Signalling and anti-proliferative effects mediated by gonadotrophin-releasing hormone receptors after expression in prostate cancer cells using recombinant adenovirus

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Abstract

Gonadotrophin-releasing hormone receptors (GnRH-Rs) are found in cancers of reproductive tissues, including those of the prostate, and gonadotrophin-releasing hormone (GnRH) can inhibit growth of cell lines derived from such cancers. Although pituitary and extra-pituitary GnRH-R transcripts appear identical, their functional characteristics may differ. Most extra-pituitary GnRH-Rs have low affinity for GnRH analogues and may not activate phospholipase C or discriminate between agonists and antagonists in the same way as do pituitary GnRH-Rs. Here we have assessed whether GnRH-Rs expressed exogenously in prostate cancer cells differ functionally from those of gonadotrophs. We found no evidence for endogenous GnRH-Rs in PC3 cells, but after infection with adenovirus expressing the GnRH-R (Ad GnRH-R) at 10 plaque forming units (p.f.u.)/cell or greater, at least 80% of the cells expressed GnRH-Rs. These sites had high affinity (K_d for [125 I]Buserelin 1.1 ± 0.4 nM) and specificity (rank order of potency: Buserelin > GnRH >> chicken (c) GnRH-II), and mediated stimulation of [3 H]inositol phosphate (IP) accumulation. Increasing viral titre from 3 to 300 p.f.u./cell increased

receptor number (2000 to 275 000 sites/cell respectively) and [3 H]IP responses. GnRH also caused a biphasic increase in the cytoplasmic Ca^{2+} concentration in Ad GnRH-R-infected cells but not in control cells. Mobilization of Ca^{2+} from intracellular stores contributed to the spike phase of this response whereas the plateau phase was dependent upon Ca^{2+} entry across the plasma membrane. This effect on Ca^{2+} and stimulation of [3 H]IP accumulation were both blocked by the GnRH-R antagonist, Cetrorelix. In addition, GnRH reduced cell number (as measured in MTT activity assays) and DNA synthesis (as measured by [3 H]thymidine incorporation) in Ad GnRH-R-infected cells (but not in control cells). This effect was mimicked by agonist analogues and inhibited by two antagonists. Thus, when exogenous GnRH-Rs are expressed at a density comparable to that in gonadotrophs, they are functionally indistinguishable from the endogenous GnRH-Rs in gonadotrophs. Moreover, expression of high affinity GnRH-Rs can facilitate a direct anti-proliferative effect of GnRH agonists on prostate cancer cells.

Journal of Endocrinology (2003) **176**, 275–284

Introduction

Gonadotrophin-releasing hormone (GnRH) is the key hormone in the regulation of reproduction and is responsible for stimulating the secretion of follicle-stimulating hormone and luteinizing hormone (Conn & Crowley 1994). At the pituitary, GnRH acts via G-protein coupled receptors (GPCRs) which cause a $G_{q/11}$ -mediated activation of phospholipase C (PLC) and a consequent inositol trisphosphate (IP_3)-mediated mobilization of Ca^{2+} from intracellular stores (Sealfon *et al.* 1997). GnRH-stimulated gonadotrophin secretion can be blocked with antagonists, or mimicked by agonists, but in the latter case, sustained stimulation causes desensitization. Thus, both agonists and antagonists ultimately reduce circulating gonadotrophins

and gonadal steroids. This 'medical castration' is exploited to treat sex hormone dependent neoplasms such as those of the prostate, ovary, endometrium or breast (Barbieri 1992, Conn & Crowley 1994). However, GnRH receptors (GnRH-Rs) (often along with GnRH) may also be expressed in these cancers (Miller *et al.* 1985, Emons & Schally 1994, Emons *et al.* 1996, 1997, Moretti *et al.* 1996, Motta *et al.* 1996, Schally 1999, Gruendker & Emons 2001, Schally *et al.* 2001). Interest in these receptors stems primarily from the fact that GnRH analogues (or cytotoxic derivatives thereof) can inhibit proliferation of cell lines derived from such cancers, implying that such direct effects may contribute to the efficacy of GnRH analogues in cancer therapy (Miller *et al.* 1985, Qayum *et al.* 1990, Limonta *et al.* 1992, Dondi *et al.* 1994, Emons & Schally

1994, Emons *et al.* 1997, Jungwirth *et al.* 1997, Bahk *et al.* 1998, Palyi *et al.* 1999, Schally 1999, Halmos *et al.* 2000, Ravenna *et al.* 2000, Schally *et al.* 2001).

