EXPERIMENTAL STUDY

Expression of growth hormone-releasing hormone in human primary endometrial carcinomas

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Abstract

Background: Hypothalamic GH-releasing hormone (GHRH) regulates GH release from the pituitary, but an ectopic production of GHRH has been detected in various non-hypothalamic tissues, especially cancers.

Objective: To investigate whether endometrial tumors produce GHRH.

Methods: Twenty-four endometrioid, three serous papillary (SP), three mixed type endometrioid/serous papillary adenocarcinomas and one malignant mixed Müllerian tumor (MMMT) were assessed for GHRH immunoreactivity by the polyclonal anti-rabbit antibody SV95 and for the expression of GHRH mRNA by *in situ* hybridization using an oligonucleotide probe.

Results: Increased GHRH immunoreactivity was detected in 15 out of 24 (63%) of the endometrioid tumors, including two out of three (66%) of the mixed type endometrioid/serous adenocarcinomas but not in the three SP or the MMMT tumor. Cytoplasmic staining was detected in all positive cases, while in three of them strong nuclear localization of GHRH was also revealed. *In situ* hybridization indicated the presence of GHRH mRNA in six cases, all characterized as positive for GHRH immunoreactivity.

Conclusion: GHRH is expressed in a subset of endometrial tumors, of the endometrioid type in particular. A paracrine/autocrine role for GHRH in the development of the disease should be considered.

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Introduction

Endometrial cancer is a widespread malignancy of the female genital tract, especially in postmenopausal women. Most cases of endometrial carcinoma are detected at early stages and therefore the clinical outcome of treatment is favorable. However, a significant number of patients with early-stage disease develop localized recurrence and distal metastases (1).

According to the World Health Organization histologic classification of endometrial carcinomas, the most common type is the endometrioid adenocarcinoma. Recent studies have associated this type of cancer with an endocrine milieu of estrogen predominance and considered them sensitive to endocrine manipulations (1), while tumors of non-endometrioid histology like serous papillary adenocarcinomas, were not responsive to endocrine manipulations (1). Genetic alterations associated with the development of the endometrioid adenocarcinomas include microsatellite instability and mutations in the *K-ras*, *PTEN* and

β -catenin genes, while non-endometrioid carcinomas often harbor p53 mutations and losses in several chromosomes (2). Mixed tumors containing both endometrioid and non-endometrioid elements have also been described.

Growth hormone-releasing hormone (GHRH) is a 40–44 amino acid peptide secreted by the hypothalamus which, upon binding to specific GHRH receptors in the anterior pituitary, stimulates the synthesis and release of growth hormone (GH). In turn, GH induces the production of insulin-like growth factor-I in the liver, this is a known mitogen for various cell types (3). In addition to its neuroendocrine function in regulating the release of GH, GHRH may play a direct paracrine/autocrine role in peripheral tissues and cancers. Thus, various normal tissues, such as placenta, ovaries, testis and lymphocytes produce biologically active GHRH (4–12). Ectopic production of GHRH has also been shown in primary tumors and/or cancer cell lines from patients with lung, prostate and pancreatic cancer, as well as gynecological malignancies (13–19).

The evidence for a possible mitogenic role of locally produced GHRH was provided by recent studies which demonstrated that the exposure of cancer cells cultured *in vitro* to GHRH stimulates proliferation while the antagonists of GHRH produce an inhibition both *in vitro* and *in vivo* (18, 20–23). These findings suggest an autocrine/paracrine role for GHRH in human tumors.

Although various studies have been performed on the role of GHRH on cancer cell lines, information on the ectopic production of this neuropeptide hormone by primary tumors is relatively sparse. In view of the endocrine nature of the endometrioid carcinomas and the recently identified mitogenic effects of GHRH in human cancers, the aim of the present study was to investigate whether endometrial tumors of the endometrioid type express GHRH. The specimens were analyzed first for GHRH-like immunoreactivity using a rabbit polyclonal antibody for GHRH (24). Then, in order to determine if positive staining is due to the local production of GHRH or the increased accumulation of the circulating GHRH by the cancer cells, the samples were analyzed by

in situ hybridization for the presence of mRNA for GHRH.

