MOLECULAR BIOLOGICAL EXAMINATION OF SOMATOTROPH PITUITARY ADENOMAS RELATED TO CLINICAL DATA FROM PATIENTS WITH ACROMEGALY

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TABLE OF CONTENTS

ABBREVIATIONS

AIP	aryl hydrocarbon receptor interacting protein
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanidine monophosphate
Cox-2	cyclooxygenase-2
D_2R	dopamine receptor 2
E-cadherin	epithelial cadherin
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
FGF	fibroblast growth factor
FIPA	familial isolated pituitary adenomas
GH	growth hormone
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GNAS	guanine nucleotide-activating α subunit
GRK2	G-protein-coupled receptor kinase 2
GSK3β	glycogen synthase kinase 3 β
IGF-1	insulin-like growth factor 1
JNK	c-Jun NH2-terminal kinase
LAR	long-acting release
MAP(K)	mitogen activated protein (kinase)
MEN-1	Multiple Endocrine Neoplasia type 1
MMP	matrix metalloprotease
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NFκB	nuclear factor κB
NMT	neuroendocrine-to-mesenchymal transition
NO	nitric oxide
NOS	nitric oxide synthase
NPV	negative predictive value
PDE4A5	phosphodiesterase-4A5
PEBP	phosphatidylethanolamine binding protein

PGE_2	prostaglandin E_2
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PKAR1A	protein kinase A type 1-α regulatory subunit
PPV	positive predictive value
ptd-FGFR4	pituitary tumour-derived fibroblast growth factor receptor 4
PTP	phosphotyrosine protein phosphatases
PTTG	pituitary tumour transforming gene
RKIP	Raf kinase inhibitory protein
SHP-1/2	Src homology domain phosphatase-1/2
SMR	standard mortality rate
SMS	somatostatin
SSTR	somatostatin receptor
TGF-β	tumour growth factor β
TNFα	tumour necrosis factor α
ZEB1/2	zinc finger E-box binding homeobox 1/2
VEGF	vascular endothelial growth factor

LIST OF PUBLICATIONS

Paper 1

Fougner SL, Borota OC, Berg JP, Hald JK, Ramm-Pettersen J, Bollerslev J. The clinical response to somatostatin analogues in acromegaly correlates to the somatostatin receptor subtype 2a protein expression of the adenoma. Clinical Endocrinology 2008 Mar;68(3):458-65.

Paper 2

Fougner SL, Bollerslev J, Latif F, Hald JK, Lund T, Ramm-Pettersen J, Berg JP. Low levels of Raf kinase inhibitory protein (RKIP) in somatotroph pituitary adenomas correlate to poor clinical response to octreotide. J Clin Endocrinol Metab. 2008 Apr;93(4):1211-6.

Paper 3

Fougner SL, Lekva T, Borota OC, Hald JK, Bollerslev J, Berg JP. The expression of E-cadherin in somatotroph pituitary adenomas is related to tumor size, invasiveness and to somatostatin analog response. Resubmitted after revision.

INTRODUCTION

Acromegaly

General

Definition and epidemiology

The first historical description of acromegaly could be the story of David and Goliath described both in The Old Testament and in the Koran. Goliath was a giant, but David defeated him by sneaking up on him, maybe in his visual field defect, and hitting him with a stone in the forehead using a sling. The connection between gigantism and the pituitary, hence with the possibility of visual disturbances, was not recognized until 1884, published in a book by Fritzsche and Klebs. Two years later, Pierre Marie introduced the name acromegaly and a clinical description of the syndrome. He had several suggestions for the aetiology; none included the pituitary (1;2).

Acromegaly is a clinical syndrome due to chronic exposure to supra-physiological levels of growth hormone (GH), in almost all cases caused by a GH producing somatotroph pituitary adenoma. When the GH overproduction occurs before the fusion of the growth plates, it results in increased and continuous length growth, termed gigantism (3). However, the disease usually develops later in life, with a median age of diagnosis at 40-50 years (4-8), men being a little younger than females (5;6;8). The incidence of acromegaly is reported to be 2-4 new cases per million inhabitants per year (4-7), but the prevalence is markedly higher, varying between 36 and 125 patients per million inhabitants in recent publications (5;7;9;10). The reports with the lowest numbers for both incidence and prevalence are from national registers of acromegaly, where the results from different regions are highly variable. This could be due to registration bias and underestimation in some of the regions, and hence an underestimation of total incidence and prevalence.

Clinical characteristics

The clinical features and symptoms of acromegaly develop slowly, and the diagnosis is usually delayed by several years. The median time from start of symptoms to diagnosis has been reported to 4 to 6 years in recent studies (7-11). This diagnostic delay did not change in patients diagnosed between 1992 and 2003, compared to 1981 – 1991 (11). A published patient's journey is an illustrating example of how long the way to correct diagnosis can be

(12). The symptoms of acromegaly are due to tumour mass or hormone hypersecretion. The symptoms caused by the expanding tumour mass are similar for all pituitary tumours, and include headache and compression of the optic nerve tract or chiasm causing visual field defects. In larger adenomas, various degrees of pituitary insufficiency can occur due to suppression of the other hormone producing cells in the pituitary. The elevated level of GH leads to increased production of insulin-like growth factor 1 (IGF-1), both in the liver causing increased systemic IGF-1, and locally. The effects of elevated GH and IGF-1 include acral and soft tissue hyperplasia, causing enlarged and swollen hands and feet, carpal tunnel syndrome, coarse facial features and macrognathia, macroglossia, sleep apnoea and deepening of the voice. Arthropathy with joint pain is common, developing to irreversible osteoarthritis in a later stage. Metabolic changes like increased insulin resistance causing impaired glucose tolerance or diabetes mellitus are not unusual. Commonly reported are also increased sweating, fatigue and physical weakness. A recent study reported higher incidence of affective disorders, particularly depressions, in patients with acromegaly, also compared to patients with other chronic diseases. Cardiac hypertrophy occurs even in patients shortly exposed to GH hypersecretion, and can develop further to cardiomyopathy and heart failure in untreated acromegaly. Arterial hypertension, arrhythmias and atherosclerosis are other manifestations (3;11;13-15). A schematic overview of symptoms is given in table 1.

Diagnosis

Single GH measurements are not reliable in the diagnostic evaluation of acromegaly due to the normal pulsatile GH secretion. However, IGF-1 is a function of the integrated 24-h serum GH level and a single measure can be used as screening procedure. In acromegaly, serum IGF-1 will be elevated compared to the normal age related reference range. The diagnosis are confirmed with an oral glucose tolerance test (OGTT), where hyperglycaemia will fail to suppress GH sufficiently in the case of acromegaly (3). A magnetic resonance imaging (MRI) scan of the pituitary will usually identify the adenoma.

Table 1. Symptoms of acromegaly

Local tumour effects
Pituitary enlargement
Visual field defects
Hypopituitarism (i.e. menstrual disturbances, infertility)
Hyperprolactinaemia with galactorrhea
Headache
Cranial nerve palsy
Systemic effects
General
Fatigue
Affective disorders
Bone and soft tissue
Acral enlargement
Coarse facial features
Arthralgia
Osteoarthritis
Carpal tunnel syndrome
Prognathism and jaw malocclusion
Visceromegaly (i.e. tongue, thyroid gland)
Skin and gastrointestinal system
Excessive sweating
Increased skin thickness
Skin tags
Colonic polyps
Cardiovascular system
Hypertension
Arrhythmias
Ventricular and septal hypertrophy
Diastolic dysfunction and heart failure
Endothelial dysfunction
Pulmonary system
Upper airway obstruction
Sleep apnea
Metabolism
Impaired fasting glucose and glucose tolerance
Insulin resistance
Diabetes mellitus

Pathology of the somatotroph pituitary adenoma

General

Growth hormone production in the normal somatotroph cell is regulated through growth hormone releasing hormone (GHRH) and somatostatin, both secreted by cells in the hypothalamus. GHRH binds to its own receptor at the surface of the somatotroph cell, and a stimulatory G-protein is activated, leading to increased production of the intracellular second messenger cyclic adenosine monophosphate (cAMP). Through subsequent activation of intracellular protein kinases, this results in cell proliferation and growth hormone synthesis and secretion (16). Somatostatin inhibits GH secretion through activation of an inhibitory G-protein after binding to the somatostatin receptor (16;17).

Tumours in the pituitary can arise from all the different cell types in the anterior lobe. GH producing adenomas, the somatotroph pituitary adenomas, are tumours of the somatotroph cell line. They are considered benign tumours, as they usually grow slowly and metastasize extremely seldom, but they can be locally invasive. The growth of these adenomas are monoclonal. Yet, mutations in classical oncogenes or tumour suppressor genes found in other neoplasms have not been detected (16-19). Pituitary tumours are generally sporadic, but up to 4-5 % of patients may have an adenoma as part of a familial syndrome like Multiple Endocrine Neoplasia type 1 (MEN1) with mutation in the MEN1 gene, Carney complex with mutation in the PRKAR1A gene or McCune-Albright syndrome with somatic mutations in GNAS (17;19;20). Familial isolated pituitary adenomas (FIPA) have also been described, representing about 1 % of all pituitary adenomas (20;21). After a genetic linkage study suggested a candidate locus on chromosome 11, a Finnish group identified germline mutations in the aryl hydrocarbon receptor interacting protein (AIP) associated with familial presentation of somatotroph and lactotroph pituitary adenomas (22;23). Till now, at least 33 different AIP mutations have been detected, and they account for approximately 15 % of the FIPAs, but higher in families with exclusively somatotroph adenomas. The penetrance of disease in the AIP mutation positive families was low in the original publication, but later studies conclude with a penetrance of at least 33 % (20;21;23;24). A recent publication suggested existence of modifier genes that can explain the observed variability in phenotype between the mutation positive persons in a family (25). The mutant AIPs have a lower ability to bind to its known interacting partner PDE4A5, thereby affecting its effect to modulate cAMP (20;21;24). In the study from Finland, the same AIP mutation was detected also in 16 % of sporadic acromegaly patients

(23). This has not been confirmed in other studies, where germline mutations have been found in none or very few of the patients with sporadic acromegaly (20;21;24). The patients with an AIP mutation seem to be younger at the onset of disease, also compared to other FIPA patients (23;24).

Gsp oncogene

Activating mutations in GNAS, the gene encoding the Gsα subunit of the G-protein linking the GHRH-R to adenylyl cyclase, are designated gsp oncogene. They are reported to exist in approximately 40% of the sporadic somatotroph adenomas. This mutation, found in the codon for amino acid 201 or 227, causes a constitutive activation of Gsα leading to increased production of the intracellular second messenger cAMP. The result is cell proliferation and growth hormone synthesis and secretion (26-28). Patients with a gsp positive adenoma do not differ in age and sex compared to patients with gsp negative adenomas. Studies correlating the gsp status to clinical variables like GH level and tumour size have not been conclusive, although most studies suggest that gsp positive adenomas are smaller than gsp negative adenomas (29-34). Most, but not all studies found a better *in vitro* or acute octreotide response in gsp positive adenomas (29;30;32-37) compared to tumours without this mutation. The two studies of long-term octreotide response in 18 and 42 patients did also conclude with a better GH response during octreotide treatment in patients with gsp positive adenomas, but no difference in IGF-1 response in the one study presenting these data (29;38).

Morphology

The somatotroph pituitary adenomas have morphologically been classified into two types; densely and sparsely granulated somatotroph adenomas. At the ultrastuctural level, the densely granulated adenomas have extensively developed rough endoplasmatic reticulum and Golgi complex, and numerous large secretory granules. Histologically, they are acidophilic and show strong immunostaining for GH. They can also show immunoreactivity for prolactin or the α-subunit of glycoprotein hormones. The sparsely granulated adenomas, on the other hand, are chromofobic and show none or only weak positivity for GH by immunohistochemistry. In the electron microscope, they have few and small secretory granules and the Golgi apparatus is often replaced by a fibrous body (39). The fibrous bodies are dense aggregates of cytokeratin filaments and are considered a marker of sparsely granulated adenomas. The fibrous bodies can be demonstrated as dot-like appearances by immunohistochemistry for cytokeratin, in contrast to the densely granulated adenomas that

display a diffuse perinuclear staining pattern (39-41). In the first study of gsp oncogene and adenoma morphology, there was a high concordance where all the eight gsp positive adenomas were densely granulated and six out of nine gsp negative adenomas were sparsely granulated (33). In a recent study, the 14 sparsely granulated adenomas were gsp negative, while five of the 12 densely granulated adenomas harboured the mutation (42). So far, no other correlations have been performed between adenoma morphology and gsp mutation. In addition, an intermediate group has been described. Both a mixed population of the two described patterns and transitional patterns of cytokeratin staining with borderline shapes with neither pure perinuclear nor dot-like appearances have been described in a substantial proportion of adenomas (40). The normal adenohypophysial cells show the perinuclear pattern in immunostaining with an anti-cytokeratin antibody (40), and it is demonstrated that densely granulated adenomas transform and develop fibrous bodies after *in vitro* exposure to pegvisomant (42). One theory can be that the gsp positive adenomas remain densely granulated, but that the gsp negative adenomas can develop fibrous bodies and a sparsely granulated morphology parallel with a dedifferentiation. Studies have indicated that the densely granulated adenomas respond better to somatostatin analogue treatment (43;44), and that the sparsely granulated adenomas are more often macroadenomas and invasive (40;41). In addition, downregulation of the adhesion protein E-cadherin is demonstrated particularly in the sparsely granulated adenomas (40;45;46).

