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Antibody array strategy for human growth factor secretome profiling of GH-secreting adenomas

Orly Ozeri[1,2,3,4] · Zvi R. Cohen[3,4,6] · Moshe Hadani[3,4,6] · Dvora Nass[5] · Ilan Shimon[1,2,3,4] · Hadara Rubinfeld[1,2,3,4]

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Abstract

Purposes To test if the antibody array strategy could be utilized to simultaneously detect the secretion of multiple growth factors by human pituitary GH-adenomas and to measure octreotide-induced alterations.

Methods Specimens of human pituitary adenomas were cultured and incubated with or without octreotide for 24 h. Conditional media were analyzed by human growth factor antibody array and VEGF concentrations were measured by ELISA. Media were also analyzed for GH concentrations. p21 expression levels were examined by Western blot of the specimens lysates.

Results The antibody arrays successfully identified growth factors secreted by GH-adenomas in vitro. Octreotide treatment induced both elevations and reductions in growth factors secretion. GH response to octreotide was measured, and in this small-sized study resistant and sensitive GH-adenomas presented with no unique secretome pattern of each of the groups. Octreotide-induced VEGF alterations analyzed by the antibody array and by ELISA were not fully matched.

Conclusions This study suggests that the broad proteomic strategy of antibody arrays may be utilized to study the growth factors secretion pattern of GH-adenomas and its regulation by somatostatin analogs or other compounds.

Keywords GH-adenomas · Growth factors · Secretome · VEGF

Introduction

The pituitary was shown to be a site of both synthesis and action of different growth factors. Growth factors regulate hormone gene expression, e.g. EGF was shown to stimulate or inhibit prolactin and GH secretion respectively in rat pituitary tumor cell lines [1–3], TGFβ was found to reduce both basal and calcium ionophore or estradiol -stimulated PRL production from rat pituitary tumor cell lines [4–6] and bFGF enhanced PRL and GH secretion in rat pituitary cultures and human adenomas [7–12]. Many studies also show the effects of growth factors on cell proliferation in pituitary tumors. Alteration of growth factors expression may therefore play a role in the regulation of GH secretion and cell proliferation by somatostain analogs. However, to our knowledge, studies regarding the effects of somatostatin analogs on the secretion of growth factors by pituitary cells are extremely scarce and constricted to VEGF. VEGF secretion induced by the adenylate cyclase activator Forskolin in human non-functioning adenomas (NFAs) was completely abrogated by both somatostatin and pasireotide, a somatostatin receptor (SSTR) ligand and their effects on cell viability were in correlation with the VEGF response [13]. Moreover, VEGF-induced increase in cell viability was completely inhibited by somatostatin and pasireotide [13]. Similarly, the dopamine agonist cabergoline was shown to inhibit VEGF secretion and cell viability in NFAs [14]. Interestingly, VEGF was down regulated in GH-adenomas treated pre-operatively with the somatostatin analog octreotide,

Orly Ozeri and Zvi R. Cohen have contributed equally to this study.

✉ Hadara Rubinfeld
hadarar@clalit.org.il

1 Institute of Endocrinology and Felsenstein Medical Research Center, Petach Tikva, Israel

2 Rabin Medical Center, Petach Tikva 49100, Israel

3 Sackler School of Medicine, Tel Aviv, Israel

4 Tel-Aviv University, Tel Aviv 69978, Israel

5 Department of Pathology, Sheba Medical Center, Tel-Hashomer 52621, Israel

6 Department of Neurosurgery, Sheba Medical Center, Tel-Hashomer 52621, Israel

suggesting that octreotide may mediate angiogenesis inhibition [15]. In models other than the pituitary, octreotide induced a significant decrease in VEGF production by neuroendocrine tumor cell lines [16] and regulation of IGF1 synthesis by octreotide was shown in rat hepatocytes [17]. Additionally, the releases of EGF, IGF1 and platelet derived growth factor B homodimer (PDGF BB) were inhibited by the somatostatin analog lanreotide in rat aortic allografts [18].

To date, a number of growth factors have been identified in adenohypophysial cells (reviewed in [19–26]). Most of the reports on pituitary growth factors describe mRNA or intracellular protein expression of a single factor. Secretome profile of pituitary adenomas was not reported yet. We therefore aimed to examine if the growth factor secretome profile of human GH-adenomas could be characterized by the antibody array strategy.