Investigation of the extra-pituitary GnRH-Rs has revealed major functional differences between GnRH-Rs in pituitary and extra-pituitary sites, in spite of the expression of identical receptor transcripts (Kakar *et al.* 1994). In radioligand binding studies (Emons *et al.* 1997), pituitary GnRH-Rs have high affinity for agonists such as Buserelin (nM K_d values) whereas most extra-pituitary expressed GnRH-Rs have low affinity (μ M K_d values). In the pituitary, GnRH-Rs are coupled via $G_{q/11}$ to PLC, causing an IP_3 -mediated mobilization of Ca^{2+} and a protein kinase C-mediated activation of MAP kinases, but there is little evidence for PLC activation by endogenous extra-pituitary GnRH-Rs (Emons *et al.* 1997, Limonta *et al.* 2001). Instead, G_i -mediated activation of protein phosphatase and inhibition of MAP kinase activity may underlie at least some of the antiproliferative effects mediated by GnRH-Rs (Moretti *et al.* 1996, Imai *et al.* 1997, Emons *et al.* 1998, Gruendker *et al.* 2001, Limonta *et al.* 2001). A further intriguing distinction is that peptides which act as competitive antagonists of pituitary GnRH-Rs have been shown to mimic effects of agonists in cancer cells (including those of the prostate), implying that the agonist/antagonist dichotomy established for pituitary GnRH-Rs may not be applicable to extra-pituitary GnRH-Rs (Emons *et al.* 1997, Limonta *et al.* 1997, 2001). In some cancers, however, locally produced GnRH may actually support (rather than inhibit) proliferation, such that agonists and antagonists can reduce proliferation by down-regulation and blockade (respectively) of GnRH-Rs (Arencibia & Schally 2000).

The stoichiometry of receptors to effectors and accessory proteins can influence receptor function and, since this can vary from cell-to-cell and with receptor number, operational characteristics of GPCRs can vary according to receptor density and cellular context. Accordingly, the reported differences between pituitary and extra-pituitary receptors could reflect an extreme degree of context-dependent signalling by this receptor. To test this possibility we have recently developed recombinant adenovirus expressing GnRH-Rs and have begun to use these to express GnRH-Rs, at a density which would be physiological for pituitary GnRH-Rs, in a human cancer-derived cell line reported to express endogenous GnRH-Rs (Everest *et al.* 2001). Here we show that recombinant adenovirus provides a simple means of expressing functional GnRH-Rs in an androgen-independent human prostate cancer cell line (PC3), that the operational characteristics of these receptors are similar to those of pituitary GnRH-Rs, and that expression of these receptors facilitates a pronounced anti-proliferative effect of GnRH-Rs agonists (but not antagonists). Accordingly, although we have found no evidence for context-dependence of GnRH-R signalling, we have shown that

GnRH-R numbers are limiting for its direct anti-proliferative effect, implying that manipulations that increase or maintain GnRH-R numbers in prostate cancer may increase the effectiveness of direct GnRH-R targeted therapy.

Materials and Methods

Materials

Peptides were purchased from Peninsula Laboratories Europe Ltd (Merseyside, UK) or from Sigma (Poole, Dorset, UK) with the exception of Buserelin and [^{125}I]Buserelin (2000 Ci/mmol) which were donated by Professor J Sandow (Aventis Pharma GmbH, Frankfurt, Germany) and Cetrorelix which was donated by Asta Medica (Frankfurt am Main, Germany). Culture media were from Sigma, sera were from First Link (Brierly Hill, Staffs, UK) and plasticware was from Gibco BRL (Paisley, UK) or Falcon (Becton Dickinson, Oxford, UK). All other reagents were purchased from standard commercial suppliers.

Cell culture and transfection

PC3 cells (European Collection of Cell Cultures and Cell Lines, Salisbury, UK) were cultured in RPMI with 10% calf serum, 50 iu/ml penicillin, 50 μ g/ml streptomycin and 2 mM L-glutamine. They were maintained at 37 °C in humidified air/CO₂ (19:1) and passaged weekly. For experiments, cells were harvested by trypsinization, then incubated for 1–7 days in culture plates as described. Cells were transfected by infection with recombinant replication-deficient adenovirus expressing sheep GnRH-Rs (Ad GnRH-R), prepared as described (Hislop *et al.* 2000, Everest *et al.* 2001). Viral titre was determined in a standard plaque formation assay and is expressed as the number of plaque forming units per plated PC3 cell (p.f.u./cell). In some experiments a control empty adenovirus was used (identical to Ad GnRH-R except that it lacks the GnRH-R sequence). Adenovirus was typically added to the cultured PC3 cells 24 h after plating.

