

Expression of Growth Hormone-Releasing Hormone (GHRH) Messenger Ribonucleic Acid and the Presence of Biologically Active GHRH in Human Breast, Endometrial, and Ovarian Cancers*

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ABSTRACT

GHRH is produced in a variety of extrahypothalamic tissues, including some neoplasms. We have previously reported that GHRH antagonists can inhibit the growth of various human cancers xenografted into nude mice. These observations suggest that locally produced GHRH might directly affect tumor cell proliferation. To investigate this possibility, we have examined the local production of GHRH in human endometrial, ovarian, and breast cancers obtained after surgery or grown in nude mice as xenografts. We have also examined whether the GHRH produced in these tumors is biologically active. RT-PCR and Southern blotting showed expression of messenger ribonucleic acid for GHRH in 17 of 22 endometrial and 17 of 22 ovarian cancer specimens and in all of the human endometrial, ovar-

ian, and breast cancer xenografts studied. Acid extracts of endometrial cancer specimens and breast cancer xenografts that expressed the GHRH gene contained immunoreactive GHRH peptide, as assessed by RIA for GHRH. The level of immunoreactive GHRH detected was equivalent to 2.7–6.4 ng GHRH-(1–29)/g tissue. Purified extract from one of these tumor samples induced a powerful stimulation of GH release from rat pituitary cells. The presence of biologically and immunologically active GHRH and messenger ribonucleic acid for GHRH in human breast, endometrial, and ovarian cancers supports the hypothesis that locally produced GHRH may play a role in the proliferation of these tumors. (*J Clin Endocrinol Metab* 84: 582–589, 1999)

THE ROLE of hypothalamic GHRH in the regulation of pituitary GH synthesis and release is well established (1). However, the presence of GHRH has also been demonstrated in various extrahypothalamic tissues, suggesting a broader biological role for this peptide. Messenger ribonucleic acid (mRNA) for GHRH, GHRH peptide, and GHRH bioactivity has been found in rat, mouse, and human placenta (2–5), suggesting a regulatory role for GHRH in placental function. GHRH immunoreactivity was also detected in extracts from stomach, duodenum, and jejunum (6, 7), pancreas (8), and liver and lung (9). GHRH was shown to stimulate digestive enzyme secretion from exocrine pancreatic preparations and insulin secretion from Langerhans cells *in vitro* (10, 11). Both GHRH gene transcription and GHRH immunoreactivity have been also demonstrated in human lymphocytes (12). Mouse hemopoietic stem cells expressing

GHRH showed increased reproductive capacity when infused into irradiated animals (13).

GHRH exerts important biological actions on germ cell maturation and hormone production in ovary and testis. GHRH gene expression (14), GHRH peptide production (15, 16), and functional receptors for GHRH have been demonstrated in these organs (17, 18). These data implicate GHRH as an intragonadal regulatory factor. Gene expression for both GHRH and GHRH receptor was detected in various rat tissues using the RT-PCR method (19).

Frohman and Szabo were the first to demonstrate ectopic production of GHRH by carcinoid and pancreatic cell tumors in 1981 (reviewed in Ref. 20). Subsequently, two groups isolated GHRH from human pancreatic tumors that caused symptomatic acromegaly (21, 22). Since 1981, GHRH has been demonstrated in many tumors arising from and composed of peptide hormone-producing endocrine cells. Carcinoid tumors, tumors of the gut, pancreatic islet cell tumors, medullary cancers of the thyroid, pheochromocytomas, and even some small cell lung carcinomas can produce GHRH, which sometimes significantly contributes to elevated serum bioactive GHRH levels resulting in GH overproduction (7, 20). In addition, endometrial cancers have been shown to exhibit a significant amount of GHRH immunoreactivity in the absence of clinical acromegaly (20). A set of malignant tumors of various origins contained detectable levels of immunoreactive GHRH, leading to the conclusion that al-

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though they may produce large amounts of GHRH, this does not always lead to acromegaly (9). Studies of GHRH mRNA expression and GHRH production in normal human breast tissues and cancer samples showed that normal and tumoral tissues can express the peptide and also release it in a significant amount (23). These observations raise the question of whether tumors produce a biologically active form of GHRH.

In the present study, to clarify the pathophysiological significance of GHRH in tumors, we investigated the expression of the GHRH gene in human breast, ovarian, and endometrial cancers obtained after surgery or grown in nude mice and determined whether biologically and immunologically active GHRH was present.

