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III. Hormonal Treatment of Prostate Cancer (in Honour of Professor H. J. de Voogt)

THE PARTIAL PURIFICATION AND CHARACTERIZATION OF GnRH-LIKE ACTIVITY FROM PROSTATIC BIOPSY SPECIMENS AND PROSTATIC CANCER CELL LINES

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Summary—We have investigated the possibility of the secretion of gonadotrophin-releasing-hormone (GnRH)-like peptides by prostatic cancer cells in culture and their presence in cytosolic preparations from human prostatic biopsy specimens. A GnRH-specific radio-immunoassay showed GnRH-like activity in concentrated cytosolic preparations and conditioned media from DU 145, an androgen-insensitive human prostatic cell line and from LNCaP, an androgen-responsive prostatic cancer cell line. GnRH immunoreactivity in culture media correlated directly with cell numbers. HPLC demonstrated that this GnRH-like material co-migrated with synthetic GnRH. This homology between synthetic GnRH and partially purified prostatic GnRH was confirmed following V8 protease and trypsin digestion which resulted in similar alterations in HPLC characteristics. The mean content of GnRH-like activity/g specimen tissue was significantly more in malignant tissue (88.5 ± 80.5 fmol) than in benign (29.6 ± 22 fmol), though more specimens of benign tissue were positive (37/54) than malignant tissue (6/22). This observation, taken with an earlier finding of GnRH-specific receptors in a hormone-sensitive cell line and human cancer specimens provides supportive evidence for the autocrine hypothesis of cell regulation.

INTRODUCTION

The long-acting superagonist analogues of gonadotrophin-releasing hormone (GnRH) are effective forms of treatment of advanced prostatic cancer [1, 2]. It has been suggested that these analogues act by down-regulating the pituitary–gonadal axis, where their constant occupancy of pituitary GnRH-receptors leads to decreased release of the gonadotrophins and reduces the serum testosterone to castrate levels [3]. An additional hypothesis is that there is a direct anti-proliferative effect of GnRH-analogues on prostatic tissue and this is supported by the observation of the presence of specific GnRH-binding sites in experimental prostatic cancer, prostatic cancer cells in culture and biopsy specimens [4, 5]. Since hypothalamic GnRH is undetectable in peripheral blood, the presence of GnRH-binding sites raises the possibility of a locally produced GnRH-like peptide and that this peptide acts as the natural ligand for prostatic GnRH-binding sites. In addition to the prostate, other extrapituitary receptors for GnRH have been previously characterized in the testis and ovary which may interact with locally produced GnRH-like peptides [6, 7]. In this study, we have investigated the production of GnRH-like peptides in prostatic cancer. We have previously described the detection of uncharacterized GnRH-like immunoreactivity in culture medium from prostatic cancer cells as well as in cytosolic preparations from biopsy specimens [5]. Here, we report the further characterization of this prostatic GnRH-like activity.

MATERIALS AND METHODS

Human prostatic cancer cell lines were purchased from the American Type Culture Collection. The DU 145 cell line is androgen-unresponsive and was established from a prostatic cancer brain metastasis [8]. LNCaP cells are hormone-sensitive and were originally cultured from a lymph node metastasis [9]. Both cell lines were routinely cultured in RPMI

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including 10% foetal calf serum and insulin (1 μg/ml). Culture medium for assay for GnRH-like activity was collected from cells cultured in RPMI with 5% steroid-depleted foetal calf serum. The foetal calf serum for this purpose was treated with 0.25% activated charcoal and 0.025% dextran T-70 (Sigma, Poole U.K.), heated at 56°C for 2 h, centrifuged at 5000 *g* for 10 min, filtered using 0.2 μm filter and frozen in aliquotes for later use. The cytosolic preparations from prostatic biopsy specimens were made as described previously [5]. Briefly, the specimens were excised clean, homogenized using a pre-cooled Ultraturrax homogenizer (BDH, Essex, U.K.) (three periods of 30 s each with an interval of 30 min on ice) and centrifuged twice at 250 *g*, discarding the pellet each time. The supernatant was then centrifuged at 10,000 *g* for 30 min and the membrane fraction preserved for further use. The cytosolic supernatants (cytosols) were acidified with 0.1% trifluoracetic acid (TFA) and centrifuged at 5000 *g* for 10 min. The resulting supernatants were passed through a C-18 Sep-Pak mini-column (Millipore, London, U.K.) prewetted sequentially with methanol and acidified water. The retained portion was eluted with 60% acetonitrile containing 0.1% TFA. A control culture medium enriched with a radiolabelled GnRH-analogue and subjected to a similar procedure showed 67% recovery of radioactivity.