Radioligand binding assays

A whole-cell suspension binding assay was used to determine number and pharmacological characteristics of expressed GnRH-Rs (Hislop *et al.* 2000, Everest *et al.* 2001). Twenty-four hours after plating at 200 000 cells/well in 6-well plates, PC3 cells were infected with Ad GnRH at 0–300 p.f.u./cell. They were then incubated for a further 24 to 48 h at 37 °C before being scraped from the plate, pelleted and resuspended in a physiological salt solution (PSS; 127 mM NaCl, 1.8 mM CaCl₂, 5 mM KCl, 2 mM MgCl, 0.5 mM NaH₂PO₄, 5 mM NaHCO₃, 10 mM

glucose, 0.1% BSA and 10 mM Hepes (pH 7.4)) which contained 1 mg/ml bacitracin. The number and affinity of GnRH-Rs expressed were determined by competition radioligand binding using [125 I]Buserelin (approximately 10^{-10} M) and 10^{-12} – 10^{-6} M unlabelled Buserelin. After 30 min at room temperature (21 °C), free and bound ligand were separated by centrifugation through oil as described (Hislop *et al.* 2000, Everest *et al.* 2001). The binding data were analysed by non-linear least square curve fitting using Prism software (GraphPad Software Inc., San Diego, CA, USA) to calculate K_d and B_{max} . Cell number was determined in parallel enabling calculation of receptor number per cell. Ligand specificity was assessed in a similar manner, except that the unlabelled competing peptide was varied. Some binding experiments were also performed using adherent cells in 96-well plates. These were seeded at 10 000 cells/well, infected 24 h later with Ad GnRH-R (100 p.f.u./cell) and then maintained for a further 24 h. The cells were then washed several times with PSS and incubated in PSS with 1 mg/ml bacitracin with varying concentrations of unlabelled competitor peptide and approximately 10^{-10} M [125 I]Buserelin for 30 min at room temperature (21 °C). The cells were washed four times with ice cold PSS and then solubilized in 0.2 M NaOH/10% SDS. Radioactivity was determined using a gamma counter.

[3 H]Inositol phosphate ([3 H]IP) accumulation

Phospholipase C activity was quantified as [3 H]IP accumulation in cells labelled with [3 H]inositol and stimulated in the presence of LiCl (McArdle *et al.* 1992a, Everest *et al.* 2001). Cells were seeded at 50 000 cells/well into 24-well plates and infected 24 h later with Ad GnRH-R (0 or 100 p.f.u./cell). After a further 24 h, 2 mCi [2- 3 H]inositol (14–16 Ci/mmol) were added. The cells were then incubated overnight (approximately 16 h) before being washed (twice) in PSS and stimulated for 30 min with 250 μ l PSS containing 10^{-2} M LiCl and the indicated concentrations of agonist. Stimulation was terminated with 1 ml water at 95 °C. After freezing and thawing, separation of [3 H]inositol phosphates and free [3 H]inositol was achieved by anion exchange chromatography, as described (Everest *et al.* 2001).

Dynamic video imaging of cytoplasmic Ca^{2+}

The cytoplasmic Ca^{2+} concentration was measured by video imaging in fura 2-loaded PC3 cells as described (McArdle *et al.* 1992b, Everest *et al.* 2001). Briefly, cells were seeded onto glass cover-slips at 50 000 cells/ml and infected with Ad GnRH-R 24 h later. After incubation for a further 24 h, they were washed in PSS and then exposed to 2 μ M fura-2/AM (in PSS) for 30 min at 37 °C. The cover-slips were then washed and loaded into a stainless steel holder fitted into a heating chamber at

37 °C. Image capture was performed within 5–25 min of loading in approximately 500 μ l PSS or in a modified PSS containing 100 μ M EGTA and no $CaCl_2$ (Ca^{2+} -free medium), and calibration was as described (Everest *et al.* 2001).

MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide) reduction

MTT reduction was used as a measure of viable cell number (Everest *et al.* 2001). The cells were routinely seeded at 2500 cells/well into 96-well plates. After 24 h they were transferred to medium with 1% FCS and the Ad GnRH-R was added at this time. After 24 h the cells were stimulated with the indicated peptide and 3 days later 50 μ l MTT solution (5 mg/ml in water) were added to the culture medium. After 4 h at 37 °C the medium was removed and 50 μ l acidified isopropanol (10^{-2} M HCl in isopropanol) were added to each well. The colour was allowed to develop for 5 min and optical density at 550 nm was determined. In some experiments cell number and incubation times were varied as described in the Figure legends.

