cases among 10); lanes 6–7, prostatic carcinoma tissues (two selected cases among 13). C – , negative control (omission of cDNA in the PCR mixture); C + , gastric mucosa used as a positive control; MW, molecular mass markers.

cancer (results not shown). In contrast, none of the cases of benign prostatic hyperplasia (Fig. 2B) or carcinoma, whether neuroendocrine or non-neuroendocrine (not shown), was immunoreactive for ghrelin. Anti-human ghrelin polyclonal serum stained a large percentage (>80%) of ghrelin-positive cells in the PC-3 cell line (Fig. 3A), whereas it did not reveal any positive cells in DU-145 (Fig. 3B) or LNCaP (Fig. 3C) cell lines. The omission of the primary antibody or preabsorption with excess of antigen gave negative immunostaining in the PC-3 cell line (data not shown).

Ghrelin in cell culture medium

Using the EIA kit, no ghrelin was found in the medium collected from the culture flasks of subconfluent prostate carcinoma cells immunoreactive for ghrelin (PC-3 cell line).

Expression of GHS-R1a and 1b mRNAs

Expression of GHS-R1a and 1b mRNAs was evaluated by RT-PCR. After RT-PCR (Fig. 4), a 65 bp and a 66 bp signal, corresponding to GHS-R1a and GHS-R1b mRNAs respectively, were observed (lane C +) in a classic ghrelin target tissue, such as the pituitary gland (8, 17). In contrast, no signal corresponding to GHS-R1a was detected in either prostatic hyperplasia (two representative cases shown in lanes 4 and 5) or prostatic cancer (lanes 6 and 7) tissues. A signal corresponding to GHS-R1b was detected in five of 10 cases of benign prostatic hyperplasia (examples of a positive and a negative case given in lanes 4 and 5), but none of the cancer cases was positive for GHS-R1b (lanes 6 and 7). Conversely, mRNA for both GHS-R1a and 1b was found in DU-145 cells (lane 1), whereas no signal for either GHS-R was observed in LNCaP (lane 2) and PC-3 cells (lane 3), as confirmed

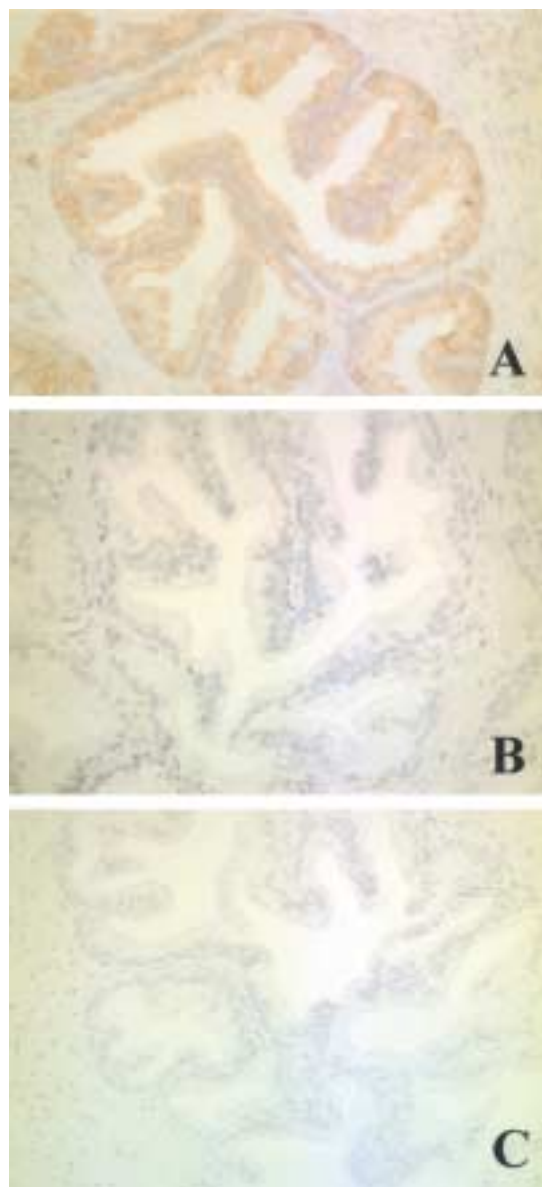


Figure 2 (A) *In situ* hybridization of ghrelin mRNA and (B) ghrelin immunoreactivity in human benign prostatic hyperplasia. (C) Negative control for *in situ* hybridization, performed using an unrelated probe. *In situ* hybridization and immunohistochemistry were conducted as described in Materials and methods. Original magnifications, $\times 400$.

by Southern blot analysis. Moreover, no signal was detected in an otherwise positive sample, when RT enzyme was omitted (lane C –).

