EXPERIMENTAL STUDY

Immunohistochemical detection of GHRH and its receptor splice variant 1 in primary human breast cancers

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Abstract

Objective: GHRH is secreted by the hypothalamus and, upon binding to specific GHRH receptors in the pituitary, stimulates growth hormone (GH) production and release from the pituitary. In addition to this neuroendocrine action, accumulated evidence implies additional roles for GHRH in carcinogenesis in non-pituitary tissues. *In vitro* and *in vivo* studies have shown that splice variant 1 (SV1) of the GHRH receptor, which is widely expressed in non-pituitary tissues and cancers, can mediate the proliferative effects of GHRH. The aim of the present study was to investigate the operation of an autocrine stimulatory loop between GHRH and SV1 in primary breast tumors.

Design: Fifty-three primary breast tumors were evaluated for GHRH and SV1 expression.

Methods: Expression of GHRH and SV1 was assessed by immunohistochemistry using anti-GHRH SV95 and anti-SV1 2317/5 polyclonal antibodies.

Results: About 40% of the specimens tested express GHRH and/or SV1 (approx. 25% each), while in 35% of these positive specimens co-expression of these antigens was detected ($P < 0.01$). Furthermore, a correlation of GHRH, but not SV1, expression was detected in lobular compared with ductal carcinomas.

Conclusions: These results constitute the first demonstration for the expression of GHRH and SV1 in primary breast cancers, and provide evidence for the operation of an autocrine stimulatory loop between GHRH and SV1 in primary cancers. Our findings indicate that GHRH analogs could have diagnostic and therapeutic applications for the management of breast cancer.

European Journal of Endocrinology 151 391–396

Introduction

Growth hormone-releasing hormone (GHRH) is secreted by the hypothalamus and, after binding to specific GHRH receptors in the pituitary, stimulates growth hormone (GH) release (1–3). GH, in turn, stimulates the production of insulin-like growth factor-I (IGF-I) in the liver, which is a mitogen for various cell types (4).

Besides this neuroendocrine action, accumulated evidence points to additional actions of GHRH in non-pituitary tissues, especially neoplasms, by direct mechanisms. This evidence includes the expression pattern of this neuropeptide which suggests that GHRH is detected in many types of non-pituitary tumors (reviewed in 5). In addition, treatment of various experimental cancers, such as lung, brain, prostate,

endometrial, ovarian and breast carcinomas, with antagonistic analogs of GHRH significantly inhibits their growth. This antitumor action of GHRH has been demonstrated in both *in vivo* experiments involving transplantable tumors in mice, and in *in vitro* cell culture conditions, in which by definition the endocrine GHRH/GH/IGF-I axis is not operational (reviewed in 4, 5). Thus, a role for GHRH as an autocrine/paracrine growth factor has been established (6–15). However, the receptor that mediates the proliferative effects of GHRH in non-pituitary tissues remains obscure. While the receptor for GHRH, in its pituitary form, is not expressed in extrapituitary tissues, a splice variant (SV) of this protein has been shown to be expressed in a wide range of non-pituitary tissues and experimental cancers (16, 17). This protein, which lacks a short extracellular portion of the full-length receptor, was

shown to be capable of mediating the mitogenic effects of GHRH and to possess, in addition to its ligand-dependent activity, ligand-independent activity in stimulating cell proliferation (18, 19). While the aforementioned evidence demonstrates that slice variant 1 (SV1) can mediate the growth-promoting effects of GHRH, to what extent the operation of such an autocrine stimulatory loop reflects the conditions of primary cancers remains poorly understood.

In the present study, we evaluated primary breast tumors, a cancer cell type which previously was shown to express biologically active GHRH and to respond significantly to the antagonists of GHRH, for expression of GHRH and SV1 (20–22). Our results indicate that the expression of GHRH and SV1 is not only detectable, but also correlated, providing an initial demonstration for the operation of this autocrine stimulatory loop in primary cancers.

Materials and methods

Specimens

Formalin-fixed paraffin-embedded tissues from 53 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 were treated by mastectomy with axillary lymph node dissection. Histological grading of invasive ductal carcinomas takes into consideration the growth pattern of the tumors as well as cytological features of differentiation. The parameters measured were the extent of tubule formation, nuclear hyperchromasia and mitotic rate, and the histological grade was expressed in three categories: well differentiated (grade I), intermediate (grade II), and poorly differentiated (grade III).

