3. Results

3.1. Assay Optimization

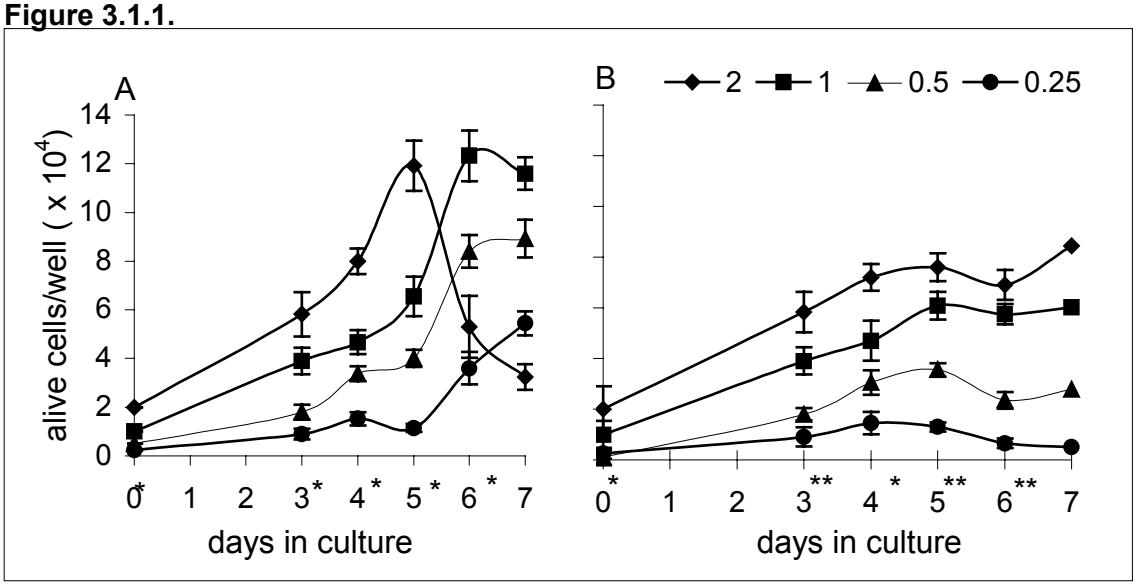
Before the effect of growth factors or human pituitary tumor fractions on GH_3 cell growth and hormone secretion could be examined, the bioassay conditions had to be optimized. This involved the evaluation of GH_3 growth characteristics and appropriate positive controls. In addition, a new assay was developed to examine cell surface IGF1R expression and its influence on apoptosis of pituitary tumor cells. These assay optimizations and development will be described in the following chapter.

3.1.1. GH_3 bioassay optimization

3.1.1.1. Determination of optimal seeding concentration

GH_3 cells proliferate to 70-75 % confluence before detachment and eventual cell death begins. A seeding concentration, which allowed GH_3 cells to remain in the exponential growth phase throughout the length of the experiment, had to be determined. In order to establish a growth profile, different concentrations of GH_3 cells were grown for 7 days in 96-well plates (Figure 3.1.1. A). From the third day on, when cell adherence was ensured, the culture medium was replaced daily (*). When

Figure 3.1.1.



Growth of GH_3 cells in C-DM/F12 (A) or in SF-DM/F12 (B). Cells were seeded at 0.25 – 2 x 10^4 cells/well and grown for 7 days. The culture medium was replaced routinely with fresh C-DM/F12 (*) or SF-DM/F12 (**) and from the third day on the number of alive and dead cells was determined in duplicate wells in the presence of Trypan Blue. Mean ± SEM is displayed.

seeded at 2 x 10^4 cells/well, the maximum concentration of alive adherent cells (approximately 12 x 10^4 cells/well) was reached after 5 days of culture. During the following days the cell number decreased, suggesting detachment and cell death. At a starting concentration of 1 x 10^4 cells/well the peak concentration was reached between day 5 and 6. At lower seeding concentrations the growth plateau was not reached within the experimental time. The average doubling time of cells in this experiment was 34 $\pm$ 1.9 hrs, which confirmed calculations from routine culture. These data suggested an optimal cell concentration between 3 to 7 x 10^4 cells/well at the time of growth factor or tumor extract addition. At this concentration all GH_3 cells would remain in the growth phase throughout the experimental time of 24 hrs.

3.1.1.2. GH_3 cell survival under serum free conditions

In order to test the ability of growth factors or pituitary tumor fractions to induce proliferation, apoptosis or hormone secretion of GH_3 cells, the culture medium had to be void of endogenous growth factors, which could interfere with the molecules of interest. A prerequisite was the ability of GH_3 cells to survive under serum free conditions. This was tested in the following experiment (Figure 3.1.1.B). Cells were grown at different concentrations for 7 days. Upon serum deprivation (**) the number of alive cells did not change for the remaining experimental time at any seeding concentration. This experiment suggested, that serum deprivation for up to 3 days leads to growth arrest without major cell detachment or death.

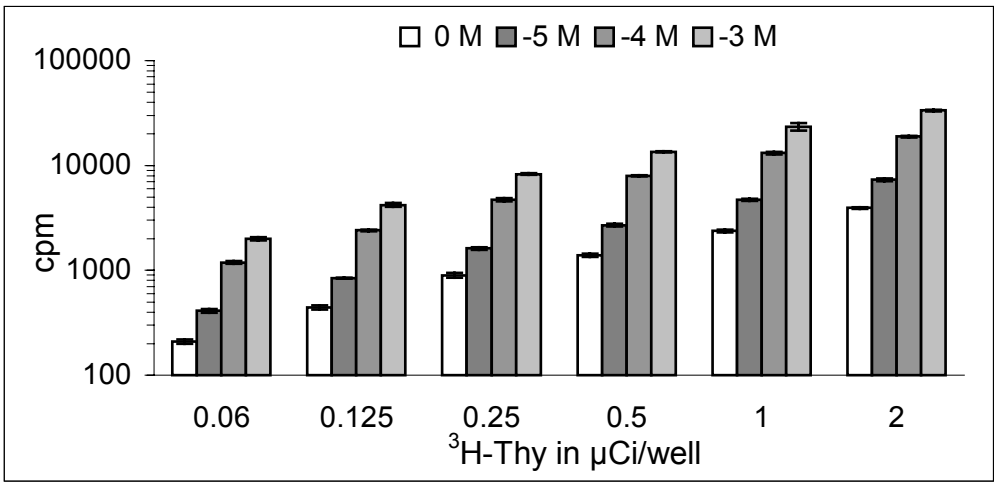
In conclusion, optimal conditions for the GH_3 bioassay included a seeding concentration of 2 x 10^4 cells/well, followed by 2 days of culture in C-DM/F12 to ensure adherence. The effect of tumor fractions or growth factors on cell proliferation, apoptosis or hormone secretion would be tested for 24 to 48 hrs in SF-DM/F12. In additional experiments it was learned that cell adherence was completed within 24 hrs after seeding (data not shown). As a result, the experimental setup was altered to culture of GH_3 cells in C-DM/F12 at 3 - 6 x 10^4 cells/well for 24 hrs before stimulation.

3.1.1.3. ^{3}H-thymidine incorporation: optimal concentration and appropriate positive control

One of the classic assays to study cell proliferation is based on the incorporation of ^{3}H-thymidine into replicating DNA. Although handling of radioactive material is necessary, this is still one of the most reliable and sensitive assays of its kind. The adaptation of this method to the presented GH_3 cell setup required finding an appropriate concentration of ^{3}H-thymidine. In addition a positive control that reliably induced proliferation had to be found. Such a substance is adenosine (personal communication Dr. M. Lewis, Dr. U. Ploeckinger).

Figure 3.1.2. shows a dose-response curve of ^{3}H-thymidine uptake into GH_3 cells, which were stimulated for 24 hrs with different concentrations of adenosine

Figure 3.1.2.



Effect of adenosine (AD) on ^{3}H-thymidine incorporation of GH_3 cells. Cells were exposed to 10^{-3} M, 10^{-4} M, or 10^{-5} M AD diluted in SF-DM/F12 or to SF-DM/F12 alone (0 M). ^{3}H-thymidine (0.06 - 2 µCi/well) was added to appropriate wells and 24 hrs later its uptake into DNA was measured. Data are expressed as mean ± SEM of quadruplicate measurements.

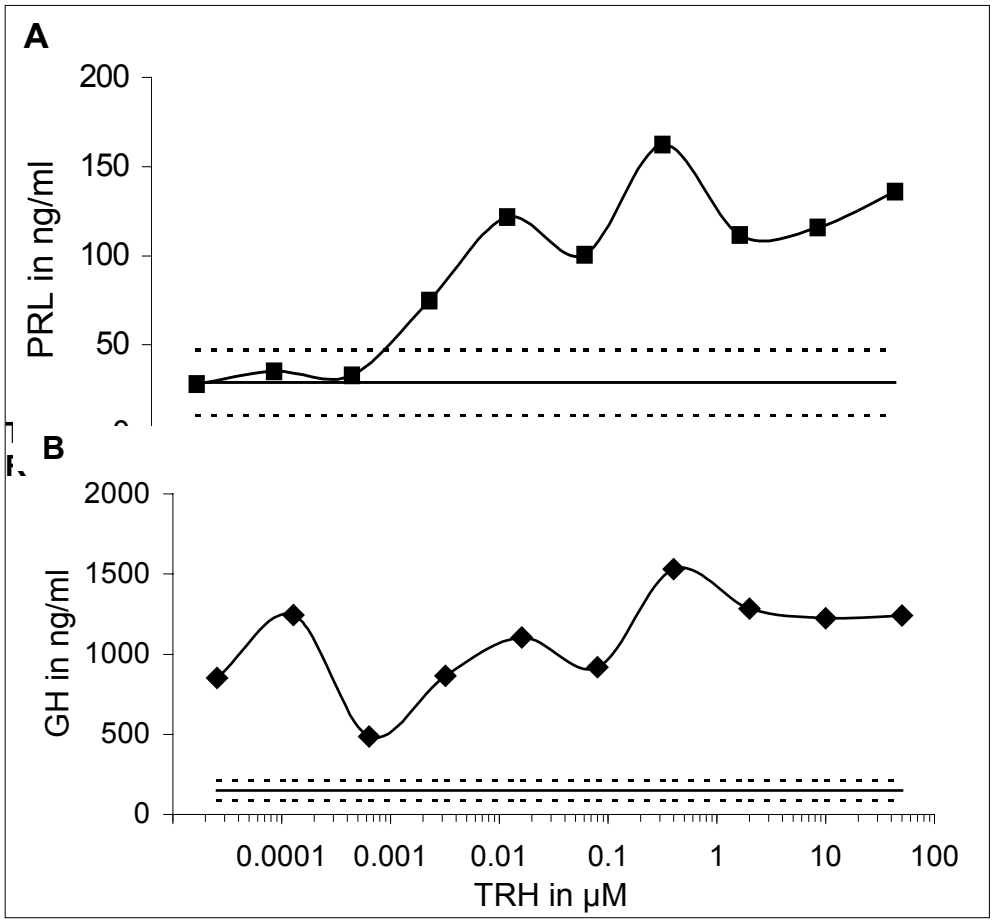
suspended in SF-DM/F12 (AD). All concentrations of AD (1 x 10^{-5} – 1x10^{-3} M) increased ^{3}H-thymidine incorporation in a dose dependent manner. The stimulation indices (cpm of cells in AD divided by cpm of cells in SF-DM/F12) were similar at all ^{3}H-thymidine concentrations tested (data not shown), suggesting a medium concentration of 0.5 µCi/well for subsequent bioassays. AD at a concentration of 1 mM (=10^{-3} M) was used as a positive control.

3.1.1.4. Determination of a positive control for stimulated hormone secretion

A number of substances, i.e. TRH, PACAP, bombesin, VIP and cortisol (35-38) have been described to induce GH and/or PRL secretion of GH_3 cells. They were tested in our setting in order to establish a positive control when examining the ability of peptides or pituitary tumor fractions to induce hormone secretion of GH_3 cells.

Dose responses of TRH induced GH and PRL secretion are summarized in Figures 3.1.3. A+B. Cells were incubated with various concentrations of TRH under serum

Figure 3.1.3.



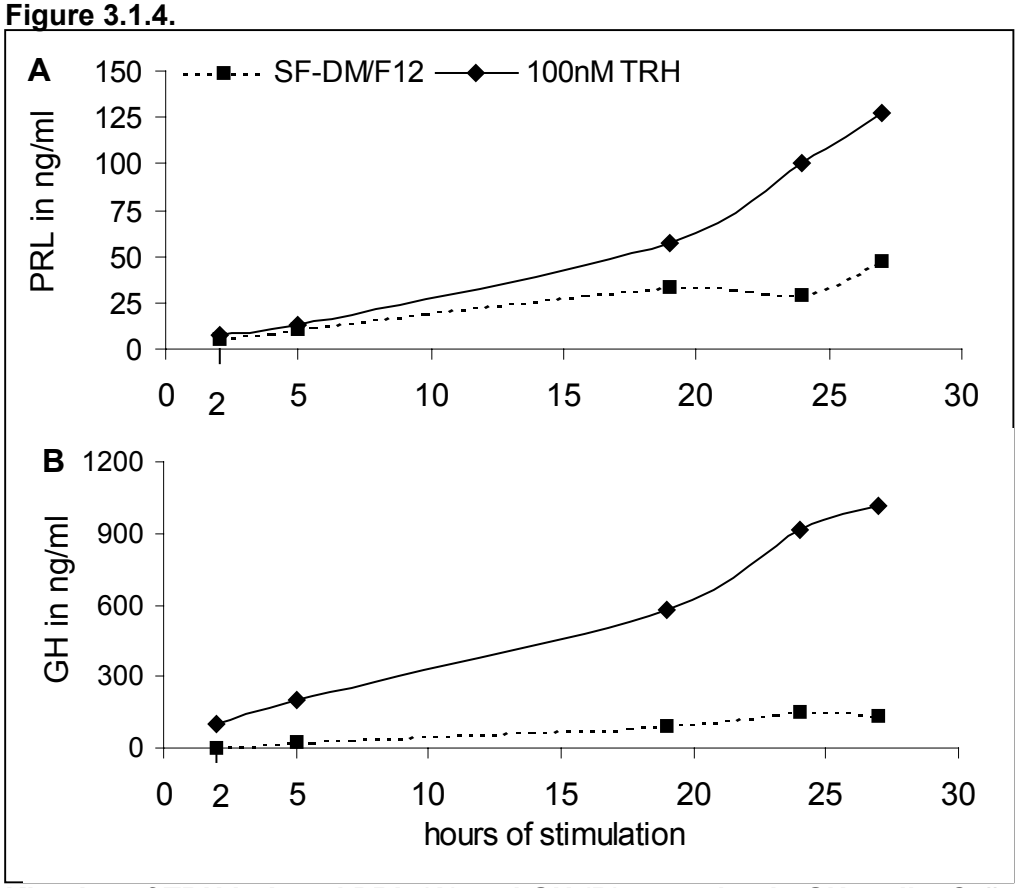
H induced PRL (A) and GH (B) secretion in GH_3 cells. Cells were incubated with different concentrations of TRH diluted in SF-DM/F12 (TRH) or in SF-DM/F12 alone (horizontal line). After 24 hrs, PRL and GH were determined from cell supernatants by RIA or ELISA. Data are expressed as mean ± SEM of quadruplicate (SF-DM/F12) or mean of duplicate (TRH) measurements.

free conditions and after 24hrs GH and PRL concentrations in the supernatants were determined by immunoassay. TRH at concentrations of approximately 1 nM to at least 50 μM stimulated PRL secretion above control levels (Fig. 3.1.3. A). GH secretion appeared to be increased at all concentrations of TRH tested (Fig. 3.1.3. B). In conclusion, 100 nM (10^{-7} M) TRH was routinely employed as a positive control to stimulate GH and PRL secretion of GH_3 cells.

