4. Discussion

4.1. Major aims and results

The role of growth factors in pituitary tumor growth and secretory function was systematically studied. The data showed, that growth factors can modulate proliferation, apoptosis and hormone secretion in permanent rat pituitary tumor cells (GH_3). The presence and action of growth- and secretion-promoting substances in human pituitary adenomas was demonstrated, even though their identity remains unknown. Evidence for the potential pathophysiological relevance of growth factors in primary human pituitary cells was presented. They may function as paracrine/autocrine apoptosis modulators with protective or augmenting responses depending on the growth factor and the individual tumor. Finally, evidence was presented that some of these apoptosis modulating effects may be mediated through the IGF1R, which is known to substantially participate in the regulation of tumor growth, protection from apoptosis and transformation in other cell systems. Taken together, the study suggests, that growth factors may affect pituitary tumor growth by modulating its proliferation and apoptosis. The IGF1R may mediate some of the effects on apoptosis.

In the following sections all findings are discussed in detail. First, the results are discussed with regard to tumor growth, followed by hormone secretion. The findings are related to those obtained with pituitary tumor fractions. Bioassay limitations are pointed out. Finally, in a first attempt to find possible growth regulatory mechanisms, the data on IGF1R expression are discussed.

4.2. Discussion of effects of growth factors on <u>proliferation and apoptosis</u> in pituitary tumor cells

4.2.1. bFGF

The fact that bFGF inhibited the proliferation of GH_3 cells, as shown by reduced ^{3}H-thymidine uptake, confirms previous findings in GH_3 cells (44). The importance of this growth factor in pituitary tumor growth was supported by the ability of bFGF to protect GH_3 cells and, even more authentic, 8 of 18 primary cultures of human pituitary adenomas from apoptosis. The responsiveness to this growth factor did not correlate with histological diagnoses, patient sex or age. Taken together with the suggestion of

folliculostelate cells as the major source of bFGF in the pituitary gland (45) and the demonstration of elevated levels of immunoreactive bFGF in the plasma of patients with pituitary adenomas (46), a role for this growth factor in the regulation of apoptosis in pituitary adenomas is likely. This role for bFGF has also been indicated in related cell types, such as the pheochromocytoma cell line PC12 (47, 48) or in embryonic neurons (49).

4.2.2. EGF

Our data suggest that EGF may also be involved in the regulation of pituitary tumor growth. This growth factor augmented ^{3}H-thymidine incorporation of GH_3 cells and protected them from apoptosis. Interestingly, in some primary cultures of human nonfunctioning adenomas the peptide induced an increase in the rate of apoptosis. Previously, either stimulatory (4, 50) or inhibitory (51, 52, 53, 40) effects of EGF on proliferation have been shown. One report utilized a different subclone (GH_4C_1) of GH cells (53), another studied a mouse corticotropic pituitary cell line, or varying medium compositions were used. These methodological and species differences might explain opposing responses of EGF to proliferation.

The protective effect of EGF on apoptosis of GH_3 cells seen in the present study was also demonstrated by Yoshinaga (54, 55). The opposing effect seen in human primary cells may be related to species differences or to the difference in tumor subtypes, since GH_3 cells are derived from a PRL and GH secreting rat pituitary tumor, whereas the primary cells were derived from patients with clinically nonfunctioning adenomas. In support of a role for EGF in tumor growth regulation are findings in related cell types, such as primary rat prostatic epithelial cells, which respond to EGF with an increase in cell growth and a decrease in cell death (56). Similar effects of EGF have been suggested in polycystic ovary syndrome (57).

4.2.3. IGF1

A role in pituitary tumor growth regulation was also suggested for IGF1. This growth factor appeared to protect GH_3 cells from apoptosis. Furthermore, a protective effect was seen in 6 of 9 primary cultures of human nonfunctioning pituitary adenomas. The responsiveness to IGF1 did not correlate to hormone secretion (based on histological

findings), patient sex or age. Our findings are in accord with others, who showed a protective effect of IGF1 from apoptosis in tilapia somatothroph pituitary cell cultures (58) and in other cell types, such as neuronal cells (59) or smooth muscle cells (60). In fact, one of the main functions of IGF1 and its receptor is to protect cells from apoptotic death (24, 42).

