CLINICAL STUDY

Molecular and functional properties of densely and sparsely granulated GH-producing pituitary adenomas

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Abstract

Objective: GH-producing pituitary adenomas display two distinct morphological patterns of cytoplasmic GH-containing secretory granules, namely the densely and sparsely granulated somatotroph adenoma subtype. It is unknown whether these morphological variants reflect distinct pathophysiological entities at the molecular level.

Methods: In 28 GH-producing adenoma tissues from a consecutive set of patients undergoing pituitary surgery for acromegaly, we studied the GH granulation pattern, the expression of somatostatin receptor subtypes (SSTR) as well as the calcium, cAMP and ZAC1 pathways in primary adenoma cell cultures.

Results: The expression of *GSP* oncogene was similar between densely and sparsely granulated somatotroph adenoma cells. There were no differences in the calcium, cAMP and ZAC1 pathways as well as in their regulation by SSTR agonists. SSTR2 was exclusively expressed in densely but not in sparsely granulated tumours (membrane expression 86 vs 0%; cytoplasmic expression 67 vs 0%). By contrast, expression of SSTR5 was only found in sparsely but not in densely granulated somatotroph adenomas (membrane expression 29 vs 0%; cytoplasmic expression 57 vs 0%).

Conclusions: Our results indicate that different granulation patterns in GH-producing adenomas do not reflect differences in pathways and factors pivotal for somatotroph differentiation and function. *In vitro*, the vast majority of both densely and sparsely granulated tumour cells were responsive to SSTR activation at the molecular level. Sparsely granulated adenomas lacking SSTR2, but expressing SSTR5, might be responsive to novel SSTR agonists with increased affinity to SSTR5.

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Introduction

The diagnosis and classification of benign and malignant neoplasms have been traditionally based on histo- and cytomorphological as well as immunohistochemical criteria. Over the last decades, however, the molecular characterisation of tumour cells has tremendously enhanced our understanding of tumour pathogenesis. This often led to reclassification of tumours into new entities and subtypes based on specific molecular characteristics and to the identification of novel targets for therapeutic intervention.

In GH-secreting pituitary adenomas, a number of molecular changes have been identified, often through analyses of familial tumour syndromes (reviewed in (1, 2, 3)). Mutations of the *GNAS1* (*GNAS*) gene, called *GSP* mutations, cause McCune–Albright syndrome and also frequently occur in sporadic GH-secreting adenomas, but despite elevated cAMP levels (4), this does not lead to a distinct pathological or clinical phenotype. Menin mutations are responsible for multiple endocrine neoplasia type I and mutations of the regulatory subunit type 1A of protein kinase A (PRKAR1A) cause Carney complex, but both alterations do not seem to play a role in the development of sporadic adenomas. Recently, mutations in the aryl hydrocarbon receptor-interacting protein (*AIP*) gene have been found to be one cause of familial isolated pituitary adenoma (5), but *AIP* mutations in sporadic adenomas appear to be very rare (6). The identification of these molecular alterations, however, did not lead to a clinically relevant subclassification of sporadic GH-producing pituitary adenomas so far.

According to electron microscopy studies, GH-secreting adenomas can be divided into two distinct variants reflecting different patterns of GH-containing secretory granules. The densely granulated subtype is characterised by a high number of large GH-containing secretory granules resembling non-tumorous somatotrophs. In contrast, the sparsely granulated counterpart shows only sparse and small secretory granules. The latter can be easily recognised by the presence

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of juxtanuclear aggregates of intermediate filaments, namely fibrous bodies, showing a distinct immunohistochemical staining pattern using antibodies against cytokeratins (7, 8). Recent findings suggest that these phenotypes could be associated with specific molecular characteristics as patients harbouring densely granulated somatotroph adenomas appear to respond better to medical treatment with somatostatin analogues (SSA) than those with the sparsely granulated tumours (9, 10, 11, 12, 13).