Immunohistochemistry

Four-mm thick sections from representative paraffin-embedded blocks of 53 breast adenocarcinomas were collected onto poly-L-lysine-coated slides and stained for GHRH and SV1 antigens. The immunohistochemical detection of GHRH was carried out with rabbit anti-GHRH SV95 (23) polyclonal antibody, diluted with 1 × phosphate buffer saline (PBS) at 1:100, using the Kwik-DAB kit (ThermoShandon, Pittsburgh, PA, USA) according to the manufacturer's instructions. The immunohistochemical detection of SV1 was performed with rabbit anti-SV1 polyclonal antibody 2317/5 diluted with 1 × PBS at 1:10⁴. The 2317/5 anti-SV1 antibody was designed and produced by J Horvath, J Varga, M Zarandi and K Groot in the laboratory of one of us (A V S); its development will be reported elsewhere. Negative controls in which the primary antibody has been omitted has also been included

in the analysis (data not shown). 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%, ++; 71–100%, ++++. Images shown were obtained by Pro-image Analysis Software (Media Cybernetics, Inc., Silver Springs, MD, USA).

Statistical analysis

Statistical analysis was performed with the chi-square test and results were considered significant when $P < 0.05$.

Results

GHRH expression was detected in 13 (25%) and SV1 in 14 (26%) out of the 53 cases tested (Table 1). Immunoreactivity was cytoplasmic in all cases and it ranged from mild (±) to intense (+++) (Figs 1 and 2). Among the 20 (38%) cases identified as positive for either GHRH or SV1 expression, in 7 of them (35% of the specimens expressing either GHRH or SV1, or 13% among the total number of specimens tested), a co-expression of the ligand and the receptor was revealed ($P < 0.01$). No association between SV1 expression and the histological type of the breast cancer was found, but GHRH was detected at a higher ratio in lobular (3 out of 6, 50%) than in ductal (10 out of 42, 23%) carcinomas. For the latter analysis, some cases in which a mixed type carcinoma was found, being both ductal and lobular and/or mucinous, as well as the single papillary specimen, were excluded (Table 1).

Discussion

GHRH is a neuropeptide that plays an essential role in the stimulation of the GHRH/GH/IGF-1 axis. The accumulated evidence attributes to GHRH a direct role in tumorigenesis and implies that in certain cases GHRH fulfills the criteria for being considered as an autocrine growth factor. This role was initially demonstrated in small cell lung carcinoma and subsequently shown in other tumors as well (5, 6). Consistent with this notion are reports claiming the ectopic production of GHRH by several non-pituitary cancers, as well as the demonstration of the potent antitumor activity of GHRH antagonists by mechanisms that frequently bypass the GH/IGF-I axis. It is noteworthy that the receptor for GHRH is absent from virtually all non-pituitary tissues tested. However, the identification of splice variants of GHRH receptors in such tissues implies that they may function as the receptors that mediate the mitogenic effects of GHRH in primary cancers. While preliminary initial characterization of the predominant splice variant of GHRH receptor, namely SV1, suggests

Table 1 Clinicopathological data and expression of GHRH and GHRH receptor splice variant SV1 in human breast carcinoma specimens.

Case no.	Age (years)	Histology*	Grade (FIGO)†	No. of metastatic lymph nodes**	GHRH immunostaining†	SV1 immunostaining†
1	72	D	II	0/19	±	++
2	72	L			—	—
3	63	D	II	10/16	+	+
4	48	D	I	1/15	—	+
5	55	D	II		—	—
6	80	D	II	2/10	—	—
7	45	D	III	9/20	—	—
8	56	L		8/17	+	—
9	50	D	II	2/15	—	—
10	42	D	III	12/14	—	—
11	61	D	III	3/28	—	+
12	51	D	III	0/9	—	—
13	63	D	III	4/11	—	—
14	76	D	III		—	+
15	37	D,P,M	II	0/15	—	—
16	69	D	III	3/10	—	—
17	67	D,L	II	5/15	—	—
18	73	D	III	0/10	++	—
19	74	L			+	—
20	70	D	III		—	—
21	72	D	I	0/13	—	++
22	70	D	III		—	±
23	77	D	III	2/18	+	—
24	72	D	III	15/16	++	+
25	62	L,M,D ^{IS}			—	—
26	58	D	III	0/19	—	+
27	56	D	III		—	—
28	70	D	II	0/10	—	—
29	68	D	II	3/24	+	+
30	56	D			—	—
31	71	D	III	12/23	—	—
32	27	D	II	2/9	—	—
33	66	L		1/28	—	+++
34	76	P		0/9	—	—
35	58	D	III	0/26	—	—
36	72	D	III	0/5	+	+
37	47	D	III		—	—
38	72	D	II	1/8	—	—
39	65	D	III	3/13	—	—
40	58	D	II	4/8	—	—
41	44	D	III		—	—
42	51	D ^{IS}		0/13	—	—
43	74	D	II	0/12	—	—
44	54	D	II	5/13	—	—
45	72	D	II	0/12	—	—
46	72	D,L	II	16/19	—	—
47	81	D	II	0/12	—	—
48	49	L		0/11	+++	—
49	64	D	II	2/16	±	+
50	76	L		1/20	—	—
51	60	D	III	0/17	—	—
52	73	D	II	1/16	+	+
53	85	D	III	4/10	+	—

* D, ductal; L, lobular; P, papillary; M, mucinous; ^{IS}, *in situ*.

