2. METHODS

2.1. Patient Information

Pituitary tumor tissue of 10 patients (T1-T10) was used in the first part of the study, examining its stimulatory activities in GH_3 cells (Results chapter 3.2.). The tumors were removed by transphenoidal resection in a University Hospital in Cardiff, England. They were 5 null cell adenomas, 3 gonadotropinomas, and 2 somatotropinomas.

Tumor tissue of 12 additional patients was included into the second and third part of the study: Identification of stimulatory activities with neutralizing antibodies (Results chapter 3.3.4.); study of their effect on apoptosis and IGF1R expression in pituitary cells (Results chapter 3.4. & 3.5.). These tumors were removed by transphenoidal resection in the University Hospital in Erlangen, at the Krankenhaus Neukölln or at the University Hospital Benjamin Franklin in Berlin. Patient demographics are summarized in Table 2.1.

Table 2.1.

Patient ID	**T03**	**T04**	**T06**	**T09**	**T18**	**T19**	**T21**	**T23**	**T24**	**T25**	**T26**	**T27**
Hist. Diagn.	Null	Null	Gon	Som	Gon	Null	Null	Null	Gon	Gon	Gon	Gon
Sex	F	m	f	m	m	m	?	m	m	m	m	f
Age	75	63	72	33	69	?	?	41	49	79	51	45

Demographics of patients, whose tumor tissue was extracted and separated by gel filtration chromatography. Histological diagnoses included null cell adenoma (Null), gonadotropinoma (Gon) and somatotropinoma (Som); (?) = unknown.

2.2. Tumor preparation

Tumors 1 through 10 were extracted and fractionated at the University hospital in Cardiff, England in the laboratory of Prof. Scanlon by Dr. Mark Lewis. Tumors 18 to 27 were prepared in our laboratory with a modified method of the one developed in Cardiff. In the following chapter both protocols will be described, but only the fractionation data of tumors prepared in our laboratory will be presented.

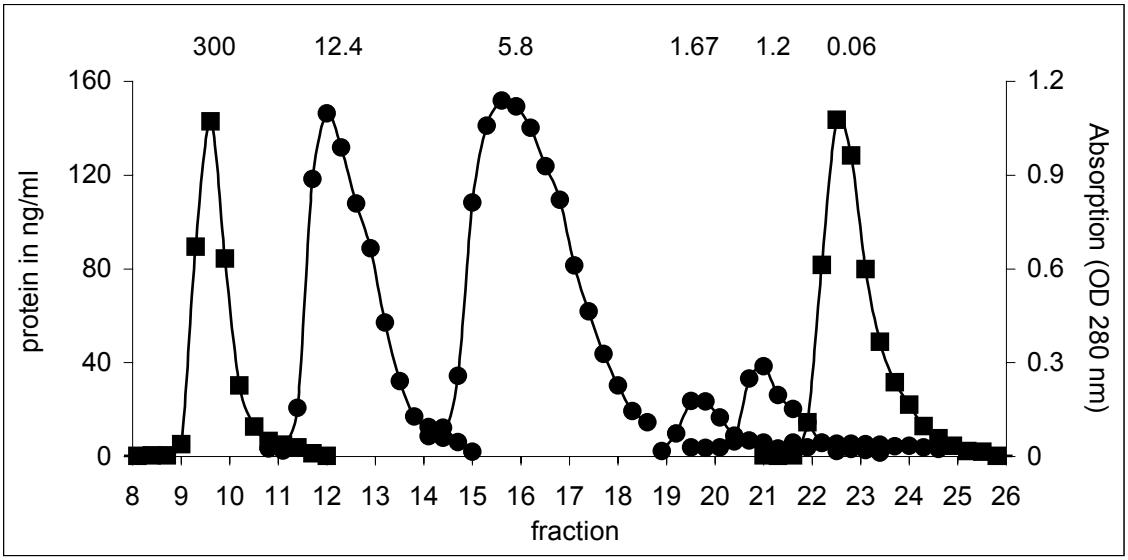
2.2.1. Tumor extraction

Immediately after surgery, tumor tissue was collected in sterile phosphate buffered saline (PBS), transported on ice to the laboratory and frozen in liquid nitrogen. Before extraction, tissue was thawed, weighed and dissected into small pieces. The tissue was homogenized in up to 3 ml cold 0.1 M hydrochloric acid containing 20 µg/ml pepstatin A. The homogenate was sonicated for 20 min and centrifuged at 10,000 rpm for 30 min at 4°C. The protein content of the supernatant was determined with coomassie blue (32) and immediately separated by gel filtration chromatography into molecules of different size.