The physiology of normal GH-secreting pituitary cells is predominantly regulated by the GH secretagogues GH-releasing hormone (GHRH) and ghrelin as well as by somatostatin, which inhibits GH secretion. GHRH activates the cAMP signalling pathway, which is crucial for growth and differentiation of somatotroph cells and regulates the synthesis and secretion of GH (14, 15). Inappropriate activation of the cAMP signalling pathway by activating *GSP* mutations is one cause for the development of GH-secreting adenomas, which exemplifies the pivotal role of this signalling pathway for somatotroph regulation (4, 15). Ghrelin, in its n-octanoylated or n-decanoylated form, binds to the GH secretagogue receptor 1a isoform (GHS-R1a), which couples to Gq and activates the Ca^{2+}-phosphatidylinositol signalling pathway leading to a rise in cytosolic free calcium ($[Ca^{2+}]_i$) (16, 17). GHRH through activation of the cAMP pathway and regulation of voltage-sensitive Ca^{2+} influx also causes an increase in $[Ca^{2+}]_i$ (18, 19), which is the trigger for GH secretion via the synaptotagmin-mediated exocytosis of GH-containing secretory granules (16, 20, 21, 22). Somatostatin, through activation of the somatostatin receptor (SSTR), suppresses both cAMP and cytosolic calcium signalling, thereby inhibiting GH secretion and proliferation (23, 24, 25). Furthermore, in rat somatotroph GH3 cells, analogues of somatostatin enhance the expression of the transcription factor *Zac1* (*Plagl1*), which negatively regulates somatotroph growth and function (26). ZAC1 has been suggested as one pathophysiological factor in the development of GH-secreting adenomas as its expression is decreased in pituitary adenomas compared with normal pituitary tissue (27, 28).

To investigate whether densely and sparsely granulated GH-secreting pituitary adenomas exhibit distinct molecular and functional characteristics, we studied the immunohistochemical pan-cytokeratin staining pattern, the expression of SSTR subtypes, *GSP* mutations and *ZAC1* as well as the effects of SSTR activation on cytosolic calcium, cAMP and ZAC1 signalling pathways. This was studied in a series of adenoma tissues from consecutive patients undergoing pituitary surgery for acromegaly at a single institution. The characterisation of distinct molecular characteristics of GH-producing adenomas could identify novel drug targets as well as facilitate individualised algorithms for the treatment of patients with acromegaly.

Subjects and methods

Tissue samples

Seventy-three consecutive patients underwent trans-sphenoidal surgery for acromegaly from April 2008 to November 2009 in the Department for Neurosurgery of the University Hospital Erlangen, Germany. Adenoma tissue was obtained from 45 patients who gave their informed consent. Ten of the 45 samples had to be excluded from further experimental analyses because the tissue preparation did not yield sufficient GH-secreting adenoma cells or the primary cell culture contained fibroblasts. The diagnosis was verified in each patient by immunohistochemical staining for pituitary hormones and by a review of the medical history. Seven of the 35 patients had received SSA or radiotherapy in the past and were excluded from further analyses. The mean age of the final cohort of 28 patients was 46.9 years ± 10.1 (S.D.); 15 patients were female, seven had a microadenoma and 21 patients had a macroadenoma. None of the patients had a family history of pituitary adenoma. Four patients had received dopamine agonist before surgery (Supplementary Table 1, see section on supplementary data given at the end of this article). Only six of 28 patients received medical therapy after surgery for persistent hormone excess; the response to SSA treatment in these patients is shown in Supplementary Table 2. Five normal anterior pituitary tissue samples were obtained during pituitary surgery performed for non-hormone-secreting adenomas and 14 normal anterior pituitaries were obtained during autopsy. All normal pituitaries were examined by tissue staining to ensure the absence of any pathological tissue.

Immunohistochemistry

Four micrometer thin sections were prepared from formalin-fixed paraffin-embedded tissue and developed using the 3,3'-diaminobenzidine (DAB) method as described previously (29). Evaluation of immunohistochemistry staining was performed by a neuropathologist (R B) unaware of the experimental results. For evaluation of the pan-cytokeratin expression pattern and detection of fibrous bodies (sparsely granulated somatotroph adenomas), we used the MAB IOPath^{+} Cytokeratin-Large-Spectrum (clone KL1; dilution 1:40; Immunotech, Marseille, France). According to the WHO classification and published studies, adenomas with a mixed appearance were classified as densely granulated adenomas (8). The monoclonal mouse antibody for SSTR2 (clone #402038) was purchased from R&D Systems, Wiesbaden, Germany (30). The mouse MABs for SSTR1, 3, 4 and 5 were provided by Novartis, and their specificity has recently been described in detail (31). The SSTR5 antibody was raised against amino acids 350–363 at the very C-terminus of the human SSTR5 (31) and therefore does not recognise recently

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described truncated SSTR5 variants that arise from alternative splicing at the C-terminus (32, 33).

Each section was graded for the percentage of stained adenoma cells (no staining, <10, 10–50, 50–90 and $>90\%$). Intensity of staining was graded in comparison to control samples from normal pituitary tissue (for SSTR1, 2, 3 and 5) and to pancreas tissue (SSTR4) (no staining, (+) barely detectable, + weakly positive below the intensity of control sample, ++ same intensity as controls, +++ stronger than controls). The absolute intensity was clearly higher for membrane than for cytoplasmic staining (30, 31). Samples were scored positive when they showed a ++ or +++ staining in more than 50% of tumour cells as described elsewhere (34). Both membrane and cytoplasmic stainings were evaluated because membrane staining has been observed only rarely for SSTR1, 3 and 5 while cytoplasmic staining for these subtypes was common in different tissues (35, 36, 37, 38).