[3 H]Thymidine incorporation

Incorporation of [3 H]thymidine into newly synthesized DNA was used as a measure of cell proliferation (Williams *et al.* 2000, Everest *et al.* 2001). Cells were seeded into 96-well plates at 2500 cells/well and 24 h later the media were replaced with medium containing 1% FCS and the required amount of Ad GnRH-R. Twenty-four hours later the cells were stimulated with the indicated peptides and 3 days later 10 μ l [3 H]thymidine were added to each well. After a further 4 h at 37 °C, the media were removed and 100 μ l trypsin/EDTA were added to each well. The cells were incubated at 37 °C for 30 min after which the plates were stored at –20 °C. Incorporated [3 H]thymidine was collected and measured as described (Everest *et al.* 2001).

Results

In preliminary experiments, transfection efficiency was assessed using a range of conventional (lipid or calcium phosphate based) techniques and flow cytometry to quantify expression of green-fluorescent protein (GFP) after transfection with a GFP vector. Using conventional techniques <20% of PC3 cells were transfected whereas >80% of the cells were transfected after infection with a recombinant adenovirus expressing GFP at >10 p.f.u./cell. These data (not shown) were very similar to those obtained in other cancer cell lines (see Hislop *et al.* 2000 and Everest *et al.* 2001 for details of methods) and recombinant adenovirus expressing GnRH-Rs were

Figure 1 GnRH-R expression in Ad GnRH-R-infected PC3 cells: binding affinity and receptor number. Main panel: PC3 cells were infected with Ad GnRH-R at 100 p.f.u./cell, then cultured for 48 h before use in suspension binding assays. Data shown are means $\pm$ S.E.M. and are pooled from seven experiments ($n=3-7$), each with duplicate or triplicate observations and normalized as a percentage of the internal control binding (without competitor). Inset: PC3 cells were infected with Ad GnRH-R at the indicated titre then cultured for 48 h and used in suspension binding experiments with [125 I]Buserelin and 0 or 10^{-6} M unlabelled Buserelin (non specific binding). The figure shows receptor number per cell, calculated as described in the Results. Data are from three independent experiments, each having triplicate observations ($n=3$).

therefore used for the remainder of this study. Whole cell radioligand binding studies with [125 I]Buserelin revealed no endogenous specific sites in control cells but after infection with Ad GnRH-R (100 p.f.u./cell) specific binding sites were clearly present (Fig. 1, main panel). Competition analysis revealed the presence of a single high affinity site with a K_d value of 1.1 ± 0.4 nM (Fig. 1). In a second series of experiments, cells were infected with Ad GnRH-R at various titres prior to single point binding assays. As adenoviral titres were increased from 3 to 300 p.f.u./ml, specific [125 I]Buserelin increased from approximately 2000 to 275 000 sites/cell. The number of receptors per cell was calculated from the specific binding and cell counts (performed in parallel) assuming a K_d of 1.1 nM and a Hill coefficient of 1 (in which case the tracer used occupies approximately 10% of the receptors at equilibrium). This calculation most likely causes a modest underestimate of receptor number at low adenoviral titre because it assumes 100% transfection efficiency. At 10 p.f.u./cell, for example, transfection efficiency is approximately 80% (estimated as described above) so receptor number (in the subset of cells that are transfected) is underestimated by approximately 25%. At higher titres transfection efficiency increases to $>90\%$ so that this error is reduced.

In order to assess ligand specificity, competition curves were constructed using [125 I]Buserelin and various concentrations of Buserelin, GnRH and chicken (c) GnRH-II

Figure 2 GnRH-R expression in Ad GnRH-R-infected PC3 cells: binding specificity. PC3 cells were infected with Ad GnRH-R at 100 p.f.u./cell, then cultured for 48 h before use in a suspension binding assay using [125 I]Buserelin and the indicated concentrations of agonists (upper panel: Buserelin (solid circles), GnRH (open circles), cGnRH-II (solid triangles)) or antagonists (lower panel: Antide (open circles), Cetrorelix (solid circles)). Data shown are means $\pm$ S.E.M. from seven experiments, each having duplicate or triplicate observations ($n=3-7$) and are normalized as a percentage of the internal control (without competitor).

(agonists at pituitary GnRH-Rs) as well as Cetrorelix and Antide (antagonists at pituitary GnRH-Rs). As shown, all five peptides competed with [125 I]Buserelin for binding (Fig. 2). The rank order of potency for the agonists (Buserelin $>$ GnRH $>$ cGnRH-II) was similar to that seen with endogenous pituitary GnRH-Rs and with type I GnRH-Rs expressed in other heterologous systems (Sealfon *et al.* 1997, Hislop *et al.* 2000, Everest *et al.* 2001). To determine whether these binding sites are functional receptors, effects on [3 H]IP accumulation and Ca^{2+} mobilization were assessed. In control cells, GnRH failed to increase [3 H]IP accumulation or cytosolic Ca^{2+} ion concentration ($[\text{Ca}^{2+}]_i$). However, after infection with Ad GnRH-R (100 p.f.u./cell) GnRH caused a clear and dose-dependent increase in [3 H]IP accumulation (Fig. 3) with an EC_{50} value of 0.86 ± 0.42 nM. Buserelin and cGnRH-II caused similar dose-dependent effects with