Although in the present study IGF1 did not affect the proliferation of GH_3 cells, an increase in proliferation was seen by Newton et. al (16). In their study, GH_3 cells were preincubated for 48 hrs under serum free conditions, before IGF1 was added for 7 days. In addition to this much longer incubation time, the preincubation deprived the cells of nutrition, most likely resulting in higher sensitivity to exogenous stimuli. Mitogenic activity of IGF1 was also described in normal rat pituitary cells (4) and in mouse corticotropic tumor cells (51). Furthermore the viability of dispersed human pituitary gonadotropinomas was increased in response to IGF1 (61). Taken together, under certain conditions, IGF1 appears to have mitogenic activity in most pituitary cells.

4.2.4. IL2

Arzt et al. showed an IL2-induced increase of ^{3}H-thymidine incorporation in GH_3 cells (62), whereas in the present study this effect was seen only in the presence of pituitary specific molecules and hormones. A possible explanation may be the source of IL2; in the present study natural and recombinant human IL2 were used, whereas Arzt et al used purified rat IL2, which might have been less pure. Alternatively, human IL2used in our study might not be homologous enough to elicit an effect in rat GH_3 cells.

Our findings in GH_3 cells are in accord with the literature, which does not indicate an apoptosis modulating function for IL2 in the pituitary or in other related cell systems. Thus this effect was not further studied in primary pituitary adenoma cells. Taken together, the role of this cytokine in pituitary cell proliferation remains inconclusive and it does not appear to modulate apoptosis.

4.2.5. IL6

The IL6-induced augmentation of ^{3}H-thymidine incorporation in GH_3 cells seen in the present study confirms findings by Arzt, who showed in addition that the number of cells was increased (62). This proliferation inducing effect was also demonstrated in the hormone inactive rat pituitary tumor cell line MtT/E (63). In a folliculostellate like mouse cell line (TtT/GF) IL6 even functions as an autocrine growth factor (65). Our data showed a marginal protective effect of IL6 on apoptosis in GH_3 cells. In primary human pituitary adenoma cells this effect remains inconclusive. A protective effect of IL6 on apoptosis in other cell types, such as neurons of the rat hippocampus (65) or myeloma cells (66) has been seen, but it remains inconclusive whether in pituitary adenomas the cytokine has a similar effect. Taken together the data suggest that IL6 participates in pituitary growth regulation.

In conclusion, all of the examined growth factors and cytokines, except IL2, appear to participate in the regulation of pituitary tumor growth, although some of them seem to have more pivotal roles than others. bFGF seems to be an important regulatory growth factor, since it can shift the balance between cell proliferation and death in both directions; it was the most potent factor in protecting pituitary cells from apoptosis and it decreased proliferation, at least in GH_3 cells. The same complexity in action is true for IGF1. EGF appears to be a growth promoting factor in GH_3 cells, were it can increase proliferation and decrease the rate of apoptosis, whereas it is pro-apoptotic, at least for primary cultures of some human pituitary adenomas. The actions of IL6 are similar to those of EGF, even though the effect of this cytokine on apoptosis appears to be heterogeneous, since it prevents apoptosis in primary cells of one patient and induces apoptosis in another patient. These complex actions merit further investigations, before their significance in pituitary tumor growth will be fully understood.

4.3. Discussion of effects of growth factors on hormone secretion in pituitary tumor cells

4.3.1. bFGF

The finding that bFGF increased PRL secretion of GH_3 cells, is in agreement with previous studies, which have shown bFGF induced PRL modulation on protein-, mRNA- and DNA levels (51, 68, 44, 52, 68, 69). Together with studies suggesting folliculostellate cells as the major source of bFGF in the pituitary gland (45), this peptide appears to regulate PRL secretion in pituitary cells.

Taking into consideration that bFGF did not alter GH mRNA levels in GH_3 cells (44), the present finding of bFGF-augmented GH secretion suggests that bFGF may regulate GH secretion rather than its transcription. In agreement with this conclusion are findings by Mormede et. al (68), who reported that bFGF stimulation did not affect the potential of GH_3 cells to release GH. They measured GH in the supernatant of cells incubated for 4 hrs under serum free conditions, following pretreatment with bFGF for 24 hrs. Interestingly, in GH_4C_1 cells bFGF reduced GH secretion (52), possibly an effect particular to this subclone of GH cells, which is characterized by a much lower rate of GH synthesis than that seen in GH_3 cells (53). Taken together, bFGF may influence not only PRL but also GH secretion of pituitary tumor cells.

