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Facilitation of decidualization by locally produced ghrelin in the human endometrium

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Ghrelin acting via the growth hormone secretagogue receptor (GHS-R) stimulates GH secretion from pituitary glands. Both ligand and receptor are present in the pituitary, hypothalamus and many peripheral tissues including the uterus. This study demonstrates the cyclical expression of GHS-R and ghrelin in human endometrium. mRNA and protein for ghrelin and GHS-R were examined using RT–PCR and immunohistochemistry. Both ghrelin and GHS-R mRNA levels were highest in the secretory phase, with lower levels in the mid-proliferative phase and even lower expression in the menstrual phase. Immunoreactive ghrelin and GHS-R were confined predominantly to glandular epithelial and stromal cells with the greatest intensity of staining in secretory phase samples, consistent with the RT–PCR data. Additionally, we examined ghrelins effect on the decidualization of human endometrial stromal cells (HESCs) combined with sex steroid and cAMP treatments using prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) production as markers of decidualization. Ghrelin administered in combination with sex steroids to HESC, resulted in an increase in PRL and IGFBP-1 production above that obtained with cAMP, or sex steroids alone ($P < 0.001$) whereas ghrelin in combination with cAMP inhibits the action of cAMP. These findings have potential clinical applications for the regulation of fertility.

Keywords: decidualization; endometrium; ghrelin; GHS-R; prolactin

Introduction

The growth hormone secretagogue receptor (GHS-R) and its ligand the stomach peptide ghrelin are the newest facets of the GH axis and have shown to be involved in GH secretion as well as exhibiting other peripheral roles. Both ghrelin and the GHS-R are also expressed in a wide range of tissues other than hypothalamus and pituitary including the brain (Nakazato *et al.*, 2001), placenta (Gualillo *et al.*, 2001), kidney (Mori *et al.*, 2000), testis (Barreiro *et al.*, 2002), small intestine (Gnanapavan *et al.*, 2002) and the uterus (Tanaka *et al.*, 2003) suggesting that ghrelin acts on the GHS-R to regulate different functions in these organs and tissues. A number of other bioactive peptides first described in either the hypothalamus or the intestine have recently been described as highly expressed in the uterus. These include GRP-like peptide (Whitley *et al.*, 1996) and intestinal trefoil factor (Kao *et al.*, 2002). Many bioactive molecules including growth factors and cytokines play an important role in the events involved in implantation. Among these events is the process of the decidualization of human endometrial stromal cells (HESCs) which involves dramatic morphological and functional differentiation of these cells.

The decidualized cells themselves produce various growth factors and cytokines which advance decidualization and regulate trophoblast invasion (Das *et al.*, 1999; Norwitz *et al.*, 2001). Decidualization is a process of continuum that is controlled by specifically coordinated activation of specific genes (Dunn *et al.*, 2003; Tabibzadeh and Babaknia, 1995). Numerous studies have demonstrated that HESC decidualize *in vitro* in the presence of certain hormones and growth factors (Tseng *et al.*, 1992). Decidual cells are particularly characterized by their secretory products such as prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) and their pavement like morphology (Petraglia *et al.*, 1992). Progesterone is the main physiological inducer of decidualization in women, and decidualization of HESC can be induced *in vitro* by the addition of progesterone or cyclic (c) AMP to cultures treated with E_2 (Huang *et al.*, 1987).

One published study has demonstrated mRNA and protein expression of ghrelin and GHS-R in non-pregnant endometrium using biopsies from women undergoing total hysterectomy for leiomyoma or carcinoma *in situ* of the uterine cervix. However, they failed to show cyclical variation and specific localization of these peptides in stromal and glandular epithelial cells. They also observed PRL mRNA increase in HESC treated with cAMP and ghrelin *in vitro* compared with cAMP treated cells. This study examined PRL mRNA and this may not necessarily be translated into protein. In contrast, our study is the first detailed and thorough study using tissues from women with no apparent endometrial dysfunction to demonstrate cyclical variation of mRNA and protein expression for both ghrelin and GHS-R. We show detailed localization in stromal and glandular epithelial cells throughout the menstrual cycle which has not been reported in the literature. Furthermore, we provide evidence for a role for exogenous ghrelin in progesterone-induced *in vitro* decidualization by measuring the secretion of PRL and IGFBP-1, classical markers of decidualization. Thus, our study highlights that ghrelin is a novel paracrine/autocrine factor that is involved in cross-talk between the endometrium and embryo during embryo implantation.

Materials and Methods

Patients and tissue collection

Endometrial biopsies, representative of all stages of the menstrual cycle, were collected by dilation and curettage from women undergoing laparoscopy. All women were 20–40 years of age, experiencing regular menstrual cycles and without any apparent endometrial disorders. The women had no steroid treatment or other medication for at least 3 months before the collection of the tissue. The stage of the menstrual cycle was initially determined by patient testimony and was subsequently confirmed by histological dating. The tissue collection procedure was approved by the appropriate Institutional Human Ethics Committee. Written informed consent was obtained from all women participating in the study. Tissue samples were fixed immediately in 4% buffered formalin overnight at 4°C, washed thoroughly with Tris-buffered saline (TBS, pH 7.6) prior to paraffin embedding. Other tissue was immediately immersed in RNA later (Ambion, TX, USA) and stored at −20°C or in Dulbecco's modified Eagles's medium (DMEM; Trace Biosciences, Sydney, Australia).