Figure 3 GnRH-R expression in Ad GnRH-R-infected PC3 cells: [^3H]IP accumulation. [^3H]IP accumulation was measured in [^3H]inositol-labelled cells, stimulated for 30 min with the indicated concentration of Buserelin (open circles), GnRH (solid circles) or cGnRH-II (triangles), in PSS with 10^{-2} M LiCl. The data shown are means $\pm$ S.E.M. from five experiments, each having been performed in triplicate. The [^3H]IP responses were normalized as a percentage of the internal control maximum response to each peptide.

EC_{50} values of 0.23 ± 0.04 nM and 2.96 ± 1.35 nM respectively, and stimulation of [^3H]IP accumulation by 10^{-9} M GnRH was abolished by 10^{-7} M Antide or Cetrorelix (not shown). In cells infected with Ad GnRH-R (100 p.f.u./cell) 10^{-8} M GnRH also caused a robust increase in $[\text{Ca}^{2+}]_i$ (Fig. 4) whereas no such increase was seen in control (uninfected) cells. In individual cells GnRH typically caused a spike-plateau type increase or a gradual increase to a sustained plateau and the magnitude of this response varied greatly from cell to cell, with some cells ($<40\%$) showing no increase and others showing increases to over 10^{-6} M (not shown). Although GnRH causes oscillatory Ca^{2+} responses in other systems, a clear oscillatory increase in $[\text{Ca}^{2+}]_i$ was seen in only one cell (e.g. $<0.5\%$ of the imaged population, not shown). When the cells were transferred to Ca^{2+} -free medium (containing 10^{-7} M GnRH) during the plateau phase of the response, the $[\text{Ca}^{2+}]_i$ was rapidly decreased (Fig. 4). When the cells were transferred to Ca^{2+} -free medium prior to stimulation with GnRH, the peptide caused only a modest and transient increase in $[\text{Ca}^{2+}]_i$; subsequent transfer to normal medium (still containing GnRH) elicited an increase in $[\text{Ca}^{2+}]_i$ to the plateau level (Fig. 4). Thus, in Ad GnRH-R-infected PC3 cells GnRH causes an initial spike-phase increase in $[\text{Ca}^{2+}]_i$ which is (at least in part) dependent upon mobilization from intracellular stores, followed by a plateau increase which is dependent upon Ca^{2+} entry across the plasma membrane. These data are characteristic of responses seen on robust activation of PLC-coupled GPCRs and are qualitatively similar to those seen in pituitary cells with endogenous or exogenous GnRH-Rs (Hislop *et al.* 2000, Stojilkovic & Catt 1995).

Figure 4 Effect of GnRH on $[\text{Ca}^{2+}]_i$ in Ad GnRH-R-infected PC3 cells. PC3 cells were infected with Ad GnRH-R at 100 p.f.u./cell, and then cultured for one day before being loaded with fura 2 and used for Ca^{2+} imaging. Upper panel: cells infected with Ad GnRH-R were stimulated with 10^{-7} M GnRH (horizontal bar). The arrow indicates a change of control medium (e.g. GnRH to GnRH). Lower panel: cells were stimulated with 10^{-7} M GnRH in normal medium and this was then replaced (indicated by the large vertical arrow) with Ca^{2+} -free medium containing 10^{-7} M GnRH (solid circles). Alternatively, the cells were transferred to Ca^{2+} -free medium (small vertical arrow) before stimulation with GnRH (horizontal bar) first in Ca^{2+} -free medium and then (indicated by the large vertical arrow) in Ca^{2+} -containing medium (open circles). The traces are the means $\pm$ S.E.M. from the pooled data of 8–10 experiments with responses of 10–50 cells averaged for each experiment. In control uninfected cells, GnRH failed to alter the $[\text{Ca}^{2+}]_i$ (not shown).