Ghrelin binding studies

[125 I]Tyr⁴-ghrelin binding was observed in both benign and malignant prostate tumours. Specific binding of ghrelin was observed in all tissue specimens examined and represented about 55–64% of total radioactivity



Figure 3 Ghrelin immunoreactivity in (A) PC-3, (B) DU-145 and (C) LNCaP cell lines. Original magnifications, $\times 400$.

bound. A considerable specific binding was also found in two (DU-145 and PC-3) of three human prostate cancer cell lines examined, in which it represented 59–71% of total radioactivity bound. Ghrelin binding was very high in the androgen-independent cell line DU-145, with values that were twofold greater ($P < 0.05$) than those of the other androgen-independent cell line

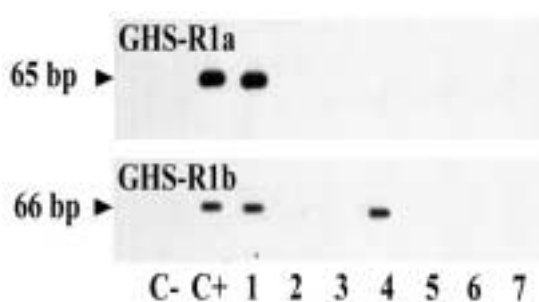


Figure 4 Expression of mRNA for GHS-R types 1a and type 1b in human prostate tumours and related cancer cell lines, demonstrated by Southern blot analysis of RT-PCR products. C -, negative control (omission of cDNA in the PCR mixture and the reverse transcriptase enzyme during retrotranscription); C +, pituitary gland used as a positive control. The 65 and 66 bp bands correspond to RT-PCR products for GHS-R1a and 1b respectively. Lanes 1, 2, 3 are for DU-145, LNCaP and PC-3; lanes 4–5, benign prostatic hyperplasias (two selected cases among 10); lanes 6–7, prostatic carcinoma tissues (two selected cases among 13).

(PC-3) or those previously found (22–31 fmol/mg protein) in the human pituitary gland (17). In contrast, negligible ghrelin binding values (less than 0.2%) were detected in the androgen-dependent LNCaP prostate cancer cells (Table 1). Experiments using increasing concentrations of [125 I]Tyr⁴-ghrelin provided evidence for a saturable specific binding in benign prostate hyperplasia, prostate carcinomas, DU-145 and PC-3, but not in LNCaP cells (Fig. 5). Scatchard analysis of these data (data not shown) demonstrated the existence of high-affinity sites with limited binding capacity (B_{max}) in benign and malignant neoplasms, and in DU-145 and PC-3 cell lines. No difference in the K_d values was observed among prostate tumours, DU-145 and PC-3 cells, whereas the number of receptors varied, showing a greater binding capacity in the DU-145 cell line (Table 1).

The specificity of [125 I]Tyr⁴-ghrelin binding to membranes from those samples of prostate tumours (carcinomas) that yielded sufficient amounts of membranes and from DU-145 or PC-3 cell lines was established by competitive binding experiments. In these, either ghrelin or des-octanoyl ghrelin was used, the latter an endogenous ghrelin derivative (5) devoid of binding affinity for GHS-R1a (38) and of GH-releasing activity *in vivo* (6, 7). Some synthetic peptidyl (hexarelin) and non-peptidyl (MK-0677) GHS, in addition to two hypothalamic neuropeptides (GHRH and SRIF) structurally

Table 1 [125 I]Tyr⁴-ghrelin binding to membranes of human prostate neoplasms and DU-145, PC-3 and LNCaP prostate cancer cell lines.