Materials and methods

Human pituitary tumors

Samples of pituitary adenomas were obtained during curative transsphenoidal surgical resection (Department of Neurosurgery, Sheba Medical Center) with informed consent, in accordance with methods and conditions approved by the local Institutional Review Board. The clinical characteristics of the adenomas are presented in Table 1.

Specimens were mechanically dispersed and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase (both from Sigma) for 45–60 min. Cell suspensions were filtered through 80 µM nylon mesh (Millipore, Bedford, MA) and cultured in low-glucose DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics.

Cohort size

We have received 29 GH-adenomas during this project. Fifteen of the 29 failed to meet the requirements of this research because of low yield of cells, and the antibody arrays of 4 adenomas were of poor quality and removed from the analysis.

Cell cultures

Approximately 7.5×10^4 cells/well were seeded in 48- well tissue culture plates (Costar, Cambridge, MA) in 0.5 ml medium and incubated for 24 h in a humidified atmosphere of 95% air-5% CO_2 at 37C. A single pituitary specimen was sufficient for 8–20 wells, depending upon the size of the specimen. Following extensive washing with FCS depleted medium, cultures were incubated with or without 10 nM octreotide in FCS depleted medium for 24 h. Conditional mediums of all control or octreotide treated wells were collected in one tube each and stored at − 80 °C for analysis by the human growth factor antibody array and for GH and VEGF measurements.

Antibody array

Culture conditioned media were analyzed for their content of growth factors by incubation with membranes of the RayBiotech C-Series Human Growth Factor Antibody Array C1 kit, AAH-GF-1 (RayBiotech, Norcross, GA, USA). Following manufacturer's instructions the membranes were incubated in blocking buffer for 30 min, followed by overnight incubation at 4C with conditioned media. Membranes were then washed five times with wash buffer and incubated for 2 h with biotin-conjugated antibodies at room temperature. Then, membranes were washed five times with wash buffer

Table 1 Clinical characteristics of patients with GH-adenomas

Adenoma	Age	Gender	Size (mm)	CS invasion	Histological diagnosis	Ki67
GH1	55	F	18	None	GH + TSH + βLH	5–7%
GH2	70	F	Macro	None	GH + scattered PRL	Rare
GH3[a]	45	M	20	Right	GH + scattered PRL + rare TSH	1%
GH4	63	M	Micro	None	GH + TSH	1%
GH5	50	M	Micro	None	GH	1–2%
GH6	54	F	13	Right	GH	2–3%
GH7	70	F	Micro	None	GH	rare
GH8	59	M	20	Right	GH + rare TSH	3–4%
GH9	54	F	Micro	None	GH + PRL + scattered TSH	2%
GH10	37	M	Micro	None	GH + scattered PRL	< 1%

F female, *M* male, *CS* cavernous sinus

[a]This patient was treated with 20 mg Sandostatin LAR injections for 8 months before surgery with no satisfactory response

and incubated for 2 h with horseradish peroxidase-conjugated streptavidin. After washing process, the human growth factors were detected by enhanced chemiluminescence reagents using a chemiluminescence imaging system (DNR Bio-Imaging Systems, Neve Yamin, Israel).

Antibody array data analysis

Data were processed by the TotalLab Quant v12.4 software and were scored according to the optical densitometry units after background subtraction and positive control normalization for comparison of results across multiple arrays.

Growth hormone

Human Growth hormone measurements were performed by the IMMULITE 2000 system (immunoradiometric assay, IRMA; Siemens Healthcare Diagnostics, Gwynedd, United Kingdom).

VEGF ELISA assay

VEGF in the conditional media was quantitatively detected by ELISA using RayBiotech Human VEGF-A ELISA kit according to manufacture instructions (RayBiotech, Norcross, GA, USA).

Protein extraction and Western blotting

Frozen tissue specimens derived from resistant and sensitive GH-adenomas were minced in liquid nitrogen followed by homogenization in RIPA buffer supplemented with protease and phosphatase inhibitors cocktails, all from Sigma-Aldrich Ltd. Equal protein aliquots were loaded on 15% SDS-PAGE, transferred into nitrocellulose membrane, blocked and incubated overnight at 4C with antibody against p21 (Santa Cruz Biotechnology) or against β-actin (Sigma–Aldrich Ltd). After 3 washes in TBS/Tween 20, the membranes were incubated with the secondary antibody for 60 min. Immunodetection was performed using the Chemiluminescent Peroxidase Substrate WesternBright Sirius (Advansta Co., Menlo Park, CA). Optical densities of the bands were measured employing the VersaDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA).

