The effects of gonadotrophin releasing hormone analogues in prostate cancer are mediated through specific tumour receptors

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Summary We have investigated the possibility of a direct regulatory effect of gonadotrophin releasing hormone (GnRH) analogues on prostatic cancer cell growth. Here we report high affinity binding (Kd = 50 nM) of a GnRH analogue resulting in biphasic growth modulation of the human androgen-sensitive prostatic cancer cell line LNCaP. In contrast, the human androgen-insensitive prostatic cancer cell line DU145 showed low-affinity (Kd = 10 μM) binding without any biological response to the GnRH analogue. A GnRH-specific radioimmunoassay demonstrated GnRH-like immunoreactivity in the concentrated culture medium from both cell lines. Seventy-six human benign and malignant tumours were assayed following surgical resection. Nineteen of 22 (86%) malignant tumours and 49 of 54 (91%) benign tumours exhibited high affinity GnRH-analogue binding. Fourteen of 19 (74%) malignant tumours and 17 of 49 (35%) benign tumours exhibiting high affinity binding contained GnRH-like immunoreactivity, suggesting that this system may be involved in prostatic epithelial cell growth in vivo.

Cancer of the prostate is the second most common cause of malignancy among males. The treatment of prostatic cancer aims to reduce serum androgen concentrations. The long-acting agonist analogues of GnRH have been recently introduced as effective alternative therapies to orchiectomy and oestrogen treatment (Waxman, 1987). Their effect on androgen dependent cell growth is thought to be mediated through down-regulation of the pituitary–gonadal axis. GnRH agonist treatment leads to an initial increase in the serum levels of LH, FSH and androgens and this may be accompanied by tumour flare (Waxman et al., 1985). However, this exacerbation of symptoms is not temporally related to changes in serum LH, FSH and androgen concentrations. The differences in the timing of these biochemical and clinical phenomena raises the possibility of a direct effect of GnRH analogues at the level of the tumour.

This present study explored the direct effects of GnRH analogues on prostatic cancer cells in culture and in vivo. GnRH analogue binding to human prostatic cancer cells grown in culture and biopsy samples was first examined. GnRH binding sites were characterised in cell lines and in human tumours. The biological effects of GnRH on prostatic cancer cells and a possible autocrine stimulatory role of GnRH-like peptides were investigated.

Materials and methods

Cell culture

The human androgen-sensitive prostatic cancer cell line LNCaP (isolated from lymph node metastasis; Horoszewicz et al., 1983) and the human androgen-insensitive prostatic cell line DU145 (isolated from brain metastasis) were obtained from the American Type Culture Collection. Cultures were maintained in exponential growth in RPMI 1640 medium containing 10% charcoal stripped fetal calf serum and 5 μg ml⁻¹ insulin. The fetal calf serum used in cell culture medium was treated with dextran and charcoal to remove steroids. Charcoal 0.25% and dextran-T70 0.025% were added to the serum, which was heated at 56°C for 2 h and then centrifuged at 4,000 r.p.m. for 10 min. The pellet was discarded, serum was filtered through 0.4 μm filters and used in culture medium. The treated serum was assayed for steroid hormones by the Hammersmith Hospital Endocrine Laboratories and found to be steroid free. Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and subcultured weekly using 0.1% EDTA to remove the cells from the plastic substratum. Under these conditions, the doubling times of DU145 and LNCaP cells were 72 and 144 h respectively.

GnRH binding assays

Exponentially growing cells were harvested with 0.1% EDTA, washed twice with PBS and counted using 0.2% trypan blue as an indicator. The cells were then resuspended in assay buffer (10 mM Tris-HCl pH 7.6, 1 mM dithiothreitol, 0.3% bovine serum albumin). One million cells per tube were incubated with 200,000 c.p.m. (0.1 nM) of 125I-buserelin (125I-D-Ser(TBU)-LHRH-ethylamide) (Hoechst AG, Frankfurt), (specific activity 53,000 kBq μg⁻¹) with or without varying concentrations of unlabelled buserelin (0.1–100 nM). Non-specific binding was estimated in the presence of 10 μM unlabelled buserelin. After 3 h incubation at 4°C, 1 ml of ice-cold assay buffer was added and the tubes were immediately centrifuged at 2,000 g for 10 min. The supernatants were discarded and the pellets were washed once with ice-cold assay buffer and counted in a gamma counter. The optimal time and temperature dependence of GnRH analogue binding was determined in both cell lines.