Growth factors and cell cycle control

Several cell cycle inhibitors are shown to be reduced in pituitary adenomas, in particular the cyclin-dependent kinase $p27^{Kip1}$. p27 regulates the progression of G_1 to S phase in the cell cycle. Reduced protein levels of p27 has been demonstrated in pituitary tumours, but mainly in corticotroph adenomas and carcinomas, and negatively correlated to the proliferation marker Ki-67 (19;47-49). Interestingly, p27 protein levels were upregulated in pituitary adenomas after *in vitro* treatment with somatostatin analogues (50). Possible mechanisms for the importance of p27 in the somatostatin analogue response are further elaborated in the somatostatin analogue and receptor section (p. 20). Another cell cycle regulator is the pituitary tumour transforming gene (PTTG) which is overexpressed in pituitary tumours, and is correlated to invasiveness and Ki-67 expression (16;19). Alterations in the expression of growth factors like epidermal growth factor (EGF) and fibroblast growth factors (FGFs) and their receptors EGFR and FGFRs might have roles in the adenoma growth. The activated phosphorylated EGFR has been found in pituitary adenomas and with higher

levels in carcinomas. One study demonstrated higher levels of EGFR in recurrent somatotroph adenomas (51;52). A truncated kinase-containing variant of FGFR4 has been found in pituitary adenomas, termed ptd-FGFR4 (53). This is detected in the cytoplasm, in contrast to wild-type FGFR4 which is located to the cell membrane, and correlated to the expression of Ki-67. One study of all pituitary adenoma types has indicated that ptd-FGFR4 is higher in macroadenomas, but a smaller study of somatotrophs could not confirm this (54;55).

Treatment of acromegaly

Rationale for treatment

Overall, mortality is increased by approximately 70 % in acromegaly, as given in two recent meta-analyses (56;57). This is mainly due to the effect of excess GH on the cardiovascular system (13;57). The correlation between acromegaly and malignancy is more controversial, but in a nationwide survey from Finland a significant higher cancer incidence (colorectal and thyroid cancer) was found in patients with acromegaly compared to the general population. For colorectal cancers, this was only in the group of patients with GH > 2.5 μg/l after treatment (58). In both meta-analyses, the patients successfully treated with normalisation of GH level below 2.5 μg/l had mortality rate close to the reference level. Standard mortality rate (SMR) for the patients with GH > 2.5 μg/l after treatment was 1.9 (95 % confidence interval 1.5-2.4), (57). Although more debated, this was analysed with respect to the IGF-1 level in one of the meta-analyses, with SMR 2.5 (1.6-4.0) for patients with elevated IGF-1 (57). Therefore, effective treatment of acromegaly with normalization of hormone levels is important to restore normal life expectancy in addition to controlling tumour growth and to relieve symptoms for the patient.

Surgery

The first attempts of surgical resection of pituitary adenomas were performed in the beginning of the 20th century, but with high mortality rates. Dr. Schloffer did the first successful removal of an adenoma by transsphenoidal, transnasal approach in 1907, and the procedure was later modified by Harvey Cushing and others. Despite only about 5 % mortality rate of Cushing's large series of transsphenoidal adenomectomy from 1910 to 1925, he also modified the transfrontal approach. From 1930 Cushing abandoned the transsphenoidal technique, and for the next 35 years it was in little use. Guiot and Hardy

developed the method further with illumination of the surgical site and an operating microscope, and from the late 1960ies the method had its renaissance (59;60). Today, transsphenoidal adenomectomy is still the primary treatment of most patients with acromegaly (61). While overall surgical cure rate has been reported as high as 52-57 % in series from particularly experienced neurosurgeons, microadenomas 75-82 % and macroadenomas 47-50% (62;63), recent reports from national or regional surveys with the same stringent criteria for cure show a marked lower cure rate. They report an overall cure rate of 30-40 %, but with large variation between the included centres (4;5;7;64). The results are best in the hands of a particularly interested neurosurgeon (65;66).

Radiotherapy

Radiotherapy of the adenoma was previously used more regularly, but today it is mostly used if tumour growth control and normalization of hormone levels cannot be obtained by surgery and medical treatment (5;61). Both conventional fractionated radiation therapy and stereotactic radiotherapy (i.e. gamma knife) are used. The mortality is shown to be particularly high in radiated patients. This can partly be due to the resistant acromegaly in these patients, but also due to consequences of radiation therapy like hypopituitarism (5;6;67;68).

Medical treatment; dopamine agonist

The first successful report of medical treatment in acromegaly was the study of L-dopa treatment of eight patients published in 1972 (69). All patients responded with GH reduction, and two patients reached normal GH levels. When bromocriptin later was shown to be a dopamine agonist, the same group demonstrated a GH lowering response to oral intake of bromocriptin in the seven patients studied (70). Today, cabergoline is the dopamine agonist most used, and the advantages are the relatively low cost and the oral administration. However, hormone levels can be controlled only in approximately one third of patients treated with a dopamine agonist in monotherapy (71-73). Nevertheless, cabergoline can be used in combination with other drugs, particularly somatostatin analogues. In patients with inadequate response to treatment with somatostatin analogues, addition of cabergoline led to normalization of hormone levels in 40-50 % of the patients (74-76). The clinical response to dopamine agonist treatment was better in patients with elevated S-prolactin in one study and in patients with adenomas showing positive immunostaining for prolactin in another study (72;76). Newer and larger studies have not found a correlation between clinical cabergoline response and baseline S-prolactin level or

prolactin immunostaining of the adenoma (73-75). The clinical response to dopamine agonist treatment is probably related to the expression of dopamine receptor 2 (D_2R) in the tumour. One study has demonstrated such a correlation between immunohistochemical expression of D_2R and the *in vitro* response to quinagolide, but there was no correlation to the *in vivo* response during an acute quinagolide test. Nevertheless, this study demonstrated that D_2R is expressed both in mixed GH/prolactin adenomas and also in pure GH secreting adenomas (77).

Medical treatment; growth hormone receptor antagonist

The structures of GH and the GH receptor (GHR) and the receptor binding parts of the GH molecule were discovered in the late 1980ies. Following, the important dimerization of the GHR upon binding of the ligand was discovered (78), and the development of mutant GH analogues and studies of the ability of these to inhibit growth in transgene mouse led to the discovery a GH antagonist (78-83). Pegvisomant is a GHR antagonist with a single amino acid substitution in position 120 to inhibit the receptor dimerization important for downstream signalling, and substitution of eight amino acids in the binding site to increase affinity. For clinical use, the molecule is pegylated to increase biological half-time (82). Pegvisomant acts in the periphery and not on the pituitary tumour like the other drugs used in the treatment for acromegaly. Therefore, measurements of GH can not be used in the clinical evaluation of treatment response. The efficacy of this compound in reducing serum IGF-1 has been reported to 71-97 %, and it significantly reduces the symptoms of acromegaly. Reported adverse effects have been reversible liver transaminase elevations and growth of the pituitary tumour; the latter being seldomly observed (84-88). In a large German observational study of more than 300 patients (83 % of all patients receiving pegvisomant treatment in Germany), tumour volume increased during treatment in only nine patients and discontinuation of the treatment was necessary in only three patients (84). The major reason for the limited use of pegvisomant so far is probably the high costs. Therefore, combination treatment of pegvisomant and somatostatin analogues is increasingly used, allowing reduced doses of pegvisomant, often with a twice-weekly administration. This is as efficient as pegvisomant monotherapy (89;90).

Somatostatin and the somatostatin receptor

Somatostatin treatment

Somatostatin analogue development

A hypothalamic peptide inhibiting the release of GH from the pituitary was detected in 1973, designated somatostatin (SMS). Both native ovine and synthetic somatostatin inhibited GH secretion in rats when administrated intravenously, and *in vitro* native somatostatin significantly diminished GH secretion in pituitary adenoma cells from a patient with acromegaly (91). The first studies of somatostatin administration in healthy individuals and acromegaly patients demonstrating GH lowering effects were performed the same year (92;93). However, native somatostatin has very short biological half-life (2-3 minutes) which complicates its use in a clinical setting, and in addition there were disadvantages with rebound hypersecretion of hormones. The development of long-acting somatostatin analogues was therefore important, and octreotide (SMS 201-995) was presented in 1982 (94). The first clinical tests with this analogue demonstrated strong GH inhibition in 6/7 and 7/8 patients in up to 10 hours after injection, with no rebound GH response and only short-time insulin lowering effect that declined after repeated dosing (95;96). Three days treatment with octreotide 2-3x daily gave reduction in GH levels by 30-79%, and two patients were treated for several months with sustained GH reduction and improvement of symptoms yet no side effects (97). Later, a long-acting release octreotide (Octreotide LAR) and the equivalent analogue lanreotide (Lanreotide Autogel) have been developed, allowing a dosing interval of approximately four weeks. Today, these somatostatin analogues are widely used in the treatment of acromegaly, and are regarded as the first-line drugs in the medical treatment of this disease (61).

Clinical treatment

The clinical response to treatment is highly variable. Some patients respond well with normalization of hormone levels and regression of the tumour, while other patients experience increasing hormone levels and tumour size. Studies of clinical biochemical response to long-term somatostatin analogue treatment have included very different patient groups and have used different definitions of disease control (98). A few studies have included only unselected *de novo* patients who have not received prior treatment. In these, biochemical control is achieved in 20, 25, 27 and 50 % of patients, with biochemical control defined as a mean GH below 2-2.5 μg/l and a normal IGF-1 for age. (99-102). Mean overall

tumour shrinkage in unselected, primarily treated patients has been reported to be 25-40 % (99-102). Meta-analyses of studies with primarily treated patients (unselected and selected) have concluded with 20 and 50% mean shrinkage (99;103), but individually the tumour volume reduction is highly variable, ranging from +13 to -100 % (104;105). There is no correlation between biochemical response and tumour shrinkage (99;101;105;106).

Somatostatin receptors and signalling

Somatostatin receptors and their expression

The somatostatin analogues exert their effects through binding to the somatostatin receptor (SSTR) localized in the cell membrane. The somatostatin receptor was first characterized in 1978 when it was shown that the receptor was necessary for somatostatin to exert its biological activities (107). In the 1980ies, studies demonstrated the existence of more than one SSTR subclass (108;109), and in the early 90ies the five receptor subtypes were cloned (110-112). These were named somatostatin receptor 1-5 (SSTR1-5). The genes for each receptor are located on different chromosomes, but with partial sequence homology. There is also a high degree of structural conservation across species. All receptor subtypes are transmembrane G-protein coupled receptors. Except for SSTR4 they are all glycosylated, which explains the observed variation in protein size on immunoblots. The SSTR2 gene encodes two variants of the protein, SSTR2a and 2b, due to alternative splicing. They differ only in the length of the cytoplasmic tail (113;114). One study indicates that SSTR2b binds with higher affinity to the downstream signalling molecules and that it may be less susceptible for desensitization (115). However, in the human pituitary and specifically in somatotroph adenomas, SSTR2a dominates (116;117). The SSTRs are widely distributed throughout many tissues and in various cancer cells, but in a species and cell specific pattern (113;114). In the human pituitary and in somatotroph adenomas, messenger RNA (mRNA) for the four subtypes SSTR1, 2, 3 and 5 are expressed, but SSTR 2 and 5 dominate (118;119). More recently, this was confirmed at the protein level in a immunohistochemical study, while another study found positive staining for all SSTRs in the somatotroph adenomas (120;121). Nevertheless, the individual patterns of subtype expression are highly variable even among the somatotroph adenomas.