2.2.2. Gel filtration chromatography

A 65 cm x 1,6 cm Superdex G30 column (Amersham-Pharmacia Biotech, Braunschweig, Germany) was calibrated with molecular weight standards. A representative calibration curve is shown on Figure 2.1. The fractionation range was approximately 15,000 kD to <1 kD with an elution volume of 113 ml. The flow rate was maintained at 0.6 ml/min with a Watson Marlow 503U peristaltic pump (Smith & Nephew Watson Marlow, Fallmouth, England). The eluent was sterile degassed

Figure 2.1.



Calibration curve of a Superdex 30 column. Blue Dextrane (300 kD), Cytochrome C (12.4 kD), insulin Lispro (5.8 kD), neurotensin (1.67 kD), angiotensin (1.2 kD) and aceton (0.058 kD) were eluted in 30 5 ml fractions. Absorbance at 280 nm (■) or protein concentration (●) was measured in each fraction.

PBS. Up to 3 ml of tumor extract were loaded onto the column and thirty 5 ml fractions were collected with a UKB Ultrorac 7000 Fraction Collector (UKB, Stockholm, Sweden). The optical density of each fraction was determined at 280 nm. In parallel, their protein content was determined with coomassie blue (32). Then fractions 9 to 24 were divided into 0.5 ml, 1 ml and 1.5 ml aliquots. Fractions 1-8, which served as negative controls in stimulatory assays, were pooled into 4 ml aliquots. All fractions were lyophilized in a Univapo 100 vacuum concentrator (UniEquip, Martinsreed, Germany) and stored at -20°C.

In Cardiff, tumor extracts T1 through T10 were fractionated on a 90 cm x 1.6 cm Sephadex 50 column. Its elution volume was approximately 180 ml, with a fractionation range of 30,000 kD to 2 kD. The flow rate was maintained at 1 ml/min. The eluent was sterile degassed 1 M acetic acid. The column was calibrated with different molecular weight markers. These included albumin (66 kD), ribonuclease (13.7 kD), insulin (5.8 kD), and somatostatin (1.75 kD). The peaks of these substances eluted into fractions 16, 22, 29, and 44 respectively. Up to 3 ml of tumor extract were loaded onto the column. Thirty-two 4 ml fractions were collected and their optical density was determined at 280 nm. They were divided into aliquots of 1 ml and 1.5 ml, lyophilized in a speed vacuum concentrator and stored at -20°C.

2.3. Cell culture

2.3.1. GH_3 and HepG2 cell lines

GH_3 cells were a kind gift of Dr. M. Lewis (Cardiff, GB). They were routinely grown in DMEM/F12 medium (Gibco, Germany) supplemented with 15 % horse serum, 2.5 % fetal calf serum (FCS, Biochrom, Berlin), 100 000 IE/L penicillin (Biochrom, Berlin), 100,000 g/L streptomycin (Biochrom, Berlin) and 0.044 mg/L Ca_2Cl (C-DM/F12). The medium was replaced every second or third day until approximately 70 % confluence was reached. At that time, the cells were subpassaged into a new culture vessel.

HepG2 cells, which were used to establish the flow cytometric method measuring cell surface IGF1R, were a kind gift of Dr. C. Grötzinger (Berlin, Germany). This human liver carcinoma cell line was routinely grown in Earle's MEM (Biochrom) supplemented with 10 % FCS, 10% sodium pyruvate (Biochrom, Berlin), 100,000 IE/L penicillin, and 100 000 g/L streptomycin. The medium was replaced every

second or third day until cell confluence was reached. At that time, cells were subpassaged into a new culture vessel.

