FUNCTIONAL HETEROGENEITY AMONG CELL TYPES IN THE NORMAL PITUITARY GLAND AND IN HUMAN AND RAT PITUITARY TUMORS

FUNCTIONELE HETEROGENITEIT BINNEN CELTYPEN VAN DE NORMALE HYPOFYSE EN VAN HYPOFYSETUMOREN BIJ DE MENS EN BIJ DE RAT

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ABBREVIATIONS

AII	angiotensin II
ACTH	adrenocorticotropic hormone (corticotropin)
α-subunit	alpha-subunit
β-LPH	beta-lipotropin
β-MSH	beta-melanotropin
β-endorphin	beta-endorphin
BSA	bovine serum albumin
CRH	corticotropin releasing hormone
DA	dopamine
DNA	deoxyribonucleic acid
FCS	fetal calf serum
FSH	follicle stimulating hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin releasing hormone
GH	growth hormone
HBSS	Hank's balanced salts solution
hGH	human growth hormone
hPRL	human prolactin
HSA	human serum albumin
IR	immunoreactive
IGF-I	insulin-like growth factor I
IRMA	immunoradiometric assay
LH	luteinizing hormone
mRNA	messenger ribonucleic acid
MEM	minimal essential medium
n	number of determinations
oRBC	ovine red blood cells
pfc	plaque forming cell
PRL	prolactin
ref.	reference
RIA	radioimmuno assay
RHPA	reverse hemolytic plaque assay
rPRL	rat prolactin
SMS 201-995	sandostatin
SRIH	somatotropin release inhibiting hormone (somatostatin)
TSH	thyrotropin
TRH	thyrotropin releasing hormone
VIP	vasoactive intestinal peptide
vs	versus

CHAPTER I

GENERAL INTRODUCTION

I.1 The anterior pituitary gland

I.1.1 Cellular composition and cellular heterogeneity

The anterior pituitary gland is heterogeneous with regard to its cellular composition. Based on morphological, ultrastructural and immunocytochemical criteria it has been established that the anterior pituitary gland is composed of at least five distinct hormone-secreting cell types (1). These cell types are divided into polypeptide hormone and glycoprotein hormone secreting cells. Polypeptide secreting cells are growth hormone secreting cells (GH cell or somatotrope), prolactin secreting cells (PRL cell or mammotrope) and adrenocorticotropin secreting cells (ACTH cell or corticotrope). Glycoprotein secreting cells are thyrotropin secreting cells (TSH cell or thyrotrope) and gonadotropin secreting cells (LH and/or FSH cell or gonadotrope). Somatotropes and mammotropes represent the largest group of anterior pituitary cells.

During a long period of time the theory of "one cell - one hormone" has been generally accepted. An exception to this has been the gonadotrope cell type. In addition to monohormonal cells, secreting LH or FSH, bihormonal cells that produced both hormones simultaneously were distinguished (2-5). During the last decade however, with the development of more sophisticated techniques, the concept of "one cell - one hormone" has been challenged. Next to LH and FSH, also GH and PRL have been shown to be secreted by the same cell (6-8) or to be co-localized within the same cell (9). This cell type has been named mammosomatotrope. Direct evidence for the existence of such mammosomatotrope cells in the adult normal human pituitary gland has been presented recently by Lloyd et al (10). These investigators estimated that the percentage of mammosomatotrope cells in the normal human pituitary ranged from 26-50% as determined by immunocytochemistry and from 29-

49% as determined by the reverse hemolytic plaque assay (RHPA). With the latter technique hormone secretion by individual pituitary cells can be detected (see chapter I.3). In neonatal and normal adult rats evidence has been obtained that transformation of GH cells into PRL cells may be responsible for the shift in the proportion of GH- and PRL cells which is observed under certain physiological conditions (7, 11-13). Therefore, the classical concept of "one cell - one hormone" appears to be out of date.

Next to the hormone secreting cell types the anterior pituitary gland also contains two types of epithelial cells: the folliculo-stellate cells, which do not contain secretion granules and the oncocytes, which probably represent degenerating secretory cells (14, 15). Their function remains unknown although it was recently shown that the folliculo-stellate cells might be related to anterior pituitary hormone secretion by a cell-to-cell communication mechanism (16).

I.1.2 Hypothalamic regulation of hormone secretion by anterior pituitary cells

Anterior pituitary hormone secretion is under direct hypothalamic control of hypothalamic regulatory factors/hormones. These regulatory factors/hormones are released from the axons of the hypothalamic neurosecretory neurons and reach the anterior pituitary via the hypophyseal portal system of blood vessels. This system of blood vessels begins in the median eminence of the hypothalamus and drains downwards into the sinusoids of the anterior pituitary gland. A summary of the various hypothalamic factors/hormones and of the corresponding anterior pituitary hormones of which they affect the secretion is given in table 1. Most of the hypothalamic hormones have a specific effect on the secretion of only one hormone (see table 1). However, some of these hormones affect the secretion of multiple hormones. For example, TRH has been shown to stimulate the secretion of both TSH and PRL although the role of TRH as a physiological prolactin releasing hormone is doubtful (17). In addition SRIH has been shown to inhibit the secretion of GH, TSH and

PRL. The complexity of the regulation of anterior pituitary hormone secretion by hypothalamic hormones is further illustrated by several studies demonstrating paracrine interactions between anterior pituitary cell types and demonstrating functional heterogeneity within cell types of the anterior pituitary. These latter two aspects will be discussed more in detail in the next paragraphs.

Table 1: Known hypothalamic hormones or factors and their effects on anterior pituitary hormone secretion.

Hypothalamic hormone or factor	Abbreviation	Effect on the secretion of	Stimulatory (+) or Inhibitory (-)
1. thyrotropin releasing hormone	TRH	TSH, PRL, (FSH)	+
2. growth hormone releasing hormone	GHRH	GH	+
3. somatotropin release inhibiting hormone (somatostatin)	SRIH	GH, TSH, PRL	-
4. gonadotropin releasing hormone	GnRH	LH, FSH	+
5. corticotropin releasing hormone	CRH	ACTH	+
6. prolactin releasing factor (vasoactive intestinal peptide?)	PRF (VIP?)	PRL	+
7. prolactin release inhibiting factor (dopamine)	PRIF (DA)	PRL	-

I.1.2.1 Paracrine interactions between anterior pituitary cell types

There are several lines of evidence that next to the direct effects of hypothalamic regulatory hormones also paracrine interactions between anterior pituitary cell types may play a role in the regulation of anterior pituitary hormone secretion (18). Several morphological studies in the rat have shown that the distribution of anterior pituitary cell types is not random. Rat gonadotropes and lactotropes are frequently found in close association with each other (19-23). Similar close associations were observed between corticotropes and somatotropes (20, 24, 25) and between

thyrotropes and somatotropes (26). Although these cell associations are suggestive for intercellular communication, functional morphological correlates have not been reported (18).

Another indication suggestive for paracrine interactions in the anterior pituitary is the local presence of biogenic amines, neuropeptides, growth factors and arachidonic acid metabolites in the pituitary gland. In addition these substances directly affect pituitary hormone release in vitro (18). Direct in vitro evidence for paracrine communication between anterior pituitary cell types has been given by the extensive studies of Denef's group. In their studies they used gradient sedimentation of dispersed cells at unit gravity, which is a cell separation technique by which the cells are separated ("enriched") according to differences in their size (for review see 27). Paracrine interactions between enriched populations could be studied by coaggregating enriched populations into three-dimensional reaggregates (28). Denef et al. showed that GnRH stimulated rat PRL release in vitro, the magnitude of this response being dependent on the proportional number and size of the gonadotropes that were present in the coaggregate cell cultures (29). Using the same technique they also showed that the PRL response to Angiotensin II (AII) may be partly mediated via gonadotrope cells (18).

With regard to intercellular communication with somatotropes Denef's group showed that other rat anterior pituitary cell types (presumably lactotropes or corticotropes) might be involved in the response of GH release to epinephrine and vasoactive intestinal peptide (VIP), provided that dexamethasone was present in the culture medium (30). Moreover, evidence was presented that the GH response to AII in vitro might be mediated by an intercellular signaling system (31).

Finally, the folliculo-stellate cell, which is an anterior pituitary cell type of which the function is unknown, might be involved as a local inhibiting modulator of rat pituitary hormone secretion in a paracrine fashion (16).

To our knowledge there are no studies so far,

providing direct evidence for paracrine regulatory mechanisms in the normal human anterior pituitary.

I.1.2.2 Functional heterogeneity among anterior pituitary cell types

Physiological heterogeneity within a rat anterior pituitary cell population has been described sixteen years ago for the first time by Hopkins and Farquhar (32). These investigators observed differential uptake of tritiated leucine into rat somatotropes by autoradiography. Using double isotope labeling of pituitary cells maintained in monolayer culture combined with autoradiography Walker and Farquhar (33) have shown that within the rat mammotrope population a subpopulation of cells exist which manufactures and secretes PRL at a very fast rate. Moreover this mammotrope subpopulation appeared to be unresponsive to TRH (33). Since the demonstration of functional heterogeneity within the somatotrope cell population by Hopkins and Farquhar two new techniques have been introduced in endocrinological research by which the field of functional heterogeneity could be approached more precisely. These techniques include cell separation of anterior pituitary cell types according to differences in size and/or density (for reviews see 27, 34) and the reverse hemolytic plaque assay, which enables the detection of hormone secretion at the single cell level (for review see 35). Both techniques will be discussed in more detail in chapter I.3. Cell separation experiments have demonstrated the existence of morphologically and functionally different subpopulations of rat mammotropes (36-38), rat somatotropes (39, 40), rat thyrotropes (41) and rat gonadotropes (42-44). In most of these studies the cells in the different gradient fractions were functionally heterogeneous in either hormone production/secretion per cell (36-39, 41, 42-44) or in their responsiveness to secretagogues such as GnRH (42, 44), VIP (37) or dibutyryl cyclic adenosine monophosphate (40). Using the reverse hemolytic plaque assay more evidence for functional heterogeneity within rat anterior pituitary cell

populations has been provided. Frawley et al. (45-47) have demonstrated the existence of subpopulations of somatotropes, which differed in their responsiveness to GHRH (45,46) and IGF-I (47). In addition, also mammotrope subpopulations which showed differential responsiveness to DA and TRH were demonstrated (48-50). It has been suggested that differences in hypothalamic input between anterior pituitary regions may be responsible for functional heterogeneity between PRL-cell types (50). Finally, using the reverse hemolytic plaque assay (RHPA), also subpopulations of gonadotropes which differed in their responsiveness to GnRH have been demonstrated (44, 51).

Little is known, so far, about functional heterogeneity among normal human anterior pituitary cell types. Only one study describes the separation of subpopulations of human mammotropes which differed in the amount of PRL secreted per cell (52). Recently it was shown by Mulchahey et al. (53) that human and monkey fetal somatotropes respond differentially to GHRH. Furthermore, glucocorticoids were shown to maintain this subpopulation of fetal somatotropes in the GHRH responsive state (53).

I.1.3 Concluding remarks

Hypothalamic releasing or release-inhibiting hormones are main regulators of anterior pituitary hormone secretion. However, the regulation of pituitary hormone secretion by these hormones is very complex. The ultimate response of anterior pituitary hormone secretion to hypothalamic regulatory hormones appears to be determined by several fine-regulatory mechanisms. First, it has been demonstrated that there is heterogeneity among anterior pituitary cell types of the amount of hormone secretion per cell. Moreover, some anterior pituitary cell types produce and secrete more than one hormone simultaneously. Secondly, it has been shown that certain anterior pituitary cell types may be functionally heterogeneous in their response to secretagogues. Finally, several hypothalamic hormones may have an effect on anterior pituitary hormone secretion via

paracrine effects between anterior pituitary cell types. Therefore, the response of anterior pituitary hormone secretion to hypothalamic regulatory hormones may be the result of a combination of the fine-regulatory mechanisms which appear to exist within the anterior pituitary. However, the real contribution of these fine-regulatory mechanisms to the overall anterior pituitary hormone secretion remains to be established.

I.2 Pituitary adenomas

I.2.1 Types of human pituitary adenomas

By means of morphological, ultrastructural and immunocytochemical criteria six types of human pituitary adenomas are distinguished. These types included somatotropic adenomas (GH-secreting adenomas), prolactinomas (PRL-secreting adenomas), corticotropic adenomas (proopiomelanocortin derivate-secreting adenomas), thyrotropic and plurihormonal adenomas (TSH- and multiple hormone-secreting adenomas), gonadotropic adenomas (LH and/or FSH or α-subunit secreting adenomas) and non-functioning adenomas (α-subunit secreting adenomas and adenomas unable to synthesize or secrete hormones). Although this classification is mainly based on the type of hormone which is present in and/or being secreted by the tumor cells, most types of pituitary adenomas appear to be heterogeneous with regard to their cellular composition. This cellular heterogeneity of pituitary adenomas will be discussed in the next paragraphs.

I.2.2 Cellular heterogeneity of human pituitary adenomas

I.2.2.1 Cellular heterogeneity of somatotropic adenomas

The percentage of GH-immunoreactive (IR) cells in somatotropic adenomas varies considerably. Trouillas et al (54) found percentages varying between 5 to 100% GH-IR cells. Moreover, Lloyd et al (10) showed that the percentage of GH-secreting cells, as determined by the reverse hemolytic plaque assay, varied in four GH-producing pituitary adenomas between 47 and 78%.

In GH-secreting pituitary adenomas other hormones apart from GH may be detected by immunocytochemistry. Most frequently prolactin and the non-related glycoprotein hormone α-subunit are simultaneously present. The percentage of GH-secreting adenomas in which also PRL-immunoreactive cells can be demonstrated varies from 45-68% (54-57). Using the protein A-gold electron microscopic immunotechnique Bassetti

et al (58) have shown that in most GH- and PRL-secreting pituitary adenomas from acromegalic patients GH and PRL are present in the same cell and even in the same secretory granule. They proposed that the frequency of mixed GH- and PRL secreting adenomas containing mammosomatotropic cells, i.e. cells containing GH and PRL simultaneously, may be higher than previously believed (59). In addition Robert et al (60) recently demonstrated the presence of mammosomatotropic cells in all cases of a group of 30 adenomas from patients with signs of GH and PRL hypersecretion. Lloyd et al (10) recently presented evidence for dual hormone (GH + PRL) secreting cells in pituitary adenomas from acromegalic patients by a reverse hemolytic plaque assay.

Using specific anti-α-subunit antisera combined with absorbtion tests with α-subunit Trouillas et al (54) showed that in 45 out 84 tumors (53%) from acromegalic patients apart from GH-containing cells also α-subunit containing cells were present. A similar percentage was reported by Landolt and Heitz (57). Recently Beck-Peccoz et al (61) presented evidence for α-subunit and GH-coexistence within the same tumoral cell in some tumors.

Less frequently (29%) immunoreactive ACTH can be demonstrated in biopsy specimens and the simultaneous presence of FSH, LH and TSH is seen only rarely (62).

I.2.2.2 Cellular heterogeneity of prolactinomas

Trouillas et al (54) showed in a group of 54 women with amenorrhea or amenorrhea-galactorrhea that all tumoral cells were positive with anti-hPRL antiserum. Moreover, in their series plurisecretion was exceptional (54). Landolt (63) however, found in about half of the biopsy specimens positive reactions with antibodies against ACTH, GH and/or LH. In addition Lloyd et al (10) recently showed by plaque assay studies and immunocytochemistry that within prolactinomas mammosomatotropic cells, i.e. cells secreting both PRL and GH, may exist.

I.2.2.3 Cellular heterogeneity of corticotropic adenomas

In most cases, adenoma cells from patients with Cushing's disease react with all the antisera against the main peptides of the proopiomelanocortin (anti-ACTH 1-24, and anti-ACTH 17-39, anti-βLPH, anti-β-endorphin and anti-β-MSH; 54). In some tumors of patients without Cushing's disease however, the percentage of cells showing positive reactions with antisera against these peptides is variable (54, 64).

I.2.2.4 Cellular heterogeneity of gonadotropic adenomas

Gonadotropic adenomas may consist of FSH-containing cells, LH-containing cells or FSH- and LH-containing cells (54). In case of FSH-LH adenomas both hormones may be present in the same tumoral cell (54). Next to LH and FSH also α-subunit is detected frequently in the adenoma cells of this type of pituitary adenoma. It remains to be elucidated however, whether, in case multiple hormones are secreted by the tumoral cells, all cells contain these hormones simultaneously or whether there are distinct cells containing LH or FSH or α-subunit.

I.2.2.5 Cellular heterogeneity of other types of pituitary adenomas

Thyrotropic and plurihormonal adenomas are very rare (54). Apart from TSH often one or two other hormones are detected by immunocytochemistry. These hormones include GH, PRL or FSH (54, 65, 66). Immunocytochemistry on sequential pituitary adenoma sections or studies with a double-immunolabeling method, revealed that two or three hormones may be secreted by different cell types (54) or by the same cell type (54, 65, 66).

In non-functioning adenomas cells reacting with antisera against the glycoprotein hormone α-subunit are frequently detected (67-70), although the percentage of α-subunit positive cells in this type of adenoma may vary (67). Non-functioning adenomas not secreting α-subunit and in which

no α-subunit is detectable by immunohistology are extremely rare (71).

I.2.3 Regulation of pathological hormone secretion by pituitary adenomas

In general hormone secretion by human pituitary adenomas shows a considerable variability in its response to various secretagogues and drugs.

In acromegaly, pathological GH secretion remains, in most instances sensitive to the physiological hypothalamic hormones, GHRH and SRIH, which also regulate normal GH secretion. In addition paradoxical stimulation of GH-secretion by GnRH (72), TRH (73), VIP (74) and CRH (75) or inhibition by dopamine or by the dopamine agonist bromocriptine (73, 76-78) may occur in acromegalic patients.

Prolactin secretion by prolactinomas remains in most instances sensitive to dopamine and dopaminergic drugs as bromocriptine. Bromocriptine-treatment decreases hyperprolactinaemia in 75% of the cases, and in 20-60% of the cases it causes tumor shrinkage to about 50% of the initial tumor volume (79). In most of the patients with a prolactin secreting pituitary adenoma TRH is ineffective in modifying plasma PRL-levels (80, 81). This absence of responsiveness of prolactinoma cells to TRH seems not to be linked to the absence of specific TRH-receptors (82). Moreover an absent TRH-response of cultured prolactinoma cells could be restored when the cells were cultured in the presence of adequate concentrations of bromocriptine (83).

ACTH secretion by anterior pituitary micro-adenomas can be stimulated by CRH and vasopressin and inhibited by high doses of dexamethasone (84). ACTH-secretion by this group of micro-adenomas appears to be insensitive to bromocriptine (84). However, ACTH secretion by (multiple) micro-adenomas, possibly originating from the intermediate lobe, is less sensitive to CRH, vasopressin and dexamethasone but can often be inhibited by bromocriptine. Especially, the latter group of micro-adenomas appears to secrete α-MSH (84).Paradoxical stimulating effects by VIP,

TRH and GnRH on ACTH release by corticotropic adenoma cells have also been demonstrated (85, 86).

LH, FSH and α-subunit secretion by gonadotrope and non-functioning adenomas shows in most instances a paradoxical response to TRH and in a minority of the cases to the physiological regulatory peptide GnRH (87,88). In addition it has been demonstrated that bromocriptine inhibits LH, FSH and/or α-subunit secretion by some of these adenomas in vivo and in vitro (87).

I.2.3.1 Paracrine interactions between pituitary adenoma cells

There is very little information so far, with regard to intercellular communication between pituitary tumor cells. In rats it has been shown that estrogen-induced pituitary tumors produce an autostimulatory peptide once the growth of the tumor cells has become insensitive to estrogens (89). At present there are no studies providing direct evidence for paracrine interactions between human pituitary adenoma cells.

I.2.3.2 Functional heterogeneity among pituitary adenoma cells

As has been indicated in the previous paragraphs pituitary adenomas may be considerably heterogeneous with regard to their cellular composition. It might be speculated therefore that also functional heterogeneity exists among pituitary adenoma cells. For example, do all cells within a mixed GH-PRL secreting adenoma respond equally to secretagogues as TRH and GHRH or to drugs as the DA-agonist bromocriptine and the somatostatin analog SMS 201-995? To our knowledge however, there are no reports so far, showing direct evidence for the existence of functional heterogeneity among cells in human pituitary adenomas.

Recently, Hymer and Motter (38) demonstrated heterogeneity in rat mammotropes prepared from diethylstilbesterol-induced prolactinomas. Using a combination of cell separation by unit gravity sedimentation and a cell blot assay they showed that the cells in the

various gradient fractions differed in the amount of secreted PRL. Moreover, they demonstrated by Western blot assays the existence of a subpopulation of prolactinoma cells containing an unique PRL variant. They concluded that there is significant heterogeneity at both the cellular and molecular level within the mammotrope population of diethylstilbesterol-induced prolactinomas (38). It is not know whether these functional differences also exist among cells of human prolactinomas. However, Bartke et al (90) have argued that these tumors bear a striking resemblance to spontaneous prolactinomas in man.

I.2.4 Concluding remarks

As has been mentioned in this chapter hormone secretion by pituitary adenomas is highly variable. Between pituitary adenomas there is a considerable variability with regard to basal secretion rates but also with regard to the responsiveness of hormone secretion to secretagogues and drugs. Moreover, there is heterogeneity within pituitary adenomas with regard to their cellular composition. Most pituitary adenomas secrete more than one hormone simultaneously and immunocytochemical studies have demonstrated that part of the adenomas (including all types of pituitary adenomas) contain multiple hormone secreting cells and/or different cell types secreting different hormones. It is not known at present to which extent the variability among pituitary adenomas in hormone secretion and the variability among pituitary adenomas in responsiveness to secretagogues and drugs may be related to the cellular heterogeneity within these adenomas. In addition there are no studies so far, providing evidence for functional heterogeneity within pituitary adenomas. For example it is unknown whether all cells within a single pituitary adenoma respond to the same extent to certain drugs. More knowledge in this field leads to a better understanding of the mechanism of action of drugs which have an inhibitory effect on pathological hormone secretion by human pituitary adenomas. This knowledge may eventually result in a better medical treatment of certain types of pituitary adenomas.

I.3 Methods

I.3.1 General introduction

As has been described in chapters I.1 and I.2 both the normal pituitary gland and pituitary tumors may consist of cellular and/or functional heterogeneous subpopulations of cells. During the last decade two techniques, which enable the study of cellular and functional heterogeneity within pituitary cell populations, have been introduced in neuroendocrinological research. These techniques include the reverse hemolytic plaque assay (RHPA) (91) and cell separation techniques.

Basal and secretagogue-induced hormone release by individual normal pituitary cells or by pituitary tumor cells can be studied using the RHPA. The RHPA gives both qualitative and quantitative information with regard to hormone secretion by individual cells and can be useful for the evaluation of quantitative differences in hormone secretion among subpopulations of cells present within particular cell suspensions.

Using cell separation techniques subpopulations of cell types, which differ in their size and/or density, can be obtained. Basal or secretagogue-induced hormone release by these cell subpopulations can be studied in vitro and may provide information with regard to functional heterogeneity between these cell-subpopulations.

Both the RHPA and a cell separation technique (percoll density gradient centrifugation) were used for the work which is presented in this thesis. The principles of both techniques will be discussed in detail in the following paragraphs. The materials that were used for these two techniques are described in chapters III, IV and VI (RHPA) and III, IV and V (percoll density gradient centrifugation).

Cell dispersion and isolation techniques, cell culture methods, specific hormone determinations by radioimmunoassay (RIA) or by immunoradiometric assay (IRMA) and the method of detection of intracellular hormone by immunocytochemistry has been described in detail in chapters III-VII.

I.3.2 Reverse hemolytic plaque assay (RHPA)

The RHPA for the detection of hormone release by individual neuroendocrine cells is a technique which is based on a classical immunological reaction of complement-mediated cytolysis of antigen-antibody bearing cells. The principle of the RHPA is schematically shown in Figure 4.1.

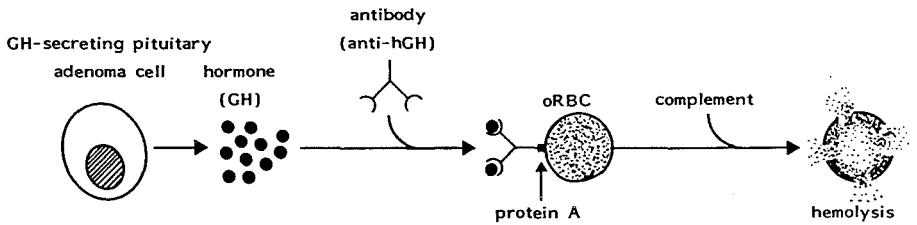


Fig. 4.1. Schematic representation of the principle of the RHPA for the detection of hormone secretion by individual pituitary (adenoma) cells.

As an example the detection of hGH secretion by human GH-secreting pituitary adenoma cells is shown. The GH-secreting pituitary adenoma cells are mixed with an excess of protein A-coated ovine red blood cells (oRBC) and incubated with an antiserum against hGH. The protein A, which is coated on the membrane of the oRBC, binds the Fc-domain of the added anti-hGH antibodies. These antibodies also complex with the hGH which is secreted by the adenoma cells. Consequently the oRBC in the vicinity of those adenoma cells secreting hGH are

bearing hGH-anti-hGH complexes. In the presence of complement (which is added in the RHPA) these oRBC will undergo cytolysis thereby forming a zone of hemolysis (plaque) around those cells secreting hGH but not around cells not secreting hGH.

In figure 4.2 a schematic presentation of the several steps included in the RHPA is shown. Pituitary (adenoma) cells are cultured in petridishes during 24 h. Thereafter, the cells are trypsinized from the petridishes. The resulting cell suspension (0.2 x 106 cells/mL culture medium (MEM) + 0.1% bovine serum albumin (BSA)) is mixed with an equal volume of a 12% (v/v) suspension of protein A-coated oRBC. The mixture of pituitary (adenoma) cells and oRBC is then infused into Cunningham chambers (92) by capillary action. The Cunningham chambers are constructed by placing a coverslip over two pieces of double-sided adhesive tape positioned on a poly-L-lysine coated glass slide. The slides were coated with poly-L-lysine by placing them in a solution of poly-L-lysine (0.25 mg/mL distilled water) during 30 min. Thereafter the slides were rinsed twice in distilled water and air dried. After infusion of the cells into the chambers the pituitary (adenoma) cells and oRBC are allowed to attach to the floor of the chamber during 45 min. at 37°C in a CO_2-incubator (5% CO_2/95% O_2). After this incubation period the chambers are rinsed twice with 30μL of MEM + 0.1% BSA in order to remove eventually unattached cells. Thereafter incubations were started by infusing antiserum (for example anti-hGH serum) without or with secretagogues. The slides are now incubated during a period of 0.5-4 hour at 37°C in the CO_2-incubator. During this period the complexing of antibodies and secreted hormone to the oRBC as described in figure 4.1 occurs. After the incubation with antiserum the chambers are rinsed twice with MEM + 0.1% BSA and complement is added to the chambers. After 45 min. of incubation at 37°C, during which time plaque formation occurs, the cells are fixed by infusing fixative (30 min. at room temperature), the coverslips are carefully removed and the cells are stained with a haematoxylin-solution in order to ensure the detection of non-plaque-forming cells. In plaque assays an incubation time

with complement of at least 30 min. is usually sufficient to achieve maximal plaque formation (91).