Signal transduction and molecular responses

Signalling through the SSTRs is complex. Binding of ligand to the somatostatin receptors activates a variety of cellular responses through activation of specific G-proteins coupled to different intracellular signalling pathways. Each receptor subtype is coupled to multiple second messenger systems, some are common for all subtypes and some are subtype specific. All receptor subtypes are potent inhibitors of adenylyl cyclase and cAMP formation, and all activate phosphotyrosine protein phosphatases (PTP). All receptors also modulate the mitogen activated protein (MAP) kinase signalling pathway, phospholipase C and the K^+ ion channels. Most also influence the Ca^{2+} ion channels, in addition to nitric oxide (NO) and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (98;113;114;122). Table 2 gives a detailed overview of the signals from each receptor subtype.

Table 2. Somatostatin receptor signalling

Transduction pathway	**SSTR1**	**SSTR2**	**SSTR3**	**SSTR4**	**SSTR5**
Adenylyl cyclase	↓	↓	↓	↓	↓
Ca^{2+} channels	↓	↓			↓
K^+ channels	↓	↑	↑	↑	↑
Phospholipase C	↑	↑	↑	↑	↑/↓
Phospholipase A_2	↓	↓		↑	
PTPs	↑	↑	↑	↑	↑
PI3 kinase/Akt	↑	↑/↓		↑	
Nitric oxide/cGMP	↓	↑/↓	↓	↓	
Na+/H+ exchange	↓		↓	↑/↓	
ERK (MAP kinase)	↑/↓	↑/↓	↑/↓	↑	↓
Responses					
GH secretion	↓	↓			↓
Cell proliferation	↓	↓	↓	↑/↓	↓
Apoptosis		↑	↑		

Patel YC (1999), Pyronnet S et al (2008) and Weckbecker G et al (2003) (113;114;122).

As mentioned above, several studies have demonstrated lack of correlation between biochemical response and tumour shrinkage during treatment with a somatostatin analogue in acromegaly. This suggests that different mechanisms are responsible for the antihormone

and the antitumour response (123). Figure 1 is a simplified map over the somatostatin receptor responses. The pathways of the responses in the somatotroph adenomas can be divided into the following categories;

1) Antisecretory response

Somatostatin inhibits the secretion, but not the production of GH (122). The inhibition of GH secretion in the somatotroph adenomas is mediated by the inhibition of adenylyl cyclase and reduction in cAMP, but probably more important, by a decrease in intracellular Ca^{2+}. The reduced intracellular Ca^{2+} is a result of opening of K^{+} channels or inhibition of Ca^{2+} channels. Reduced secretion could also be mediated by a serine/threonine phosphatase. It has been suggested that SSTR1, 2 and 5 are responsible for the antisecretory effects of somatostatin (113;122;124).

2) Antitumour responses

   a) Indirect antitumour effects

Somatostatin inhibits tumour growth by inhibition of growth factors like IGF-1 and EGF, in addition to inhibition of angiogenesis (114;122). The somatotroph pituitary adenomas express increased levels of angiogenic markers like FGF-2 and vascular endothelial growth factor (VEGF) (125), and *in vitro* treatment with the somatostatin analogue pasireotide led to decreased VEGF and reduced cell viability in non-functioning pituitary adenomas. This effect was not mediated by SSTR5 despite the high affinity of pasireotide to SSTR5, because the responder group did not express this receptor subtype (126). It has also been shown that proliferating, but not resting, endothelium express SSTR2 and 5, and *in vitro* treatment with both SSTR2 and 5 preferring analogues resulted in reduced proliferation of the endothelial cells (127). SSTR3 can also be involved, as one study demonstrated that SSTR3 mediated inhibition of angiogenesis through inhibition of MAPK and endothelial nitric oxide synthase (eNOS) (128).

   b) Direct antitumour effects

      i) Induction of cell cycle arrest (cytostatic response)

All five somatostatin receptors can mediate the antiproliferative response of somatostatin. The intracellular pathways involved are differently regulated according to receptor subtype and cell environment (114). However, all SSTRs are shown to activate PTPs and the altered cellular phosphorylation pattern triggers the further signalling (113). Activation of the PTP SHP-1 is demonstrated to be a critical step for SSTR2-mediated antiproliferative signalling (129). The PTP SHP-2 is probably involved in the interaction between SSTR2 and SHP-1 (130). The activated SHP-1 dephosphorylates different signalling molecules, including nitric

oxide synthase (NOS), leading to increased p27. p27 is a cyclin-dependent kinase inhibitor that has an important role regulating entry into and exit from the cell cycle (50;131;132). SSTR5 can also increase p27, and both SSTR2 and 5 activation lead to reduction of cyclin D1, a cell cycle progression protein (133). Activated SHP-1 also induces increased cGMP via activation of NOS and the resulting nitric oxide, which also results in inhibition of cell proliferation (131). The similar response is seen downstream of SSTR5 activation (134). Via receptor tyrosine kinases, SSTR2, 3 and 5 inhibit the MAPK pathway which leads to inhibited cell proliferation (50;113;134).

ii) Induction of apoptosis (cytotoxic response)

Apoptosis is induced through activation of SSTR2 and SSTR3, both using mechanisms dependent on SHP-1 activation. SSTR3 promoted apoptosis is mediated through activation of wild-type tumour suppressor protein p53 and the pro-apoptotic protein Bax. Independent of this, apoptosis is triggered by intracellular acidification due to activation of an acidic endonuclease in a SHP-1 dependent manner (135-137). SSTR2 also induces apoptosis dependent of SHP-1, but independent of p53 (138). SSTR2 sensitizes the cells to apoptosis induced by death ligands (like TNFα) through stimulation of NFκB by upregulating their receptors and inhibition of the MAPK JNK (139;140). Activation of SSTR2 also results in PI3K inactivation and induction of apoptosis (141).

Internalization and degradation

The G-protein-coupled receptors have the ability to regulate their responsiveness to continuous agonist exposure, with desensitization and uncoupling from the G-proteins and receptor internalization. After internalization, the receptors are either degraded or recycled back to the cell membrane. These mechanisms also apply to the human SSTRs, however, the regulation is subtype specific (113). SSTR3 and 5 display the highest degree of internalization, while SSTR1 do not internalize (142). While SSTR3 is a subject of degradation after internalization, SSTR2a is often recycled back to the cell membrane. The internalization of SSTR2a requires binding to β-arrestin after phosphorylation of SSTR2a by G-protein-coupled receptor kinase 2 (GRK2) (143).

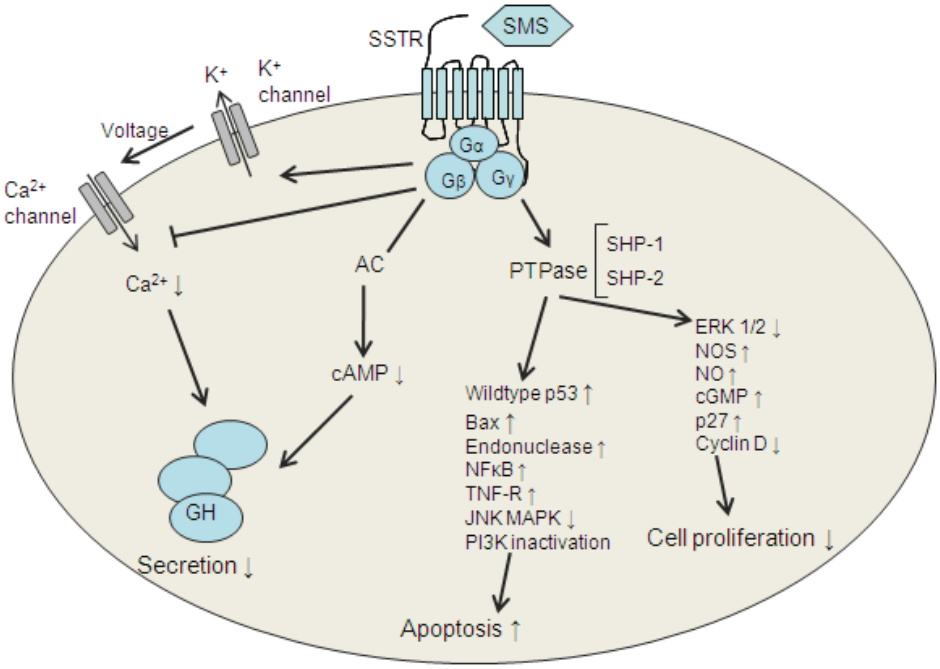

Figure 1. A simplified map over the main responses downstream of the somatostatin receptor. Modified from Weckbecker et al 2003 (122) .

Tachyphylaxis

Tachyphylaxis is the adaptation or escape of somatostatin effect after prolonged treatment. From a clinical point of view, tachyphylaxis is not a problem in the somatostatin analogue treatment of acromegaly, only very few cases have been reported (142;144;145). In the treatment of carcinoid tumours, however, tachyphylaxis is frequently observed. It has been suggested that expansion of carcinoid tumour cell clones lacking SSTR2 may be the explanation of tachyphylaxis rather than downregulation of the receptor, partly because of the relatively long time before the escape of effect (142).

Factors influencing the SMS response

As outlined above, the clinical response to treatment with a somatostatin analogue is highly variable. Most studies have concluded that the gsp positive adenomas show a better SMS response than the gsp negative adenomas, but only two of these studies have analysed the long-term octreotide efficacy. Since the somatostatin analogues available today bind with highest affinity to SSTR2, it is expected that the adenoma expression of this receptor is

important for the SMS response. However, most studies have examined the mRNA level of SSTR2a, and not the protein expression. The mRNA level is not necessary correlated to the protein level of the receptor (133;146). The first studies demonstrated a positive correlation between the response in an acute octreotide test and the uptake of radiolabelled octreotide (147;148). Later, three of four studies (9-16 patients) have found a positive correlation between the SSTR2 mRNA level and the reduction in GH during an acute somatostatin suppression test (36;37;118;149) and the effect of somatostatin analogue on GH secretion *in vitro* (36;118). In the two studies of long-term octreotide response (11 and 23 patients) only one found a positive correlation between SSTR2 mRNA level and the percentage reduction of GH and IGF-1 (150;151). Prior to our study, only one paper had correlated the SMS response to the protein expression of SSTR2a. This study of 22 patients found that adenomas with more than 50% of cells positively stained for SSTR2a by immunohistochemistry, responded better to an acute octreotide challenge (152).

New somatostatin analogues

Octreotide and lanreotide both have a high binding affinity for SSTR2, less affinity for subtype 5 and lowest affinity for subtype 3 (113). Recently, a new somatostatin analogue has been developed. This analogue, pasireotide (SOM230), has a broader receptor affinity with strongest binding to SSTR5, yet relatively high affinity for subtype 2 and 3, and lower for receptor subtype 1 (153;154). The acute response to pasireotide was significantly better than the octreotide response in three of 12 patients and comparable responses in eight patients (155). Yet, no studies of clinical long-term efficacy of pasireotide have been published, but the hopes are that this drug can be efficient in patients with inadequate response to the traditional somatostatin analogues. Similarly, studies indicate that pasireotide could get a role in the treatment of non-functioning and corticotroph pituitary adenomas which have very limited responses to octreotide and lanreotide (126;156-158). However, a very recent *in vitro* study has demonstrated that pasireotide antagonizes some of somatostatin's actions on the intracellular signalling pathways downstream of SSTR2a. pasireotide may therefore not be considered as a pure somatostatin mimetic (159). Chimeric analogues binding to both SSTR2 and D_2R (BIM-23A387) or SSTR2, SSTR5 and D_2R (BIM-23A761) are also under development. *In vitro* studies have suggested a synergistic effect with these analogues, larger than the responses seen with subtype specific analogues and combinations of these, and good response also in cells from partial octreotide

responders (160-162). This could be mediated by the receptor homo- and heterodimerization induced by these multiple ligands, which lead to increased coupling to and inhibition of adenylyl cyclase (163). Another theory is that the different interaction between the ligand and its receptor allows prolonged stabilization of its active conformation or alters the rate of SSTR internalization (160).