Spontaneous mutations, which may lead to morphological or physiological alterations in the cell population, potentially occur with every cell division. In order to limit such cellular alterations, a new batch of cells was thawed every 3 months. The fresh cells belonged to a pool of frozen cells of the same passage number, which were frozen in aliquots at the beginning of the project.

2.3.2. Primary pituitary tumor cells (PP)

Human pituitary tumors were removed by transphenoidal resection from 27 patients. Patient demographics are summarized in table 2.2. Fourteen patients were

Table 2.2.

Patient ID	**PP31**	**PP33**	**PP34**	**PP35**	**PP36**	**PP42**	**PP43**	**PP47**	**PP50**	**PP51**
Clin. Diagn.	Acro	NFA	NFA	Prol	Acro	NFA	NFA	Acro	NFA	Cush
Hist. Diagn.	Som	Gon	Gon	Prol	Som	Pluri	Null	Som	Gon	Cor
Sex	m	f	f	m	m	m	f	m	m	f
Age	29	59	54	63	37	67	77	31	55	59

Patient ID	**PP57**	**PP58**	**PP59**	**PP65**	**PP66**	**PP68**	**PP69**	**PP70**	**PP71**	**PP72**
Clin. Diagn.	NFA	NFA	NFA	NFA	NFA	NFA	Acro	Acro	Acro	NFA
Hist. Diagn.	Gon	Gon	Gon	Pluri	Pluri	Gon	Som	Som	Som	Gon
Sex	f	m	f	f	m	m	f	f	f	m
Age	78	54	57	67	61	43	24	61	39	43

Patient ID	**PP73**	**PP75**	**PP76**	**PP77**	**PP78**	**PP80**	**PP81**			
Clin. Diagn.	NFA	NFA	NFA	NFA	NFA	NFA	NFA			
Hist. Diagn.	Gon	Null	Gon	Null	Gon	Gon	Null			
Sex	m	f	f	f	f	m	m			
Age	59	72	63	56	62	67	61			

Demographics of patients, whose tumor tissue was prepared into primary culture. Clinical diagnoses included acromegaly (Acro), nonfunctioning adenoma (NFA), prolactinoma (Prol) and cushing syndrome (Cush). Histological diagnoses included

somatotropinoma (Som), gonadotropinoma (Gon), plurihormonal (Pluri), null cell adenoma (N) and corticotropinoma (Cor).

women (mean age 59.1) and 13 patients were men (mean age 51.5). Tumor subtypes included 6 somatotropinomas, 1 prolactinoma, 1 corticotropinoma, 3 plurihormonal adenomas, 12 gonadotropinomas and 3 null cell adenomas.
Tumor tissue was suspended in C-DM/F12 and transported to the laboratory. Tumors were obtained at two different hospitals. The time between surgery and culture of primary tumor cells varied from 30 min to 2 days dependent on the location of the hospital. Cell viability was always tested and above 90 %. Primary cultures were prepared according to a modified procedure by Auernhammer et al. (33). Tumor tissue was weighed and dispersed in digestion buffer, containing 0.01 mg/ml Collagenase Type I (CellSystems Biotechnologie, St. Katharinen, Germany), 0.1 mg/ml Soybean Trypsin Inhibitor (Sigma, Deisenhofen, Germany), 1 mg/ml Hyaluronidase (Sigma), 4 mg/ml BSA (Sigma) and 0.01 mg/ml DNAse II (Sigma). The tissue was minced with forceps and a scalpel and enzymatically digested as needed for up to 75 min at 37°C. During digestion, cell aggregates were periodically triturated. Thereafter, the debris was allowed to settle on the bottom of 15 ml tubes and the cell suspension was washed twice in C-DM/F12. If necessary, erythrocytes were lysed with NH_4Cl-Tris buffer and washed in SF-DM/F12. The cells were resuspended in culture medium (C-PPM, pH=7.4), containing Dulbecco's MEM (Gibco, Eggenstein, Germany), 10 % FCS, 100,000 IE/L penicillin, 100,000 g/L streptomycin, 0.1 mM glutamine (Biochrom), 1 % nonessential amino acids (Biochrom), 10 mM HEPES (Sigma), 30 pM triiodothyrionine (Sigma), 10 ng/L Insulin (Sigma), 20 µg/L Selen (Sigma), 5 mg/L Transferrin (Sigma), 2.2 g/L $NaHCO_3$ (Sigma), and 98 mg/L lyophilized MEM (Biochrom) and plated for bioassay.