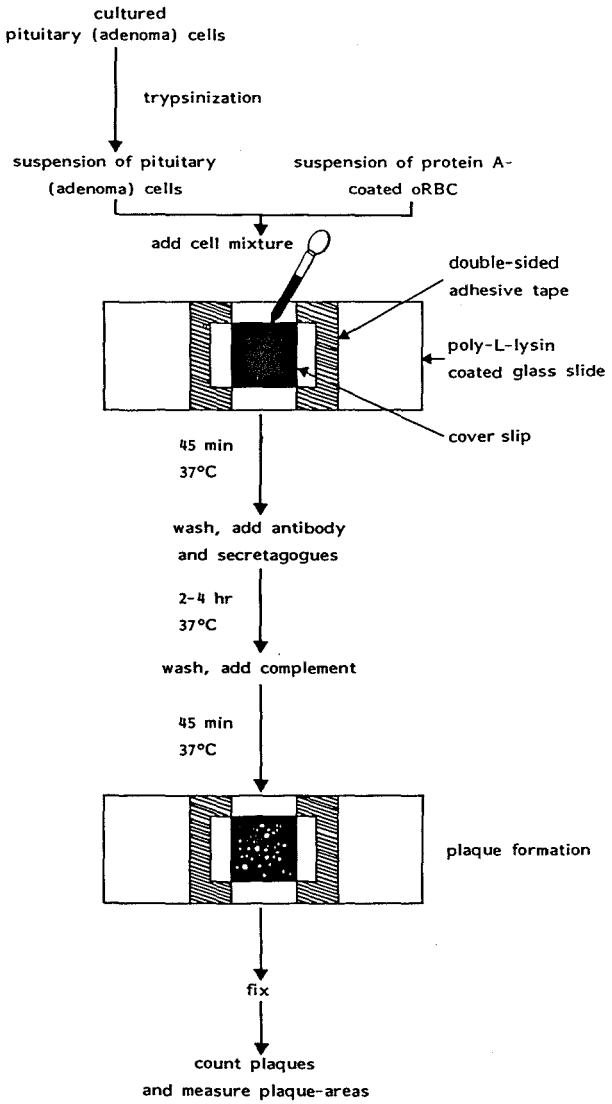
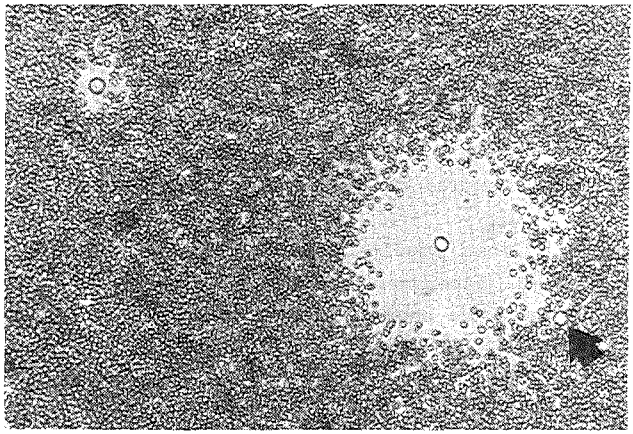


Fig. 4.2. Schematic representation of the several steps included in the reverse hemolytic plaque assay.

A



B

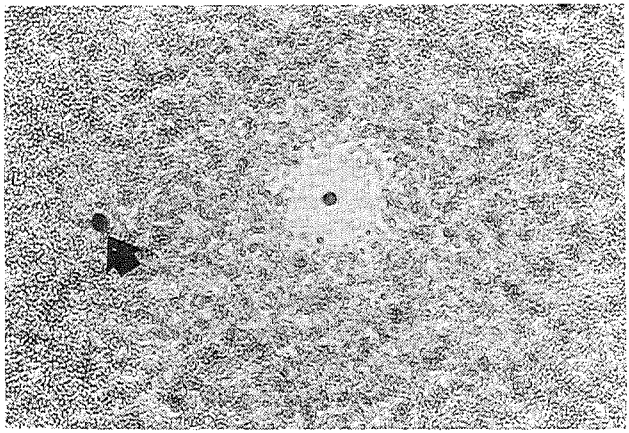


Fig. 4.3. Photomicrographs (magnification 400x) of plaque forming cells of human GH-secreting pituitary adenoma cells prior to (A) and after (B) staining with haematoxylin (HE). Note that the cells not forming plaques (black arrows) are better visible after staining with HE.

Figure 4.3 shows that non-secreting cells can be detected easily within the layer of oRBC when the preparations are counterstained with haematoxylin. After staining the pituitary (adenoma) cells and oRBC are embedded in a glycerin-gelatin mixture (1g gelatine dissolved at 37°C in 6 mL distilled water, mixed with 7 mL glycerin during 30 min; use at 37°C) and stored until microscopic evaluation of the plaques.

I.3.2.1 General considerations of the RHPA

Most studies in which the RHPA was used to study functional heterogeneity within populations of particular cell types have been performed using anterior pituitary cells from rats (44-51). Only one study so far, describes functional heterogeneity within the human and monkey fetal somatotrope cell population with regard to GHRH-responsiveness (53). Functional heterogeneity within somatotrope, lactotrope and gonadotrope cell populations has been demonstrated by evaluation of frequency distributions of individual plaque areas under basal and/or secretagogue induced conditions (45, 49) or by evaluating the percentage of plaque-forming cells (pfc) as a function of the incubation time (48, 50, 51) or by evaluating both parameters (46, 47, 53). It is important to note, however, that information with regard to functional heterogeneity of a particular cell-subpopulation can only be derived from frequency distribution curves of individual plaque areas when the percentage pfc is maximal under basal conditions (which is dependent on the length of the incubation time in the RHPA) or when secretagogue-treatment of the cells does not alter the percentage pfc (at incubation time with maximal plaque formation). When the percentage pfc is not maximal under basal conditions or decreases/increases in the presence of particular secretagogues part of the cells of a cell population is excluded from the frequency distribution curves and no conclusions with regard to functional heterogeneity can be drawn. Therefore, in each experiment using the RHPA the time-relationship with the percentage pfc has to be

determined. An example for this time-relationship is shown in figure 4.4. This figure shows the percentage of rPRL-pfc as a function of the incubation time in a suspension of normal female rat anterior pituitary cells as was used for the experiments described in chapter III of this thesis. The percentage of rPRL-pfc gradually increases as a function of the incubation time to 46% after 120 min. of incubation in control slides.

In the presence of 100 nM TRH maximal percentual plaque formation was already achieved after 30 min. of incubation and no further increase in the percentage pfc was seen at prolonged incubation times (44% at 120 min. of incubation). Since there was no significant difference in the percentage pfc at 120 min. between control and TRH-treated slides it was concluded that the percentual plaque formation was maximal at this incubation time (see chapter III).

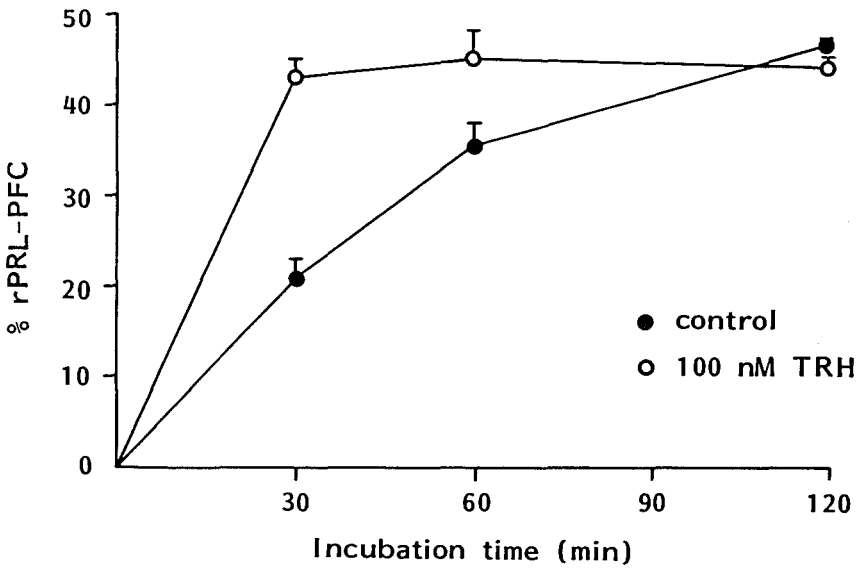


Fig. 4.4. Percentage of prolactin plaque forming cells in the absence or in the presence of TRH as a function of the incubation time. Dispersed anterior pituitary cells derived from female rat pituitaries were used.

I.3.2.2 Technical aspects of the RHPA

The RHPA enables quantitative detection of hormone release by pituitary cells. Several investigators (35,49, chapter VI of this thesis) have shown a significant correlation between plaque size and hormone secretion rates by cohort cultured cells in vitro. In addition Allaerts et al. (93) recently demonstrated a quantitative relationship between plaque size and the amount of secretion of PRL in rat pituitary cells using an analytical approach of the diffusion phenomenon in the RHPA. Their study confirmed the validity of the assumptions underlying the RHPA, providing that the cell density is low, the incubation time moderately long and the concentration of specific antiserum sufficiently high. The RHPA which is used for the investigations described in chapters III, IV and VI of this thesis meets these criteria. The species-origin of the specific antiserum which is used in the RHPA is also an important factor since protein A has different binding capacity for immunoglobulins from different species. For example, protein A shows low reactivity with mouse and goat immunoglobulins whereas it shows high reactivity with rabbit and guinea pig immunoglobulins (94). It should be advised therefore, to use rabbit or guinea pig antisera in the RHPA. Finally it should be mentioned that the complement which is used in the RHPA may contain non-specific immunoglobulins which may cause background cytolysis of the oRBC in the RHPA. Our experience suggests that the best results were obtained when the complement was stripped prior to use in the RHPA from immunoglobulins by incubation with protein A-coated Sepharose as described by Leong et al. (8).

I.3.3 Cell separation using percoll density gradient centrifugation

I.3.3.1 Introduction

Several methods have been applied for the separation of anterior pituitary cell types. The most commonly used methods are unit gravity sedimentation of

anterior pituitary cells on BSA-gradients, centrifugal elutriation of anterior pituitary cells and density gradient centrifugation of anterior pituitary cells using density gradients prepared from BSA or percoll (for reviews see 27,34). With unit gravity sedimentation and centrifugal elutriation cells are separated mainly on differences in their size. Using density gradient centrifugation cells are separated based on differences in their density. With all the three techniques for cell separation comparable results have been obtained with regard to the enrichment of specific rat anterior pituitary cell types. These results are summarized in table 4.1.

As has been mentioned in chapter I.1 functional heterogeneity between subpopulations of specific cell types has also been demonstrated using cell separation based on differences in cell density or cell size. For the study of functional heterogeneity within the normal rat pituitary lactotrope population (chapter III), within a rPRL-secreting pituitary tumor (chapter IV) and within human GH-secreting pituitary adenomas (chapter V) we have chosen for the use of percoll density gradient centrifugation because the technique yields results which are comparable to the other two techniques and because it has some advantages over these techniques. Percoll has some properties which makes it an ideal material for the separation of living cells. These properties are summarized in table 4.2.

The main advantages of percoll gradient centrifugation over the other two methods for cell separation are 1) the technique is very simple and cheap since no specific instruments are required. In contrast unit gravity sedimentation requires special sedimentation chambers and centrifugal elutriation requires an expensive elutriator. 2) Percoll density gradient centrifugation requires very short centrifugation times (< 30 min.). In contrast unit gravity sedimentation requires much longer sedimentation times varying from 1.25 up to 5 h. which may affect cell viability (34).

Table 4.1 A comparison of the results obtained with unit gravity sedimentation, centrifugal elutriation and density gradient centrifugation of adult normal rat anterior pituitary cells.

Method	Pituitary donor	Cell type	Cell enrichment (factor : x)	Reference
Unit gravity sedimentation	random cycle	mammotrope	1.9	36,109-110
	estrus		1.9	36,110
	diestrus		1.9	36,110
	36-45 days	somatotrope	2.2-2.6	101,104,109
	random cycle		2.0	110
	300-400 g		1.7	112
	adult		2.3	113,116
	300-400 g	gonadotrope	4.9-6.1	102,103,112
	adult		2.0	105,113,114,116
	200-250 g	thyrotrope	8.0	41,106
	adult		2.0	113,116
	400 g	corticotrope	3.5	115
Centrifugal elutriation	200-250 g	mammotrope	2.0	99,111
	200-250 g	gonadotrope	3.4-5.0	99,111
Density gradient centrifugation using percoll:	random cycle	mammotrope	1.6-1.7	37,117
	random cycle	somatotrope	2.0-3.3	37,117
	150-200 g		3.0	40
	400 g	corticotrope	3.8	115
using BSA:	65 days	somatotrope	2.2-2.5	39,95
	250 g		2.5	39,97,98,100,101,108
			2.5	107

Adapted and extended from Hymer and Hatfield (34).

Table 4.2 Properties of percoll and advantages for the separation of cells.

Properties	Advantages
1. Low osmolarity	Precise adjustment to physiological conditions with neglible interference from the medium.
2. Non-toxic	Separation of cells without loss of viability
3. Impermeable to biological membranes	No interference with the buoyant denstity of the cells and easy removal of the medium from recovered cells
4. Spontaneous gradient formation during centrifugation at high speed	Easy preparation of continuous percoll gradients.
5. Percoll gradients are stable over a broad temperature range	Cell separation can be performed at room temperature (minimum loss of cell viability).

In addition percoll density gradient centrifugation can be performed at room temperature whereas the separation of cells on BSA-density gradients is carried out at 4°C (34). Such low temperatures may be deleterious to the viability of particular pituitary cell types (96).

I.3.3.2 Separation of pituitary (adenoma) cells on continuous percoll density gradients.

Percoll density gradients were used for the separation of normal rat pituitary cells, rat pituitary tumor cells and pituitary adenoma cells from acromegalic patients as described in chapters III, IV and V respectively. Prior to the cell separation procedure continuous percoll density gradients are preformed by centrifugation of 8 mL of a percoll solution with a particular density at 25.300 x g during 30 min (room temperature). During this centrifugation-step the gradients are generated. 2 mL of a single pituitary (tumor) cell suspension is then layered on a preformed density gradient and the gradient plus cells is centrifuged at 800 x g during 20 min. Thereafter the

fractions are collected from the top of the gradient and washed two times in order to remove the Percoll. The subpopulations of cells with different densities in the various fractions are then cultured and basal- or secretagogue-induced hormone release can be studied in vitro.

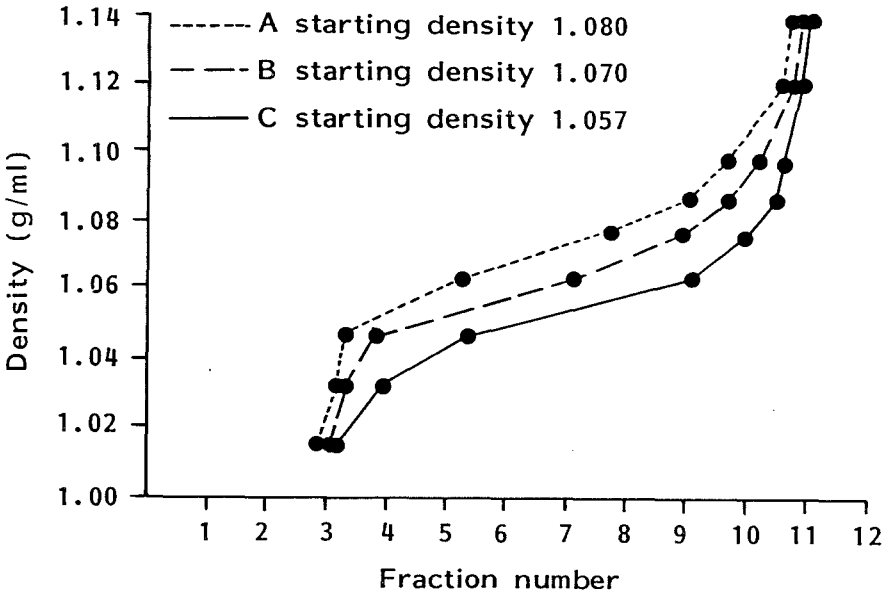


Fig. 4.5. Density distribution profile in three preformed Percoll density gradients. The different gradients were preformed by centrifugation of 8 ml of a Percoll solution with a particular density (A, B or C) in 12 ml Sorvall polypropylene tubes (8 mL Percoll solution per tube) during 30 min. at 25,300 x g in a Sorvall SS-34 rotor. Each point in the curves represents the position of marker beads with a particular density (see chapter 1.3.3.2).

In each experiment aliquots of nine different Percoll density marker beads, each having its own particular density in Percoll, were added to a control tube. This tube was treated the same as all other tubes. After the second centrifugation-step the distance of the marker beads to the meniscus of the fluid was measured. Figure 4.5 shows the position of the marker beads relative to the fraction numbers from the Percoll gradients that were used for the experiments described in chapters III (gradient A), IV (gradient B) and V (gradient C). Note that the linear part in the gradients allows to separate cells having only slight differences in their density.

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CHAPTER II

SCOPE OF THESIS

Hormone secretion by the anterior pituitary gland is under control of hypothalamic regulatory factors/hormones (see chapter I.1) and peripheral hormones. Apart from the direct effects of these hormones on anterior pituitary hormone secretion several fine- regulatory mechanisms may play a role in determining the ultimate response of hormone secretion by the anterior pituitary to the hypothalamic or peripheral feedback hormones. These fine- regulatory mechanisms include paracrine effects between anterior pituitary cell types (see chapter I.1.2.1) and functional heterogeneity within anterior pituitary cell types (see chapter I.1.2.2). Whether these fine- regulatory mechanisms may also occur within human pituitary adenomas is not known (see chapter I.2.3).

It has been suggested that differences in the hypothalamic input, due to a different intrapituitary localization of cell types, may be a basis for normal rat mammotrope heterogeneity. In addition it is not known whether a similar "mechanism" may cause functional heterogeneity as has been demonstrated within the normal rat somatotrope population (see chapter I.1.2.2). One of the aims of this thesis was to investigate whether normal rat mammotropes and somatotropes are functionally heterogeneous with respect to basal hormone release and responsiveness to secretagogues when cultured in the absence of hypothalamic influence, in order to provide additional evidence for the above hypothesis. In chapter III the results of experiments are described in which rat mammotrope and somatotrope subpopulations, separated according to differences in their density, were cultured during 7 days in the absence of hypothalamic hormones. Subsequently, these subpopulations were tested for their responses to the hypothalamic hormones which normally regulate the secretion of PRL and GH.

As has been mentioned in chapter I.2 functional heterogeneity of cells (cell types) within experimental rat

pituitary tumors and within human pituitary tumors has not been studied extensively yet. The second aim of this thesis was to investigate whether functional heterogeneity exists among cells of pituitary adenomas. We have used for our studies two types of pituitary adenomas, an experimental prolactin-secreting rat pituitary tumor and human GH-secreting pituitary adenomas. In chapters IV, V and VI the results of experiments concerning intratumor heterogeneity of an experimental rat pituitary tumor (chapter IV) and of GH-secreting human pituitary adenomas (chapters V and VI) are described. In chapter VII the results of a study concerning heterogeneity between GH- secreting pituitary adenomas from acromegalic patients are presented.

CHAPTER III

LONG-TERM CULTURE OF RAT MAMMOTROPH AND SOMATOTROPH SUB-POPULATIONS SEPARATED ON CONTINUOUS PERCOLL DENSITY GRADIENTS: EFFECTS OF DOPAMINE, TRH, GHRH AND SOMATOSTATIN.

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III.1 Abstract

Normal adult female rat mammotroph and somatotroph subpopulations were separated on continuous percoll density gradients according to differences in their density. Viable cells were recovered in 16 fractions. The cells from each fraction were cultured during 7 days after which period 4 hour incubations were performed. rPRL secretion per cell increased towards the higher density fractions. No major difference in TRH-, dopamine- and somatostatin-responsiveness was observed between mammotrophs that were recovered in the different gradient fractions. In addition no differences in somatostatin-responsiveness between the somatotroph cells in the different gradient fractions were observed. However, somatotrophs that were recovered in the highest density region of the gradient appeared to be more responsive to GHRH than the lower density somatotrophs. In the various gradient fractions there were no paradoxical effects of TRH and dopamine on rGH release and of GHRH on rPRL release. Conclusions: 1) In long-term cultures there is no evidence for functionally different subpopulations of mammotrophs and somatotrophs, separated according to differences in their density, with regard to dopamine- and TRH-responsiveness and with regard to somatostatin-responsiveness respectively. 2) There is no evidence for

a (mammosomatotroph?) subpopulation of cells showing paradoxical responses of PRL- or GH release to GHRH and dopamine or TRH respectively.

III.2 Introduction

Functional heterogeneity within the normal rat pituitary mammotroph and somatotroph population has been demonstrated by several techniques. Using the reverse hemolytic plaque assay the existence of subpopulations of somatotrophs and mammotrophs has been demonstrated, which differed in their responsiveness to growth hormone-releasing hormone (GHRH) and/or insulin-like growth factor I (IGF-I) (1-3) and thyrotropin-releasing hormone (TRH) and/or dopamine (DA) (4-6) respectively. By autoradiography, Walker et al. (7) identified a subpopulation of mammotrophs which preferentially released newly synthesized PRL. In addition Hopkins and Farquhar (8) observed differential incorporation of tritiated leucine into male somatotrophs and concluded that this cell population was functionally heterogeneous. Finally, cell separation experiments have demonstrated the existence of morphologically and functionally different subpopulations of mammotrophs (9-11) and somatotrophs (12-13). Another form of functional heterogeneity within these cell populations is the existence of mammosomatotrophs, which are cells that contain (14) and secrete (15-17) PRL and GH simultaneously. Boockfor and Frawley (6) recently presented evidence that PRL release from mammotrophs and mammosomatotrophs is regulated differently and that the ratio of these two cell types may dictate, in part, the manner in which a specific region of the pituitary responds to regulatory hypothalamic input (6).

Virtually all the studies indicated have in common that the pituitary tissue or the dissociated cells were used either directly in the experiments or that short-term cultures (up to 3 days) were used. In the present study we used Percoll density gradient separation of pituitary cells followed by long-term culture (7 days) in order to investigate whether 1) functional heterogeneity exists in

mammotroph and somatotroph cell subpopulation, separated according to differences in their density, when the cells are deprived from hypothalamic influence because of the longer period of culture, 2) evidence could be provided for a (mammosomatotroph?) cell population showing paradoxical responses of PRL- or GH release to GHRH and DA or TRH respectively.

III.3 Materials and Methods

Animals and cell dispersion

Female wistar rats, weighing 180-200 g, were kept in an artificially illuminated room (08.30-20.30 h) with food and water ad libitum. The animals, in any stage of the estrous cycle, were killed between 09.00 and 10.00 h by decapitation. The pituitary glands were removed within 5 min. after killing, the neurointermediate lobe was discarded, and the anterior lobes were collected in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS; GIBCO, Europe), supplemented with 10 g/l human serum albumin, penicillin (10^5 U/l), streptomycin (100 mg/l), fungizone (0.5 mg/l) and sodium bicarbonate (0.4 g/l). The anterior pituitary lobes were dissociated with Dispase, a neutral protease from Bacillus polymyxa (grade II, final concentration 2.4 x 10^3 U/l; Boehringer Mannheim, Mannheim, W. Germany) as described in detail elsewhere (18). Viability of the cells, as determined by trypan blue exclusion was greater than 80%.

Separation of dispersed cells on continuous density gradients

A 90% Percoll solution was made by mixing 9 parts of Percoll stock (Pharmacia; Uppsala, Sweden) with 1 part of 10 times concentrated Ca- and Mg-free HBSS. This 90% iso-osmotic Percoll solution was further diluted to 57% with Phosphate Buffered Saline (pH 7.4). Of this 57% Percoll solution 8 mL was then added to polypropylene tubes (Sorvall; 12 mL - 16 x 102 mm). Continuous sigmoidal shaped density gradients were pre-formed by centrifugation at 25.300 x g in

a Sorvall SS-34 rotor during 30 min. at 20°C. Approximately 1.5 x 10^7 cells in 2 mL HBSS + HSA were layered on the preformed gradients and then centrifuged at 800 x g during 20 min. at 20°C. The gradients were fractionated using an Auto Densi-Flow IIC (Searle; Bachler Instruments, Fort Lee, New Jersey, USA: US Pat. no. 3682305). Fractions of 30 sec. (approximately 400 µl per fraction; 24 fractions) were collected from the top of the gradients. Each fraction was then diluted 2 fold with HBSS + HSA and centrifuged during 5 min. at 600 x g. After two washsteps in HBSS + HSA the cells were resuspended in culture medium (see below). Counting of the cells in each fraction was done with a Bürker counting chamber. Viability of the cells was determined by trypan blue exclusion.

Recovery of the cells from the gradients was 90 ± 4% (n = 10 experiments). The exact density of each fraction was determined by measuring the refractive index, which has a linear relation with the density of the Percoll solution.

Cell culture

The cells of the original cell suspension and of each fraction were cultured at a density of 10^5 cells per well per 1 mL in 48-well plates (Costar, Cambridge, Massachusetts, USA). On day 4 of culture the medium was changed and after another medium change on day 7 of culture 4 hour incubations without or with secretagogues were performed in quadruplicate. The culture medium consisted of Minimal Essential Medium with Earle's salts (MEM) supplemented with non-essential amino acids, sodium pyruvate (1 mmol/l), 10% fetal calf serum, penicillin (10^5 U/l), streptomycin (100 mg/l), fungizone (0.5 mg/l), L-glutamine (2 mmol/l) and sodium bicarbonate (2.2 g/l final concentration). The medium was adjusted to pH 7.4 with 1 mol/l NaOH. This medium obtained as a 10-times concentrated solution, and supplements were purchased from GIBCO (Paisley, Scotland).
Dopamine (DA) and somatostatin (SRIH) were obtained from Sigma Chemical Company (St. Louis, MO, USA); TRH from Hoechst (Amsterdam, the Netherlands) and growth hormone releasing hormone (GHRH)

from Universal Biologicals (Cambridge, U.K.).
Intracellular hormone concentrations at the end of the incubation period were measured in cell extracts obtained by lysis of the cultured cells in distilled water containing 1 g/l bovine serum albumin (Sigma), followed by repeated freeze-thawing, as described in detail elsewhere (18).

Reverse hemolytic plaque assay (RHPA)

The RHPA was performed as described in detail elsewhere (19). In short, the cells of the Percoll gradient fractions were trypsinized from Petri dishes at day 1 of culture and resuspended in MEM containing 1 g/l bovine serum albumin (BSA; Sigma).

A suspension of 200.000 cells/ml was mixed with an equal volume of a 12% suspension of protein A-conjugated ovine red blood cells (oRBC). This cell mixture was infused into Cunningham chambers, which were prepared on poly-L-lysine coated glass slides. After 1 hour of incubation at 37°C in a CO_2-incubator the chamber was rinsed twice with MEM + 1 g BSA/l. Thereafter incubations were started by infusing anti-rat PRL serum (1:50 final dilution in MEM + BSA). The anti-PRL serum was a kind gift of Dr. D.A. Leong (University of Virginia, Charlottesville, U.S.A.). Specificity of this antiserum was described previously in detail (16). After 2 hours of incubation the chambers were filled with immunoglobulin-stripped guinea pig complement (for method see 16) at a dilution of 1:40 in MEM + BSA. After 45 minutes of incubation with complement, during which time plaque formation occurred, the reaction was stopped by infusing fixative (2% glutaraldehyde in phosphate buffered saline, pH 7.4). The pituitary cells were stained with hematoxylin-solution in order to ensure the detection of non plaque-forming cells. All incubations were performed on duplicate slides and 100 cells per slide were counted. The choice of an incubation time of 2 hours was based on several experiments using unfractionated cell suspensions from female rat anterior pituitary cells. In these experiments (data not shown) 2 hours of incubation were needed in order to obtain the maximal percentage of plaque-forming cells.

Hormone assays

Rat GH (rGH) and rat prolactin (rPRL) concentrations in the culture media and cell extracts were determined using double-antibody RIA's as described elsewhere (18,20). Rat TSH (rTSH) in the culture media and cell extracts was measured by a double antibody RIA using materials and protocols supplied by the distribution officer of the NIDDK. rTSH concentrations are expressed in rTSH reference preparation RP-2. Rat FSH (rFSH) and rat LH (rLH) concentrations were determined as described in detail elsewhere (21). ACTH was measured by a commercial RIA-kit purchased from Eurodiagnostics (Apeldoorn, the Netherlands). All hormone concentration are expressed in μg/l.

Statistical analysis

The statistical significance of the differences between mean values was determined using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made by the Newman-Keuls test (22). All data are expressed as mean $\pm$ SEM. When the responsiveness to secretagogues was compared between the gradient fractions all values were expressed in percentage of control release from corresponding cells prior to statistical analysis. Each experiment is representative of at least three independent experiments unless indicated otherwise.

III.4 Results

Cell distribution and hormone profiles in the Percoll gradient fractions

In Fig. 1 the distribution of the percentages of recovered viable cells in the gradient fractions is shown. The majority of the cells was recovered in fractions 8-12 (density range: 1.026-1.057 kg/l). The rest of the cells was equally distributed in the fractions 13-19 (density range: 1.062-1.082 kg/l). The method of separation on the Percoll

gradients was highly reproducible since the values represent the mean of 10 independent experiments.