Raf kinase inhibitory protein (RKIP)

RKIP and its functions

Raf kinase inhibitory protein (RKIP) is a member of the phosphatidylethanolamine binding protein (PEBP) family of evolutionarily conserved proteins without significant homology with other proteins. RKIP is widely expressed in different tissues, localized to the cytosol and at the plasma membrane (164). The protein was isolated only a decade ago, when it was shown to inhibit the MAP kinase signalling pathway Ras/Raf-1/MEK/ERK (165). RKIP modulates the pathway by binding to Raf-1, and inhibits Raf-1 phosphorylation and activation (165-167). This intracellular signalling pathway is involved in the control of cell proliferation and differentiation, cell death and apoptosis (165;168). RKIP can also inhibit MEK/ERK via indirect inhibition of B-Raf (169), a protein shown to be overexpressed in non-functioning pituitary adenomas (170). Protein kinase C (PKC) phosphorylates RKIP, which then dissociates from Raf-1 and instead binds to and inhibits G protein-coupled receptor kinase 2 (GRK2) (171). This leads to decreased internalization and degradation of G-protein coupled receptors, and internalization of SSTR2a is shown to be dependent of GRK2-mediated phosphorylation of the receptor (143). Activation of PKC leads to enhanced signalling of the G-protein receptors both by removing an inhibitor from Raf-1, resulting in an enhanced MAPK signalling, and by blocking receptor internalization and degradation, thereby prolonging the receptor signal (171). Figure 2 gives an overview over these RKIP functions.

Through its inhibition of the MAPK pathway, RKIP regulates the spindle checkpoint of the mitosis. Loss of RKIP with increased MAPK activity leads to suppression of the Aurora B kinase activity. The cells move faster through mitosis with partial suppression of the spindle checkpoint, resulting in increased probability for accumulation of chromosomal abnormalities (172). Moreover, when the NF-κB pathway is activated by for instance TNFα

or other cytokines, RKIP antagonizes this activation through its interaction with upstream kinases (173).

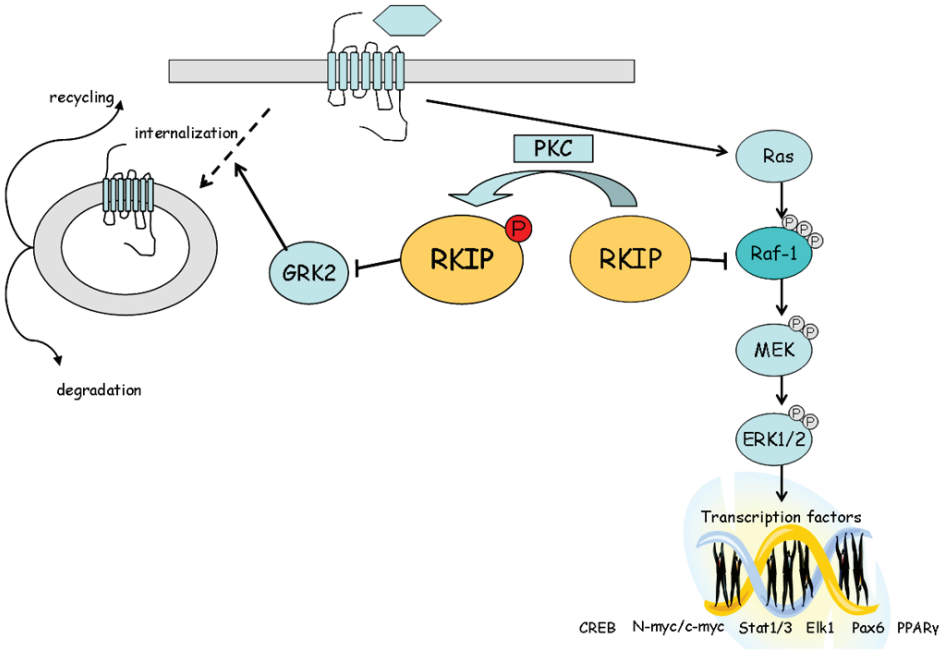

Figure 2. RKIP signalling

RKIP and cancer

In several cancer types, RKIP has been suggested to be a metastasis suppressor gene, where loss of RKIP is associated with metastasis development. Metastatic tissue has significantly lower RKIP levels compared to the primary tumour, poorly differentiated tumours have also low levels of RKIP, and benign tissue has the highest levels (174-179). Loss of RKIP predicted metastatic recurrence and was the strongest predictor for reduced survival in a study of colorectal cancer (180). Reduced RKIP has been correlated to poor survival also in gastrointestinal stromal tumours (GIST) (181). In prostate cancer, injection of cancer cells with RKIP overexpression into mice prostatic gland reduced the number of mice that developed metastasis by 70 % (174), whereas RKIP overexpression in malignant melanoma cells markedly reduced their invasion potential *in vitro* (176). Loss of RKIP has also been linked to resistance to chemotherapeutic drugs in prostate and breast cancer cell lines (182).

E-cadherin and epithelial-to-mesenchymal transition (EMT)

E-cadherin

Cadherins are a large family of more than 100 different glycoproteins that mediate cell-cell adhesion dependent on Ca^{2+}. All have the characteristic extracellular cadherin repeats. The classical cadherins type I include epithelial (E) and neuronal (N) cadherin that mediate strong cell-cell adhesions, in addition to interactions with the actin cytoskeleton (183;184). Anterior pituitary cells have an epithelial phenotype, where appropriate cell-to-cell adhesion and polarity are fundamental. Expression of the protein E-cadherin is typical for the epithelial cells, providing a physical link to both the adjacent cells and to the intracellular cytoskeleton. The extracellular domain of E-cadherin of one cell binds to an E-cadherin molecule of an adjacent cell. The intracellular domain of the protein is linked to the actin cytoskeleton via a protein complex with α-, β- and p120 catenin and EPLIN (epithelial protein lost in neoplasm) (183;185-187).

Regulation of E-cadherin

The level of E-cadherin in a cell is regulated at several levels. E-cadherin gene expression can be directly regulated via promoter hypermethylation, resulting in reduced promoter activity. Indirectly, the gene expression can be inhibited by increase in E-cadherin-specific transcriptional repressors like Snail, Slug, ZEB1 and ZEB2 (184;185). These transcriptional inhibitors, particularly Snail, are again regulated by different signalling pathways, such as receptor tyrosine kinases like EGFR or TGF-β, or by Ras-MAPK, NFκB, VEGF or prostaglandin E_2 and others (183;188). Newly synthesized E-cadherin requires binding to β-catenin for transport to the cell membrane. When localized to the cell membrane, E-cadherin is subject for posttranscriptional regulation like phosphorylation, ubiquitination and degradation (183). P120 catenin stabilizes E-cadherin to the cell membrane, and loss of E-cadherin–p120 binding results in rapid endocytosis of the E-cadherin complex. Once internalized, E-cadherin needs to be recycled back to the cell membrane to escape degradation (183;184). The cytoplasmic tyrosine kinase Src leads to tyrosine phosphorylation of E-cadherin and β-catenin, thereby dissociating the complex, and E-cadherin is then available for ubiquitination mediated by the Hakai protein resulting in degradation (189). Glycogen synthase kinase-3 β (GSK3β), on the other hand, phosphorylates the serine residues of E-cadherin, increasing the binding affinity to β-catenin.

The binding of E-cadherin to β-catenin stabilizes β-catenin in the cytoplasm. In addition, GSK3β targets the remaining cytoplasmic β-catenin for degradation by phosphorylating the serine residues. If free β-catenin accumulates in the cytosol, it is translocated to the nucleus where β-catenin acts as a transcriptional coactivator and modulates expression of a large number of genes involved in cell proliferation, migration and invasion. In epithelial cells, GSK3β is normally activated and Src inactivated, which maintains E-cadherin and β-catenin in complex at the cell membrane (183-185;190).

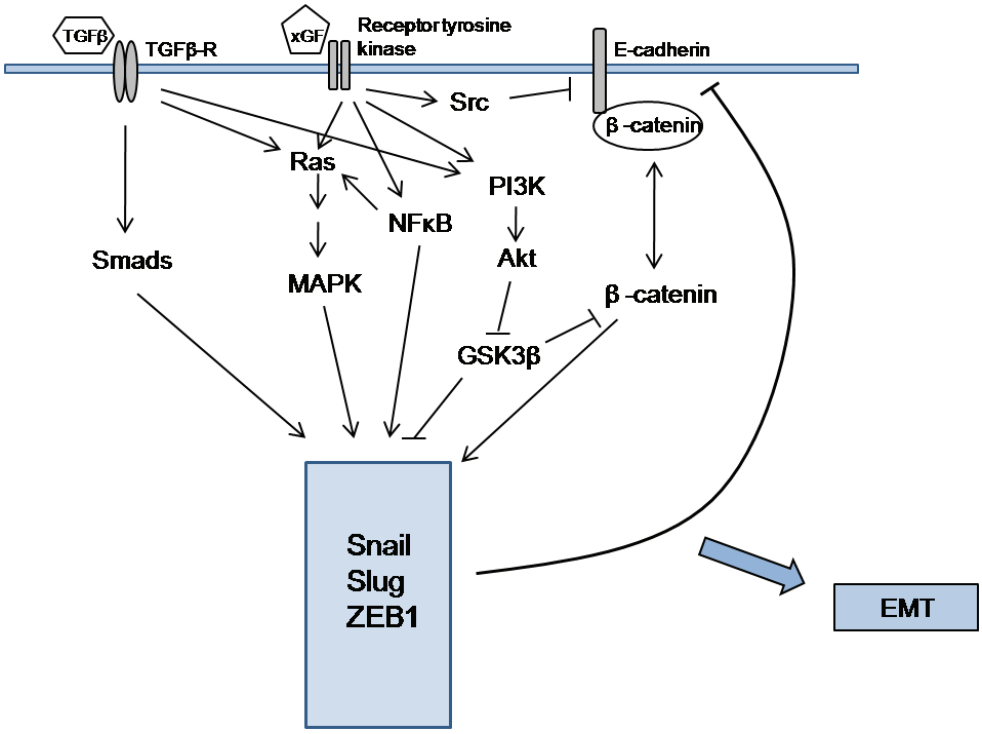

Figure 3. A simplified overview over some of the regulating pathways of E-cadherin, Snail and EMT. Modified after Guarino M et al (2007) and Gavert N et al (2008) (185;191).

Cleavage of E-cadherin and nuclear translocation

The extracellular domain of E-cadherin is proteolytically cleaved by matrix metalloproteases (MMPs), disrupting the cell-cell adhesion (185). Subsequently, the remaining E-cadherin can be cleaved by presenilin-1/γ-secretase at the transmembrane-cytoplasm interface, generating a soluble E-cadherin fragment in the cytosol (192). The p120 catenin promotes this cleavage, and p120 also mediates nuclear translocation of this

fragment (193;194). Using an antibody directed against the intracellular domain of E-cadherin, this E-cadherin fragment has been found in the nucleus in malignant cells of different origins, including the endocrine pancreas (195).

Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is a biological process describing the morphological and molecular process where epithelial cells lose their characteristics with intercellular adhesion and gain mesenchymal properties with increased cell motility. The EMT process is required during the embryonic development, both during gastrulation and formation of various tissues including the neural crest, and is involved in wound healing. It is believed that a reactivation of parts of this embryonic EMT program could underlie the mechanism of tumour invasion. For tumours of epithelial origin, loss of adhesion and increased cell motility providing the tumour the ability to invade locally is necessary for the subsequent development of distant metastases. Other characteristics of EMT, like activation of specific signalling pathways, are also often seen in carcinogenesis (185;191). Functional loss of E-cadherin is a key molecular change in and a hallmark of EMT (185;188), leading to destabilization of the epithelial architecture.

E-cadherin and EMT in tumours

Studies of several cancer types have demonstrated that E-cadherin often is lost in malignant carcinomas of epithelial origin. Loss of E-cadherin is most pronounced in poorly differentiated and invasive tumours, and correlates to tumour grade, metastasis and poor prognosis. An increase in the expression of transcriptional repressors of E-cadherin, like Snail, has also been demonstrated (196-199).

In the pituitary, immunohistochemistry has confirmed a high E-cadherin expression in normal pituitary cells (46;200). In pituitary tumours, E-cadherin expression has been studied only with immunohistochemistry, where reduced E-cadherin expression has been found in a proportion of adenomas (200-204). In GH secreting adenomas, reduced E-cadherin expression has been demonstrated particularly in sparsely granulated adenomas with prominent fibrous bodies (40;45;46;203). In a large percentage of these adenomas, hypermethylation of the E-cadherin promoter was present (46;203). Because of the correlation to fibrous bodies in the adenomas, it has been suggested that fibrous bodies are developed in relationship with the dysfunction of adhesion molecules in these adenomas (40). Reduced E-cadherin expression correlated to tumour size and invasiveness in one (203), but not in four other studies (45;46;201;204). Nuclear expression of E-cadherin in

pituitary adenomas has been analysed in one study, and was present in a large proportion of adenomas, particularly in non-functioning adenomas, and in 4 of 10 somatotroph adenomas. The nuclear staining correlated to loss of membranous E-cadherin and to tumour invasiveness (200).