Results

Detection of growth factors secretion by antibody arrays

We first aimed to examine if the strategy of antibody array is sensitive enough to allow the detection of growth factors in the conditional media of GH-adenomas. Conditional media were analyzed by antibody arrays including 41 growth factors (Table 2) and visible spots were produced. As this is a semi-quantitative detection we did not attempt to compare the basal secretion between adenomas but rather to establish if this strategy can be utilized for differential secretion profiling upon a specific treatment. For this purpose each adenoma was treated with octreotide or left untreated.

In vitro effect of octreotide on GH release by GH-adenomas

We first examined the hormonal response of cultured GH-adenomas to octreotide by measuring GH secretion. Specimens of 10 human pituitary adenomas were cultured and incubated with or without 10 nM octreotide for 24 h. In five adenomas, GH1-5, no suppression or minimal reduction in GH (< 25%) were obtained following octreotide treatment, whereas five adenomas, GH6-10, responded with GH reductions of 66–93% (Fig. 1). Accordingly, adenomas GH1-5 were entitled 'resistant' and adenomas GH6-10 were entitled 'sensitive'. Hormonal control, namely GH and IGF1 reductions following treatment with somatostatin analogs is achieved in approximately 50% of acromegaly patients [27]. Thus, the in vitro response to octreotide in 50% of the GH-adenomas included in our study is in agreement with the reported clinical response rate. Moreover, the in vitro response of adenoma GH3 is consistent with the in vivo resistance to SSA treatment of this patient before surgery.

The growth factors secretome of GH-adenomas

We next examined if octreotide induces changes in growth factors secretion that can be detected by the antibody array. Conditional media of the 10 GH-adenomas were analyzed by growth factor antibody arrays and interestingly we observed both elevations and reductions in growth factors secretion upon octreotide treatment.

An antibody array of an octreotide resistant adenoma—GH1, and of octreotide sensitive adenoma—GH8 are shown in Fig. 2. Data were analyzed as detailed in Methods and spot signal optical densities of octreotide/untreated ratios were calculated. Changes of 25% or more were defined as reductions (Fig. 2, blue frames) or increases (Fig. 2, red frames). As for the small number of samples this study will be presented as illustrative with no statistical analysis.

Octreotide-induced alterations of growth factors secretion

Growth factors that were downregulated or upregulated in 5 or more of the 10 tested GH adenomas following octreotide treatment were listed in Table 3. Four families, EGF, IGF, neurotrophins and PDGF are represented by 3 or more

Table 2 Human growth factor antibody array map

	A	B	C	D	E	F	G	H	I	J	K	L
Each antibody is spotted in duplicate vertically												
1	POS	POS	NEG	NEG	AREG	bFGF	b-NGF	EGF	EGFR	FGF-4	FGF-6	FGF-7
2												
3	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF1	IGF1SR
4												
5	IGF2	M-CSF	MCSFR	NT-3	NT-4	PDGFR alpha	PDGFR beta	PDGFA A	PDGFA B	PDGFBB	PLGF	SCF
6												
7	SCFR	TGF alpha	TGF beta	TGF beta2	TGF beta3	VEGFA	VEGFR2	VEGFR3	VEGFD	BLANK	BLANK	POS
8												

