Overexpression of the insulin-like growth factor I receptor in human pheochromocytomas

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Abstract

In order to determine the role of the IGF-I receptor (IGF-IR) in human pheochromocytomas we have compared the expression of the IGF-IR in normal tissues and in pheochromocytomas with regard to the IGF-IR mRNA levels and ligand binding. By semiquantitative reverse transcription polymerase chain reaction (RT-PCR), the mRNA of the IGF-IR could be detected in all samples of normal adrenomedullary cells (n=13) and pheochromocytomas (n=16). However, pheochromocytomas exhibited 2·8-fold higher mean IGF-IR mRNA levels than normal adrenomedullary cells (2·8±0·5×10^5 molecules/µg RNA vs 7·8±1·2×10^5 molecules/µg RNA; $P<0·001$). This overexpression of the IGF-IR in pheochromocytomas could be confirmed at the protein level by binding studies. Radioligand assays and Scatchard analysis revealed a single class of high affinity IGF-IR binding sites with a similar dissociation constant (K_d: 0·32±0·1 nmol/l vs 0·22±0·08 nmol/l) for both normal adrenomedullary cells and pheochromocytomas. However, specific ^{125}I-labeled IGF-I binding and the calculated receptor concentration were significantly elevated in pheochromocytomas as compared with normal adrenomedullary cells (58·3±5 vs 24·3±12 nmol/kg protein; $P<0·05$). In summary, our results demonstrate significant overexpression of the IGF-IR in human pheochromocytomas. This suggests a possible role of the IGF system in the pathogenesis of adrenal neoplasia and thus IGF-IR may be a target for future therapeutic approaches.

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Introduction

Previous studies by our own group and others have shown a critical role for the insulin-like growth factor (IGF) system in either normal adrenocortical cells or adrenocortical tumors (Weber *et al.* 1997, Boulle *et al.* 1998, Fottner *et al.* 1998, 2001). In malignant adrenocortical carcinomas, overexpression of IGF peptides (mainly IGF-II), receptors (IGF-IR) and binding proteins (IGFBP-2) has been observed. In contrast, expression of IGF-I peptides and receptors appears to be unaltered in adrenocortical hyperplasia and adenomas.

The IGF system has also been shown to play a potent regulatory role in cell proliferation and maintenance of sympathetic ganglia and adrenal medulla. In adult adrenal medulla and sympathetic ganglia, gene expression of IGF-I and IGF-II and their receptors was proven at the mRNA level (Haselbacher *et al.* 1987, Gelato & Vassalotti 1990, Ilvesmäki *et al.* 1993). IGFs have been shown to promote chromaffin cell survival and proliferation *in vivo* (Frödin & Gammeltoft 1994); moreover IGF-I enhances catecholamine synthesis (Hwang & Choi 1996) and secretion (Dahmer *et al.* 1990) in these cells.

Interestingly, alterations in the IGF system also seem to play an important role in tumors originating from the adrenal medulla or sympathetic ganglia, like pheochromocytomas or neuroblastomas. Overexpresion of the IGF-I receptor in neuroblastoma cells results in resistance to apoptosis leading to unregulated growth (Singleton *et al.* 1996). IGF-II (Sullivan *et al.* 1995) and IGFBP-2 (Menouny *et al.* 1997) are widely expressed in human neuroblastomas possibly enhancing and/or modulating IGF-I receptor activation. Overall, alterations in the IGF system have been shown to modulate neuroblastoma growth *in vitro*, thus underlining its importance in the growth-maintenance of these highly malignant tumors (reviewed in Zumkeller & Schwab 1999).

Overexpression of IGF-II mRNA and peptide has been described in human pheochromocytomas (Haselbacher *et al.* 1987, Gelato & Vassalotti 1990). However, no information about the abundance of the IGF-I receptor in human pheochromocytomas compared with normal adrenomedullary tissue is available to date. In order to evaluate whether the IGF-IR is overexpressed in human pheochromocytomas, we therefore investigated the expression of the IGF-IR in normal adult adrenomedullary tissue and in pheochro-

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mocytomas with regard to mRNA levels and ligand binding *in vivo*.