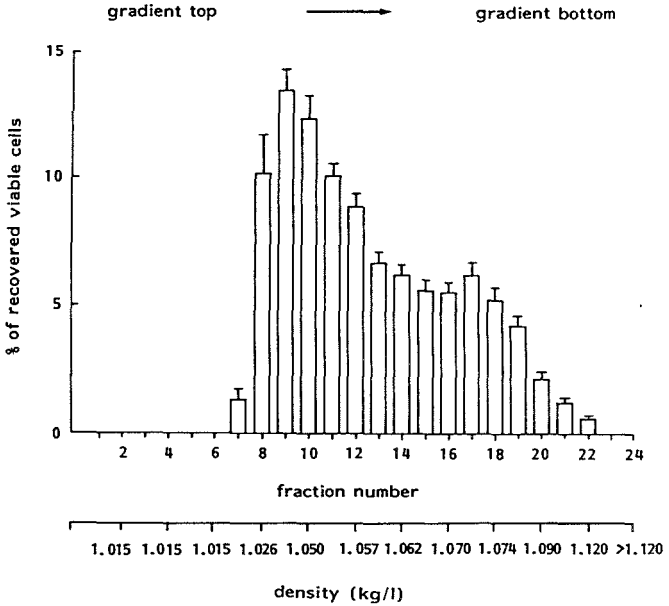

Fig. 1 Distribution of recovered viable cells after separation of female rat anterior pituitary cells on a continuous Percoll density gradient. Data represent the mean ± SEM of 10 independent experiments. Cell recoveries averaged 90 ± 4% in these experiments.

In three experiments we determined rPRL, rGH, rFSH, rLH, rTSH and ACTH in the media and cell extracts of the gradient fractions and of the original cell suspension. Fig. 2 shows the data from a representative experiment. The interrupted horizontal line which is drawn in each hormone profile represents the amount of hormone release (left part of the figure) and the amount of intracellular hormone (right part) of the original (unseparated) cell suspension. The unseparated cells were cultured 7 days under the same conditions as the fractions, before measurement of hormone release. All values are expressed in μg hormone/l per 10^5

cells. Most of the rPRL was recovered in the low density region (1.026-1.053 kg/l) while most of the rGH was recovered in the high density region (1.072-1.099 kg/l). As compared to the original cell suspension rPRL and rGH showed in these regions a 2- and 3-fold enrichment, respectively. Similar distribution patterns of rPRL and rGH were observed in cell extracts of each fraction (10^5 cells) directly after isolation (not shown). The profiles of rFSH, rLH and rTSH were virtually superimposable and showed a peak in the middle density region (fractions 12-16, density range 1.057-1.070 kg/l). In contrast most of the ACTH was recovered in the low density region (1.053-1.062 kg/l).

We also determined by the reverse hemolytic plaque assay the percentage rPRL-secreting cells in 1 min gradient fractions (800 μl per fraction). The results of a representative experiment are shown in table 1. rPRL release and intracellular rPRL concentrations decrease from the low density fractions to the high density fractions. The ratio of rPRL release: intracellular rPRL remains constant over all the fractions. In the fifth column the percentage of rPRL-secreting (plaque-forming) cells is shown. The percentage of rPRL-secreting cells decreases from 79.1% in fraction 4 to 1.7% in fraction 9. Interestingly the amount of rPRL release per cell (last column in table 1) increased from the low density fractions 4 and 5 to the higher density fractions 6, 7 and 8.

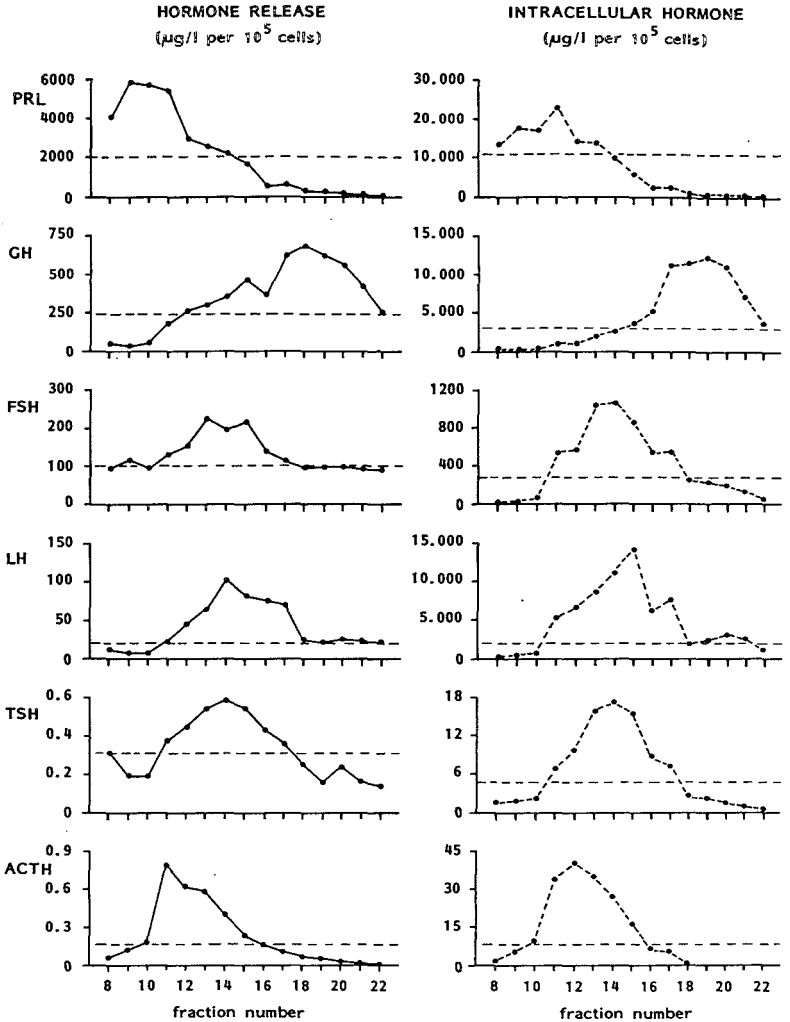

Fig.2: Distribution profiles of anterior pituitary hormones in the gradient fractions after separation of female rat anterior pituitary cells on a continuous Percoll density gradient. The cells from the various fractions were cultured for 7 days in MEM + 10% FCS (10^5 cells per well). On day 7 of culture 4 hour incubations were performed in quadruplicate (medium; ●——●). At the end of the 4 hour incubation period the cells were lysed in distilled water containing 0.1% BSA (intracellular hormone; ●----●). In the fractions 1-7 and 23-24 not enough cells were recovered (see Fig. 1) in order to perform secretion studies on basal hormone release. The horizontal interrupted line in each profile represents the hormone release (left part) and the intracellular hormone concentration from the original (unseparated) cell suspension.
Values are means; n = 4 wells per group; standard errors were always less than 10%.

Table 1

Heterogeneity of rPRL release per cell in percoll gradient fractions of female rat anterior pituitary cells.

fraction number	rPRL release (µg/l per 10^5 cells per well)	intracellular rPRL (µg/l per 10^5 cells per well)	ratio release/ intracellular rPRL	number (%) rPRL-secreting cells/well	rPRL release per cell (pg/cell)
4 (7 ± 8)	2351 ± 208	7723 ± 386	0.30	79.100 (79.1%)	29.7
5 (9 ± 10)	1683 ± 126	7253 ± 298	0.23	66.700 (66.7%)	25.2
6 (11 ± 12)	2555 ± 94	10111 ± 311	0.25	38.000 (38.0%)	67.2
7 (13 ± 14)	756 ± 27	2680 ± 206	0.28	12.600 (12.6%)	60.0
8 (15 ± 16)	113 ± 8	507 ± 12	0.22	1.700 (1.7%)	66.5
9 (17 ± 18)	82 ± 6	274 ± 6	0.30	- (not done)	-

Female rat anterior pituitary cells were separated on a continuous percoll density gradient. After separation the gradients were fractionated in 1 min. fractions (approximately 800 µL per fraction; 12 fractions). The fraction numbers shown in the first column in the table represent the combination of two 30 sec fractions. The fraction numbers of the 30 sec fractions are in parenthesis. Culture conditions were the same as described in Fig. 2 The number of rPRL-secreting cells per well (column five) was calculated from the percentages of rPRL-plaque forming cells (values in parenthesis in column five) as determined by the reverse hemolytic plaque assay (see materials and methods).

Effect of secretagogues on rPRL and rGH release by the Percoll gradient fractions

We also studied the effect of maximally active concentrations of TRH, DA, GHRH and SRIH (18,23) on rPRL and rGH release by the gradient fractions. In the fractions 1 to 7 and 20 to 24 not enough cells were recovered to perform extensive secretion studies. In Fig. 3 the effect of 100 nmol/l TRH and 500 nmol/l DA on the gradient fractions 8 to 16 is shown. Fractions 17 to 19 are not shown because the amount of rPRL release was to low to measure accurately. TRH significantly stimulated rPRL release by all fractions (all $p < 0.01$ vs. control). The fractions were significantly different in responsiveness to TRH as determined by ANOVA ($p < 0.05$). The maximal difference in TRH-responsiveness (on a percentage base) between the fractions was 64% (fraction 8 vs. fraction 15). 500 nmol/l DA significantly inhibited rPRL release by all fractions (Fig. 3; all $p < 0.01$ vs. control). Again the fractions were significantly different in responsiveness as determined by ANOVA ($p < 0.001$). The maximal difference between the fractions in DA-responsiveness was 18% (fraction 11 vs. fraction 16). The small differences in TRH- and DA- responsiveness among the fractions were not the same, fraction by fraction, in independent experiments. TRH and DA did not affect rGH release by gradient fractions 8 to 16 (not shown). Fig. 4 shows the effect of 10 nmol/l GHRH and 10 nmol/l SRIH on rGH release by the gradient fractions 10 to 19. Fractions 9 and 10 are not shown because the amount of rGH release by these fractions was to low to measure accurately. 10 nmol/l GHRH significantly stimulated rGH release by all fractions (all $p < 0.01$ vs. control). The fractions were significantly different in GHRH-responsiveness ($p < 0.001$ by ANOVA). The maximal difference in GHRH-responsiveness between the fractions was 243%. In this experiment the higher density fractions were significantly more responsive to GHRH than the lower density fractions. Similar results were found in two other experiments. However, in three experiments we observed no such differences in GHRH-

responsiveness between the low- and high-density fractions. In the latter experiments all fractions responded equally well to GHRH (data not shown). 10 nmol/l SRIH significantly inhibited rGH release by all gradient fractions (Fig. 4; all $p < 0.01$ vs. control). The fractions were significantly different in SRIH-responsiveness ($p < 0.001$ by ANOVA). The maximal difference between the gradient fractions was 26% (fraction 16 vs. fraction 19). In independent experiments these differences were not the same, fraction by fraction. GHRH did not affect rPRL release by the gradient fractions 10 to 19 (not shown).

III.5 Discussion

In the present study we separated normal female rat anterior pituitary cell types according to differences in their density on continuous Percoll density gradients. We studied in 7-day monolayer cultures the effects of TRH, DA, GHRH and SRIH on rPRL and rGH release by mammotrophs and somatotrophs respectively, present in the various gradient fractions. Previous studies have demonstrated that Percoll density gradient centrifugation of anterior pituitary cells is a simple and rapid method for the enrichment of pituitary cell types (13, 24-26). Two groups of investigators so far, have described the enrichment of normal female rat mammotrophs and somatotrophs on Percoll density gradients (10,13,26). Our present study basically confirms the latter two studies with regard to the densities of the various anterior cell types. However, in contrast to these studies we did not study hormone release by two or three cell bands (10,13,26) but fractionated the total gradient in order to study the hormonal secretion behaviour of mammotrophs and somatotrophs over a broad density range. We found that the mammotrophs and somatotrophs in the gradient fractions retained their functional integrity. rPRL release by all fractions was significantly inhibited by DA and stimulated by TRH. We showed that the gradient fractions differed in the amount of rPRL released per rPRL-secreting cell.

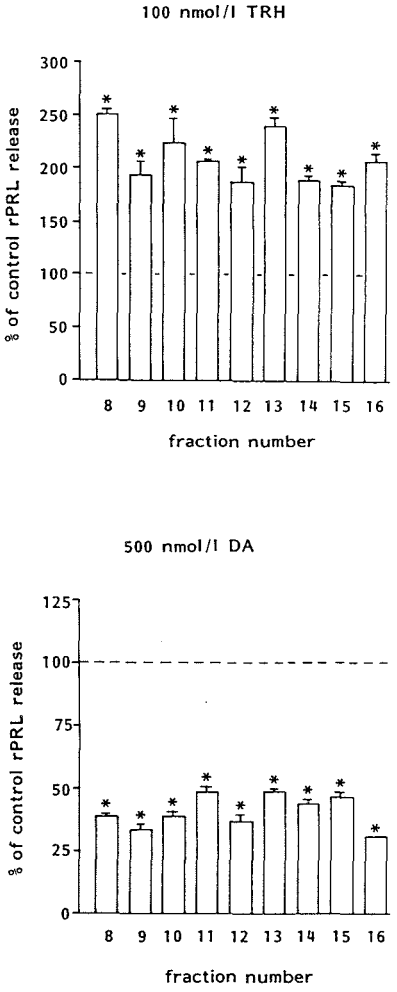

Fig. 3 Effect of 100 nmol/l TRH and 500 nmol/l DA on rPRL release by the gradient fractions after separation of female rat anterior pituitary cells on a continuous Percoll density gradient. The values for each fraction are expressed as percentages of control release from corresponding fractionated cells (without TRH or DA). Culture conditions were the same as described in Fig. 2. Values are means ± SEM, n= 4 wells per group.

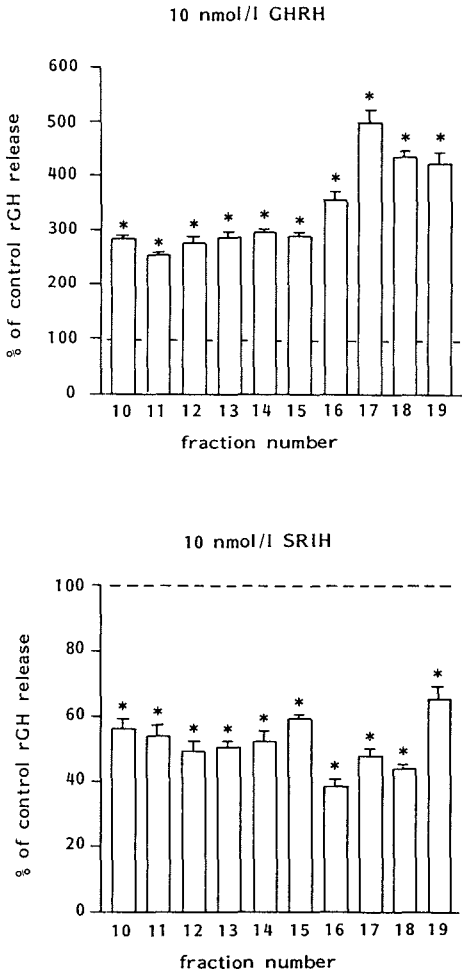

Fig. 4 Effect of 10 nmol/l GHRH and 10 nmol/l SRIH on rGH release by the gradient fractions after separation of female rat anterior pituitary cells on a continuous Percoll density gradient. The values for each fraction are expressed as percentages of control release from corresponding fractionated cells (without GHRH or SRIH). Culture conditions were the same as described in Fig. 2. Values are means ± SEM; n = 4 wells per group.

Interestingly we found no major differences among these gradient fractions in their responsiveness to DA and TRH. Therefore, we have no evidence for functional heterogeneity within the normal adult female mammotroph population in long-term culture with regard to TRH- and DA-responsiveness. In agreement with our data Velkeniers et al. (10) did also not observe differential DA-responsiveness between Percoll gradient fractions of female anterior pituitary cells, which differed in the amount of rPRL production. Walker and Farquhar (7) showed in double isotope labeling experiments using 48 h pituitary monolayer cultures that rPRL is synthesized and secreted very rapidly by a minor subpopulation of the PRL-cells. In addition these cells also appeared to be unresponsive to TRH. These investigators proposed that these non-responsive cells are already synthesizing and releasing rPRL at a maximal rate and hence cannot respond further, or alternatively, they may possess few or no receptors for TRH, or the receptors could be masked in some way. The results of our study are in favour of the latter two possibilities concerning the receptor status of the cells. In our experiments the cells of the gradient fractions all responded equally to TRH and DA, despite their differences in rPRL secretion per cell. This seems contradictory to the experiments by Walker and Farquhar (7). However, in contrast to their study, in which 48 h cultures were used, we used 7-day cultures. It might be suggested therefore, that the differences in TRH-responsiveness between mammotroph subpopulations, which have been demonstrated in short-term cultures (1-3 days) by Walker and Farquhar (7) and by others using the reverse hemolytic plaque assay (4-6), may disappear in long-term culture. During this period the cells are deprived from their hypothalamic regulatory input. In this respect the recent study by Boockfor and Frawley (6), who showed that rPRL release by PRL-secreting cells in different anterior pituitary regions is regulated differently by TRH and DA, is of potential interest. In their study they provided evidence that the hypothalamic influence might be a basis for mammotroph heterogeneity (6). Unfortunately we were not able to perform reproducible secretion studies after a

short period of culture since cell attachment needed a culture period of at least 3 days in our culture system (18). The results from these experiments were highly variable, both within and among the experiments. Although further investigations with regard to the comparison of functional heterogeneity within the mammotroph cell population in short- and long-term culture are needed we propose that deprivation of a possible functionally heterogeneous mammotroph population from hypothalamic input might result in a homogeneous mammotroph cell population. Thus, functional heterogeneity between mammotroph subpopulations might be a temporary state of these cells which is able to change under different physiological conditions. An other explanation for the homogeneous response of the cells to TRH or DA may be that, following 7 days of culture, possible differences between various fractions could have been masked either by cell proliferation resulting in a redistribution of cell functions or by a redistribution of cell function within individual cultures. We cannot exclude the latter possibilities. Snyder et al. (12), who separated male rat anterior pituitary cells on BSA-density gradients obtained subpopulations of somatotrophs which differed in their responsiveness to secretory agents as thyroxine and hydrocortisone. In three experiments we found that the high-density somatotrophs were significantly more responsive to GHRH than the lower density somatotrophs while we observed no differences in SRIH-responsiveness between these somatotroph subpopulations. However, the difference in GHRH-responsiveness was not consistently found in all experiments. In some experiments rGH-release by all fractions responded equally to GHRH. As yet we do not have an explanation for these inconsistent results. The differential GHRH-responsiveness which we found in part of the experiments is well in agreement with data by Frawley and Neill (1) who showed, using the reverse hemolytic plaque assay, that GHRH-treatment of short-term cultured adult female anterior pituitary cells caused a shift in the frequency distribution of individual GH-plaque areas from an unimodal to a bimodal distribution. Thus, they provided evidence for a subpopulation of somatotrophs preferentially responsive to

this secretagogue (1).

We found no paradoxical effects of DA and TRH on rGH secretion by the gradient fractions containing mammotrophs and of GHRH on rPRL secretion by the gradient fractions containing somatotrophs. Recently, several studies have demonstrated the existence of mammosomatotroph cells, which are cells containing (14) and secreting (15-17) both PRL and GH. In addition PRL secretion by mammotrophs and mammosomatotrophs might be regulated differentially (6). No studies have been performed so far, on possible effects of DA or TRH and of GHRH, on PRL and GH secretion respectively, by mammosomatotroph cells. Our results do not support the possibility that these secretagogues affect PRL or GH release paradoxically. Although, we cannot conclude from our experiments in which gradient fractions mammosomatotroph cells are present, we have no evidence from our experiments for a mammosomatotroph population which is regulated differently as compared to the pure GH- and PRL-secreting cells. However, the long-term culture of the cells might have caused a shift in the number of mammosomatotrophs since changes in the physiological environment of the cells may cause a shift in the proportion of GH-, PRL and GH + PRL-secreting cells (17).

The conclusions which can be drawn from our experiments are: 1) mammotroph and somatotroph subpopulations separated according to differences in density are after long-term culture not functionally heterogeneous with regard to DA/TRH- and SRIH- responsiveness respectively. 2) there is no evidence for a (mammosomatotroph?) subpopulation of cells showing paradoxical responses of PRL- or GH release to GHRH and DA or TRH respectively.

III.6 References

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CHAPTER IV

PERCOLL DENSITY GRADIENT CENTRIFUGATION OF RAT PITUITARY TUMOR CELLS: A STUDY OF FUNCTIONAL HETEROGENEITY WITHIN AND BETWEEN TUMORS WITH RESPECT TO GROWTH RATES, PROLACTIN PRODUCTION AND RESPONSIVENESS TO THE SOMATOSTATIN ANALOG SMS 201-995.

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IV.1 Abstract

Tumor cells prepared from PRL- secreting rat pituitary 7315b tumors of increasing weight were separated on continuous Percoll density gradients, according to differences in their density. It was investigated whether the cell subpopulations obtained by density gradient separation showed differences in protein content per cell, PRL production per cell, growth rates and responsiveness to the somatostatin analog SMS 201-995 in vitro. In addition we studied PRL release by individual 7315b tumor cells, using the reverse hemolytic plaque assay (RHPA).

The tumor cells from the tumors of increasing weight were recovered within a narrow density range (1.060-1.070 g/mL) and showed a normal distribution profile. Between the subpopulations there were no differences with respect to the parameters mentioned above. Moreover, no differences were found with respect to these parameters, between tumor cells derived from tumors of increasing weight. In agreement with the above data we found no evidence for subtypes of adenoma cells being preferentially responsive to SMS 201-995, using the RHPA. Conclusions: 1) the transplantable PRL- secreting rat pituitary tumor 7315b consists of a functionally homogeneous cell population; 2) growth of this tumor in vivo does not lead to the induction of functionally heterogeneous cell subpopulations within this tumor; 3) the escape of this tumor from the tumor growth- inhibitory effect of SMS 201-995

as has previously been demonstrated in vivo, may not have been the result of clonal selection of somatostatin-unresponsive cells.

IV.2 Introduction

Most murine and human solid tumors show intratumor heterogeneity. Tumor cell populations can be heterogeneous for many phenotypic characteristics such as karyotype, biochemical profile (e.g. levels of various enzymes), hormone receptor content and drug- or radiosensitivity [1]. Little is known however, with respect to functional heterogeneity within pituitary tumors. Although it has been demonstrated that both human and rat pituitary tumors may consist of subpopulations of cells secreting more than one hormone simultaneously [2-7], a possible differential responsiveness of these tumor cell subpopulations to hypothalamic regulatory hormones or to drugs has not been investigated extensively. In addition it is not known whether, in case cell proliferation occurs in vitro, all cells within a pituitary tumor cell suspension show similar growth rates. Knowledge of intratumor heterogeneity of pituitary tumors can be helpful in the understanding of the effects of certain drugs on the growth of the tumor cells or on the regulation of hormone release by these tumor cells in vivo and in vitro. The somatostatin analog SMS 201-995 has previously shown to inhibit the growth of the transplantable prolactin (PRL)- secreting rat pituitary tumor 7315a in vivo [8]. However, this tumor rapidly "escaped" from the tumor growth- inhibitory effect of SMS 201-995. In this study [8] it could not equivocally be excluded whether this "escape" might have been due to clonal selection of somatostatin-unresponsive tumor cells. The aim of the present study was to investigate whether functional heterogeneity exists within and between 7315b PRL- secreting rat pituitary tumors of increasing weight. For this purpose we used Percoll density gradient centrifugation of acutely dispersed 7315b tumor cells in order to obtain subpopulations of tumor cells with different densities. It was investigated whether the subpopulations of tumor cells differed in a) growth rates b)

protein and PRL- content of the cells c) responsiveness of the tumor cells to the inhibitory effect of the somatostatin analog SMS 201-995. Since pituitary tumor cells do not store large amounts of hormone and because the density of the cells may be dependent on the mass of protein hormone present in the cytoplasm [9] the method of density gradient separation may not be an appropriate method for the study of functional heterogeneity among cells of the 7315b tumor with respect to PRL release. Therefore, we also investigated PRL release by 7315b tumor cells at the single cell level, using the reverse hemolytic plaque assay (RHPA) [10].

IV.3 Materials and methods

Animals, tumor growth in vivo and preparation of dispersed tumor cells

The origin of the transplantable PRL- secreting 7315b rat pituitary tumor is described in detail elsewhere [11]. This tumor originates from the ACTH-PRL- secreting 7315a tumor but has lost its ability to secrete ACTH as well as its receptors for glucocorticoids. It is unchanged with respect to PRL secretion, estrogen- and progesterone receptor content [11]. Female buffalo rats (Harlan, Madison, USA) weighing 160-180 g were inoculated subcutaneously between the scapulae with 0.2 mL of a suspension of the 7315b tumor. This suspension was prepared by mincing twenty grams of tumor tissue in 50 mL sterile saline (9 g NaCl/L). The rats were kept in an artificially illuminated room (09.00-21.00 h) with food and water ad libitum. 2, 3 And 4 weeks after inoculation of the tumor cell suspension rats were killed by an overdose of ether anesthesia and tumors of 7, 11 and 40 grams were removed and collected in sterile saline.

The 7315b pituitary tumor cells were isolated by mechanical dispersion as described in detail elsewhere [11]. Viability of the resulting cell suspension was always greater than 90%. The cells were resuspended either in culture medium (for culture of the original, unfractionated cell suspension) or in Hank's Balanced Salt Solution (HBSS) supplemented with

10 g/L human serum albumin (HSA), penicillin (10^5 U/L), streptomycin (100 mg/L), fungizone (0.5 mg/L) and sodium bicarbonate (0.4 g/L). The latter cell suspension was used for Percoll gradient separation.

Separation of dispersed cells on continuous Percoll density gradients

A 90% Percoll solution was made by mixing 9 parts of Percoll stock solution (Pharmacia Fine Chemicals Uppsala, Sweden) with 1 part of a 10 times concentrated calcium- and magnesium-free Hank's Balanced Salt Solution (GIBCO, Europe). This 90% iso-osmotic Percoll solution was further diluted to 50% (density 1.070 g/mL) with Phosphate Buffered Saline (pH 7.4). 8 mL of the 50% Percoll solution was then added to polypropylene tubes (Sorvall; 12 mL - 16 x 102 mm). To one tube, which was treated the same as all other tubes, calibration density marker beads (no's 2-9, Pharmacia, Uppsala, Sweden) were added. Gradients were pre-formed by centrifugation at 25,300 x g in a Sorvall SS-34 rotor during 30 minutes at 20°C. For cell-separation approximately 1.5 x 10^7 to 2 x 10^7 cells in 2 mL HBSS + HSA were layered on the pre-formed gradients and then centrifuged at 800 x g during 20 min. at 20°C. The gradients were fractionated using an Auto Densi-Flow II C (Searle, Bachler Instruments, Fort Lee, New Jersey, U.S.A.; US Pat. No. 3682305). 12 fractions (833 μL per fraction) were collected from the top of the gradients. Each fraction was then diluted (2 times) with HBSS + HSA and centrifuged for 5 min. at 600 x g. The cells were washed two times more with HBSS + HSA and finally resuspended in culture medium (see below). Counting of the cells in each fraction was done with a Bürker counter chamber. Viability of the cells was determined by trypan blue exclusion.

After centrifugation calibration of the formed density gradients was done by measuring the distance of each density marker bead to the meniscus of the Percoll fluid. Cell recovery from the gradients yielded 86 $\pm$ 8% (mean $\pm$ SE; n=3 independent experiments)

Cell culture

The culture medium used in all experiments is Minimal Essential Medium with Earle's salts (MEM) supplemented with MEM non-essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin (10^5 U/L), streptomycin (100 mg/L), fungizone (0.5 mg/L), L-glutamine (2 mmol/L) and sodium bicarbonate (2.2 g/L final concentration). The medium was adjusted to pH 7.4 with 1 mol/L NaOH. The 7315b pituitary tumor cells were seeded at a density of 25,000 viable cells per well in 1 ml of culture medium in 24-well plates (Costar, Cambridge, Massachusetts, USA) without or with SMS 201-995. After 6 days of culture the media and cells were collected and stored at -20°C until analysis. Medium and supplements were purchased from Grand Island Biological Co. Europe (Paisley, Scotland). SMS 201-995 was a gift by Sandoz (Basel, Switzerland).