For subcultivation or bioassays, GH_3 cells were brought into suspension by incubation with 0.1 % EDTA solution for 5 min at 37°C. HepG2 and primary pituitary tumor cells were incubated with 0.5 g/L Trypsin and 0.2 g/L EDTA for 6 min at 37°C. EDTA or Trypsin/EDTA were removed by centrifugation at 300 x g for 5 min at RT, and the cells were resuspended in fresh culture medium.

2.4. Bioassays

2.4.1. Preparation of tumor fractions, peptides and antibodies

Lyophilized pituitary tumor fractions were thawed and resuspended in 2 ml SF-DM/F12. The pH was adjusted to 7.4 with 1 N NaOH or 1 N HCl and occasionally suspensions were sterilized through a 0.22 μM filter, before incubation with GH_3 for bioassay.

Peptides tested in GH_3 cells included hnIL6 (Sigma), hrIL2 (Sigma), hrEGF (R&D Systems, Wiesbaden, Germany), hrIGF1 (Biomol, Hamburg, Germany), and hrbFGF (Sigma). They were stored in aliquots at –20°C or –80°C. For bioassay, they were diluted in SF-DM/F12, adjusted to a pH of 7.4 and sterilized through a 0.2 μM filter.

Neutralizing antibodies were preincubated for 2hrs at RT with peptides (hrbFGF, hrEGF, hrIGF1, hrIL2, and hnIL6) at their optimal stimulatory concentration. The antibodies included mouse monoclonal anti human bFGF (20 μg/ml - 1.25 μg/ml, Biomol), rabbit monoclonal anti mouse EGF (20 μg/ml - 1.25 μg/ml, Biomol), goat monoclonal anti human IGF1 (25 μg/ml – 1.5 μg/ml, Sigma Aldrich) goat monoclonal anti human IL2 (8 μg/ml – 0.5 μg/ml, R&D Systems), and rabbit monoclonal anti human IL6 (20 μg/ml – 1.26 μg/ml, R&D Systems). An isotypic control at a concentration comparable to the highest concentration of the specific antibody was included in every experiment (rabbit IgG, Dako, Hamburg, Germany; mouse IgG, Dako; goat IgG, DPC Biermann, Bad Nauheim, Germany).

2.4.2. ^{3}H-thymidine incorporation, cell numbers and hormone secretion in GH_3 cells stimulated with growth factors, their neutralizing antibodies or tumor fractions

^{3}H-thymidine incorporation, cell counts and hormone secretion of GH_3 cells were measured upon stimulation with human pituitary tumor fractions, known growth factors, or growth factors in the presence and absence of neutralizing antibodies. All experiments were performed under the same conditions, unless described differently. In the one experiment ^{3}H-thymidine incorporation was assessed in one 96 well plate, whereas cell counts and hormone secretion (GH, PRL) were measured in a parallel plate.

GH_3 cells were seeded in C-DM/F12 at 3 or 6 x 10^4 cells/well in 96 well plates. After 24 hrs, supernatants were removed and adherent cells were rinsed with 200 µl serum free DMEM/F12 (SF-DM/F12) to remove remaining serum and endogenously produced growth factors. Peptides or tumor fractions suspended in SF-DM/F12 were added to the cells in replicates of at least 4. Eight negative (SF-DM/F12 alone) and positive controls (1 mM adenosine or 0.1 M thyrotropin releasing hormone (TRH)) were included in appropriate plates. ^{3}H-thymidine (0.5 µCi/well) was added to those plates assigned for proliferation. After 24 hrs all cells and supernatants were harvested.

For hormone measurements, the 96-well plates were centrifuged at 300 x g for 5 min. Supernatants were transferred in aliquots of 70-80 µl to new 96-well plates and stored at –20°C until hormone measurement.

Cells of the same plate were brought into suspension with 0.1 % EDTA (as described above) and counted in duplicate on a Z1 coulter counter (Coulter Electronics, Germany).