† See text for details of scoring.

** Positive/total found lymph nodes.

that it is capable of mediating the stimulatory effects of GHRH in non-pituitary tissues upon exogenous expression, it is unclear whether it functions as such in primary tumors. If the latter hypothesis were correct, it would be expected that the expression of this receptor

splice variant SV1, and its ligand GHRH, would be correlated in primary cancers. Therefore, we have undertaken an immunohistochemical analysis for the detection of GHRH and SV1 expression in primary breast cancers, a tumor type previously shown to

Figure 1 GHRH immunoreactivity in primary human breast tumors: (a) invasive lobular carcinoma; (b) invasive ductal carcinoma. Arrows indicate positively stained cells.

Figure 2 SV1 immunoreactivity in primary human breast tumors: (a) invasive lobular carcinoma; (b) invasive ductal carcinoma. Arrows indicate positively stained cells.

respond to GHRH antagonists. Our results confirmed the hypothesis that GHRH and SV1 are co-expressed in primary breast cancers. Although only a limited number of specimens, about 25%, expressed either GHRH or SV1, 35% of these specimens were positive for both ($P < 0.01$), which corresponds to 13% among the total number of specimens tested. It has to be mentioned that given the methodology employed in the present study, our results might be underestimates and that molecular analyses including RT-PCR and/or *in situ* hybridization might have revealed a higher incidence of GHRH and SV1 positivity. Considering that SV1 is acting as a membrane receptor, in a manner analogous to that of GHRH receptor, the cytoplasmic staining detected in our analysis might be interesting and suggestive of additional roles of SV1. However, this finding should be considered with caution since immunohistochemistry followed by light microscopy is not the most appropriate technique for localization studies at the sub-cellular level. Confocal analyses in combination with immunofluorescence or other analogous approaches should be followed in

order to investigate with high accuracy the precise intracellular localization of SV1.

That certain cases were positive for GHRH alone, besides the fact that it may be due to the reduced sensitivity of our assay in detecting the splice variant receptors for GHRH, is consistent with the notion that GHRH can act through other receptors, namely the receptor for the vasoactive intestinal peptide or the pituitary adenyl cyclase activating polypeptide. Thus, GHRH, even in the absence of SV1, may bind to other homologous receptors, eliciting mitogenic responses. Furthermore, the cancer cells probably are not the only targets for the locally produced GHRH, and an effect on the tumoral stroma should also be considered. This is in agreement with previous findings showing that an agonist of GHRH can stimulate the proliferation and increase the levels of c-myc mRNA in normal human dermal fibroblasts (24). In addition, a recent study involving the analysis of GHRH expression in primary endometrial carcinomas by a combination of *in situ* hybridization analysis and immunohistochemistry demonstrated that while the GHRH mRNA-producing

cells exhibited a mosaic pattern, the peptide itself was detected in a more uniform pattern implying a paracrine, most likely a stromal, action (25).

SV1 was detected in certain cases in which GHRH expression was absent. Recent findings suggest that SV1, besides its ligand GHRH-dependent activity, also possesses ligand-independent activity, consisting of the stimulation of cell proliferation in the absence of GHRH (19). Consequently, this receptor may play a role in the development of the disease even without reaching its maximal stimulation which occurs only in the presence of ligand binding.

Our results show that GHRH expression is detected predominantly in the lobular as compared with the ductal carcinomas. While the number of specimens tested is limited and does not permit reliable statistical analysis and therefore has to be confirmed by further studies, it raises interesting possibilities regarding our understanding of the mechanisms of pathogenesis of the disease.

Collectively, this study provides the first demonstration that GHRH and splice variant SV1 of its receptor are expressed in primary breast cancers and implies that the operation of an autocrine stimulatory loop between this ligand and the corresponding receptor is associated with the development of the disease. These findings also suggest that approaches to therapy of breast cancer based on antagonists of GHRH merit an investigation.

Acknowledgements

We thank Dr Gabor Halmos for critical reading of the manuscript. We are also grateful to P Kollia-Lariou and K Ploumi for help in specimen collection and processing.

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Received 3 May 2004

Accepted 24 May 2004