Reverse hemolytic plaque assay (RHPA)

The reverse hemolytic plaque assay was performed as described in detail elsewhere [10]. In short, freshly dispersed (unseparated) and 6-day cultured cells (which had not attached to the floor of the wells) were harvested, centrifuged and resuspended in MEM + 0.1% bovine serum albumin (BSA; SIGMA). A suspension of 150,000 cells/mL was mixed with an equal volume of a 12% suspension of protein A-coated ovine red blood cells (oRBC). This cell mixture was infused into Cunningham chambers, which were prepared on poly-L-lysin coated glass slides. After 1 h of incubation at 37°C in a CO_2-incubator the chambers were rinsed twice with MEM+BSA. Thereafter incubations were started by infusing rabbit anti-rat PRL serum (1:50 final dilution in MEM+BSA) without or with 10 nmol/L SMS 201-995. The anti-rat PRL serum was a kind gift of Dr. D.A. Leong (University of Virginia, Charlottesville, USA). Specificity of this antiserum was described previously in detail [12]. After 24 h of incubation at 37°C in the CO_2-incubator the chambers were filled with immunoglobulin-stripped guinea pig complement [12] at a dilution of 1:20 in MEM+BSA. The choice of an incubation time

of 24 h was based on several experiments using increasing incubation times (not shown). After 45 min. of incubation with complement, during which time plaque formation occurred, the reaction was stopped by infusing fixative (2% glutaraldehyde in phosphate buffered saline, pH 7.4). The 7315b cells were stained with hematoxylin in order to ensure the detection of non-plaque forming cells. All incubations were performed on duplicate slides and 100 cells per slide were counted. Plaque areas were measured with a calibrated ocular micrometer. A Leitz Diavert micoscope was used at 400-fold magnification.

Assays

Rat PRL concentrations in the culture media was measured by a double antibody RIA using materials and protocols supplied by the distribution officer of the NIADDK. All results are expressed in rat PRL reference preparation (RP-1).

The DNA content of the tumor cells was determined as described in detail elsewhere [13]. The method is based on a DNA dependent fluorescence enhancement of a fluorochrome. In short, freshly isolated tumor cells (25,000 cells) or the cultured tumor cells at the end of the incubation period (the latter cells did not attach to the floor of the wells) were collected and washed twice with an ice-cold saline solution. The remaining cell pellet was stored at -20°C until analysis. The cells were extracted with 300 µL ammonia solution (1 mol/L)- Triton X100 (0.2 % v/v) by sonification during 5 seconds at amplitude 15 (Soniprep 150; MSE). Thereafter 2 mL assay buffer (100 mmol/L NaCl, 10mmol/L EDTA, 10mmol/L Tris; pH 7.0) was added. The remaining solution was centrifuged at 2000xg during 5 min and 100 µL aliquots of the supernatant were mixed with 1.5 mL Hoechst dye H33258 (100 µg/L). Fluorescence was measured after 15 min with the excitation and emission wavelengths set at 350 nM and 455 nM respectively. Fluorescence of experimental samples was referenced to a standard curve of calf thymus DNA (type II, no D-3636; Sigma Chemical Company, StLouis, MO, USA).

The protein content of the tumor cells was estimated using the reagent kit from Bio-Rad (Richmond, CA) with BSA (Sigma) as standard. For estimation of the protein content of the cells the same cell extracts were used as those used for DNA- determination.

Analysis of data

The statistical significance of the differences between mean values was determined using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made using the Newman-Keuls test [14]. All data are expressed as mean $\pm$ SEM. We considered a difference between mean values to be statistically significant when $p<0.01$.

"Growth rates" of the cells were calculated as follows: The number of doublings in 144 h is b-a in which a= $\log_2$ (DNA t=0h) and b= $\log_2$ (DNA t=144h). This method of calculation of the growth rate of cells has been previously described by Patterson [15].

IV.4 Results

Fig. 1 shows the distribution of the recovered viable cells after separation of 7315b tumor cell suspensions derived from tumors of increasing weight on continuous Percoll density gradients. In the figure the density profiles of the gradients are also indicated. The profiles of the recovered cells from the different tumors were completely similar in that they all showed a normal distribution with the majority of the cells being recovered in a narrow density range between densities of 1.060 and 1.070 g/mL. Since in the fractions 4 to 6 and 9 to 11 not enough cells were recovered in order to perform culture studies we used pooled fractions. With these pooled fractions the experiments as described below were performed. The pools are indicated in Fig. 1 by I, II and III on the x-ordinate.

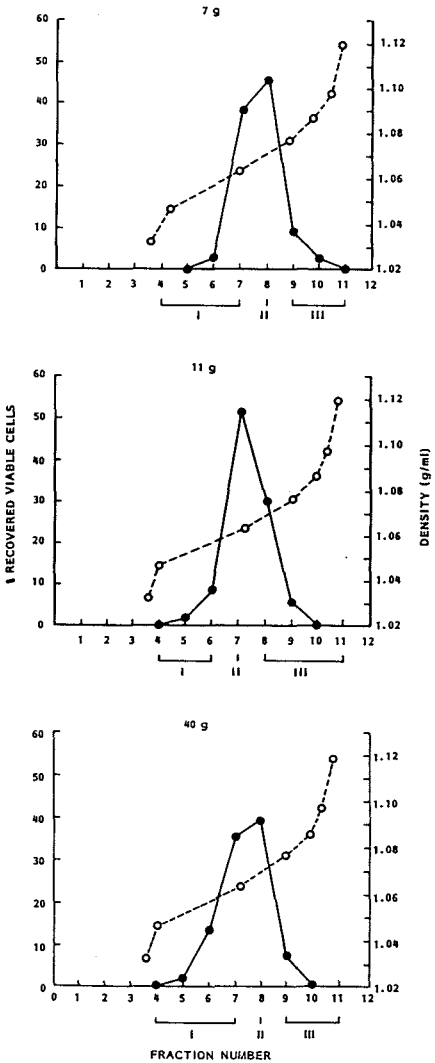

Fig.1 Profiles of recovered viable cells in the gradient fractions after separation of 7315b tumor cells derived from tumors of increasing weight (7, 11 and 40 grams) on continuous Percoll density gradients (●——●). The density profile of the Percoll gradient is indicated by the interrupted line (O-----O). Each point on this density profile represents the position of density marker beads each having a specific density in the gradient. The symbols I, II and III which are indicated on the x-ordinate represent the pooled fractions that were used in the 6-day culture studies (pool I, II and III-cells).

In none of the tumors there were statistically significant differences in the amount of protein per ng DNA between pool I, II and III-cells. This is shown in Table 1. In addition we found no statistically significant difference of protein per DNA between the tumors of different weight (Table 1).

Table 1: Protein content per ng DNA of percoll gradient fractions (directly after isolation) of 7315b tumors with increasing weight.

	7 g	11 g	40 g
	protein/DNA (µg/ng)	protein/DNA (µg/ng)	protein/DNA (µg/ng)
Original suspension	348±32	550±30	482±57
Pool I-cells	534±37	683±58	542±22
Pool II-cells	426±17	568±46	532±22
Pool III-cells	441±35	609±31	550±42

Values in mean ± SE; n=4.
Cell suspensions derived from 7315b tumors with increasing weight were separated according to differences in their density on continuous Percoll density gradients. Several gradient fractions were pooled in order to obtain pool I, II and III-cells (see also legends to Fig. 1). Aliquots from each suspension containing 25,000 viable cells were analyzed for protein and DNA-content.

Table 2 shows that the amount of DNA significantly increases from t = 0 h to t = 144 h of culture (in all instances $p < 0.01$) indicating that the cells are growing during this period. In order to compare the "growth rates" of the different tumors (7, 11 and 40 grams) and pool I, II and III-cells we calculated for each suspension the number of doublings which took place during the 144 h culture period. Calculation of the number of doublings was done according to the method described in the materials and methods section.

Table 2: Number of doublings in the original cell suspensions and in the Percoll gradient fractions after 6 days (144 h) of culture

Tumor weight	Cell suspension	DNA (t = 0 h) (ng)	DNA (t = 144 h) (ng)	"growth rate" (number of doublings in 144 h)
7 g	Original susp.	332 ± 10	948 ± 21[a]	1.51 ± 0.03
	Pool I-cells	267 ± 6	456 ± 13[a]	0.77 ± 0.05
	Pool II-cells	418 ± 20	1597 ± 53[a]	1.93 ± 0.05[b,c]
	Pool III-cells	386 ± 23	858 ± 25[a]	1.15 ± 0.04[b]
11 g	Original susp.	293 ± 6	1007 ± 51[a]	1.77 ± 0.07
	Pool I-cells	203 ± 12	368 ± 12[a]	0.85 ± 0.05
	Pool II-cells	270 ± 4	913 ± 27[a]	1.76 ± 0.04[b]
	Pool III-cells	240 ± 10	902 ± 50[a]	1.89 ± 0.09[b]
40 g	Original susp.	440 ± 9	1407 ± 96[a]	1.65 ± 0.10
	Pool I-cells	300 ± 7	672 ± 37[a]	1.16 ± 0.08
	Pool II-cells	360 ± 19	873 ± 51[a]	1.26 ± 0.09
	Pool III-cells	309 ± 17	875 ± 29[a]	1.50 ± 0.05[b]

Cell suspensions derived from 7315b tumors of increasing weight were separated on continuous Percoll density gradients (see legend to Fig 1). 25,000 cells of the original cell suspension and of the pooled fractions were inititally seeded (in six-fold) and subsequently grown for 6 days (t = 144 h) in MEM + 10% FCS. The DNA content of the cells was determined directly after isolation and after 6 days of culture. "Growth rates" were calculated as described in the materials and methods section. Values in mean ± SE; n = 4 for DNA (t = 0 h) and n = 6 for DNA (t = 144 h).
[a] $p < 0.01$ vs DNA (t = 0 h); [b] $p < 0.01$ vs. number of doublings in 144 h of pool I-cells; [c] $p < 0.01$ vs. number of doublings in 144 h of pool III-cells.

Fibroblast contamination and influence of the proliferation of these cells on the "growth rate"-data of the 7315b cells can be excluded because fibroblasts (if present) attached to the floor of the wells while the 7315b cells did not (see materials and methods). The cultured 7315b cells could be harvested by removing the culture medium plus cells thereby not collecting eventually present fibroblasts. We found no statistically significant differences between the tumors of 7, 11 and 40 grams with regard to the latter parameter. In addition there were also no major differences between pool I, II and III-cells with respect to the number of doublings in 144 h except for the fact that pool I-cells showed in virtually all instances a significant lower "growth rate" than pool II and III-cells ($p < 0.01$ for pool I vs pool II and III-cells). Only in the 40 grams tumor pool I and II-cells had no different "growth rates". Analysis of the viability of pool I, II and III-cells at time of plating and after 24 h of culture revealed that pool I-cells always showed a slightly lower viability than pool II and III-cells (data not shown). However, between 0 h and 24 h there were no differences in the viability of the cells. Routine light microscopic evaluation of cytospin preparations showed no differences between the original cell suspension and pool I, II and III-cell suspensions. In all instances the majority of cells were tumor cells with only a few cells being white blood (host) cells (less then 2% of the cell population).

Because PRL is the major protein hormone which is produced by the 7315b tumor cells we also determined the amount of intracellular PRL in the cells of the original cell suspension and of pool I, II and III-cells, directly after isolation and after 6 days of culture. Since the number of cells significantly increases during culture and because the "growth rates" of the pool I, II and III-cells showed significant differences we expressed the amount of intracellular PRL as the ratio over DNA. The results are shown in Table 3.

Table 3: Rat prolactin (PRL) content per ng DNA of the original cell suspension and of Percoll gradient fractions of 7315b tumors of increasing weight directly after isolation and after 6 days of culture (t=144 h).

	ratio PRL : DNA (pg : ng)					
	7 g tumor		11 g tumor		40 g tumor	
	directly after isolation	after 144 h of culture	directly after isolation	after 144 h of culture	directly after isolation	after 144 h of culture
Orig. Susp.	3.0 ± 0.5	52.0 ± 1.8[a]	3.5 ± 0.2	48.1 ± 2.7[a]	3.7 ± 0.2	44.0 ± 1.6[a]
Pool I-cells	4.2 ± 0.6	59.5 ± 3.5[a]	4.5 ± 0.2	41.4 ± 1.8[a]	3.5 ± 0.2	37.4 ± 3.0[a]
Pool II-cells	3.7 ± 0.1	44.7 ± 1.9[a,b]	4.4 ± 0.1	49.2 ± 4.5[a]	2.8 ± 0.2	36.3 ± 2.6[a]
Pool III-cells	3.3 ± 0.4	60.0 ± 2.5[a]	4.9 ± 0.6	56.5 ± 3.6[a]	3.3 ± 0.2	27.4 ± 2.0[a]

Values in mean ± SE; [a] $p < 0.01$ vs. PRL : DNA directly after isolation; [b] $p < 0.01$ vs. pool I and III-cells.
See legend to Table 2.

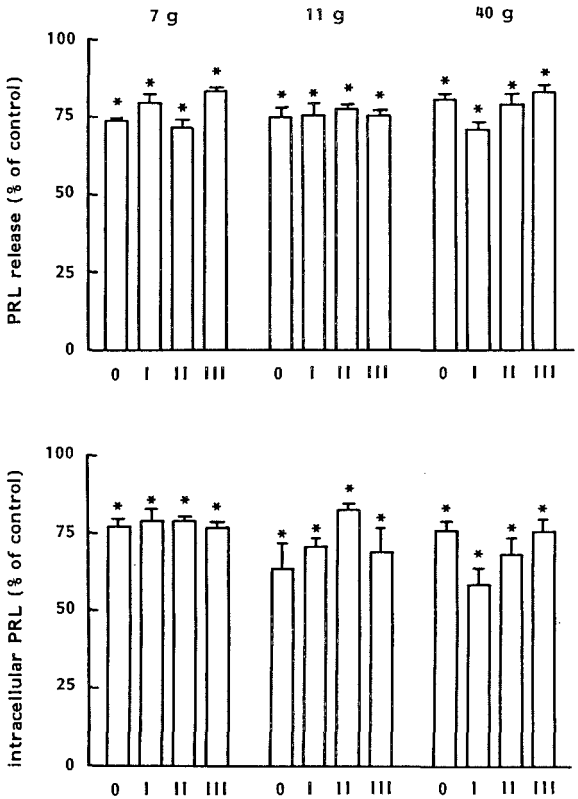

Fig.2 The effect of 10nM SMS 201-995 on the release of PRL in 6 days by the original cell suspension (O) and by pool I, II and III-cells (see legend to Fig.1) of 7315b tumors of increasing weight (7, 11 and 40 grams). The cells were initially seeded as 25,000 cells per well (in four-fold) and grown in MEM + 10% FCS for 6 days. At the end of the culture period the media were collected and stored at-20°C until analysis of PRL. The values are expressed as the percentage of PRL release in untreated wells. *p<0.01 vs. control release.

Again no statistically significant differences were observed between the 7, 11 and 40 grams tumors and between pool I, II and III-cells. The ratio of PRL over DNA after 6 days of culture was in all instances significantly higher (approximately 10-fold; $p < 0.01$ in all instances) than the PRL:DNA ratio directly after isolation indicating that the cells contain virtually no PRL directly after isolation and that they restore their capacity of PRL storage during the 6-day culture period.

Finally, we also determined the responsiveness of the cells in the original cell suspension and in pool I, II and III to 10 nM SMS 201-995. This concentration of the drug had no effect on the growth of the cells (data not shown) but significantly inhibited the amount of intracellular PRL and PRL release by the cells over the six day culture period (in all instances $p < 0.01$ vs. control PRL release; Fig. 2). There were no statistically significant differences in SMS 201-995- responsiveness between the cell suspension of the 7, 11 and 40 grams tumor and between pool I, II and III-cells.

In order to further investigate functional heterogeneity among 7315b tumor cells with respect to PRL release we also studied PRL release by individual cells in unseparated 7315b cell suspensions using the RHPA. Freshly dispersed 7315b cells did not secrete sufficient amounts of PRL to produce plaques in the 24h incubation. This was probably due to the low amount of intracellular PRL directly after cell isolation (see table 3). However, using 6-day cultures of 7315b cells 58 $\pm$ 4% of the tumor cells formed plaques. 10 nmol/L SMS 201-995 did not significantly affect the percentage of plaque forming cells (56 $\pm$ 8%). However, the drug significantly ($p<0.01$ vs control without SMS 201-995) inhibited the mean plaque area (mpa) by 47% (mpa of control cells being 4584 $\pm$ 226 μM^2 and mpa of cells incubated with 10 nmol/L SMS 201-995 being 2421 $\pm$ 23 μM^2). The mpa represents the mean amount of PRL released by 100 cells. Fig. 3 shows the frequency distributions of the individual PRL plaque areas produced by unseparated 7315b tumor cells in the absence (closed symbols) or in the presence (open symbols) of 10 nmol/L SMS 201-995. The frequency distribution shows under control conditions an

unimodal mode with a slight skewness toward the larger plaques. In the presence of 10 nmol/L SMS 201-995 the frequency distribution shifts toward the smaller plaques. However, the shape of the frequency distribution was not affected by treatment of the cells with SMS 201-995 which may indicate that the drug did not preferentially inhibit any population of 7315b tumor cells.

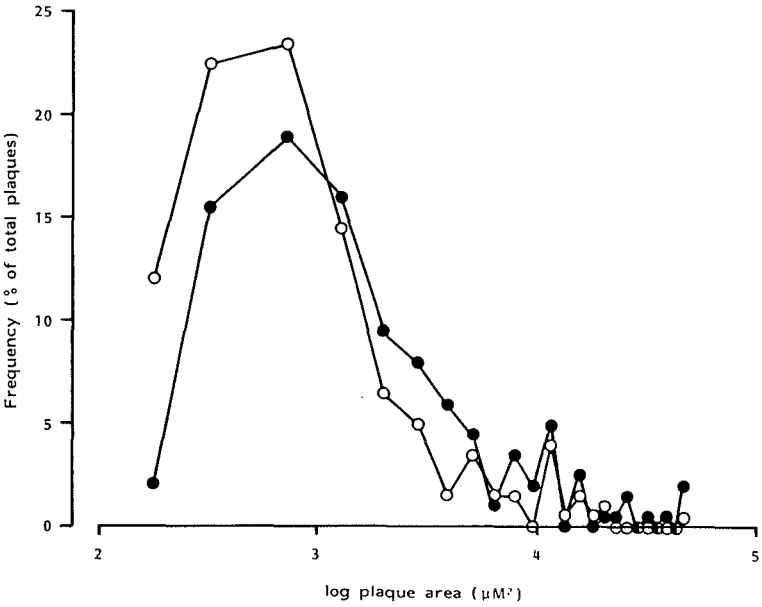

Fig. 3 Frequency distributions of individual PRL plaque areas by unseparated 7315b rat pituitary tumor cells, in the absence (closed symbols) or in the presence (open symbols) of 10 nmol/L SMS 201-995. Each point in the frequency distribution represents the mean result from 2 slides (100 plaques were measured per slide).

IV.5 Discussion

It is well established that most murine and human tumors show intrinsic cellular heterogeneity [1]. Until now, however, little is known whether human pituitary tumors or transplantable rat pituitary tumors such as the GH_3, MtT/W15 and 7315 a or b tumors also show functional heterogeneity with regard to cell growth- characteristics and/or responsiveness to regulatory hormones or steroids. Three of these experimental tumors have been shown to produce more than one hormone simultaneously. GH_3 and MtT/W15 tumors contain both single (GH or PRL) and dual hormone secretors (GH and PRL) as determined by the reverse hemolytic plaque assay [5-7], while 7315a tumors produce both PRL and ACTH [8]. Boockfor et al. [6] have demonstrated that cultures of GH_3 cells are also functionally heterogeneous. They showed that chronic treatment of cultures of GH_3 cells with TRH, estradiol or cortisol caused reciprocal shifts in the proportions of GH and PRL cells present. Although their data were suggestive of interconversion of GH cells into PRL cells and vice versa they could not preclude the possibility that other processes (such as cell proliferation) were involved. The present study was undertaken in order to evaluate whether functional heterogeneity of cells within the transplantable "pure" PRL-secreting pituitary tumor 7315b exist, with respect to growth rate, PRL production and responsiveness to the somatostatin analog SMS 201-995. We separated freshly dispersed 7315b tumor cells derived from tumors of increasing weight on continuous Percoll density gradients, according to differences in their density. The technique of density gradient centrifugation has previously been shown to provide a useful tool for the study of intratumor cell heterogeneity [16,17]. The cell distribution of the 7315b cells derived from tumors of increasing weight on the Percoll gradients showed a normal distribution profile with the cells being recovered within a narrow density range. This homogeneous distribution indicates that the cells within the 7315b tumor are physiologically homogeneous. In this respect the observations by Grdina et al. [18] showing that the distribution of tumor cells obtained

from density gradient centrifugation reflects the heterogeneity within tumors is of interest. In agreement with the homogeneous profile with respect to the density of the cells we found no differences between the Percoll gradient fractions with respect to protein content per cell, PRL production per cell and responsiveness to the inhibitory effect of the somatostatin analog SMS 201-995. In addition we found no differences between tumors of increasing weight (7, 11 and 40 grams) with respect to these parameters and with respect to the growth rates of the tumor cells in vitro. Therefore it can be concluded that the 7315b transplantable PRL- secreting pituitary tumor is homogeneous with regard to its cellular composition and that growth of 7315b tumors in vivo does not lead to the induction of heterogeneous subpopulations as has been described for most human and murine solid tumors [1]. The lack of heterogeneity between the gradient fractions in responsiveness to the somatostatin analog SMS 201-995 may exclude clonal selection of somatostatin-unresponsive cells leading to "escape" of the tumor from the tumor growth- inhibitory effect of this drug as has been described previously [8].

Since the 7315b pituitary tumor cells do not store large amounts of PRL the method of density gradient separation may not have been an appropriate method for the study of functional heterogeneity among 7315b tumor cells with respect to PRL release. Therefore, we also used an alternate technique, the RHPA, which has been previously shown to detect functional subtypes of hormone secreting cells in unseparated, normal rat anterior pituitary cell suspensions [19-22]. The results of our experiments using the RHPA for the detection of PRL release by individual 7315b cells basically confirmed our cell separation data. In agreement with the latter data we found no evidence for a differential responsiveness to SMS 201-995 among cells of the 7315b tumor. Only with respect to basal PRL release we demonstrated that there was a minor population of adenoma cells secreting a high amount of PRL.

The only difference that was observed in our study between the Percoll gradient fractions was a slightly lower growth rate of the cells in the gradient fraction with the

lowest density. The most likely explanation for the lower growth rate of the low density fraction is a lower viability of the cells (or part of the cells) in this gradient fraction related to a limited life-span of the cells. In agreement with this is our observation that this fraction had a slightly lower viability than the higher density fractions. An other possibility may be that this particular fraction represents a subpopulation of tumor cells in a non-proliferating or quiescent state [23,24] caused by nutrient deprivation of the tumor cells in vivo. However, the latter explanation is less plausible for two reasons: first, studies using density gradient centrifugation of murine fibrosarcoma cells have previously shown that these particular subpopulations are recovered in the high density region of density gradients [16,17], while secondly we observed no shifts in the density distribution profile of tumor cells derived from tumors of increasing weight and no differences between these tumors with respect to their growth rates in vitro.

In conclusion our study demonstrates that the transplantable PRL- secreting rat pituitary tumor 7315b consists of a homogeneous cell population as measured by density distribution profile, protein content of cells, growth rates and responsiveness to the somatostatin analog SMS 201-995. Only with respect to PRL production per cell there may be a small fraction of cells within the tumor cell population secreting a high amount of PRL. Whether this tumor homogeneity is representative for human pituitary tumors in general remains to be established.

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CHAPTER V

HETEROGENEITY OF PITUITARY ADENOMA CELL SUBPOPULATIONS FROM ACROMEGALIC PATIENTS OBTAINED BY PERCOLL DENSITY GRADIENT CENTRIFUGATION.

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V.1 Abstract

Pituitary adenoma cells from 6 acromegalic patients were separated on continuous Percoll density gradients according to differences in their density. Two adenomas produced GH only in culture, the other 4 adenomas produced either GH and PRL (one adenoma) or GH and α-subunit (one adenoma) or GH, PRL and α-subunit (two adenomas). The cell subpopulations obtained by this technique differed in the amount of hormone production per 10^5 cells: GH release decreased from the low density fractions to the higher density fractions in 5 of 6 adenomas. Intracellular GH levels completely followed this profile. In the mixed GH/ α-subunit adenomas the α-subunit profile completely paralleled the GH profile, whereas in the mixed GH/PRL adenomas the PRL profile showed a pattern different from that of GH (and α-subunit). In neither of the adenomas did we find any differences between the subpopulations with respect to the responsiveness of GH, PRL or α-subunit release to GHRH, TRH and the somatostatin analogue SMS 201-995. Conclusions: 1. Within pituitary adenomas from acromegalic patients heterogeneity exists with respect to hormone production per cell. 2. The cell subpopulations obtained by density gradient centrifugation are not different in their responsiveness to SMS 201-995, GHRH or TRH. 3. Because GH and α-subunit release by the fractions from the mixed GH/α-subunit secreting adenomas were completely parallel, further evidence for co-release of GH and α-subunit by the same

tumoural cells is provided.

V.2 Introduction

GH-secreting pituitary adenomas from acromegalic patients frequently contain and secrete also PRL and glycoprotein hormone α-subunit. This has been demonstrated by several in vitro studies (1-6) and by morphological techniques (1, 7-13). However, pituitary adenomas from acromegalic patients are very heterogeneous with respect to their cellular composition. Melmed et al. (11) recently classified pituitary adenomas from patients with acromegalic features into nine morphological categories. Pituitary adenomas from acromegalic patients may be composed of distinct populations of GH-secreting and PRL-secreting cells or of cells containing both hormones simultaneously (11). Using the protein A-gold electron microscopic technique Bassetti et al. (14) recently demonstrated in 5 mixed GH- and PRL-secreting pituitary adenomas the presence of numerous mammosomatotropes, i.e. cells containing simultaneously GH and PRL (from 50-80% of the whole cell population). Using the same technique Beck-Peccoz et al. showed a similar coexistence within tumours of acromegalic patients of GH and α-subunit in the same tumoural cell (15).

Until now, evidence for cellular heterogeneity within tumours from acromegalic patients has been based on morphological and immunocytochemical techniques. To our knowledge, however, it is not known whether these different cell populations are also functionally different in their responsiveness to stimulatory and/or inhibitory hormones like growth hormone releasing hormone and somatostatin. Using density gradient centrifugation to fractionate anterior pituitary cell suspensions into discrete subpopulations, several investigators have demonstrated the existence of functionally different rat and ovine somatotrope subpopulations (16-18). In the present study we used Percoll density gradient centrifugation in order to separate pituitary adenoma cells from acromegalic patients according to differences in density. We investigated whether the

different cell subpopulations obtained by Percoll gradient centrifugation differed with respect to a) hormone release and cellular content b) responsiveness to GHRH, TRH and the somatostatin analogue SMS 201-995.

V.3 Subjects and Methods

Patients and preparation of dispersed tumour cells.

Tumour specimens from 6 untreated acromegalic patients (of both sexes) were obtained by transsphenoidal operation as described in detail previously (19). The viability of the resulting cell suspensions as determined by trypan blue exclusion was always greater than 80%.

Separation of dispersed adenoma cells on continuous Percoll density gradients.

A 90% Percoll solution was made by mixing 9 parts of Percoll stock solution (Pharmacia Fine Chemicals Uppsala, Sweden) with 1 part of a 10 times concentrated calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) (GIBCO, Europe). This 90% iso-osmotic Percoll solution was further diluted to 41% with phospate buffered saline (PBS) (pH 7.4). 8 ml of the 41% Percoll solution was then added to polypropylene tubes (Sorvall; 12 ml, 16 x 102 mm). To one tube, which was treated in the same way as all other tubes, calibration density marker beads (No's 2-9, Pharmacia, Uppsala, Sweden) were added. Gradients were pre-formed by centrifugation at 25300 x g in a Sorvall SS-34 rotor during 30 minutes at 20°C. After centrifugation, calibration of the formed density gradients was done by measuring the distance of each density marker bead to the meniscus of the Percoll fluid. The density range in the gradient was 1.014 kg/l (gradient top) - 1.082 kg/l (gradient bottom). For cell-separation approximately 1.5 x 10^7 to 2 x 10^7 cells in 2 ml HBSS + HSA were layered on the pre-formed gradients and then centrifuged at 800 x g during 20 min. at 20°C. The gradients were fractionated using an Auto Densi-Flow II C (Searle, Bachler Instruments, Fort Lee,

NJ, US Pat. No. 3682305). 60 sec. fractions (position 2 of the speed, approximately 800 μL per fraction) were collected from the top of the gradients. Each fraction was then diluted (2 times) with HBSS + HSA and centrifuged for 5 min. at 600 x g. The cells were washed two times more with HBSS + HSA and finally resuspended in culture medium (see below). Counting of the cells in each fraction was done with a Bürker counter chamber. Viability of the cells was determined by trypan blue exclusion.

Cell recovery from the gradients yielded 61, 74, 86, 99, 95 and 87% for the cell suspensions from patients No. 1, 2, 3, 4, 5 and 6 respectively.