Primary culture of pituitary adenoma cells

Adenoma tissue was minced with a scalpel suspended in DMEM and treated with collagenase A at a final concentration of 1 mg/ml for 1 h at 37 °C. Undissociated tissue was removed, cells were collected by centrifugation, washed twice and resuspended in DMEM (10% fetal bovine serum, Glutamax (Invitrogen), antibiotics and non-essential amino acids). Cytospins of cell preparations were stained for GH and showed more than 95% of GH-positive cells. Approximately 200 000 cells were seeded for RT-PCR analysis and 20 000 cells were put on 22 mm glass coverslips for $[Ca^{2+}]_i$ measurement. Cells were allowed to attach overnight, washed once with DMEM and checked for fibroblast contamination by phase-contrast microscopy. GH content of cell culture supernatant was determined (Immulite, Siemens, Erlangen, Germany) to assure the presence of GH-producing adenoma cells. Primary cell cultures were serum starved overnight before further experimental analyses.

Measurement of intracellular free calcium ($[Ca^{2+}]_i$)

Cells cultured on coverslips for 48 h were loaded with 5 μM Fura-2/AM (Invitrogen) and placed in a temperature-controlled superfusion chamber at 37 °C perfused with buffer containing 20 mM HEPES (pH 7.4), 130 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose and 0.1% BSA. $[Ca^{2+}]_i$ was recorded in single cells of average size and healthy appearance as described (39). Cells were treated for 2 min with superfusion buffer containing 45 mM KCl to cause membrane depolarisation and voltage-sensitive calcium influx and thereafter cells were allowed to re-equilibrate in superfusion buffer (see above) for 17 min (Supplementary Figure 1, see section on supplementary data given at the end of this article). Then an agonist selective for SSTR2, 3 and 5 (octreotide, Bachem, Bubendorf, Switzerland) or a pan-SSTR agonist (SRIF-14, Bachem) was administered for 3 min at a concentration of 100 nM that causes full activation of the respective receptors to determine the effect on resting $[Ca^{2+}]_i$. Still in the presence of the respective SSTR agonist, a second 2-min stimulation with 45 mM KCl was performed to test the effect of SSTR activation on Ca^{2+} influx through voltage sensitive calcium channel (VSCC). Cells were considered as being responsive to the respective SSTR agonist if $[Ca^{2+}]_i$ decreased by at least 5 nM upon treatment. For each adenoma sample, at least eight cells were analyzed. Cells that did not respond to 45 mM KCl with an increase in $[Ca^{2+}]_i$ were excluded.

Quantitative RT-PCR and gsp genotyping

Cells cultured for 48 h after isolation were treated with SRIF-14 (100 nM), octreotide (100 nM), forskolin (10 μM, Calbiochem Merck, Darmstadt, Germany), H89 (10 μM, Calbiochem) or control medium for 8 h. Total RNA was isolated (RNA-Gold, PeqLab, Erlangen, Germany) and reverse transcribed using anchored oligo-dT primers and superscript III reverse transcriptase (Invitrogen) along with RNA from the 19 normal anterior pituitary tissues. Quantitative RT-PCR analyses for glyceradehyde-3-phosphate-dehydrogenase (*GAPDH*), the cAMP target gene-inducible cAMP early repressor (*ICER*), *SSTR1* to *SSTR5* and *ZAC1* were based on previously described primers (27, 40, 41) and were performed on a Roche Lightcycler 480 using Kapa-SYBR-Fast mixes (PeqLab). The primers for *SSTR5* do not detect recently described truncated *SSTR5* variants (32, 33). Cp values were determined with the second-derivative maximum method (42). Individual adenoma samples were considered responsive when the mRNA levels in the treated sample showed at least a twofold suppression (*ICER*) or induction (*ZAC1*) compared with untreated controls. *GSP* mutation status was determined by PCR and direct sequencing of exons 7–10 of the *GNAS1* gene.

Statistical analyses

The relative mRNA copy numbers per 100 000 copies of *GAPDH* and the corresponding CI were calculated by the formula $2^{(Cp_{GAPDH}-Cp_{target\ gene})}\times 10^5$. Differences in $[Ca^{2+}]_i$ and mRNA levels were tested with paired or unpaired *t*-tests as appropriate using $[Ca^{2+}]_i$ and Cp values that were normally distributed. Fisher's exact test was used to compare the proportions of granulation pattern, hormone expression *GSP* mutation status and immunohistochemical expression of SSTR. All statistical tests were performed in Sigma-Plot version 11.