Materials and Methods

Collection of samples from patients

Twenty-two endometrial cancer specimens and 22 ovarian cancer specimens were collected at the time of initial surgical treatment at Tulane Medical Center and Medical Center of Louisiana (New Orleans, LA). The local institutional (Tulane and Louisiana State University) review board has approved the collection and use of these specimens for the current analysis. The uterine specimens were taken from the surgical field after resection and were opened with a scalpel to expose the endometrial cavity, and grossly visible endometrial tumor samples approximately 1 cm³ in size were excised with the scalpel. Samples of similar size were obtained from primary ovarian malignant tumors in the surgical field by sharp excision. All samples were flash-frozen in liquid nitrogen and stored at -70 C until analysis was performed. Histological sections of each sample were examined to confirm the presence of malignant tumor with minimal admixed nonmalignant tissue. Samples with significant amounts of nonmalignant tissue were not used. All histological slides were examined by Tulane pathologists and reviewed by one of us (W.R.R.) to confirm the diagnosis.

Clinicopathological data (Tables 1 and 2) were compared to GHRH mRNA levels determined by the RT-PCR method. Statistical analysis was performed using Student's *t* test.

Collection of human tumor xenografts

Estrogen-independent human breast cancer cell lines MDA-MB-231 and MDA-MB-468, estrogen-dependent human breast cancer cell lines ZR-75-1 and T-47-D, human endometrial cancer cell line HEC-1-A, and human ovarian cancer cell line OV-1063 were obtained from the American Type Culture Collection (Manassas, VA). The estrogen-independent, but sensitive, human mammary cancer cell line MCF-7-MIII was provided by Dr. R. Clarke (Georgetown University Medical Center, Washington DC). Tumors were initiated by sc injection of the respective cell line into the flanks of female athymic (NCR/c *nu/nu*) nude mice obtained from the NCI (Frederick, MD) and were maintained by transplantation. Hormone-independent doxorubicin-resistant human mammary cancer xenograft MX-1 originating from surgical explant (NCI-Frederick Cancer and Development Center, Frederick, MD) was grown in nude mice. Tumors weighing 0.4–0.6 g were removed, immediately snap-frozen in liquid nitrogen, and stored at -70 C until use.

RT-PCR and Southern blot

Total RNA was extracted from tissues using the RNA Isolation Kit (Stratagene, La Jolla, CA) and was quantified spectrophotometrically at 260 nm. Three micrograms of total RNA were reverse transcribed with 200 U Superscript II ribonuclease H⁻ reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. PCR for GHRH was carried out in a final volume of 100 μ L containing 20 mmol/L Tris-HCl (pH 8.8), 10 mmol/L KCl, 10 mmol/L (NH₄)₂SO₄, 2 mmol/L MgSO₄, 1% Triton X-100, 1 mg/mL nuclease-free BSA, 2.5 U *Taq* Extender PCR Additive (Stratagene), 200 μ mol/L of each deoxy-NTP (Stratagene), and primers at a concentration of 0.3 μ mol/L each, and 2.5 U *Taq* 2000 (Stratagene) were added after 5 min of denaturation at 94 C in a thermal cycler (Stratagene). Primers were designed according to the sequence of the human GHRH (hGHRH) gene (24) as follows: sense, 5'-ATT TGA GCA GTG CCT CGG AG (nucleotides 30–49); and antisense, 5'-TTT GTT CTG CCC ACA TGC TG (nucleotides 331–350). Then, 40 cycles of PCR were performed using the following step program: 94 C for 30 s, 60 C for 30 s, and 72 C for 1 min, with a final 7-min extension at 72 C. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining on an UV transilluminator.

RNA from liver tissue of MT-hGHRH transgenic mice developed as

TABLE 1. Clinicopathological data and GHRH mRNA findings for 22 human endometrial cancer samples

Patient no.	Age (yr)	Survival (months)	Histological type	Histological grade	Stage	GHRH mRNA
1	65	18 (DOD)	Papillary	3	IVb	++
2	79	19 (D)	Endometrioid	2	Ia	+
3	63	9 (DOD)	Endometrioid	1	Ib	++
4	69	11 (DOD)	Papillary	3	IVb	++
5	58	58 (L)	Endometrioid	1	Ia	+
6	78	58 (L)	Papillary serous	3	IVb	+++
7	63	65 (L)	Endometrioid	1	Ib	+
9	78	55 (D)	Endometrioid	3	Ib	++
10	62	62 (L)	Endometrioid	2	Ia	++
11	66	10 (DOD)	Endometrioid	3	IIIc	++
12	54	23 (D)	Endometrioid	2	IIIc	+++
13	62	28 (D)	Endometrioid	1	Ia	±
14	65	46 (L)	Endometrioid	1	Ia	-
15	43	14 (L)	Endometrioid	3	IVb	-
16	70	55 (DOD)	Endometrioid	1	Ia	-
17	83	24 (D)	Endometrioid	1	Ia	-
18	85	36 (D)	Endometrioid	3	Ib	++
19	29	36 (L)	Endometrioid	1	Ia	+
20	66	55 (L)	Endometrioid	1	Ia	-
21	61	10 (D)	Papillary	3	IVb	++
22	54	38 (L)	Endometrioid	1	Ia	+
23	70	32 (L)	Endometrioid	1	Ib	++