AIMS OF THE STUDIES

The major aim of our studies was to relate clinical data from patients with acromegaly to protein and DNA analyses of the adenoma tissue from these patients. In particular, we wanted to explore potential determinants for the variable clinical response to treatment with somatostatin analogues.

The detailed aims for each study were as follows;

Paper 1:

- To examine the protein expression of somatostatin receptor 2a in a relatively large cohort of somatotroph adenomas
- To analyse the adenomas with respect to gsp oncogene status
- To examine if the adenoma expression of SSTR2a and the gsp oncogene status was correlated to clinical octreotide efficacy, both the acute and the long-term response
- To correlate both SSTR2a expression and gsp status to clinical variables like preoperative medical treatment, preoperative hormone levels and tumour size and invasiveness

Paper 2:

- To study the protein level of Raf kinase inhibitory protein (RKIP) in the somatotroph pituitary adenomas
- To examine if the adenoma expression of RKIP was correlated to clinical octreotide efficacy, both the acute and the long-term response
- To correlate the adenoma RKIP expression to preoperative hormone levels, tumour size and invasiveness
- To correlate the RKIP protein level to the adenoma expression of SSTR2a and to the presence of gsp oncogene

Paper 3:

- To evaluate the protein expression of E-cadherin in a large cohort of somatotroph pituitary adenomas, both the protein level (Western blot) and the immunohistochemical expression and distribution
- To examine if E-cadherin nuclear expression is present in somatotroph adenomas
- To correlate the E-cadherin expression to tumour size and invasiveness
- To evaluate the protein expression of E-cadherin in relation to acute and long-term SMS responses
- To correlate E-cadherin expression to the expression of SSTR2a and RKIP in the adenomas and to gsp oncogene status
- To evaluate the expression of E-cadherin in relation to preoperative medical treatment, and the *in vitro* response in primary cell cultures to octreotide treatment

SUMMARY OF PAPERS

Paper 1

The somatostatin analogue octreotide exerts its biological responses through binding to the transmembrane somatostatin receptors (SSTR), and reduced expression of SSTR subtype 2 has been suggested to explain poor octreotide responses in acromegaly. Adenoma SSTR2 expression had previous been studied only at the mRNA level, and the results comparing this to octreotide efficacy had been contradictory. Some studies, but not all, found a better somatostatin analogue response in gsp positive adenomas. The objective of this study was to determine adenoma SSTR2a protein expression and gsp status in a large group of acromegaly patients, and relate this to the clinical effect of octreotide. Seventy-one patients were included, and 23 patients had received octreotide treatment prior to transsphenoidal surgery. The adenoma SSTR2a expression was examined by immunohistochemistry of paraffin sections and Western blot of proteins extracted from frozen adenoma tissue. Gsp status was determined by PCR of extracted DNA. An acute octreotide test had been performed prior to medical treatment, and the change in IGF-1 level after 6 months preoperative octreotide treatment was available in 20 of the preoperatively treated patients.

The acute octreotide response in non-pretreated patients and the long-term response in preoperatively treated patients were significantly better in the adenomas with a large proportion of cells with immunohistochemical reactivity for SSTR2a. The SSTR2a protein level (Western blot), however, did not correlate with the octreotide response. The preoperatively treated group had lower SSTR2a protein level, and there was a tendency towards fewer adenomas with a large percentage of positively stained cells in this group. Gsp oncogene was detected in 43% of the adenomas, and we found no differences in octreotide responses in gsp positive adenomas compared to adenomas without the gsp mutation. However, the gsp positive adenomas had higher SSTR2a protein expression (both modalities). Neither SSTR2a expression nor gsp status of the adenomas correlated to preoperative hormone levels or tumour size.

To conclude, the clinical effect of octreotide correlates with the proportion of cells positive for SSTR2a by immunohistochemical staining, rather than the total protein level in the adenoma cells or the gsp status. There may be a downregulation of SSTR2a during treatment.

Paper 2

The clinical hormone response to octreotide treatment is highly variable, and has been shown to correlate with adenoma protein expression of SSTR2a, a G-protein coupled transmembrane receptor. However, in our previous study (paper 1), there were adenomas with poor long-term octreotide response despite a high proportion of SSTR2a positive cells. Inhibition of the MAP kinase signalling pathway is one of the mechanisms responsible for the antiproliferative effects of octreotide. Non-phosphorylated Raf kinase inhibitory protein (RKIP) binds to and inhibits Raf1 kinase and thereby reduces MAP kinase signalling, while phosphorylated RKIP inhibits G protein receptor internalization and degradation due to inhibition of G protein receptor kinase 2. The purpose of this study was to examine RKIP protein levels in pituitary somatotroph adenomas, and relate these to clinical characteristics and response to octreotide treatment in patients with acromegaly. In 51 patients with active acromegaly, the somatotroph adenoma was frozen short time after surgery. The RKIP level was analysed by Western blot of proteins extracted from these tumours. An acute somatostatin test was performed in 46 of the patients, always prior to SMS treatment. The biochemical long-term efficacy of SMS treatment was available in 21 patients with measurements of the IGF-1 level before and 6 months after medical treatment, and tumour shrinkage during treatment could be evaluated in 16 patients.

There were no associations between the tumour RKIP level and the patient age, baseline hormone levels or tumour size. The RKIP level in the adenomas correlated significantly to both the acute and the long-term octreotide responses on serum levels of GH and IGF-1, respectively. In multiple regression analyses, both the RKIP level and the SSTR2a expression were significant determinants for both the GH reduction in the acute test and the IGF-1 reduction after approximately 6 months. No correlation between adenoma RKIP level and tumour size reduction during SMS treatment was found, but only 16 patients had data available for this analysis. There was no correlation between RKIP level and previously analysed SSTR2a protein expression or gsp status.

In conclusion, in addition to the SSTR2a expression, the adenoma RKIP protein level seems to be important for the clinical effect of SMS treatment, where low levels of RKIP correlate to poor clinical response to SMS.

Paper 3

Loss of the transmembrane adhesion protein E-cadherin and nuclear translocation of its intracellular domain have been associated with poorly differentiated and invasive tumours, metastasis and poor prognosis in carcinomas. This has also been demonstrated in a proportion of somatotroph pituitary adenomas, correlating to tumour invasiveness in some studies. Our objective was to investigate the protein expression of E-cadherin in somatotroph adenomas, and relate this to tumour characteristics and to the clinical SMS response, and in addition to the previously analysed SSTR2a and RKIP expression. We examined the adenoma E-cadherin protein expression by Western blot analysis (61 patients) and by immunohistochemistry (80 patients), the latter with antibodies against both extracellular and intracellular domains of E-cadherin. Tumour size and invasiveness was analysed on MRI scans. An acute octreotide test was performed in 50 patients not preoperatively treated with SMS, and reduction of IGF-1 and tumour shrinkage was analysed in 26 and 23 patients treated with SMS prior to surgery. Primary cell cultures from the adenomas of five patients were cultured with and without octreotide present.

This study demonstrated that membranous E-cadherin is frequently reduced in somatotroph adenomas. Low E-cadherin levels (Western blot) correlated to large adenomas, but to tumour invasiveness only in the preoperatively treated group. There was also a positive correlation between E-cadherin level and tumour shrinkage following SMS pretreatment, and a trend towards positive correlation to IGF-1 reduction. Reduced membranous E-cadherin correlated to large and invasive adenomas and to acute octreotide response. Nuclear E-cadherin staining was found in 9 patients (11 %), and correlated to large tumours and to poor biochemical and tumour response to preoperative SMS treatment. Patients treated with SMS prior to surgery had significantly lower E-cadherin level compared to not pretreated patients, but no different immunohistochemical E-cadherin expression. The *in vitro* results suggested a downregulation at the mRNA level in non-treated patients (significant in one of two adenomas), but no additional effect of octreotide on E-cadherin in cells from SMS treated patients. There was also a positive correlation between E-cadherin expression and the SSTR2a and RKIP expression.

To conclude, this study demonstrates that E-cadherin is downregulated and redistributed in a substantial proportion of somatotroph adenomas, with translocation to the nucleus in some adenomas. This was associated with large and invasive tumours and with poor clinical SMS efficacy. The reduction of E-cadherin expression may be a marker of epithelial dedifferentiation in these adenomas.

DISCUSSION

Methodological considerations

Patient cohort

All patients included in our studies had active acromegaly verified by typical symptoms, biochemical analyses and a visible pituitary tumour on MRI scans. None had received radiation therapy prior to surgery.

Our studies are cross-sectional, although some of the patients were included in prospective studies prior to surgery. From 1996, the intention was to collect adenoma tissue during surgery from all patients with acromegaly evaluated at the Section of Endocrinology, Rikshospitalet. Adenoma tissue was stored at -70 °C, in addition to the tissue in paraffin sections. Unfortunately, tumour tissue was not collected for all patients. Until 2005 there was none from Section of Endocrinology to ensure that all somatotroph pituitary adenomas were included, and the collection of tissue relied on the neurosurgeon to remember this during the surgery. We know that some adenomas operated in the period after the former main neurosurgeon retired and until the next was proper established, was not collected. Probably also some microadenomas have not been included due to little tumour mass. Tumour tissue for the pathological examination and diagnosis had priority. There may therefore be a selection bias leading to fewer microadenomas in the study cohort. Nevertheless, the study cohort consists of the majority of patients with acromegaly diagnostically evaluated and surgically treated at Rikshospitalet in this period.

Another likely selection bias is the decision to treat patients preoperatively with SMS compared to direct surgery. Patients might have been selected for preoperative medical treatment because they had a large and invasive adenoma. However, more than half of these patients included in our studies received pretreatment as participants in clinical randomized studies. This was the case for 15 of 23 patients in study 1, 10 of 15 in study 2 and 15 of 29 in study 3. There is probably a selection bias for the remaining pretreated patients, being most important for the findings in paper 3, as discussed later.

A main strength of our studies is the large cohort of well characterized patients, where none had received radiation therapy that could have influenced the tumour protein expression. We also found it important to record all preoperative treatment, to ensure that we could analyse the preoperatively treated group separately if the protein expression was

significantly different in this group. This was the case for both SSTR2a and E-cadherin. In study 1 and 3, we therefore evaluated only preoperative long-term SMS treatment for assessment of long-term SMS efficacy.

The acute octreotide test

In patients not treated with somatostatin analogues (in paper 1 and 3 preoperatively treated), we used the response from an acute octreotide stimulation test as a measure of SMS response. The clinical usefulness of the acute tests has been debated as different studies have diverse conclusions (205-207). One study concluded with a positive predictive value (PPV) of 94 % and a negative predictive value (NPV) of 100 % (206), while another found PPV of 82 % and NPV of 50 % (207), both using the same definitions of GH levels. However, the interpretation of both studies is complicated by the fact that several patients received radiation therapy either prior to or during the study. Nevertheless, since this is the only measure of SMS efficacy in these patients, we chose to use the acute suppression test.

We chose to mainly use the percentage reduction of GH measured between two and four hours after the test dose. Following the initiation of the POTA study (Preoperative Octreotide Treatment in Acromegaly) in 1999 (4), the procedure was improved with the measurement of three pre-octreotide GH values (-60, -30 and 0 minutes) instead of only baseline (0 minutes), and then measurements both two, tree and four hours after injection compared to only two and four hours after. We have observed that the first pre-test GH value is often higher than the next two, probably due to stress reaction during needle installation. In addition, the test dose was decreased from 100 µg octreotide subcutaneous to 50 µg. However, in our opinion this change in test dosage has limited effect, as also shown in a previous publication (207). Probably, maximal GH suppression is achieved also with a 50 µg test dose.

GH and IGF-1 measurements

The biochemical diagnosis and evaluation of patients with acromegaly is based on the levels of GH and IGF-1 in serum. However, measuring levels of these two hormones is not without problems. Both are measured by immunoassays, with high variability between different assays and between different laboratories (208-210). In our studies, GH and IGF-1

were measured at the Hormone Laboratory, Aker University Hospital, in eight patients. For the rest of the patients, the assays were performed at our hospital (Rikshospitalet).