Preparation of tumour cell membranes and cytosolic fractions

After transurethral resection prostatic biopsy specimens were immediately frozen at ~70°C, and subsequently cleaned and homogenised in PBS using an Ultraturrax homogenizer (Janke and Kunkel IKA Laborotechnik, FR Germany) for three periods of 30 s each with an interval of 3 min on ice between each treatment. The debris and nuclear material were removed by centrifuging twice at 2,000 g for 10 min, discarding the pellet each time. The resultant supernatants were centrifuged at 10,000 g for 30 min at 4°C. The pellets were resuspended in PBS and either used immediately for binding experiments or stored frozen at ~70°C. The supernatants were stored frozen at ~70°C until use. The protein content was determined by Bradford's method (Bradford, 1976). Two hundred μg of membrane proteins per tube were used to assess the amount of 125I-buserelin binding.
Biological response studies

Exponentially growing cells were harvested using 0.1% EDTA, counted with 0.2% trypan blue and washed once with PBS. One million cells per flask were seeded in T-25 flasks in 5 ml of RPMI 1640 medium containing 10% charcoal stripped fetal calf serum. Fresh medium containing various concentrations of buserelin with or without a 100-fold excess of the GnRH antagonist D-pGlu\(^6\), D-Phe\(^2\), D-Trp\(^3\)\(^8\) GnRH (Sigma, UK) was added every third day. After 10 days the cells were harvested with 0.1% EDTA, washed once with PBS and counted with 0.2% trypan blue. Total DNA was estimated by Burton's method (Burton, 1956).

GnRH radioimmunoassay

The cells were cultured in RPMI 1640 medium containing 10% charcoal stripped fetal calf serum. The medium was collected after 2 weeks, without refeeding, acidified with 0.1% trifluoroacetic acid and centrifuged at 5,000 g for 10 min. The pellet was discarded and the supernatant was passed through C-18 Sep-Pak cartridge (Millipore, UK) (prewetted with methanol) followed by acidified water. The retained portion was eluted with 60% acetonitrile in water. There was 70% recovery of a control solution of buserelin added to fresh culture medium after a similar extraction procedure. The cytosol preparations from human prostatic samples were similarly concentrated. The eluates were examined for GnRH-like activity using GnRH radioimmunoassay kit (Amersham, UK). The sensitivity of the assay was 0.25 fmol per tube, inter-assay variation 7% and intra-assay variation 12%. Bradykinin, oxytocin, TRH, concentrated culture medium containing 10% dextran-charcoal heat-inactivated FCS and concentrated culture medium containing 10% normal FCS did not show any GnRH-immunoreactivity under the same conditions (Figure 1).

Results

GnRH receptor expression in prostatic cancer cell lines

\(^{125}\text{I}-\text{buserelin binding to membrane preparations from both prostatic cell lines is shown in Figure 2. The LNCaP cells showed high affinity binding of buserelin with 50\% inhibition obtained at 50 nM concentration of unlabelled peptide. The binding was saturable and specific as the structurally unrelated peptides bradykinin, oxytocin and TRH did not displace labelled buserelin in binding assays. A Scatchard plot of the same data indicated a single class of binding sites with } K_d = 40 nM (Figure 3). The binding of buserelin to DU145 cells was inhibited only by very high concentrations of unlabelled buserelin (K_d = 10 \mu M) indicating the presence of only very low affinity binding sites in these cells. The difference in specific binding of buserelin to both cell lines was more evident when a protease substrate (L-cystine-bis-(4-nitroanilide)) was included in the binding assays. This protease substrate has been shown to compete with GnRH for GnRH-degrading sites (Kuhl & Baumann, 1981; Kuhl & Taubert, 1975). The addition of this protease substrate to the assay tubes in a final concentration of 10 \mu M reduced the specific binding by 68\% in DU145 but by only 28\% in LNCaP cells (Figure 4).

The biological effect of buserelin on prostatic cancer cells

Treatment of LNCaP cells with buserelin for 10 days stimulated the growth of the cells by as much as 140\% relative to the control cultures as measured both by cell numbers and total DNA content (Figure 5). This stimulatory effect was observed only at lower concentrations of buserelin (1–10 nM)
which correlated well with the measured binding affinity. The growth of the cells was slightly inhibited at higher concentrations of GnRH analogue (100 nM). The androgen-insensitive cells did not show any response to buserelin treatment. No effects on cell growth were seen in short term culture. The stimulatory and inhibitory effects of buserelin on androgen-sensitive LNCaP cells were partially blocked when 100-fold excess of GnRH-antagonist was added to the culture medium (Figure 6).