GHRH mRNA expression was determined by RT-PCR and Southern blotting. DOD, Dead of disease; L, living; D, died of other cause. The differences in GHRH mRNA levels were estimated by comparing the intensities of the autoradiographic signals (+, low; ++, medium; +++, high intensity, after 3 h of film exposure time; ± denotes autoradiographic signal obtained after 3 days of film exposure time only; and, - denotes the lack of signal even after longer film exposure time).

TABLE 2. Clinicopathological data and GHRH mRNA findings for 22 human ovarian cancer samples

Patient no.	Age (yr)	Survival (months)	Histological type	Histological grade	Stage	GHRH mRNA
1	50	24 (DOD)	Mucinous	3	IIIc	+++
2	68	38 (DOD)	Serous	3	IIIc	+
5	51	35 (DOD)	Serous	3	IIIb	+++
6	68	29 (DOD)	Serous	3	IV	+++
7	70	18 (DOD)	Serous	3	IV	+++
10	65	28 (DOD)	Serous	3	IIIa	-
11	69	48 (DOD)	Serous	3	IIIc	+
12	62	33 (DOD)	Serous	3	IIIc	-
14	51	40 (L)	Serous	2	IIIc	-
15	48	55 (L)	Serous	2	IIIc	-
16	63	20 (DOD)	Serous	2	IIIc	+++
17	58	47 (DOD)	Serous	3	IV	++
18	60	20 (DOD)	Endometrioid	IIIc	Ia	++
22	56	26 (DOD)	Serous	2	IIIc	++
23	49	6 (DOD)	Mucinous	3	IIIc	±
24	73	14 (DOD)	Serous	2	IIIc	++
25	70	32 (DOD)	Serous	3	IIIc	+
26	68	31 (DOD)	Serous	3	IIIb	+
27	64	39 (DOD)	Serous	3	IIIc	++
29	58	12 (DOD)	Serous	3	IIIc	++
30	54	30 (L)	Serous	3	IIIc	+
31	51	12 (L)	Serous	3	IV	+

GHRH mRNA expression was determined by RT-PCR and Southern blotting. DOD, Dead of disease; L, living; D, died of other cause. The designation of symbols is as in Table 1.

previously described (1) served as a positive control. RT without reverse transcriptase and PCR without complementary DNA (cDNA) were performed to test the possibilities of genomic DNA contamination and cross-contamination of the samples, respectively.

Determination of the expression of the β -actin gene served as an internal control for the amount of RNA template in each RT-PCR aliquot. For the detection of human or mouse β -actin, mRNA primers and conditions were used as previously described (25, 26).

GHRH PCR products were transferred to a nylon membrane (Hybond N⁺, Amersham, Arlington Heights, IL), and the DNA was linked onto it by baking. Blots were prehybridized in a buffer containing 4 × SSC (standard saline citrate) buffer, 2.5 × Denhardt's solution (Sigma Chemical Co., St. Louis, MO), 0.1% SDS, 5 mmol/L ethylenediamine tetraacetate, and 0.1 mg/mL denatured salmon sperm DNA (Sigma Chemical Co.) at 50 C for 4 h. Blots were hybridized with 100 ng ³²P 5'-end-labeled oligonucleotide probe specific for GHRH at 50 C for 16 h in a hybridization buffer containing 5 × SSC, 0.5 × Denhardt's solution, 0.02 mol/L Tris-HCl, and 0.1 mg/mL sonicated salmon sperm DNA. The probe was designed according to the sequence of the hGHRH cDNA (24) as follows: 5'-GAC CCT CAG GAT GCG GCG GTA TGC AGA TGC (nucleotides 166–195). It was radiolabeled in a reaction mixture of 200 ng oligonucleotide DNA, 8 μ L [³²P]ATP 3000 Ci/mL, 10 mCi/mmol, and 10 U T₄ polynucleotide kinase (Amersham). After incubation at 37 C for 1 h, the reaction was terminated by adding 2 μ L 0.5 mol/L ethylenediamine tetraacetate. Unincorporated radioactivity was separated using a NuTrap column (Stratagene). After hybridization, blots were washed under stringent conditions and were exposed to Biomax MS Kodak film (Eastman Kodak Co., Rochester, NY) for exposure times of 2 h to 3 days at -70 C.