3.1.1.5. Time course of stimulated hormone secretion

PRL and GH secretion of GH_3 cells is most likely regulated through different pathways. Whereas PRL is stored in intracellular vesicles and released only as needed, GH is directly secreted without significant intracellular storage (18). Thus,

Figure 3.1.4.



Kinetics of TRH induced PRL (A) and GH (B) secretion in GH_3 cells. Cells were cultured with 100 nM TRH in SF-DM/F12 or SF-DM/F12 alone for up to 24 hrs. PRL and GH were determined from cell supernatants by RIA or ELISA. Data are expressed as mean of duplicate measurement

the appropriate time of stimulation for sufficient PRL and GH detection had to be determined. Kinetics of TRH induced hormone secretion was studied. As shown in Figure 3.1.4., PRL and GH secretion were detectable within the first five hours upon stimulation with TRH and increased steadily within the following 27 hours. In the presented setting it could not be distinguished between stored and immediate hormone release upon TRH stimulation. From these data it was concluded that stimulation for 24 hrs would be appropriate to measure hormone secretion of GH_3 cells.

3.1.2. Cytometric detection of apoptosis

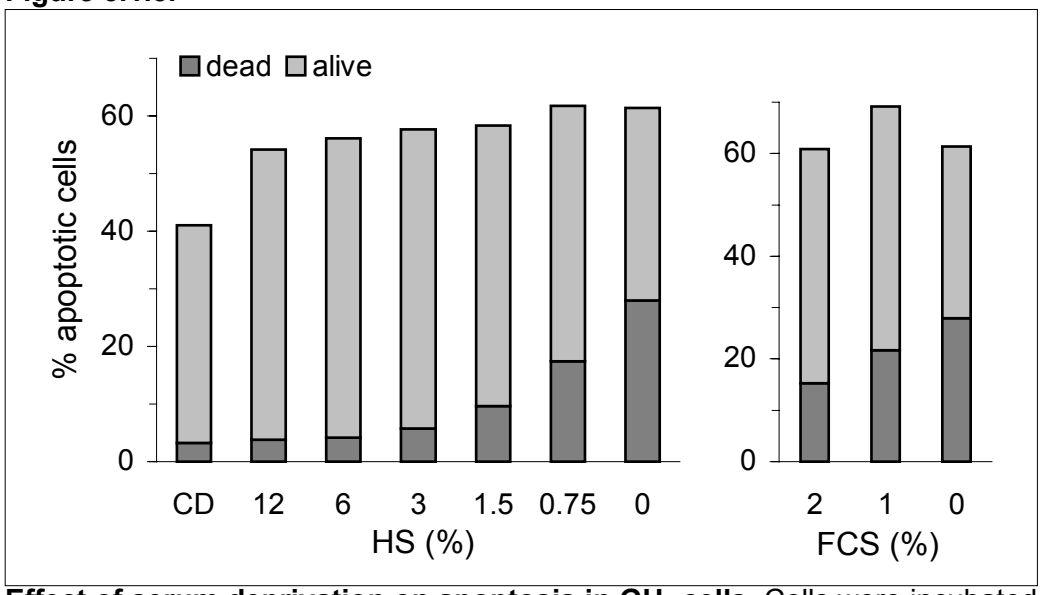
Changes in the cell surface plasma membrane are one of the earliest features of cells undergoing apoptosis (39). The membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V, which is a 35-36 kD Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS, is conjugated to Fluorescein Isothiocyanate (FITC) for easy identification of apoptotic cells by flow cytometry. Since translocation of PS to the external cell surface also occurs during necrosis, Annexin V-FITC (AnnF) is typically used in conjunction with a vital dye such as propidium iodide (PI) to distinguish apoptotic from necrotic cells. In preliminary studies, optimal assay conditions, including the AnnF and PI titres, and staining conditions, were established (data not shown).

3.1.2.1. Induction of apoptosis by serum deprivation

Apoptosis can be easily induced in cell cultures by removal of growth factors, which are generally provided by fetal calf serum (FCS) and/or horse serum (HS). The effect of serum deprivation on GH_3 cells is shown in Figure 3.1.5. Cells cultured in C-DM/F12 served as a negative control. Approximately 37% and 3% of these cells were AnnF positive (alive apoptotic) and AnnF/PI positive (dead apoptotic), respectively. They represent cells undergoing spontaneous apoptosis, and to a lesser degree, unspecific binding to phospholipids other than PS. SF-DM/F12 supplemented with 12% HS (in the absence of FCS), which corresponds to the HS concentration routinely added in C-DM/F12, induced the least amount of apoptosis as compared to

further serum removal. Removal of HS induced a dose dependent increase of apoptotic cells. A similar effect was observed when cells were cultured in SF-DM/F12 supplemented with different concentrations FCS in the absence of HS.

Figure 3.1.5.



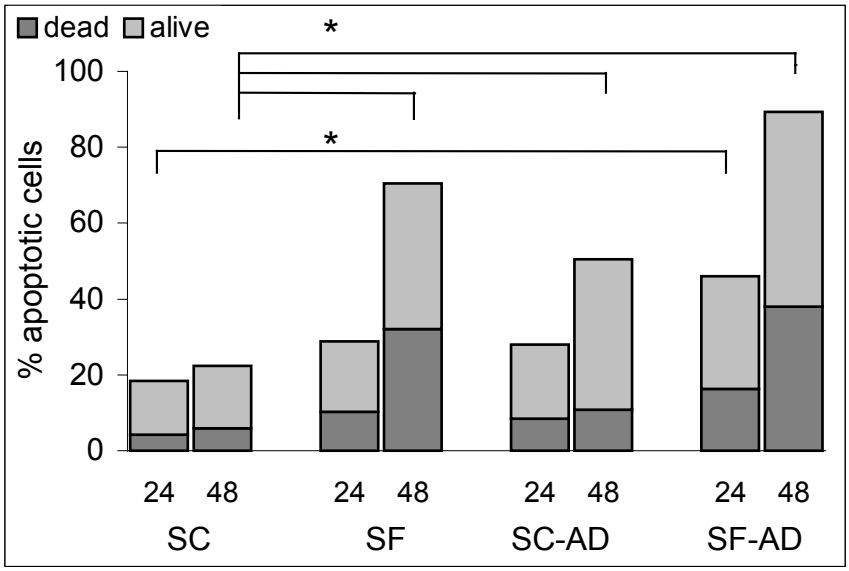
Effect of serum deprivation on apoptosis in GH_3 cells. Cells were incubated in C-DM/F12 (CD) or SF-DM/F12 supplemented with different concentrations HS or FCS. 24hrs later, they were stained with AnnF/PI and analyzed by flow cytometry. The percentage of alive (AnnF positive) and dead (AnnF/PI positive) apoptotic cells are displayed.

3.1.2.2. Induction of apoptosis with adenosine

In addition to inducing proliferation in GH_3 cells, Adenosine has recently been reported to also induce apoptosis at high concentrations (40). To verify this effect in our system, GH_3 cells were exposed for 24 or 48 hrs to C-DM/F12 (SC), SF-DM/F12 (SF) or to either medium supplemented with 1mM adenosine (AD). Summarized results of three such experiments are shown in Figure 3.1.6. After 24hrs, the percentage of apoptotic cells was significantly increased following serum deprivation in combination with adenosine treatment (SC-SFAD). After 48hrs apoptosis was significantly increased following serum deprivation (SC-SF), adenosine treatment (SC-SCAD) or a combination of both (SC-SFAD).

These results demonstrate that serum deprivation and 1 mM adenosine induce apoptosis in GH_3 cells after 48 hrs. A combination of both treatments did not appear

Figure 3.1.6.



Apoptosis in GH_3 cells. Cells were incubated in SC, SF, or in SC or SF supplemented with 1mM adenosine. After 24 or 48 hrs, cells were stained with AnnF/PI and analyzed by flow cytometry. The percentages of alive (AnnF) and dead (AnnF/PI) apoptotic cells from three separate experiments are depicted. Statistical significance comparing apoptosis of all treatments are presented. *= $p<0.05$, Mann Whitney U test

to potentiate the effect. Thus, in subsequent experiments apoptosis was induced by 48 hrs of serum deprivation.

3.1.3. Development of a new method for the semi-quantitative detection of cell surface IGF1R

Currently, IGF1R regulation is studied by measuring its transcribed or translated form or in receptor transfection models. Receptor expression is routinely detected with ^{125}I-IGF1 competition assays. This method, although simple and fast, harbors a number of disadvantages, such as exposure to radioactivity, high cost of ^{125}I-IGF1, the requirement of large numbers of cells, and inherently large assay variation. Therefore, a new method was developed, which is based on the cytometric detection of fluorescent antibodies bound to cell surface IGF1R. In flow cytometry intra assay variation is small and reproducibility great. Another advantage is the ability to simultaneously label cells with markers for more than one epitope. This allows, for instance, the simultaneous detection of IGF1R expression and apoptosis by AnnF/PI

when studying growth characteristics of tumor cells. Another combination of interest is the detection of IGF1Rs and intracellular cytokeratin on the same cell. Cytokeratins are intracellular filamentous proteins, which are only expressed in endothelial cells, such as pituitary tumor cells.

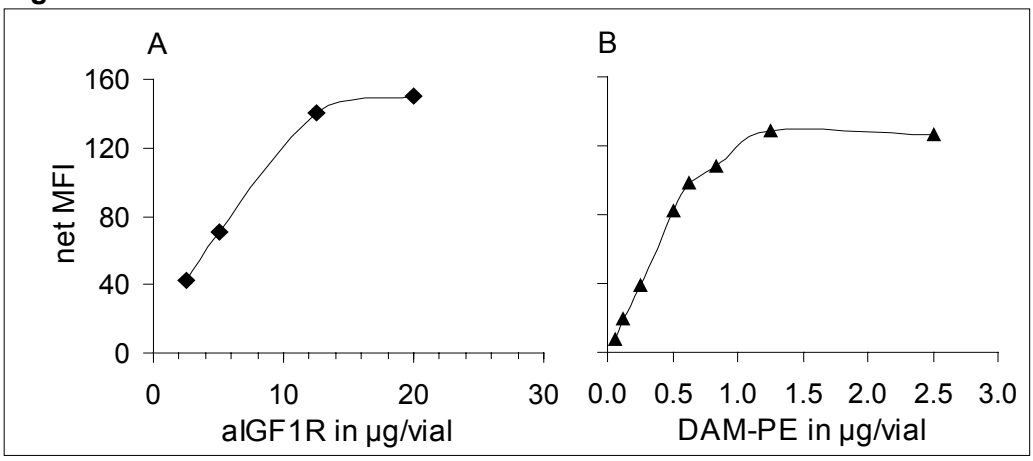
In the following chapter the flow cytometric detection of IGF1R expression will be described and compared to the classic detection method with ^{125}I-IGF1. The establishment of multi staining protocols will not be illustrated in detail.

3.1.3.1. Cytometric measurement of IGF1R expression

There are few anti IGF1R antibodies (aIGF1R) commercially available. Most appropriate for flow cytometric studies would be an aIGF1R, which is monoclonal and directly conjugated to a fluorochrome (i.e. FITC, PE or PerCP). Unfortunately, when these studies began, only un-conjugated aIGF1R could be purchased, which led to the development of an indirect receptor staining method. Primary mouse aIGF1R would be visualized with a secondary anti mouse phytoerythrin (PE) conjugated antibody. In preliminary studies two aIGF1R were compared and the one with the highest receptor affinity was selected (data not presented). The chosen antibody was a monoclonal mouse aIGF1R, isotype IgG, with neutralizing activity. The suitable secondary antibody was a donkey anti mouse PE conjugate (DAM-PE), preabsorbed against mouse, human, rat, horse, goat, sheep, rabbit and bovine immunoglobulins to minimize naturally occurring cross-reactivity.

Optimal titers of both antibodies were determined. GH_3 cells were stained with different concentrations of both antibodies and analyzed by flow cytometry. Some cells were stained with an isotypic control antibody in parallel (primary antibody of same isotype as specific antibody, but directed against an epitope not present in these cells). Figure 3.1.7. shows results of such an experiment. Net mean fluorescence intensity (net MFI) against antibody concentration is depicted. Net MFI was calculated by subtracting MFI of isotypic controls from that of specific aIGF1R-DAMPE staining. Saturated binding was obtained at approximately 15 µg/vial aIGF1R (Fig.3.1.7. A) and 1.25 µg/vial DAM-PE (Fig.3.1.7.B), respectively. The required aIGF1R concentration was found to be rather high, since characteristically 10 fold lower antibody titers are employed in these kinds of experiments. This

Figure 3.1.7.

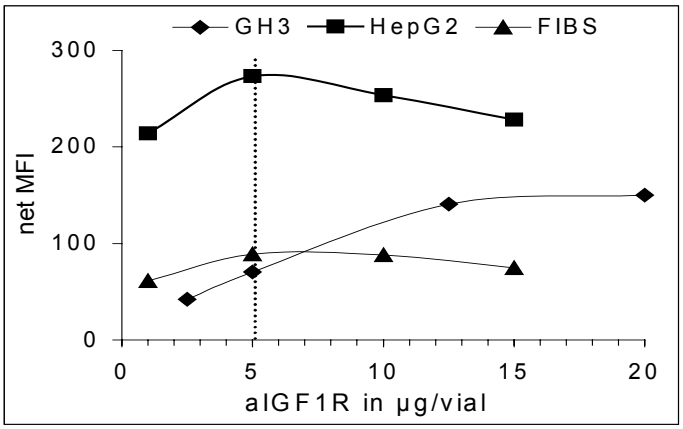


Titration curves of primary antiIGF1R (A) and secondary DAM-PE (B). GH_3 cells were stained with different concentrations primary mouse anti IGF1R antibody (aIGF1R) followed with 2.5 µg/vial DAM-PE (A) or with 0.5 µg/vial aIGF1R followed with different concentrations of DAM-PE (B). Depicted are antibody concentrations versus net MFI (specific mean fluorescence intensity - isotype MFI).

suggested that either the affinity of the aIGF1R or receptor density on GH_3 cells was rather low.

To test the latter possibility, different cells known to express high levels of IGF1R were stained. Such cells are HepG2, a human liver carcinoma cell line, and primary human fibroblasts isolated from a neuroendocrine tumor of the gut (FIBS, Figure 3.1.8.). The concentration of aIGF1R required to obtain saturated staining of IGF1R

Figure 3.1.8.