Cell culture

The cells from the initial cell suspension or from the fractions after separation of the initial cell suspension on Percoll density gradients were cultured at a density of 10^5 cells per well per 1 ml of culture medium in 48-well plates (Costar, Cambridge, MA). The cells were cultured at 37°C in a water-jacketed incubator in humidified air with 5% CO_2. On day 4 of culture, after which the cells had formed a monolayer, the medium was changed and 24-h incubations without or with secretagogues or drugs were performed in quadruplicate. The culture medium was used in all experiments was Minimal Essential Medium with Earle's salts (MEM) supplemented with non-essential amino acids, sodium pyruvate (1 mmol/l), 10% fetal calf serum (FCS), penicillin (10^5 U/l), streptomycin (100 mg/l), Fungizone (0.5 mg/ml), L-glutamine (2 mmol/l) and sodium bicarbonate (2.2 g/l final concentration). The medium was adjusted to pH 7.4 with 1 mol/l NaOH. Medium, obtained as a 10 times concentrated solution, and supplements were purchased from Grand Island Biological Co. Europe (Paisley, Scotland). Since all wells contained the same number of initially seeded tumour cells the results of the experiments were expressed as μg GH or PRL or α-subunit/l per 10^5 cells.

GHRH was obtained from Universal Biologicals (Cambridge, UK); TRH from Hoechst (Amsterdam, The Netherlands). SMS 201-995 was a gift from Sandoz (Basel, Switzerland). Intracellular hormone concentrations were measured in cell extracts obtained by lysis of the cultured cells in distilled water containing 0.1% bovine serum albumin (Sigma) as described in detail elsewhere (20).

Immunocytochemistry

Immunocytochemical identification of GH-, PRL- and ACTH-containing cells was performed using an indirect immunohistoperoxidase method (21).
Cytospin preparations were prepared from the dispersed adenoma cells. These preparations were fixed in methanol + 0.3% H_2O_2 (in order to suppress endogenous peroxidase activity). Thereafter the preparations were treated with 10% non-immune pig serum (code X901, Dakopatts, Denmark) in phosphate-buffered saline (PBS, pH 7.4) to prevent non-specific background staining. The preparations were then incubated for 30 min. at 37°C with rabbit anti-GH serum (1:600), rabbit anti-PRL serum (1:1000) and rabbit anti-ACTH serum (1:500), all obtained from Dakopatts. The antisera were diluted in PBS + 5% bovine serum albumin (BSA). The slides were washed twice with PBS and incubated during 30 min. at 37°C with porcine peroxidase-conjugated anti-rabbit Ig (code P217, Dakopatts). This second antibody was used in a 1:100 dilution in PBS + 5% BSA. After two PBS washes the slides were stained in the dark with a 0.075% solution of di-amino-benzidine-tetrahydrochloride (SIGMA) in PBS, activated with 0.075% H_2O_2. Finally the cells were counterstained with a hematoxylin solution.

Hormone assays

GH and PRL in the culture media and cell extracts were measured by immunoradiometric assays for human growth hormone (HGH-IRMA) and human prolactin (PRL-IRMA) respectively, purchased from Euro-diagnostics (Apeldoorn, The

Netherlands). The intra- and inter-assay variation was <7% and <13% respectively for the HGH-IRMA and <10% and <15% respectively for the PRL-IRMA.

α-subunit in the culture media and cell extracts was measured by a double-antibody RIA using antibodies purchased from UCB (Brussels, Belgium), and MRC 78/554 (potency: 1 mIU/mg) as standard preparation. Intra- and inter-assay variation was <6% and <11% respectively. Crossreactivities (in ng/ng) with LH MRC 68/40 (potency 6.6 mIU/ng) and with a purified FSH preparation from KABI (Stockholm, Sweden) were 3 and 3.6%, respectively.

Statistical analysis.

The statistical significance of the differences between mean values was determined using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made using the Newman-Keuls test (22). All data are expressed as mean ± SEM.

V.4 Results

In vivo data and immunocytochemistry

All the patients included in this study had elevated plasma GH levels (Table 1). Patients No. 1, 3 and 5 showed clearly elevated plasma PRL levels, whereas patients 2 and 4 had mild hyperprolactinemia. Immunocytochemistry revealed the presence of GH-containing cells in the adenoma cell suspensions from all adenomas, the percentages varying from 15 to 95 (Table 1). Immunostaining was not performed on the adenoma of patient No. 1.

Significant numbers of PRL-containing cells were found in the adenoma cells from patients No. 2 and 3. No ACTH-containing cells were detected in the adenoma cell suspensions. We found no relation between the percentage of GH- or PRL-containing cells and the serum GH and PRL levels, respectively.

Table 1: Plasma GH- and plasma PRL levels of the acromegalic patients included in this study and immunocytochemical data of dispersed pituitary adenoma cells from these patients.

Patient no.	Age (years)	Sex (M/F)	Plasma GH (μg/l)	Plasma PRL (μg/l)	Immunocytochemistry % of cells positive for GH	PRL	ACTH
1	32	F	19 ± 1	74 ± 4	not available		
2	28	F	26 ± 6	19 ± 1	95	50	0
3	39	M	143 ± 9	101 ± 8	80	5	0
4	43	M	27 ± 2	27 ± 1	65	<1	0
5	44	F	69 ± 6	108 ± 30	15	<1	0
6	41	M	82 ± 6	11 ± 1	80	<1	0

Hormone levels are the means of two plasma samples taken at two different days at 08.00 h.

Values are means ± SEM.

M = male; F =female

Normal GH plasma values ranged from 0.2-0.5 μg/l and the upper limit of normal plasma PRL levels were 12 μg/l in men and 15 μg/l in women.

Distribution of GH, PRL and α-subunit in the gradient fractions

Figs. 1 and 2 show the profiles of the percentages of recovered cells from the Percoll density gradients, the profiles of GH, PRL and α-subunit release per 24-h by the gradient fractions and the cellular content at the end of the 24-h incubation period.

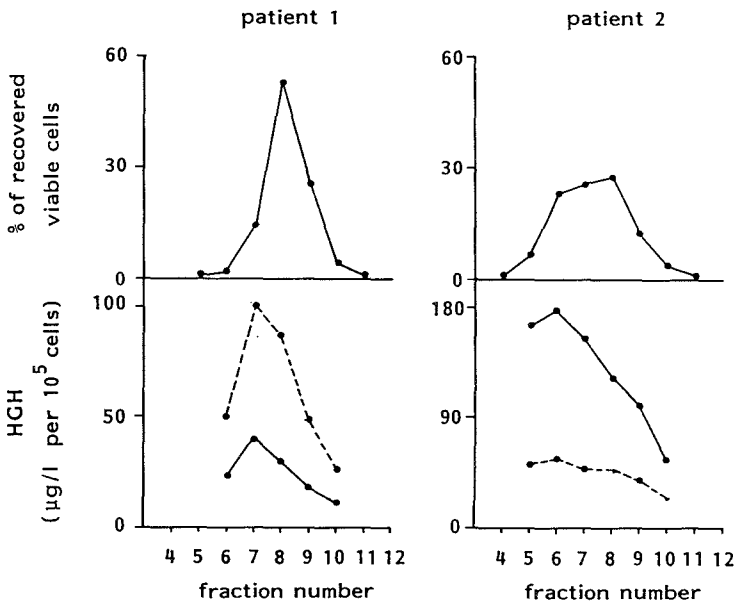

Fig. 1: Separation of pituitary adenoma cells from two acromegalic patients on continuous Percoll density gradients. The upper panel shows the profile of the percentages of recovered vital cells from the Percoll gradients, the lower panel shows the profile of GH release per 24-h (●——●) and the cellular content at the end of the 24-h incubation period (●---●). The cells were cultured in MEM + 10% FCS. After 4 days of culture the medium was changed and 24-h incubations were performed. N=4 wells per group. Values are means: SEM always less than 10%.

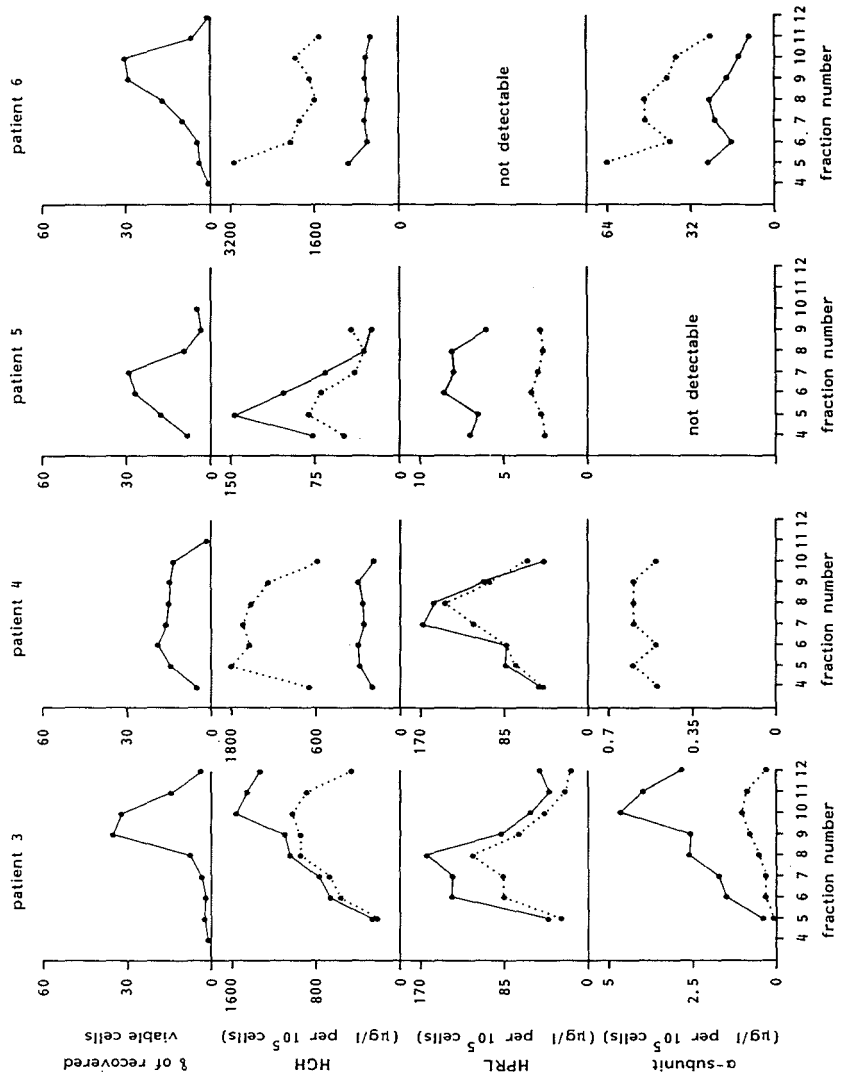

Fig. 2: Separation of pituitary adenoma cells from four adenomas secreting GH/PRL/α-subunit (patient No. 3 and 4), GH/PRL (patient No. 5) or GH/α-subunit (patient No. 6). See legends to Fig. 1. The amount of α-subunit release by the adenoma cells of patient No. 4 was to low to be measured.

After centrifugation, the gradients were fractionated from the top of the gradients into 12 fractions. The density increased from the top to the bottom of the gradient and amounted from 1.014 kg/l in fraction 4 up to 1.082 kg/l in fraction 11. As can be seen in Figs. 1 and 2, the pituitary adenoma cells from all 6 patients were recovered between these densities. However, there was a difference between the 6 patients with respect to the density of the fractions in which the majority of adenoma cells was recovered. The densities of these fractions varied from approximately 1.045 kg/l (patients No. 2 and 5) to approximately 1.056 kg/l (patients No. 3 and 6). The initial cell suspensions (cell suspensions before separation) from the two latter patients had a considerable higher amount of intracellular GH (GH content of the cells directly after isolation), 3833 ± 120 and 4939 ± 333 μg/l per 10^5 cells, respectively, as compared with those from patients No. 2 and 5 (68 ± 3 and 627 ± 51 μg/l per 10^5 cells, respectively). This indicates that the cellular GH content is a major factor for the density of the pituitary adenoma cells.

Two adenomas produced GH only in culture, namely those from patients 1 and 2. The profile of GH release and cell content of the gradient fractions is shown in Fig. 1. GH release and cell content per 10^5 cells decreases from the top fractions (low density) to the bottom fractions (high density). From the adenomas which in addition to GH also produced PRL (patients No. 3, 4 and 5) and/or α-subunit (patients No. 3, 4 and 6), a similar GH profile is seen in the gradients from patients No. 5 and 6 (Fig. 2). Interestingly, patient No. 3 shows a completely opposite pattern with the bottom fractions having the highest GH production per 10^5 cells. Fig. 2 also shows that the PRL profiles (release and intracellular) in the gradients from patients No. 3, 4 and 5 do not follow the GH profiles. In contrast the α-subunit profiles (patients No. 3, 4 and 6; Fig. 2) closely follow the GH profiles in all cases. The amount of α-subunit secreted by the adenoma cells of patient No. 4 was to low to be measured which is in line with the significant difference between the amounts of intracellular GH and GH release of the

cells of this patient.

Effect of SMS 201-995, GHRH and TRH on hormone release by the gradient fractions

The effect of 10 nmol/l SMS 201-995, 10 nmol/l GHRH and 100 nmol/l TRH on GH, PRL and α-subunit release by the different gradient fractions was studied. SMS 201-995 significantly inhibited GH release by the original cell suspension and the gradient fractions 6, 7, 8 and 9 of patient No. 1. The percent inhibition varied from 12 to 21 ($p<0.01$ vs control GH release). GHRH stimulated GH release by 20 to 49% ($p<0.01$ vs control GH release). There was no statistically significant difference between the fractions with respect to their responsiveness to either SMS 201-995 or GHRH. TRH did not significantly affect GH release by the cells of this patient. SMS 201-995 also significantly inhibited GH release by the original cell suspension and the gradient fractions 5, 6, 7, 8 and 9 of patient No. 2 (in all instances $p<0.01$ vs control GH release). The percent inhibition varied from 20 to 41. Fraction 5, 6 and 7 were significantly less responsive to SMS 201-995 than fraction 8 and 9 ($p<0.01$ for fraction 5 and 6 vs fraction 8 and 9 and $p<0.05$ for fraction 7 vs fraction 8 and 9). GHRH and TRH did not significantly affect GH release by the cells of this patient.

Fig.3 shows the effect of 10 nmol/l SMS 201-995 and 100 nmol/l TRH on GH and α-subunit release by the fractions and the original cell suspension of patient No. 6. All effects are expressed as percentage of control hormone release.

Control hormone release is shown in Fig.2. In all fractions SMS 201-995 significantly inhibited GH and α-subunit release to 39-51% (fraction 8 and fraction 11, respectively) of control GH release and to 46-67% (fraction 10 and fraction 11, respectively) of control α-subunit release. GH release by fraction 11 was significantly less inhibited by SMS 201-995 than GH release by fractions 5 and 8 ($p<0.05$). Between the other fractions there were no statistically significant

differences in responsiveness to SMS 201-995. With respect to α-subunit release, fractions 10 and 11 differed significantly in their responsiveness to SMS 201-995 ($p<0.01$). TRH significantly stimulated GH and α-subunit release in all fractions and the original cell suspension. There were no statistically significant differences between the various fractions with respect to the responsiveness to 100 nmol/l TRH. GHRH, 10 nmol/l, did not significantly affect GH- and α-subunit release by the cells of patient No. 6.

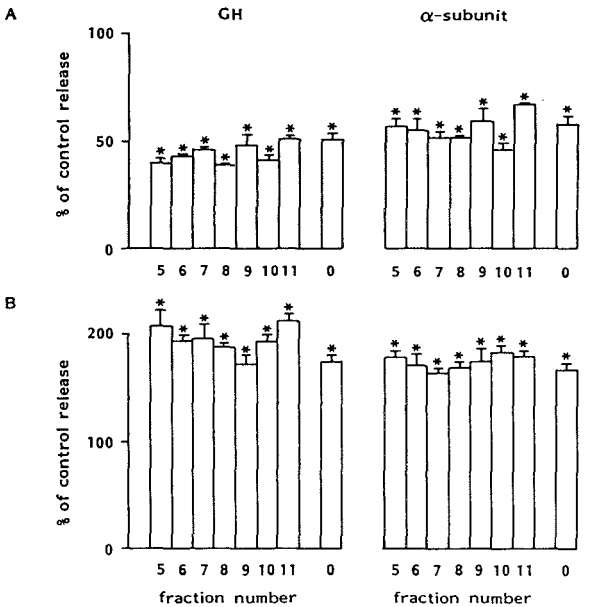

Fig. 3:
The effect of 10 nmol/l somatostatin analogue SMS 201-995 (A) and 100 nmol/l TRH (B) on GH-and α-subunit release by Percoll gradient fractions of adenoma cells of patient 6. The pituitary adenoma cells were separated on continuous Percoll density gradients which were subsequently fractionated. The symbol 0 represents the original (unfractionated) cell suspension. The fractions 1-4 and 12 are not shown as either no cells were recovered in these fractions or the number of recovered cells was insufficient to perform incubation studies (see Fig. 2). The cells from the different fractions were cultured in MEM + 10% FCS for 4 days. Thereafter the medium was changed and 24-h incubations without or with SMS 201-995 or TRH were performed in quadruplicate. N=4 wells per group. Values are expressed as percentage of control release (without SMS 201-995 or TRH), the values are means ± SEM. *$p<0.01$ vs control GH release.

In Fig. 4 the effect of 10 nmol/l SMS 201-995 on GH, PRL and α-subunit release by the gradient fractions and the original cell suspension of patient No. 3 is shown. SMS 201-995 significantly inhibited hormone release in all instances ($p<0.01$ vs control hormone release). There were no statistically significant differences between the fractions with respect to the inhibitory effect of SMS 201-995 and GH, PRL and α-subunit release. TRH and GHRH did not significantly affect GH, PRL and α-subunit release by the cells of this patient.

SMS 201-995, 10 nmol/l, GHRH, 10 nmol/l and TRH, 100 nmol/l, did not significantly affect GH and PRL release by the original (unfractionated) cell suspension and by the gradient fractions of patients No. 4 and 5 (not shown).

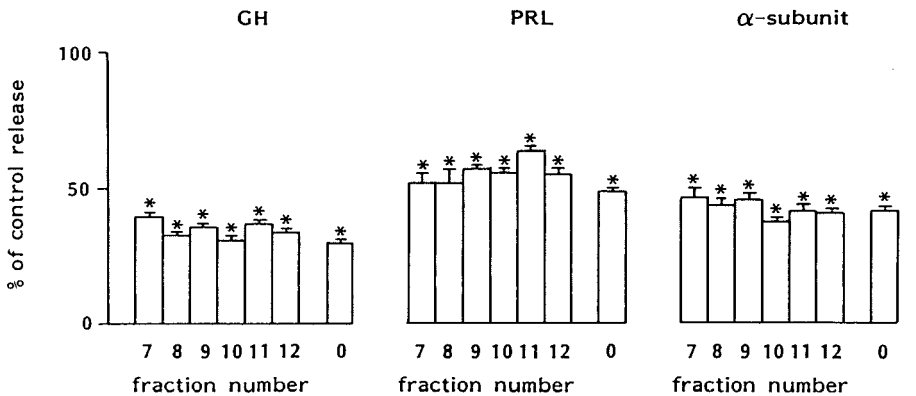

Fig. 4: The effect of 10 nmol/l somatostatin analogue SMS 201-995 on GH, PRL and α-subunit release by Percoll gradient fractions of adenoma cells of patient No. 3. The fractions 1-6 are not shown since not enough cells were recovered in these fractions in order to perform secretion studies. See legends to Fig. 3.

V.5 Discussion

Pituitary adenoma cells from acromegalic patients were separated on continuous Percoll density gradients. We investigated whether the cell subpopulations obtained by this technique differed in hormone production and in their responsiveness to GHRH, TRH and the somatostatin analogue SMS 201-995. We showed that the different cell subpopulations, separated according to differences in their densities, were heterogenous in the amount of GH secretion and cellular GH content per 10^5 cells. From two adenomas, which produced GH only in culture, GH release per 24 h decreased from the fractions with a low density towards the fractions with a higher density. In addition, the cellular GH content showed a similar decrease towards the higher density fractions. The ratio of medium GH to cellular GH content was roughly the same for all fractions (not shown). This indicates that the different cell subpopulations obtained by density gradient centrifugation are not different with regard to the ratio of storage and release of GH. We found that the majority of the cells of adenomas with a high initially amount of intracellular GH were recovered in the gradients at higher densities than the adenoma cells from adenomas with a lower amount of intracellular GH. An explanation of this finding may be that the density of the cells is largely dependent on the mass of protein hormone present in the cytoplasm (18). Our observation that in 5 of 6 adenomas, the lower density fractions had the highest GH production (medium + intracellular GH) seems, however, contradictory to this statement. The most likely explanation of this latter observation is an increase in the number of cells not secreting or synthesizing GH towards the higher density fractions. In favour of this possibility are studies by Lloyd et al. (23) who showed that the percentage of GH-secreting cells, as determined by the reverse hemolytic plaque assay, varied in 4 GH-producing pituitary adenomas between 47 and 78%, whereas the percentage of GH-immunoreactive cells in these adenomas, as detected by immumnocytochemistry, varied between 54 and 98. Similarly Reubi et al (24) showed that the number

of GH-immunoreactive cells in a group of 10 GH secreting adenomas varied from one third to more than 50%. We also separated cells from 4 pituitary adenomas which in addition to GH produced PRL (3 of 4 patients) and/or α-subunit (3 of 4 patients). In three cases GH release and cellular content by the various gradient fractions followed basically the same pattern as the gradient fractions from the pure GH-secreting adenomas. In one patient, however, the pattern was completely opposite, the high density fractions having the highest GH production per 10^5 cells.

The PRL profile from the fractions of the adenomas which also produced PRL (three patients) did not parallel the GH profile. In contrast, the glycoprotein hormone α-subunit profile completely paralleled the GH profile in all three patients. This might indicate that the different fractions of the mixed GH/PRL adenomas either represent mammosomatotrope subpopulations having different capacities of the amount of GH and PRL produced per cell or represent mixtures of two different cell types producing GH or PRL. On the basis of the immunocytochemical data we could exclude neither of these possibilities. Only in the cell suspension from patient No. 2 did we find that the percentage of GH-containing cells plus the percentage of PRL- containing cells exceeded 100% indicating that this adenoma contains mammosomatotropes. However, the adenoma cells from this patient did not contain or secrete PRL in culture. Moreover, this patient had only mild hyperprolactinemia. In our study we found no relation between the percentages of GH- and PRL-cells as detected by immunocytochemistry and the plasma GH- and PRL-values, respectively. This discrepancy between immunocytochemical data and plasma GH and PRL levels is in agreement with earlier findings by others (12, 25). Data with respect to the frequency of mammosomatotropic cells within pituitary adenomas from acromegalic patients are conflicting so far. Bassetti et al (14) showed that the frequency of mixed GH- and PRL-secreting adenomas containing mammosomatotropic cells may be higher than previously believed (11). Robert et al. (26) recently demonstrated the presence of mammosomatotropic cells in all of a group of 30 adenomas from

patients with signs of GH and PRL hypersecretion, although the number of immunostained cells varied from case to case. As the profiles of GH and α-subunit were completely parallel in all instances, the glycoprotein hormone α-subunit appears to be produced by the same tumoural cells that also produce GH. This conclusion is further substantiated by a study from Beck-Peccoz et al (15) who, using the protein A-gold particle immunotechnique, demonstrated the presence of both GH and α-subunit in the same secretory granule from adenoma cells from acromegalic patients. We exclude the possibility that either the prolactin or the α-subunit we measured was derived from normal tissue contaminating the adenoma tissue since we found no ACTH-containing cells by immunocytochemistry and since no radioimmunoassayable ACTH was present in the culture media or cell extracts.

We also investigated the responsiveness of the various adenoma cell subpopulations obtained by Percoll density gradient centrifugation to GHRH, TRH and SMS 201-995. The subpopulations of cells obtained from the two pure GH-secreting adenomas all responded to approximately the same extent to the inhibitory effect of the somatostatin analogue SMS 201-995 and to the stimulatory effect of GHRH. Although we observed statistically significant differences in the responsiveness between some of the subpopulations in one patient, these differences were only minor on a percentage base (the maximal difference in SMS 201-995 responsiveness was 21%). Therefore we feel that these minor differences do not justify the conclusion of the existence of functional heterogeneity within these adenomas with respect to their responsiveness to SMS 201-995 and GHRH. Furthermore, the cell subpopulations in the different fractions from an adenoma which secreted GH and α-subunit simultaneously also showed close parallel responses to both SMS 201-995 and TRH. Finally, in one adenoma which secreted GH, α-subunit and PRL in culture and in which the GH and PRL profile of the gradient fractions showed a completely different pattern, no differences were observed between the various fractions with respect to the responsiveness of the release of all three hormones to SMS

201-995. These data may explain the parallel responses of GH and α-subunit release in acromegalic patients in vivo and in vitro (2, 15) and the parallelism of the responses of GH and PRL release in vivo (14, 27-31) and in vitro (32-34).

We conclude that heterogeneity exists within pituitary adenomas from acromegalic patients with respect to hormone production per cell. However, the different cell subpopulations separated according to differences in density are not different in their responsiveness to secretory agents such as SMS 201-995, GHRH and TRH.

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CHAPTER VI

HETEROGENEITY OF GH RELEASE BY INDIVIDUAL PITUITARY ADENOMA CELLS FROM ACROMEGALIC PATIENTS AS DETERMINED BY THE REVERSE HEMOLYTIC PLAQUE ASSAY : EFFECTS OF SMS 201-995, GROWTH HORMONE RELEASING HORMONE (GHRH) AND THYROTROPIN-RELEASING HORMONE (TRH).

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VI.1 Abstract

We used the reverse hemolytic plaque assay to study the dynamics of GH secretion by individual pituitary adenoma cells from 8 acromegalic patients. There was a considerable variation between the adenomas with respect to the percentages of GH secreting cells (25-78.5%) and also with respect to the amount of GH released per individual pituitary adenoma cell (mean plaque areas varying from 901 to 3559 μM^2).

The GH plaque area frequency distributions from the adenoma cells were not normally distributed, but revealed a preponderance of small plaques, defined as those with areas smaller than the mean plaque area. The large plaques, that is those with areas larger than the mean plaque area, constituded 24-38% of the total cell population from different tumors and accounted for a large fraction (63-80%) of the total plaque area (the total amount of GH released by the adenoma cells). The somatostatin analog SMS 201-995 caused a shift in the GH plaque area frequency distributions towards smaller plaques, but had no effect on the overall percentages of GH plaque forming cells in 3 of the 5 adenomas in which it was studied. This finding suggests that the adenoma cells from these patients that formed large plaques were preferentially inhibited by SMS 201-995. GHRH (studied in two adenomas) and TRH (studied in one adenoma) had no

preferential effect on any subpopulation of adenoma cells.

We conclude that GH secretion by individual somatotroph adenoma cells is highly variable both within and between adenomas and that SMS 201-995 has a preferential inhibitory effect on a subpopulation of adenoma cells in some adenomas.

VI.2 Introduction

GH release by normal somatotrophs is heterogeneous. Using density gradient centrifugation of anterior pituitary cells, several investigators have demonstrated the existence of subpopulations of rat and ovine somatotrophs which differ in their responsiveness to secretory agents as dibutyryl cyclic adenosine monophosphate and somatostatin (1-3). The existence of functional heterogeneity within the normal rat somatotroph cell population has been confirmed using the reverse hemolytic plaque assay (RHPA) (4-8), which allows detection of hormone secretion from single cells (4, 8). Using this technique Mulchahey et al. (9) found that fetal human somatotrophs are heterogeneous, both in their responsiveness to GHRH and to glucocorticoids. Whether such functional cellular heterogeneity might also exist within pituitary adenomas from acromegalic patients is not known. Reubi et al. recently reported that in some GH-secreting adenomas a significant proportion of somatotrophs did not contain SRIH receptors (10). Therefore, it might be expected that in GH-secreting adenomas somatotroph subpopulations exist which are differentially responsive to SRIH or its analogs, such as SMS 201-995. In this study we evaluated the dynamics of GH secretion by individual pituitary adenoma cells from acromegalic patients using the RHPA. In addition, the effects of SMS 201-995, GHRH and TRH on GH release by these cells were studied.