Tissue or cell line	Specific binding (% radioactivity added/0.1 mg protein)	B_{max} (fmol/mg protein)	K_d (nmol/l)
Prostate neoplasms			
Benign hyperplasias ($n = 6$)	1.00 (0.60–1.70)	24.0 (12.0–32.0)	0.55 (0.40–0.60)
Carcinomas ($n = 3$)	1.50 (0.70–2.80)	38.0 (22.0–44.0)	0.55 (0.39–0.86)
Prostate carcinoma cell lines			
Du-145 ($n = 4$)	3.21 \pm 0.22*	62.0 \pm 5.0*	0.47 \pm 0.05
PC-3 ($n = 4$)	1.40 \pm 0.06	33.3 \pm 3.4	0.59 \pm 0.07
LNCaP ($n = 4$)	0.17 \pm 0.03	n.d.	n.d.

Values are median (range) or mean \pm s.e.m. n , number of cases or experiments; n.d., not detectable. * $P < 0.05$ compared with PC-3 cells or prostate carcinomas.

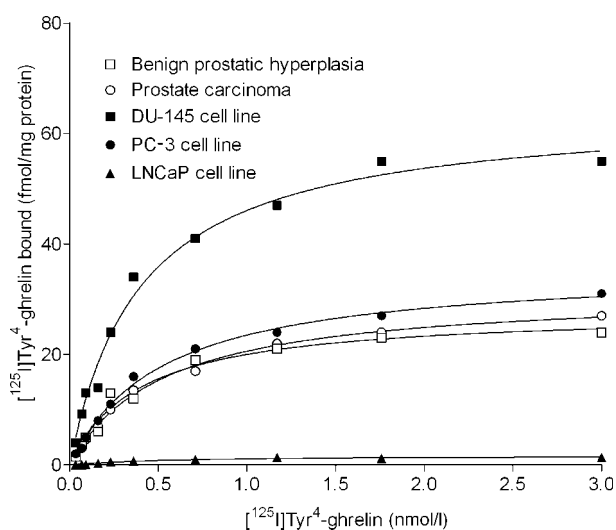


Figure 5 Representative saturation of [125 I]Tyr 4 -ghrelin binding to membranes obtained from a benign prostate hyperplasia, a prostate carcinoma and prostate cancer cell lines DU-145, PC-3 and LNCaP. Experiments were performed by incubating a fixed amount of membrane protein (100 μ g/tube) with increasing concentrations of radiolabelled ghrelin, either alone (total binding) or together with unlabelled ghrelin (2.0 μ mol/l) (non-specific binding). Specific binding values were obtained by subtracting non-specific binding from total binding.

unrelated to ghrelin, were also studied in these competitive binding experiments. [125 I]Tyr 4 -ghrelin bound to membranes of prostate carcinomas (Fig. 6A) and DU-145 cells (Fig. 6B) was completely displaced in a dose-dependent manner by unlabelled ghrelin, hexarelin, MK-0677 and, surprisingly, by des-octanoyl ghrelin, all of which exhibited high-affinity binding values (Table 2). In contrast, no competition was observed in the presence of GHRH or SRIF. The specificity of displacement observed in the PC-3 cell line was quite similar to that observed in the DU-145 cells (data not shown).

Effects of ghrelin, des-octanoyl ghrelin and synthetic GHS on prostate cancer cell proliferation

Both acylated and des-acylated ghrelin, in addition to hexarelin and MK-0677, inhibited the IGF-I-stimulated incorporation of thymidine in DU-145 cells, at concentrations close to their binding affinities reported in Table 2 (Fig. 7). The calculated 50% effective doses (mean \pm S.E.M. of four separate experiments) were 8.0 ± 1.4 nmol/l for ghrelin, 13.1 ± 2.0 nmol/l for des-octanoyl ghrelin, 21.9 ± 3.1 nmol/l for hexarelin and 41.0 ± 4.4 nmol/l for MK-0677. In contrast, no inhibition was observed in the presence of GHRH, which was unable to displace [125 I]Tyr 4 -ghrelin from membranes of DU-145 cells. In addition, no change in thymidine incorporation was observed when the different compounds were incubated with DU-145 cells growing in the absence of IGF-I (i.e. under basal conditions).