For GH, we did not perform renewed analyses, but used the value measured at the clinical evaluation. During the years, different immunoassays were used. However, at every change of method at Rikshospitalet a cross-calibration was performed and a factor added if necessary. In our studies, we used this GH level mainly for correlation to baseline characteristics which were minor parts of the studies. In the acute octreotide test, all GH analyses were performed at the same laboratory with the same assay, and we used mainly the percentage reduction in GH, abrogating the problem.

For IGF-1, renewed measurements were performed in stored serum samples in one run to minimize inter assay variability. This was available in approximately half of the patients. The new IGF-1 level proved to correlate well to the previous values ($r>0.86$). Like for GH, these values were used only in minor parts of our study. We focused on the reduction in IGF-1 after SMS treatment, and for this evaluation the original IGF-1 value was used. As for GH, we used the reduction of IGF-1 instead of absolute value. IGF-1 values both prior to and after treatment were analysed at the same laboratory with identical assays except for one patient. For this patient, one of the IGF-1 values was adjusted after cross-calibration of the methods.

As mentioned, for evaluation of biochemical response to SMS treatment we used mainly the reduction in IGF-1 level. This is a good clinical parameter of biochemical response. However, it has been demonstrated that octreotide may have additional non-pituitary effects on the GH-IGF-1 axis (211;212). This could have an influence of our results since our objective was to evaluate the adenoma antihormone responses to treatment. Yet, this would probably have led to false negative results in our studies of adenoma protein expression.

MRI analyses

Tumour volume and invasiveness were measured retrospectively on MRI scans. I performed the measurements under guidance from an experienced neuroradiologist (JKH), always blinded for patient identity or clinical data. The first analyses were performed together, while later the neuroradiologist was consulted if any question of the adenoma border could be raised. However, for most of the patients, the neuroradiologist observed the measurements, making sure he agreed. We think it was important that the same person made

all measurements to be certain that it was done identically for all patients and for both MRI scans for the patients long-time treated with SMS. T1 weighted images were used for the analyses. The sagittal view was used for analysis of maximal vertical height and anterior-posterior length, and the coronal view for measurement of maximal transverse diameter (width). In the sagittal plane, the diameters were laid perpendicular to each other. We used the formula height x length x width x 0.5 (213) for assessment of the tumour volume, and the SIPAP grading system for estimation of tumour invasiveness (214).

If a scan was not available for re-analysis, tumour characteristics were not registered, because we observed that primary measurements were often not done systematic. In this way, we ensured that all tumour analyses were done as uniform as possible.

Protein expression analyses (Western blot and immunohistochemistry)

Previous studies of pituitary tumours have often analysed the adenoma mRNA levels. When we started our study, this was the case for all published studies of SSTR2a expression related to the clinical SMS efficacy (36;37;118;149-151). However, it is the proteins that exert the cellular responses, and the protein expression is often subject to posttranslational regulation. Indeed, studies have also demonstrated lack of correlation between SSTR mRNA and protein expression (133;146). We therefore aimed to evaluate the protein expression of somatotroph adenomas in our studies.

Western blot

One method to evaluate protein expression is the Western blot analysis. We performed Western blot on proteins extracted from frozen tissue collected during surgery. The level of the desired protein was quantified relative to the level of a reference protein, which is comparable to the method for mRNA analysis. Both methods rely on the hypothesis that the chosen reference protein or mRNA is not regulated in the adenoma cells. This is a possible weakness of these methods, since we can not be certain on that. However, we chose reference proteins that are generally used in protein analyses of other tissues and in mRNA analyses in studies of pituitary adenomas. Due to technical reasons, we used either β-actin or GAPDH as reference protein depending of the size of the studied protein. In paper 2, we used both β-actin and α-tubulin as reference proteins. The antibody staining was visualized by chemiluminescence in a camera using visible light, providing a digital image of the membrane. The advantage of this camera is the possibility of setting exact exposure time, to ensure identical exposure of all membranes. A digital software was used

for measuring signal intensity and size for each band, and allowed for subtraction of background signal. We ensured that the exposure time was short enough to avoid saturation of signals, which would have led to a lower dynamic range with false low levels for bands with strong staining intensity.

A possible bias is the variation between each run of the Western blots, since only 13 adenomas could be analysed in one membrane. Due to this, we aimed to treat the membranes identically to minimize variation, and we included a reference lane between blots to evaluate the variation. In paper 2, where Western blot was the only method of protein expression analysis, most adenomas were analysed in more than one blot. The mean protein level was used in the analyses.

A disadvantage of Western Blot's is that it does not display the localization of the protein, only the protein level in the extract of frozen tissue. In addition to adenoma tissue, there might be contamination of blood and other tissues expressing SSTRs, like endothelium and fibrous tissue, in the collected samples. This could have interfered with our results. However, the genotyping of Gsα gave distinct and equal peaks for both the mutant and the wild-type nucleotide in electropherograms from gsp positive adenomas, suggesting that the tissue samples consisted mainly of adenoma cells.

One advantage of Western blot analysis is the possibility to ensure specificity of the antibody. The extracted proteins are separated by size, and the size of all proteins binding to the antibody is visualized. The antibodies used in paper 2 and 3 gave only one stained band at the expected protein size. The antibody used in paper 1, however, caused staining of several bands in the Western Blot membrane, but testing demonstrated that the additional bands were unspecific and due to binding of the secondary antibody.

Immunohistochemistry

Immunohistochemistry, on the other hand, displays the localization of the stained protein. It is therefore possible to evaluate the expression only in the pituitary adenoma cells and not in neighbouring tissue. In addition, the method allows an evaluation of cellular localization of the protein. We included immunohistochemical analyses in paper 1 and 3. The evaluation of the cellular localization was particularly significant in paper 3, since both preserved membranous and gained nuclear localization of E-cadherin was important to register. On the other hand, it is not possible to evaluate the specificity of the antibody using immunohistochemistry. Except for the E-cadherin antibody raised against the intracellular

domain, we therefore performed these analyses using the same antibody in both immunohistochemical and Western blot analyses.

A disadvantage of the immunohistochemistry method is that the adenomas have to be semiquantitatively classified according to immunoreactivity, and qualitatively according to localization before comparisons with clinical data can be performed. For the proteins we analysed, there were no defined classification systems available. Thus, we had to establish our own classification system. The analyses are therefore subjective and based on the observer's judgment. Comparisons between different publications are also difficult, as discussed further in the next section. In our studies, the adenoma scoring was performed by only a single neuropathologist. In paper 1, however, adenomas difficult to score were also examined by a second, independent pathologist. These adenomas were scored identically by the independent researchers, both blinded for all clinical data. The classification criteria decision was based on both the a priori evaluation on what would be important for the clinical behaviour of the adenomas and on the classification criteria used in previous studies, but also on the actual staining patterns of the adenomas. The latter was of particular relevance for paper 3, where the pathologist made a detailed description of the staining pattern for each adenoma prior to the final definition of the classification criteria. For instance regarding the E-cadherin antibody directed against the intracellular domain, we observed that the majority of adenomas displayed strong membranous staining in all cells. Among adenomas with reduced membranous staining, some showed nuclear positivity. Importantly, no statistical analyses were performed prior to the final definition of classification criteria.

Primary cultures

We established a method for making primary cell culture of pituitary adenomas. The reason for this was that we wanted to be able to perform more mechanistical studies. The original objective was to alter the expression of RKIP in the cells and evaluate if this led to altered octreotide sensitivity *in vitro*. However, the attempts to increase RKIP expression using expression vector or reduce the expression with siRNA were not successful. To our knowledge, there are no publications using these methods on primary pituitary cell cultures. This can be due to the fact that the methods to modify protein expression *in vitro* are most efficient on dividing cells; cells from human pituitary adenomas rarely proliferate *in vitro*.

We did not perform specific procedures to eliminate fibroblasts from the primary cultures. However, the morphology of the cultured cells was relatively homogenous, and the

cultures showed two different patterns as previously described (215;216). Most adenomas consisted of individual or small groups of rounded cells free floating or only weakly attached to the well, similar to the described morphology for densely granulated adenomas. One adenoma consisted mainly of spindle shaped cells attached to the well, which has been described for sparsely granulated adenomas (215). The cultured cells also produced relatively high levels of GH which made dilution of the medium necessary for the GH measurements by ELISA. Still, we can not exclude the possibility that the primary cultures to some extent were contaminated by fibroblasts.

SSTR2a expression and gsp oncogene (Paper 1)

In this study, we examined the protein expression of somatostatin receptor 2a in a relatively large cohort of somatotroph pituitary adenomas. Immunohistochemistry was performed in 63 patients and Western blot in 53 patients. To our knowledge, this was one of the first studies of SSTR2a protein levels in somatotroph pituitary adenomas, and the first to correlate SSTR2a protein expression to clinical long-term response to octreotide treatment.

We demonstrated that adenomas with a large proportion of SSTR2a positive cells responded significantly better to octreotide, both in an acute test and during long-term treatment. In not preoperatively treated patients, the SSTR2a immunohistochemical expression correlated to the octreotide response in the acute test. In patients treated with octreotide prior to surgery, there was a correlation to the percentage IGF-1 reduction after approximately 6 months treatment. Adenomas with >75 % SSTR2a positive cells responded significantly better to octreotide compared to adenomas with less than 75 % of the cells positively stained. This is in accordance with a Japanese study investigating the acute octreotide test and SSTR2a immunohistochemistry in 22 patients (152). A more recent study found correlation between the SSTR2a immunohistochemical expression and the acute octreotide response and the *in vitro* octreotide response in 20 and 24 patients, respectively. They also reported correlation between the SSTR2a positivity and the level of IGF-1 after 6 months postoperative SMS treatment stratified into three groups (77). However, I could not reproduce significance using the given data. These two studies also grouped the adenomas after the proportion of positive cells, but used positivity in 50 % of cells as the lower level for highly positive adenomas and in 10-20 % of cells as the upper level for negative adenomas, while we chose to use 75 and 25 %, respectively. The levels for grouping the adenomas are arbitrary, but could influence the results if very few adenomas thereby are

assigned to one of the groups. The immunohistochemical grading in our study was determined independent of group sizes and prior to all analyses, but showed to provide relatively equal groups although most of the somatotroph adenomas displayed high proportions of SSTR2a positive cells.

Our study demonstrated correlation between the clinical octreotide response and the proportion of SSTR2a positive cells in the adenoma, but no correlation to the SSTR2a protein level assessed by Western blot. We therefore suggested that the response to octreotide treatment may be correlated to the proportion of adenoma cells expressing SSTR2a, and to a lesser extent to the total amount of SSTR2a in the adenoma cells. A previous study of an octreotide sensitive and a partially sensitive adenoma also found correlation between GH reduction in an acute test and the percentage of cells labelled for SSTR2 mRNA by *in situ* hybridization (118). There might be a threshold limit of receptor in each cell, where the maximal inhibitory effect of somatostatin is achieved. This could explain the lack of correlation to mean level of SSTR2a in the adenomas. Another explanation could be that the total protein level may not represent the receptor level in the cell membrane available for ligand binding. The Western blot analysis do not discriminate between membrane bound SSTR2a and SSTR2a internalized into cytoplasm. This receptor is often internalized after ligand binding, but in contrast to for instance SSTR3 which undergoes degradation once internalized, SSTR2a is recycled back to the cell membrane (142;143). In the immunohistochemical analysis, intracytoplasmic immunoreactivity was observed in a relatively large number of adenomas with varying intensity although most were weak. Unfortunately, we did not analyse this prior to publication, but our data suggests that the pretreated patients significantly more often have adenoma cells with cytoplasmic staining for SSTR2 compared to the not preoperatively treated patients. This is in accordance with the fact that SSTR2a often is internalized after chronic ligand exposure. In our study, cytoplasmic staining was noted, but our chosen grading system did only partly discriminate between cytoplasmic and membranous staining. In the case of only weak cytoplasmic staining in more than 75 % of the cells, the adenoma was a grade 2 adenoma despite the high proportion of stained cells. Since SSTR2a is shown to rapidly recycle back to the cell membrane instead of degradation, we thought it was appropriate not to neglect the cytoplasmic positivity. However, only in very few cases the cytoplasmic staining was strong enough to mask the membranous staining pattern.

We observed that adenomas from preoperatively treated patients had significantly lower SSTR2a protein level assessed by Western blot compared to the direct surgery group.