To assess the amount of ^{3}H-thymidine incorporation, cells were harvested on a semiautomatic harvester (Wallac, Freiburg, Germany), lysed with dH_2O and the DNA transferred onto cellulose, which was dried, sealed in a plastic envelope and dispersed in dry scintillator. ^{3}H-thymidine was counted on a Wallac ß-counter (Wallac, Freiburg, Germany).

2.4.3. Stimulation of PP with growth factors

PP were seeded at a density of 1-4 x 10^5 cells/ml on Poly-D-Lysine (Sigma) coated 12- or 24-well plates. After cell adherence, C-PPM was routinely changed every second or third day until bioassay. Assays were performed within the first 10 days of culture. Cell supernatants were removed and PP were stimulated under various conditions for 24 or 48 hrs. Dependent on the amount of available cells, single, duplicate or triplicate wells were prepared. At the end of the incubation time, cells were trypsinized and stained to assess apoptosis or IGF1R expression by flow cytometry.

2.5. Hormone measurements

2.5.1. Rat GH radioimmunoassay (rGH RIA)

rGH released into the culture medium was measured in a specific radioimmunoassay using materials kindly provided by the National Pituitary Agency, NIDDK (Torrance, CA, USA). The tracer was labeled with a modification of the chloramine T method (34). Unlabeled GH and free ^{125}Iodide were separated from ^{125}I-GH (tracer) on a G50 Sephadex column saturated with bovine serum albumin. rGH standards, controls or samples were incubated with tracer and monkey anti GH antiserum for 24 hrs at room temperature. Donkey anti monkey secondary antibody (Antibodies Inc., Davis, CA, USA) was added for the last 4 hrs. The antigen-primary-antibody-secondary-antibody complex was precipitated by centrifugation at 1500 x g for 30 min at 4°C. Unbound tracer was decanted with the supernatant. The amount of radioactivity inversely corresponding to the amount of rGH present in the samples was detected on a 1470 Wizard automated gamma-counter (Wallac Distribution GmbH, Freiburg, Germany).

2.5.2. Rat Prolactin (rPRL) ELISA

rPRL in culture supernatants was measured with a commercially available ELISA according to manufacturer's instructions (DPC Biermann, Bad Nauheim, Germany). Assay standards, controls and culture supernatants at previously determined dilutions were measured in parallel in 96 well plates. When assay reactions were completed, the absorption at 450 nm was measured in a Dynex MRX plate reader (kindly provided by DPC Biermann). Data was analyzed with reader specific software (Revelation from Dynex, Stuttgart, Germany)

2.6. Flow cytometry

All staining procedures were performed at 4°C in the dark. The average cell concentration was 1-5 x 10^5 cells per vial. Cells were always stained with 50 µl antibody solution, then resuspended in 500 µl buffer and washed once (centrifugation at 300 x g for 5 min, decantation of supernatant, suspension of cells in buffer).

2.6.1. Detection of apoptosis with Annexin V-FITC/Propidium iodide (AnnF/PI)

Cells were stained with a commercially available Annexin V-FITC kit (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) according to modified manufacturer's instructions. Briefly, suspended cells were washed once in binding buffer (BB, 10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM Ca_2Cl) and for 15 min incubated with 50 µl Annexin V-FITC, diluted 1:10. Some cells were stained with Annexin-FITC suspended in Ca^{2+} free BB as a control for unspecific binding. All cells were washed in BB and incubated for 1 min with 50 µl of a 1 µg/ml propidium iodide solution (PI), before they were suspended in 250-500 µl BB and analyzed by flow cytometry. Early alive apoptotic (AnnF positive) and dead apoptotic (AnnF/PI positive) cells were analyzed.