Subjects and methods

Specimens

Formalin-fixed paraffin-embedded tissues from 31 patients who had been surgically treated were collected from the Surgical Pathology archives of the Department of Pathology, Aretaieion Hospital, University of Athens, Athens. The study was approved by the Hospital Ethics and Scientific Research Committee. All patients had been treated by total extrafascial hysterectomy, bilateral salpingo-oophorectomy, and pelvic and para-aortic lymph node sampling. Patients were staged according to the International Federation of Gynecology and Obstetrics (FIGO) classification, and their tumors were graded as well (G1), moderately (G2) or poorly (G3) differentiated. Data on tumor histotype, FIGO grade, age of the patient and invasion of the myometrium are shown in Table 1. For both immunohistochemistry and *in situ* hybridization, 4 mm thick sections were used.

Table 1 The age of patients, histotype, FIGO stage, invasion of the myometrium (%), percentage of GHRH-positive cells in immunohistochemistry (IHC) and GHRH *in situ* hybridization (ISH) positivity.

Case no.	Age of patient (years)	Histotype	FIGO grade	Invasion of myometrium (%)	GHRH expression	
					GHRH IHC	ISH
1	50	E	G2	100	+++ ⁿ	+
2	53	E	G2	80	+++	+
3	70	E	G1	100	+++	+
4	86	E	G2	N/A	+++ ⁿ	+
5	68	E	G1	N/A	+++	–
6	63	E	G2	83	+++	–
7	N/A	E	G1–G2	50	+++	–
8	60	E	G1	33	+++	+
9	62	E+SP	G1	67	++	–
10	72	E	G2	67	+++ ⁿ	–
11	64	E	G1	67	++	+
12	72	E	G1	25	++	–
13	54	E+SP	G1	33	++	–
14	64	E	G1	33–50	++	–
15	43	E	G1	0	++	–
16	67	E	G2	50	++	–
17	87	E	G2	25–33	++	–
18	90	MMMT	N/A	100	+	–
19	68	SP	N/A	67–75	+	–
20	49	E	G2	50	+	–
21	69	E	G1	100	+	–
22	55	E	G1	0	+	–
23	69	SP	G2	33	±	–
24	59	E	G1	50	±	–
25	53	E	G2	0	±	–
26	67	E	G2	83	±	–
27	N/A	E	N/A	33	±	–
28	66	SP	G3	N/A	–	–
29	73	E	G2	50	–	–
30	48	E	G1	N/A	–	–
31	66	E+SP	G2	N/A	–	–

N/A, not available; E, endometrioid; SP, serous papillary; MMMT, mixed malignant Mullerian tumor; ⁿ, nuclear staining.

Immunohistochemistry

Serial sections from representative paraffin-embedded blocks of 31 endometrial adenocarcinomas were collected onto poly-L-lysine-coated slides and stained for GHRH antigen. The immunohistochemical detection of GHRH was performed with the rabbit anti-GHRH polyclonal antibody SV95 (24) diluted with 1 × phosphate-buffered saline (PBS) at 1:200, using the Kwik-DAB kit (ThermoShandon, Pittsburgh, PA, USA) according to the manufacturer's instructions. Specimens were evaluated for positive staining and classified according to the percentage of positive cells into the following categories: 1–10% (±), 11–30% (+), 31–70% (++) and 71–100% (+++).