RIA for GHRH

Three different human cancer xenografts (ZR-75-1, MX-1, and MCF-7-MIII) and three human endometrial cancer specimens were used for studying GHRH contents by RIA. Tissues were homogenized in 2 mol/L acetic acid supplemented with protease inhibitors (1 mg/100 mL phenylmethylsulfonyl fluoride, 200 μ g/100 mL Trasylol, and 200 μ g/100 mL pepstatin) with an Ultra-Turrax tissue homogenizer (IKA, SE, Wilmington, NC) on ice. The homogenates were placed in a boiling water bath for 10 min and then centrifuged at 10,000 × g for 10 min. The supernatants were lyophilized and subsequently dissolved in RIA buffer.

GHRH was measured using [¹²⁵I]GHRH-(1–40) (Bachem, Torrance, CA) as labeled hormone and anti-GHRH-(1–40) (SV-95) antibody generated in our laboratory in a final dilution of 1:70,000, which cross-reacts

100% with GHRH-(1–29) as described previously (27). GHRH(1–29)NH₂ was used as a standard. The range of the standard curve was 0.025–50 ng/tube (bound/free ratio, 23%; nonspecific binding, 4.3%). The inter- and intraassay coefficients of variation were 11.5% and 7.8%, respectively.

In vitro GHRH bioactivity of tumor extracts

For studying GHRH bioactivity, three human breast cancer ZR-75-1 xenografts, weighing a total of 1.4 g, were minced while frozen and placed immediately in boiling water for 10 min to inactivate the proteases. Acetic acid was then added to a final concentration of 2 mol/L, and the tissue was homogenized and stirred for 30 min at 4 C. The homogenate was centrifuged at 10,000 × g for 10 min. The supernatant was passed through a Mega Bond Elut^R C₈ cartridge (12 cc, 2 g; Varian, Walnut Creek, CA) previously equilibrated with 2 mol/L acetic acid. Thereafter, the column was washed with 2 mol/L acetic acid and eluted with 70% aqueous acetonitrile. Acetonitrile was evaporated, and the aqueous solution was lyophilized.

The purified extract of ZR-75-1 cells was tested for GHRH bioactivity in a dispersed rat pituitary superfusion system. The details of this method have been described previously (28). Briefly, anterior pituitary fragments from young male Sprague-Dawley rats (150–250 g BW) were digested with collagenase for 50 min followed by gentle mechanical dispersion. The cell clusters were mixed with Sephadex G-10 and loaded on superfusion columns. Medium 199 supplemented with 0.1% BSA, 50 mg/L penicillin G, and 87 mg/L gentamicin was perfused through the columns at a speed of 20 mL/h. After an overnight recovery period, the cells were exposed periodically to test compounds dissolved in fresh medium immediately before application. Three-minute stimulations were performed with 0.2, 0.5, and 1 nmol/L GHRH-(1–29)NH₂ (DOB-2-048-1, synthesized in our laboratory); a mixture of 1 nmol/L GHRH-(1–29) and 20 nmol/L somatostatin (SS-14), and 25 mmol/L KCl. One-milliliter fractions were collected from the effluent medium for subsequent GH assay. In a separate experiment, stimulations with 1 nmol/L GHRH-(1–29), 1 nmol/L vasoactive intestinal peptide (VIP; California Peptide Research, Inc., Napa, CA), and the mixture of 1 nmol/L GHRH-(1–29) and 1 nmol/L VIP were performed in a set-up similar to that described above.

The rat GH (rGH) content of the collected fractions was determined by RIA using materials provided by Dr. A. F. Parlow, National Hormone and Pituitary Program, NIDDK (Torrance, CA; rGH-RP-2/AFP/3190B,

rGH I-6/AFP-5676B, and anti rGH-RIA-5/AFP-4115). RIA results were analyzed as previously described (28).

Results

RT-PCR and Southern blot

RT of RNA from liver tissue of MT-hGHRH transgenic mice, followed by PCR amplification with specific primers for GHRH, produced a fragment of the expected size of 322 bp as seen under UV light after ethidium bromide staining. A PCR product of the same size could be observed in 15 of 22 endometrial cancers (Fig. 1A), 12 of 22 ovarian cancers (Fig. 2A), 5 of 6 human breast cancer xenografts, and also in OV-1063 ovarian cancer and HEC-1-A endometrial cancer xenografts (Fig. 3A). Southern blotting with a specific radiolabeled oligonucleotide probe demonstrated that the 322-bp fragment was the genuine transcript of the GHRH gene. Southern blotting also revealed additional tumors that expressed GHRH. Sixteen of 22 endometrial (Fig. 1B) and 16 of 22 ovarian cancer samples (Fig. 2B) could be demonstrated to contain GHRH mRNA after 3 h of film exposure time. Longer film exposure (3 days) revealed an additional endometrial and an ovarian cancer sample with a positive GHRH signal (not shown). GHRH message could not be detected in 5 endometrial and 5 ovarian cancer specimens even after extended exposure times. All of the human cancer xenografts investigated expressed the GHRH gene. Human breast cancer xenograft ZR-75-1 had the strongest GHRH signal (Fig. 3). In the case of MCF-7-MIII tumor, a GHRH signal could only be verified after 3 days of exposure (not shown).