2.6.2. Detection of cell surface IGF1R receptors by flow cytometry

2.6.2.1. IGF1R labeling

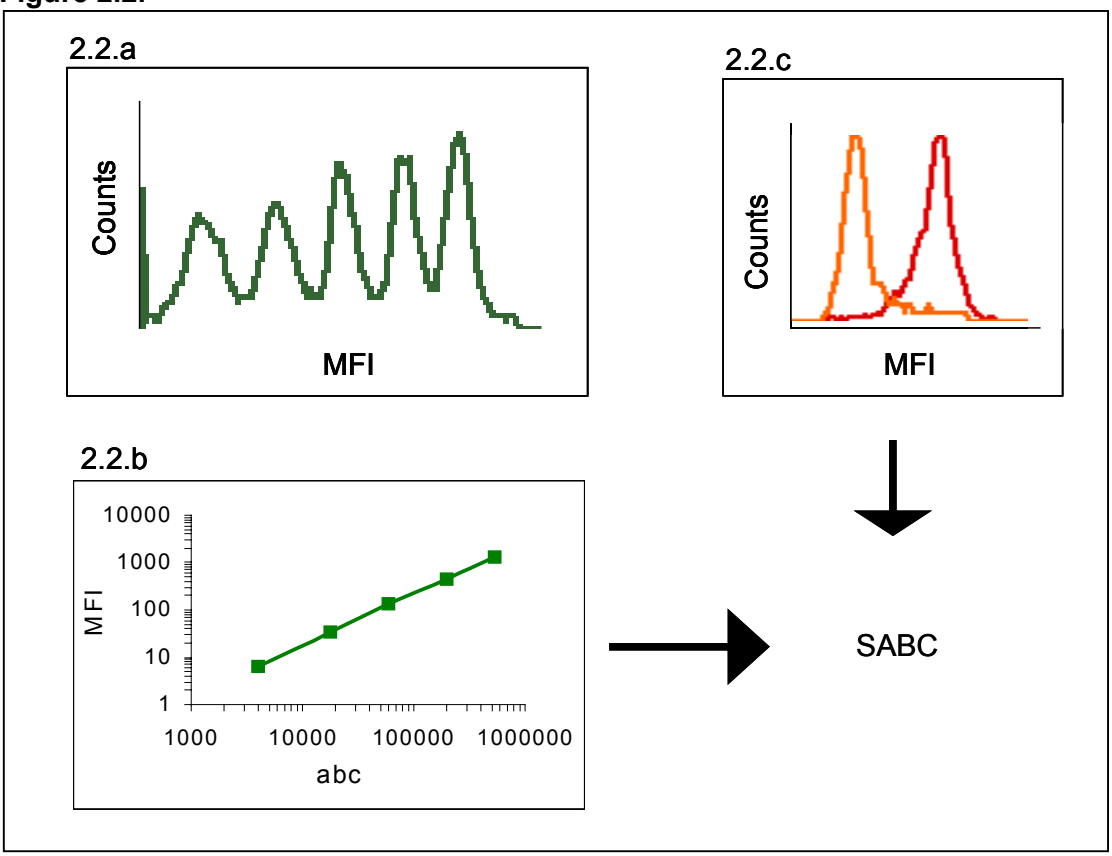
Suspended cells were washed once in FACS buffer (PBS, 0.87 % sodium chloride, 0.5 % bovine serum albumin, 0.1 % EDTA, and 0.02 % sodium azide) and incubated for 30 min with 5 µg/ml monoclonal mouse anti human IGF1R antibody (aIGF1R, R&D Systems) or 5 µg/ml monoclonal mouse negative control antibody (DAKO). After another wash, 50 µg/ml secondary donkey anti mouse phycoerythrin conjugated F(ab')2 fragment (DAM-PE, Acris, Herford, Germany) was added to the cells or to 50 µl QIFIT beads for 15 min. Unbound antibody was washed off once and the cells and beads were resuspended in 250-500 µl FACS buffer for flow cytometric analysis.

2.6.2.2. Quantification of aIGF1R

In order to study IGF1R regulation with this method, quantification of receptor expression was necessary. Direct comparison of fluorescence intensities (MFI) is insufficient, because of day-to-day differences in staining and instrument settings and unknown stoichiometry of receptor–antibody interactions. Quantification of cell surface IGF1Rs was achieved by including so called QIFIT beads. They consist of a series of synthetic beads, 10µm in diameter and coated with different, but well-

defined quantities of mouse monoclonal antibody molecules (anti-human CD5). The beads mimic cells with different antigen densities, which have been labeled with a primary mouse monoclonal antibody, isotype IgG. Beads and cell specimens, which have been stained with aIGF1R, were labeled in parallel, with secondary DAM-PE. If the primary aIGF1R was used at saturating concentrations, the number of bound primary antibody molecules corresponded to the number of antigenic sites present on the cell surface. The secondary antibody was also used at saturating concentrations. Consequently, the fluorescence intensity correlated with the number of bound primary antibody molecules on the cells and on the beads. After analysis on the flow cytometer (Figure 2.2.a), the bead-data were used for the construction of a

Figure 2.2.