In situ hybridization

In situ hybridization for the detection of the mRNA for GHRH was performed as follows. Tissue sections were de-paraffinized twice in xylene for 5 min and hydrated through a standard series of ethanol to double-distilled H₂O (ddH₂O). Endogenous peroxidase was inhibited with 3% H₂O₂ for 15 min at room temperature and specimens were washed with ddH₂O and then 1 × PBS. Subsequently specimens were permeabilized with 0.5% Triton-X 100 for 5 min on ice, washed with 1 × PBS for 10 min three times and incubated with 20 µg/ml proteinase K in Tris-EDTA for 30 min at 37 °C. Sections were then rinsed three times with 1 × PBS and twice with 2 × SSC (10 min each). Pre-hybridization was performed at 37 °C for 10 min in a buffer containing 25% formamide, 1 mg/ml yeast tRNA, 2 × SSC and 10% dextran sulfate. For hybridization, the slides were incubated for 16 h at 42 °C in the pre-hybridization buffer supplemented with the digoxigenin-labeled oligonucleotide riboprobe (5'-CUC-GCUCUUGGUUGCUCUCU-3') at 5 ng/µl. The slides were then washed with 2×, 1× and 0.5× SSC for 30 min each, rinsed with 1 × PBS containing 0.1% Triton-X 100 for 5 min and incubated for 1 h at room temperature with anti-digoxigenin-peroxidase conjugate (Roche), diluted in PBS at 1:500. After washing with PBS, the slides were exposed to the DAB chromogen solution for 5 min, washed with ddH₂O, counterstained with hematoxylin and mounted. All reagents were obtained from Sigma unless otherwise stated.

Negative controls consisting of the substitution of the primary antibody with 1 × PBS or hybridization with a digoxigenin-labeled sense riboprobe were also included for immunohistochemistry and *in situ* hybridization respectively.

Results

In the present study, endometrial tumors, predominantly of the endometrioid type, were analyzed for increased GHRH-like immunoreactivity using a polyclonal

antibody for GHRH, and for the expression of mRNA for GHRH by *in situ* hybridization. Our results showed that in 15 out of 24 (63%) of the endometrioid tumors and two out of three (66%) of the mixed type endometrioid/serous tumors, a strong GHRH-like immunoreactivity (++, +++ specimens) was detectable by means of immunohistochemistry (Table 1 and Fig. 1). Among 17 GHRH-immunopositive specimens, six revealed the expression of GHRH mRNA by *in situ* hybridization, confirming that the source of the GHRH-like immunoreactivity is GHRH produced locally by the cancer cells (Fig. 2). Among these six specimens, positive for GHRH mRNA, case no. 1 had metastases in the lymph nodes and extension in the endocervix. There was no healthy endometrium in the section tested to allow for the evaluation of GHRH mRNA expression in normal tissue. Case no. 2 exhibited atypical complex hyperplasia in the non-neoplastic tissue. The remaining four cases corresponded to menopausal women, 60–72 years of age. Normal endometrium was absent from the sections available.

Discussion

We have shown that endometrial cancers are the source of ectopic production of GHRH. Among the 31 cases examined, 17 were positive for GHRH immunoreactivity. In six of these 17 GHRH-immunopositive cases, the expression of mRNA for GHRH was also detected by *in situ* hybridization. It must be mentioned that the term 'ectopic' may not be applicable for GHRH, as well as other hypothalamic neuropeptides, i.e. luteinizing hormone-releasing hormone, because their production in non-hypothalamic tissues may be associated with physiological functions and not necessarily only with pathological conditions. This is in agreement with a recent study by Khorram *et al.* (17) who demonstrated the expression of mRNA for GHRH in normal endometrial tissue. According to the same study, the levels of GHRH mRNA were significantly increased in secretory endometrium compared with proliferative endometrium (17). In the present study, only three specimens were obtained from patients younger than 50 years. Most patients, 20 out of 29 cases examined for whom age was available, were older than 60 and presumably menopausal (Table 1).

The failure to detect mRNA for GHRH in the remaining 11 specimens, which were positive for GHRH-like immunoreactivity, may be due to a combination of factors, such as the relatively higher half-life of the GHRH peptide in these tumors, as compared with the corresponding mRNA, as well as high local accumulation of GHRH which is produced by other sources. Thus, the sensitivity of the immunohistochemical detection of GHRH was greater compared with *in situ* hybridization. These findings suggest that GHRH is probably implicated in the pathogenesis of the disease by autocrine or

Figure 1 Immunohistochemical detection of GHRH in endometrioid adenocarcinomas of the uterus. Immunohistochemical positivity is mainly shown in the glands, while the endometrial stroma is negative for GHRH (a–e). In (b), the arrows indicate nuclear staining. (f) Negative control (primary antibody has been omitted).