Negative controls yielded no detectable signals, indicating that PCR products were generated from cDNA and not from genomic DNA or contamination. PCR amplification with human or mouse β -actin-specific primers generated products of the expected sizes from all samples, confirming that no degradation occurred in the RNA preparations.

GHRH RIA

RIA was performed on acid extracts of three human breast cancer xenografts and three endometrial cancer specimens. All samples contained variable, but measurable, amounts of immunoreactive GHRH (Table 3). No cross-reactivity was observed with VIP, eliminating the possibility that the immunoreactivity was due to this peptide related to GHRH.

GH-releasing activity of the purified tissue extract in vitro

Dispersed rat pituitary cells were stimulated with KCl, GHRH-(1-29), a combination of GHRH-(1-29) and SS, and the purified extract of ZR-75-1 breast cancer xenografts (dissolved in 2.5 mL medium) in the superfusion system. Pulsatile stimulation with the tissue extract induced a significant, transitory elevation of GH in the effluent medium similar in shape to that following the stimulation with GHRH-(1-29). Based on the size of the net integral response (area under the curve above the baseline), the GH-releasing potency of the extract was similar to that of 2-3 nmol/L GHRH-(1-29). GHRH immunoreactivity in this extract and in the 2 nmol/L standard were the same, as determined by RIA. During the infusion of the tumor extract (Fig. 4, *black column*), there was a significant increase in GH release in contrast to the absence of effects observed during simultaneous GHRH and SS-14 infusion, indicating that a significant quantity of SS-14 was not contained in the tumor extract (Fig. 4). In our system, stimulation with 1 nmol/L VIP evoked a GH response amounting to 1/10th that induced by equimolar concentration of GHRH-(1-29), and when VIP was added together with GHRH, there was an additive effect on the GH response.

Comparison of GHRH mRNA expression of human cancer specimens with clinicopathological data

No significant correlation was found between GHRH mRNA levels in human cancer specimens and the clinicopathological data, such as histological grade of the tumor, tumor stage, and clinical outcome of the disease (Tables 1 and 2).

Discussion

These results are the first to demonstrate that GHRH is expressed in most endometrial and ovarian cancer specimens and in all xenografts of ovarian, endometrial or breast tumors studied. The biological relevance of this finding is supported by the fact that immunoreactive and bioactive GHRH was detected in the samples studied. It is likely that the mRNA for GHRH found in these samples is translated into a functional peptide. Our results are consistent with those of Losa *et al.*, who used RIA and detected GHRH in 4 human breast and 3 ovarian cancer specimens (9). Although Asa *et al.* could

FIG. 1. GHRH gene expression in 22 individual human endometrial cancer specimens (no. 1-7 and 9-23). PCR products were visualized either on ethidium bromide-stained agarose gel (A) or by Southern blot hybridization after 3 h of exposure (B). RT-PCR resulted in selective amplification of the predicted 322-bp fragment of the human GHRH gene (M, 100-bp DNA ladder).

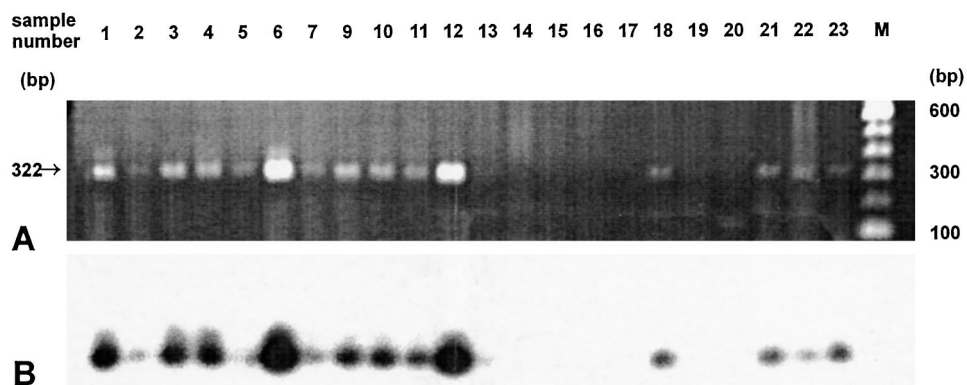


FIG. 2. GHRH gene expression in 22 individual human ovarian cancer specimens. RT-PCR amplification of the 322-bp sequence representative of GHRH mRNA was detected on ethidium bromide-stained agarose gel (A) or by Southern blot hybridization (B). Lanes 1–22, Human ovarian cancer samples; lane 23, MT-hGHRH tissue (positive control); M, 100-bp DNA ladder.