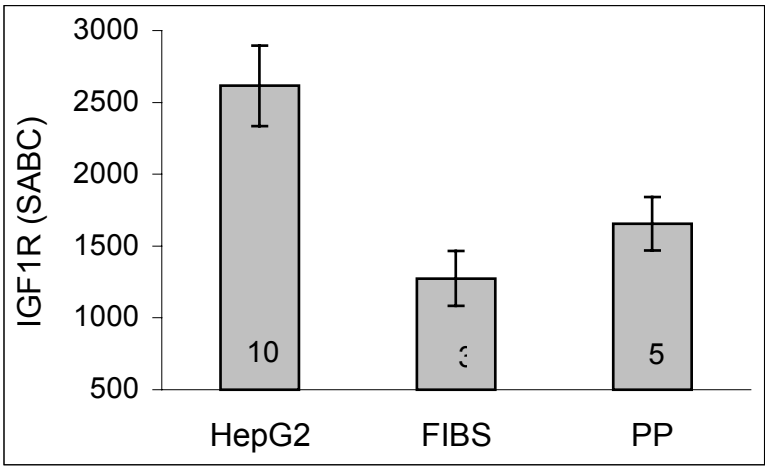
Titration of antiIGF1R on different cell types. Cells were stained with different concentrations aIGF1R and 1.25 µg/vial DAM-PE as described. Net MFI are depicted. The dotted line represents the saturating aIGF1R concentration for HepG2 and FIBS.

on HepG2 and FIBS was with 5 µg/vial approximately 3 fold lower than that required for GH_3 cells. From these results it was concluded, that this method is not sensitive enough to study IGF1R expression and regulation on GH_3 cells. This might be due to low affinity of the anti human IGF1 antibody to rat IGF1Rs. Thus, these cells were not used in any of the following experiments to study IGF1R expression.

3.1.3.2. Detection of cell surface IGF1R expression in different cells

Cell surface IGF1R expression on HepG2 and cytokeratin positive (PP) and negative (FIBS) human primary pituitary tumor cells was quantified with QIFIT beads. Summarized results of up to 10 such experiments are presented in Figure 3.1.9. It is apparent that the IGF1R density varied up to threefold between cell type. HepG2 cells had the highest specific antigen binding capacity (SABC), followed by that of PP, which was still higher than that of FIBS.

Figure 3.1.9.

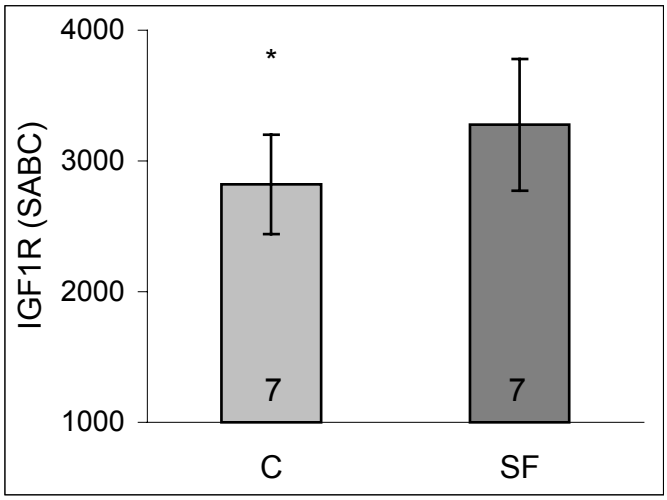


IGF1R expression in different cell types. Cells were cultured for 24 hrs in serum containing medium and stained with aIGF1R-DAMPE/aCyt. Quantified IGF1R expression (SABC) of cytokeratin positive (HepG2, PP) and cytokeratin negative (FIBS) cells are presented. Data of multiple separate experiments are presented as mean +/- SEM.

3.1.3.3. Detection of IGF1R expression in HepG2 cells after serum deprivation

IGF1R expression was assessed in HepG2 cells, which had been incubated for 24 hrs with serum containing or serum free media. Data of 7 experiments are summarized in Figure 3.1.10. The data show that IGF1R expression was significantly increased in HepG2 cells exposed to serum free media.

Figure 3.1.10.



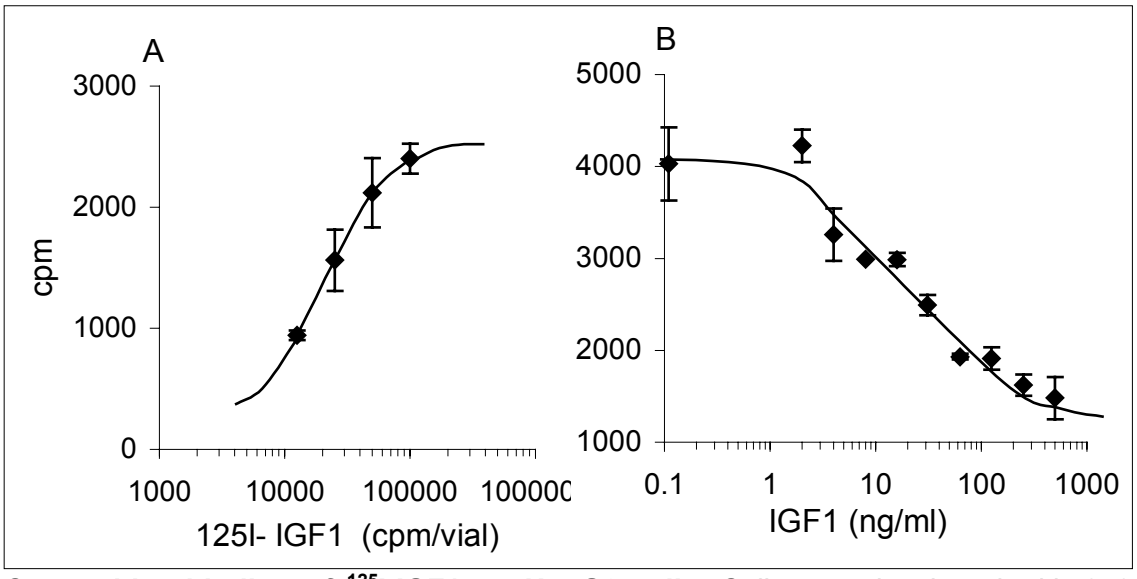
IGF1R expression in HepG2 cells. Cells were incubated in serum containing (C) or serum free culture (SF) media and IGF1R expression was detected by flow cytometry. Quantified data (SABC = specific antigen binding capacity, as measured with aIGF1R and DAM-PE) of multiple separate experiments are presented as mean +/- SEM. Statistical analysis was computed with nonparametric Wilcoxon paired t test, * $p<0.05$.

3.1.3.4. Detection of IG1R binding sites with ^{125}I-IGF1 in HepG2 cells after serum deprivation

The classic method to measure receptor expression is a modification of Scatchard analysis, where receptor binding is detected with ^{125}I-labeled ligand (41). In this competitive binding assay, 'cold' IGF1 is employed to detect unspecific binding. We established this method with HepG2 cells in order to verify the newly developed cytometric IGF1R expression method. In preliminary experiments, assay conditions,

such as the required amounts of unlabeled IGF1 and ^{125}I-IGF1 were determined. Figure 3.1.11. A shows a dose response curve of cells incubated with excess IGF1

Figure 3.1.11.



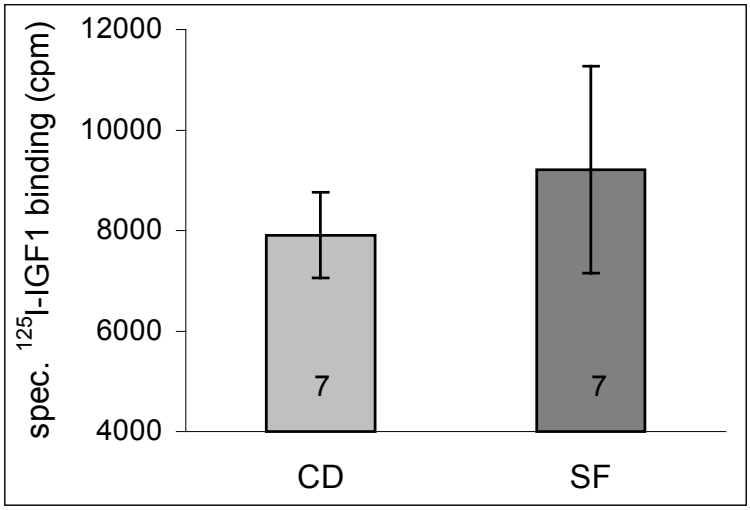
Competitive binding of ^{125}I-IGF1 on HepG2 cells. Cells were incubated with 250 ng/ml unlabeled IGF1 and increasing concentrations of ^{125}I-IGF1 (A) or with 50,000 cpm ^{125}I-IGF1 and increasing concentrations unlabeled IGF1 (B). After 3 hrs cell associated ^{125}I-IGF1 was determined. Data are expressed as mean ± SEM per 1 x 10^5 cells (A), per 4 x 10^5 cells (B).

(250 ng/ml) and increasing concentrations of ^{125}I-IGF1. In Figure 3.1.11. B a dose response curve of 'cold' IGF1 incubated with 50,000 cpm ^{125}I-IGF1 is depicted. These experiments suggested that the optimal concentrations of ^{125}I-IGF1 and 'cold' IGF1 were 50,000 cpm and 250 ng/ml, respectively. Final assay conditions are described in Methods.

IGF1 binding sites were determined in HepG2 cells, which had been incubated for 24 hrs in serum free (SF) or serum containing (CD) culture medium (Figure 3.1.12.). There was no significant difference in specific ^{125}I-IGF1 binding between the two groups, although ^{125}I-IGF1 binding tended to be increased after serum deprivation in 3 of 7 experiments.

In conclusion, the data indicate that the flow cytometric detection of IGF1R expression is more reproducible and more suitable to study small differences in receptor expression.

Figure 3.1.12.



IGF1 binding on HepG2 cells after serum deprivation. Cells were cultured in serum containing (CD) or serum free (SF) medium and 24 hrs later modified scatchard analysis was performed. Specific ^{125}I-IGF1 binding per 1 x 10^6 cells are expressed as mean ± SEM of 7 separate experiments.

3.2. Effect of growth factors on ^{3}H-thymidine incorporation, apoptosis and hormone secretion in GH_3 cells

A major goal of this study was to evaluate the importance of growth factors for pituitary tumor growth and development. In the following chapter, the effects of such growth factors on growth (proliferation and apoptosis) and secretion of rat GH_3 cells are discussed (chapter 3.2.). To evaluate whether such substances can be detected in human pituitary tumors, the effects of pituitary tumor fractions on GH_3 cell growth and secretion were examined next (chapter 3.3.). Finally, the physiological significance of such factors was examined in primary human pituitary tumor cells (chapter 3.4.).

The effect of several growth factors on proliferation, apoptosis or hormone secretion of GH_3 cells was assessed. The peptides were selected because they have been shown to stimulate hormone secretion and/or proliferation in normal or even tumorous pituitary cells (see introduction). GH_3 cells were cultured with peptides diluted in SF-DM/F12 as summarized in Table 3.2.1. Cells cultured with SF-DM/F12 alone served as negative controls. After 24 hrs ^{3}H-thymidine incorporation, apoptosis or hormone secretion were measured. All experiments were repeated at least once.

Table 3.2.1

Growth Factor	species	MW	Conc. tested
bFGF	human recom.	4 kD	1000-0.1 ng/ml
EGF	human recom.	6 kD	100-0.01 nM
IGF1	human recom.	7.6 kD	1000-0.01 ng/ml
IL 2	human natural	15-17 kD	100-0.01 U/ml
IL 6	human natural	26-29 kD	300-0.01 U/ml

Summary of growth factors potentially participating in pituitary tumor development and growth. Depicted are species, molecular weight (mw) and concentration ranges tested.

3.2.1. ^{3}H thymidine incorporation

No significant increase in ^{3}H-thymidine incorporation was observed with any of the peptides (data not shown). Thus, the possibility of a requirement for co-factors was explored. ^{3}H-thymidine incorporation was assessed in the presence of different concentrations of serum. To evaluate the possibility of a delayed reaction, incubation was prolonged for up to 86 hrs. Results of these experiments are summarized in Table 3.2.2.

Table 3.2.2.

		24hrs			48hrs				72hrs	86hrs
		0%	1%FCS	5%HS	0%	1%FCS	5%FCS	10%FCS+	0%	0.5%FCS
bFGF	ng/ml	-	-	-	-	-	-	-	-	-
EGF	nM	-	-	1	10	-	1	-	-	-
IGF1	nng/ml	-	-	-	-	-	-	-	-	-
IL2	U/ml	-	-	-	-	-	-	1	-	-
IL6	U/ml	n.d.	-	30,300	-	-	30,300	300	-	-

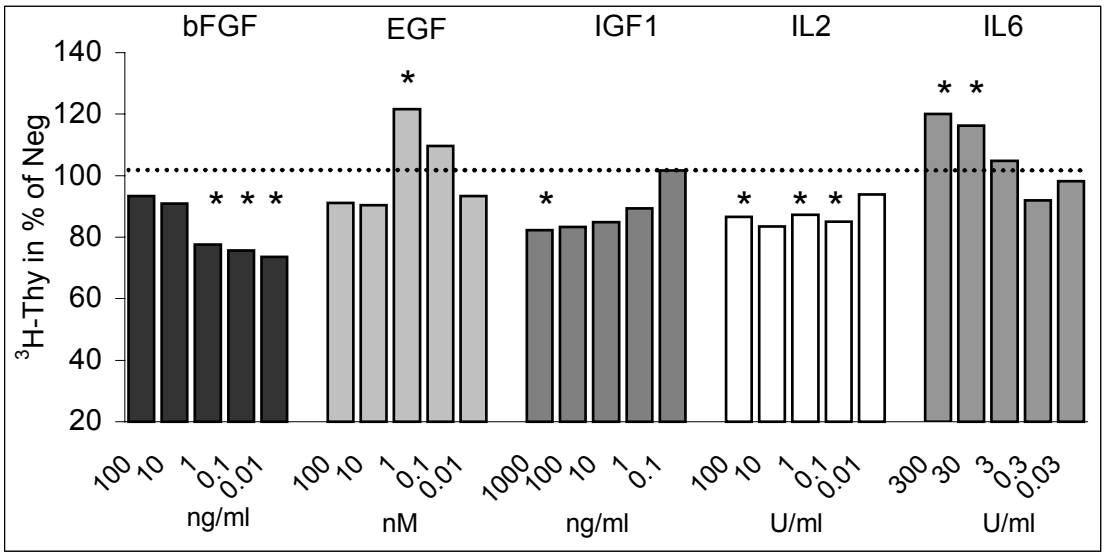
Peptide-induced ^{3}H-thymidine uptake of GH_3 cells. Cells were cultured under various conditions in the presence and absence of peptides. Peptide concentrations, which significantly ($p<0.05$ by t test) increased ^{3}H-thymidine uptake above that of unstimulated cells, are indicated. Inhibitory concentrations are not shown. HS = horse serum; FCS = fetal calf serum; 10FCS%+ (enriched) = 10 % FSC plus transferrin, insulin, and T3; n.d. = not done

In the absence of serum for up to 86 hrs, no significant increase in ^{3}H-thymidine incorporation was observed with any peptide except for EGF (10 nM, 48 hrs of exposure), negating the possibility for a delayed peptide reaction. In the presence of serum, EGF, IL2 and IL6 induced ^{3}H-thymidine incorporation above baseline. In the presence of 5 % HS or 5 % FCS 1 nM EGF increased ^{3}H-thymidine uptake after 24 or 48 hrs, respectively. IL2 (1 U/ml) augmented ^{3}H-thymidine incorporation, when cells where incubated for 48 hrs in 'enriched' medium. Incubation for 24 or 48 hrs in the presence of 5 % HS or FCS appeared to be necessary for IL6 induced ^{3}H-thymidine incorporation. IGF1 and bFGF did not induce ^{3}H-thymidine incorporation above baseline under any condition tested. The data suggested that EGF, IL2 and

IL6 require co-factors to increase ^{3}H-thymidine incorporation above baseline and that this increase was more likely to be significant after 48 hrs of stimulation.