In addition, there was tendency towards a lower proportion of grade 3 adenomas (>75 % of positive cells) in the pretreated group. When dividing the study population in two groups; adenomas with less than and more than 75 % positive cells, the difference was significant (p=0.043). This suggests some degree of downregulation of SSTR2a receptors during octreotide treatment. A change in the adenoma SSTR2a expression during treatment might also explain the observed discrepancy between the results of the acute and long-term effect of octreotide in the pretreated patients, because the acute test was performed prior to treatment. Clinical tachyphylaxis would probably not be observed if downregulation of the receptor occurs and a new expression pattern is established within weeks of treatment. Evaluation of the patient's final clinical effect of treatment is usually done after a few months of treatment. The fact that tachyphylaxis is not a clinical problem in the treatment of acromegaly is therefore not necessarily in contrast with our observation. Another explanation can be that octreotide treatment leads to a decrease in SSTR2a positive cell mass, and hence a relative expansion of tumour cell clones lacking SSTR2a (142). In a proportion of somatotroph adenomas, the adenoma cells show heterogeneity in SSTR2a expression, both in previous (118;121;217) and in our study. Not in accordance with this theory, in one of the few reported patients with tachyphylaxis in acromegaly, re-introduction of a somatostatin analogue after short-time withdrawal revealed resensitization to treatment (145).

We demonstrated in this study that the response to octreotide was significantly associated with the immunohistochemical proportion of adenoma cells stained positively for SSTR2a. Nevertheless, there were both patients with few positive cells and a good response especially in the acute octreotide test, and patients with almost no clinical effect of octreotide despite a high proportion of positive adenoma cells. This suggests that other receptors and mechanisms are involved in determining the clinical efficacy of somatostatin analogues in the somatotroph adenomas. As reviewed, octreotide also binds to SSTR5, and a single study found correlation between the effect of octreotide and the combined SSTR2 and 5 mRNA level in the adenoma (37). Unfortunately, our testing of the available SSTR5 antibody did not give reliable staining of the membranes, and data on SSTR5 protein expression could therefore not be presented in this study. The same SSTR5 antibody was used in another immunohistochemical study, but no correlation between SSTR5 expression and response to an acute octreotide challenge was found (152).

We detected a gsp mutation in 43 % of the 63 patients, a frequency in accordance with most other published studies. In contrast to several previous publications, the gsp

positive adenomas in our cohort were not smaller and the proportion of microadenomas was comparable to the gsp negative adenomas. However, most of the previous studies had included relatively few patients, and not all studies used MRI scans for tumour size measurements. Similarly, the by far largest study of acute octreotide response in 100 patients did not confirm a better octreotide efficacy in gsp positive adenomas found in smaller study populations, and none has demonstrated difference in IGF-1 response after long-term treatment (29;35;38). In our study of a relatively large group of patients, no difference in octreotide response was found, neither for the acute nor the long-term efficacy. However, there was a significantly higher proportion of adenomas with low immunohistochemical expression of SSTR2a among the gsp positive adenomas. Despite this, these tumours did not show a reduced octreotide efficacy. In two of the studies demonstrating a better response for gsp positive adenomas in an acute octreotide test, equal mRNA levels of SSTR2a were found in these adenomas (36;37). This suggests that other mechanisms in addition to SSTR2a are responsible for the effect of octreotide in gsp positive adenomas. A potential positive effect of gsp oncogene could have been masked in our study by a lower proportion of adenoma cells expressing the SSTR2a protein in the gsp positive adenomas.

Raf kinase inhibitory protein (RKIP) (Paper 2)

This study demonstrated a significant correlation between the protein level of RKIP in somatotroph adenomas and the response to octreotide in patients with active acromegaly. Both the acute growth hormone response and the long-term effect on IGF-1 were reduced in patients harbouring tumours with low RKIP levels.

As discussed, the main factor influencing the efficacy of somatostatin analogue treatment in acromegaly is the tumour expression of somatostatin receptors, in particular receptor subtype 2. In paper 1, we demonstrated a correlation between octreotide response and adenoma SSTR2a expression immunohistochemically, where adenomas with a high proportion of SSTR2a positive cells responded significantly better to octreotide treatment. Nevertheless, the observed exceptions suggested that additional mechanisms downstream of the SSTRs can be involved in determining the clinical effect of somatostatin analogues in somatotroph adenomas. In the present study, we found a strong correlation between RKIP protein level in the adenoma assessed by Western blot and both acute and long-term clinical response to octreotide. However, the RKIP level did not correlate to the SSTR2a protein

expression assessed by Western blot analysis or by immunohistochemistry. In the multiple regression models, both SSTR2a immunohistochemical grading and the RKIP level were significant determinants of the octreotide response. When we looked at the observed exceptions, we found that the patients with poor octreotide response despite a high proportion of SSTR2a positive cells seemed to have low RKIP levels. Similarly, the patients with few SSTR2a positive cells, but relatively good octreotide response, had high RKIP levels. This suggested a model where SSTR2a expression is a permissive factor for octreotide response in acromegaly, with RKIP as an independent modulator of the efficacy of somatostatin analogues.

Despite the fact that RKIP, in addition to SSTR2a, was a significant determinant of SMS efficacy in our multiple regression models, we do not know if RKIP itself is crucial or whether RKIP is related to an associating unknown factor responsible for the effects. Yet, there are potential mechanisms for RKIP to be of importance for the somatostatin analogue effect. First, RKIP inhibits the MEK-ERK signalling pathway, which is also inhibited by activation of several SSTR subtypes including 2 and 5 (113;165). Synergistic inhibitory effect on the MEK-ERK pathway is therefore a possible mechanism for the association between adenoma RKIP levels and the clinical efficacy of octreotide. Second, phosphorylation of RKIP by protein kinase C activates RKIP binding to and inhibition of G-protein coupled receptor kinase 2 (GRK2) (171). Hence, internalization and degradation of the somatostatin receptor is reduced. However, there was no correlation between total RKIP level and the level of SSTR2a in our study. In our subsequent studies following this publication, we sought to examine the relevance of RKIP phosphorylation. We tested an antibody against phospho-RKIP thoroughly which seemed to bind specifically to the phosphorylated form of the protein. However, Western blot analyses of the level of phospho-RKIP in the adenomas revealed correlation to total RKIP, but there were no correlations to SSTR2a expression or to the clinical SMS response (unpublished).

RKIP has previously been found to be important for the responses to medical treatment. Both breast and prostate cancer cell lines resistant to chemotherapeutic drugs expressed low levels of RKIP. However, increasing RKIP levels by transfection of an expression vector resensitized the cells to drug-induced apoptosis. And in previously drug sensitive cell lines, downregulation of RKIP led to resistance to the chemotherapeutic drug (182). Rituximab, a monoclonal antibody against the B-cell-specific CD20, is used in combination with chemotherapy for instance in the treatment of non-Hodgkin's lymphoma. *In vitro*, both rituximab and a newer, humanized CD20 antibody has been shown to sensitize

non-Hodgkin's lymphoma cell lines to chemotherapeutic drug-induced apoptosis, via upregulation of RKIP. There was a concomitant decrease in Snail protein expression (218;219). Both modulation of the MEK-ERK pathway and inhibition of the NF-κB pathway were suggested as possible mechanisms for these drug-sensitizing effects.

We found no correlation between RKIP level and tumour shrinkage. However, available MRI scans for this analysis was limited to 16 patients, wherefore a type II statistical error can not be ruled out. As discussed above, several studies have demonstrated lack of correlation between biochemical effect and tumour size reduction during long-term treatment of acromegaly (99;101;105;106), explained by the different mechanisms involved in the antitumour and the antihormone responses of SSTR signalling. It is possible that RKIP modulates only the mechanisms responsible for the antihormone response to SMS analogues.

Studies have demonstrated that RKIP acts as a metastasis suppressor gene in both prostate cancer and breast cancer, and both studies suggested increased angiogenesis and vascular invasion as possible mechanisms (174;177). In ovarian cancer, an *in vitro* study found that RKIP in addition to inhibition of metastasis also inhibited cell proliferation by altering cell cycle progression (178). This is in concordance with the study showing that RKIP regulates the mitotic checkpoint (172). In our study of pituitary adenomas, the RKIP level did not correlate to tumour size and invasiveness. Despite the often locally invasive growth of the somatotroph tumours, this could be due to the general benign nature of these adenomas. Nevertheless, our studies are not mechanistic, and only associations can be found with this study design.

E-cadherin and EMT (Paper 3)

In the present study, we examined the protein expression of E-cadherin in the somatotroph adenomas. This was done with an anti-E-cadherin antibody directed against the extracellular domain of the protein in Western blot analysis and immunohistochemistry. In addition, an antibody directed against the intracellular domain was used for immunohistochemical analyses to detect internalization and nuclear translocation of the cytoplasmic component of the protein after proteolytic cleavage. We demonstrated that the E-cadherin protein expression in somatotroph pituitary adenomas is variable, and that a low Western blot E-cadherin level, lost membranous and gained nuclear localization correlate to large tumours,

and partly to tumour invasiveness. The E-cadherin protein expression was also associated with the somatostatin analogue response, in particular to tumour shrinkage.

Our study confirms the variable expression of E-cadherin in somatotroph pituitary adenomas that has been found previously (40;45;46;200;201;203). In our large cohort, we found a significant negative correlation between E-cadherin protein expression and tumour size and also partly to tumour invasiveness. Large and invasive adenomas seemed to have lost their membranous E-cadherin expression. This is in contrast with most previous studies, however, these have included several types of pituitary adenomas and the methods for evaluation of tumour size and invasiveness have not been similar (45;46;201;204). As discussed, loss of E-cadherin is a hallmark of EMT and malignant progression in several cancers of epithelial origin. Loss of E-cadherin could be only a marker of EMT, but a causal role of E-cadherin in tumour progression has been demonstrated *in vivo* in a mouse model of pancreatic β–cell carcinogenesis (220). It has been suggested that loss of E-cadherin not only means loss of epithelial adhesion, but also mediates direct activation of signalling pathways to cause invasive phenotypes independent of β-catenin (220;221). This is also supported by *in vitro* data from thyroid carcinoma cell lines demonstrating that cell lines from anaplastic thyroid carcinomas lack E-cadherin expression, and that re-expression of E-cadherin in these cells resulted in growth arrest by upregulation of p27 (222). Although the somatotroph adenomas are not malignant, their growth are often locally invasive. A role for E-cadherin in the growth of these tumours can not be ruled out. Alternatively, a theory could be that E-cadherin is lost during progressive growth parallel to, or as a marker of, other molecular changes allowing the excessive growth in the large and invasive adenomas.

We demonstrated a correlation between adenoma expression of E-cadherin and the clinical response to treatment with a somatostatin analogue. The acute SMS response correlated to E-cadherin expression analysed by all modalities. For the long-term response, the E-cadherin level from Western blot did not correlate significantly to the IGF-1 reduction following treatment ($p=0.054$). The immunohistochemical analyses revealed that adenomas with present nuclear E-cadherin staining show a poor long-term SMS response, both biochemically and with respect to tumour shrinkage. This correlation to somatostatin analogue efficacy can be a result of the observed positive correlation between E-cadherin expression and the expression of SSTR2a in the adenoma, since the expression of this receptor is important for the clinical efficacy of SMS treatment. A coupling between E-cadherin and the somatostatin receptor signalling pathway has been suggested previously. Ligand binding to SSTR2 has been shown to activate the tyrosine phosphatase SHP-1,

which dephosphorylates E-cadherin and restores E-cadherin function (223). In a human neuroendocrine cell line, blocking of SSTR signalling decreased the membranous E-cadherin level and resulted in morphological changes of the cells (224). However, in this study we found a reduced E-cadherin protein level in patients preoperatively treated with SMS, compared to the non-pretreated patients. This suggests a downregulation of E-cadherin protein in the adenoma cells following SMS treatment, and is contradictory to what would be expected from these *in vitro* studies. One could suspect that our results could be caused by sample selection bias, where the patients were selected for preoperative SMS treatment because they had large and invasive adenomas. However, similar result was found when we analysed only the patient cohort that had been randomized to preoperatively SMS treatment or not in a prospective clinical study (the POTA study) (4). Additionally, our *in vitro* results indicate downregulation at the mRNA level in non-treated patients, whereas we found no additional effect of octreotide on E-cadherin in tissue from SMS pretreated patients. Indeed, in this cohort we have also documented a decreased SSTR2a expression in the preoperatively treated patients (paper 1). There seems to be a parallel regulation of SSTR2a and E-cadherin in the adenomas. This could reflect loss of epithelial differentiation with loss of the SSTR2a normally expressed in the somatotroph cells. The implication of a downregulation of the level of these proteins, yet no alteration of immunohistochemical cellular distribution and expression, is unclear.