VI.3 Materials and methods

Patients and cell culture

Pituitary adenoma tissue from 8 untreated acromegalic patients, 4 women, 4 men, age range 38-58 y, was obtained by transsphenoidal operation as described previously (11). The aspirated adenoma tissue was washed twice with calcium-and magnesium- free Hank's Balanced Salt Solution (HBSS) supplemented with 10 g/L human serum albumin, penicillin (1x 10^5 U/L), streptomycin (100 mg/L), fungizone (0.5 mg/L) and sodium bicarbonate (0.4 g/L final concentration). Thereafter the tissue was incubated for approximately one hour with Dispase (grade II, 2.4 x 10^3 U/L; Boehringer Mannheim, Mannheim, W. Germany) and dispersed using an all-glass Dounce tissue grinder (Wheaton Scientific, Millville, USA). In order to remove the 100- to 1000-fold excess of erythrocytes the remaining cell suspension was layered on Ficoll-Isopaque (density 1.077 g/cm^3, prepared by the University Hospital Dijkzigt Pharmacy; Rotterdam, the Netherlands) and centrifuged at 500 x g for 20 min. The interphase containing the adenoma cells (11) was washed twice with HBSS + 1% HSA. The viability of the resulting cell suspension as determined by trypan blue exclusion was always greater than 80%.

The cells were cultured in Petri dishes (10^6 cells per dish) in 3 mL culture medium at 37°C in a water-jacketed incubator in humidified air with 5% CO_2. The culture medium consisted of Minimal Essential Medium with Earle's Salts (MEM) supplemented with non essential amino acids, sodium pyruvate (1 mmol/L), 10% fetal calf serum (FCS), penicillin (1 x 10^5 U/L), streptomycin (100 mg/L), fungizone (0.5 mg/L), L-glutamine (2 mmol/L) and sodium bicarbonate (2.2 g/L final concentration). The medium was adjusted to pH 7.4 with 1 mol/L NaOH. This medium, obtained as a 10-times concentrated solution, and supplements were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, UK).

For use in the RHPA the cells were harvested from the Petri dishes after day 1 of culture by mild

trypsinization. Cells which had attached to the Petri dishes were rinsed twice with HBSS without HSA. Thereafter 1 mL trypsin-EDTA solution [0.05% (w/v) trypsin and 0.02% (w/v) EDTA; Flow laboratories] was added to the Petri dishes. After 15-30 minutes of incubation at 37°C the cells were collected in culture medium (MEM + 10%FCS), washed twice in MEM + 10%FCS (in order to inactivate trypsin-activity), and resuspended in MEM containing 0.1% bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO, U.S.A.). The viability of the cells at this stage was always greater than 95%. The cells were used in the RHPA in a concentration of 2×10^5 cells per mL.

Reverse hemolytic plaque assay (RHPA)

The RHPA was performed as described in detail elsewhere (8). The trypsinized adenoma cells were mixed with an equal volume of a 12% suspension of protein A-conjugated ovine red blood cells (oRBC) and infused into Cunningham chambers, which were prepared on poly-l-lysine coated glass slides. After incubation for 1 h at 37°C in a CO_2-incubator with humidified air (5% CO_2) the chamber was rinsed twice with MEM + 0.1%BSA. Thereafter incubations were started by infusing anti-HGH serum (1:40 final dilution in MEM + 0.1%BSA) without or with SMS 201-995 (gift from Sandoz, Basel, Switzerland), GHRH (Universal Biologicals, Cambridge, UK) or TRH (Hoechst, Amsterdam, the Netherlands). The GH antiserum was obtained from UCB-Pharma (Brussels, Belgium; HGH antiserum for immunocytochemistry, raised in rabbits, No i515/002).

The slides then were incubated for a period of 1-4.5 hour in a CO_2-incubator (5% CO_2- 95%O_2). After the incubation period with antiserum (without or with secretagogues) the chambers were rinsed twice with MEM + 0.1% BSA and refilled with guinea pig complement (Flow Laboratories) at a dilution of 1:40 in MEM + 0.1% BSA. During the incubation with complement plaque formation occurred. After 45 minutes of incubation, the time required for maximal plaque formation, the reaction was stopped by filling the chambers with fixative (2% glutaraldehyde solution in

phosphate-buffered saline, pH 7.4) for approximately 30 minutes. The adenoma cells were stained with hematoxylin-solution in order to ensure the detection of nonplaque-forming cells. After staining of the adenoma cells the slides were stored until analysis. Prior to use in the RHPA the complement was stripped of immunoglobulins by treatment with protein A-conjugated sepharose CL-4B (Pharmacia, Uppsala, Sweden) as described by Leong et al (12) and stored at -80°C until use.

The specificity of plaque formation was established by the following criteria: a) no plaque formation occurred when either the GH antiserum or complement were omitted, b) no plaque formation occurred when normal rabbit serum was used in a dilution of 1:40 instead of the GH antiserum, c) no plaque formation occurred when a working dilution of the GH antiserum was absorbed with HGH covalently linked to Sepharose 4B beads (Pharmacia) before use in the RHPA. For this purpose, cyanogen bromide-activated Sepharose 4B beads (Pharmacia) were swelled and washed with 200 mL HCl (1 mmol/L). 3 mg biosynthetic human growth hormone (Lilly Research Centre Ltd. Surrey, England) was reconstituted in 600 µL 0.1 mol/L $NaHCO_3$ buffer, pH 8.3 containing 0.5 mol/L NaCl and mixed with 600 µL gel (packed volume). The resulting gel suspension was incubated and gently shaken for 2 h at room temperature. Thereafter the residual active groups were blocked by incubation of the gel for 2 h at room temperature in 0.2 mol/L glycine, pH 8.0. The GH-conjugated sepharose beads were washed five times in a cycle of 0.1 mol/L acetate buffer, pH 4.0 followed by 0.1 mol/L $NaHCO_3$ buffer, pH 8.3, each containing 0.5 mol/L NaCl. The gel was stored in 150 mmol/L NaCl containing 0.01% merthiolate. Absorption of the GH antiserum with the GH-coupled sepharose beads was performed as described in detail elswhere (12). d) dilutions of the GH antiserum of up to 1:100 did not bind [^{125}I]-labeled LH, TSH, LH-alpha, FSH, PRL and ACTH (1-39) (tested by UCB Pharma, Brussels, Belgium).

Finally, we determined viability of the cells in the Cunningham chambers at the end of the incubation period by infusing trypan blue solution into the chambers. The

viability of the cells determined in this way always was more then 95%.

Cohort incubation studies were performed by plating 0.5×10^5 adenoma cells (the same cells that were used in the RHPA) per well per 1 mL culture medium in quadruplicate (without or with the secretagogues as described above) in multiwell plates (Costar, Cambridge, MA, USA) at the same time that the RHPA incubations were started. At the end of a 1-4.5 h incubation period the media were collected, centrifuged at 600 x g and the supernatants were stored at -20°C until GH measurement. GH was measured by immunoradiometric assay using kits purchased from Euro-diagnostics (Apeldoorn, the Netherlands). The intra- and interassay coefficients of variation were <7% and <13%, respectively.

Immunocytochemistry

Cytospin preparations were made from the trypsinized cells that were used in the RHPA. These preparations were fixed in methanol + H_2O_2 (in order to suppress endogenous peroxidase activity). Immunocytochemical identification of GH-containing cells was performed with an indirect immunohistoperoxidase method (13). Thereafter the preparations were treated with 10% non-immune pig serum (code X901, Dakopatts, Denmark) in phosphate-buffered saline (PBS, pH 7.4) to prevent non-specific background staining. The preparations then were incubated for 30 min. at 37°C with rabbit anti-GH serum (code A 570, Dakopatts, Denmark). The anti-GH serum was used in a 1:600 dilution in PBS + 5% bovine serum albumin (BSA). The slides were washed twice with PBS and incubated during 30 min. at 37°C with porcine peroxidase-conjugated anti-rabbit Ig (code P217, Dakopatts, Denkmark). This second antibody was used in a 1:100 dilution in PBS + 5% BSA. After two PBS washes the slides were stained in the dark with a 0.075% solution of di-amino-benzidine-tetrahydrochloride (SIGMA) in PBS, activated with 0.075% H_2O_2. Finally the cells were counterstained with a hematoxylin solution.

Data analysis

The pituitary adenoma cells (plaque forming and nonplaque forming were viewed and counted with the aid of a Leitz Diavert microscope at 400-fold magnification. Plaque areas were measured with a calibrated ocular micrometer. All incubation studies were performed on duplicate slides and 100 cells per slide were counted and measured. The results are expressed as the mean $\pm$ SE. All data were analyzed within experiments. Statistical analysis was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test (14). Differences between mean values were considered to be significant when $p<0.05$. As the frequency curves of the mean plaque areas were not normally distributed all of the plaque area data were transformed to log-normal prior to statistical analysis.

VI.4 Results

Fig. 1a shows the rate of plaque formation as a function of incubation time by the adenoma cells of four patients (patients 1, 2, 3 and 4). Six slides incubated only with GH antiserum were included in the RHPA in all patients. These slides were incubated for different periods. Plaque areas were always measured after incubation times of 1 and 2 hours (2 slides per incubation time) and the third incubation time was chosen according to the results of the first two incubation times (with respect to percentage of cells forming plaques and individual plaque areas). The percentages of GH-plaque forming cells from all patients significantly increased with increasing incubation times. As shown in Fig. 1a the cells from patient 1 had a considerably lower rate of plaque formation than the cells from patients 2, 3 and 4, cells from the latter three patients having comparable rates of plaque formation. The percentages of cells forming plaques were 33% at 3 hours of incubation for patient 1, 70% at 3.5 hours for patient 3 and 78.5% and 71% at 4 hours for patients 2 and 4, respectively.

We also measured the plaque areas at the different incubation times. Fig. 1b shows the mean plaque areas as a function of the incubation time in the same 4 patients.

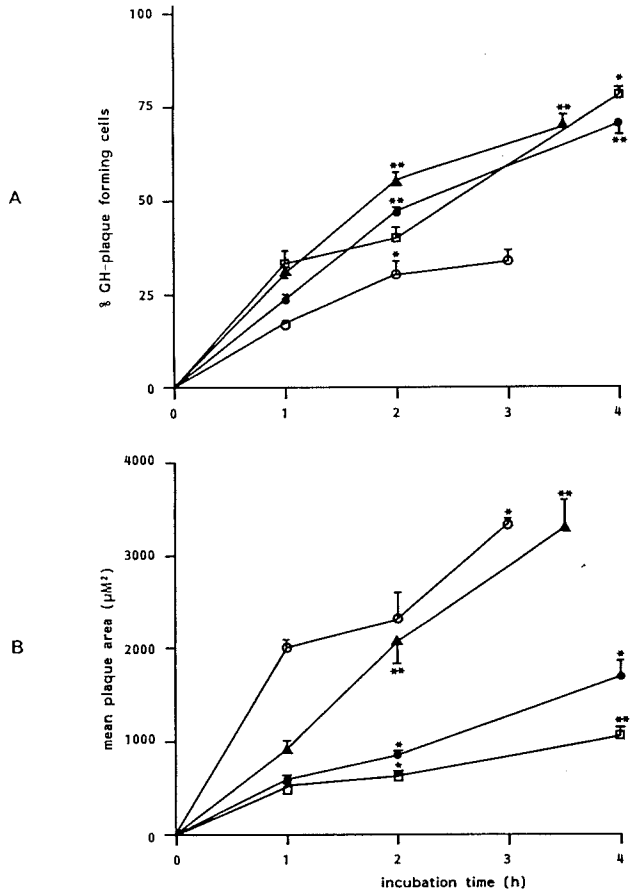

Fig 1 a, b: The dynamics of GH secretion by individual adenoma cells from four acromegalic patients as measured by the rates of GH plaque formation (Fig. 1a) and the increases in mean plaque areas (Fig. 1b) at different times of incubation. 1a: The mean (± SE) percentages of plaque-forming cells at different times of incubation from individual adenoma cells of patient 1 (○), 2 (□), 3 (▲) and 4 (●). 1b: The mean (± SE) plaque areas at different times of incubation in the same four patients (for symbols see 1a).
100 Cells per slide were counted or measured; n = 2 slides per incubation time $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with the previous time of incubation.

The mean plaque area also significantly increased as a function of incubation time. Interestingly, the cells from patient 1, which had the lowest rate of plaque formation, had the highest mean plaque areas. In contrast, the cells from patients 2 and 4 had considerably lower mean plaque areas at all incubation times as compared to the mean plaque areas of the cells from patient 3, despite similar rates of plaque formation in these three patients. These results indicate that these tumors differ not only with respect to the percentages of hormone secreting cells but also with respect to the mean amounts of hormone released per pituitary adenoma cell. This variability is also evident from the data presented in table 2 which will be discussed below. The mean plaque areas produced by the adenoma cells from five different acromegalic patients varied from 901 to 3559 μM^2 (Table 2).

Fig. 2 shows the frequency distributions of the GH plaque areas (which represent the amount of GH secreted per pituitary adenoma cell) from the 8 acromegalic patients. The GH plaque areas of all patients were not normally distributed. The frequency distributions of all patients indicated that only a small portion of the cells accounted for a large fraction of the mean plaque areas. The vertical interrupted lines in the frequency distributions indicate the logarithm of the mean plaque area; these lines are present in the right side of the frequency distribution. In order to further illustrate that only a minority of the tumor cells was responsible for a large fraction of the total amount of GH released by the tumor cells, we also calculated from the frequency distributions the percentages of plaques with individual plaque areas smaller than the mean plaque area (small plaques) and the percentages of plaques with individual plaque areas larger than the mean plaque area (large plaques). The results are shown in Table 1. The percentages of large plaques in the tumor cell suspensions of the 8 patients varied from 24 to 38% of the total cell population, but the large plaques accounted for 63% (patient 5) to 80% (patient 8) of the total plaque area (representing the total amount of GH released per 100 cells, which may be

considered as a measure of the total amount of GH secreted by the tumor).

We also investigated the effects of SMS 201-995, GHRH and TRH on the mean plaque areas and on the percentages of GH plaque-forming cells of patients 2, 3, 5, 7 and 8.

Figure 3 shows a photomicrograph (at low magnification) of the plaques from patient 8 (data in Table 2) in control cells (left panel), cells exposed to GHRH (middle panel) and cells exposed to SMS 201-995 (right panel). Note the large differences in individual plaque areas. 10 nmol/L SMS 201-995 significantly reduced the mean plaque areas in all patients (Table 2, $p < 0.05$ vs control in patients 5 and 7, and $p < 0.01$ vs control in patients 2, 3 and 8). In patient 2 SMS 201-995 reduced the mean GH plaque area in a dose-dependent manner, the effect of 0.1 nmol/L SMS 201-995 being significantly less than that of 10 nmol/L ($p < 0.01$). In the 2 other patients in which the effects of 0.1 and 10 nmol/L SMS 201-995 was studied, it reduced the mean plaque area either maximally (patient 8) or only at a concentration of 10 nmol/L (patient 3). 10 nmol/L SMS 201-995 also significantly reduced the percentage of plaque- forming cells in 2 of these 5 patients (patients 3 and 8).

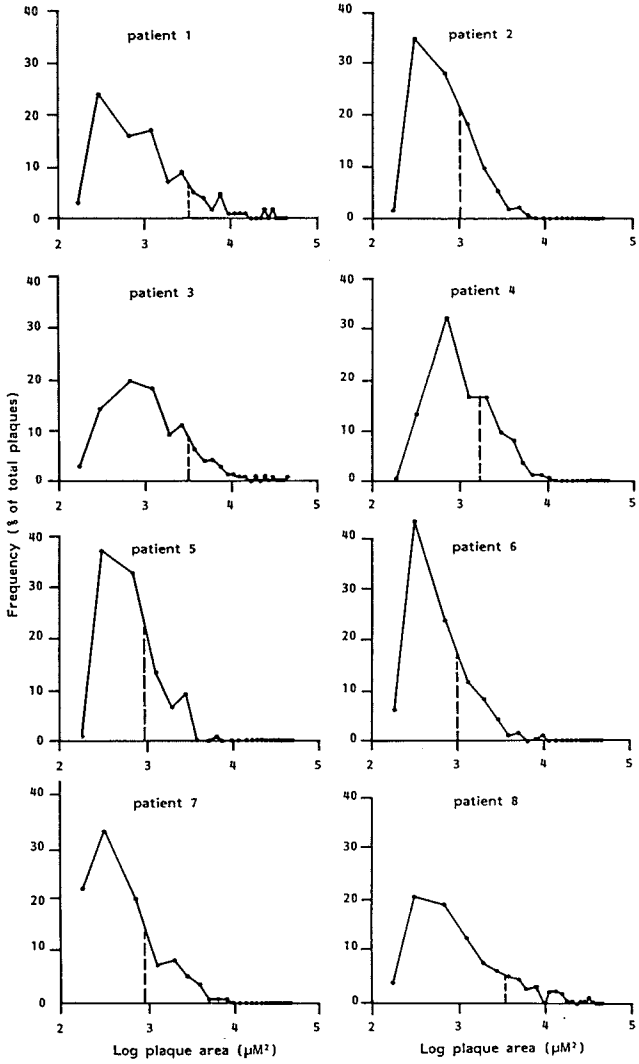

Fig. 2: Frequency distributions of GH plaque areas (GH secreted per GH-secreting pituitary adenoma cell) from eight acromegalic patients (patients 1-8). The plaque areas were measured at the incubation times of maximal (percentage) plaque formation. Each point in the frequency distribution curve represents the mean results from 2 slides (100 plaques were measured per slide). The vertical interrupted lines in each frequency distribution curve indicate the mean plaque area.

Table 1. Heterogeneity of plaque areas of individual adenoma cells from 8 acromegalic patients.

Patient	% small plaques	% large plaques	total plaque area of small plaques	total plaque area of large plaques	contribution of large plaques to the total plaque area
1	76.0 ± 3.8	24.0 ± 3.8	799 ± 52	2546 ± 407	76 %
2	63.5 ± 2.5	36.5 ± 2.5	305 ± 24	909 ± 222	75 %
3	74.5 ± 1.5	25.5 ± 1.5	901 ± 41	2391 ± 303	73 %
4	62.0 ± 3.0	38.0 ± 3.0	468 ± 27	1230 ± 198	72 %
5	70.5 ± 3.5	29.5 ± 3.5	348 ± 31	584 ± 73	63 %
6	72.5 ± 4.5	27.5 ± 4.5	310 ± 18	672 ± 142	68 %
7	75.0 ± 5.0	25.0 ± 5.0	284 ± 26	617 ± 139	68 %
8	72.0 ± 3.0	28.0 ± 3.0	715 ± 25	2844 ± 132	80 %

Total plaque areas are expressed in $\mu M^2 \times 10^2$. All data were derived from two slides, at the incubation times with maximal plaque formation. 100 cells per slide were counted and measured. The sum of the total plaque areas of the small and the large plaques is the total plaque area by 100 plaques. Plaques were designated as small plaques when they had individual plaque areas smaller than the mean plaque area (see Table 2, and in Figures 2, 3 and 4 as indicated by the vertical interrupted lines); large plaques had individual plaque areas larger than the mean plaque area.

Table 2 The effects of SMS 201-995, GHRH and TRH on the mean plaque areas and the percentages of GH plaque-forming pituitary adenoma cells from acromegalic patients.

	Treatment	Mean plaque area (μM^2)	Percentage of GH-plaque-forming cells
Patient 2	control	1050 ± 81	78.5 ± 1.5
	SMS201-995 (0.1 nmol/L)	830 ± 55[a]	78.5 ± 1.5
	SMS201-995 (10 nmol/L)	642 ± 8[b]	87.0 ± 1.0[b]
Patient 3	control	3293 ± 341	70.0 ± 3.0
	SMS 201-995 (0.1 nmol/L)	4586 ± 631	77.0 ± 1.0
	SMS 201-995 (10 nmol/L)	1711 ± 14[b]	61.5 ± 1.5[a]
	GHRH (10 nmol/L)	6723 ± 433[b]	76.5 ± 2.5
	SMS 201-995 (10 nmol/L) + GHRH (10 nmol/L)	2860 ± 275	66.0 ± 2.0
Patient 5	control	931 ± 42	47.0 ± 2.0
	SMS 201-995 (10 nmol/L)	473 ± 38[a]	40.0 ± 3.0
Patient 7	control	901 ± 112	25.0 ± 0.0
	SMS 201-995 (10 nmol/L)	448 ± 37[a]	19.5 ± 3.5
	GHRH (10 nmol/L)	985 ± 87	26.0 ± 2.0
Patient 8	control	3559 ± 157	77.0 ± 4.0
	SMS 201-995 (0.1 nmol/L)	1624 ± 278[b]	57.0 ± 4.0[a]
	SMS 201-995 (10 nmol/L)	1542 ± 556[b]	57.0 ± 1.0[a]
	GHRH (10 nmol/L)	7934 ± 905[b]	83.0 ± 0.0
	TRH (100nmol/L)	7267 ± 632[b]	75.0 ± 3.0

n=2 slides per treatment; 100 cells per slide counted and measured.

[a]$P<0.05$ vs control; [b]$P<0.01$ vs control.

10 nmol/L GHRH significantly increased the mean plaque area in 2 of the 3 patients in whom it was studied to 204% (patient 3) and 230% (patient 8) of control values, but had no significant effect on the percentage of plaque-forming cells in either patient. This result indicates that in these patients the percentage of GH plaque-forming cells was maximal at the incubation time in which the effects of the drugs and secretagogue was studied. The stimulating effect of GHRH on the mean plaque area in patient 3 was completely inhibited by 10 nmol/L SMS 201-995. We also studied the effect of 100 nmol/L TRH on the cells of patient 8. TRH significantly increased the mean plaque area of the cells of this patient to approximately the same extent (to 204% of control value) as did 10 nmol/L GHRH.

Fig. 4 shows the frequency distributions of the GH plaque areas of patients 5 and 7 under control conditions and in the presence of 10 nmol/L SMS 201-995. The frequency distribution shifted towards the smaller plaques in the presence of SMS 201-995 and there was a decrease in the number of plaques with large plaque areas, especially in patient 5. SMS 201-995 (10 nmol/L) did not decrease the percentage of GH-secreting cells in these patients under these conditions (Table 2). The frequency distribution of the GH plaque areas of patient 2 showed a comparable shift towards the smaller plaques in the presence of 10 nmol/L SMS 201-995 (not shown), but there was no significant decrease in the percentages of plaque forming cells (Table 2).

Fig. 5 shows that in patient 8 10 nmol/L SMS 201-995 caused a shift towards the smaller plaques. However, in this patient the number of GH plaque-forming cells decreased in the presence of SMS 201-995, indicating that also the smaller plaques were inhibited by SMS 201-995. SMS 201-995 had the same effect on the frequency distribution of GH plaque areas of patient 3. 10 nmol/L GHRH caused in both patients (3 and 8) a comparable increase in large plaques and a decrease in the small plaques, an effect completely abolished by 10 nmol/L SMS 201-995 (Fig. 5, bottom left). TRH (100 nmol/L) caused a shift in the frequency distribution in patient 8 comparable to the effect of GHRH (Fig. 5).

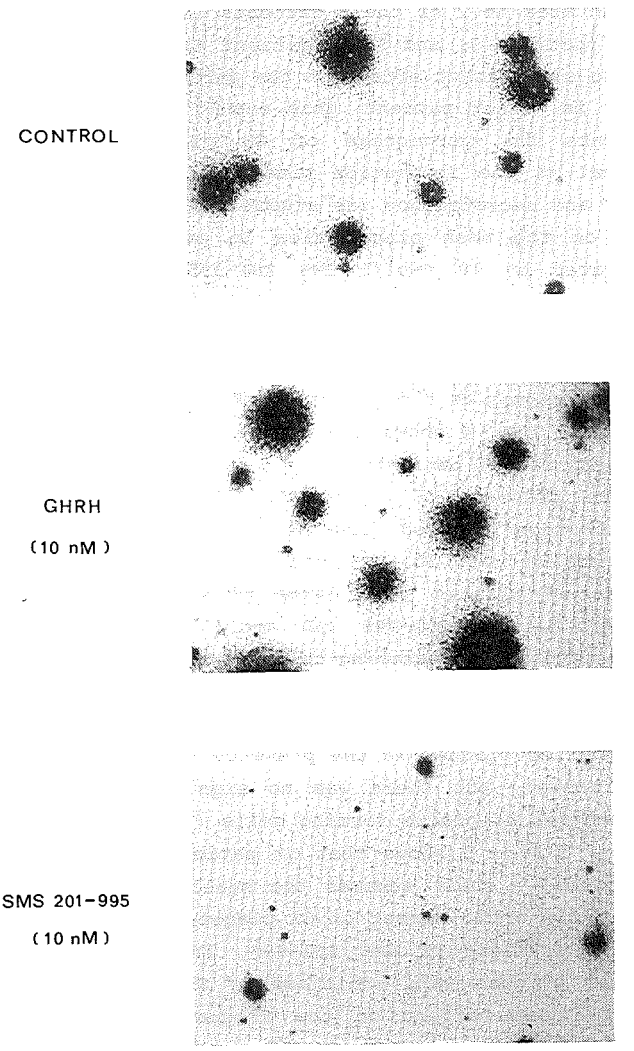

Fig. 3: Photomicrograph of GH plaques of patient 8 (black areas in the monolayer of oRBCs) at low magnification (40x) in a control slide (upper panel); incubation with GHRH (middle panel), and incubation with SMS 201-995 (lower panel). The incubation time was 2h.

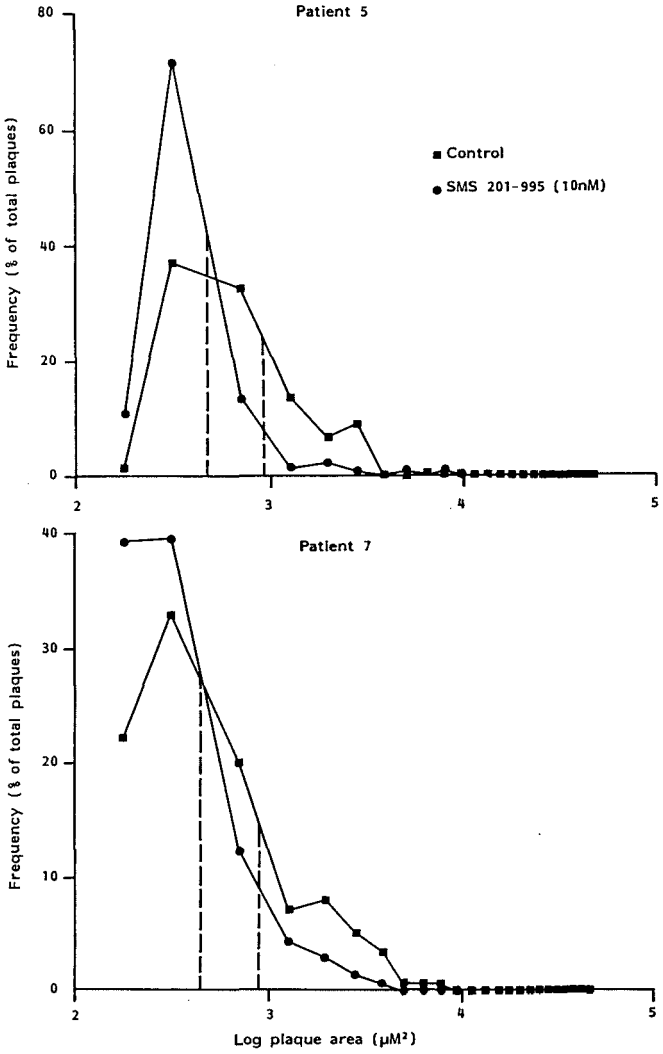

Fig. 4: The effect of 10 nmol/L SMS 201-995 on the frequency distributions of GH plaque areas from patients 5 and 7. See Legend to Fig. 2.

Finally, we validated our RHPA system by immunocytochemical localization of GH in the same cells which were used in the RHPA (adenoma cells from patients 3 and 4; Table 3).

Table 3

A comparison between the percentages of GH plaque-forming cells and the percentages of cells immunoreactive for GH of pituitary adenoma cells from two acromegalic patients.

	% of GH plaque-forming cells	% of GH-containing cells detected by immunocytochemistry
patient 3	70.0 ± 3.0	67.5 ± 3.5
patient 4	71.0 ± 3.0	80.0 ± 2.0

Mean ± SE

n = 2 slides, 100 cells counted per slide.

There were no significant differences between the percentages of cells which stained for GH and which formed plaques at the times of maximal plaque formation. Moreover, the intensity of the GH immunostaining of the different cells varied widely, from slightly to heavily stained cells (not shown). In addition, we performed cohort incubation studies in multiwell plates with the same cells and under the same conditions as were used in the RHPA (patients 1, 2, 3 and 4). When the (log) amounts of GH released into the medium in the multiwell plates (expressed in fg/cell) were correlated with the (log) mean plaque areas at the different incubation times (patients 1, 2 and 4) or with the effects of SMS 201-995 and GHRH on the mean plaque areas (patient 3), the correlation coefficients were 0.99 (patient 1), 0.98 (patient 2), 0.93 (patient 3) and 0.99 (patient 4). These results clearly demonstrate that the plaque areas were representative of the amounts of GH released by the tumor cells.