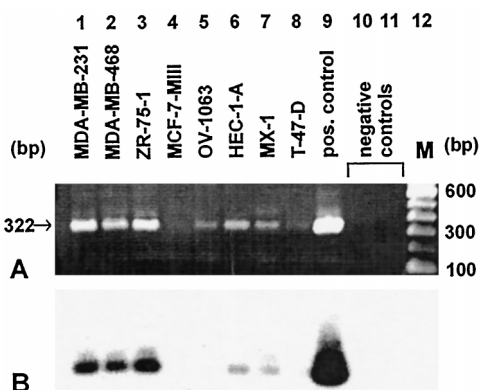
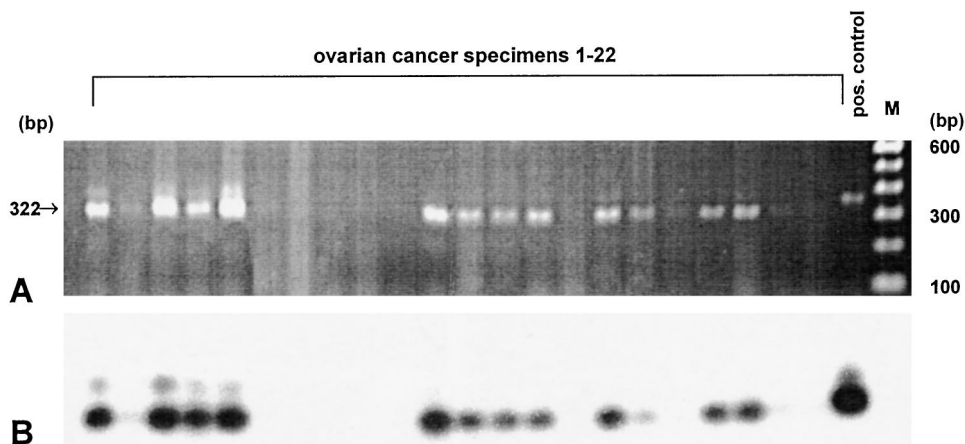


FIG. 3. GHRH gene expression in human breast cancer (lanes 1–4, 7, and 8), ovarian cancer (lane 5), and endometrial cancer (lane 6) xenografts. PCR products were stained with ethidium bromide (A) and detected with Southern blot hybridization after 3 h of exposure time (B). Lane 9, MT-hGHRH tissue (positive control). M, 100-bp DNA ladder. RT-PCR resulted in the selective amplification of a fragment of the expected size (322 bp). No PCR products were amplified from the negative controls (lanes 10 and 11).

TABLE 3. Results of RIA for GHRH on acid extracts from three different human breast cancer xenografts and three different endometrial cancer specimens compared with their GHRH mRNA levels

Source of tumor	Type	GHRH mRNA	GHRH activity [equivalent to ng GHRH (1–29)/g tissue]
Human cancer xenografts grown in nude mice	ZR-75-1	+++	2.7
	MX-1	+	2.7
	MCF-7-MIII	±	3.2
Human endometrial cancer specimens	#6	+++	6.4
	#12	+++	5.0
	#13	±	4.6

GHRH mRNA expression was determined by RT-PCR and Southern blotting. The designation of symbols is as in Table 1.

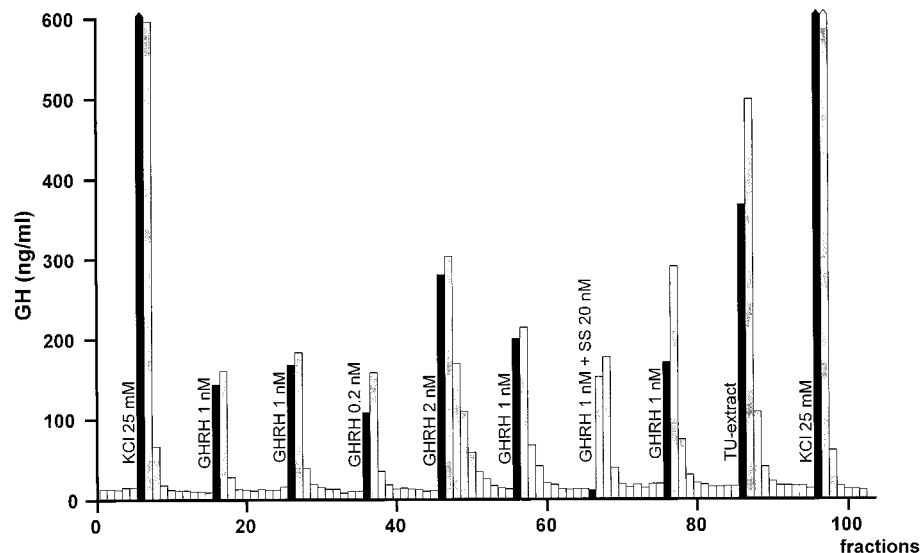
not detect GHRH in endometrial cancers using immunohistochemistry (29), Frohman *et al.* reported measurable levels of the peptide in 15 such tumors by RIA (20). The report of Benlot *et al.*, who showed GHRH gene expression in human breast cancers (23), is also consistent with our observations.