The concentrated cytosols and culture medium were lyophilized under vacuum and reconstituted in PBS. Native GnRH was chromatographed on reverse-phase HPLC using a C-18 column (Beckman, Palo Alto, Calif., U.S.A.) and an acetonitrile concentration gradient. Samples were similarly chromatographed and fractions corresponding to GnRH eluates were collected and a GnRH-radioimmunoassay carried out. The HPLC fractions were analysed for GnRH-like activity by radioimmunoassay. The assay was based on the competition between ^{125}I-labelled GnRH (sp. act. 2000 μCi/μg) and unlabelled hormone (GnRH, samples) for anti-GnRH antibodies directed against the carboxyl terminal of GnRH. One of the antibodies (RPN 1811) was purchased commercially (Amersham Chemicals, Amersham, U.K.) and the second anti-GnRH antibody (F86) was a gift from Dr R. N. Clayton (CRC Northwick Park Hospital, London, U.K.). The primary antibodies were used in a final dilution of 1:1000 and 1:7000 for RPN 1811 and F86, respectively. The binding capacity ranged from 35 to 48% with non-specific binding ranging from 3 to 8%. The inter-assay variation was 7% and intra-assay variation was 12%. The sensitivity of the assay was improved by increasing the time for hormone-antibody binding to 48 h and the lower limit of detection was 0.25 fmol/tube. Bound ligand was separated by adding a second antibody (R 0881, Sigma, U.K.) in a final dilution of 1:200 for 24 h at 4°C, followed by centrifugation (5000 *g*, 30 min, 4°C) to precipitate the hormone-antibody–antibody complex. The specificity of the assay was determined by the fact that structurally unrelated peptides did not significantly complete in the assay.

Native GnRH was treated with purified proteolytic enzymes cleaving different bonds in the peptide sequence. The indicated amounts of GnRH or sample were incubated in 1% NH_4CO_3 buffer (pH 8.0) in a ratio of 10:1 for 24 h with V8 protease or for 4 h with trypsin at 37°C. The digested and undigested peptide was chromatographed on reverse-phase HPLC and fractions collected. The fractions were examined by RIA to observe the changes in recognition of the molecule by the specific antibody. The conconcentrated, partially purified GnRH-like activity was also subjected to a similar procedure and then fractionated.

RESULTS

Peptides structurally unrelated to GnRH, including bradykinin, thyrotropin-releasing hormone and oxytocin, were not recognized by the anti-GnRH antibody in the radioimmunoassay, as shown in Fig. 1. The possibility of artefactual competition of binding was investigated by using controls which included RPMI with 10% charcoal–dextran treated heat inactivated foetal calf serum, RPMI alone and 60% acetonitrile containing 0.1% TFA brought to pH 7.4 with 1 M NaOH in radioimmunoassay buffer (PBS containing 0.1% sodium azide, 0.1% EDTA and 0.25% normal rabbit serum). No displacement of antibody bound ^{125}I-labelled GnRH-was observed by any of the three mixtures. When concentrated culture media from DU 145 and LNCaP prostatic cell lines were used in the RIA, they displaced antibody bound ^{125}I-labelled GnRH. This displacement was proportional to the concentration of the medium added to the assay (Fig. 1). The observed RIA activity was also dependent on the number of cells present at the time of medium collection.