More detailed results of the peptide response in the presence of 5 % FCS are presented in Figure 3.2.1. ^{3}H-thymidine incorporation was significantly augmented by 1 nM EGF and 30-300 U/ml of IL6. IGF1, IL2 and bFGF did not have any stimulatory effect on ^{3}H-thymidine incorporation. In fact, IGF1 at concentrations of 1000 ng/ml

Figure 3.2.1.



Effect of growth factors on ^{3}H-thymidine incorporation of GH_3 cells. Cells were incubated for 48 hrs with different concentrations of peptides in the presence of SF-DM/F12 and 5% FCS, or with SF-DM/F12 and 5% FCS (Neg) alone. Data are presented as % of Neg (horizontal line) of triplicate measurements. Positive controls (1 mM adenosine) significantly increased ^{3}H-thymidine uptake (not shown). * = $p<0.05$ (t test computed on raw data against Neg).

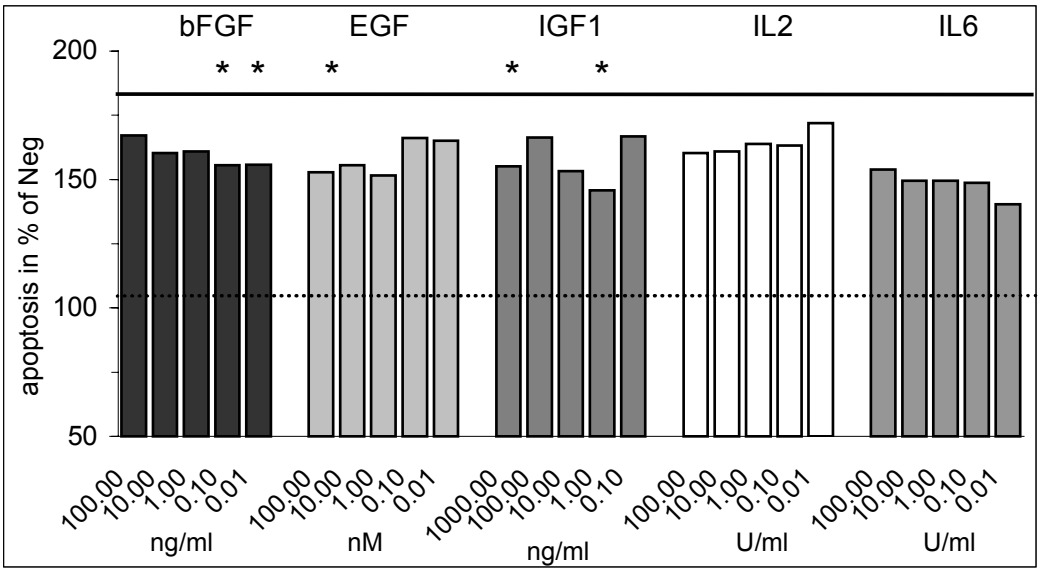
significantly decreased ^{3}H-thymidine incorporation. The same was true for bFGF at concentrations between 1 and 0.01 ng/ml and IL2 at concentrations of 100, 1 and 0.1 U/ml.

3.2.2. Apoptosis

Apoptosis was assessed in GH_3 cells after 24 hrs of stimulation with growth factors in the presence of SF-DM/F12 and 5% serum. The serum was added to reduce the amount of spontaneous apoptosis. Cells incubated in C-DM/F12 or SF-DM/F12 and 5% FCS alone served as negative (Neg) and positive (Pos) controls; experiments are

presented in Figure 3.2.2. The percentage of apoptosis was increased by 74% in cells incubated with SF-DM/F12 (solid horizontal line) as respectively. Data were normalized against Neg and compared to cells incubated in C-DM/F12 (dotted horizontal line). bFGF significantly reduced apoptosis at concentrations of 0.1 and 0,01ng/ml. EGF significantly reduced apoptosis at a concentration of 100 nM. IGF1 significantly reduced apoptosis at concentrations of 1000 and 1 ng/ml. IL2 did not

Figure 3.2.2.



Effect of growth factors on apoptosis in GH_3 cells. Cells were incubated with various concentrations of peptides suspended in SF-DM/F12, with SF-DM/F12 alone (Pos, solid line), or with C-DM/F12 (Neg, dotted line). After 24 hrs apoptosis was determined by AnnF/PI staining. Data are presented as % of Neg of 5 separate experiments. * = $p<0.05$ (Wilcoxon test for paired samples, computed on raw data against Pos).

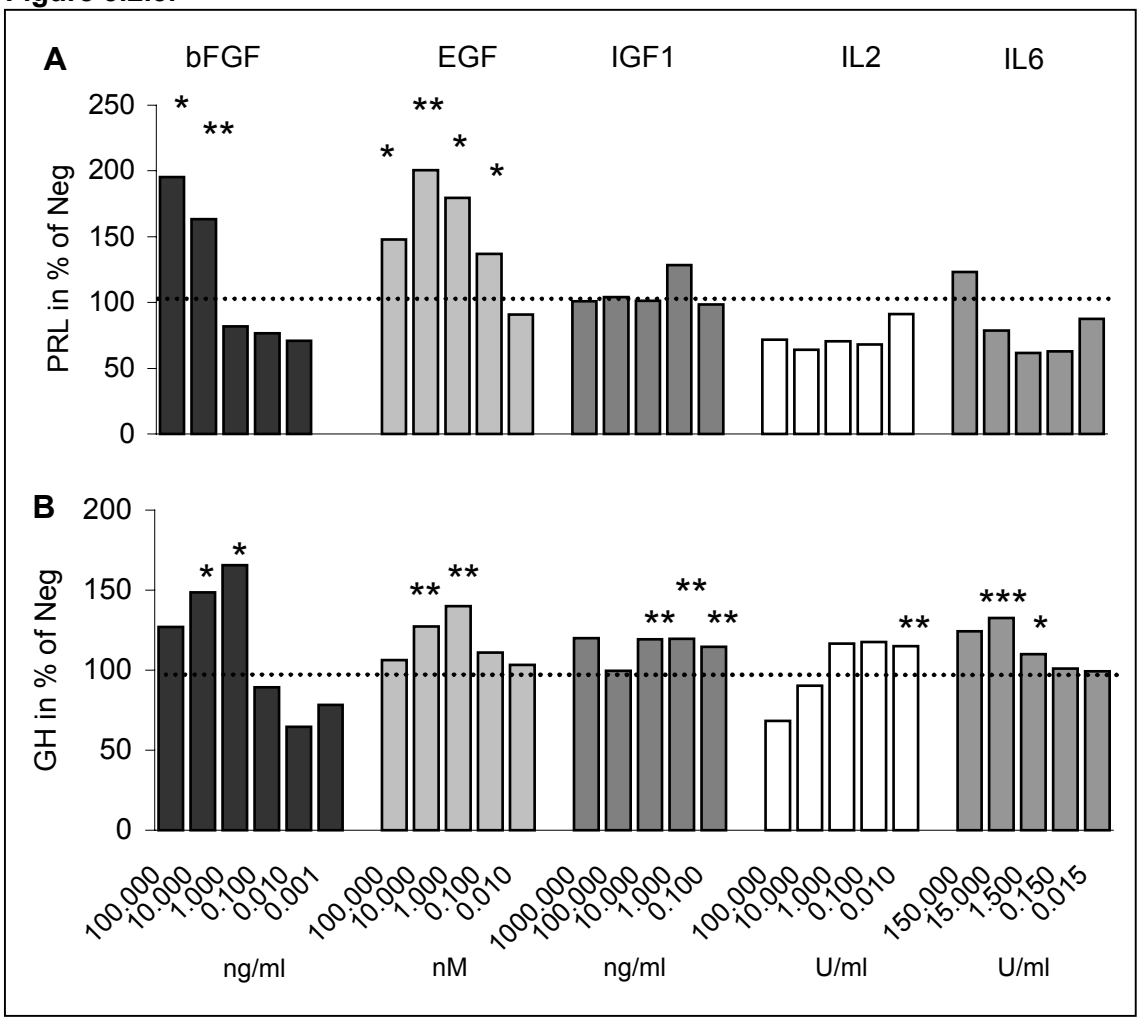
protect cells from apoptosis. Although there appeared to be a protective effect of IL6 in some experiments, it was not statistically significant.

3.2.3. Hormone secretion

Hormone secretion was assessed after 24 hrs of stimulation with growth factors under serum free conditions (Figure 3.2.3. A+B). PRL secretion was significantly increased by bFGF at concentrations between 100 - 10 ng/ml and by EGF at concentrations between 100 - 0.1 nM. IGF1, IL2 and IL6 did not have any effect on

PRL secretion. GH secretion was increased by bFGF (10 - 1 ng/ml), EGF (10 - 1 nM), IGF1 (10 - 0.1 ng/ml), IL2 (0.01 U/ml) and IL6 (15 - 1.5 U/ml).

Figure 3.2.3.



Effect of growth factors on hormone secretion of GH_3 cells. Cells were incubated for 24 hrs in the presence and absence of peptides (Neg). PRL (A) and GH (B) were measured in the supernatants. Data are presented as % of Neg (horizontal line) of duplicate measurements. Positive controls (0.1 µM TRH) significantly stimulated hormone secretion (not shown). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$ (t test computed on raw data against Neg)

The effects of growth factors on GH_3 cells are schematically summarized in Table 3.2.3.

Table 3.2.3.

		bFGF	EGF	IGF1	IL2	IL6
⇕	^{3}H-Thymidine uptake					
⇓	Apoptosis					
⇑	Hormone secretion GH					
↑	Hormone secretion PRL					
[ ]	**Summary of the effects of growth factors on GH_3 cells** = not statistically significant					

3.3. Detection of growth and secretion promoting activities in human pituitary tumor fractions

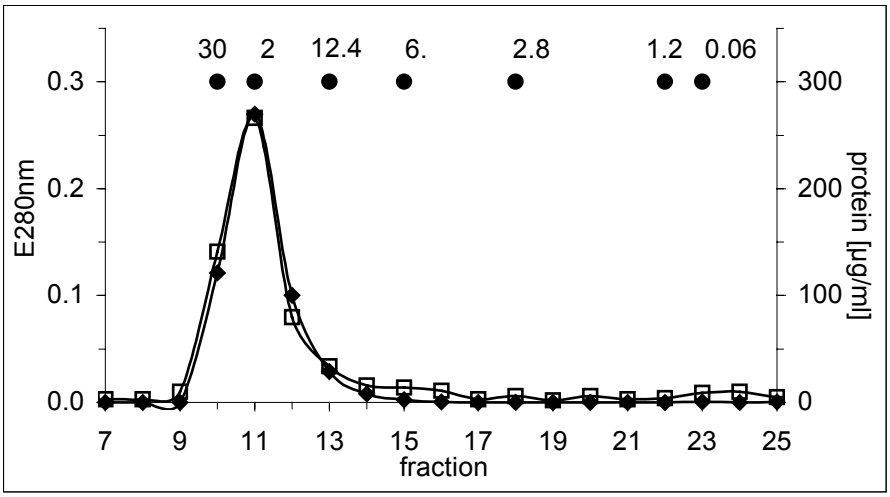
The previous data suggested, that growth factors and cytokines may affect pituitary cell growth and secretion. Even though the presence of these molecules in the normal pituitary has been demonstrated, it remains unclear, whether such peptides have pathophysiological relevance. To study, whether they can be separated from pituitary tumor, tumors were homogenized and separated into fractions of different molecular weight. The effect of tumor fractions on ^{3}H-thymidine, apoptosis and hormone secretion of GH_3 cells was assessed. Results are presented in the following chapter.

3.3.1. Pituitary tumor fractionation

The majority of human pituitary tumors examined in this study (T01-T10) were homogenized and fractionated by our collaborators in Cardiff, England. These fractionation data are not discussed in this report.

In our laboratory, tumors 18 to 27 were homogenized and fractionated on a Superdex 30 column. Representative data of one tumor (T18) are shown in Figure 3.3.1. The majority of detectable protein eluted into fractions 10 through 16. The remaining

Figure 3.3.1.



Elution of a human pituitary tumor extract on a Superdex 30 column. Protein concentrations of fractions 7 to 25 were determined spectrophotometrically at 280nm (open squares) and colorimetrically (closed circles). Elution peaks of 7 molecular weight standards (in kD) are superimposed.

fractions contained proteins at concentrations below the detection limit. Peptide recovery was up to 80% (of 3.32 mg protein applied, 2.67 mg were recovered in fractions 10-25). Note, that the numbering of subsequently presented fractions differs, since all bioassays, except those measuring apoptosis were performed with tumors fractionated in Cardiff.