Since it has previously been shown that GnRH analogues can directly inhibit the proliferation of PC3 cells, we assessed whether infection with Ad GnRH-R was able to enhance or facilitate any such effect. We found no effect of GnRH or Buserelin (both at 10^{-7} M) on MTT reduction in control uninfected PC3 cells or in PC3 cells infected with an empty adenovirus at 100 p.f.u./cell (Fig. 5) but after infection with Ad GnRH-R, GnRH and Buserelin both reduced MTT activity by 40–60%. In subsequent experiments viral titre and duration of

Figure 5 Effect of Buserelin on MTT activity in PC3 cells: dependence on Ad GnRH-R infection. Cells were infected with empty adenovirus (empty Ad; 100 p.f.u./cell), with Ad GnRH-R (100 p.f.u./cell) or were uninfected (no Ad), then cultured with 10^{-7} M of either GnRH (open bars) or Buserelin (solid bars) (control cells received no peptide) and MTT activity was determined on the last day of culture. The data are from five separate experiments (means $\pm$ S.E.M., $n=5$) each with four replicate observations. For pooling, the data were normalized as a percentage of the MTT activity seen in control cells receiving no peptide and the control values did not differ significantly from each other. * $P<0.05$ compared with internal control value of 100% (dotted line).

Figure 6 GnRH-R-mediated effects on MTT activity in PC3 cells: dependence on time and Ad GnRH-R titre. Left panel (open bars): cells were infected with Ad GnRH-R (100 p.f.u./cell) and this was removed by aspiration and washing immediately ($t=0$) or after 1, 4 or 72 h, as indicated. Approximately 24 h later, 0 or 10^{-10} M Buserelin were added and the cells were cultured for 72 h prior to measurement of MTT activity. Right panel (solid bars): cells were cultured, Ad infected and stimulated with 0 or 10^{-10} M Buserelin, as described above (sustained exposure to Buserelin) except that the Ad titre was varied from 0 to 100 p.f.u./cell, as indicated. The data are from four separate experiments (means $\pm$ S.E.M., $n=3-4$) each of which had four replicate observations. For pooling, the data were normalised as a percentage of the MTT activity seen in control cells receiving no peptide. * $P<0.05$ compared with internal control value of 100%.

exposure to Buserelin were varied. As shown in Fig. 6, treatment of Ad GnRH-R-infected cells for 1 or 4 h with 10^{-10} M Buserelin had little effect on MTT activity,

whereas continuous exposure to Buserelin for 3 days reduced MTT activity by approximately 50%. Moreover, the inhibitory effect of 3-day treatments with 10^{-10} M Buserelin increased as viral titre was increased from 20 to 100 p.f.u./cell. This reflects dependence on receptor number (rather than on percentage of cells transfected) because we have estimated that at least 80% of cells are transfected under these conditions (above). In related experiments (not shown), the inhibitory effect of Buserelin was found to be essentially independent both of cell number at plating (between 1500 and 4500 cells/well) and of period of stimulation (between 1 and 6 days). Accordingly, all subsequent experiments were performed using 3 days of continuous exposure to test peptides after seeding at 2500 cells/well and infection at 100 p.f.u./plated cell.

In order to explore ligand specificity, the effects of Buserelin, GnRH and Cetrorelix (antagonist at pituitary GnRH-Rs) on MTT activity were determined. As demonstrated in Fig. 7 (upper panel), each of the agonists produced a dose-dependent reduction in cell viability, with Buserelin being more potent and effective than GnRH. Chicken GnRH-II also reduced MTT activity and its effect was comparable to that of GnRH (not shown). In contrast, Cetrorelix did not measurably influence MTT activity and similar data were obtained with Antide (not shown). Similar experiments were performed using [3 H]thymidine incorporation (Fig. 7, lower panel). Again, Buserelin and GnRH caused dose-dependent reductions whereas Cetrorelix failed to do so. The effects on [3 H]thymidine incorporation were clearly more pronounced than those on MTT activity (e.g. 10^{-8} M Buserelin reduced MTT activity by 50% and [3 H]thymidine incorporation by 80%). In a final series of experiments, the influence of Antide and Cetrorelix on the inhibitory effect of Buserelin was assessed. As shown in Fig. 8, Buserelin caused the expected reduction in MTT activity ($EC_{50} < 10^{-11}$ M). Antide and Cetrorelix had no measurable effect alone but clearly inhibited the Buserelin effect, shifting the Buserelin dose-response curve rightward and thereby increasing the EC_{50} to $> 10^{-9}$ M.

Discussion

In addition to their expression in the pituitary, GnRH-Rs are also found in several hormone-dependent cancers (or cell lines derived from such cancers) where they can mediate effects on cell division or death, but their identity and mechanism of action remain controversial. Although identical type I GnRH-R transcripts have been found in pituitary and extra-pituitary sites, their operational characteristics may differ in different sites (Conn & Crowley 1994, Stojilkovic *et al.* 1994, Imai *et al.* 1997, Sealfon *et al.* 1997, Grosse *et al.* 2000, Hislop *et al.* 2000, Gruendker