Prostatic cancer cells secrete GnRH-like material

The observation that GnRH binding resulted in growth stimulation raised the possibility of the secretion of GnRH-like peptides by LNCaP cells themselves. We therefore examined this using a specific radioimmunoassay. The GnRH radioimmunoassay detected GnRH-like immunoreactivity in concentrated medium collected after two weeks culture of both cell lines. The concentrated culture media from 2 and 20 million LNCaP cells showed the presence of 3 and 30 fmol of GnRH-like immunoreactivity respectively (Figure 1). Twenty million DU145 cells cultured for 2 weeks secreted 35 fmol GnRH-like immunoreactivity. The elution profile of this immunoreactive peptide on high performance liquid chromatography was identical to native GnRH.

Discussion

Extrapituitary receptors for GnRH have been described in normal testis and ovary (Clayton et al., 1979, 1980). There have also been reports of the presence of GnRH-binding sites in breast cancer cells (Eidne et al., 1987), pancreatic tumours (Szende et al., 1989) and induced rat prostatic cancers (Kadar et al., 1988). The binding of GnRH to breast cancer cells has been shown to result in growth modulation (Millar et al., 1985; Eidne et al., 1987). In this present study the observation of high affinity binding sites in prostatic cancer cell lines and prostatic biopsy specimens suggests that GnRH and its analogues may exert their effects directly on tumour tissue. However, the biological effect observed in short term culture at therapeutic concentrations (1–10 nM) of GnRH analogue is stimulation of growth rather than inhibition. The initial direct stimulatory effect of GnRH analogues on prostatic cancer cells may therefore explain the lack of a temporal correlation of hormonal change with the clinical observation of tumour flare. It is possible that the long-term and continued occupancy of its binding sites by GnRH-analogues leads to desensitisation and down-regulation of receptors resulting in growth inhibition.
Table 1  Fresh frozen, transurethrally resected benign and malignant prostatic tissues examined for GnRH binding

| Tissue                | No. | $K_d \times 10^{-9} \text{M}$ | Capacity (fmol mg$^{-1}$) | GnRH-RIA $+$ ve | GnRH-RIA $-$ ve | GnRH activity fmol g$^{-1}$ tissue |
|-----------------------|-----|-------------------------------|---------------------------|----------------|---------------|----------------------------------|
| Benign prostatic hypertrophy |     |                               |                           |                |               |                                  |
| GnRH-R $+$ ve         | 49/54 | 46±43                         | 390±218                   | 32/49          | 17/49         | 26.16±20.2                     |
|                       | (90%) | (9.2%)                        |                           | (65.5%)        | (34.7%)       |                                  |
| GnRH-R $-$ ve         | 5/54  | 10±14                         | 301±180                   | 5/19           | 14/19         | 77±71.4                         |
|                       | (85%) | (100%)                        |                           | (26.3%)        | (73.7%)       |                                  |
| Prostatic cancer      |     |                               |                           |                |               |                                  |
| GnRH-R $+$ ve         | 19/22 | 10±14                         | 301±180                   | 5/19           | 14/19         | 77±71.4                         |
|                       | (85%) | (100%)                        |                           | (26.3%)        | (73.7%)       |                                  |
| GnRH-R $-$ ve         | 3/22  | 13.6%                         | 301±180                   | 1/3            | 2/3           | 100                             |
|                       | (65.5%) |                           |                           | (33.3%)        | (66.6%)       |                                  |

The cytosolic preparations from tissues were concentrated and assayed for GnRH-like activity in GnRH-RIA. Results are summarised from 76 prostatic tissues.

The finding of specific GnRH-binding sites in prostatic cancer cells which mediate growth, and the observation of the secretion of GnRH-like peptides by these cells, suggests that GnRH-like peptides may play an autocrine stimulatory role in this system. It is not yet known whether GnRH itself has mitogenic activity nor whether its has synergy with other stimulatory growth factors, and this requires further investigations. The results of our study provide impetus for the development of GnRH antagonists for use in prostatic cancer treatment.

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