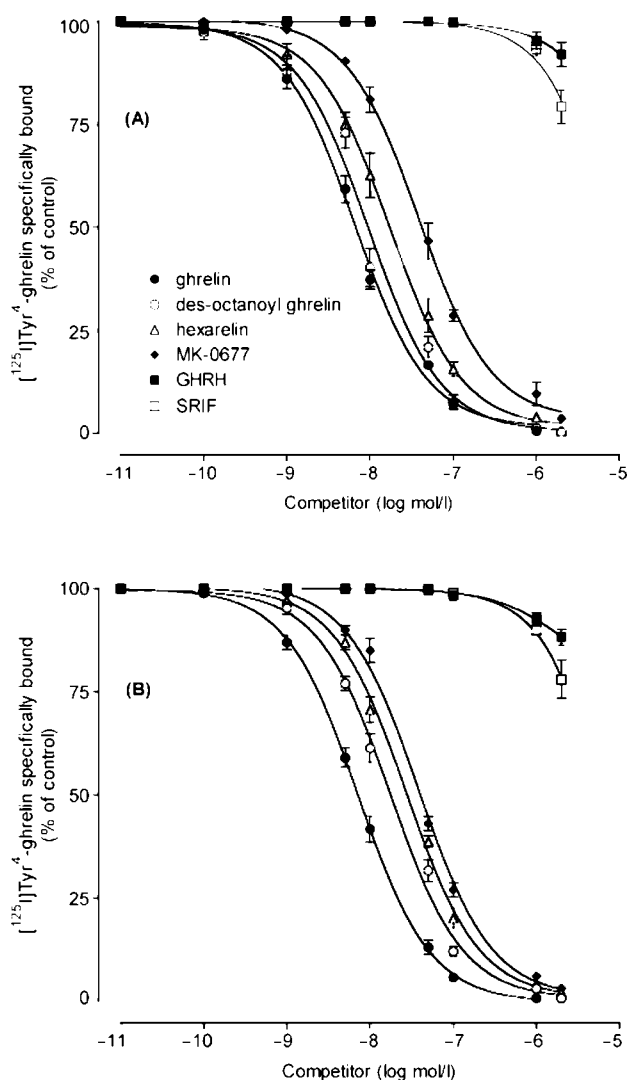


Figure 6 Displacement of [125 I]Tyr 4 -ghrelin bound to membranes from (A) prostate carcinomas and (B) DU-145 prostate cancer cell line, by different unlabelled competitors. Binding assays were conducted as described in Materials and methods. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor). Values are mean \pm S.E.M. of three separate experiments.

Table 2 IC $_{50}$ values of ghrelin, des-octanoyl ghrelin, hexarelin and MK-0677 for [125 I]Tyr 4 -ghrelin binding to membranes of prostate carcinomas and DU-145 prostate cancer cells.

Competitor	IC $_{50}$ (nmol/l)	
	Prostate carcinomas	DU-145 cells
Ghrelin	7.6 \pm 0.6	7.9 \pm 0.4
Des-octanoyl ghrelin	11.3 \pm 1.2	15.7 \pm 0.9
Hexarelin	19.0 \pm 1.7	25.0 \pm 3.9
MK-0677	36.7 \pm 4.4	38.9 \pm 2.0

Values are mean \pm S.E.M. of three separate experiments.