RNA extraction and cDNA synthesis

Total RNA was extracted from endometrial biopsies from each of the menstrual (days 2–5), mid-proliferative (days 8–12) and mid-secretory (days 19–21) phases of the cycle ($n = 8$/phase) using an RNeasy™ Minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Genomic DNA was removed from samples using RNase-free DNase I (DNase-free™ Kit; Ambion) for 30 min at 37°C. The final concentration of RNA was determined spectrophotometrically, and the RNA quality was evaluated by gel electrophoresis (1% agarose) and by the ratio of optical density (OD) 260:280 (1.8–2.0). Total RNA (2 μg) was reverse transcribed (RT) at 46°C for 1.5 h in 20 μl reaction volume using 100 ng random hexanucleotide primers and 6 IU AMV reverse transcriptase (Roche, Castle Hill, Australia) in the presence of cDNA synthesis buffer, 1 mmol/l dNTPs, 10 mmol/l + dNTPs, 10 mmol/l dithiothreitol, 10 IU ribonuclease inhibitor (RNasin, Promega, Annandale, NSW, Australia). The resultant cDNA mixtures were heated at 95°C for 3 min before storage at −20°C. Negative controls omitted reverse transcriptase.

Semi-quantitative PCR

Semi-quantitative PCR for mRNA expression of GHS-R and ghrelin was performed using a conventional PCR block cycler (Hybaid, Middlesex, UK). RNA loading was monitored by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. A 20 μl aliquot of RT product was amplified in a total volume of 25 μl using 2.0 μl of RT single strength PCR buffer [10 mmol/l + Tris–HCl, 1.5 mmol/l + $MgCl_2$, 50 mmol/l + KCl, pH 8.3 (Roche), 2.5 mmol/l + dNTPs (Gibco, Melbourne, Vic Australia), 15 pmol/l sense and antisense primers and 2.5 IU Taq polymerase (Roche). The PCR for ghrelin, GHS-R and GAPDH was performed using specific primers Gualillo *et al.* (2001), Hattori *et al.* (2001) and Nie *et al.* (1999), respectively. The first stage for all three genes involved 95°C for 5 min, 95°C for 30 sec, and the annealing temperature of 58°C (36 cycles), 52°C (36 cycles) and 60°C (28 cycles) was used for ghrelin, GHS-R and GAPDH, respectively. The final stage was 72°C for 10 min. Annealing temperatures and cycle number were optimized for each primer set to ensure that amplification was in the exponential increasing phase for density analysis. The PCR for GAPDH was performed on the same aliquots as those used for ghrelin and GHS-R. GAPDH has been used as a loading control in many previous studies examining gene expression in the human endometrium (Nie *et al.*, 1999; Kressin *et al.*, 2001; Dheenadayalu *et al.*, 2002; Wolber *et al.*, 2003; Staar *et al.*, 2005; Ledee *et al.*, 2006). PCR products were analysed by electrophoresis on a 1% agarose gel (Roche) and stained with ethidium bromide. Bands of interest were excised from the gel, purified and directly sequenced to confirm their identity.

Immunohistochemistry

The specificity of staining of the ghrelin and GHS-R antibodies has been previously assessed by preabsorption of the antibody with full-length human ghrelin (Kojima *et al.*, 1999) or GHS-R peptide (Howard *et al.*, 1996); this completely abolished staining. Paraffin sections (5 μm) of endometrial biopsies from the menstrual (days 2–5), mid-proliferative (days 8–12) and mid-secretory (days 19–21) phases of the cycle ($n = 8$/phase) were dewaxed in histosol and rehydrated through descending grades of ethanol. Sections were subjected to trypsin for antigen retrieval. Endogenous peroxidase activity was quenched by immersion in 3% H_2O_2 for 10 min. Sections were then incubated with blocking solution containing 10% swine serum, 2% human serum and 0.1% Tween-20 in TBS for 30 min. Primary antibodies were applied, diluted to 0.6 μg/ml (polyclonal rabbit α human GHS-R; kind gift from Merck Laboratories), or 0.8 μg/ml (polyclonal rabbit α human ghrelin; kind gift from Kojima Masayasu) in blocking solution, and left at 4°C overnight. Antibody was detected by sequential application of biotinylated swine anti-rabbit IgG (Vector Laboratories, Burlingame, USA) in blocking solution, and an avidin–biotin complex conjugated to horse-radish peroxidase (HRP) (Vectastain® Elite ABC Kit, Vector Laboratories). The substrate used was diaminobenzidine (Zymed, San Francisco, USA), and nuclei were counterstained with Harris hematoxylin (Sigma). At the time of these studies, there was no commercially available blocking peptide so that no immunoreactivity in the endometrium would be seen after the absorption of ghrelin antiserum. The specificity of the antibody has been confirmed previously by the use of a blocking peptide (Kojima *et al.*, 1999) by the suppliers of the ghrelin antibody as kind gift. Therefore, a negative control was included for each tissue whereby the primary antibody was substituted with matching concentration of rabbit IgG on a sequential section. A section from a single block of secretory phase endometrium was included in each staining run, for quality control.