For further purification, the samples were fractionated on HPLC. Synthetic GnRH, when

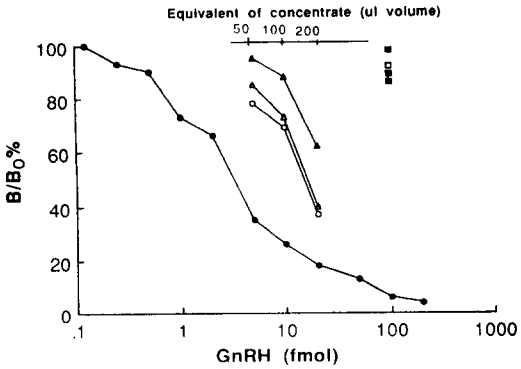

Fig. 1. The GnRH-like activity from prostatic biopsy specimens and from cell lines was partially purified and examined in GnRH-radioimmunoassay, as described under "Materials and Methods". The specificity of RIA was determined by using bradykinin (■), oxytocin (□) or TRH (▨) in the assay. HPLC purified cytosolic preparations from biopsy specimen (○——○) or culture medium from DU 145 (△——△) or LNCaP (△——△) cells were lyophilized and redissolved in 1 ml PBS in different concentrations (50, 100, 200 μl).

chromatographed using an acetonitrile gradient, was eluted as a single compact peak emerging at 18–19 min. When the fractions were checked in the GnRH-radioimmunoassay, approx. 76% of the GnRH loaded was recovered in the combined peak fractions. The concentrated, lyophilized samples were similarly fractionated and assayed. Although a scatter of GnRH-like activity was observed, most of the immunoreactivity was present in the fractions corresponding to the elution position of synthetic GnRH. Those fractions from cell lines and biopsy specimens containing the highest amounts of GnRH-activity were collected, pooled, lyophilized and preserved for further use. Digestion of GnRH with V8 protease, which cleaves at the carboxyl side of glutamic acid, produced two fractions detected separately on HPLC (Fig. 2). Although one of these segments was not recognized in the

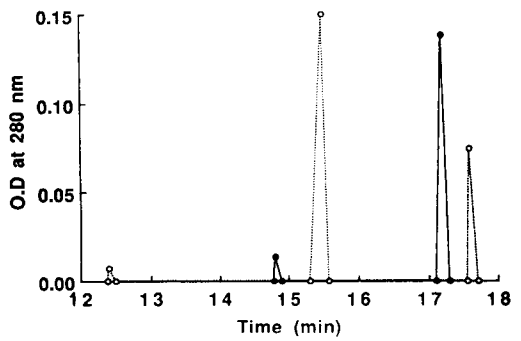

Fig. 2. Native GnRH (10 nmol) was digested with either V8 protease (●——●) for 24 h or with trypsin (○——○) for 4 h at 37°C, as described under "Materials and Methods". The digested GnRH was then chromatographed on HPLC. The undigested GnRH was eluted at 18–19 min.

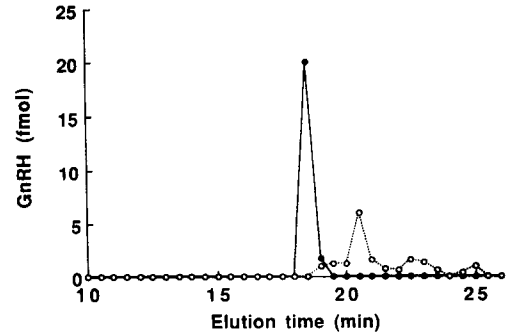

Fig. 3. GnRH (100 fmol) was digested either with V8 protease (●——●) or with trypsin (○——○), as described under "Materials and Methods". The digests were fractionated on HPLC and analysed for GnRH immunoreactivity of the fractions. The eluted fractions from HPLC of similarly treated but undigested GnRH showed maximum immunoreactivity at 18–19 min.

radioimmunoassay, this enzyme did not significantly affect the immunoreactivity of the peptide which was concentrated in the fraction containing the larger slightly more hydrophobic fragment (Fig. 3). However, there was a slight shift by approx. 1 min of RIA activity to the left. Partial digestion of GnRH with trypsin yielded three fragments on HPLC eluting at 12.4, 15.5 and 17.6 min, however they were not recognized by the GnRH antibody in the RIA (Fig. 3). A similar pattern was observed when samples from tumours or conditioned media were digested with V8 and trypsin followed by detection of GnRH-like activity in HPLC fractions (Fig. 4).