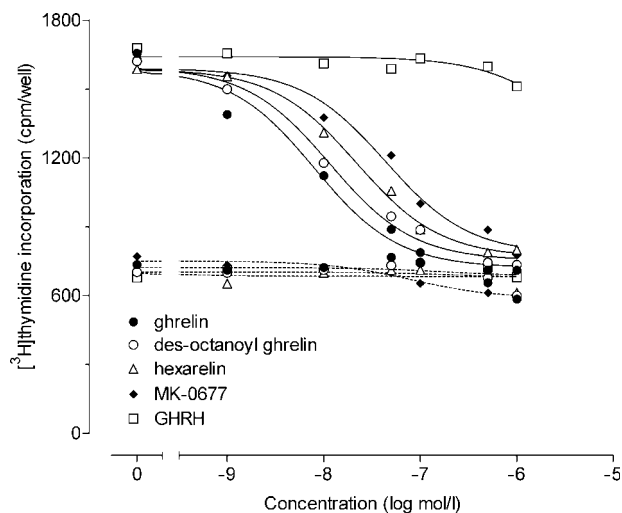


Figure 7 Effect of ghrelin, des-octanoyl ghrelin, hexarelin, MK-0677 and GHRH on basal (dashed line) and IGF-I-stimulated (solid line) incorporation of [³H]thymidine into DNA by human DU-145 prostate cancer cells. DNA synthesis was estimated by incorporation of [³H]thymidine after a 20 h incubation with or without 20 ng/ml IGF-I in the absence or presence of different concentrations of the indicated compounds. Data are the average of duplicate assay determinants and similar results were obtained in at least two other independent measurements.

The biological effect of ghrelin and des-octanoyl ghrelin on cell growth was determined at different concentrations and at various hours of treatment. Figure 8A shows the dose-dependent effect of ghrelin in all the three cell lines tested at 48 h of treatment. Interestingly, low peptide concentrations (10 and 100 pmol/l) stimulated cell growth in PC-3 cells only, but were ineffective in DU-145 cells (which were inhibited by ghrelin at concentrations ranging from 1 nmol/l to 1 μ mol/l). In contrast, no change in cell growth was observed when LNCaP cells were exposed to different concentrations of ghrelin. Similar findings concerning the cell growth of the three cell lines studied were obtained when they were incubated with des-octanoyl ghrelin for 48 h (Fig. 8B). Figure 9 shows the time-course of the effect of acylated and des-acylated ghrelin on cell growth of DU-145 and PC-3 cell lines at a concentration (10 pmol/l) that stimulated PC-3 cell proliferation or at a concentration (1 μ mol/l) that inhibited the cell growth of both cell lines. When added at 1 μ mol/l concentration, both ghrelin and des-octanoyl ghrelin were associated, at 48 h, with a significant decrease in the numbers of DU-145 and PC-3 cells, compared with controls; by 72 or 96 h the decrease in cell numbers was even more evident (Fig. 9). With a low concentration of peptide (10 pmol/l), both acylated and des-acylated forms of ghrelin were associated, after 48 h of treatment, with a significant increase in cell numbers only in PC-3 cells, and this effect was even more evident at 72 or 96 h. In contrast, no change in cell numbers was observed when DU-145 cells were incubated for periods

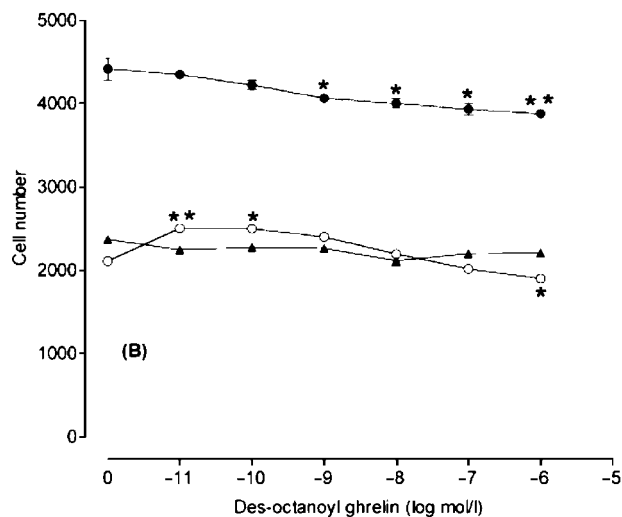
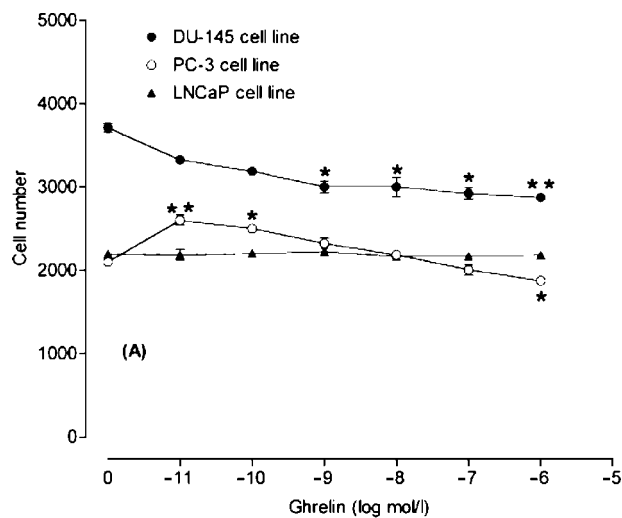