4.3.2. EGF

Our findings of EGF-stimulated PRL secretion of GH_3 cells are in agreement with previous reports (52, 69, 70). The effect of EGF on GH secretion in GH_3 cells appears to be heterogeneous, since the stimulatory effect seen in this study contradicts an inhibitory effect seen before (71), although the difference might be due to a subclone of GH_3 cells (GH_3/D6 cells) used in the reference study or to a different method used to detect newly synthesized GH (two-dimentional electrophoresis of ^{35}S-methionine-labeled hormone). Also in contrast are findings of an EGF-induced decrease of GH secretion in GH_4C_1 cells (52, 70), although those differences may again be related to the particular subclone of GH cells (53). The hypothesis of a variable response to EGF in different pituitary cell types is strengthened by findings in normal rat pituitary cells, in which no effect of EGF on GH secretion was seen (72).

4.3.3. IGF1

The unresponsiveness of IGF1 to PRL secretion in GH_3 cells seen after 24 hrs in the present study, are not necessarily opposing previous findings of an IGF1-induced decrease of PRL mRNA after 48 hrs (43), since the difference may be specific to the length of incubation. Findings in normal teleost pituitary cells, where IGF1 increased PRL synthesis (73), suggest either species heterogeneity or diverse regulation in normal and tumorous cells.

The present finding of increased GH secretion induced by IGF1 has previously been seen in GH_3 cells (74), even though it seems to contradict the classical negative feedback mechanism known to take place in normal and adenomatous somatotrophes (13, 14, 75-78). Although other investigators did see a dose-dependent IGF1-induced decrease of GH in GH_3 cells (72, 79), they used partially purified IGF1, which may have been contaminated with other substances. Nevertheless, a report of an IGF1-induced decrease in GH mRNA of GH_3 cells (43) is in apparent contrast to the findings of this study and cannot be further explained.

4.3.4. IL2

The present finding of IL2-augmented GH secretion of GH_3 cells is in agreement with data by Arzt et. al who showed that IL2 increased GH secretion in normal rat pituitary cells and in GH_3 cells (62). Their findings of no effect of IL2 on PRL secretion in normal and tumorous rat pituitary cells are also in agreement with the present study. Furthermore, they could demonstrate the presence of IL2 receptors on GH_3 cells. Thus, in the pituitary IL2 appears to modulate GH but not PRL secretion.

4.3.5. IL6

The present finding of an augmentation in GH secretion induced by IL6 confirm other data in GH_3 cells (62, 80), even though one of these previous studies also showed unresponsiveness in normal rat pituitary cells.

Both investigators also reported that IL6 induced an increase in PRL secretion, even though this effect could not be confirmed in the present study. The differences may result from different detection systems. A commercial ELISA was used in the present

study, which is less prone to false positives than the radioimmunoassay used in both other reports. Even though less likely, the absence of an IL6-induced response to PRL secretion seen in the present study may have resulted from the source of IL6 used, which was natural human IL6, as compared to recombinant human IL6 employed in the previous study. In both cases the amount of IL6 used was 100 U/ml.

In conclusion, all investigated growth factors can apparently participate in the modulation of hormone secretion in pituitary cells, although each growth factor appears to have an individual pattern. With regard to PRL secretion, bFGF and EGF seem to have stimulatory effects, whereas the effect of IGF1 seems to be variable dependent on species or transformational stage of the cell. IL2 does not appear to be involved in the regulation of this hormone and the effect of IL6 remains inconclusive. GH secretion can apparently be upregulated by bFGF, IL2 and IL6, whereas EGF may have variable effects. The classical suppressive effect of IGF1 in response to high GH levels possibly does not occur in GH_3 cells, although it can be observed in normal and tumorous somatothroph cells.

Taking into consideration the individual patterns of action with regard to proliferation and apoptosis, the complexity of growth factor-induced actions becomes apparent and is reminiscent of the complexity of their action seen in the immune response. Further investigations are necessary to decipher their role in pituitary tumorigenisis.