The fact that the relative level of expression of mRNA for

GHRH did not correlate with GHRH content is of interest. Although GHRH could be detected by RIA in all of the samples tested for protein translation, GHRH contents did not always reflect GHRH mRNA levels. These data are in accordance with the observation that GHRH secretion by human breast cancer samples in a perfusion system did not correspond to their GHRH contents (23). Moreover, higher release was inversely correlated to GHRH tissue contents (23). These apparent differences could be due to tumor heterogeneity, because we used different parts of the tumor in the case of xenografts for molecular biology and biochemical determinations. Other possible explanations for this dichotomy could include tissue-specific rates of degradation of mRNA for GHRH and GHRH peptide or an asynchronism in gene expression and protein translation, which has been observed in other endocrine systems (30).

Superfusion studies revealed that the potency of the GHRH purified from ZR-75-1 human breast cancer xenografts was equivalent to 2–3 nmol/L GHRH-(1–29) in tests on GH release from dispersed rat pituitary cells. This result is in good correlation with the concentration of GHRH in the tissue extract determined by RIA. These findings indicate that most of the GHRH produced is bioactive. VIP has also been suggested to be an autocrine growth factor in breast cancer (31), raising the possibility that the presence of VIP could contribute to the response seen in our superfusion system. However, we found that equimolar concentrations of VIP evoked significantly lower GH release from rat pituitary cells, about 1 order of magnitude less than that caused by GHRH. This is consistent with a report on cultured bovine hypophysial cells, where VIP was much less potent in releasing GH than GHRH (32). These results taken together with the findings that VIP does not cross-react in our GHRH RIA and a good agreement between GHRH detected by RIA and superfusion indicate that VIP could only contribute to a minor degree, if at all, to the GH-releasing activity of tumor extracts. In addition, the purification procedure used makes it unlikely that small molecules such as monoamines and amino acids that are able to provoke a GH response were present in the purified fractions eluted with 70% acetonitrile that were used in our studies. Our work supports the view that GHRH mRNA detected in ZR-75-1 breast cancer xeno-

FIG. 4. GH release from dispersed rat pituitary cells tested in superfusion system. The columns represent GH concentrations in consecutive 3-min fractions collected during the experiment. The durations of stimulations and post-stimulatory responses are indicated by *black* and *shaded* columns, respectively. The cells were stimulated for 3 min with 25 mmol/L KCl, various concentrations of GHRH, the mixture of GHRH and SS, or purified extract of ZR-75-1 tumor.



grafts could serve as a template for the synthesis of GHRH in the tumor, which we detected biologically in superfusion system and immunologically by RIA. Moreover, the size and specificity of the PCR product, the immunoreactivity of the peptide product, and its high bioactivity make it likely that GHRH detected in the cancer samples studied is similar or identical to GHRH of hypothalamic origin.

The human breast cancer cell line ZR-75-1 (33), human mammary tumors, and normal tissues (23) have been shown to produce SS. SS is also present in neuroendocrine tumors (29). As SS is a powerful inhibitor of GHRH-induced GH release, the possibility existed that the actions of GHRH in our superfusion system could have been antagonized by locally produced SS. However, we have previously demonstrated that the GH-releasing effect of GHRH is detected despite the presence of large amounts of SS during stimulation (28), which may be explained by the fact that in the superfusion system the inhibitory effect of SS disappears immediately after exposure of cells to the test material is stopped, whereas the effect of GHRH becomes evident. The response to the ZR-75-1 tumor extract in Fig. 4 shows that the amounts of SS-like bioactivity present in our purified extract were too low to affect our results.