Semi-quantitative analysis of immunohistochemistry

Positive immunostaining was scored semi-quantitatively by two independent observers. An intensity score for each cellular compartment (stromal cells and glandular epithelium) was assigned from 0 (no staining) to 3 (maximal staining intensity) with reference to positive and negative controls. The scoring method used to analyse the immunohistochemical data is the standard method for a complex reproductive tissue such as the endometrium, and although subjective, such a system is noted in numerous publications (Kilpatrick *et al.*, 1999; Dimitriadis *et al.*, 2000; Jones *et al.*, 2005). Results are expressed as mean ± SEM.

Stromal cell isolation and in vitro decidualization

HESCs were isolated from tissue by enzymatic digestion and filtration as described previously (Dimitriadis *et al.*, 2002). This produces >95% pure stromal cell cultures, determined by the analysis of vimentin and cytokeratin expression (Marsh *et al.*, 1994). Briefly, endometrial tissue was finely minced with scissors and digested with bacterial collagenase type III (Worthington Biochemical Corporation, Freehold, NJ, USA) at a concentration of 45 IU/ml in the presence of 3.5 μg/ml deoxyribonuclease (Boehringer Manneheim Biochemica, Manneheim, Germany). After 40 min of agitation at 37°C, the digested tissue was filtered sequentially through 45 and 10 μm nylon filters to remove glands, layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 160*g* to pellet erythrocytes. Stromal cells were collected from the Ficoll interface and resuspended in 1:1 mixture of DMEM/F12 with 10% CT-FCS (Trace Biosciences, Sydney, Australia) and 1% antibiotics (penicillin, streptomycin and fungizone; Commonwealth Serum Laboratories, Melbourne, Australia). The cells were transferred to 24-well dishes at a density of 2.5×10^5 cells/well and grown for 2–4 days until confluent, with medium changes every 48 h. Once cells were confluent, experiments were conducted in medium containing 2% CT-FCS with medium change every 48 h. After 4 days, fresh medium was added (experimental day 0) with the additions of either estradiol 17β (E) 10^{-8} mol/l (Sigma), human *n*-octanoylated ghrelin (G) 10^{-7} M (kind gift from Kojima Masayasu), E + G, 8-Bromo cAMP (Sigma) 0.5 mM + G, cAMP alone, E + medroxyprogesterone acetate (MPA) or E + MPA + G. A test group of MPA alone was not included in the experiments because MPA is a weak inducer of decidualization (Gellersen and Brosens, 2003). Estradiol is used in combination with MPA as it up-regulates the progesterone receptor (PR) accelerating decidualization compared with MPA alone. Estradiol alone has no effect on decidualization. The supernatant was harvested every 48 h, centrifuged at 160*g* to remove non-adherent cells and stored at −20°C. The supernatant from three triplicate wells per treatment were pooled. Cells for each treatment group were photographed for morphological analysis at required times. Each experiment was repeated three times with different cell preparations. Confluent wells of HESCs were used in the decidualization study. Under these

experimental culture conditions, the confluent monolayers of HESC do not proliferate. This has been predetermined in a pilot study. In addition, Fig. 1 shows that the cells remain as confluent monolayers at the end of the experiment. Therefore, it is unlikely that differences seen in PRL secretion are due to differences in cell number. The most appropriate way of expressing the secretion data is secretion/cell number; however, this is extremely difficult to do, as decidualized stromal cells are difficult to trypsinize as they set down a matrix. We did trypsinize and count decidualized and non-decidualized cells from the same wells but not all the wells; therefore, we could not group all the data in this manner.

PRL ELISA assay

The production of immunoreactive PRL by cultured HESC was measured quantitatively in duplicate using ELISA kits (Bioclone Australia, Pty Ltd, Marrickville, NSW, Australia) according to the manufacturer's instructions. The conditioned media collected and stored at -20°C from cell cultures at day 8 were thawed and concentrated 5-fold using a vacuum dryer. A quality control sample (culture medium from a single endometrial cell culture) was included in every assay. The lower detection limit of the assay was 50 mIU/l. The inter- and intra-assay CV were 5.3% and 3.0%, respectively. Results were expressed as SEM of mIU/l PRL.

IGFBP-1ELISA assay

The production of immunoreactive IGFBP-1 in culture medium was assessed in duplicate using an Elisa kit (Diagnostic Systems Laboratories, Inc Webster, TX USA) according to the manufacturer's instructions. The conditioned media collected and stored at -20°C from cell cultures at day 8 were thawed and concentrated 5-fold using a vacuum dryer. Standards, controls and unknown samples were incubated in microtitration wells coated with an anti-IGFBP-1 antibody. After incubation and washing, the wells were treated with anti-IGFBP-1 detection antibody labelled with the enzyme, HRP. After a second incubation and a washing step, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620 nm. The absorbance measured was directly proportional to the concentration of IGFBP-1 present. The inter- and intra-assay CV were 6.2% and 3.6%, respectively.