4.4. Discussion of the demonstration of growth factor-like activities in human pituitary tumors

A central objective of this study involved uncovering growth factor-like activities from human pituitary tumors in order to provide evidence for their relevance in tumorigenesis. Preliminary studies had shown that biologically active growth- or secretion-stimulatory activities could not be detected in unseparated tumor extracts (personal communication Dr. M. Lewis, Dr. U. Plöckinger). Stimulatory and inhibitory activities may have counteracted, thereby masking the effect on proliferation and hormone secretion of GH_3 cells. Thus, gel filtration chromatography was chosen as a tool to crudely separate such factors from tumor extracts. Unlike most other

separation techniques, gel filtration chromatography does not require denaturing solvents, enabling the study of biologically active molecules.

A limiting factor of this technique is the potential of similar size peptides to elute into the same fraction. Therefore, data analysis of stimulatory activities considered only their frequency within all examined tumors. Reliable analysis of the magnitude of stimulatory activities was unfeasible, because a distinction could not be made whether they resulted from a single substance, from the additive effect of several substances, or from the combined effect of stimulatory and inhibitory substances. In addition to such biological phenomena, some only marginally positive (i.e. Fig 3.3.4. PRL activities in fractions 23 and 24) activities, may have been false positives (statistical type I errors).

The most abundant proliferation inducing activity, which eluted into fraction 19, corresponded to a molecular weight of approximately 30 kD. Human IL6, which has a molecular weight of 26 – 29 kD, was a potential candidate. However, even though in initial experiments IL6 did increase ^{3}H-thymidine incorporation in GH_3 cells (Table 3.2.2.), these results could not be confirmed in preliminary neutralization experiments (Figure 3.3.7.). In further neutralization experiments (Figure 3.3.9.) did none of the previously twice tested and positive tumor fractions increase ^{3}H-thymidine incorporation anymore. Moreover, in the presence of neutralizing aIL6 tumor-fraction-induced ^{3}H-thymidine incorporation was not decreased as expected, but further increased. It is unlikely that GH_3 cells lost responsiveness to IL6 due to prolonged culture, since different freshly thawed batches of GH_3 cells were used for antibody titration- and neutralization experiments. It is more likely, that even though the recombinant cytokine and the lyophilized tumor fractions were stored in aliquots at –80°C, their activity was lost over time. Since preparation of fresh tumor fractions and their testing for stimulatory activities would have exceeded the time limit of this project, the experiments were not repeated. Taken together, the identity of the 30 kD proliferation inducing activity separated from human pituitary tumor extracts remains unknown.

The second most abundant proliferation inducing activity eluted into fraction 26, which corresponded to a molecular weight of approximately 6 kD. Growth factors of that molecular weight, which are found in the pituitary, are EGF with 6 kD and IGF1

with 7.6 kD. Both growth factors were plausible candidates for the proliferation inducing activity, since the proliferation augmenting effect of EGF on GH_3 cells could be demonstrated in the present study and Newton et. al have previously shown the mitogenic effect of IGF1 on GH_3 cells (16). Furthermore, preliminary neutralization experiments suggested that the mitogenic activity of EGF could be reduced in the presence of aEGF (Figure 3.3.7.). Nevertheless, the final neutralization experiments remained inconclusive, since the previously twice positive tumor fractions did not augment ^{3}H-thymidine incorporation of GH_3 cells anymore and none of the neutralizing antibodies had an effect. Again, the most plausible explanation for these experiments is the loss of activity due to prolonged storage of tumor fractions.

The same fraction did not always increase ^{3}H-thymidine incorporation in parallel with the number of cells (Figure 3.3.2.). The most likely reason for false positive cell counts is well-to-well variation of the seeding concentration. Since the Coulter Counter has a reproducibility of >99%, variations due to this counting technique are less likely.

The lack of parallel increases in cell counts and ^{3}H-thymidine incorporation may also be explained with the greater sensitivity of the ^{3}H-thymidine technique due to its incorporation into the DNA before cell replication is completed. In lieu of an average cell-doubling rate of approximately 30 hrs, exposure of tumor fractions for more than 24 hrs might have increased the likelihood of agreement between the two methods.