Materials and methods

Materials and sample collection

Recombinant human IGF-I was purchased from Boehringer (Mannheim, Germany), (3-[^{125}I]iodotyrosyl) IGF-I (human recombinant, specific activity 2000 Ci/mmol) was purchased from Amersham Buchler GmbH & CoKG (Braunschweig, Germany). Molecular biology reagents for reverse transcription (RT)-PCR were obtained from Promega (San Diego, CA, USA) and Gibco BRL (Eggenheim, Germany).

According to the guidelines of the ethics committee of the Ludwig-Maximilians-University of Munich, normal adrenal tissue was obtained from patients undergoing surgical treatment for renal neoplasia with concomitant ipsilateral adrenalectomy. Immediately after surgical removal, the tissue was dissected by the pathologist and a sample of fresh, non-necrotic adrenal tissue was provided. All adrenal glands were found to be normal after morphological and histopathological examination. Due to the large amount of protein necessary for the binding studies, tissue from only five adrenal glands provided enough material for membrane preparation and consecutive binding assays. If only small parts of the intact adrenal gland were available after pathological analysis, the tissue was used for mRNA isolation. Pheochromocytomas were obtained from patients undergoing surgical treatment of adrenal neoplasia. All patients gave written informed consent. Immediately after surgical removal, samples of normal adrenal tissue and of pheochromocytomas were dissected by the pathologist and a sample of fresh non-necrotic representative adrenal tissue was provided. All adrenal glands were found to be normal after morphological and histopathological examination. For the tumor samples, necrotic and ulcerative portions were removed when necessary and the presence of at least 90% tumor cells was verified histologically. For the preparation of adrenomedullary tissue samples, adrenal glands were freed from the periadrenal fat tissue, incised longitudinally and the adrenal medulla was separated from the cortex under a dissecting microscope. For RNA extraction and membrane preparation, tissue samples were snap frozen in liquid nitrogen and stored at -70 °C until further analysis.

The mean age of the pheochromocytoma patients at the time of surgery was 43·5 years and the female/male ratio was 1·2:1. One of the pheochromocytomas was classified as malignant due to the presence of distant metastases (lung and local intestinal lymph-node metastases). The mean tumor size was $3{\cdot}4 \pm 1{\cdot}2$ cm. Two of the 17 histologically verified pheochromocytomas occurred as part of multiple endocrine neoplasia type 2 (MEN 2) and three occurred as part of von Hippel-Lindau disease. The mean age of the patients undergoing adrenalectomy due to renal neoplasia was 64·3 years and the female/male ratio was 1:1·4.

RT-PCR

For measurement of IGF-IR levels in 13 normal adrenal glands and 16 pheochromocytomas, a commercially available competitive quantitative RT-PCR method (Clontech Laboratories, Palo Alto, CA, USA) was used, as previously described in detail (Weber *et al.* 2002). The MIMIC PCR technique utilizes an exogenous internal standard (MIMIC), which competes for the same primers as the target IGF-I receptor DNA. With knowledge of the amount of MIMIC DNA added in serial dilutions to the amplification reactions, the amount of the target template could be determined, and thus the amount of initial IGF-IR. The competitive internal standard, which contains the identical primer binding sites used to amplify the IGF-IR DNA was generated by amplifying a BamH1/EcoR1 fragment of v-erB with two composite primers. In these composite primers (40-mer), the first 20 nucleotides are complementary to IGF-II or the IGF-IR and the following 20 nucleotides are complementary to v-erB. The internal standard was synthesized, purified and quantified by spectophotometry as described by the manufacturer (Clontech Laboratories). Amplification of the competitive internal standard generated a 288 bp fragment. The primers used to amplify the human IGF-IR and the internal standard respectively were: sense 5′ACAGAG AACCCCAAGACTGAGG3′, antisense: 5′TGATGTT GTAGGTGTCTGCGGC3′, corresponding to nucleotides 2095–2116 (exon 10) and 2341–2320 (exon 11) of the human IGF-IR cDNA sequence (Ullrich *et al.* 1986). Amplification of the target DNA with these intron-overlapping primers yielded one specific 247 bp fragment of the IGF-IR, thereby excluding amplification of contaminating DNA. The PCR products obtained were confirmed by sequencing. In pilot experiments, the exponential phase of the amplification was determined for the target DNA and the internal standard. Subsequently, a cycle number that was in the middle of the linear amplification range (21–27 cycles) was chosen. In the system used, the efficiencies of amplification of target cDNA and competitive internal standard DNA were equal (Gilliland *et al.* 1990, Alms *et al.* 1996, Becker-Andre *et al.* 1989, Kutoh *et al.* 1998, Zhang *et al.* 1998).