Statistical analysis

Data were expressed as mean $\pm$ SEM. Data were analysed by ANOVA followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

Determination of mRNA for GHS-R and ghrelin in human endometrium

Both GHS-R and ghrelin mRNA were detected by semi-quantitative RT–PCR in human endometrium (Fig. 1A). Three samples were examined for each of the menstrual, mid-proliferative and mid-secretory phases. Densitometric analysis showed that the mean intensity of the bands (corrected for loading with GAPDH) for both ghrelin and GHS-R increased as the menstrual cycle progressed with maximal levels being observed in the secretory phase (Fig. 1B and C, respectively). In each case, differences were significant between all three phases ($P < 0.05$).

Immunoreactive ghrelin in human endometrium

Immunoreactive ghrelin was detected in all endometrial samples ($n = 24$). In the menstrual phase, pale stain was seen, predominantly in glandular epithelial cells, although some staining was also detected in the stroma (Fig. 2A). In the mid-proliferative phase samples, stronger intensity of staining was observed in the glandular epithelium (Fig. 2C). Where luminal epithelium was present, staining was less intense and less consistent than in the glandular epithelial cells. The most intense immunoreactivity was observed in the glands in the mid-secretory phase (Figs 2E and 3A). The intensity of stain in the glands appears significantly less in the menstrual compared with the mid-secretory phase, with staining intensity being between these two extremes during the proliferative phase (Fig. 3A). Staining in the stroma was of low intensity and with no cyclical variation. Blood vessels and leukocytes were always devoid of staining.

Immunoreactive GHS-R in human endometrium

Immunoreactive GHS-R was also detected in all endometrial samples ($n = 24$). No staining was detected in luminal epithelium (where present), blood vessels or leukocytes. Positive staining was found in both glandular epithelial and stromal cells throughout the cycle (Figs 2B, 2D, 2F and 3B). However, the maximal staining intensity was seen in the glandular epithelium during the secretory phase and this appeared stronger than that in glands during the menstrual and mid-proliferative phases. Stromal staining was moderate, and did not change throughout the cycle.

Effect of exogenous ghrelin on PRL and IGFBP-1 secretion during HESC decidualization

We determined the effect of ghrelin on cAMP or progesterone-induced decidualization of HESC using PRL and IGFBP-1 as markers following these experimental regimes; medium, E, G, E + G, cAMP + G, cAMP, E + MPA and E + MPA + G. Culture media from day 8 was used to quantitate PRL and IGFBP-1 production. Cells treated with the decidualizing stimuli, cAMP, E + MPA and E + MPA + G for 8 days secreted

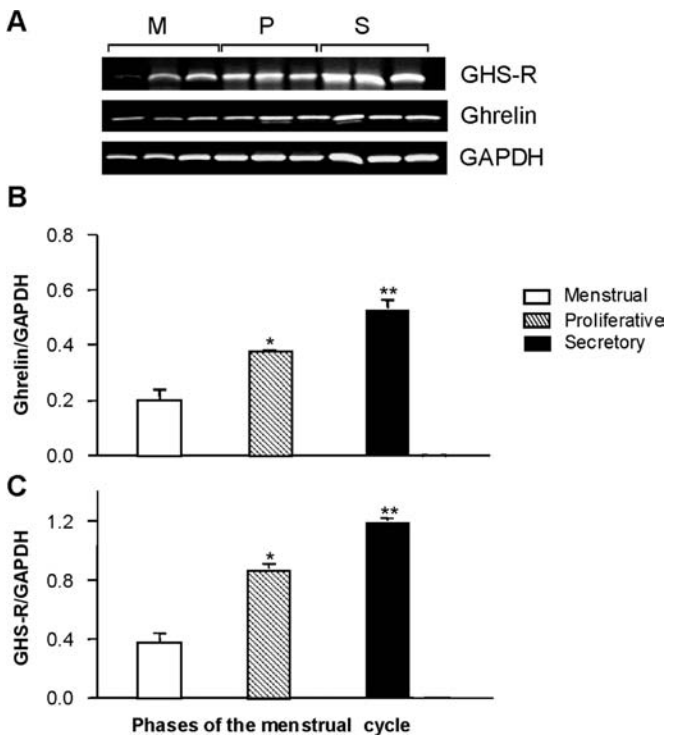

Figure 1. Expression of the GHS-R and ghrelin in endometrial biopsies. Gene expression was determined by reverse-transcription–polymerase chain reaction (RT–PCR) with primers specific for the GHS-R and ghrelin (**A**) Letters at the top refer to the phase of the cycle, (M) menstrual, (P) proliferative and (S) secretory. Densitometric representation of bands for GHS-R (**B**) and ghrelin (**C**) corrected for loading by GAPDH analysis. $^{**}P < 0.05$ compared with other phases