The biopsy specimens taken both from benign hypertrophy and malignant prostatic tissues contained detectable GnRH-like activity. Although more specimens from BPH (68.5%) were positive than those from malignant prostate (27.2%) (Table 1). The mean content of GnRH-like activity/g tissue was less in BPH

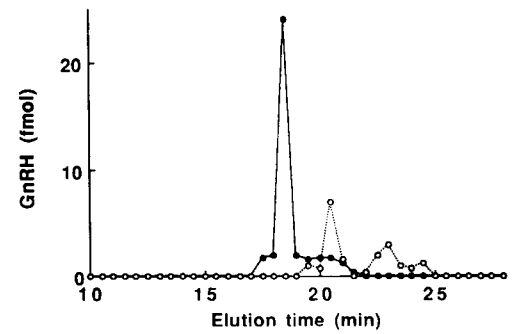

Fig. 4. Partially purified prostatic GnRH-like activity (100 fmol) was digested either with V8 protease (●——●) or with trypsin (○——○), as described under "Materials and Methods". The digests were fractionated on HPLC and analysed for GnRH immunoreactivity of the fractions. The fractions from similarly treated but undigested GnRH showed maximum immunoreactivity at 18–19 min.

Table 1. Total GnRH immunoreactivity per million cells or per g biopsy tissue

	n	RIA + ve	RIA activity (fmol)
DU 145	4	4/4	8.5 (±4.1)
LNCaP	2	2/2	12 (±2.8)
BPH	54	37/54	29.6 (±22)
Ca prostate	22	6/22	88.5 (±80.5)

(29.6 ± 22 fmol) than in malignant tissue (88.5 ± 80.5 fmol). These findings may not reflect the true *in vivo* situation as transurethal resection may induce significant tissue damage.

DISCUSSION

The hypertrophied or malignant human prostate contains GnRH-like immunoactivity, as demonstrated in a GnRH specific radioimmunoassay using two different antibodies, raised against GnRH. The assay was specific as it did not detect peptides structurally unrelated to GnRH, and it can therefore be assumed that the activity detected has complete or partial structural similarity to GnRH. This assumption was confirmed by the observation that this material chromatographed with native GnRH on reverse-phase HPLC and also that the pattern of cleavage by the two different proteolytic enzymes is shared with synthetic GnRH. Human prostatic cancer cell lines, androgen-responsive and androgen-unresponsive, also secreted GnRH-like activity into their culture medium, in amounts proportional to cell numbers.

The significance of these observations in the prostate may be that GnRH plays an autoregulatory role on the rate of cell proliferation. The presence of specific GnRH-receptors and the biphasic effects of a GnRH-analogue on the proliferation of prostatic cancer cells [5] suggests that GnRH-like peptides found in this work may regulate cell growth in an autocrine manner in prostate. Whether this is a significant event in the process of carcinogenesis or merely an epiphenomenon is unclear. Our results suggest that the observation is significant, and provides a paradigm for peptide treatment of human cancer where a structurally modified growth factor has been used to regulate a human cancer. The kinetics of secretion of GnRH may be relevant to these effects. In the hypothalamus GnRH-release is pulsatile, and interference with this mechanism by GnRH-analogues leads to desensitization of GnRH-receptors. It is possible that prostatic cells also secrete GnRH-like peptides in a pulsatile pattern and that the inhibition by GnRH superagonists is a consequence of the receptors being persistently occupied by the modified and very stable ligand. In the natural state GnRH-degrading enzymes, which are particularly abundant in the malignant prostate [5], may contribute to the maintenance of cellular responsiveness through rapid degradation of secreted GnRH-like activity.

GnRH-like peptides have previously been demonstrated in human milk [10], follicular fluid [11], ovarian extracts [7] and testicular tissue [6]. All these tissues and the prostate are sex-steroid responsive. It is possible that steroids regulate the secretion and hence activity of these peptides in responsive tissues. The future investigation of this interaction may provide insights into the functional role of GnRH-like peptides in normal and malignant hormone responsive tissues.

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