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Results

Immunohistochemical cytokeratin staining

Twenty-one adenoma samples displayed a cytokeratin staining pattern characteristic for densely granulated somatotroph adenomas. Seven tumours showed distinct fibrous bodies and were classified as sparsely granulated variants. Twenty adenomas expressed GH only, whereas in five densely and in three sparsely granulated tumours, co-expression of prolactin could be demonstrated using immunohistochemistry (Table 1).

cAMP signalling pathway

Eighteen adenomas expressed wild-type *GNAS1* sequence in the region examined. Ten of the 28 adenoma samples had a mutated *GNAS1* gene. Eight adenomas harboured a R201C mutation and two a Q227L mutation (Table 2). Ten of the 21 densely granulated and none of the seven sparsely granulated adenomas had *GSP* mutations ($P=0.03$) (Table 1).

Activation status of the cAMP signalling pathway was assessed by measuring the expression of the cAMP target gene *ICER*, which is downstream of Gsα, protein kinase A (PKA) and the cAMP-responsive element binding protein (40, 43). This approach integrates the activity of the cAMP signalling pathway at the transcriptional level and therefore in principle allows for the detection of yet unknown activating mechanisms of this pathway also distal to *GSP* mutations. The differences in the *ICER* expression between *gsp*-positive and *GNAS1* wild-type adenomas were consistent with previously published results showing a higher basal but lower stimulated cAMP signalling pathway activity in *gsp*-positive adenomas (Supplementary Figure 2, see section on supplementary data given at the end of this article) (4).

In densely and sparsely granulated adenomas, basal *ICER* expression was similar (Fig. 1A). To further characterise the activation status of the cAMP signalling pathway, adenoma cells were treated with forskolin (10 μM) or with the PKA inhibitor H89 (10 μM). Forskolin (10 μM) stimulated *ICER* expression in both densely and sparsely granulated adenomas about fourfold ($P<0.001$) and treatment with H89 (10 μM) significantly suppressed *ICER* expression compared with controls by about 70% ($P<0.001$) (Supplementary Figure 3, see section on supplementary data given at the end of this article). There were no differences in the effects of forskolin and H89 between the two adenoma subtypes indicating a similar degree of activation of the cAMP signalling pathway (Supplementary Figure 3).

Treatment for 8 h with either the pan-SSTR agonist SRIF-14 (100 nM), which activates all SSTR subtypes, or with the SSA octreotide (100 nM), which stimulates SSTR subtypes 2, 5 and to a lesser extent SSTR3, caused a marked decrease in *ICER* mRNA expression in the majority of tumour samples (Table 2) and significantly suppressed mean *ICER* levels ($P<0.001$) (Fig. 1A). Both, SRIF-14 and octreotide were equally effective and there were no differences between densely and sparsely granulated adenomas (Fig. 1A).

Calcium signalling pathway

The average resting $[Ca^{2+}]_i$ in single cells obtained from 28 adenoma samples was 75 nM ($n=275$ cells from 28 adenoma cell preparations (8–15 cells per adenoma sample), 95% CI 63–88 nM). Many cells showed spontaneous Ca^{2+} transients and Ca^{2+} oscillations with varying frequency and amplitude (Supplementary Figure 4, see section on supplementary data given at the end of this article). Upon treatment with octreotide (100 nM), a decrease in $[Ca^{2+}]_i$ could be observed, which on average amounted to 27 nM ($n=105$ cells from 25 adenomas, 95% CI 17–37 nM) and spontaneous Ca^{2+} transients and Ca^{2+} oscillations were suppressed (Fig. 1B, and Supplementary Figure 4). SRIF-14 (100 nM) caused a similar drop in $[Ca^{2+}]_i$, which was not significantly different from the effects of octreotide (Fig. 1B). As depicted in Table 2, most adenomas responded to octreotide and/or SRIF-14 (defined as a decrease in $[Ca^{2+}]_i$ by at least 5 nM). There were no differences observed between cells obtained from densely or sparsely granulated adenoma samples in the effects of octreotide or SRIF-14 on resting $[Ca^{2+}]_i$ (Fig. 1B).

Voltage-sensitive Ca^{2+} influx is an important component of resting and stimulus-induced Ca^{2+} signalling in somatotroph cells (19, 44). We therefore investigated the effect of octreotide or SRIF-14 on the KCl

Table 1 Expression of *GSP*-mutated *GNAS1*, pituitary hormones and somatostatin receptor (SSTR) subtype in 21 densely and seven sparsely granulated GH-secreting adenomas. All adenomas were negative for SSTR4.