For RNA extraction, 50 mg tissue specimens were incubated with 1 ml cell lysis buffer (Trizol, Gibco, Grand Island, NY, USA) for 5 min, then total cellular RNA was isolated using the acid-guanidinium isothiocyanate phenol-chloroform extraction method as

described by Chomczynski & Sacchi (1987). The assessment of RNA integrity was evaluated by inspection of the 28S and 18S ribosomal RNA bands using gel electrophoresis, and the concentration and purity of the RNA were further determined by ultraviolet spectrophotometry; RNA was stored at −70 °C until analyzed. For reverse transcription of extracted RNA to cDNA, 1·0 μg total RNA template was incubated for 60 min at 37 °C in 20 μl reaction volume containing 1 × 1st strand buffer (50 mM Tris/HCl, 75 mM KCl, 3 mM $MgCl_2$), 0·5 mM of each deoxynucleotide, 1·8 μg random primer, 10 mM dithiothreitol (DTT), 20 U ribonuclease inhibitor RNasin (1·0 U/μl), and 240 U Moloney murine leukemia virus reverse transcriptase (12 U/μl). The reaction was stopped by incubating at 95 °C for 5 min, and samples were placed on ice or stored at −20 °C for further analysis. Subsequently, PCRs were performed in a thermal cycler (Gene Amp PCR System 2400, Perkin-Elmer, Weiterstadt, Germany and icycler, BioRad, Munich, Germany): 2 μl RT product and internal MIMIC standard in serial dilution were amplified in a volume of 50 μl, containing 1 × PCR buffer (10 mM Tris–HCl, pH 9·0, 50 mM KCl), 1·0 mM $MgCl_2$, 200 μM each deoxynucleotide, 0·6 pmol/μl each primer, and 0·06 U/μl Taq DNA polymerase. The first denaturation step (95 °C for 6 min) was followed by 29 cycles with a 1 min denaturing step at 95 °C, a 1 min annealing step, starting at 70 °C and decreasing by 0·5 °C with each cycle to a minimum of 65 °C, and a 1·5 min elongation step at 72 °C. As a final extension step, the reaction was heated to 72 °C for 6 min and then cooled. PCR products were electrophoresed on a 1·5 agarose-gel with a 1 Kb DNA ladder followed by ethidium-bromide staining. The stained gel was analyzed with a computerized scanner and image analyzing software (NIH Image, version 1·61, National Institute of Health, Bethesda, MD, USA). Routinely, negative controls without input RNA or with omitted RT-step were included. For quantification of the target mRNA levels, equal amounts of target cDNA were amplified with different dilutions of known amounts of MIMIC DNA. After RT-PCR, the ratios of MIMIC to target band intensity were determined, and the concentration of a 1:1 MIMIC/target ratio was calculated as described (Kutoh *et al.* 1998). For each sample, an initial estimate of IGF-IR mRNA was performed with a single dose of internal standard DNA, followed by a narrow titration of internal standards around this estimated value, according to the method of Alms *et al.* (1996). Results were expressed as number of molecules per μg total RNA. The RNA of each sample was reverse transcribed and analyzed by RT-PCR in duplicate in two separate experiments. Using this method, the intra-assay coefficient of variation for IGF-IR mRNA quantification was 3%, and the interassay coefficient of variation was 11%.