Figure 7 GnRH-R-mediated effects on MTT activity and [^3H]thymidine incorporation in PC3 cells: ligand specificity. Upper panel: the cells were cultured, Ad GnRH-R infected (100 p.f.u./cell) and stimulated as in Fig. 6 (sustained exposure to peptide) except that the indicated concentration of Buserelin (solid circles), GnRH (open circles) or Cetorelix (triangles) were used. The figure presents data from five or more separate experiments (means $\pm$ S.E.M.) each with four replicate observations. For pooling, the data were normalized as a percentage of the MTT activity in control cells receiving no peptide. The effects of Buserelin were statistically significant at all concentrations tested ($P < 0.05$) and the effects of GnRH were statistically significant at 10^{-8} and 10^{-7} M concentrations ($P < 0.05$). Lower panel: the cells were treated as in the upper panel, except that [^3H]thymidine incorporation (rather than MTT activity) was measured on the last day of culture. Statistically significant ($P < 0.05$) inhibition was seen with all concentrations of Buserelin and with 10^{-9} – 10^{-7} M GnRH.

et al. 2001, Limonta *et al.* 2001). Perhaps the most puzzling observation is that antagonists can mimic the antiproliferative effect of GnRH-R agonists on some hormone-dependent cancer cell lines. In some models it appears that locally produced GnRH actually supports cell proliferation so that this paracrine or autocrine effect can be reduced by receptor blockade or by agonist-induced receptor down-regulation (Arencibia & Schally 2000). In others, it is thought that GnRH-R activation inhibits proliferation (or causes cell death), in which case the identical effects of agonists and antagonists would imply

Figure 8 GnRH-R-mediated effects on MTT activity in PC3 cells: antagonist effects. The cells were cultured, Ad GnRH-R infected (100 p.f.u./cell) and stimulated as described in Fig. 6 (sustained exposure to peptide) except that the indicated concentrations of Buserelin were used either alone (solid circles) or with 10^{-7} M Cetorelix (upright triangles) or 10^{-7} M Antide (inverted triangles). The data are from four separate experiments (means $\pm$ S.E.M., $n = 4$) each of which had four replicate observations. For pooling, the data were normalized as percentage inhibition of control MTT activity (that seen in control cells receiving no peptide).

that the agonist/antagonist dichotomy defined largely with pituitary GnRH-Rs does not hold for receptors of extra-pituitary sites (Emons *et al.* 1997, Limonta *et al.* 1997, 2001).

In order to explore the context-dependence of GnRH-R function, we have previously generated recombinant adenovirus expressing mammalian GnRH-Rs and have previously used these to express GnRH-Rs in human mammary cancer cells (Everest *et al.* 2001). Although we were unable to detect endogenous GnRH-Rs, adenoviral infection enabled us to express GnRH-Rs in these cells at a range of densities encompassing physiological levels for pituitary GnRH-Rs. Using this approach we found that although mammalian GnRH-Rs can mediate a direct inhibition of proliferation in human mammary cancer cells, they are functionally indistinguishable from their pituitary counterparts (Everest *et al.* 2001). The obvious limitation of this work is that we may have simply chosen the wrong model system to reveal the context-dependence of GnRH-R signalling. For this reason, we have extended these studies to other hormone-dependent cancer cell lines. Here we have explored the functional characteristics of GnRH-Rs expressed in a human prostate cancer cell line (PC3 cells) by means of recombinant adenovirus. PC3 cells were used because a large proportion of human prostate cancers appear to express GnRH-Rs (Qayum *et al.* 1990, Schally 1999, Halmos *et al.* 2000, Schally *et al.* 2001) and because evidence exists that GnRH-Rs expressed by prostate cancer-derived cell lines can mediate direct inhibition of proliferation but differ

functionally from those of the pituitary. Androgen-independent cells were used because of the suggestion that GnRH-R activation can cause transactivation of steroid receptors (Turgeon & Waring 1994) and adenovirus-mediated transfection was used because it is vastly more efficient than standard transfection techniques in these cells.

Intact cell binding assays revealed no endogenous binding sites in control cells, but specific [125 I]Buserelin binding was clearly increased after infection with Ad GnRH-R. Curve fitting revealed high affinity sites (K_d 1.1 nM for Buserelin), and increasing viral titre from 3 to 300 p.f.u./cell increased the number of binding sites through a range (approximately 2000 to 275 000 sites/cell) that encompasses the physiological range for pituitary GnRH-Rs (20 000–70 000 sites per gonadotroph) (Clayton & Catt 1981, Loumaye & Catt 1983, Everest *et al.* 2001). These sites had high affinity for Buserelin, GnRH, Cetrorelix and Antide, and lower affinity for cGnRH-II, and were functional, because GnRH failed to increase the cytoplasmic Ca^{2+} concentration or to stimulate [3 H]IP accumulation in control cells, but did so after infection with Ad GnRH-R. The rank order of potency for stimulation of [3 H]IP accumulation was identical to that seen in competition binding experiments (Buserelin = GnRH > cGnRH-II). Similar results were obtained in Du-145 cells (an androgen-dependent human prostate cancer derived cell line) and in MCF7 breast cancer cells (Everest *et al.* 2001). In all three systems we were unable to detect endogenous GnRH-Rs but infection with Ad GnRH-R facilitated expression of high affinity phospholipase C-coupled receptors showing identical ligand specificity to that shown here.