Quantification of anti IGF1R antibody binding. 2.2.a represents a histogram depicting the mean fluorescence intensity (MFI) of 5 QIFIT bead populations. In 2.2.b the resulting QIFIT calibration curve with antigen binding capacity (abc) versus MFI is shown. In 2.2.c the MFI of cells stained with aIGF1R-DAMPE (right peak) and that of cells stained with an isotypic control antibody (left peak) are shown.

calibration curve (Figure 2.2.b), expressing MFI of the different bead populations against their antigen binding capacity (ABC, corresponds to the number of primary

antibody bound to the beads). The cell specimen were also analyzed on the flow cytometer (Figure 2.2.c) and ABC (antigen density) of cells labeled with specific aIGF1R and of those labeled with isotype control antibody were calculated. The specific ABC (SABC = ABC – ABC of isotype control) was deduced by interpolation on the calibration curve.

2.6.2.3. Two-color IGF1R and cytokeratin labeling

Cytokeratins are intracellular filament proteins, which are only expressed in cells of endothelial origin, thus distinguishing specific pituitary tumor cells from fibroblasts. To separate IGF1R expression on fibroblasts from that on endothelial tumor cells, cells were first stained with aIGF1R and DAM-PE, as described above. The cells were fixed for 10 min with 4% paraformaldehyd / 50 mM PBS (w/v) at pH 7.3 and washed once in FACS buffer. After two more washes in 1 ml permeabilisation buffer (PB, 50mM PBS (pH7.3), 1% FCS, 0.1% NaN_3, 0.1% Saponin), the cells were incubated for 30 min with 10 µg/ml monoclonal FITC conjugated anti human pan-cytokeratin antibody (aCyt-FITC, DAKO) or with 10 µg/ml monoclonal FITC conjugated negative control antibody (DAKO). Unbound antibody was washed off once and the cells were resuspended in 250-500 µl FACS buffer for flow cytometric analysis.

2.6.2.4. Three-color AnnF/PI and IGF1R labeling

For simultaneous detection of apoptosis and IGF1R, the cells were first stained with aIGF1R. Then DAM-PE and Annexin V-FITC were added in parallel to the cells and the above-described staining protocol was followed.

Flow cytometric analysis of all of the above procedures was performed on a FACS Calibur (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser. Appropriate instrument settings and compensation, if necessary, were determined with unstained cells and cells stained with a single fluorescence. Data were analyzed with Cell Quest software (Becton Dickinson).

2.7. ^{125}I-IGF1 competitive binding assay

Suspended cells (0.3-1 x 10^6 per vial, but constant for all vials in one experiment) were incubated at least in duplicate with 50,000 cpm ^{125}I-IGF1(Amersham Pharmacia, Freiburg, 2000 Ci/mmol) and 250 ng/ml of unlabeled IGF1 for 2 hrs at 20°C in a final volume of 100 µl 50 mM Hepes buffer (pH 8.0) supplemented with 1 % BSA, 120 mM NaCl, and 1.2 mM $MgSO_4$. The cells were washed once in 500 µl Hepes buffer to remove unbound ^{125}I-IGF1. Preliminary experiments had shown that repeated washing reduced specific ^{125}I-IGF1 binding and that the maximum ^{125}I-IGF1 binding occurred within the first two hours of incubation and did not increase significantly thereafter. Cell associated radioactivity was determined on a gamma-counter (Wallac, Freiburg). Data was expressed as specific ^{125}I-IGF1 binding by subtracting non-specific binding (cells incubated with unlabeled IGF1 and ^{125}I-IGF1) from total binding of ^{125}I-IGF1(cells incubated in the absence of unlabeled IGF1).