	Cytokeratin staining pattern		
Parameters	Densely granulated (%) $n=21$	Sparsely granulated (%) $n=7$	***P* value**
GSP mutation	48	0	0.030
Pituitary hormones			
GH	76	57	0.371
GH/PRL	24	43	
SSTR1			
Cytoplasmic	19	43	0.318
Membrane	5	0	1.000
SSTR2			
Cytoplasmic	67	0	0.006
Membrane	86	0	<0.001
SSTR3			
Cytoplasmic	52	29	0.396
Membrane	0	0	1.000
SSTR5			
Cytoplasmic	0	57	0.002
Membrane	0	29	0.056

P value was determined by Fisher's exact test. NS, not significant.

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Table 2 Clinical data, histology and signalling pathways in 28 adenoma tissues. GNAS1, genotype of the *GNAS1* gene; *ICER*, changes in relative mRNA expression levels of the cAMP target gene *ICER* after treatment with SRIF-14 (100 nM) or octreotide (100 nM) for 8 h as determined by quantitative RT-PCR compared with controls. $\Delta[Ca^{2+}]_i$ absolute change in $[Ca^{2+}]_i$ after treatment with SRIF-14 (100 nM) or octreotide (100 nM) for 3 min. *ZAC1*, changes in *ZAC1* mRNA expression after treatment with SRIF-14 (100 nM) or octreotide (100 nM) for 8 h as determined by quantitative RT-PCR compared with controls.

							ICER		**$\Delta[Ca^{2+}]_i$ (nM)**		***ZAC1***	
Patient	**Age**	**Sex**	**Adenoma size**	**Hormone expression**	**Granulation pattern**	***GNAS1***	SRIF-14 (%)	OCT (%)	SRIF-14	OCT	SRIF-14 (%)	OCT (%)
D1	49	F	Micro	GH	Densely	R201C	**−88**	**−92**	**−12**	**−14**	89	101
D2	45	F	Macro	GH	Densely	wt	−31	−33	ND	**−20**	**351**	**427**
D3	54	M	Macro	GH	Densely	R201C	**−87**	**−71**	**−30**	**−26**	37	82
D4	62	M	Macro	GH	Densely	wt	**−86**	**−96**	ND	**−24**	**215**	163
D5	45	F	Macro	GH	Densely	wt	34	−29	**−6**	**−10**	93	58
D6	28	M	Macro	GH	Densely	wt	**−56**	−45	−1	**−27**	**222**	**286**
D7	59	M	Macro	GH	Densely	wt	**−65**	**−58**	**−32**	**−19**	89	71
D8	48	F	Macro	GH	Densely	Q227L	**−90**	**−93**	ND	**−77**	104	119
D9	44	F	Macro	GH	Densely	wt	−36	−31	ND	**−23**	107	98
D10	42	M	Macro	GH	Densely	R201C	−14	−15	**−13**	**−5**	100	129
D11	46	F	Micro	GH	Densely	R201C	**−56**	+44	ND	**−46**	198	92
D12	40	F	Macro	GH	Densely	R201C	ND	**−51**	ND	**−29**	105	145
D13	36	M	Micro	GH	Densely	wt	−42	**−64**	ND	**−42**	140	140
D14	47	M	Macro	GH	Densely	wt	**−81**	**−92**	ND	**−6**	114	142
D15	29	M	Macro	GH	Densely	R201C	ND	**−63**	ND	**−88**	**232**	**304**
D16	43	F	Macro	GH	Densely	wt	**−83**	**−92**	1	0	**241**	**292**
D17	49	F	Macro	GH/PRL	Densely	wt	−37	**−69**	**−9**	ND	96	62
D18	63	F	Micro	GH/PRL	Densely	R201C	**−81**	**−90**	**−10**	ND	70	88
D19	53	M	Macro	GH/PRL	Densely	wt	**−90**	**−90**	−1	−2	165	161
D20	43	M	Macro	GH/PRL	Densely	Q227L	**−81**	**−94**	ND	**−19**	98	103
D21	64	M	Micro	GH/PRL	Densely	R201C	**−87**	**−88**	ND	**−66**	81	75
Responsive (*n*)							13	15	7	17	5	4
S1	65	F	Macro	GH	Sparsely	wt	**−95**	**−85**	−2	−1	181	**246**
S2	41	M	Macro	GH	Sparsely	wt	**−64**	**−54**	**−13**	**−60**	104	128
S3	58	M	Macro	GH	Sparsely	wt	−42	**−55**	ND	−1	ND	187
S4	35	F	Macro	GH	Sparsely	wt	ND	**−80**	ND	**−35**	**514**	**442**
S5	34	F	Micro	GH/PRL	Sparsely	wt	−38	−37	**−5**	0	84	71
S6	49	F	Macro	GH/PRL	Sparsely	wt	**−86**	**−84**	ND	**−85**	107	76
S7	41	F	Micro	GH/PRL	Sparsely	wt	**−98**	**−98**	**−22**	**−15**	**235**	**275**
Responsive (*n*)							4	6	3	4	2	3

Bold type indicates responsive samples as defined in the Subjects and methods section; F, female; M, male; Micro, microadenoma; Macro, macroadenoma; OCT, octreotide; ND, not determined; PRL, prolactin.