Figure 8 Dose-response relationship of (A) ghrelin and (B) des-octanoyl ghrelin on the proliferation of DU-145, PC-3 and LNCaP human prostate cancer cell lines. Cells were incubated in the absence (control) or presence of increasing concentrations of ghrelin and des-octanoyl ghrelin (ranging from 10 pmol/l to 1 μ mol/l). Cell numbers were determined at 48 h by a standardized absorbance assay (crystal violet). Values represent the mean \pm S.E.M. of three separate experiments. * P < 0.01, ** P < 0.001 compared with control.

up to 96 h with a low concentration (10 pmol/l) of the above-mentioned peptides.

Discussion

We have shown here for the first time that ghrelin and GHS receptors are expressed in human prostatic carcinomas and benign hyperplasias. We have also shown that ghrelin and some of its analogues produce different effects on cell proliferation in three different prostatic carcinoma cell lines.

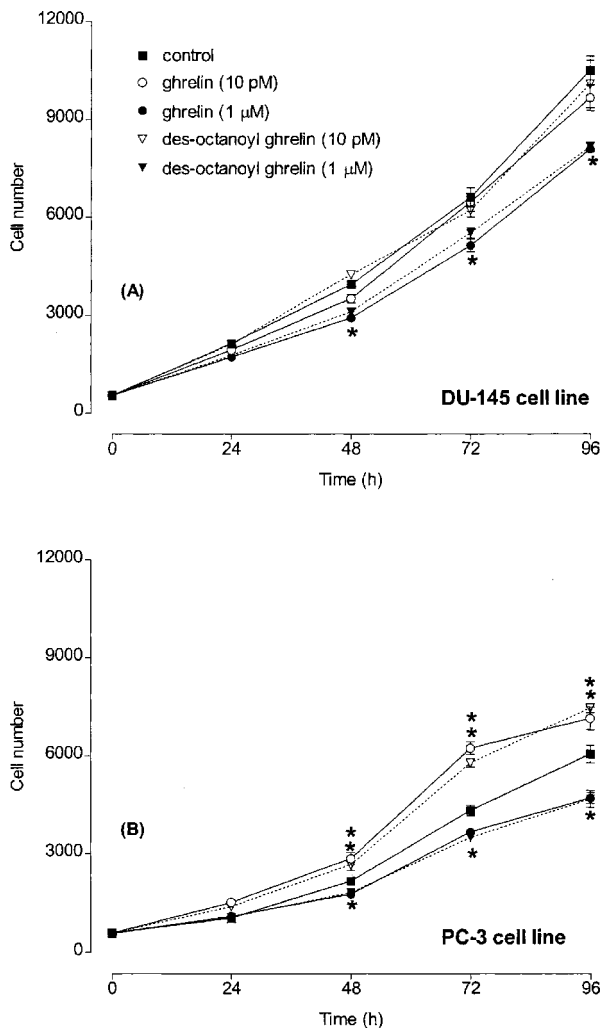


Figure 9 Time-course of the effect of ghrelin (—) and des-octanoyl ghrelin (---) on cell proliferation of (A) DU-145 and (B) PC-3 human prostate cancer cell lines. Cells (500/well) were grown for 96 h in the absence (control) and presence of the indicated compounds; the culture medium was changed every 48 h. The cell number was determined at the indicated time by a standardized absorbance assay (crystal violet). Values are means \pm S.E.M. of three separate experiments. * $P < 0.01$, ** $P < 0.001$ compared with control.