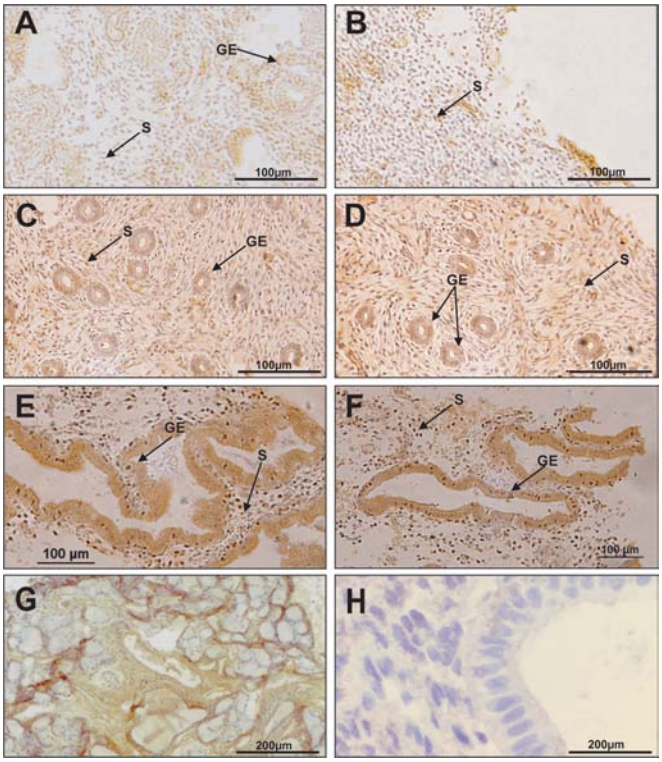

Figure 2. Immunohistochemical localization of ghrelin and the GHS-R in the normal human endometrial stroma (S) and glandular epithelial (GE) cells. GHS-R protein expression (**A**, **C** and **E**) and ghrelin (**B**, **D** and **F**) in human endometrium (A + B) menstrual phase, (C + D) proliferative phase and (E + F) secretory phase. Note the alteration in the intensity of immunostaining from the menstrual phase to secretory phase endometrium. (**G**) Ghrelin in human tonsil tissue (positive control) and (**H**) negative control. Scale bars (A–F), 100 μm; (G–H), 200 μm

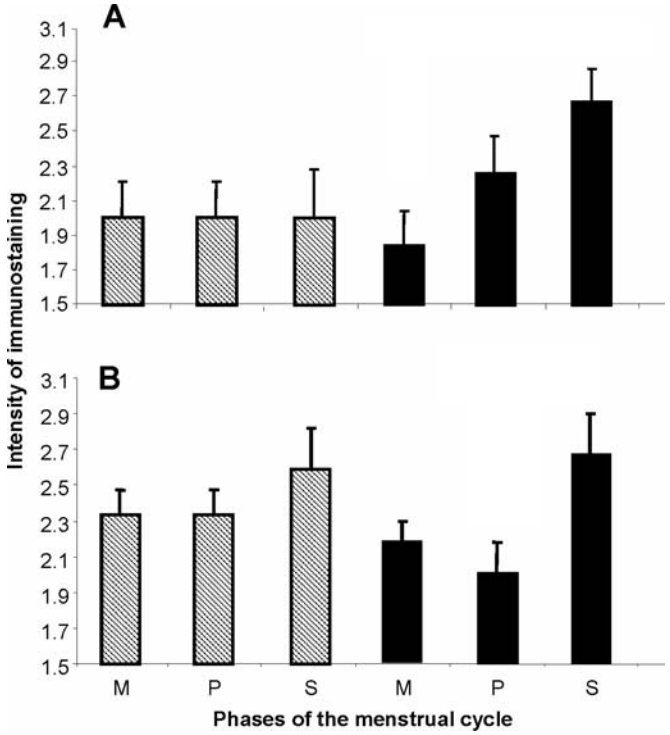

Figure 3. Histogram displaying immunolocalization and relative staining intensity of immunostaining (mean ± SEM) for (**A**) ghrelin and (**B**) GHS-R in endometrial stromal cells (shaded bar) and glandular epithelial cells (black bar). Data are presented from menstrual cycle: menstrual (M), proliferative (P) and secretory (S) stages of the cycle

higher amounts of PRL and IGFBP-1 compared with E, G, E + G and cAMP + ghrelin ($P < 0.001$, Fig. 4A and B) and underwent characteristic morphological changes. Ghrelin in combination with E + MPA significantly stimulated higher amounts of PRL and IGFBP-1 compared with all other treatment groups ($P < 0.001$, Fig. 4A and B). A synergistic effect on PRL secretion was observed with the combined treatment of G + E + MPA, compared with E + MPA alone. In contrast, an inhibitory effect on PRL and IGFBP-1 secretion was seen with treatment of ghrelin in combination with cAMP ($P < 0.001$, Fig. 4A and B).