Exploring possible GnRH-R-mediated effects on proliferation, no effect of sustained stimulation with Buserelin or GnRH on cell number (as revealed by MTT assays) was observed in either control cells receiving no adenovirus or in cells infected with a control 'empty' adenovirus. This is in apparent contradiction of earlier work showing direct inhibition of PC3 cell proliferation by GnRH analogues (Limonta *et al.* 1992, Palyi *et al.* 1999) but is in accord with the lack of endogenous receptor binding and signalling (data herein) and with previous work showing no inhibition of proliferation (Bahk *et al.* 1998). The reasons for such discrepancies are unknown but could relate to culture conditions, particularly since these have been shown to cause selection of functionally distinct sub-populations of PC3 cells (Festuccia *et al.* 2000). Clearly, however, the cells and protocols used herein reveal no evidence of endogenous GnRH-Rs and this model does not provide an opportunity to compare endogenous and exogenous GnRH-Rs. Instead, it enables functional characterization of the latter, in isolation. Doing so, we found that GnRH and Buserelin both caused a pronounced and potent reduction in cell number, after infection of PC3 cells with Ad GnRH-R (Fig. 5). This effect was dependent upon

viral titre (e.g. receptor number) and required sustained stimulation, since no inhibition was seen after stimulation for only 1 or 4 h (Fig. 6). It was also ligand-specific because Buserelin was more potent than GnRH (minimum effective concentrations of 10^{-11} and 10^{-9} M respectively) and Cetrorelix had no measurable effect, even at 10^{-7} M (Fig. 7). Qualitatively similar data were obtained by measurement of [3 H]thymidine incorporation on the last day of culture (Fig. 7) except that the effect was more pronounced (e.g. 80% maximal inhibition of [3 H]thymidine incorporation as compared with 50% maximal reduction in MTT activity). The greater reduction of [3 H]thymidine incorporation indicates that it is not simply a consequence of the reduced cell number. Indeed, it is more likely that agonists have inhibited cell proliferation (thereby reducing cell number) and that the greater reduction in [3 H]thymidine incorporation occurs because this assay compounds the reduction in cell number and proliferation rate. Similar data were obtained in the androgen-dependent Du-145 prostate cancer cell line (not shown) and in MCF7 cells (Everest *et al.* 2001).

An intriguing distinction was observed between the concentration–response relationships seen with different assays. Thus, Buserelin was 100–1000 times more potent than GnRH at inhibition of proliferation (Fig. 7) but was barely distinguishable from GnRH in binding assays or at stimulation of [3 H]IP accumulation (Figs 2 and 3). *In vivo*, the high potency of Buserelin (as compared with GnRH) is attributed to its resistance to degradation and the same may hold true in the proliferation assays, since sustained stimulation with agonist is needed for this effect (Fig. 6).

In summary, we have shown that recombinant adenovirus provides an efficient means of expressing functional GnRH-Rs in human prostate cancer cells and that varying viral titre enables the control of receptor number over a range encompassing physiological levels of pituitary GnRH-Rs. We have found no evidence for context-dependent GnRH-R signalling but have shown, instead, that human prostate cancer cells certainly are capable of expressing high affinity GnRH-Rs which are functionally indistinguishable from endogenous gonadotroph GnRH-Rs or from GnRH-Rs transfected into gonadotroph lineage cells. We were unable to detect endogenous GnRH-Rs or effects of GnRH on proliferation in control cells, and infection with Ad GnRH-R did not increase proliferation rates (as might be expected if endogenous GnRH were exerting a trophic effect). Instead, infection with Ad GnRH-R facilitated a receptor-mediated inhibition of proliferation, as evidenced by a reduction in cell number and rate of [3 H]thymidine incorporation per cell. This effect was inhibited (rather than mimicked) by GnRH-R antagonists and was increased as adenoviral titre (i.e. receptor number) was increased. Thus, GnRH-R activation is inhibitory for proliferation of these cells and strategies designed to increase or maintain GnRH-Rs may

therefore be useful in maximizing direct antiproliferative effects mediated by extra-pituitary GnRH-Rs.

Acknowledgement

This work was supported by the Wellcome Trust (059795 to C A M).

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Received 9 October 2002

Accepted 25 October 2002