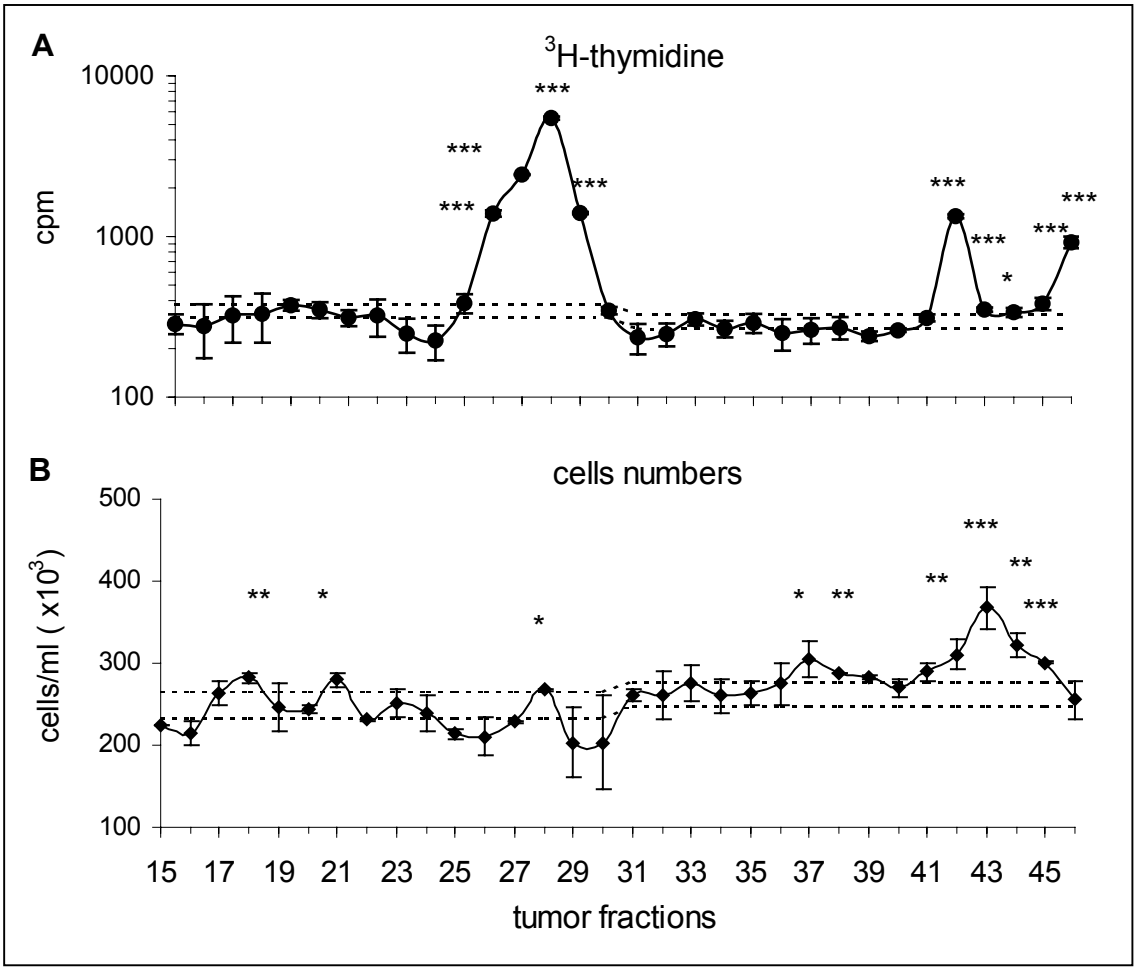
3.3.2. Effect of human pituitary tumor fractions on ^{3}H-thymidine incorporation, cell numbers, apoptosis and hormone secretion in GH_3 cells

Lyophilized chromatography fractions of 10 human pituitary tumors were dissolved in SF-DM/F12 and incubated with GH_3 cells. After 24 hrs ^{3}H-thymidine incorporation, cell numbers, apoptosis and hormone secretion were measured. Cells incubated in the absence of tumor fractions served as negative controls. In the following figures data of one representative tumor are presented. Since this study focused on mechanisms promoting tumor growth, data analysis emphasized on growth and secretion augmenting activities, whereas inhibitory activities are not further discussed.

3.3.2.1. ^{3}H-thymidine incorporation and cell numbers

Figure 3.3.2. shows that ^{3}H-thymidine incorporation (A) was significantly augmented by fractions 26-29 and 42-46, whereas cell numbers (B) were slightly, but significantly increased by fractions 18, 21, 28, 37-38, and more prominently by fractions 42-45.

Figure 3.3.2.

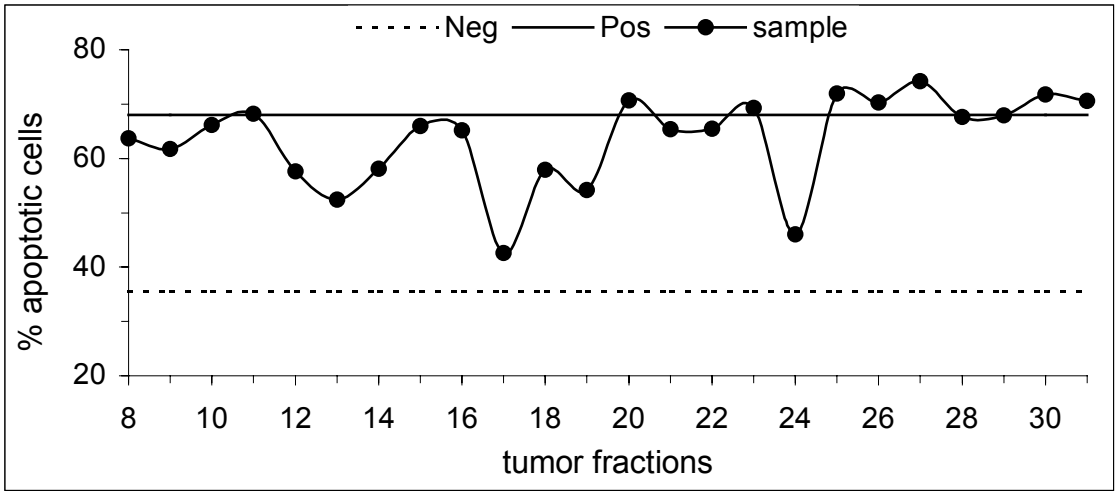


Pituitary tumor fraction-induced ^{3}H-thymidine incorporation and cell numbers of GH_3 cells. Cells were stimulated for 24 hrs with pituitary tumor fractions 15 to 46 of one tumor (T08). ^{3}H-thymidine uptake (mean of triplicates ± SD) or cell numbers (mean of duplicates ± SD) were compared to those of negative controls (cells + SF-DM/F12 alone, Neg). * = $p<0.05$, ** = $p<0.01$, *** $p<0.001$ (t test). Note that fractions 15-30 and 31-46 were tested in different microtiter plates, resulting in the difference of Neg ± SD between fractions 30 and 31 (this is also true for Figure 3.3.4.).

3.3.2.2. Apoptosis

The effect of human pituitary tumor fractions on apoptosis of GH_3 cells is presented in Figure 3.3.3. Apoptosis following treatment with pituitary fractions was compared to that under serum free conditions (Pos). Cells incubated in C-DM/F12 represent the

Figure 3.3.3.



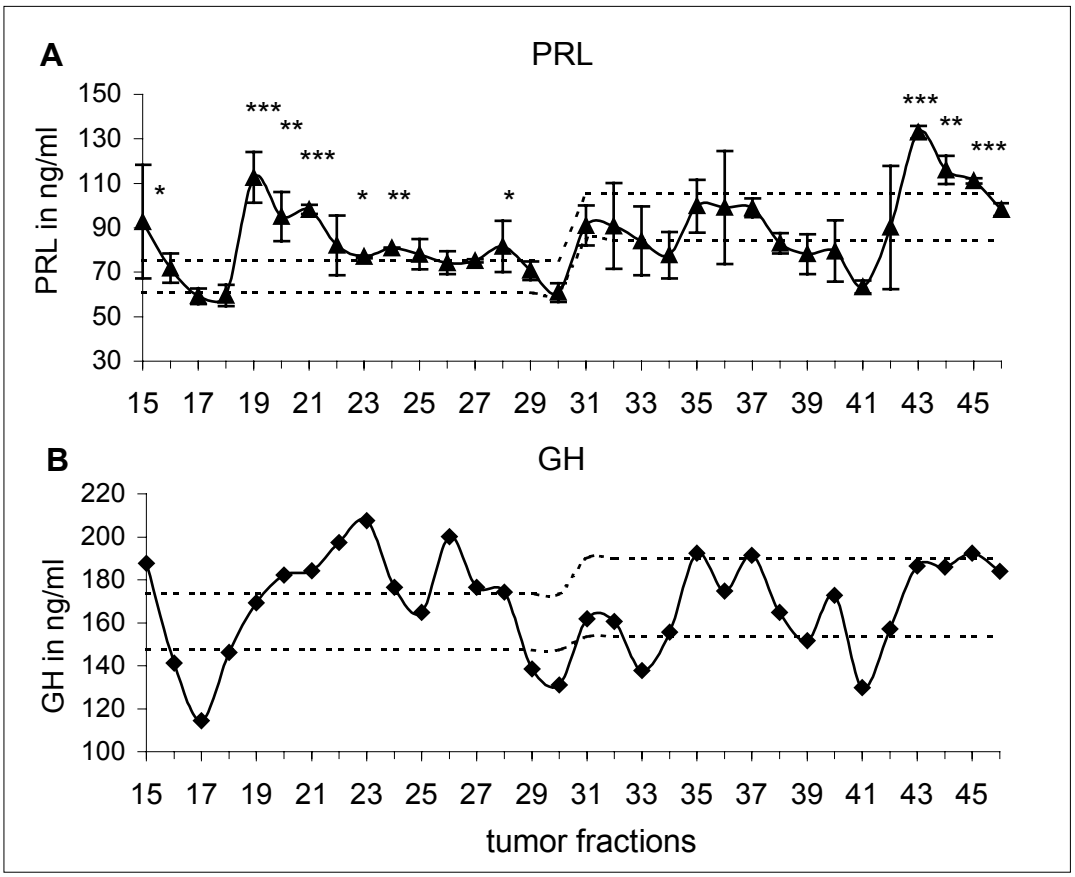
Pituitary tumor fraction-induced protection from apoptosis of GH_3 cells. Cells suspended in SF-DM/F12 were incubated with fractions 8-31 (sample) of T19. Cells in C-DM/F12 or SF-DM/F12 served as negative (Neg) and positive (Pos) controls, respectively. After 24hrs the cells were stained with AnnF/PI and analyzed by flow cytometry. The percentage of alive (AnnF-positive) and dead (AnnF/PI positive) apoptotic cells are displayed.

percentage of spontaneous apoptosis and served as negative controls (Neg). The majority of tumor fractions (8-11, 15-16, 20-23, 25-31) induced apoptosis comparable to that induced by SF-DM/F12 alone (68 %), However, the percentage of apoptosis induced by fractions 12-14, 17-19 and 24 was more comparable to that induced by C-DM/F12 (35.5 %), suggesting that these fractions contained substances which protected GH_3 cells from apoptosis.

3.3.2.3. Hormone secretion

Hormone secretion induced by fractions 15-46 of tumor 8 was assessed. PRL secretion (Figure 3.3.4. A) was significantly increased by fractions 15, 19-21 23-24,

Figure 3.3.4.



Hormone secretion of GH_3 cells induced by pituitary tumor fractions. Cells were stimulated for 24 hrs with pituitary tumor fractions 15- 46 of tumor 8. PRL and GH were measured in the supernatants of duplicate wells and compared to negative controls (cells + SF-DM/F12 alone, horizontal lines). Data are expressed as mean of duplicate measurements ± SD. *= $p<0.05$, **= $p<0.01$ (t-test). In the case of GH, positive were those samples larger than Neg + SD.

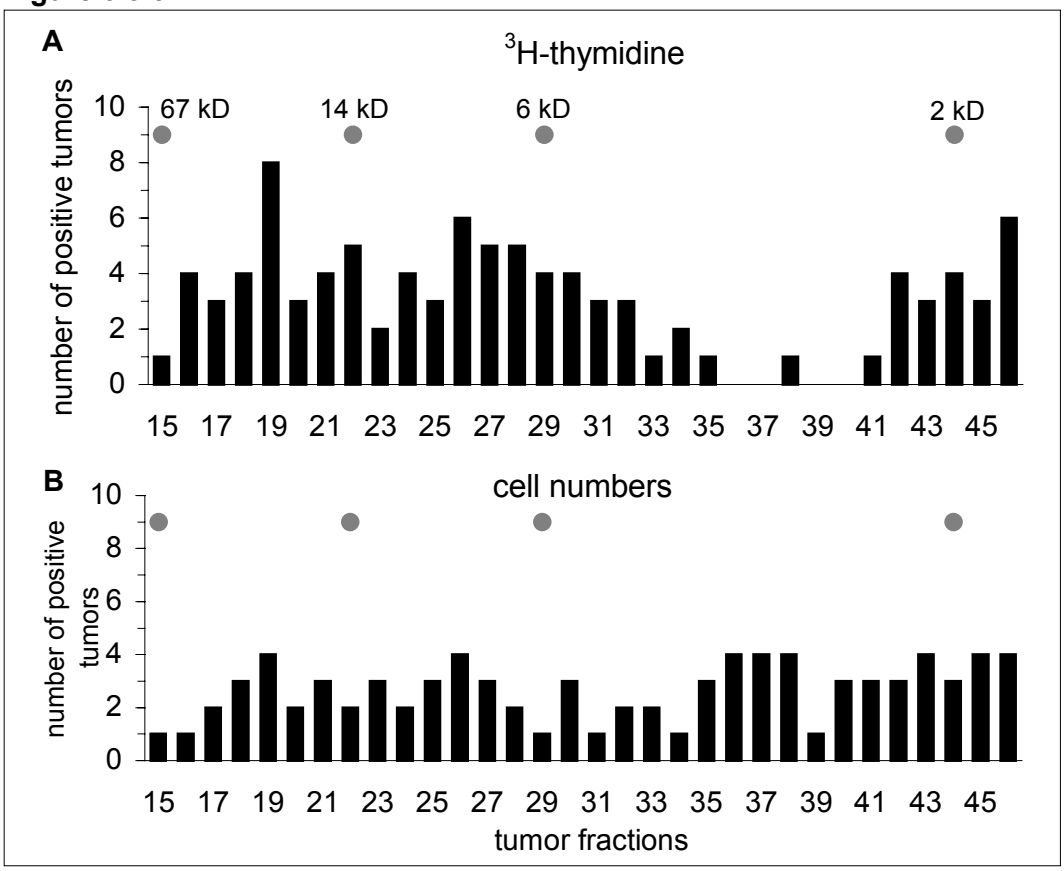
27 and 43-45. Of these fractions the secretion was augmented most prominently by fractions 19-21 and 43-45. GH secretion (Figure 3.3.4. B) was augmented by fractions 15, 20-24, 26-28, 35, 37 and 45. Of these, the secretion was most prominently augmented by fractions 23, 24 and 26.

Taken together, the results suggest that a number of substances can be separated from a human pituitary tumor, which induce proliferation and hormone secretion or protect GH_3 cells from apoptosis. Some tumor fractions appear to augment both,

proliferation and hormone secretion, whereas others elicited only one of the responses tested. The significance of the magnitude of stimulation will be discussed later.

Data of proliferation and hormone secretion induced by all 10 pituitary tumors are presented in subsequent figures and tables. The effect of tumor fractions on apoptosis was examined in only 3 tumors and was not evaluated statistically. Data were grouped by tumor fraction to visualize those effects prominent in all tumors. Regardless of the degree of stimulation, those fractions, which significantly induced stimulatory activity, were considered. Figure 3.3.5. shows that the highest prevalence of proliferation inducing activity was found in fraction 19, which increased ^{3}H-thymidine incorporation (A) or cell numbers (B) in 8/10 or 4/10 tumors respectively.

Figure 3.3.5.