The types of cells in the tumor that produce GHRH have not been identified. It is known that lymphocytes can infiltrate the tumors, and lymphocytes have been reported to produce GHRH (12). However, we do not believe that the primary source of GHRH is immune cells, because mRNA for hGHRH and peptide were detected in human cancer xenografts in which stromal cells of human origin are not present, thus supporting the view that the GHRH-producing capability of these tumors originated within the cancer cells themselves.

Under physiological conditions, GHRH in the hypophyseal portal blood may be in a high (nanomolar) range depending on its pulsatile release, whereas GHRH immunoreactivity in the peripheral plasma is low or undetectable (34, 35). Many factors contribute to the rapid clearance of GHRH. Firstly, GHRH produced in the hypothalamus and released into the portal circulation is diluted at least 100-fold after

perfusing the pituitary (35). Secondly, GHRH is rapidly inactivated in the circulation due to proteolytic cleavage by plasma dipeptidylpeptidase IV. After bolus iv injection, a substantial portion of GHRH-(1-44) is converted to a biologically inactive form [GHRH-(3-44)] within 1 min while it remains fully immunoreactive (36). The findings reported in the literature together with our results indicate that GHRH expression could be quite common in cancer. A rapid breakdown of GHRH in the circulation also favors its exclusive local intratumoral role, which should be distinguished from the rare cases of clinical acromegaly caused by ectopic GHRH secretion.

Our laboratory has previously demonstrated that blockade of GHRH action by potent antagonistic analogs of GHRH can powerfully inhibit the growth of various human tumors xenografted into nude mice (reviewed in Ref. 37). Thus, administration of GHRH antagonists MZ-4-71 and/or MZ-5-156 suppressed the growth of Caki-I renal adenocarcinoma (38), SK-ES-1 and MNNG/HOS osteosarcomas (39), PC-3 and DU-145 prostate cancers (40), and H-69 small cell and H-157 nonsmall cell lung carcinomas (41). Our results also indicate that GHRH antagonists can also inhibit the proliferation of human colorectal, pancreatic, and mammary cancers xenografted into nude mice and MXT hormone-independent breast cancers in BDF mice (unpublished data). The antiproliferative effects of GHRH antagonists could be based on several mechanisms. By inhibiting GH release, GHRH antagonists decrease the secretion of insulin-like growth factor I (IGF-I) by the liver and other tissues, which may lead to tumor growth inhibition. GHRH antagonists also decrease the growth of IGF-I- and/or IGF-II-dependent cancers by lowering autocrine or paracrine IGF-I and IGF-II production in tumors (37). The inhibition of IGF-II production and growth of DU-145 prostate cancers in mice treated with MZ-5-156 was linked to a reduction in the expression of mRNA for IGF-II in tumors (42). GHRH antagonists could exert some of these effects directly by blocking the effects of locally produced GHRH. The relevance of the latter mechanism is supported by the study of Benlot *et al.* (23), who showed that although there is no significant difference in mRNA expres-

sion for GHRH or tissue GHRH content between normal breast tissue and breast cancer, tumor samples released significantly more GHRH than normal samples during both static incubation and perfusion. It was speculated from these data that GHRH hypersecretion might represent a major disorder in breast cancer, leading to enhanced epithelial proliferation (23). Thapar *et al.* found a large number of somatotroph pituitary tumors in which overexpression of the GHRH gene was associated with neoplastic progression and clinical aggressiveness, reflecting the undifferentiated nature of the tumor (43).

To prove the pathophysiological significance of GHRH in tumorigenesis, demonstration of its specific receptors on tumors is required. GHRH receptor mRNA identical to that in the pituitary is found in the placenta, testis, ovary, and kidney, all of which also express GHRH (16, 19, 44, 45). GHRH receptor gene expression was also found in many other extrapituitary organs, although at lower levels (19). At present, GHRH receptors in tumor tissue that mediate the response to GHRH are still unidentified. Alternately, other members of this receptor family, such as VIP and pituitary adenylate cyclase-activating peptide receptors, which have been found in tumors and are still able to bind and respond to GHRH, may mediate the actions of GHRH. This would be in accordance with the concept that there are multiple receptors for several peptides in this family, which, in addition to GHRH, includes glucagon, secretin, VIP, gastric inhibitory peptide, and pituitary adenylate cyclase-activating peptide. In fact, several members of the GHRH family and the simultaneous presence of their receptors mediating special paracrine functions have been shown in the testis (45).

In conclusion, this report demonstrates that human endometrial, ovarian, and breast cancers synthesize GHRH. These findings appear to provide a mechanistic basis for our previous observations that GHRH antagonists can inhibit the proliferation of various human cancers xenografted into nude mice. Collectively, these results suggest a possible pathophysiological role for GHRH and support the merit of further investigation of antagonistic analogs of GHRH as potential therapeutic agents.

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