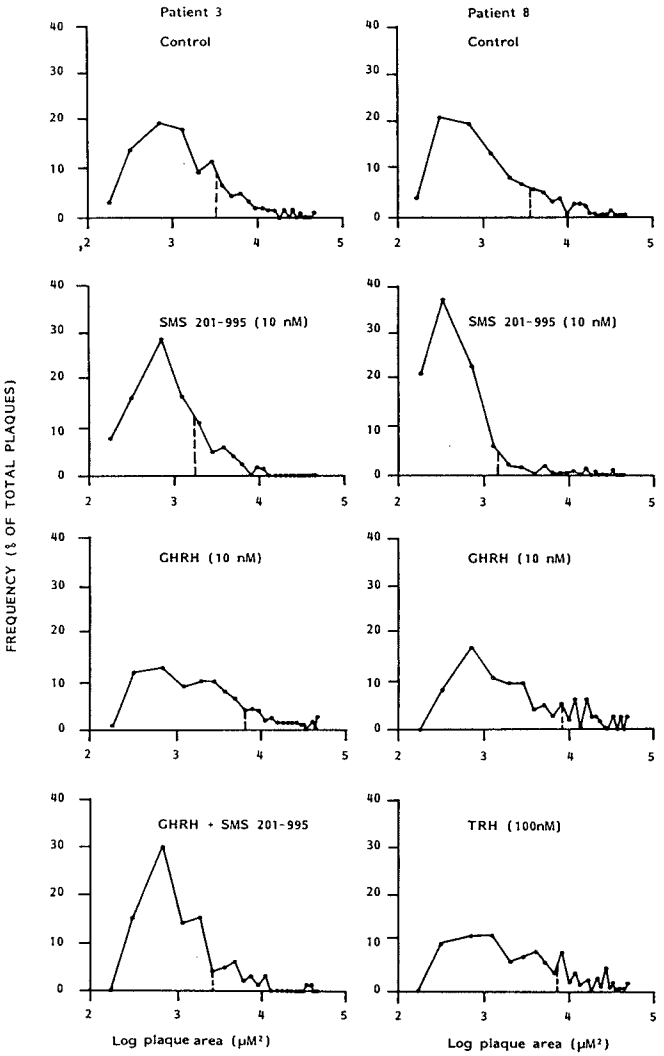

Fig. 5: The effect of SMS 201-995, GHRH, TRH and GHRH + SMS 201-995 on the frequency distribution of GH plaque areas of patients 3 and 8. See Legend to Fig. 2.

VI.5 Discussion

We studied the dynamics of GH secretion by individual pituitary adenoma cells from 8 acromegalic patients, using the reverse hemolytic plaque assay (RHPA) to detect hormone release from single cells (4, 8). The pituitary adenomas varied considerably with respect to the percentages of GH-secreting cells (from 25% to 78.5%) and with respect to the mean plaque areas (representing the mean amounts of GH released per cell, which varied from 901 to 3559 μM^2). This variability of GH secretion by adenoma cells from different acromegalic patients is in agreement with several in vivo and in vitro studies (15-17). Interestingly, among the adenomas several contained a large number of GH-secreting cells having a relatively low mean plaque area and others contained a small number of GH secreting cells having a relatively high mean plaque area. Therefore, variability of GH secretion by pituitary adenomas in vivo and in vitro may be due to differences in either the number of GH-secreting cells within the adenomas, the amount of GH secreted per cell, or both. Although the incubation time and/or the detection level in the RHPA might have been limiting factors, not all cells within the adenomas released GH. As the RHPA assay data are in agreement with the immunocytochemical data, it appears that within individual GH-secreting adenomas there are significant numbers of cells that do not produce GH. In addition Reubi et al (10) reported that in tissue sections from GH-secreting adenomas the number of GH-immunoreactive cells varied from 33% to more than 50%. At present the function of these non-GH-producing cells is unknown. In addition to the considerable variation in GH secretion between these tumors we also found considerable heterogeneity of the amount of GH released per individual adenoma cell. Only a minority (24-38%) of the GH-secreting adenoma cells accounted for a large portion of total hormone production (63-80%).

In some adenomas SMS 201-995 caused a shift in the GH plaque area frequency distribution towards the smaller plaques, but had no effect on the percentage of GH plaque

forming cells, which might indicate that in these adenomas the cells forming large plaques were inhibited preferentially by SMS 201-995. Further studies using sequential plaque assays with and without SMS 201-995 might provide further evidence concerning the presence of a subpopulation of adenoma cells preferentially responsive to the SRIH analog. The suggested, although not completely proven, difference in sensitivity to SMS 201-995 between cells from a single adenoma may be related to differences in the number of SRIH receptors per cell rather than to differences in intracellular mechanisms, since Reubi et al (10) recently found that in some GH-secreting adenomas a significant proportion of cells did not contain SRIH receptors.

Although acromegalic patients respond well to treatment with SMS 201-995 (18-26), pituitary tumor size decreases slightly in only about half of the acromegalic patients so treated (21, 23, 27). This slight decrease in tumor size might well reflect a decrease in the size of individual tumor cells (caused by lower hormonal synthesis and hormone content) rather than being the result of a cytotoxic or vascular effect of the drug (28). Our results fit well with this concept. First, we found that only a minority of the cells (approximately 30%) within these adenomas accounted for a large portion of the GH that was released. Second, we found that these cells might be preferentially inhibited by SMS 201-995 in some adenomas. Therefore the effect of SMS 201-995 on this minor cell subpopulation within adenomas from acromegalic patients may lead to only a slight decrease in tumor size despite a significant GH-release- inhibiting effect.

GHRH and TRH did not have preferential effects on a subpopulation of adenoma cells. Interestingly, in one patient TRH and GHRH caused comparable shifts in the frequency distributions of the GH plaque areas. These data suggest that in this adenoma TRH and GHRH had effects on the same population of adenoma cells.

In conclusion, the results of this study demonstrate heterogeneity of GH release by adenoma cells from acromegalic patients at the single cell level. A minority of

the adenoma cells accounted for a large portion of the total amount of GH released. In addition, we found that SMS 201-995 preferentially inhibited a subpopulation of adenoma cells in some adenomas.

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CHAPTER VII

GLYCOPROTEIN HORMONE ALPHA-SUBUNIT AND PROLACTIN RELEASE BY CULTURED PITUITARY ADENOMA CELLS FROM ACROMEGALIC PATIENTS: CORRELATION WITH GH RELEASE.

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Clinical Endocrinology, 1989; 30: 601-611

VII.1 Abstract

In vitro data of pituitary adenoma cells from 28 acromegalic patients were evaluated. Next to GH, PRL was produced by 16 adenomas (57%) and alpha-subunit by 15 adenomas (54%) while there was a significant higher incidence of tumours producing PRL and alpha-subunit simultaneously.

From 26 pituitary adenomas enough cells were obtained in order to perform secretion studies. Percentual basal hormone release [medium:(medium+intracellular hormone)]x100% of GH and alpha-subunit by 11 adenomas showed a close correlation while such a correlation for GH and PRL was present only in a subgroup of 10 of 13 adenomas. The responses of GH and alpha-subunit release to 10nM SMS201-995, 10nM bromocriptine, 100nM TRH and 10nM GHRH were closely related in that a response or an absent response of GH release to the four secretagogues virtually always was attended with a response or an absent response respectively of alpha-subunit release. Such a relationship was less evident with respect to the effects of SMS201-995, bromocriptine, TRH and GHRH on GH and PRL release.

We conclude that basal- and secretagogue-induced alpha-subunit release by cultured pituitary adenoma cells from acromegalic patients closely follows the pattern of GH release while such a relationship for GH and PRL is present only in a subgroup of the adenomas secreting GH and PRL simultaneously.

VII.2 Introduction

Pituitary adenomas from patients with acromegaly are heterogeneous with respect to hormone production. Together with GH other hypophysial hormones may be released (Kovacs and Horvath, 1986). Most frequently a combination with hypersecretion of prolactin (PRL), both in vivo (Franks et al., 1975; Moriondo et al., 1980; DePablo et al., 1981; Lamberts et al., 1982) and in vitro (Guyda et al., 1973; Peillon et al., 1978; Mashiter et al., 1979) has been demonstrated in acromegaly. Interestingly, also alpha-subunit hypersecretion may occur frequently in patients with acromegaly. Macfarlane et al. (1980) demonstrated alpha-subunit hypersecretion in vivo in approximately one-third of a group 46 acromegalic patients. Moreover White et al. (1986) showed in an in vitro study, using adenoma tissue from 32 acromegalic patients that together with GH also PRL (21 tumours) and alpha-subunit (22 tumours) is being secreted simultaneously. Recently Beck-Peccoz et al. (1985) presented evidence for alpha-subunit and GH-coexistence in the same tumoural cell using the protein A-gold particle immunotechnique. Furthermore, GHRH stimulated alpha-subunit release both in vivo and in vitro in patients with elevated alpha-subunit levels indicating co-release of GH and alpha-subunit. A similar parallellism of the response of GH and alpha-subunit to TRH and bromocriptine has been shown by Ishibashi et al. (1987).

Coexistence of two hormones within the same secretory granule in tumour cells from acromegalic patients has also been shown for GH and PRL (Bassetti et al., 1986) whereas the secretory responses of GH and PRL in acromegalic patients with mixed GH- and PRL secreting adenomas often show a close parallellism (Lamberts et al., 1979, 1982, 1983, 1985; Goldman et al., 1984; Bassetti et al., 1986). In addition, stimulation of PRL release by GH/PRL secreting tumours by GHRH has been shown in vitro (Webb et al., 1983; Ishibashi et al., 1985; Serri, 1987).

So far, most evidence for the close relationship between GH- and alpha-subunit release and GH- and PRL release has been derived from either in vivo studies or from in vitro

studies using tumour specimens from only few patients. In the present study we investigated in an in vitro study using cultured adenoma cells from a large group of acromegalic patients (28 patients), the interrelationship between GH, PRL and alpha-subunit with respect to 1) the incidence of the simultaneous presence of these hormones in media from the cultured adenoma cells and/or in cell extracts 2) basal hormone release 3) the effects of TRH, GHRH, bromocriptine and SMS201-995 on the release of the three hormones.

VII.3 Subjects and Methods

Patients and preparation of dispersed tumour cells

Tumour specimens from twenty-eight untreated acromegalic patients (of both sexes) were obtained by transsphenoidal operation as described in detail previously (Oosterom et al., 1984). Thyroid function was normal in all patients. Briefly, the aspirated adenoma tissue contaminated with blood was washed twice with calcium - and magnesium-free Hank's Balanced Salt Solution (HBSS) supplemented with 1% human serum albumin, penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (0.5 µg/ml) and sodium bicarbonate (0.4 g/l final concentration). Thereafter the adenoma tissue and blood cells were incubated for approximately one hour with dispase (grade II, 2.4 U/ml; Boehringer Mannheim, Mannheim, W. Germany) and dispersed using an all-glass Dounce tissue grinder (Wheaton Scientific, Millville, U.S.A.). In order to remove the 100- to 1000-fold excess of erythrocytes the remaining cell suspension was layered on Ficoll-Isopaque (density 1.077 g/cm^3, prepared by the University Hospital Dijkzigt Pharmacy; Rotterdam, the Netherlands) and centrifuged at 500 x g for 20 min. The interphase, containing the adenoma cells (Oosterom et al., 1984) was washed twice with HBSS +1% HSA. The viability of the resulting cell suspension as determined by trypan blue exlusion was always greater than 80%.

Cell culture

The cells were cultured at a density of 10^5 cells per well per 1 ml of culture medium in 48-well plates (Costar, Cambridge, Massachussets, USA). The cells were cultured at 37°C in a water-jacketed incubator in humidified air with 5% CO_2. On day 4 of culture, after which the cells had attached, the medium was changed and 4 or 24 hour incubations without or with secretagogues or drugs were performed in quadruplicate. The culture medium that was used in all experiments was Minimal Essential Medium with Earles Salts (MEM) supplemented with non-essential amino acids, sodium pyruvate (1 mM), 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (0.5 µg/ml), L-glutamine (2 mM) and sodium bicarbonate (2.2 g/L final medium concentration). The medium was adjusted to pH 7.4 with 1N NaOH. Medium, obtained as a 10 times concentrated solution, and supplements were purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Since all wells contained the same number of initially seeded tumour cells the results of the experiments were expressed as µg/l GH or PRL or alpha-subunit/ml per 10^5 cells per well.

GH-releasing hormone (GHRH) was obtained from Universal Biologicals (Cambridge, U.K.); TRH from Hoechst (Amsterdam, the Netherlands). SMS 201-995 and bromocriptine-mesylate were gifts from Sandoz (Basel, Switzerland).

Intracellular hormone concentrations were measured in cell extracts obtained by lysis of the cultured cells in distilled water containing 0.1% bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, MO, U.S.A.) as described in detail elsewhere (Oosterom et al., 1983).

Hormone assays

GH and PRL in the culture media and cell extracts were measured by immunoradiometric assays for human growth hormone (HGH-IRMA) and human prolactin (PRL-IRMA) respectively, purchased from Euro-diagnostics (Apeldoorn, the Netherlands). The intra- and inter- assay variation was <7%

and <13% respectively for the HGH-IRMA and <10% and<15% for the PRL-IRMA.

Alpha-subunit in the culture media and cell extracts was measured by a double-antibody RIA using antibodies purchased from UCB (Brussels, Belgium), and MRC 78/554 (potency: 1mIU/mg) as standard preparation. The sensitivity of the alpha-subunit assay, defined by a 10% fall in relative binding, was 0.2 ng/ml. Intra- and interassay variation was <6% and <11% respectively. Crossreactivities (in ng/ng) with LH MRC 68/40 (potency 6.6 mIU/ng), with a purified FSH preparation from KABI (Stockholm, Sweden) and with TSH (UCB, Brussels, Belgium; catalog number i001) were 3, 3.6 and 3.6% respectively. Crossreactivity with LH, FSH and TSH was parallel in the alpha-subunit assay.

Statistical analysis

The statistical significance of the differences between mean values of the in vitro data was determined using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made by the Newman-Keuls test (Snedecor and Cochran, 1980).Measures of correlation were made using the Spearman rank correlation coefficient. Other statistical tests that were used are mentioned in the text. All data are expressed as mean $\pm$ SEM.

VII.4 Results

In vitro data of pituitary adenoma cells from 28 acromegalic patients were evaluated. We found 9 adenomas to be positive for GH only (32% of the group of 28 adenomas), 4 adenomas were positive for GH + PRL (14%), 3 adenomas were positive for GH + alpha-subunit (11%) and 12 adenomas were positive for GH + PRL + alpha-subunit (43%). There was a significant higher incidence of tumours containing all three hormones ($p<0.01$ using Fischers extact probability test; Siegel, 1956) which indicates that PRL and alpha-subunit are mostly present simultaneously. A tumour was considered

positive for GH, alpha-subunit and/or PRL when the hormone(s) were detectable in a 96 hour poolmedium (10^5 cells per well, medium from at least four different wells) and/or in the intracellular hormone content directly after isolation (10^5 cells per 1 ml of distilled water containing 0.1% BSA; repeated freeze-thawing). We excluded the possibility that PRL and/or alpha-subunit were derived from contaminating normal tissue since no ACTH, LH or FSH was detected in the poolmedia and cell extracts.

In table 1 the amount of GH and/or PRL and alpha-subunit released into the medium by cells from 26 adenomas in 4 or 24 hour incubation periods is shown. Also the intracellular hormone content at the end of the incubation period is shown. Only data from 26 patients are shown as from the adenomas from the 2 other patients (GH-containing only) not enough cells were isolated in order to perform incubation studies. The values in table 1 are expressed in $\mu g/l$ per 10^5 initially plated cells. We also calculated for each hormone the percentual basal release (% basal release) in all patients as follows : [medium : (medium + intracellular hormone)] x 100%.

Table 1. Hormone release and cellular content from 26 cultured pituitary adenomas from acromegalic patients, expressed per 10^5 cells.

Patient no	Incubation time(h)	GH (µg/l ± SE) medium	GH intracellular	% basal release	Alpha-subunit(µg/l ±SE) medium	Alpha-subunit intracellular	% basal release	PRL (µg/l ± SE) medium	PRL intracellular	% basal release
1	4	745 ± 24	20996±891	3	-	-	-	-	-	-
2	4	73 ± 2	393± 8	16	-	-	-	-	-	-
3	4	137 ± 6	903± 51	13	-	-	-	-	-	-
4	24	76 ± 4	80± 7	49	-	-	-	-	-	-
5	24	622 ± 12	not done	-	-	-	-	-	-	-
6	4	34 ± 1	65± 2	34	-	-	-	-	-	-
7	24	86 ± 6	87± 10	50	-	-	-	-	-	-
8	24	889 ± 40	1631± 56	35	25.7±1.0	44.9±1.5	36	-	-	-
9	24	458 ± 17	379± 5	55	6.5±0.2	3.7±0.2	64	-	-	-
10	4	163 ± 21	397± 25	29	0.5±0.1	0.9±0.2	36	-	-	-
11	4	365 ± 12	368± 9	50	-	-	-	56±2	40± 3	58
12	24	184 ± 9	70± 3	72	-	-	-	9.9±0.9	5.2±0.1	66
13	24	126 ± 1	55± 1	70	-	-	-	13±1	5.6±0.2	70
14	24	24 ± 1	56± 2	30	-	-	-	ND	ND	-
15	24	549 ± 26	4874±205	10	not done	1.1±0.1	-	ND	ND	-
16	24	1473 ± 42	555± 27	73	25.3±0.9	2.9±0.1	89	203±6	84±6	71
17	24	676 ± 12	2059±107	25	20.2±0.6	30.8±1.8	39	7.6±0.4	2.8±0.1	73
18	24	314 ± 6	88± 2	78	3.4±0.3	1.3±0.1	72	1680±52	45±2	97
19	24	308 ± 15	1362± 33	18	0.2±0.1	3.5±0.3	5	126±3	18±2	87
20	4	302 ± 18	577± 63	34	0.8±0.1	2.1±0.2	28	20±3	27±2	42
21	4	173 ± 5	88± 7	66	0.8±0.1	0.3±0.0	73	209±11	22±2	90
22	24	108 ± 5	113± 5	49	0.4±0.0	0.3±0.0	57	79±3	13±2	86
23	4	43 ± 2	394± 17	10	0.3±0.1	2.1±0.1	12	8.4±0.4	46±2	15
24	24	120 ± 6	54± 7	69	ND	ND	-	8.2±0.5	4.2±0.6	66
25	4	498 ± 17	2600±100	16	ND	ND	-	155±4	368±21	30
26	24	46 ± 1	131± 4	26	ND	ND	-	ND	ND	-

% basal release= [medium:(medium+intracellular)]x100%

ND= not detectable but alpha-subunit and/or PRL detectable in 96h medium.

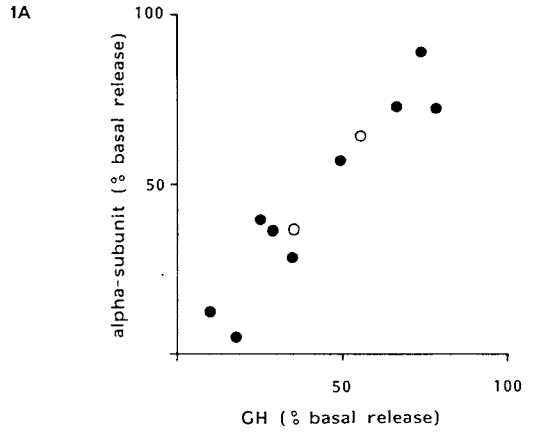
- = not detectable and no alpha-subunit and/or PRL detectable in 96 h medium.

Some adenomas that secreted PRL and/or alpha-subunit in the 96 h media or had detectable intracellular amounts of PRL and/or alpha-subunit (table 1) did not produce detectable amounts of PRL and/or alpha-subunit in the 4 or 24 h incubation experiments. This is indicated in table 1 by ND (not detectable). The 26 patients are considerably heterogeneous with respect to hormone production. Medium values for GH, alpha-subunit and PRL vary from 24 -1473, 0.2 - 25.7 and 7.6 - 1680 μg/l per 10^5 initially plated cells respectively. There was no statistically significant difference in GH production between the group of tumours containing GH only and the group of tumours containing GH, PRL and/or alpha-subunit (unpaired T-test). A similar heterogeneity within the group of 26 patients is evident with respect to percentual basal hormone release with values varying from 3 - 78% for GH, 5 - 89% for alpha-subunit and 15 - 97% for PRL. Again there was no significantly difference in percentual GH release between both groups of tumours (unpaired T-test).

As there is much evidence for co-release of GH and alpha-subunit and also for GH and PRL we determined the relationship between the % basal release of these hormones. This is shown in fig. 1A and fig. 1B. There is a very close correlation between % basal GH- and % basal alpha-subunit release (r_s = 0.8932 ; $p < 0.001$) while no significant correlation could be demonstrated between % basal GH- and % basal PRL release (r_s = 0.3970 ; not significant). However, when the three tumours having a relatively high % basal PRL release as compared to % basal GH release (patients 17, 19, and 22; see table 1) are omitted from the calculations a highly significant correlation between % basal GH release and % basal PRL release is evident (r_s = 0.8576 $p < 0.005$). Interestingly the tumour cells from patients 17, 19 and 22 all produced next to PRL also alpha-subunit (fig. 1B, closed circles), with the latter hormone having a close correlation with % basal GH release (fig. 1A).

In some adenomas GH and PRL concentrations were measured after 4 and 24 h incubation. In all instances GH and PRL concentrations were significantly higher in the 24 h

incubation media. Moreover there was no discrepancy between the % basal release of GH and PRL at 4 and 24 h of incubation.



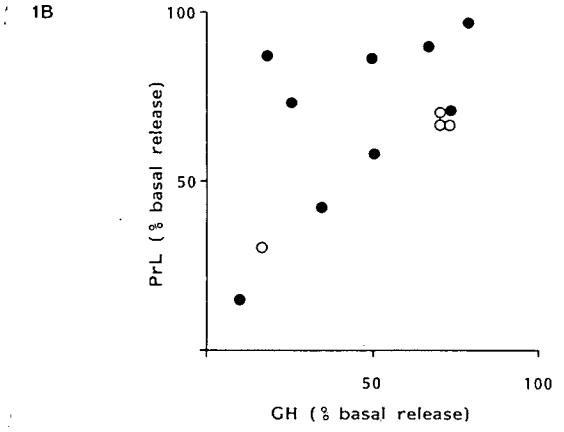


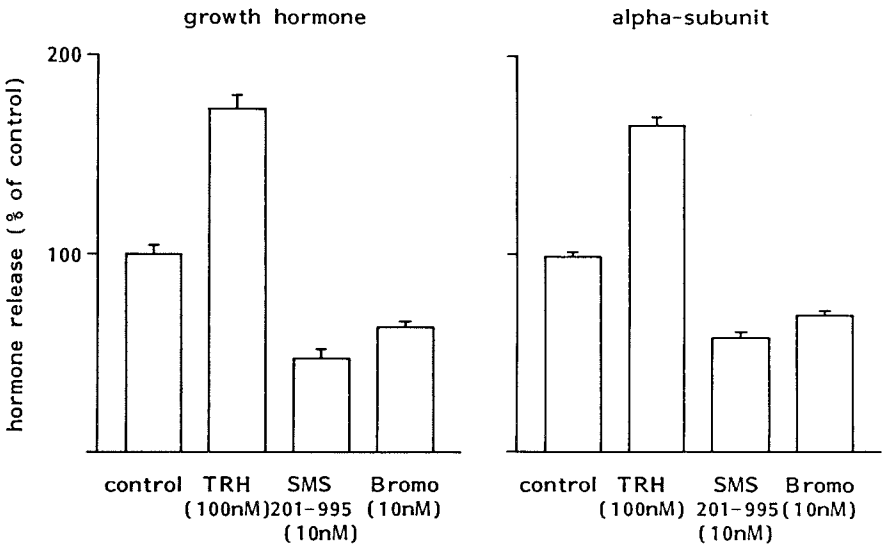
Figure 1A: The relationship between % basal GH- and % basal alpha-subunit release (=[medium : (medium + intracellular)]x100%) by cultured pituitary adenoma cells of 11 acromegalic patients. r_s=0.8932; p<0.001. Open symbols: adenomas secreting GH + alpha-subunit; closed symbols: adenomas secreting GH + PRL + alpha-subunit. Each dot represents a single patient.

1B: The relationship between % basal GH- and % basal PRL release by cultured pituitary adenoma cells of 13 acromegalic patients. r_s=0.3970; not significant. Open symbols: adenomas secreting GH + PRL; closed symbols: adenomas secreting GH + PRL + alpha-subunit. Each dot represents a single patient.

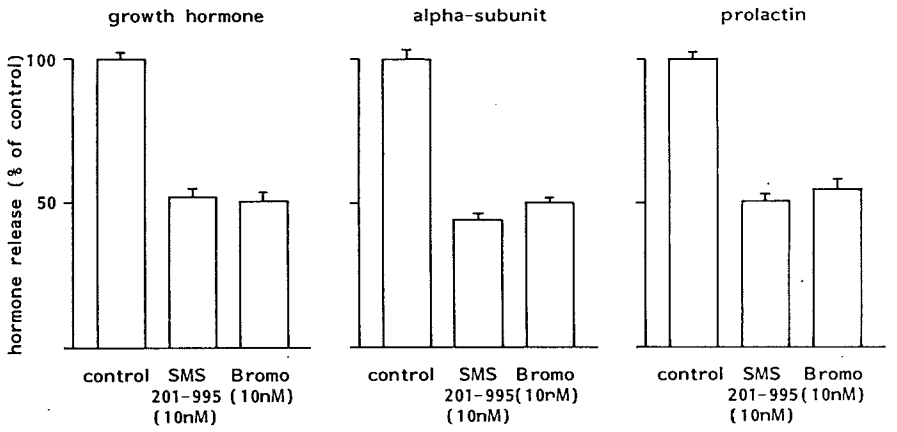
Within our group of patients we saw a similar relation of GH, alpha-subunit and PRL release with respect to the response of hormone secretion to SMS201-995 (10nM), bromocriptine (10nM), TRH (100nM) and GHRH (10nM). Using Fishers exact probality test (Siegel, 1956) we found that a statistically significant effect of GH release by the cultured tumour cells to 10nM SMS201-995 (9 adenomas studied) and 10nM GHRH (9 adenomas studied) is always attended with a significant effect on alpha-subunit release while an absent response of GH release is always attended with an absent response of alpha-subunit release ($p<0.05$). Within the group of cultures in which the effects of 10nM bromocriptine (6 adenomas studied) and 100nM TRH (7 adenomas studied) was studied we found parallel effects on GH and alpha-subunit release in 5 of 6 and 6 of 7 cultures respectively. However, the relationship was not statistically significant because of the low number of cultures in which these effects were studied. Investigation of the same relationship for GH and PRL as described above revealed that only a significant relationship was observed for the effect of 10nM SMS201-995 ($p<0.05$).

In order to further illustrate that there is in all instances a parallelism of the responses of GH and alpha-subunit release to TRH, SMS201-995 and bromocriptine and that this is not always the case for GH and PRL release we selected from the patients from table 1 three examples. These are shown in the figures 2A, B and C. The responses to TRH, SMS201-995 and bromocriptine are expressed as percentages of the hormone release in control wells. The absolute amount of GH, PRL and/or alpha-subunit secreted by the cultured tumour cells (control wells) from these patients is shown in table 1. GH and alpha-subunit release by cultured tumour cells from patient 8 (figure 2A) are significantly stimulated by 100nM TRH to 173 and 165% of control value respectively and significantly inhibited to 47 and 58% of control respectively by 10nM SMS201-995 and to 63 and 69% of control value respectively by 10nM bromocriptine ($p<0.01$ vs control for both hormones in all instances).