Morphological changes of HESC following treatments

The morphological characteristics of the cells after different treatments were also assessed. At day 8 of culture, confluent cells without treatment appeared spindle shaped, characteristic of non-decidualized stromal fibroblasts (Fig. 5A). Cells treated with ghrelin alone (Fig. 5B) resembled the control cells. Cells treated with cAMP (Fig. 5C), E + MPA (Fig. 5D), cAMP + G (Fig. 5E) and E + MPA + G (Fig. 5F) acquired a pavement-like morphology indicative of decidualization.

Discussion

This study is the first detailed description of the expression, synthesis and distribution of GHS-R and ghrelin in normal human endometrium, an unusual adult tissue in that it undergoes extensive remodelling with each reproductive cycle. mRNA and protein expression for both receptor and ligand were low during the menstrual phase, moderate in proliferative phase and increased markedly during the secretory phase, with by far the most dominant staining observed in glandular epithelial cells in the secretory phase of the cycle, coincident with the period of endometrial receptivity to implantation (Tabibzadeh and Babaknia, 1995). Furthermore, utilizing an established *in vitro* model of human stromal cell decidualization *in vitro*, we clearly demonstrate that the addition of exogenous ghrelin in combination with sex steroid hormone to HESC significantly accelerates and promotes decidualization. This study is the first to support the newest facets of the GH axis to have a functional role in implantation in primates.

Ghrelin regulates many physiological functions in addition to its action as a potent GH-releasing agent both *in vivo* and *in vitro* (Wren *et al.*, 2002). Other actions in normal tissues include stimulation of lactotroph and corticotroph secretion (Broglio *et al.*, 2003a,b), orexant activity coupled with control of energy expenditure (Weikel *et al.*, 2003) and control of gastric motility and acid secretion (Masuda *et al.*, 2000). Furthermore, ghrelin has been shown to stimulate cell proliferation (Maccarinelli *et al.*, 2005) and has anti-apoptotic effects (Kim *et al.*, 2004) in a number of cell lines indicating that it is involved in tissue regeneration and remodelling.

Extensive cellular proliferation, differentiation and degeneration occur in most tissues and organs of the body only during development or in pathological conditions. In contrast, these processes are crucial in the adult human endometrium, as it remodels during each menstrual cycle to provide a suitable environment for implantation of a blastocyst. For implantation to be successful, the endometrium must acquire optimal conditions for blastocyst attachment and intrusion for a few days in each reproductive cycle. In the absence of a blastocyst, the endometrium degenerates and the process of cell growth and differentiation recurs. This remodelling process requires participation of many biologically active systems. Many molecular factors involved

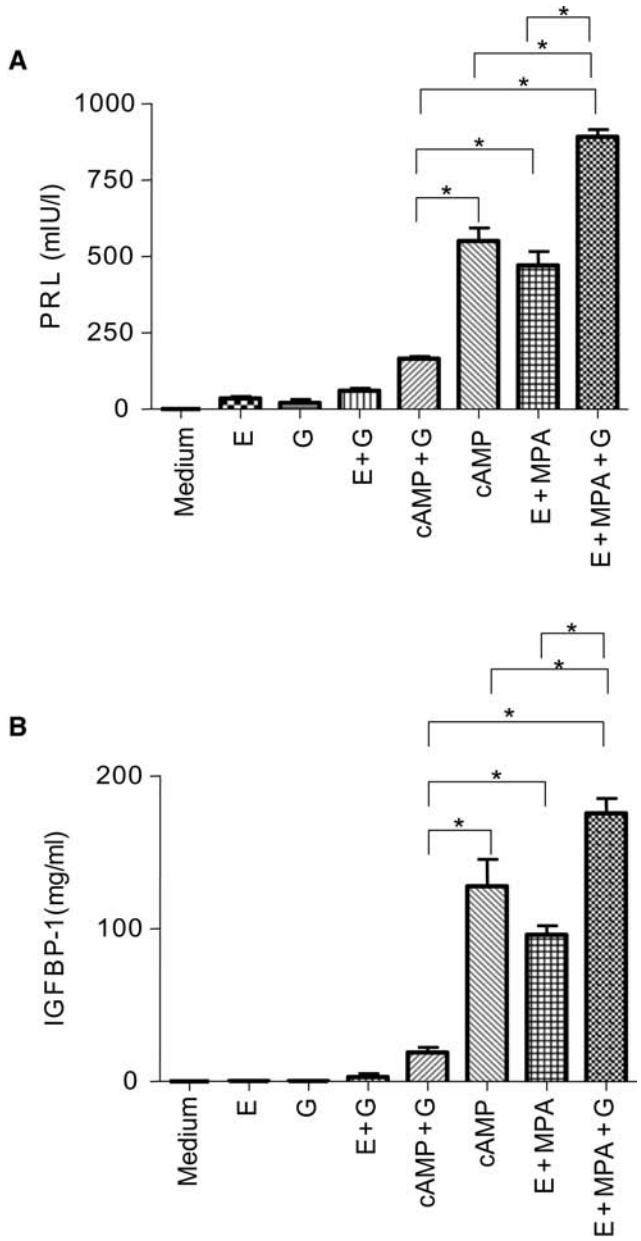

Figure 4. Effect of exogenous ghrelin on HESC (**A**) PRL and (**B**) IGFBP-1 secretion during decidualization. Confluent stromal cells were cultured for 8 days and treated with medium, E, G, E + G, cAMP + G, cAMP alone, E + MPA, E + MPA + G with medium changes occurring every 2 days. Each value represents the mean ± SEM of triplicate cultures from three separate experiments. *$P < 0.001$ cAMP + G versus E + MPA, E + MPA + G; cAMP versus E + MPA + G; E + MPA versus E + MPA + G