IGF-I binding studies

^{125}I-IGF-I binding studies were performed with membrane preparations of 5 normal adrenomedullary tissue samples and 17 pheochromocytomas as previously described (Weber *et al.* 1997). Briefly, tissue samples were homogenized mechanically in homogenization buffer (0·25 M sucrose, 0·25 mg/l antipain and 100 mg/l phenylmethyl sulfonyl fluoride (PMSF) and centrifuged at 600 ***g*** for 10 min. The supernatant was centrifuged at 10 000 ***g*** for 30 min, adjusted to a final concentration of 0·1 mol/l NaCl and $0{\cdot}2 \times 10^{-3}$ mol/l Mg_2SO_4, centrifuged twice at 100 000 ***g*** for 90 min and resuspended in membrane buffer (50 mM Tris–HCl, pH 7·4; 0·25 mg/l antipain and 100 mg/l PMSF). Aliquots of 80 μg membrane protein were incubated for 3 h at room temperature together with ^{125}I-IGF-I (20 000 c.p.m.) and increasing concentrations of unlabeled IGF-I in 400 μl binding buffer (Medium 199 containing 0·2% bovine serum albumin, 150 mM NaCl and 1·2 mM $MgSO_4$). Membrane bound radioactivity was measured and receptor kinetics were calculated by Scatchard analysis (Scatchard 1949) using a standard software program (Ligand, NICHHD, NIH, Bethesda, MD, USA).

Statistical analysis

All data are expressed as means ± S.E.M. Comparative data were analyzed by multivariate analysis and paired *t*-test with significance defined as $P<0{\cdot}05$, unless otherwise stated.

Results

IGF-I receptor mRNA expression in human pheochromocytomas

The expression of IGF-IR mRNA in human pheochromocytomas was compared with normal adrenal medulla by quantitative RT-PCR of tissue samples from 16 patients with pheochromocytomas and from 13 normal adrenal glands. Amplification of cDNA with primers located in exons 10 and 11 yielded one specific PCR product of 247 bp (Fig. 1). Sequence analysis showed that these products were identical with the cDNA sequence of the human IGF-IR (data not shown). No products were detected when the RT reaction or the input RNA were omitted. Furthermore, the same size and intensity of the amplification product was observed when the RNA was treated with DNase prior to RT-PCR, excluding possible amplification of contaminating DNA. Expression of the human IGF-IR gene was detected in the tissues of all patients. In the normal adrenal medulla, IGF-IR was expressed at $2751 \pm 541 \times 10^3$ molecules/μg RNA, while significantly higher