Figure 2 Detection of GHRH mRNA by *in situ* hybridization in endometrioid adenocarcinomas of the uterus. Nuclear positivity was sparse, featuring a clonal pattern of the expression of the mRNA for GHRH.

paracrine mechanisms. None of the specimens which were found to be negative for GHRH-like immunoreactivity was positive in *in situ* hybridization, indicating that the cross-reactivity of the anti-GHRH antibody used in our study, with other GHRH-like antigens expressed by the tumors tested, cannot account for the positive staining.

The expression of GHRH was demonstrated only in the endometrioid tumors, while the three serous adenocarcinomas and the mixed Müllerian tumor included in the present evaluation were negative for GHRH-like immunoreactivity. Although the number of specimens, particularly of the serous tumors, was limited, these findings suggest that an ectopic expression of GHRH might be associated with the development of the endometrioid type of endometrial cancers only. This is consistent with the endocrine nature of the endometrioid adenocarcinoma, as compared with other types of endometrial cancers (1).

In the GHRH-immunopositive specimens, GHRH was predominantly localized in the cytoplasm, probably because of the production of GHRH by the endoplasmic reticulum in the GHRH-producing cells or because of the internalization of this neuropeptide after receptor binding, which eventually results in the degradation of GHRH. The latter possibility applies to both GHRH-producing and non-producing cells and is supported by the observation that, in 11 out of 17 immunopositive specimens, mRNA for GHRH was not detected. Furthermore, in the specimens found to be positive for both GHRH mRNA and GHRH peptide, immunohistochemistry revealed a relatively uniform pattern of staining but, in *in situ* hybridization, the positivity was frequently variable from one cell to another and featured a clonal pattern. This pattern points to the paracrine action of this neuropeptide on cells adjacent to the GHRH-producing cells. The cancer cells may not be the only targets for the locally produced GHRH, and an effect on the stroma of the tumor should also be considered. This is in agreement with recent findings showing that JI-38, an agonist of GHRH, can stimulate the proliferation and increase the levels of c-myc mRNA in normal human dermal fibroblasts (25). Interestingly, in three cases a strong nuclear staining was apparent in a mosaic pattern, i.e. not involving all tumoral cells. This could be due to the deregulation of the sorting machinery of the cancer cells resulting in the misplacement of GHRH in the nucleus. An additional intriguing possibility, which remains to be validated experimentally, is that GHRH in some tumors may have some action not yet identified.

The receptors that mediate the effects of GHRH on endometrial tumor cells have not been identified so far. However, it has recently been shown that primary human tumors (G Halmos, A V Schally & R Busto, unpublished observations) and various human cancer cell lines (26–29), express splice variants of GHRH receptors which encode for alternate forms of the

receptor protein. Among these splice variants, SV1 appears to be the main isoform and is the most frequently expressed (26–29). We have recently shown that 3T3 fibroblasts transfected with SV1 can respond to the mitogenic effects of GHRH analogs (30). Thus, the expression of SV1 in 3T3 induced the responses to GHRH and GHRH antagonists (30). In addition, GHRH receptor belongs to a seven-transmembrane family of receptors, which also includes the receptors for vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide, calcitonin and others (31). These receptors, especially the receptors for VIP, are known to regulate cell proliferation (32). Considerable homology exists among the aforementioned receptors, as well as their corresponding ligands; cross-reaction between GHRH and these receptors would therefore not be unexpected (31).

The present study identifies, for the first time, the endometrial tumors of the endometrioid type as a source of ectopic production of GHRH. Considering the mitogenic effects of GHRH in various tumors (4), our work could also implicate neuropeptide GHRH in the pathogenesis of this disease. The present findings also suggest that approaches to therapy of endometrial cancer based on antagonists of GHRH merit investigation.

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