It is also conceivable that ^{3}H-thymidine was unspecifically incorporated into the DNA during its breakup, which occurs during apoptosis. And indeed, later experiments confirmed that 1 mM adenosine induced apoptosis, as measured with AnnF. Even though, the AnnF data were first significant after 48 hrs, it is likely that the detection of radioactive thymidine is more sensitive than that of fluorescent Annexin-V. In concurrence with this hypothesis is a report by Lewis et al., demonstrating that adenosine (0.1 mM) increased DNA laddering of GH_3 cells (40). In particular very large increases of ^{3}H-thymidine incorporation (see Figure 3.3.2.) may be linked to DNA laddering since it seems likely that ^{3}H-thymidine can unspecifically attach to DNA fragments.

Despite of these assay limitations and even though the identity of the proliferation-augmenting activities extracted from pituitary tumors remains unknown, the results

are in agreement with studies by Renner et. al (4), who demonstrated the presence of proliferation-inducing activities in supernatants of primary cultures of human pituitary adenomas. Furthermore, they were able to show that these activities could be reduced with antibodies specific against TGF-α, EGF, IGF1 and bFGF.

4.4.1. Discussion of findings on human pituitary extract fractions as related to known growth factors

In addition to proliferation-inducing activities some tumor fractions contained activities, which stimulated hormone secretion of GH_3 cells. Five of ten fractionated pituitary tumors contained GH-inducing activities with approximate molecular weights of 14, 4 and $\leq$ 2 kD, respectively. One may speculate on IL2 (15 - 17 kD), bFGF (4 kD) and possibly EGF (6 kD) as potential candidates, especially since these growth factors did augment GH secretion in GH_3 cells. The identity of these activities was not unveiled due to technical problems with the GH-RIAs (monkey GH antiserum could not be purchased any longer).

The most prominent PRL-inducing activities eluted into fractions, corresponding to a molecular weight of approximately 14 and $\leq$ 2 kD. None of the growth factors examined in this study qualify as potential candidates, since their molecular weights do not coincide with that of the detected activities.

GH and PRL secretion were not always induced by identical tumor fractions. This observation is in agreement with the results of growth factor-induced hormone secretion in GH_3 cells, since IGF1, IL2 and IL6 increased GH but not PRL secretion. Furthermore, it has recently been suggested that different pathways regulate PRL and GH secretion of pituitary cells (73).

Some tumor fractions augmented both, proliferation and hormone secretion of GH_3 cells. Whether this was caused by one single substance or multiple ones of similar molecular weight remains inconclusive, despite the fact that the stimulation data with known growth factors imply that one peptide may have multiple functions in pituitary growth regulation.

4.5. Discussion of findings on human pituitary tumor fractions as related to clinical and histological diagnoses

One study objective was to assess whether clinical or histological dissimilar pituitary tumors differ with regard to molecules regulating their growth and secretion. The grouping of bioassay data into hormone active and hormone inactive tumors, based on their immuno-histological staining pattern did not reveal different stimulatory patterns (Tables 3.3.2. and 3.3.3.). In both groups stimulatory activities were present in all molecular weight ranges, except in the 60 and 4 kD range of fractions of hormone inactive tumors, which induced PRL secretion. None of the hormone active tumors contained PRL secretion-inducing activities in the 60 and 4kD range. Taken together these data indicate that even though in vivo null cell adenomas do not secrete hormones, they contain substances, which are capable of promoting GH and PRL secretion of GH_3 cells. This might be related to differences in the responsiveness to such substances between human null cell adenoma cells and GH_3 cells.

Although it would have been very interesting to uncover potential differences between invasive and non-invasive pituitary tumors with regard to growth requirements and –regulation, such data analysis was not possible. The classification of invasive tumors is based on their infiltration into the dura and other surrounding tissues. This information becomes available mainly through neuro-surgical or -pathological observations, but was unfortunately not available to us in all cases.