The similar phenomenon of parallel regulation could also be true for E-cadherin and RKIP. We demonstrated in paper 2 that the adenoma RKIP protein level was correlated to the biochemical response to SMS treatment. The known E-cadherin transcription repressor SNAIL has also been shown to inhibit RKIP transcription (225) which could explain the observed associations between RKIP and E-cadherin. RKIP inhibits the NF-κB system which again leads to reduced SNAIL (173;226). Overexpression of RKIP in a prostate cancer cell line reduced SNAIL, increased E-cadherin (both at protein level) and induced an epithelial-like phenotype (227).

Most previous studies have used antibodies against the extracellular domain of E-cadherin for protein analyses. In our immunohistochemical analyses, we chose to use both this antibody, but in addition an antibody directed against the intracellular domain of the protein, because we wanted to study nuclear translocation of E-cadherin. A nuclear accumulation of the intracellular domain of E-cadherin has been demonstrated in several cancer types (228-230). We found nuclear accumulation of E-cadherin combined with loss of membranous E-cadherin in a few adenomas (9/80), but in a lower proportion of

adenomas than in a recent published study (200). Compared to this study, which described nuclear staining of all intensities, we found weak nuclear positivity in all of these adenomas except one with moderate immunoreactivity. The adenomas with nuclear E-cadherin positivity can represent a dedifferentiated phenotype of the somatotroph adenomas. They had very low E-cadherin protein level assessed by Western blot, they were larger compared to the adenomas with membranous positivity in all cells, and they displayed a poor acute and long-term SMS response. There was a high degree of concordance between the immunohistochemical results using the two different antibodies. However, several adenomas showed membranous positivity in all cells using the intracellular domain antibody, but had lost membranous E-cadherin using the extracellular domain antibody. This discordance could reflect the known cleavage and loss of the extracellular domain by MMPs prior to internalization of the protein. The cytoplasmic staining observed exclusively with the intracellular domain antibody could also be due to internalization of only the intracellular domain of the protein.

General discussion

The clinical response to treatment with somatostatin analogues in acromegaly is highly variable. Previous genetic studies have not found mutations in the gene for SSTR2, but a point mutation on the SSTR5 gene was found in one octreotide resistant patient (231). Moreover, known polymorphisms in the receptors SSTR2 and 5 were not associated to SMS responsiveness (232). Other studies have indicated that densely granulated adenomas respond better to octreotide treatment compared to sparsely granulated adenomas (43;44), and that gsp positive adenomas display a better GH response during long-term octreotide treatment (29;38). In our studies, we analysed the gsp status of the adenomas. However, our main objective was to examine proteins involved in the SMS response, in order to examine factors behind the variability in SMS efficacy. We confirmed, as expected, that the protein expression of somatostatin receptor 2a in the somatotroph adenoma was of importance for the clinical efficacy of SMS treatment. However, we demonstrated patients in our cohort with a poor SMS response despite high SSTR2a expression, and vice versa. This led us to the hypothesis that mechanisms downstream of the receptor might modify the receptor response. Due to the published studies of RKIP in cancers discussing its modulating effect on the MAPK pathway and its role in chemotherapeutic sensitivity, this protein could be of interest. And, indeed, our study showed that the protein expression of RKIP in the adenomas

was a marker of biochemical SMS response, in addition to the SSTR2a expression. The lack of associations between SSTR2a expression and total RKIP or phospho-RKIP suggest that RKIP does not regulate the SMS response through regulation of GRK2 and internalization and degradation of SSTR2a, at least not in the somatotroph cells. Modulation of the MAPK pathway resulting in synergistic inhibition of this signalling system by SSTR2a activation and RKIP action is probably more likely. As discussed, this pathway is considered a probable mechanism for RKIP's drug-sensitizing effects, in addition to inhibition of the NF-κB pathway (182;218;219). With respect to NF-κB, a study of a murine fibroblast cell line showed that SSTR2a signalling sensitized the cells to TNFα induced apoptosis via activation of NF-κB (139). In the rat somatotroph cell line GH3, inhibition of NF-κB led to decreased cell viability (233). However, despite the fact that active NF-κB most commonly suppress apoptosis, the signalling system can promote cell death in response to certain death-inducing signals and in certain cell types (234). The contradictory effect on NF-κB in the referred studies can therefore be explained by the different species and cell types studied. For the SMS response, inhibition of both the MAPK and NF-κB pathways has mainly been considered as mechanisms for the antitumour effect and not the antisecretory response (113;122). Thus, the regulatory pathways responsible for the association of RKIP to the acute SMS response are therefore unknown. Further studies of RKIP in somatotroph adenomas are necessary to evaluate if the expression of RKIP is causal for good SMS response or whether RKIP is related to an associating, so far unidentified factor responsible for the effects. The multiple regression analysis only identifies RKIP as a marker independent of SSTR2a. A natural first approach would be to alter the RKIP expression in somatotroph cells *in vitro* and examine the SMS response.

The studies concluding with upregulation of RKIP as a mechanism for the drug sensitizing effect of rituximab in non-Hodgkin's lymphoma also demonstrated a concomitant downregulation of the transcription repressor Snail (218;219). Snail is known to be a strong repressor of E-cadherin and an inducer of the EMT process (185), see figure 3 (p. 26). In addition, after demonstrating that E-cadherin and RKIP was coexpressed in several cancer cell lines and that RKIP was negatively correlated to Snail in metastatic prostate cancer cells, Beach et al demonstrated that Snail also inhibits RKIP expression (225). Activated NF-κB increases the expression of Snail (235). Snail has been coupled to resistance to chemotherapeutic drugs both in melanoma cells, pancreatic cancer and lung adenocarcinoma cells (236-238). It has been suggested that a regulatory NF-κB-Snail-RKIP circuitry is established in cancer cells, maintaining a drug resistant phenotype (239). Despite

the benign nature of the somatotroph adenomas, we hypothesized that these mechanisms may be present in the somatotroph pituitary adenomas, particularly in the SMS resistant tumours. We therefore wanted to analyse the protein expression of Snail and E-cadherin in the adenomas. However, testing of two different anti-Snail antibodies did not give reliable staining of the Western blot membrane. The E-cadherin antibody, on the other hand, was specific. Consequently, only the E-cadherin expression could be evaluated in this study. Our finding of a positive correlation between E-cadherin and RKIP is in concordance with a previous publication (225). Adenoma E-cadherin expression correlated positively to biochemical SMS response, but also to tumour shrinkage following SMS treatment. It would have been of interest to analyse whether E-cadherin was an independent predictor of SMS response. However, a multiple regression analysis could not be performed due to the high correlation between the included variables. The E-cadherin protein expression also showed a strong correlation to the SSTR2a protein expression in the adenomas.

As reviewed, activation of SSTR2 leads to activation of the tyrosine phosphatase SHP-1, an important step for several downstream pathways in SSTR2 signalling (129;138). This PTP dephosphorylates E-cadherin at tyrosine residues, which stabilizes and restores E-cadherin function, while tyrosine phosphorylation leads to degradation (189;223). An *in vitro* study of pancreatic cancer cells demonstrated that SSTR2 positive cells had reduced tyrosine phosphorylation of E-cadherin, yet unchanged E-cadherin protein level. Moreover, an allograft of these cells displayed reduced invasiveness after SMS treatment compared to cells not expressing SSTR2 (223). Another study of a neuroendocrine tumour cell line showed that octreotide treated cells had high membranous E-cadherin immunoreactivity, while treatment with SSTR2 blocking antibodies was followed by reduced E-cadherin and morphological changes referred to as neuroendocrine-to-mesenchymal transition (NMT) (224). These studies show that there are several possible mechanisms for interplay between the regulation of SSTR2 and E-cadherin. However, they do not support our results of E-cadherin downregulation during SMS treatment and SSTR2 activation. Our studies were designed to show associations and not mechanisms. Yet, our small *in vitro* study of primary cultured adenoma cells supported our results of reduced E-cadherin protein levels in adenomas from preoperatively SMS treated patients. On the other hand, in our cohort there was also a reduced protein expression of SSTR2a in the pretreated patients. In the future, the *in vitro* E-cadherin response to octreotide stimulation should be studied in a larger group of adenomas, with correlation to SSTR2a protein level of these cells and their secretory GH response. This could clarify if regulation of E-cadherin during octreotide treatment is

universal or restricted to a subgroup of adenomas, for instance the octreotide resistant adenoma cells.

TGF- β has been shown to be a growth inhibitory cytokine in neuroendocrine cells, and that the SSTR pathway is a determinant for this response. Any disruption in the activation of TGF- β or SSTR, including the mentioned blocking of SSTR by inhibiting antibodies, led to NMT (224). In addition, it has been demonstrated that TGF-β induces EMT in breast cancer cells via activation of NF-κB (240), but also via increase in cyclooxygenase-2 (Cox-2) and prostaglandin E_2 (PGE_2). Stimulation with TGF-β led to increased Cox-2 expression which again stimulated PGE_2 production (241). In non-small cell lung cancer, activation of Cox-2-dependent pathways was followed by decreased E-cadherin. *In vitro* treatment with the Cox-2 inhibitor celecoxib increased E-cadherin expression, while treatment of these cells with PGE_2 decreased E-cadherin via modulation of the transcriptional repressors Snail and ZEB-1 (242). Studies of gastric cancers have demonstrated increased apoptosis with a combination treatment of octreotide and a Cox-2 inhibitor (243;244). The tumour expression of SSTR2 mRNA was also higher in the celecoxib treated adenomas compared to the not treated patients (243). In patients with gastric cancer randomized to direct surgery or to pretreatment with celecoxib, the tumours of the latter group displayed increased expression of E-cadherin assessed by immunohistochemistry and increased apoptotic index (245). This leads to the hypothesis that inhibition of Cox-2 results in reversibility of an EMT process, with a concomitant increase in the expression of SSTR2. Cox-2 is usually not expressed in normal tissue, but is frequently overexpressed in neoplasms including pituitary adenomas (246). Future studies of this pathway can therefore be of interest.

There have been several reports on agents regulating some of the above mentioned mechanisms for the EMT process and drug resistance. Proteasome inhibitors and nitric oxide donating agents have been shown to inhibit the NF-κB-Snail-RKIP circuitry, suppressing EMT and increasing chemotherapy sensitivity (226;227;247;248). As mentioned, rituximab leads to drug sensitization through upregulation of RKIP. However, in the treatment of acromegaly, the acceptance of drug side effects will be lower than in the treatment of an invasive and metastasizing cancer.

Our results of reduced E-cadherin expression in a proportion of adenomas and the correlation to large and invasive tumours and poor response to somatostatin treatment, may reflect an EMT-like process and dedifferentiation in these tumours. The correlation of E-cadherin and SSTR2a expression suggests a simultaneous loss of SSTR2a during this

process. It is particularly this group of acromegaly patients that hopefully can benefit from future studies on modulators of the EMT process and potentially drug sensitizing agents in pituitary adenomas.

CONCLUSION

To conclude, our studies demonstrated that;

- The somatostatin receptor subtype 2a was downregulated at the protein level in a substantial proportion of somatotroph adenomas, and this was associated with reduced biochemical response to SMS treatment in the patients with acromegaly.
- There was no difference in octreotide responsiveness in gsp positive compared to gsp negative adenomas.
- The protein level of Raf kinase inhibitory protein (RKIP) in the somatotroph adenomas was correlated to the biochemical response to somatostatin analogue treatment, where a low RKIP level was associated with poor SMS efficacy. The RKIP level did not correlate to SSTR2a protein expression of the adenomas. This suggests RKIP as a modulator of the signalling downstream of SSTR binding.
- Reduced E-cadherin protein level and reduced membranous localization of E-cadherin were apparent in a subgroup of GH producing pituitary adenomas, and some of these adenomas also displayed nuclear translocation of the intracellular domain of the protein. This correlated to large and invasive adenomas, and to poor biochemical SMS response and little tumour shrinkage following treatment.
- Reduced E-cadherin protein expression was associated with reduced protein expression of SSTR2a and RKIP, and may be a marker of epithelial dedifferentiation and an EMT-like process in these adenomas

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