2A


growth hormone
alpha-subunit
hormone release (% of control)
200
100
control
TRH (100nM)
SMS 201-995 (10nM)
Bromo (10nM)

2B


growth hormone
alpha-subunit
prolactin
hormone release (% of control)
100
50
control
SMS 201-995 (10nM)
Bromo (10nM)

2C

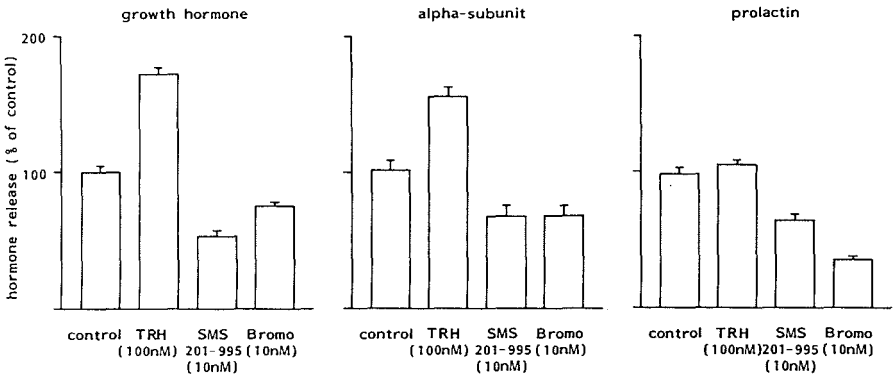


Figure 2A: The effect of TRH, SMS201-995 and bromocriptine on growth hormone- and alpha-subunit release by cultured pituitary adenoma cells from patient no.8.

2B: The effect of SMS201-995 and bromocriptine on growth hormone-, alpha-subunit- and prolactin release by cultured pituitary adenoma cells from patient no.16.

2C: The effect of TRH, SMS201-995 and bromocriptine on growth hormone-, alpha-subunit- and prolactin release by cultured pituitary adenoma cells from patient no.22.
The effects are expressed as percentage of control release ± SEM; n=4 wells per group.

Figure 2B shows the effects of SMS201-995 (10nM) and bromocriptine (10nM) on GH, alpha-subunit and PRL release by cultured tumour cells of patient 16. GH, alpha-subunit and PRL release are significantly inhibited by SMS201-995 to 52, 44 and 51% of control value respectively and by bromocriptine to 51, 50 and 55% of control respectively, demonstrating a complete parallellism of the responses of GH, alpha-subunit and PRL release by the cultured cells of this patient.

Figure 2C (patient 22) is an example of the disparate responses of GH and PRL release we observed in some instances. Again GH and alpha-subunit release show a close parallellism in the responses to TRH (100nM), SMS201-995 (10nM) and bromocriptine (10nM). However, TRH did not significantly alter PRL release by the cells of this patient while 10nM bromocriptine inhibited PRL release (to 35% of control value) significantly more than GH and alpha-subunit release (to 75 and 68% of control value respectively; $p<0.01$ vs the effect of bromocriptine on GH and alpha-subunit release). Interestingly, patient 22 was one of the three cultures with a relatively high basal PRL release as compared to basal GH release (table 1; figure 1B). Moreover, in the two other cultures (patient 17 and 19) also disparate responses of GH and PRL release to 10nM SMS201-995 and 10nM GHRH (patient 17) and to 10nM GHRH (patient 19) were observed (data not shown).

VII.5 Discussion

Two recent studies have shown that glycoprotein hormone alpha-subunit secretion by pituitary adenomas from acromegalic patients closely follows the pattern of GH secretion, both in vivo and in vitro (Beck-Peccoz et al., 1985; Ishibashi et al., 1987). In the same study Ishibashi et al. (1987) demonstrated in patients with prolactinomas a similar parallellism of the responses of PRL and alpha-subunit release in vivo to the stimulatory effect of TRH and to the suppressive effect of bromocriptine and concluded that alpha-subunit responses in patients with pituitary adenomas are generally parallel with those of the concomitantly produced hormones. Although the in vivo studies by Beck-Peccoz et al. (1985) and by Ishibashi et al. (1987) were performed using large groups of acromegalic patients (22 and 58 patients respectively) the same papers describe in vitro studies using tumour specimens from only few patients (5 patients and 1 patient respectively).

Also for GH and PRL a similar parallellism of the responses may exist (Lamberts et al., 1979, 1982, 1983, 1985;

Goldman et al., 1984; Bassetti et al., 1986). However, Serri et al (1987) recently suggested the existence of two populations of acromegalic patients with hyperprolactinaemia, one group with correlations between GH and PRL secretion, and the other without.

The present in vitro study confirms, in a large group of acromegalic patients, that basal- and secretagogue- or drug- induced GH release by cultured pituitary adenoma cells from acromegalic patients is always parallelled by simultaneous alpha-subunit release and mostly, but not always by simultaneous PRL release. Percentual basal alpha-subunit release and percentual basal GH release (for calculation see results section) by cultured pituitary adenoma cells from 11 acromegalic patients were significantly correlated which indicates that GH and alpha-subunit might be released from the same secretory granule as has been proposed by Beck-Peccoz et al. (1985). In line with this are our present data demonstrating a significant relation between GH and alpha-subunit with regard to the number of responders of hormone release to SMS201-995 and GHRH in that a significant inhibition and stimulation of GH release, respectively, always was attended with a significant effect on alpha-subunit release, while an absent response of GH release to these secretagogues was always attended with an absent response of alpha-subunit release. Next to a stimulation of alpha-subunit release by GHRH in acromegalic patients with elevated alpha-subunit levels Beck-Peccoz et al. (1985) also demonstrated in these patiens coexistence of both GH and alpha-subunit within the same secretory granules using the protein A-gold immunotechnique.

In contrast to the observed correlation between basal- and secretagogue- induced GH and alpha-subunit release no such correlation was observed for percentual basal GH- and percentual basal PRL release when we considered a group of thirteen adenomas by which GH and PRL were produced in culture simultaneously. However, when three adenomas having a relatively high percentual basal PRL release as compared to percentual GH release were omitted from the calculations a significant correlation was found between the % basal

release of both hormones. Therefore, the group of adenomas producing GH and PRL simultaneously appears not to be homogeneous. Bassetti et al. (1986) recently demonstrated the presence of numerous mammosomatotrophs (50-80% of the whole cell population) in all adenomas from a group of five acromegalic patients with hyperprolactinemia. Moreover, GH and PRL were shown to be present in the same secretory granule (Bassetti et al., 1986). These data may well explain the significant correlation between basal GH and PRL release in the ten out of thirteen adenomas observed in our study. The three other cultures with a relatively high basal PRL release as compared to GH release may consist of either mixed tumours with little mammosomatotrophs and a prevalency of distinct cells secreting GH or PRL separately.

We also demonstrated a significant higher incidence of tumours producing next to GH, PRL and alpha-subunit simultaneously. In view of this observation the suggestion of a role of LH- alphasubunit in the induction of fetal lactotroph differentiation is of potential interest (Begeot et al., 1984). However, White et al., (1986) did not demonstrate such a relationship for the simultaneous presence of GH, PRL and alpha-subunit in a recent in vitro study using pituitary adenoma tissue from thirty-two acromegalic patients. As yet we do not have an explanation for the discrepancy between their results and ours. Alpha-subunit hypersecretion is also found in patients with prolactinoma (McComb et al., 1984; Ishibashi et al., 1987) and ACTH-producing adenoma (Landolt and Heitz, 1986; Ishibashi et al., 1987). The existence of a variety of combinations of coexistence of alpha-subunit and chemically non-related hormones in pituitary adenomas suggests that pituitary adenomas may develop from a common pluripotent stem cell (McComb et al., 1984).

In conclusion, our study demonstrates in a large group of pituitary adenomas from acromegalic patients that GH release by these adenomas in vitro is significantly correlated with basal- and secretagogue- induced alpha-subunit release while such a correlation for GH and PRL was observed in a subgroup of the GH/PRL secreting adenomas.

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CHAPTER VIII

GENERAL DISCUSSION

VIII.1 Introduction

In chapters III to VII the results of our investigations dealing with several aspects of functional heterogeneity among cell types of the normal rat pituitary gland, among cells of PRL-secreting rat pituitary tumors and among cells of human GH-secreting pituitary adenomas are presented. In this chapter the results of our investigations are discussed in relation to the physiological significance of functional heterogeneity among pituitary (adenoma) cell types.

VIII.2 The normal (rat) pituitary gland

During the last decade compelling evidence for the existence of functional heterogeneity among rat anterior pituitary cell types has been presented. Anterior pituitary cell types may be functionally heterogeneous in their response to hypothalamic hypophysiotropic hormones or in the amount of hormone released per cell. In addition these two types of functional heterogeneity may be related to each other (see chapter I.1.2.2). Differences in the amount of hormone release per cell can be of significant importance in determining the total amount of hormone which is released by a total population of a specific anterior pituitary cell type. An example of this has been given by Luque et al (1) who demonstrated the existence of two subpopulations of lactotropes within anterior pituitary lobes of proestrus rats, one secreting small amounts of PRL and one secreting large amounts of PRL. In their study they showed that approximately 65% of the lactotropes were cells forming large plaques and that they accounted for about 91% of the total plaque area (total amount of PRL secreted). Since differences in the amount of hormone secreted per cell have also been demonstrated for other anterior pituitary cell types (chapter I.1.2.2) it may be suggested that this kind of functional

heterogeneity among cell types has a significant influence on anterior pituitary hormone levels in vivo. The physiological significance of secretion behaviour of individual cells with regard to the hormone level in vivo is clearly illustrated by data from Hoeffler and Frawley (2) who showed, using the RHPA, that the sexual differences of GH release in rats (mature males vs. females) can be attributed to the secretory capacities of the individual somatotropes.

The second form of functional heterogeneity among anterior pituitary cell types, differential responsiveness to secretagogues, may also play an important role in determining hormone levels and responses in vivo. In the study of Luque et al (1) evidence was presented that the lactotrope subpopulation secreting small amounts of PRL (approximately 35% of the total lactotrope population) may be totally unresponsive to dopamine whereas the plaque areas by the lactotrope subpopulations forming large plaques were significantly inhibited. Boockfor and Frawley (3) showed that PRL-cells, derived from different anterior pituitary regions showed differential responsiveness to TRH and DA. They proposed that hypophysiotropic input might be responsible for the regional differences in lactotrope-responsiveness (see also chapter I.1.2.2). This hypothesis may be supported by the results from our experiments described in chapter III. The results from these experiments showed no differences in TRH- and DA-responsiveness among long-term cultured PRL-cell subpopulations separated on Percoll density gradients according to differences in their density. Since most data with respect to functional heterogeneity among lactotrope subpopulations were derived from experiments using freshly dispersed, short-term cultured cells, and because Boockfor and Frawley suggested that the regional differences in lactotrope-responsiveness might be related to differences in hypophysiotropic input (3), we proposed in our study (chapter III) that long-term deprivation of possible functionally heterogeneous subpopulations of lactotropes from their hypothalamic regulatory input might result in a homogeneous cell population. Moreover, it has been suggested that paracrine interactions between neighboring cells may play a role in

determining functional heterogeneity among specific anterior pituitary cell types (4). Similar processes as mentioned above may also play a role in the expression of functional heterogeneity as has been observed among other anterior pituitary cell types (chapter I.1.2.2). In conclusion we propose that functional heterogeneity among anterior pituitary cell types may play an important role in determining hormone levels and hormone responses to secretagogues in vitro and in vivo. In addition the existence of this functional heterogeneity may reflect an important component of the endocrine regulatory process.

VIII.3 Pituitary adenomas

As has been discussed in the previous paragraphs heterogeneity in hormone release and in responsiveness to secretagogues among pituitary cell types may play an important role in the regulation of anterior pituitary hormone secretion. Until now, it is not known whether such functional heterogeneity also exists among cells within pituitary adenomas. In this chapter we will discuss the results of our experiments using an experimental transplantable prolactin-secreting rat pituitary tumor (chapter IV). In addition our data from experiments using dispersed human GH-secreting pituitary adenoma cells are discussed.

In line with the well-established cellular heterogeneity of human pituitary adenomas (chapter I.2.2) most experimental pituitary tumors also show cellular heterogeneity (5-7). The transplantable prolactin-secreting rat pituitary 7315b tumor which we used for the experiments described in chapter IV originates from the bihormonal ACTH-PRL secreting 7315a tumor which has lost its ability to secrete ACTH as well as its receptors for glucocorticoids (8). The results from our study using the 7315b tumor provide evidence for functional homogeneity among cells of this tumor as measured by growth rates, protein content of the cells, PRL production per cell and responsiveness of the cells to the somatostatin analog SMS 201-995. Moreover, we demonstrated that 7315b tumors in various stages of their

growth (tumors of increasing weight) also showed homogeneity with respect to the above parameters. This tumor homogeneity of the 7315b tumor which we found is in contrast to the observed tumor cell heterogeneity of most other murine and human solid tumors (9). It remains to be established therefore, whether the tumor homogeneity as was observed for the experimental 7315b pituitary tumor might be representative for human prolactin secreting pituitary tumors in general. Our observation that the subpopulations of 7315b tumor cells (subpopulations with different densities) showed similar responses to SMS 201-995 *in vitro* suggests that the escape of this tumor from the growth-inhibitory effect of SMS 201-995 as has previously been demonstrated *in vivo* (10) may not have been the result of clonal selection of somatostatin-unresponsive cells. When this "escape" is related to down-regulation of somatostatin-receptors it might be speculated that the down-regulation of somatostatin-receptors which occurs during chronic exposure to SMS 201-995 *in vivo* might be prevented by a regime of non-continuous exposure to somatostatin (analogs). These observations may have clinical significance since preliminary studies point to a slowly occurring desensitization of human endocrine pancreatic tumors during chronic SMS 201-995 therapy (11).

As mentioned in chapter I.2.2 most human pituitary tumors show cellular heterogeneity. Tumors consisting of bihormonal cells or of two cell types secreting different hormones are frequently observed (see chapter I.2.2.1 to I.2.2.5 of this thesis). A major aim of this thesis was to investigate whether the cellular heterogeneity which is frequently observed in GH-secreting pituitary adenomas from acromegalic patients is also reflected by functional heterogeneity among cells of this type of pituitary adenomas.

Pituitary adenomas from acromegalic patients frequently secrete apart from GH also PRL and the glycoprotein hormone α-subunit. α-Subunit appears to be co-localized with GH in the same cell type (12). In contrast PRL may be co-localized with GH within the same cell type but also distinct cell types secreting GH or PRL may exist within an GH/PRL- secreting adenoma. The percentage of GH/PRL- and

GH and/or PRL-cells within a particular adenoma may vary considerably (13). In line with the observed co-localization of GH and α-subunit within the same cell both basal and secretagogue-induced GH- and α-subunit release are parallel in vivo and in vitro (12, 14, chapter VII). However, basal and secretagogue- induced GH- and PRL release also frequently show parallel responses (15-20, chapter VII) despite of the considerable cellular heterogeneity among cells within a particular adenoma with respect to GH and PRL release. These observations may suggest that the cellular heterogeneity among GH-secreting pituitary adenoma cells is not directly reflected by functional heterogeneity among these cells. Additional evidence for this conclusion is provided by the results of the two other studies presented in this thesis (chapter V and VI). First, subpopulations of dispersed pituitary adenoma cells from acromegalic patients with different densities showed completely similar responses to secretagogues as TRH and GHRH and also to the somatostatin analog SMS 201-995 despite differences in the amount and/or type of hormone (GH, PRL, α-subunit) that was secreted by these subpopulations (chapter V). Secondly, using the reverse hemolytic plaque assay, we showed that TRH and GHRH had no preferential effect on any GH-secreting subpopulation of adenoma cells, despite considerable differences in the amount of GH being secreted by individual adenoma cells. Only with respect to the effect of SMS 201-995 there was in some adenomas evidence for a preferential effect of the drug on cells secreting large amounts of GH (chapter VI). As mentioned we demonstrated in the latter study that there is considerable heterogeneity in the amount of GH release among cells within a particular GH-secreting adenoma. In this study we showed that only a minority of the cells (24-38% of the total GH-secreting cell population) accounts for a large fraction (63-80%) of the total amount of GH released by an adenoma. In preliminary experiments we also identified GH-messenger RNA in individual adenoma cells by experiments using in situ hybridization. Interestingly we found the plaque assay data being completely in agreement with the in situ-data since the frequency distributions of individual

plaque areas paralleled the frequency distributions of mRNA-levels in individual adenoma cells (number of silver grains per cell). An example of the results of these experiments is given in Figure 8.1.

It can be concluded therefore that the heterogeneity with respect to GH-release among the adenoma cells is related to differences in the transcription process of the GH-gene among the individual adenoma cells. Our observations may have clinical importance because the differences in GH-production among the adenoma cells within a particular adenoma may provide an explanation for the observation that treatment of patients with GH-secreting pituitary adenomas with the somatostatin analog SMS 201-995 causes only slight reduction of tumor size in about half of the acromegalic patients so treated (23-25). This is in contrast to well-known tumor shrinkage induced by bromocriptine treatment of patients harbouring prolactinomas. The latter drug causes tumor shrinkage in these patients by blocking the transcription of the prolactin gene on mRNA (26-27), ultimately leading to shrinkage of the individual cell volume. In case of the GH-secreting pituitary adenomas the drug SMS 201-995 may significantly affect only those cells with a high GH-gene transcription rate and it appears that the latter cells form a minority of the total tumor cell population (Figure 8.1, chapter VI). Therefore, treatment of this type of pituitary adenoma with SMS 201-995 might lead to only a slight decrease in tumor size.

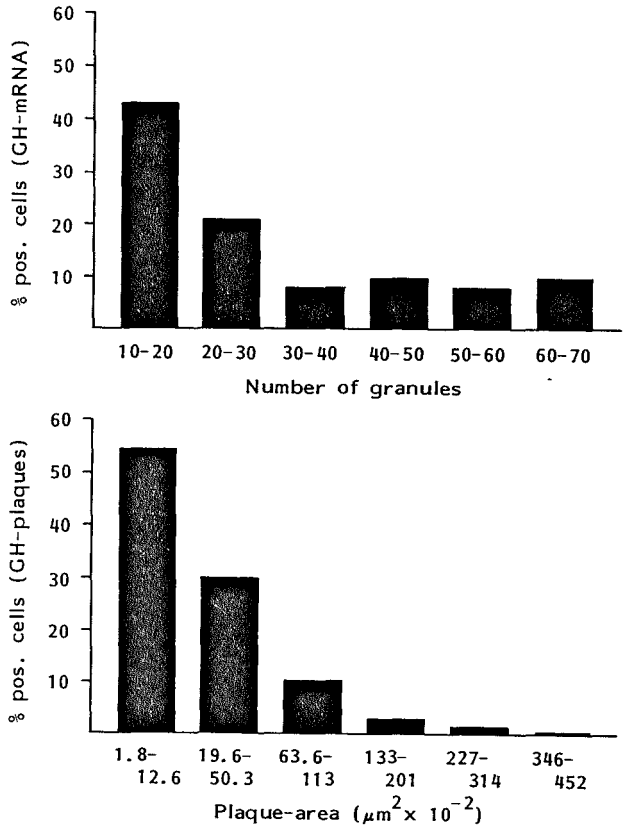

Fig. 8.1. A comparison between the frequency distribution profiles of the amount of GH-mRNA and the GH-plaque areas in individual cells of a GH-secreting pituitary adenoma. GH-mRNA was determined by in situ hybridization as described in detail elsewhere (21). The probe that was used for in situ hybridization was ^{35}S-labeled GH cDNA (DNA complementary to human GH-mRNA). This probe was a kind gift from Dr. Martial (see also ref. 22). mRNA-levels in individual cells were determined by counting the number of silver grains per cell. The reverse hemolytic plaque assay was performed as described in detail in chapter VI.

VIII.4 Concluding remarks

In the previous chapters it may have become clear that functional heterogeneity in hormone secretion and in responsiveness to secretagogues among cells of the normal pituitary gland may reflect an important component of the endocrine regulatory process. The new techniques which have been developed over the last decade, i.e. detection by in situ hybridization of mRNA encoding for specific hormones in single cells and detection by the RHPA of hormone release at the single cell level will certainly provide in the near future a more detailed insight in functional heterogeneity among several hormone-secreting cell types. In addition future investigations concerning functional heterogeneity among cells of human pituitary adenomas may provide a better understanding of the mechanism of action of certain drugs on for example tumor size reduction, but also on the regulation of hormone secretion by these tumors. The latter may have become clear from our investigations dealing with functional heterogeneity among human GH-secreting pituitary adenoma cells.

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SUMMARY

Functional heterogeneity among hormone secreting cell types may play a significant role in the regulation of hormone secretion. The aim of this thesis was to study several aspects of functional heterogeneity among cell types of the normal rat pituitary gland and among cells of human and rat pituitary adenomas. In addition an evaluation was made regarding the physiological and/or clinical significance of the results of these studies.

In chapter I a survey is given of the literature dealing with cellular and functional heterogeneity of pituitary (adenoma) cells.

In chapter III experiments are described that show that normal rat mammotrope and somatotrope subpopulations, separated on density gradients according to differences in their density, are after long-term culture not functionally heterogeneous with respect to TRH-, DA- and SRIH-responsiveness. It is discussed that the long-term culture of the cells deprives them from their hypothalamic regulatory input which might result in homogeneous cell populations. Therefore, we proposed that functional heterogeneity between mammotroph subpopulations might be a temporary state of these cells which is able to change under different physiological conditions. In addition our results may support the hypothesis that differences in hypothalamic input between anterior pituitary regions may be responsible for functional heterogeneity between PRL-cell types.

Chapter IV deals with functional heterogeneity among cells of an experimental transplantable PRL-secreting rat pituitary tumor. The results of the experiments described in this chapter show no functional heterogeneity among the tumor cells as measured by growth rate of the cells *in vitro*, protein content of the cells, prolactin production and responsiveness of the cells to the somatostatin analog SMS 201-995. In addition we found no differences between tumors of increasing weight with respect to these parameters. We concluded that growth of 7315b tumors *in vivo* may not lead to the induction of heterogeneous cell subpopulations as has

been described for most human and murine solid tumors.

In chapters V and VI experiments using two different approaches for the study of functional heterogeneity among cells of human GH-secreting pituitary adenoma cells are described. The results from the experiments described in chapter V demonstrate that subpopulations of human GH- secreting pituitary adenoma cells, separated according to differences in their density, are heterogeneous with regard to the amount and/or type of hormone (GH, PRL, α-subunit) being secreted in vitro. However, despite these differences in hormone secretion we found no differences between the subpopulations of adenoma cells with regard to their responsiveness to secretagogues as TRH, GHRH and the somatostatin analog SMS 201-995. The results from the experiments described in chapter VI are well in agreement with these observations. For the latter experiments we used the reverse hemolytic plaque assay for the detection of GH secretion by individual human GH- secreting pituitary adenoma cells. The results of these experiments show that GH secretion among individual GH- secreting adenoma cells may vary significantly within a particular adenoma. We also showed that a minority of the adenoma cells within a particular adenoma accounts for a large fraction of the amount of GH being released by the total adenoma cell population. In agreement with the observations described in chapter V is our finding that both TRH and GHRH had no preferential effect on GH secretion by any subpopulation of adenoma cells despite the differences in the amount of GH secretion among the individual adenoma cells. Only with regard to the effect of SMS 201-995 we provided evidence for a preferential inhibitory effect of the drug on a subpopulation of adenoma cells in some adenomas. The results from the experiments described in chapter VII provide additional in vitro evidence for a close parallel in the responses of GH, PRL and α-subunit release which is observed in acromegalic patients in vivo. The results from the latter three studies (chapters V, VI and VII) suggest that the cellular heterogeneity among GH- secreting human pituitary adenoma cells is not directly reflected by functional heterogeneity

among these cells. Further investigations are needed to evaluate whether the homogeneous response among cells of this type of pituitary adenoma is representative for other types of human pituitary adenomas.

SAMENVATTING

Functionele heterogeniteit binnen hormoon secernerende celtypen kan een belangrijke rol spelen bij de regulatie van de hormoonsecretie. Het doel van het in dit proefschrift beschreven onderzoek was om verscheidene aspecten van functionele heterogeniteit binnen celtypen van de normale rattehypofyse en binnen celpopulaties van menselijke en rattehypofysetumoren te bestuderen. Bovendien werd een evaluatie gemaakt van de fysiologische en/of klinische betekenis van de resultaten van deze studies.

In hoofdstuk I wordt een overzicht gegeven van de literatuur met betrekking tot cellulaire en functionele heterogeniteit van hypofyse (tumor) cellen.

In hoofdstuk III worden experimenten beschreven die aantonen dat normale ratte mammotrope en somatotrope subpopulaties, met behulp van dichtheidsgradienten gescheiden op basis van verschillen in hun dichtheid, na langdurig kweken niet functioneel heterogeen zijn met betrekking tot TRH-, DA-, en SRIH-gevoeligheid. In dit hoofdstuk wordt gesuggereerd dat het langdurig kweken van de cellen buiten de invloed van de normale hypothalame regulatoire influx resulteert in homogene celpopulaties. Verondersteld wordt daarom dat functionele heterogeniteit tussen mammotrope subpopulaties een tijdelijke status van deze cellen is die kan veranderen onder verschillende fysiologische condities. Bovendien ondersteunen onze resultaten de hypothese dat verschillen in de hypothalame influx tussen hypofysevoorkwab gebieden een oorzaak kan zijn van de functionele heterogeniteit tussen prolactine- celtypen.

Hoofdstuk IV heeft betrekking op functionele heterogeniteit van een experimentele, transplanteerbare, PRL-secreterende rattehypofysetumor. De resultaten van de in dit hoofdstuk beschreven experimenten tonen aan dat er geen functionele heterogeniteit met betrekking tot de groeisnelheid van de cellen in vitro, de eiwit content van de cellen, PRL productie en de gevoeligheid van de cellen voor de somatostatine analoog SMS 201-995 voorkomt tussen cellen van deze tumor. Bovendien waren er met betrekking tot deze

parameters geen verschillen tussen tumoren met een verschillend gewicht. Geconcludeerd wordt dat de groei van 7315b tumoren in vivo niet leidt tot het ontstaan van heterogene celsubpopulaties zoals dit beschreven is voor de meeste menselijke solide tumoren en voor solide tumoren bij knaagdieren.

In de hoofdstukken V en VI worden experimenten beschreven waarbij gebruik is gemaakt van twee verschillende technieken voor het bestuderen van functionele heterogeniteit tussen cellen van menselijke GH- secernerende hypofysetumoren. De resultaten van de in hoofdstuk V beschreven experimenten tonen aan dat subpopulaties van menselijke GH- secernerende hypofysetumorcellen, gescheiden op basis van verschillen in hun dichtheid, heterogeen zijn met betrekking tot de hoeveelheid en/of het type hormoon (GH, PRL, α-subunit) dat gesecerneerd wordt in vitro. Echter, ondanks deze verschillen in hormoon secretie werden geen verschillen tussen de tumorcel-subpopulaties aangetoond met betrekking tot de gevoeligheid voor stoffen als TRH, GHRH en de somatostatine analoog SMS 201-995. De resultaten ven de in hoofdstuk VI beschreven experimenten zijn goed in overeenstemming met deze bevindingen. Voor laatstgenoemde experimenten werd de reverse hemolytic plaque assay gebruikt om GH secretie door individuele menselijke GH secernerende hypofysetumorcellen aan te tonen. De resultaten van deze experimenten tonen aan dat er tussen individuele GH secernerende tumorcellen een aanzienlijke variabiliteit bestaat met betrekking tot de hoeveelheid gesecerneerd GH. Er werd tevens aangetoond dat slechts een gering deel van de tumorcellen van een tumor voor een aanzienlijk deel bijdraagt aan de totale hoeveelheid GH die door een totale tumorcelpopulatie wordt gesecerneerd. In overeenstemming met de bevindingen zoals die beschreven worden in hoofdstuk V werden geen verschillen gevonden tussen subpopulaties met betrekking tot de gevoeligheid van de cellen voor TRH en GHRH. Dit laatste ondanks de gevonden verschillen in hoeveelheid GH secretie. Alleen met betrekking tot het effect van SMS 201-995 waren er bij enkele tumoren aanwijzingen voor een preferentieel remmend effect van het geneesmiddel op een subpopulatie van tumorcellen. De resultaten van de

experimenten die in hoofdstuk VII beschreven worden leveren aanvullend in vitro bewijs voor een sterk parallellisme van de GH, PRL en α-subunit secretie zoals deze wordt waargenomen bij acromegale patienten in vivo. De resultaten van de laatsgenoemde drie studies (hoofdstukken V, VI en VII) suggereren dat de cellulaire heterogeniteit tussen GH secernerende menselijke hypofysetumorcellen niet gereflecteerd wordt door een fuctionele heterogeniteit tussen deze cellen. Verdere onderzoekingen zijn noodzakelijk om te kunnen evalueren of de homogene gevoeligheid tussen cellen van dit type tumor representatief is voor de andere typen hypofysetumoren.

NAWOORD

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