POS positive control spot, *NEG* negative control spot, *BLANK* blank spot, *AREG* amphiregulin, *bFGF* basic fibroblast growth factor, *bNGF* beta nerve growth factor, *EGF* epidermal growth factor, *EGFR* epidermal growth factor receptor, *FGF4* fibroblast growth factor 4, *FGF6* fibroblast growth factor 6, *FGF7* fibroblast growth factor 7, *GCSF* granulocyte colony stimulating factor, *GDNF* glial cell line derived neurotrophic factor, *GMCSF* granulocyte macrophage colony stimulating factor, *HBEGF* heparin binding EGF like growth factor, *HGF* hepatocyte growth factor, *IGFBP1* insulin like growth factor binding protein 1, *IGFBP2* insulin like growth factor binding protein 2, *IGFBP3* insulin like growth factor binding protein 3, *IGFBP4* insulin like growth factor binding protein 4, *IGFBP6* insulin like growth factor binding protein 6, *IGF1* insulin like growth factor 1, *IGF1SR* insulin like growth factor soluble receptor, *IGF2* insulin like growth factor, *MCSF* macrophage colony stimulating factor, *MCSFR* macrophage colony stimulating factor receptor, *NT3* neurotrophin 3, *NT4* neurotrophin 4, *PDGFRα* platelet derived growth factor receptor alpha, *PDGFRβ* platelet derived growth factor receptor beta, *PDGFAA* platelet derived growth factor subunit A homodimer, *PDGFAB* platelet derived growth factor subunits A and B heterodimer, *PDGFBB* platelet derived growth factor subunit B homodimer, *PLGF*, placenta growth factor, *SCF* stem cell factor, *SCFR* stem cell factor receptor, *TGFα* transforming growth factor alpha, *TGFβ* transforming growth factor beta, *TGFβ2* transforming growth factor beta 2, *TGFβ3* transforming growth factor beta 3, *VEGFA* vascular endothelial growth factor A, *VEGFR2* vascular endothelial growth factor receptor 2, *VEGFR3* vascular endothelial growth factor receptor 3, *VEGFD* vascular endothelial growth factor D

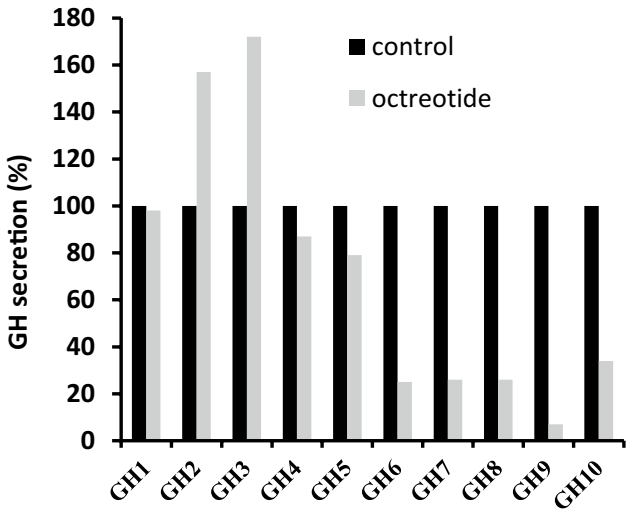

Fig. 1 In vitro effects of octreotide on GH secretion by GH-adenomas. Human GH adenomas were harvested during transsphenoidal procedures and cells were then cultured at approximately 7.5×10^4 cells/well for 24 h. Following extensive washing with FCS depleted medium, cultures were incubated with or without 10 nM octreotide in FCS depleted medium for 24 h. Medium was then collected and stored at -80C for later GH measurements, one test for each sample. Results are presented compared with control (100%)

members. Comparing the secretome profile of octreotide resistant and sensitive GH-adenomas revealed no unique pattern of each of the groups (Table 4). In each group, two adenomas have shown elevations and two have shown reductions in the majority (≥ 21) of the 41 tested growth factors (Table 4). In order to identify growth factors that have shown a trend of change in each of the resistant and sensitive groups, we picked up those who have been downregulated or upregulated in the majority of each group. Five growth factors, EGF, FGF6, HGF, NT-3 and NT-4 showed an opposite response in the resistant versus the sensitive adenomas in the majority of each group (Table 5).

VEGF concentrations

In an attempt to validate the antibody array analysis, VEGF concentrations in the conditioned media were measured by enzyme-linked immunosorbent assay (ELISA) in six adenomas (Fig. 3). In untreated cultures VEGF concentration range was ~48–606 pg/ml. In two adenomas, GH2 and GH6 VEGF concentrations were not influenced by octreotide. GH1, GH9 and GH4, GH5 showed VEGF reductions and elevations, respectively, in response to octreotide. Adenomas GH1,4 and 5 showed similar response to octreotide, as was