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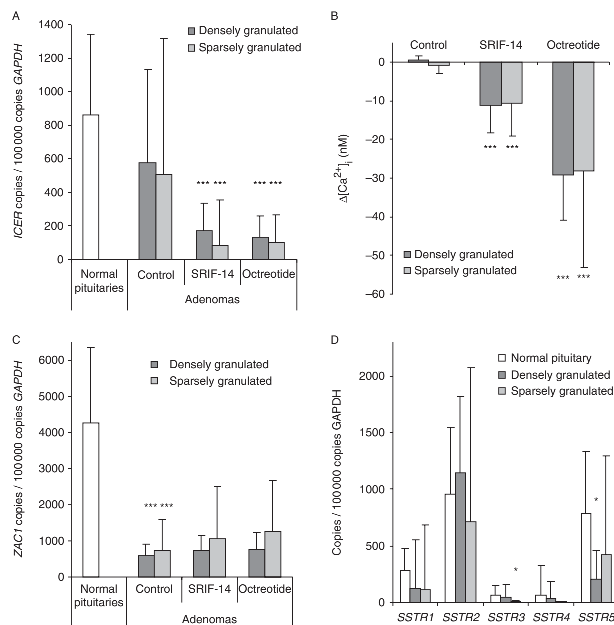

Figure 1 Signalling pathways and somatostatin receptors in 21 densely and seven sparsely granulated GH-secreting adenoma cell preparations and 19 normal pituitaries. (A) cAMP signalling pathway: mRNA expression levels of the cAMP target gene *ICER* as determined by quantitative RT-PCR. Comparison between normal pituitaries, untreated adenoma samples (control) and samples treated with SRIF-14 (100 nM) or octreotide (100 nM) for 8 h, ***$P<0.001$ vs control. (B) Calcium signalling pathway: effect of SRIF-14 (100 nM) or octreotide (100 nM) on intracellular free calcium levels ($[Ca^{2+}]_i$) (bars represent 20–104 cells). ***$P<0.001$ vs control. (C) *ZAC1* expression: *ZAC1* mRNA levels as determined by quantitative RT-PCR. Comparison between normal pituitaries, untreated samples (control) and samples treated with SRIF-14 (100 nM) or octreotide (100 nM) for 8 h, ***$P<0.001$ vs normal pituitaries. (D) *SSTR* subtype expression: levels of *SSTR* subtype mRNA as determined by quantitative RT-PCR. Comparison between normal pituitaries, densely and sparsely granulated adenomas. *$P<0.01$ vs normal pituitaries. All values are mean±95% CI.

(45 mM)-induced rise in $[Ca^{2+}]_i$, which is caused by membrane depolarisation with consecutive activation of Ca^{2+} influx through VSCC (18). KCl (45 mM) increased $[Ca^{2+}]_i$ by 34 nM ($n=275$ cells from 28 adenoma cell preparations, 95% CI 22–46 nM). Pretreatment for 3 min with octreotide (100 nM, $n=105$ cells from 25 adenomas) or SRIF-14 (100 nM, $n=51$ cells from 15 adenoma preparations) had no effect on the KCl (45 mM)-induced peak rise in $[Ca^{2+}]_i$ compared with vehicle-treated control cells neither in cells obtained from densely nor from sparsely granulated adenomas (Supplementary Figure 5, see section on supplementary data given at the end of this article). Thus, the inhibition of resting $[Ca^{2+}]_i$ by SSTR activation in somatotroph adenoma cells does not appear to be caused by a direct inhibitory action on voltage-sensitive Ca^{2+} influx. SSTR-induced suppression, however, of cytosolic calcium signalling might occur indirectly by the inhibition of the cAMP signalling pathway whose activation has been shown to promote voltage-sensitive Ca^{2+} influx in pituitary cells (18, 19, 44).

ZAC1 expression and modulation by SSTR agonists

ZAC1 expression was detectable by quantitative RT-PCR in all tumour samples. The mean expression level of *ZAC1* in the 28 GH-secreting adenomas (631 copies/100 000 copies GAPDH, 95% CI 438–909 copies) was significantly lower than in the 19 normal pituitaries (4262 copies/100 000 copies GAPDH, 95% CI 2856–6360 copies) ($P<0.001$) (Fig. 1C). There was no significant difference between the *ZAC1* levels in densely and sparsely granulated somatotroph adenomas.