in embryo implantation have been reported, including ovarian steroid hormones, cytokines, adhesion molecules and neuropeptides (Brar *et al.*, 2001; Irwin *et al.*, 2001; Dimitriadis *et al.*, 2002; Makrigiannakis *et al.*, 2004). A number of small peptides known originally for their roles in the brain and intestine are present in the endometrium during the window of receptivity; these include intestinal trefoil factor (CPE-1R) (Kao *et al.*, 2002) and the neuropeptides, corticotrophin releasing factor (CRF) (Makrigiannakis *et al.*, 2004) and vasoactive intestinal peptide (VIP) (Kao *et al.*, 2002). These, like ghrelin may play critical roles in endometrial cell–cell interactions during the complex process of embryo implantation.

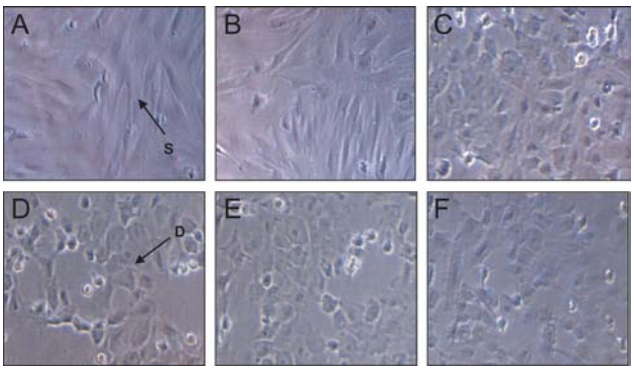

Figure 5. Morphology of HESC. Phase contrast morphology of cultured HESCs following different treatment regimes. Cells were cultured for 8 days with medium changes every 48 h and examined by phase contrast microscopy. Stromal cell (S) treatments were (**A**) medium; (**B**) G; (**C**) cAMP; (**D**) E + MPA; (**E**) cAMP + G; (**F**) G + E + MPA. Note the stromal cell transformation to decidual cells (D) in (C–F)

Ghrelin (Kojima *et al.*, 1999), the endogenous ligand for the GHS-R (Howard *et al.*, 1996), is abundantly expressed in the stomach (Date *et al.*, 2001; Matsubara *et al.*, 2004) and the hypothalamus (Kamegai *et al.*, 2000; Seoane *et al.*, 2003; Park *et al.*, 2005). This study details its expression in the human uterus. Ghrelin and the GHS-R are expressed by glandular epithelial and stromal cells of the human endometrium in increasing amounts from the endometrial menstrual through proliferative to the secretory phase, similar to the expression pattern seen for the neuropeptide CRF (Makrigiannakis *et al.*, 2004), the cytokine interleukin-11 (IL-11) (Dimitriadis *et al.*, 2000) and activin A (Jones *et al.*, 2000). The high level of expression of both the ligand and receptor in glandular epithelium demonstrated here during the mid-secretory phase, when the endometrium becomes receptive to embryo implantation, suggests a possible autocrine role for ghrelin in epithelial transformation during this period. These findings are in agreement with the findings of another study (Tanaka *et al.*, 2003). However, in that previous study, ghrelin was not detected in the proliferative phase or in stromal cells throughout the menstrual cycle (Tanaka *et al.*, 2003). Such discrepancies may be due to differences in tissues and controls used for immunohistochemistry.

The cyclical variation in ghrelin and GHS-R immunoreactivity suggests direct or indirect regulation by ovarian steroids. This may reflect different functions at different stages of the menstrual cycle and actions on the differentiation process in various cells. However, local factors such as cytokines are likely to be involved, as these are well-known to stimulate the secretion of other cytokines (Dimitriadis *et al.*, 2000) and growth factors (Jones *et al.*, 2002b). Importantly, the GHS-R is expressed in the same glandular epithelial and stromal cells as those secreting the ligand suggesting autocrine/paracrine actions of ghrelin in these cells. It remains to be evaluated whether epithelial-derived ghrelin is secreted apically into the uterine lumen or basally into the stroma, although the glandular expression indicates likely secretion into the uterine lumen. GHS-R is expressed in stromal cells suggesting autocrine/paracrine actions of ghrelin in these cells. Should ghrelin be released basally from the uterine epithelium, a paracrine effect on the underlying stroma is possible. In addition, ghrelin from the epithelial or stromal cells may have indirect effects via other factors on leukocytes (Salamonsen and Lathbury, 2000). The mid-secretory phase of the cycle is defined as days 19–21. Decidualization of stromal cells begins at approximately day 23 of the secretory phase of the cycle; therefore, we would not expect to see decidualized stromal cells in these tissues. In addition, no signals were detected in

decidualized stromal cells from ectopic pregnancies in a previous study (Tanaka *et al.*, 2003).