We have previously reported a detailed analysis of the distribution of GHS binding sites in tumoural (22–24) and non-tumoural human tissues (39–41) using a radiolabelled peptidyl GHS, such as [125 I]Tyr-Ala-hexarelin. More recently, we have demonstrated, using [125 I]Tyr⁴-ghrelin as a ligand, that ghrelin receptors are present in the human hypothalamus and pituitary gland and that ghrelin needs octanoylation to bind with high affinity to pituitary GHS-R (6, 17). By using [125 I]Tyr⁴-ghrelin, in addition to RT-PCR and *in situ* localization techniques, we have now investigated the presence of mRNA for ghrelin and types 1a and 1b GHS-R in 10 cases of prostatic benign hyperplasia and

10 cases of prostatic carcinoma, and in prostatic cancer cell lines, two androgen-independent (DU-145 and PC-3) and one androgen-dependent (LNCaP).

Interestingly, we found that ghrelin mRNA was present in all the prostatic benign (hyperplastic) and malignant (carcinomatous) tissues. However, none of these tissues expressed ghrelin at the peptide level. This suggests that hyperplastic and neoplastic prostatic cells either are unable to synthesize ghrelin (despite containing the specific mRNA) or do not retain the peptide in the cytoplasm once they have synthesized it. Additional possibilities could be insufficient sensitivity of the EIA kit or that the culture conditions used were not adequate for the ghrelin cell secretory process. In the cell lines, ghrelin was present (at both the mRNA and the peptide level) exclusively in the PC-3 cells, in contrast to what has previously been reported by Jeffery *et al.* (29), who also identified ghrelin expression in DU-145 and LNCaP cells. In addition, the concentration of ghrelin in the culture medium of the PC-3 cell line immunoreactive for ghrelin was not relevant, suggesting that this cell line is able to synthesize, but does not secrete, ghrelin.

On the basis of these observations, the functional significance of ghrelin in the human prostate remains an open question: neither prostatic tissue nor cell lines have significant amounts of ghrelin peptide. Thus only systemic or exogenous ghrelin could affect the functionality of prostatic primary tissue or cancer cell lines. However, at present, other sources of ghrelin in the reproductive tract cannot be excluded. In this sense, it was shown that Leydig cells are the primary site of expression and production of ghrelin in the rat testis, suggesting that this peptide could influence testicular function, in addition to that of other organs of the reproductive system, including the prostate (13). Moreover, a novel proghrelin isoform has been identified in mouse testis (42) and, more recently, in the human prostate, where its expression is upregulated in cancer tissues when compared with normal histological tissue (43). Nevertheless, the functional role of this novel peptide remains unexplored.

Regarding the expression of ghrelin receptors, no GHS-R1a mRNA was detected by RT-PCR in any of the tissues examined, whereas mRNA for GHS-R1b was found in 50% of benign prostatic hyperplasias. However, although no mRNA for GHS-R1a was detected in the prostatic tissues, a significant binding was observed in membranes obtained from both hyperplastic and cancerous prostatic tissues. These results suggest that, in these tumours, a ghrelin receptor subtype different from the classical GHS-R1a or 1b could be present. In agreement with the findings in the prostatic cancer tissues, in the prostatic carcinoma cell lines also, a discrepancy was found between the RT-PCR data on ghrelin receptors and binding. No mRNA for either GHS-R1a or 1b was present in LNCaP and in PC-3 cells, whereas it was found in DU-145 cells.

[¹²⁵I]Tyr⁴-ghrelin binding was very high in DU-145 cells, lower in PC-3 cells and absent from LNCaP cells, the binding showing properties typical of ligand–receptor interaction, namely high affinity, saturability and specificity. The most significant finding was the observation that the binding of the radioligand was inhibited in a dose-dependent manner by unlabelled natural (ghrelin) and synthetic GHS (hexarelin and MK-0677) and, surprisingly, by des-octanoyl ghrelin, a natural form of ghrelin devoid of GHS-R1a binding affinity (38) and GH-releasing activity *in vivo* (6, 7). This displacement pattern implies that the binding sites are specific for both acylated and des-acylated forms of ghrelin, and for peptidyl (hexarelin) and non-peptidyl (MK-0677) GHS—a finding that further suggests the presence of a GHS-R subtype different from the GHS-R1a. Considering that des-octanoyl ghrelin (which does not interact with GHS-R1a) was able to displace [¹²⁵I]Tyr⁴-ghrelin in the same manner as ghrelin, our data suggest that non-classical type 1a GHS-Rs are mainly expressed in prostate neoplasms and related cell lines. As observed by Scatchard analysis of saturation binding curves, the demonstration of a class of ghrelin binding sites only in DU-145 cells that also expressed both GHS-R1a and 1b may be attributable to the fact that the GHS-R1b is unable to bind ghrelin (3) and that the prostatic ghrelin binding sites possess a binding affinity (K_d values) quite close to that of GHS-R1a (6, 17, 38).