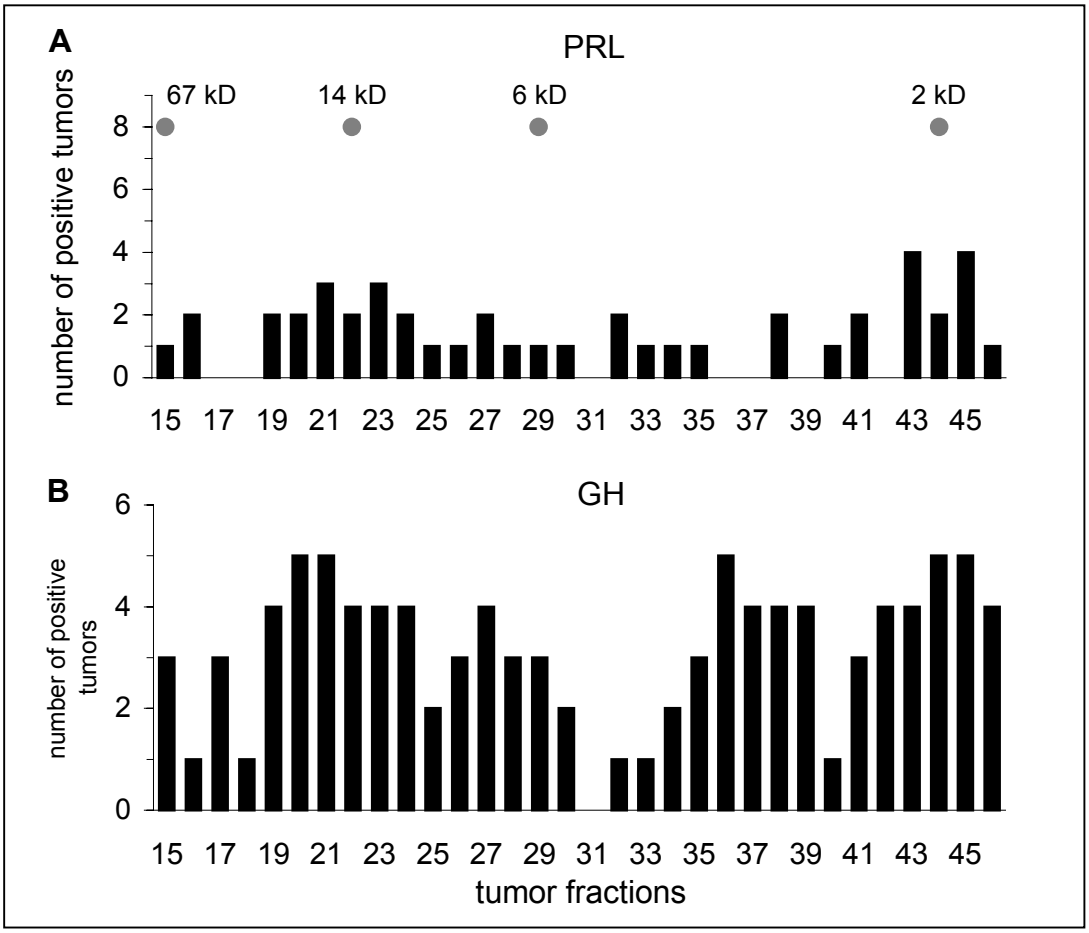


^{3}H-thymidine- and cell number-augmenting activities in chromatography fractions of 10 human pituitary tumors. Bars indicate the number of tumors, which contained stimulatory activity in the respective fraction

According to molecular weight standards this fraction corresponds to approximately 30 kD. Pronounced proliferation-inducing activity also eluted in fractions 26 and 46, both of which augmented ^{3}H-thymidine uptake and cell numbers in 6/10 and 4/10 experiments, respectively. The size of these 2 fractions is approximately 8 kD and 2 kD or smaller.

Figure 3.3.6. shows that PRL secretion (A) was increased by a number of fractions, but only a small subset of the tumors. Most frequently secretory activities eluted into fractions 21 (3/8), 23 (3/9), 43 (4/9), and 45 (4/9), which correspond to approximately

Figure 3.3.6.



Hormone secretion-inducing activities in chromatography fractions of 10 human pituitary tumors. Bars indicate the number of tumors, which contained secretory activity in the respective fraction.

14 kD and 2 kD. The highest frequency of GH inducing substances (B) was found in fractions 20, 21 (14 kD), 36 (4 kD), 44 and 45 (2 kD).

Taken together, the results indicate that proliferation and secretion inducing substances of various sizes can be identified in a subset of pituitary tumors. The molecular weights of the most abundant proliferation inducing substances are not identical to those augmenting secretion.

In a further analysis the data were then grouped according to weight ranges, since dependent on the amount of one substance present in the tumor extract, one molecule may elute into more than one fraction. The ranges were defined as follows: fractions 15-17 = 60 kD, fractions 18-20 = 30 kD, fractions 21-25 = 14 kD, fractions 26-32 = 6 kD, fractions 33-40 = 4 kD and fractions 41-46 = 2 kD. For statistical analysis, a group was considered positive, when at least one of its fractions contained significant stimulatory activity, regardless of its degree. Results of the 10 tumors are summarized in Table 3.3.1.

Table 3.3.1.

	Pituitary adenomas			
	Proliferation		Secretion	
	Thy	CC	PRL	GH
	N=10	N=10	N=9	N=10
60 kD	60	30	22	50
30 kD	80	50	44	60
14 kD	90	50	44	50
6 kD	80	50	44	70
4 kD	60	60	22	80
2 kD	80	80	78	80

Percentage of tumor fractions from extracts of all pituitary adenomas containing stimulatory activities. Tumor fractions that induced proliferation (Thy, CC) or secretion (PRL, GH) were divided into different molecular weight ranges (in kD) and are presented as a percentage of all tumors examined (=10)

Both proliferation and hormone stimulatory activities were found in the majority of tumors throughout all molecular weight ranges. At least 60% of all tumors contained ^{3}H-thymidine inducing activity (Thy) in each molecular weight range. All except the 60

kD range, possessed cell count (CC) increasing activities in the majority of all tumors. PRL secretion inducing activities were found predominantly in the 30 kD, 6 kD, and 2 kD range. At least 50% of all tumors contained GH secretion inducing activities in each molecular weight range.

3.3.3. Stimulatory activities in tumor fractions of null cell adenomas (NCA) and of hormone secreting adenomas

In order to examine whether the abundance of stimulatory activity was associated with a specific pituitary tumor subtype, the data were further grouped by histological diagnoses. There were 5 null cell adenomas (NCA, tumor histology does not reveal any hormone or its mRNA, Table 3.3.2.) and 5 hormone active adenomas (Table 3.3.3.), which consisted of 3 gonadotropinomas, 1 mamasomatotropinoma, and one plurihormonal tumor. Table 3.3.2. shows that the majority of all NCAs contained ^{3}H-thymidine augmenting activities in all molecular weight ranges. Cell count augmenting activity was present in the majority of the 30 kD, 4 kD and the 2 kD ranges. Hormone secretion inducing activities (PRL and GH) were found throughout all molecular weight ranges in the majority of NCAs.

Table 3.3.2.

	Null Cell Adenomas			
	Proliferation		Secretion	
	Thy	CC	PRL	GH
	N=5	N=5	N=4	N=5
60 kD	60	20	50	60
30 kD	80	60	75	60
14 kD	80	40	50	60
6 kD	80	40	75	60
4 kD	60	60	50	80
2 kD	80	80	100	80

Percentage of tumor fractions from extracts of null cell adenomas containing stimulatory activities. Tumor fractions that induced proliferation (Thy, CC) or secretion (PRL, GH) were divided into different molecular weight ranges (in kD) and are presented as a percentage of all tumors examined.

The results of 5 hormone active tumors were similar, except that only one of 5 tumors contained proliferation-augmenting activity of approximately 4kD. PRL secretion inducing activity was most prominent in the 2 kD range, whereas there were no such activities of 60 kD or 4 kD and only 1 or 2 of 5 tumors were positive in the 30 to 6 kD ranges.

Table 3.3.3.

	Hormone Active Adenomas			
	Proliferation		Secretion	
	Thy	CC	PRL	GH
	N=5	N=5	N=5	N=5
60 kD	60	40	0	40
30 kD	80	40	20	60
14 kD	100	60	40	40
6 kD	80	60	20	80
4 kD	20	60	0	80
2 kD	60	80	60	80

Percentage of tumor fractions from extracts of hormone secreting adenomas containing stimulatory activities. Tumor fractions that induced proliferation (Thy, CC) or secretion (PRL, GH) were divided into different molecular weight ranges (in kD) and are presented as a percentage of all tumors examined (=5).

Taken together these results demonstrate that proliferation and hormone secretion augmenting activities can be isolated from hormone active and hormone inactive human pituitary tumors. The array of activities was similar in hormone inactive and active tumors, except for substances stimulatory for PRL secretion.

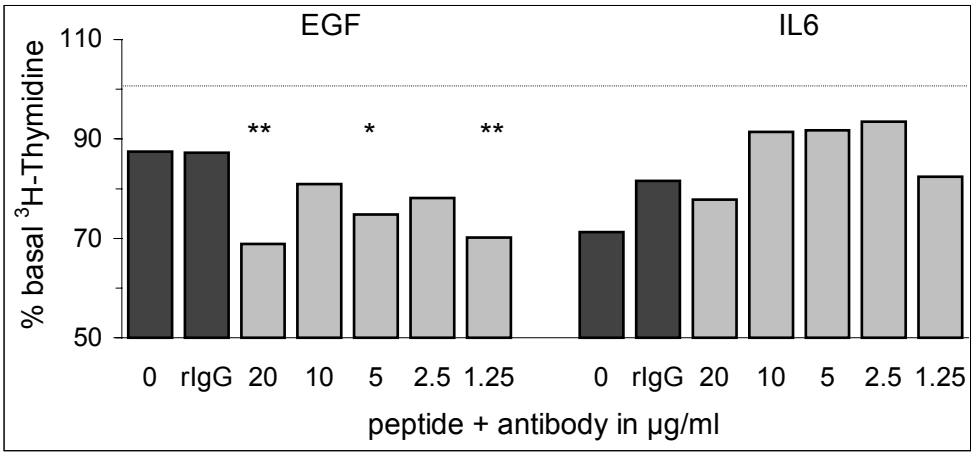
3.3.4. Neutralization of pituitary tumor induced stimulatory activities

The substances separated by gel filtration chromatography might be identical to known growth- or secretion promoting polypeptides. If so, it should be possible to counteract their effects by preincubation with specific neutralizing antibodies. Before tumor activities could be neutralized, optimal antibody concentrations had to be determined.

3.3.4.1. Determination of optimal antibody concentrations, which neutralize growth factor-induced stimulation in GH_3 cells

The neutralizing activity of antibodies directed against a number of growth factors was tested in GH_3 cells. Peptides were preincubated for 2 hrs with various concentrations of their corresponding antibody or with an isotypic control antibody. Cells stimulated with SF-DM/F12 (baseline) or with peptide alone served as negative and positive controls, respectively. Peptide-antibody-induced ^{3}H-thymidine incorporation and hormone secretion were assessed after 48 or 24 hrs, respectively. Representative data of three separate ^{3}H-thymidine experiments are depicted in Figure 3.3.7. Although neither EGF nor IL6 significantly increased ^{3}H-thymidine

Figure 3.3.7.

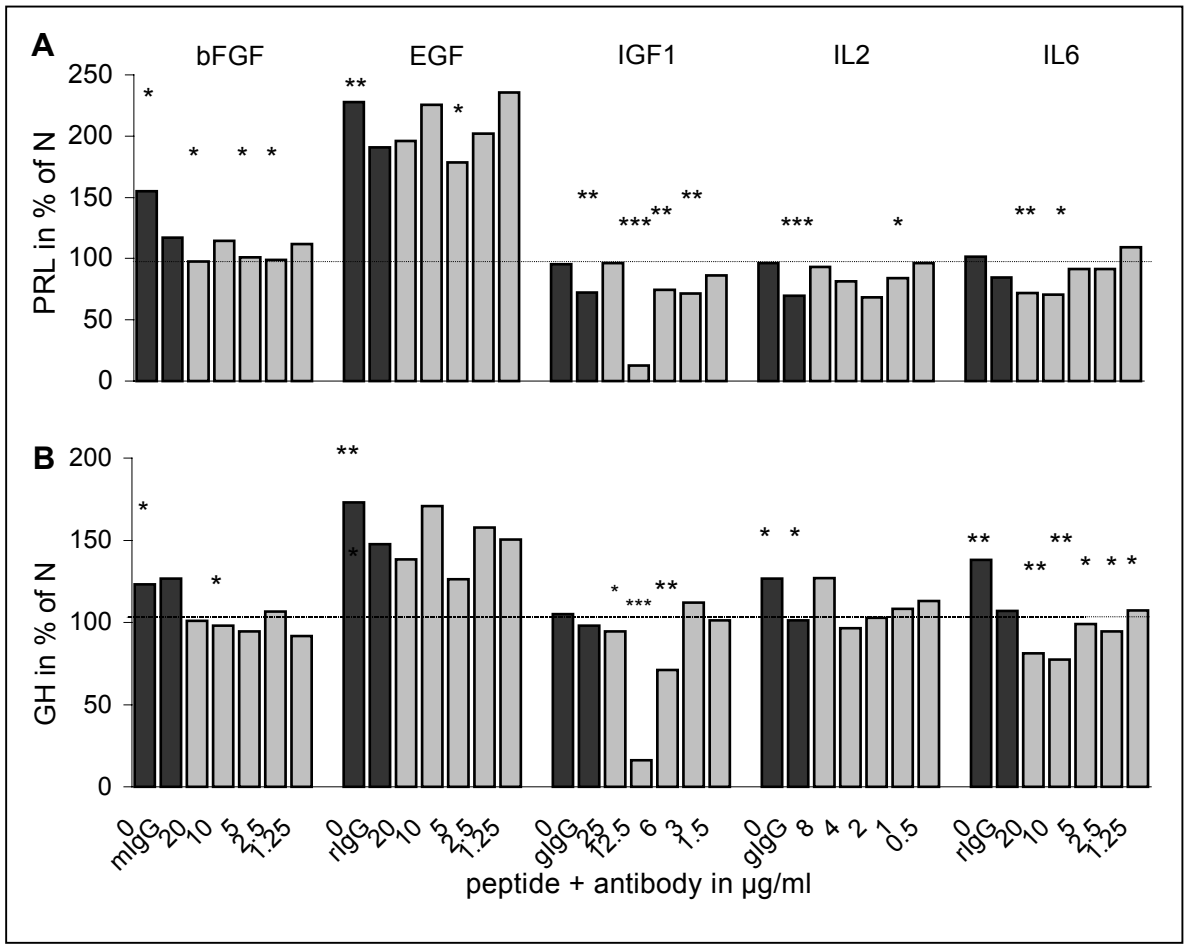


Effect of neutralizing growth factor antibodies on ^{3}H-thymidine incorporation of GH_3 cells. Cells in the presence of 5 % HS were stimulated with peptide, antibody+peptide mixture, or SF-DM/F12 alone (negative controls). ^{3}H-thymidine incorporation was assessed in quadruplicates after 48 hrs. Data are expressed as % of negative controls (horizontal line). *=$p<0.05$, ** $p<0.01$ (statistical analyses between negative controls and peptide or between peptide and antibody+peptide were computed on raw data, t test).

incorporation above baseline (horizontal line), ^{3}H-thymidine incorporation was significantly lower in the presence of neutralizing rabbit aEGF (20, 5, 2.5µg/ml) than in its absence (EGF+SF-DMF/12). The effect of aEGF was specific since rabbit IgG (rIgG) did not have any effect on EGF induced ^{3}H-thymidine incorporation. In contrast, there was no significant effect of rabbit aIL6 (20-1.25 µg/ml) or its corresponding isotypic control on IL6 induced ^{3}H-thymidine incorporation.

The effect of peptide antibodies on hormone secretion was assessed after 24 hrs (Figure 3.3.8.). Mouse abFGF appeared to decrease bFGF induced PRL and GH

Figure 3.3.8.