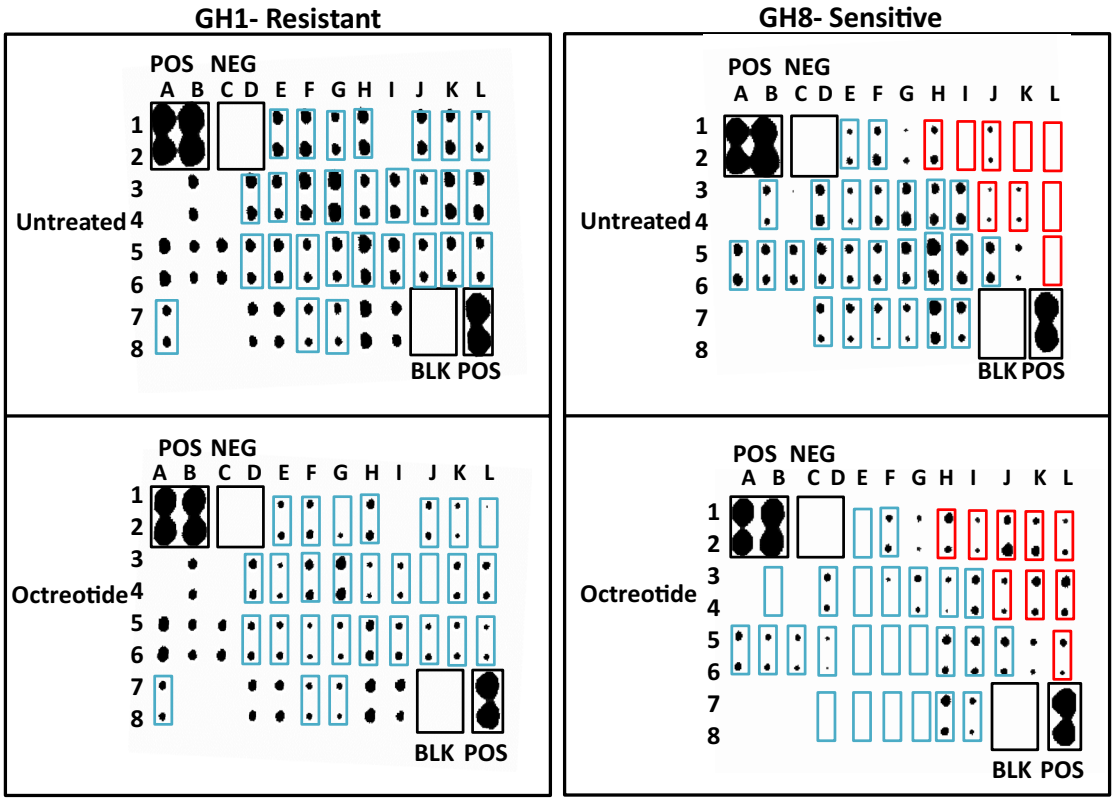

Fig. 2 Growth factors antibody arrays of adenomas GH1 and GH8. The conditioned medium of untreated (upper array) and 10 nM octreotide treated (lower array) cultures of GH1 and GH8 adenomas were collected and subjected to human growth factor antibody array. Blue and red frames indicate reduced and increased expression of growth factors, respectively, following octreotide treatment

Table 3 Growth factor secretion changes induced by octreotide in half or more of GH-adenomas

Family	Reductions in ≥5 adenomas	Elevations in ≥5 adenomas
EGF	AREG	
	EGF	EGF
	HBEGF	
FGF	bFGF	
	FGF6	FGF6
HGF		HGF
IGF	IGF1	
	IGFBP1	
	IGFBP2	
	IGFBP4	
VEGF		VEGF
		VEGFR2
CSF		MCSF
		MCSFR
Neurotrophins	NGF	
		NT3
		NT4
PDGF	PDGFAA	
	PDGFBB	
		PDGFβ
TGF		GDNF
SCF		SCFR

observed with the antibody array. However, in adenomas GH2, GH6 and GH9 the results derived from the antibody array and the ELISA did not match (Table 6).

The secretome patterns versus clinical or cellular characteristics

The clinical characteristics of the adenomas included are presented in Table 1. Interestingly, when GH-adenomas were categorized by elevations > reductions of growth factors secretion (GH4, 5, 9, 10) or the opposite (GH1-3, 6–8) (Table 4), all the adenomas in the first group were microadenomas (< 10 mm) and all the adenomas in the second group (reductions > elevations) beside GH7 were macroadenomas (≥ 10 mm).