Treatment with octreotide (100 nM) or SRIF-14 (100 nM) for 8 h resulted in an average 1.25-fold increase in *ZAC1* expression levels ($n=28$ adenoma, $P<0.05$). However, when analysing densely and sparsely granulated adenomas separately, the octreotide- or SRIF-induced changes in *ZAC1* expression were no longer significant, and there were no obvious differences between the two groups (Fig. 1C). This might be explained by the high variability of the responses with less than half of the adenoma samples demonstrating a rise in *ZAC1* expression (defined as a more than twofold increase in *ZAC1* mRNA levels) in response to SSTR activation (Table 2).

Expression of SSTR subtypes

SSTR2 mRNA was the most abundant subtype in adenomas according to quantitative RT-PCR analysis (Fig. 1D). *SSTR2* mRNA levels in both densely and sparsely adenomas were not different from those in normal pituitaries (Fig. 1D). Immunohistochemistry, however, revealed that protein expression of SSTR2 was restricted to densely granulated adenomas, both for membrane (86 vs 0%; $P<0.001$) and for cytoplasmic localisation (67 vs 0%; $P=0.006$) (Table 1).

SSTR5 mRNA was the second most abundant *SSTR* subtype expressed in adenomas. Densely but not sparsely granulated tumours had significantly lower *SSTR5* mRNA levels than normal pituitaries ($P<0.01$) (Fig. 1D). In the immunohistochemical analysis, membrane and cytoplasmic expression of SSTR5 protein was exclusively observed in the sparsely granulated adenoma cells (29 vs 0%; $P=0.056$ and 57 vs 0%; $P=0.002$) (Table 1).

The expression of mRNA for *SSTR1*, *3* and *4* was clearly lower than that for *SSTR2* and 5 (Fig. 1D). Immunohistochemical membrane staining for these subtypes was also rare or absent, but cytoplasmic staining for SSTR1 and 3 was frequently detected in both adenoma subtypes, with no difference between them (Table 1). SSTR4 protein could not be detected by

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immunohistochemistry, which is consistent with previous reports (45).

Detection of *SSTR* mRNA was compared with SSTR immunohistochemical protein staining, which revealed concordance rates between 57 and 89% comparable to published results from various other tissues (46, 47, 48) (Supplementary Table 3, see section on supplementary data given at the end of this article).

Discussion

The main goal in the molecular characterisation of benign and malignant neoplasms is to unravel the mechanisms underlying the abnormal growth behaviour and to identify targets to stop or retard enhanced cell proliferation. In addition, in the treatment of endocrine neoplasm such as acromegaly, the control of excess hormone secretion is another important goal. Fortunately, there are often physiological mechanisms that can be exploited to develop therapeutic strategies to inhibit abnormal hormone secretion, such as the inhibition of GH secretion by administration of SSA. The pathophysiology, however, behind the development, abnormal growth and function of somatotropinomas is less well understood.

Somatotropinomas can be subclassified into a densely and a sparsely granulated phenotype (8). There appear to be functional differences between the two subtypes as densely granulated adenomas respond better to medical treatment with currently available SSA than the sparsely granulated variant (9, 10, 11, 12, 13). The two GH-producing adenoma subtypes can be easily distinguished using antibodies against cytokeratins (7, 8). Cytokeratins are the building blocks of the cytoplasmic intermediate filaments, which are structural components of the cell that provide mechanical stability. There is some evidence that intermediate filaments may have a role in exocytosis and secretion but their role in cellular signalling and function is largely unknown (49). Thus, the histomorphological phenotypes may be associated with distinct molecular properties leading to distinct clinical characteristics.

We found no differences in the cAMP or cytosolic Ca^{2+} signalling pathways between densely and sparsely granulated GH-secreting adenoma cells. Pharmacological stimulation of SSTR either by the pan-agonist SRIF-14 or by octreotide inhibited the cAMP pathway or suppressed the cytosolic Ca^{2+} levels in most adenoma cells irrespective of their granulation pattern. The expression of the transcription factor *ZAC1*, which negatively regulates somatotroph growth and function (26) and that has been suggested as one pathophysiological factor in the development of GH-secreting adenomas, was significantly lower in the adenoma cells compared with normal pituitary tissue, which is consistent with previous reports (27, 28). Furthermore, we could demonstrate for the first time in human somatotroph adenomas that SSTR activation can up-regulate *ZAC1* expression as has been reported before for rat GH3 cells (26). The overall up-regulation of *ZAC1* by SSTR activation, however, was rather small with a high variability in the stimulation of *ZAC1* expression between individual adenoma preparations. The precise role of *ZAC1* in SSTR agonist control of growth and hormone secretion of somatotroph adenomas remains to be defined. There were no differences in basal or SSTR-stimulated *ZAC1* expression between densely and sparsely granulated adenoma cells. Together, our studies provide no evidence for an impact of the cytokeratin staining pattern on the investigated intracellular signalling mechanisms that are known to be central to somatotroph cell regulation.