The effects of ghrelin and des-octanoyl ghrelin on cell proliferation also differed among the three cell lines examined. Ghrelin and des-octanoyl ghrelin were ineffective in modulating the growth of LNCaP cells, a cancer cell line that is devoid of ghrelin binding sites. In contrast, both peptides inhibited, in a dose-dependent manner, the proliferation of the ghrelin-receptor-positive cell lines DU-145 and PC-3. Surprisingly, a biphasic effect of ghrelin and des-octanoyl ghrelin was observed only in the PC-3 cell line. Low concentrations of ghrelin or des-octanoyl ghrelin (10–100 pmol/l) were able to stimulate cell growth, whereas higher doses (10 nmol/l to 1 µmol/l) caused a significant inhibition. This promoting effect on cell proliferation in the PC-3 cell line is in agreement with what was reported by Jeffery *et al.* (29). However, those investigators reported no study performed using high peptide concentrations and no data on the inhibiting effect of ghrelin and des-octanoyl ghrelin. The biphasic profile reported here is similar to that described in neoplastic conditions for other molecules, such as somatostatin and its analogues (44). The discrepant behaviour of the three cell lines in relation to cell proliferation in the presence of ghrelin and des-octanoyl ghrelin requires further investigation. Possible interpretations include a different responsiveness to Fas-mediated apoptosis (45) or to androgen-induced cell growth (30, 32), and a difference in expression of signal transducers and activators of transcription (46).

As ghrelin and des-octanoyl ghrelin produced an almost identical inhibition of IGF-I-stimulated incorporation of thymidine and cell growth, and as it is known that des-octanoyl ghrelin does not bind to the GHS-R1a, this inhibitory effect may involve specific binding sites other than GHS-R1a. In our previous papers on breast and lung tumours, we have already suggested the existence of an alternative ghrelin/GHS receptor in neoplastic tissues, because in that case also the biological and pharmacological data did not support GHS-R1a as the receptor involved (23, 24). The hypothetical existence of a different GHS-R1a subtype or a mutated GHS-R, or both, is also supported by the finding that a number of closely interrelated GHS-R subtypes exist in various tissues (19, 47, 48) and that some artificial variants of the GHS-R1a, such as the E124-Q mutant, lose the capacity of binding non-peptidyl GHS (19). Although we have found that ghrelin and GHS-R are co-expressed at the mRNA and protein levels in prostatic cancer cell lines, our data obtained on benign prostatic hyperplasia and prostatic carcinoma could not confirm the autocrine/paracrine role of ghrelin in prostatic tumours.

In conclusion, this study shows that ghrelin and its receptors are highly expressed in the human hyperplastic and neoplastic prostate tissues, in addition to the DU-145 and PC-3 prostate cancer cell lines. Moreover, acylated and des-acylated ghrelin, in addition to synthetic GHS, inhibit DNA synthesis and proliferation of the above cell lines through binding to a novel, as yet unidentified, receptor common for ghrelin and des-octanoyl ghrelin. Taken together, these data suggest a possible role of ghrelin and its des-acylated form in the control of neoplastic cell survival and proliferation.

Acknowledgements

We thank Professors G Bussolati and F De Matteis (University of Turin) for their suggestions and for critically reviewing the manuscript.

This work was supported by grants from the Italian Ministry of Education and University (MIUR, Rome, Italy) (Confin 2002 2002063821.004 to G Muccioli), University of Turin (ex 60% to G Muccioli and H Papotti) the Associazione Italiana per la Ricerca sul Cancro (AIRC, Milan, Italy), the project 'Oncology' – Compagnia di San Paolo/FIRMS (Turin, Italy) and the Fondazione per lo Studio delle Malattie Endocrine e Metaboliche (SMEM Foundation, Turin, Italy).

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Received 28 July 2003

Accepted 23 October 2003