During the mid-late secretory phase, human endometrium starts the process of decidualization, in preparation for invasion by the trophoblast if pregnancy occurs. Decidualization involves the remodelling of the endometrium, with the differentiation of stromal cells to enlarged, rounded decidual cells and the influx of specific leukocytes (Petraglia *et al.*, 1992). This process is controlled by the activation of specific genes (Tabibzadeh and Babaknia, 1995) and results in the coordinated expression of numerous new products (Tang *et al.*, 1994). Many paracrine factors that are expressed throughout the cycle, including prostaglandins (Frank *et al.*, 1994), IL-11 (Dimitriadis *et al.*, 2002), CRF (Makrigiannakis *et al.*, 2004), activin A (Jones *et al.*, 2002a) and relaxin (Sherwood, 2004; Dimitriadis *et al.*, 2005) promote decidualization. Importantly in the present study, ghrelin both increased and accelerated the secretion of PRL and IGFBP-1 during progesterone-induced decidualization, suggesting a role in the progression of decidualization possibly by synergizing with progesterone. The specificity of this response to decidualization was confirmed by detecting IGFBP-1 secretion, highlighting the effect of ghrelin is not only on PRL directly. Our data show the secretion levels of PRL and IGFBP-1 and in contrast to a previous study are more relevant to the function of ghrelin, than the detection of PRL mRNA levels, since mRNA levels may not necessarily reflect protein levels. We have also demonstrated that the functional differentiation of HESC was accompanied by morphological differentiation, where stromal cells change from elongated fibroblast-like cells to more larger and rounder cells (Petraglia *et al.*, 1992). Similar actions have been observed for IL-11 (Dimitriadis *et al.*, 2002), activin A (Jones *et al.*, 2002c), CRF (Makrigiannakis *et al.*, 1999a,b) and RLX (Dimitriadis *et al.*, 2005; Ivell *et al.*, 2005). Analysis of cell morphology however did not detect differences in the extent of decidualization.

The initiation of the decidual process requires elevated intracellular cAMP levels and sustained activation of the protein kinase A (PKA) pathway (Gellersen and Brosens, 2003). GHRP-2, a synthetic GHS activating the GHS-R, increases intracellular cAMP and activates PKA in ovine pituitary cells (Wu *et al.*, 1996) and ghrelin significantly increases plasma cAMP in healthy volunteers (Nagaya *et al.*, 2001), suggesting this increase is due to the accumulated increase of intracellular cAMP in these volunteers after ghrelin administration. Surprisingly, cAMP in combination with ghrelin produced lower levels of PRL and IGFBP-1 compared with cAMP treatment alone; suggesting that ghrelin is blocking the action of cAMP in HESC. It may be that ghrelin increases intracellular cAMP to the extent that PRL and IGFBP-1 secretion cannot be sustained. Stimulation of HESC with cAMP for prolonged periods results in increased and then decreased PRL secretion (Gellersen and Brosens, 2003). Alternatively, ghrelin may affect cAMP stability via phosphodiesterase (PDE).

A synergistic effect was seen when decidualizing cells were treated with G + E + MPA compared with E + MPA alone; a possible explanation is that ghrelin may enhance progesterone-induced decidualization by sensitizing endometrial stromal cells to the action of progesterone (Lane *et al.*, 1994; Telgmann *et al.*, 1997) similar to the molecular mechanisms proposed for CRF in stromal cells. Importantly, ghrelin increased and accelerated the secretion of PRL by decidualizing cells suggesting a feedback role in the progression of decidualization. The increase and accelerated response in decidualization following the treatment of HESC with ghrelin was not accompanied by a change in the cell number, since decidualization experiments were performed on confluent cells suggesting that the function of ghrelin during decidualization does not involve stromal cell proliferation.

In conclusion, this is the first study to report the presence of both mRNA and protein for the GHS-R and ghrelin in human endometrium. The increase in GHS-R and ghrelin expression and immunocytochemistry staining intensity in the secretory phase suggests that this system may have a role in the preparation of the endometrium for the process of implantation. This present study also clearly demonstrates that *in vitro* ghrelin induces decidualization of HESCs as indicated by their morphological changes and secretion of PRL and IGFBP-1 into the medium suggesting that endometrially-derived ghrelins are involved in the functional differentiation that occurs during progesterone-mediated decidualization of HESCs. Importantly, it is interesting that ghrelin has a differential effect on decidualization according to the type of stimulus (i.e. cAMP or E + MPA) used to decidualize the cells, thus identifying differences in the two main pathways of decidualization. The interaction of ghrelin with other cytokines such as IL-11 and ligands coupled to the cAMP pathway such as PGE_2 and relaxin needs to be examined. The contribution of ghrelin to decidualization may prove to be critical in the successful establishment of pregnancy.

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