Cellular senescence was shown to be accompanied by increased secretion of various secretory proteins, such as inflammatory cytokines, chemokines and growth factors, depending on cell type and the senescence trigger [28]. Senescence in pituitary tumors was shown lineage specific, showing p21/p53 mediated senescence in GH secreting tumors and p15/p16 mediated senescence in other tumor types [29]. p21 expression levels were examined by Western blot in 8 of the 10 GH-adenomas for which tissues were available for lysate purification (Fig. 4). In GH1, 4–6, p21 expression was high suggesting senescence phase of these adenomas. However, these adenomas did not present any unique pattern of growth factors elevations/reductions in response to octreotide.

Discussion

In this study we present the detection of growth factors in conditional media of GH-adenomas by antibody arrays. The advantages of this strategy are sensitivity and the simultaneous detection of multiple proteins. Due to the small samples number in this study, general suggestions regarding the secretome patterns are discussed but significant conclusions regarding the regulation of growth factors secretion by octreotide should await for further studies.

Overall summary of the growth factors secretion changes following octreotide treatment revealed that 8 of the 10 tested adenomas have shown a pattern of elevations or reductions in the majority (≥ 21) of the 41 tested growth factors (Table 4). This pattern may suggest a regulation of the growth factors exocytosis mechanism by octreotide rather than a specific effect on the synthesis of each growth factor. Considering somatostatin as inhibitory factor, upregulation of growth factors secretion was rather unexpected. Although stimulatory effect of somatostatin analogs on GH release was reported, it was shown in vitro, in primate non-human pituitary cultures and by low concentrations only [30–32]. It was suggested to act via SSTR5 and cAMP [32] and by the nitric oxide synthase/nitric oxide (NOS/NO) signaling pathway [30]. We have previously shown that nitric oxide stimulates GH secretion in cell cultures of human fetal pituitaries and GH-secreting adenomas [33]. Moreover, NOS/NO signaling pathway was shown to mediate GHRH- [34] and Ghrelin- [35] induced GH release by somatotrophs. Thus, the mechanism of growth factors stimulation by somatostatin analogs is yet to be discovered and the involvement of the NOS/NO signaling pathway should be considered.

Table 4 Growth factor secretion changes induced by octreotide

	Resistant					Sensitive				
	GH1	GH2	GH3	GH4	GH5	GH6	GH7	GH8	GH9	GH10
Reductions	28	36	17		1	21	11	25		1
Elevations			2	37	27	3	9	9	30	31

Table 5 Growth factor secretion changes induced by octreotide in the majority of GH resistant or sensitive adenomas

Family	Resistant Reductions in ≥3 of 5	Resistant Elevations in ≥3 of 5	Sensitive Reductions in ≥3 of 5	Sensitive Elevations in ≥3 of 5	Opposite response
FGF	bFGF		bFGF		
	FGF6			FGF6	FGF6
IGF	IGF1				
	IGFBP1				
	IGFBP2				
	IGFBP4				
EGF	EGF			EGF	EGF
	AREG		AREG		
	HBEGF				
Neurotrophins	NGF				
	NT-3			NT-3	NT-3
	NT-4			NT-4	NT-4
HGF	HGF			HGF	HGF
PDGF				PDGFRβ	
	PDGF AA				
	PDGF BB				
VEGF				VEGF	
				VEGFR2	
	PLGF				
CSF				MCSF	
		MCSFR		MCSFR	
TGF		GDNF			
SCF				SCFR	