Effect of neutralizing growth factor antibodies on PRL (A) and GH (B) secretion of GH_3 cells. Cells were stimulated with peptide, antibody+peptide mixture, or SF-DM/F12 (negative control) as described. PRL and GH were measured after 24 hrs. Data are expressed as % of negative control (horizontal line). *=p<0.05, ** p<0.01, *** p<0.001 (statistical analyses between negative controls and peptide or between peptide and antibody+peptide were computed on raw data, t test).

secretion at all concentrations tested, although the differences were significant only at 20, 5, and 2.5 µg/ml abFGF for PRL and at 10 µg/ml abFGF for GH secretion. bFGF alone significantly stimulated PRL and GH secretion; mouse IgG (mIgG) did not affect bFGF induced hormone secretion.

Rabbit aEGF significantly reduced PRL but not GH secretion at a concentration of 5 µg/ml although the effect was not very pronounced. The effect appeared to be

specific, since rIgG did not affect hormone secretion. Both PRL and GH secretion were significantly increased in the presence of EGF alone.

Although IGF1 did not induce PRL or GH secretion above baseline, goat aIGF1 at concentrations between 12 - 3 µg/ml or 25 - 6 µg/ml significantly decreased IGF1 induced PRL or GH secretion, respectively. There was a slight but significant neutralizing effect with the isotypic goat IgG control antibody (gIgG) on PRL, but not on GH secretion.

Goat aIL2 at 1 µg/ml significantly decreased PRL secretion. GH secretion appeared to be decreased between 4 and 0.5 µg/ml of aIL2, although this decrease was not statistically significant. IL2 alone significantly increased GH, but not PRL secretion. IL2 induced PRL and GH secretion were reduced with gIgG.

IL6 induced GH secretion was significantly neutralized at all rabbit aIL6 concentration tested, whereas PRL secretion was significantly decreased with 20 and 10 µg/ml of aIL6. IL6 alone significantly increased GH, but not PRL secretion. rIgG did not have any effect.

The results indicate that bFGF- and EGF-induced PRL and bFGF- and IL6-induced GH secretion were neutralized with specific antibodies. Because of unspecific effects of gIgG, the effects of goat aIGF1 and aIL2 on PRL secretion remained inconclusive. Even though exogenous human IGF1, IL2 or IL6 did not augment PRL or (in case of IGF1) GH secretion, baseline hormone secretion may be dependent on intrinsically produced peptide, which was neutralized with antibodies.

Table 3.3.5. summarizes the antibody concentrations, which were subsequently used in an attempt to neutralize pituitary tumor fraction induced activities.

Table 3.3.5.

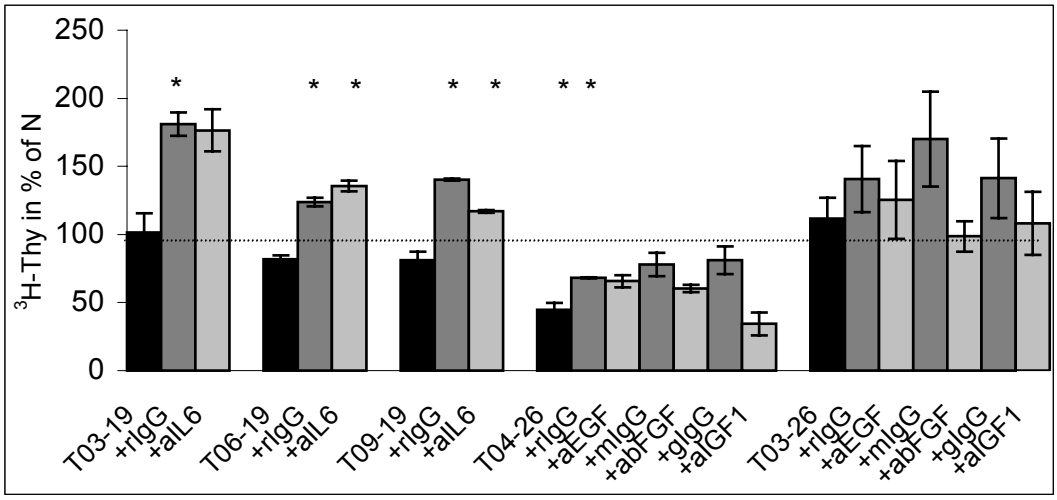
Antibody		aIGF1	abFGF	aEGF	aIL6
Isotype		gIgG	mIgG	rIgG	rIgG
Concentration	µg/ml	12	10	5	10

Antibodies used for neutralization of peptide-induced stimulations. The isotypic control antibodies were generated in goat (gIgG), mouse (mIgG) and rabbit (rIgG). Optimal concentrations for neutralization are presented.

3.3.4.2. Effects of neutralizing antibodies specific against growth factors on pituitary tumor fraction-induced ^{3}H-thymidine incorporation in GH_3 cells

Those pituitary tumor fractions, which contained proliferation-inducing activities in the majority of tumors tested (see Figure 3.3.5), were incubated with GH_3 cells in the presence of neutralizing antibodies against growth factors of according molecular weights. Cells stimulated with SF-DM/F12 alone served as negative controls (baseline). Isotypic control antibodies were used to determine unspecific interactions. ^{3}H-thymidine incorporation was assessed after 48 hrs. Data of one representative experiment are presented in Figure 3.3.9. Here, tumor fractions 19 (~30 kD) of 3

Figure 3.3.9.



Effects of neutralizing growth factor antibodies on ^{3}H-thymidine-inducing activities of pituitary tumor fractions. GH_3 cells were stimulated with tumor fractions, tumor fraction+antibody mixture, or SF-DM/F12 (Neg) as described. ^{3}H-thymidine incorporation was measured after 48 hrs. Data are expressed as % of N (horizontal line). $p<0.05$ (*) were accepted as significant (computed on raw data, Neg against peptide, peptide against peptide+antibody, t test).

tumors were treated with rabbit aIL6 or rabbit IgG. The molecular weight of human IL6 is 26 - 29 kD. Similarly, fractions 26 (~ 6 kD) of 2 tumors were co-incubated with aEGF, aIGF1 or their corresponding isotypic controls. The molecular weights of EGF and IGF1 are 6 and 7.6 kD, respectively. Unfortunately, none of the previously positive fractions induced increased ^{3}H-thymidine incorporation, when compared to negative controls. In fact, fraction 26 of T04 (T04 - 26) significantly decreased ^{3}H-thymidine incorporation. In the presence of aIL6 ^{3}H-thymidine incorporation was

significantly increased in two of three fractions. However, similar effects were observed in the presence of isotype controls and thus these data remain inconclusive. There was no neutralizing effect of aEGF, abFGF or aIGF1 on fractions 26 from 2 tumors. And again in contrast to previous data, in the absence of antibody these fractions did not increase ^{3}H-thymidine incorporation above that of negative controls.

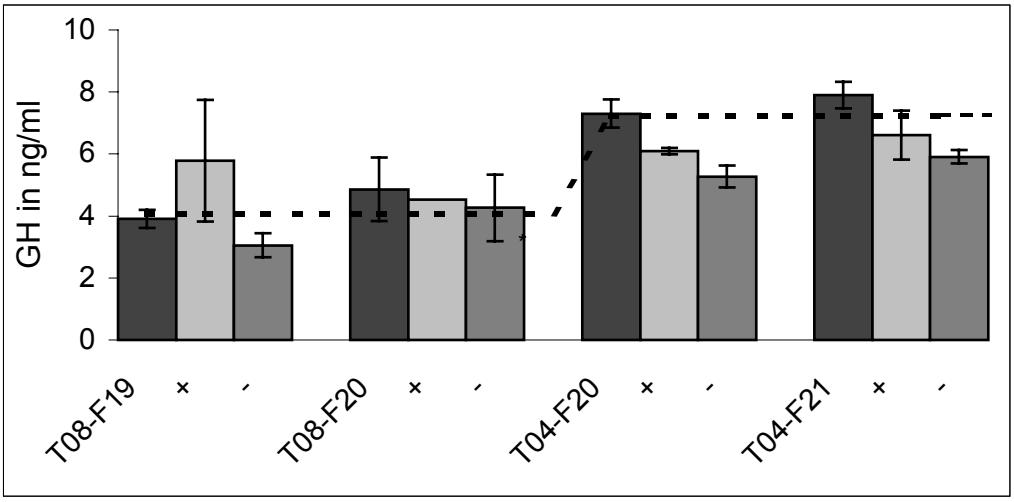
3.3.4.3. Effects of neutralizing antibodies specific against growth factors on pituitary tumor fraction-induced hormone secretion

The effect of peptide antibodies on pituitary tumor fraction-induced PRL secretion was not determined, because the previously used commercial ELISA was discontinued without availability of a replacement. The development of a new method, such as a rat PRL radioimmunoassay, which would have required labeling of rat PRL with ^{125}Iodine, surpassed the scope of this project.

Fractions of several tumors, that belonged to the molecular weight range in which the majority of GH-inducing activities where previously found (F19 - F23, ≈ 30-14 kD, see Fig 3.3.5), were used in neutralization studies. The corresponding growth factor for which a neutralizing antibody was available and previously tested with GH_3 cells was IL6 (26 - 29 kD). Fractions of 3 different tumors were incubated with GH_3 cells in the presence of anti-IL6 or its corresponding isotypic control (rabbit IgG). Cells stimulated with SF-DM/F12 alone serve as negative controls (baseline). GH secretion was assessed after 24 hrs. Data of one representative experiment are presented in Figure 3.3.10. None of the previously positive (GH-inducing) fractions induced GH secretion above baseline levels (dotted line) in this experiment. Similarly, anti-IL6 (+) or its isotypic control (-) had no effect on tumor fraction-induced GH secretion. The experiment was repeated several times with different fractions, but similar results. The data suggest, that these previously positive (GH-inducing) fractions lost their GH-inducing activity, possibly due to prolonged storage.

Taken together, the neutralization studies were unsuccessful because of the loss of stimulatory activity of tumor fractions, possibly due to prolonged or improper storage.

Figure 3.3.10.



Effects of antiIL6 on GH-inducing activities of pituitary tumor fractions. GH_3 cells were stimulated with tumor fractions, tumor fraction+anti-IL6 (+), tumor fraction+rIgG (-), or SF-DM/F12 (horizontal line) as described. GH-secretion was measured from the supernatant after 24 hrs.

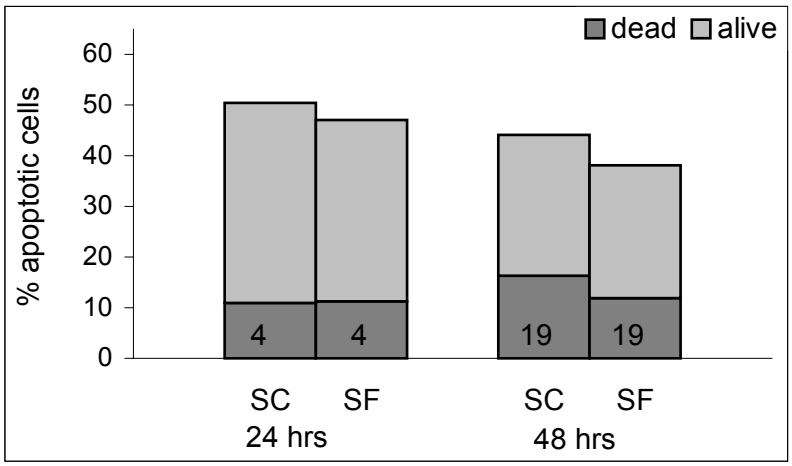
3.4. Functional effects of growth factors on primary human pituitary cells

To assess physiological significance of the above described data, the effects of growth factors were examined in primary human pituitary tumor cells (PP). The cell-doubling rate of these cells in culture is so low that proliferation studies are rather unfeasible. Thus, we examined apoptosis, as another possible contributing factor in pituitary tumor growth.

3.4.1. Effect of serum deprivation on apoptosis in PP

The aim of the first experiment was to assess whether growth factor removal by serum deprivation would induce apoptosis in PP. Cells were cultured in the presence (SC) or absence (SF) of serum. After 24 or 48 hrs, the cells were brought into suspension and stained with AnnF/PI. Data of PP from 19 patients are presented in Figure 3.4.1. Serum deprivation for 48 hrs did not alter the percentage of apoptotic cells in 19 PP. Thus, in contrast to GH_3 cells, serum deprivation for up to 48 hrs does not increase apoptosis in PP.

Figure 3.4.1.



Effect of serum deprivation on apoptosis of PP. Primary pituitary tumor cells were cultured in SC or SF and after 24 or 48 hrs they were stained with AnnF/PI. The percentage of apoptotic (AnnF & AnnF/PI positive) cells of 19 PP is displayed.

3.4.2. Effect of growth factors on apoptosis in PP

Next, the effect of several exogenous growth factors on apoptosis was studied in primary cultures of pituitary adenomas. In the absence of serum, cells were incubated with bFGF, EGF, IGF1, or IL6 for 48 hrs and the percentage of apoptotic cells was compared to that of cells incubated with serum free media alone. Growth factor concentrations stimulatory for GH_3 cells were used. Table 3.4.1. shows the results of PP from 18 patients. Overall, growth factor-induced apoptosis varied from –22% to +20% (mean: 6.1%, data not shown). The percentage of apoptosis was decreased by bFGF, EGF, IGF1 and IL6 in 8/18, 5/17, 6/9, and 3/12 PP, respectively. Some PP remained unresponsive to growth factors (bFGF: 6/18, EGF: 9/17, IGF1: 1/9, IL6: 7/12), whereas others responded with increased apoptosis (4/18, 3/17, 2/9, and 2/12, respectively. There was no apparent correlation of growth factor responsiveness to patient age, sex or histological diagnoses, although only limited interpretations were possible, since most of the tumors were gonadotropinomas.

Taken together, the data indicate that in contrast to GH_3 cells, GF removal for up to 48 hrs reduces, rather than induces apoptosis in primary cultures of human pituitary adenomas. Addition of exogenous Growth factors has variable effects, increasing apoptosis in some, but further decreasing apoptosis in other PP.

Table 3.4.1.

	PP	Histol	**bFGF**	*PP*	Histol	**EGF**	*PP*	Histol	**IGF1**	*PP*	Histol	**IL6**
	69	Som	-	*71*	Som	-	*69*	Gon	-	*71*	Som	-
	59	Pluri	-	*73*	Gon	-	*65*	Gon	-	*65*	Gon	-
	58	Gon	-	*80*	Gon	-	*68*	Gon	-	*80*	Gon	-
	50	Gon	-	*77*	Null	-	*57*	Gon	-	*69*	Som	0
	57	Gon	-	*81*	Null	-	*50*	Gon	-	*78*	Pluri	0
	42	Gon	-	*69*	Som	0	*58*	Gon	-	*66*	Pluri	0
	72	Gon	-	*66*	Pluri	0	*59*	Pluri	0	*76*	Gon	0
	80	Gon	-	*50*	Gon	0	*66*	Pluri	+	*75*	Null	0
	71	Som	0	*72*	Gon	0	*42*	Gon	+	*77*	Null	0
	66	Pluri	0	*42*	Gon	0				*81*	Null	0
	73	Gon	0	*57*	Gon	0				*73*	Gon	+
	68	Gon	0	*76*	Gon	0				*68*	Gon	+
	65	Gon	0	*68*	Gon	0						
	77	Null	0	*75*	Null	0						
	78	Pluri	+	*78*	Pluri	+						
	76	Gon	+	*59*	Pluri	+						
	75	Null	+	*58*	Gon	+						
	81	Null	+									
-			8/18			5/17			6/9			3/12
0			6/18			9/17			1/9			7/12
+			4/18			3/17			2/9			2/12

Effect of growth factors on apoptosis of PP from several human pituitary adenomas. Primary pituitary tumor cells were cultured for 48 hrs with SF-PPM or with 10 ng/ml bFGF, 1 nM EGF, 500 ng/ml IGF1, or 50 U/ml IL6 and stained with AnnF/PI. The data of individual PP are presented as apoptosis decreasing (-) or augmenting (+) effects, when in the presence of growth factors the percentage of apoptotic cells (AnnF and AnnF/PI positive) differed by at least 3% from that of cells cultured under SF conditions alone. Differences of less than 3% (0) were interpreted as unresponsive to growth factors. Values are based on duplicate or triplicate measurements. The histological diagnoses (Histol) of PP are abbreviated as Som (somatotropinoma), Pluri (plurihormonal adenoma), Gon (gonadotropinoma) and Null (null cell adenoma).