All tissues were responsive to pharmacological stimulation of SSTRs in at least one of the tested pathways and 21 of the 28 samples were responsive in at least two of the three pathways. This indicates that most GH-producing adenomas could be amenable to SSTR-based treatment as long as the agonist used reaches a high-enough concentration *in vivo* to efficiently stimulate the SSTR subtypes that are expressed on the respective adenoma.

In vitro studies indicate that most biological effects of SSA in GH-producing cells are mediated by SSTR2 and SSTR5 (50, 51). To test whether this also holds true for densely and sparsely granulated adenomas, we compared the effects of SRIF-14, which activates all five SSTR subtypes, with the effects of octreotide (100 nM) that activates SSTR subtypes 2 and 5 and to a lesser extent SSTR3 at this concentration (52). There were no differences in the actions of SRIF-14 and octreotide and no differences between densely and sparsely granulated adenomas. As the expression of SSTR2 and SSTR5 was most abundant both at the mRNA and at the protein level, it appears most likely that the observed effects of SRIF-14 and octreotide were mainly through activation of SSTR subtype 2 and/or 5. This is in line with previous studies showing that SSTR subtypes 1 and 4 are not required for the inhibitory effects of somatostatin on somatotroph adenoma cells (50, 51) and applies to both densely and sparsely granulated somatotroph adenomas.

While there were no differences in the tested pathways and their regulation by SSTR stimulation, there was, however, a significant difference in the pattern of SSTR subtype expression between densely and sparsely granulated adenomas. By immunohistochemistry, SSTR2 protein was exclusively observed in densely granulated tumours whereas sparsely granulated ones exclusively expressed SSTR5. In densely but not in sparsely granulated tumours, *SSTR5* mRNA levels were significantly lower compared with normal pituitaries, while for *SSTR2* mRNA, no differences could be observed. Overall *SSTR* mRNA expression profiles corresponded well but not perfectly to protein expression, probably because mRNA expression may not always lead to translation into detectable protein

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expression. (46, 47, 48). Therefore, determining protein expression by immunohistochemistry appears to be the preferable measure for defining the adenoma cell SSTR status.

Whether there is a causal relationship between the patterns of SSTR subtype expression and the GH secretory granulation pattern or *vice versa* remains an open question. Although we observed no differences in the SSTR-induced actions on the tested pathways between the two adenoma subtypes, this does not preclude that SSTR2 and SSTR5 may differentially regulate other intracellular signals that determine the pattern of GH-containing secretory granules. Recently described truncated SSTR5 variants have been described with different signalling behaviour compared with full-length SSTR5 and further expand the spectrum of possible regulatory interactions (32, 33). The SSTR5 antibody used in this study was raised against the C-terminus of SSTR5, which is not present in these truncated SSTR5 variants. Further studies are therefore required to evaluate the impact of these SSTR5 variants on signalling, differentiation and therapeutic response in pituitary GH-secreting adenomas and their subtypes.

Clinical studies have shown a correlation of the expression of SSTR2 with patients' responses to treatment with SSA like octreotide or lanreotide that reach serum concentrations that effectively stimulate SSTR2 but only modestly activate SSTR5 (34, 52, 53, 54, 55, 56, 57). As shown in this study and by others' (13), densely granulated adenomas predominantly express SSTR2, which explains the clinical observation that densely granulated adenomas respond better than sparsely granulated ones to treatment with the currently available SSA octreotide or lanreotide (9, 10, 11, 12, 13).

To overcome the narrow SSTR spectrum activated by octreotide or lanreotide *in vivo*, novel SSA with enhanced affinity to SSTR5 have been developed and one of them has been recently proven to be an effective clinical treatment option (58, 59, 60). As most adenomas in our study responded to pharmacological SSTR stimulation at the molecular level *in vitro*, we would speculate that patients with sparsely granulated somatotropinomas, which lack SSTR2 but express the SSTR5 subtype, might benefit from treatment with novel SSA that have a higher affinity to SSTR5 (60). Whether this is the case remains to be proven in clinical studies correlating the therapeutic response to these compounds with the histomorphological phenotype and SSTR subtype expression pattern.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-13-0134.

Declaration of interest

B Mayr, R Buslei and M Theodoropoulou have nothing to declare.

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