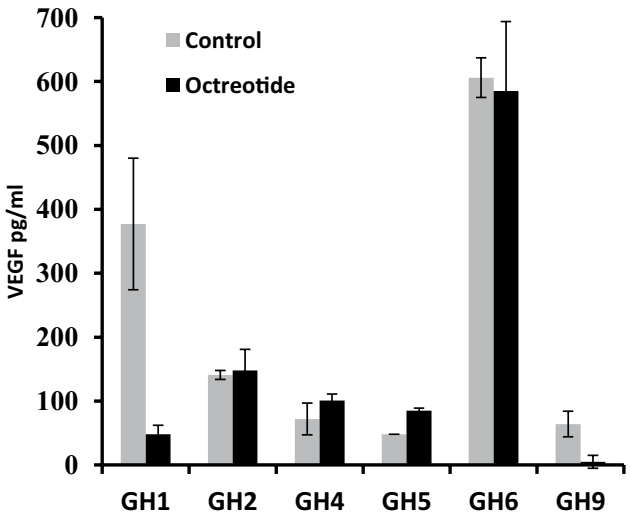

Fig. 3 Octreotide-induced changes in VEGF secretion of GH-adenomas. The conditioned medium of untreated and 10 nM octreotide treated cultures of the indicated adenomas were collected and subjected to VEGF ELISA. Each of the bars represents the mean ± SD of VEGF levels (pg/ml) in 1–3 wells, compared with control wells

Table 6 Octreotide-induced changes in VEGF secretion of GH-adenomas

Adenoma	ELISA	Antibody array
GH1	Decrease	Decrease
GH2	No change	Decrease
GH4	Increase	Increase
GH5	Increase	Increase
GH6	No change	Decrease
GH9	Decrease	Increase

VEGF inhibition following in vitro somatostatin treatment was reported in half of non-secreting adenomas tested and no upregulations were observed [13]. In our study, similar downregulation ratio of 4 out of 10 adenomas was observed, whereas octreotide induced VEGF upregulation in 5 adenomas. This discrepancy may reflect differences in the study model, namely non-secreting versus GH-adenomas and somatostatin versus octreotide which possess different receptor subtypes binding affinities. Measurements of VEGF secretion were performed by the antibody array and also by ELISA. Matching was not perfect, showing the same trend of change in 3 adenomas

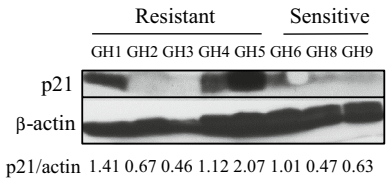

Fig. 4 Expression levels of p21 in resistant and sensitive GH-adenomas. Levels of p21 were examined by Western blot analysis. Equal protein loading was examined by the detection of β-actin. Densitometric values of p21/β-actin ratio are indicated below the blot

(GH1, 4, 5) but the opposite change in one adenoma (GH9) and in two adenomas downregulation was detected with the antibody array and no change was observed with the ELISA (GH2, 6). This inconsistency may be the result of technical concern of replicates limited number, the amount of conditioned medium of each sample was sufficient only for one antibody array and 1–3 replicates in the ELISA test. Overall, others and our findings suggest regulation of VEGF by somatostatin analogs. The signaling mechanism involved, and the functional significance and contribution to the response to somatostatin analogs should be further investigated.

The GH adenomas in each of the resistant and sensitive groups did not demonstrate homogenous and distinctive pattern of growth factors changes following octreotide treatment. This and the high number of growth factor secretion changes in the resistant group suggest that the mechanisms underlying somatostatin effects on GH and growth factor secretion are differential or partially divergent. Somatostatin inhibits GH secretion by activation of SSTR2 and 5. All the SSTR subtypes however may mediate inhibitory action on adenylate cyclase and subsequent decrease in cAMP production and consequent inhibition of exocytosis. Therefore, octreotide may affect growth factor secretion by SSTRs other than 2 and 5 and/or induce other signaling cascades.

Most reports have found no significant correlation of tumor regression and GH suppression in patients harboring GH-adenomas during somatostatin analog treatment [36–38]. Therefore, the role of growth factors in the antisecretory and antiproliferative regulation of somatostatin analogs is not necessarily identical and should be assessed in both activities. The limited cell number yield of each GH-adenoma did not enable cell proliferation measurements in our study. We also did not observe unique secretome pattern in the small number of GH-adenomas which seem to be senescent. Thus, the crosstalk of the secretome profile, senescence degree, and sensitivity to octreotide in both antisecretory and antiproliferative actions remains at this point a challenging issue. Interestingly, testing associations of growth factors secretome patterns with clinical characteristics, we noted a trend of growth factors elevations following octreotide treatment in microadenomas (< 10 mm) and reductions in macroadenomas (≥ 10 mm).

In conclusion, the role of growth factors secretion in pituitary tumorigenesis and in the response to somatostatin analogs is intriguing and may be further studied by the strategy of antibody arrays.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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