4.6. Comparison of apoptosis induction in GH_3 cells and PP by serum deprivation

When evaluating the role of growth factors in apoptosis of pituitary tumor cells, an interesting observation was made. As expected, growth factor removal by serum deprivation induced apoptosis in GH_3 cells in a time- and dose dependent manner (Figures 3.1.5 and 3.1.6), but failed to induce apoptosis in PP (Figure 3.4.1). It is unlikely that serum deprivation is an unsuitable tool to induce apoptosis in primary cells, since it is a commonly used approach. Accordingly, it has been demonstrated in PC12 tumor cells (81), in melanoma cell lines (82), in a human neuroblastoma cell line (83), in human ovarian surface epithelium cells (84), in preadipocytes (85), and in

granulosa cells (86). It is unlikely that the lack of apoptosis induction in PP resulted from unresponsiveness of these cells to growth factor removal, since the addition of exogenous growth factors did modulate apoptosis, as demonstrated in Figure 3.4.2. On the contrary, it is conceivable that primary cells endogenously produced substances, such as growth factors, which protected them from apoptosis. And indeed, Renner et al. (4) have previously shown that primary human pituitary tumor cells do produce growth factors, such as TGF-α, EGF, IGF1 and bFGF in culture. In accordance, apoptosis was increased in the present study (Table 3.4.3.) in primary cells of some human pituitary tumors treated for 48 hrs with neutralizing antibodies specific against bFGF, EGF or IL6. Additional experiments with neutralizing anti-growth factor antibodies would be necessary to decipher the possible role of endogenously produced growth factors in apoptosis modulation of primary pituitary tumor cells. Alternatively, their role could be studied by sequestering such molecules with growth factor antagonists or binding proteins, or by preventing their production (i.e. in the presence of antisense-oligonucleotides).

4.7. Discussion of the new method to detect cell surface IGF1 receptors by flow cytometry

The new flow cytometric detection of cell surface IGF1R expression has several advantages over the standard detection method with radiolabeled ligand. Handling of radioactive material is unnecessary, it is less labor-intensive, requires shorter incubation times and is more suitable when working with small numbers of cells. Most importantly though only this technique allows to semi-quantitatively distinguish between small differences of receptor densities. This was shown with HepG2 cells treated under serum free conditions, were only the new (Figure 3.1.10) but not the standard method (Figure 3.1.12) induced a significant increase in IGF1R expression.

One limiting factor of the semi-quantitative detection of IGF1R expression is the sensitivity of the standard curve, which is determined by the number of antibodies coated on the surface of QIFIT-beads. Thus, the receptor expression on cells with a receptor density close to or lower than the least detectable of the standard curve cannot be determined reliably. This was the case for GH_3 cells (Figure 3.1.8.), which are known for their low number of cell surface IGF1R (76). Due to this phenomenon

the cell line was not suitable to study the in vitro regulation of IGF1R expression or its possible role in apoptosis. Thus, such experiments were carried out in primary human pituitary tumor cells from different patients.

4.8. Discussion of IGF1R regulation in PP

The first unexpected observation was the increase of IGF1R expression following serum deprivation of PP (Figure 3.5.1). This was previously seen only in other cell systems, such as in erythrocytes from patients with reduced serum IGF1 levels (87) and in rat epithelial cells after induction of diabetes (88). In these studies IGF1R gene expression (human erythrocytes) and mRNA levels (epithelial cells) rather than receptor cell surface expression were measured. In PP this finding is of particular interest considering that upon serum deprivation these cells did in addition not undergo apoptosis. Taking into account that in other cell systems one of the major functions of IGF1 and its receptor is to protect cells from apoptosis (89, 90, 91), our data provide evidence that this may also be the case in pituitary tumor cells. Further experiments are necessary to decipher such an apoptosis-protective function of IGF1R up regulation in PP. In such experiments neutralizing IGF1R antibodies might be employed to induce apoptosis following serum deprivation. Alternatively, IGF1R oligonucleotides could be used to prevent IGF1R up regulation thereby possibly inducing increased apoptosis.

We were further able to show that in PP the IGF1R can be down regulated by high ligand concentrations in a dose- and time dependent manner (Figures 3.5.3 and 3.5.4). Our data are in agreement with those in rat pituitary GC cells (41), were preincubation with IGF1 caused an approximately 50% reduction of the number of IGF1 cell surface receptors. We also showed evidence that this receptor regulation is ligand specific, since bFGF and EGF did not down regulate the IGF1R in several PP (Figure 3.5.2).