3.4.3. Effect of neutralizing growth factor antibodies on apoptosis in PP

In light of the responsiveness to exogenous growth factors, one possible explanation for reduced apoptosis in response of SF may be the action of endogenous apoptosis-modulating growth factors. To test this possibility, various PP were incubated under SF conditions in the absence or presence of neutralizing antibodies specific against bFGF, EGF, IGF1 or IL6. Apoptosis (AnnF and AnnF/PI positive cells) was measured after 48 hrs (Table 3.4.3). Anti-bFGF augmented apoptosis in 3/10 PP, elicited no

response in 5/10 PP and actually decreased apoptosis in 2/10 PP. Anti EGF and anti-IL6 decreased apoptosis in the majority of PP (6/10, 4/8, respectively), with the effect of EGF being statistically significant ($p<0.05$). Although the majority of PP remained unresponsive to anti-IGF1 (5/8), apoptosis was decreased in 3/8 PP. The data suggest that the response of endogenous growth factors on apoptosis varies in PP, with some growth factors, like bFGF, EGF or IL6 having a protective effect, whereas they may augment apoptosis of others.

Table 3.4.3.

	PP	Histol	**a-bFGF**	*PP*	Histol	**a-EGF**	*PP*	Histol	**a-IGF**	*PP*	Histol	**a-IL6**
	70	Som	**+**	*76*	Gon	**+**	*68*	Gon	**0**	*68*	Gon	**+**
	68	Gon	**+**	*70*	Som	**0**	*73*	Gon	**0**	*81*	Null	**+**
	73	Gon	**+**	*68*	Gon	**0**	*72*	Gon	**0**	*70*	Som	**0**
	71	Som	**0**	*81*	Null	**0**	*80*	Gon	**0**	*76*	Gon	**0**
	76	Gon	**0**	*71*	Som	**-**	*81*	Null	**0**	*71*	Som	**-**
	72	Gon	**0**	*72*	Gon	**-**	*70*	Som	**-**	*80*	Gon	**-**
	77	Null	**0**	*73*	Gon	**-**	*71*	Som	**-**	*77*	Null	**-**
	81	Null	**0**	*80*	Gon	**-**	*75*	Null	**-**	*75*	Null	**-**
	80	Gon	**-**	*77*	Null	**-**						
	75	Null	**-**	*75*	Null	**-**						
+			3/10			1/10			0/8			2/8
0			5/10			3/10			5/8			2/8
-			2/10			6/10			3/8			4/8

Effect of neutralizing growth factor antibodies on apoptosis of PP from several human pituitary adenomas. Primary pituitary tumor cells were cultured for 48 hrs with SF-PPM in the absence or presence of anti-bFGF, anti-EGF, anti-IGF1 or anti-IL6 and stained with AnnF/PI. The data of individual PP are presented as apoptosis decreasing (-) or augmenting (+) effects, when in the presence of antibodies the percentage of apoptotic cells (AnnF and AnnF/PI positive) differed by at least 3% from that of cells cultured under SF conditions alone. Differences of less than 3% (0) were interpreted as unresponsive to antibodies. Values are based on duplicate or triplicate measurements. The histological diagnoses (Histol) of PP are abbreviated as Som (somatotropinoma), Pluri (plurihormonal adenoma), Gon (gonadotropinoma) and Null (null cell adenoma).

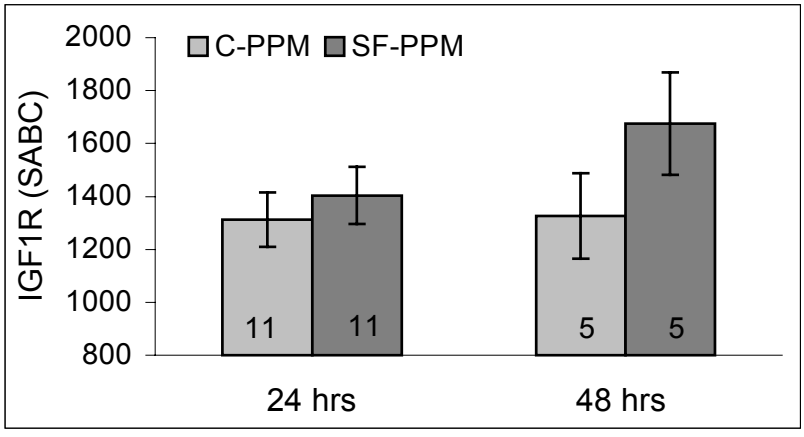
3.5. IGF1R studies in PP

Over-expression or activation of the IGF1R has been described as another protective mechanism from apoptosis in tumor cells (42). In order to evaluate whether this receptor is involved in the lack of apoptosis induction by serum deprivation of PP, IGF1R expression was examined. Unfortunately, GH_3 cells are not suitable to examine IGF1R expression, due to their low IGF1R density (chapter 3.1.3.1.) and an alteration in the IGF1 signal transduction pathway (43). Thus, IGF1R expression was assessed only in primary human pituitary tumor cells.

3.5.1. Effect of serum deprivation on IGF1R expression in PP

IGF1R expression was measured in several PP following serum deprivation (Figure 3.5.1.). After 24 hrs IGF1R expression appeared to be slightly increased (by 7%) in 11 PP treated with SF-PPM, with a further augmentation after 48 hrs (26 %) in 5 different PP. The increase was not statistically significant, although in 4 of 5 individual PP there was a significant increase following serum deprivation for 48 hrs.

Figure 3.5.1.



Effect of serum deprivation on IGF1R expression in PP. Primary pituitary tumor cells were cultured for 24 of 48 hrs in C-PPM or SF-PPM and stained with aIGF1R/DAMPE. IGF1R expression in SABC of 11 and 5 PP is expressed as mean ± SEM.

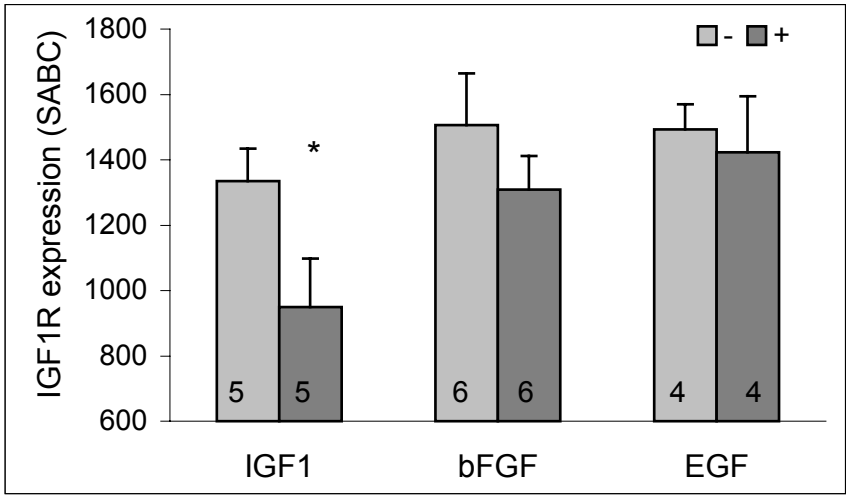
The data suggest that the IGF1R in PP may be upregulated upon serum deprivation. In light of the in parallel observed protection from apoptosis in response to SF (see

Figure 3.4.2.), the data further substantiate the question whether the IGF1R up regulation is part of a mechanism to protect PP from apoptosis.

3.5.2. Effect of growth factors on IGF1R expression in PP

The observed up regulation of the IGF1R also led to the question, whether it is part of a ligand induced regulatory mechanism. Thus, the effect of IGF1 and other growth factors on IGF1R expression in PP was examined. Figure 3.5.2. shows that high concentrations of IGF1 significantly reduced IGF1R expression in all PP examined. In contrast, exposure to EGF or bFGF did not significantly modulate IGF1R expression.

Figure 3.5.2.



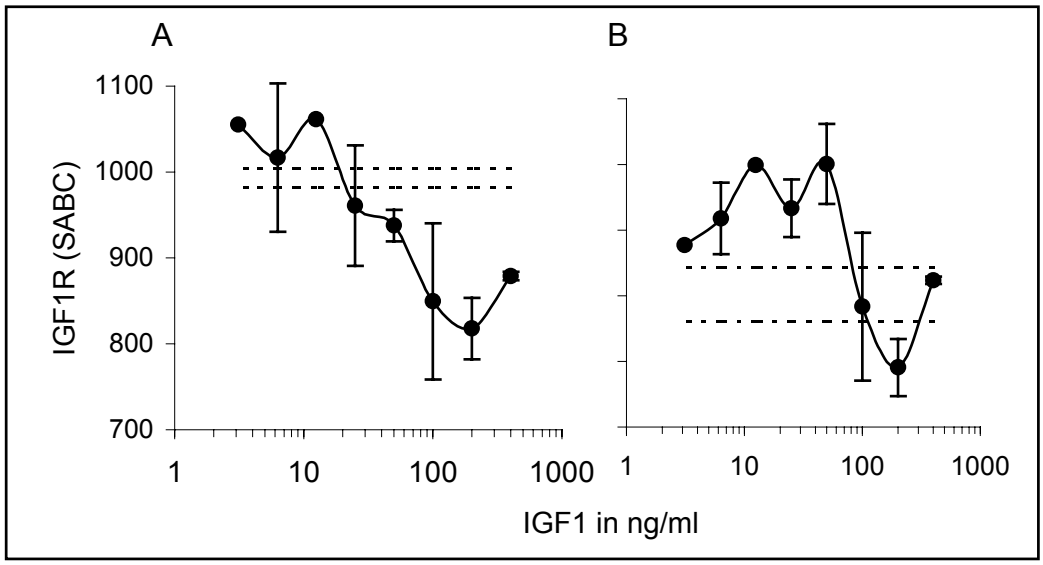
Effect of growth factors on IGF1R expression in PP. Cells were exposed to 500 ng/ml IGF1, 10 nM EGF or 10 ng/ml bFGF suspended in SF-PPM (+), or to SF-PPM alone (-). After 24 hrs they were stained with aIGF1R-DAMPE. IGF1R expression in SABC of multiple PP are expressed as mean ± SEM. * = $p<0.05$ (nonparametric Wilcoxon paired analysis, except EGF with $n<5$)

These results indicate that in PP the IGF1R can be down regulated by high concentrations of its own ligand, whereas other growth factors, such as bFGF and EGF do not appear to modulate IGF1R expression.

3.5.3. Concentration- and time dependent IGF1R modulation

The pronounced effect of 500 ng/ml IGF1 on its receptor also imposed the question whether the regulatory effect was concentration- and time dependent. PP were exposed to different concentrations of IGF1 suspended in SF-PPM or to SF-PPM alone. After 24hrs, cells were stained with aIGF1R-DAMPE and anti cytokeratin-FITC, in order to differentiate endothelial tumor cells from cells of non-endothelial lineage, such as fibroblasts in the primary cultures. Figure 3.5.3. shows data of one representative experiment after flow cytometric analysis. There appeared to be a

Figure 3.5.3.

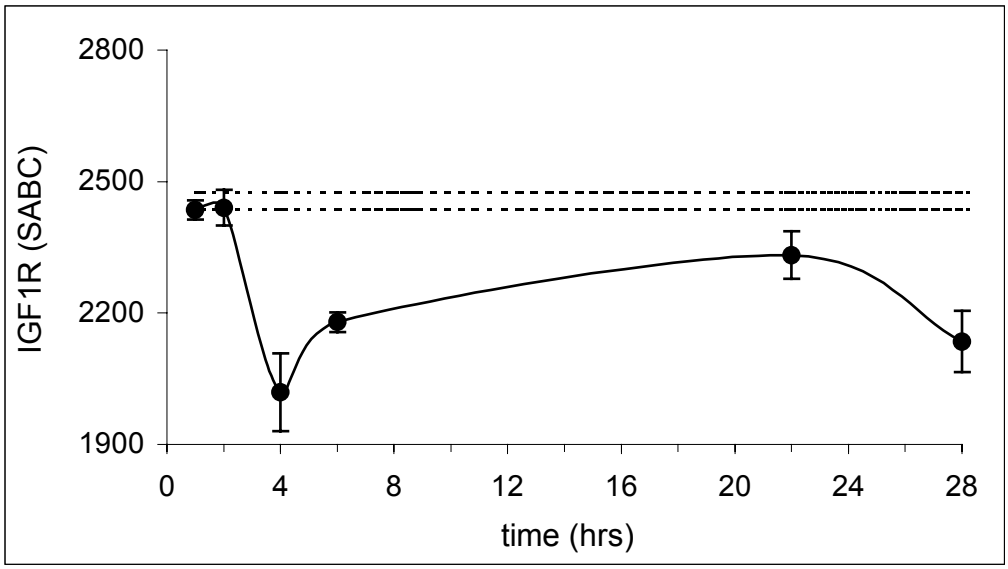


Dose-dependent ligand mediated IGF1R regulation in PP. Cells of one PP were incubated for 24 hrs with different concentrations IGF1 and stained with aIGF1R, as described. IGF1R expression (SABC) as compared to that in unstimulated cells (horizontal lines) in cytokeratin positive (A) and cytokeratin negative (B) cells are expressed as mean of duplicates ± SEM.

biphasic ligand effect with a decrease of IGF1R expression in cytokeratin positive (endothelial) PP at IGF1 concentrations between approximately 50 and 1000 ng/ml (A). In cytokeratin negative cells (B), the effect was shifted to the right with an increase of IGF1R expression at concentrations between 2 and 50 ng/ml and a decrease at higher concentrations.

Time dependent down-regulation of the IGF1R by its ligand is presented in Figure 3.5.4. In this representative PP receptor down regulation started between 2 and 4 hrs of treatment with high concentrations of IGF1 and lasted for at least 24 hrs.

Figure 3.5.4.



Time-dependent ligand mediated IGF1R regulation in PP. Cells of one PP were incubated for up to 28 hrs with 500ng/ml IGF1 and stained with aIGF1R-DAMPE. IGF1R expression (SABC) as compared to that in unstimulated cells (horizontal lines) are expressed as mean of triplicates ± SEM.

Taken together, the data suggest that in primary pituitary cells IGF1R expression can be regulated by its own ligand in a time- and dose-dependent manner.