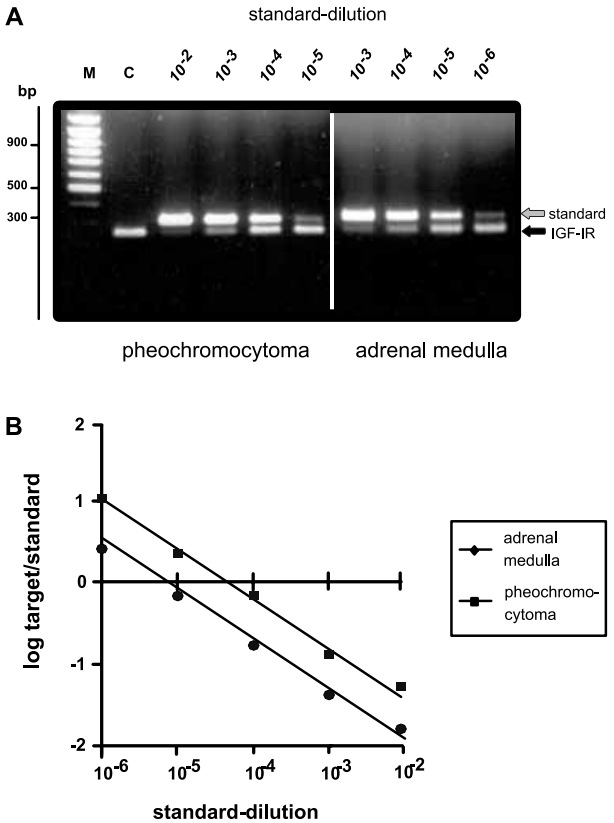


Figure 1 Representative result of the RT-PCR analysis of normal adrenomedullary tissue and a human pheochromocytoma. (A) Ethidium bromide staining of amplification products separated on an agarose gel. One microgram total RNA was reverse transcribed and amplified together with serial dilutions of internal control DNA using the same IGF-IR primers. The figure shows a typical titration experiment performed using 10^{-2}- to 10^{-6}-fold dilutions of the internal standard (100 amol/l) and fixed amounts of the target cDNA. M, marker; C, control. (B) Representative linear regression analysis of the internal standard titration curve obtained with the DNA from a pheochromocytoma (■) and normal adrenomedullary tissue (●). The values on the ordinate are the log of the internal standard/target cDNA ratio. The values on the abscissa are dilutions of the internal standard on a log scale. The titration point corresponds to the intersection with the 0 axis.

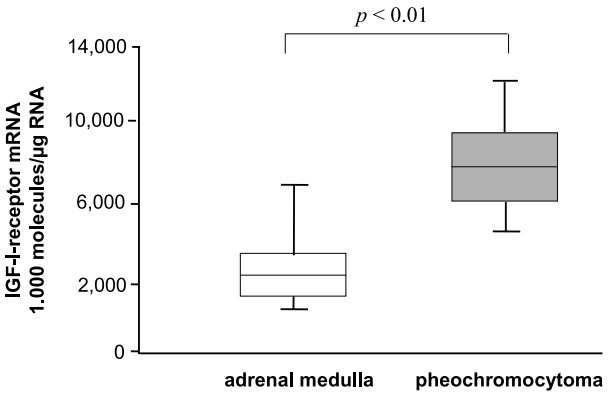


Figure 2 Mean IGF-IR expression 1000 molecules/µg RNA in human pheochromocytomas (shaded bar; *n*=16) and normal adrenomedullary tissue (open bar; *n*=13).

levels were observed in tumor samples, with a mean expression of $7772 \pm 1203 \times 10^3$ molecules/µg RNA ($P<0{\cdot}001$). Figure 1 shows a representative result of the quantitative RT-PCR analysis from two samples of normal adrenomedullary and pheochromocytoma tissue. When the IGF-IR mRNA levels of pheochromocytoma tissue samples were analyzed (Fig. 2), in contrast to normal adrenal glands, a wide heterogeneity in IGF-IR mRNA levels was observed ($2303 - 20\,520 \times 10^3$ molecules/µg RNA) (Table 1). However, IGF-IR mRNA levels of pheochromocytoma tissue samples showed 2·83-fold higher IGF-IR levels than normal adrenomedullary tissue ($P<0{\cdot}01$). Seventy-five percent of human pheochromocytomas showed a more than 2-fold overexpression of the IGF-IR as compared with normal adrenal medulla. IGF-IR expression was unrelated to tumor size, age, sex and catecholamine secretion. However, the highest IGF-IR mRNA level of all investigated tissue specimens was seen in the only malignant pheochromocytoma investigated in this series.

IGF binding to normal adrenomedullary and pheochromocytoma tissue

Binding kinetics of ^{125}I-IGF-I to membranes from five normal adrenal medullas and 17 pheochromocytomas was investigated. The mean specific binding of ^{125}I-IGF-I to membranes from normal human adrenal medulla was $3{\cdot}6 \pm 0{\cdot}9\%$. ^{125}I-IGF-I binding could be effectively displaced by unlabeled IGF-I with a 50% displacement (ED_{50}) at $6{\cdot}1 \pm 2{\cdot}0$ ng/ml. In contrast, significantly higher concentrations of IGF-II were necessary for a 50% displacement, and insulin was effective only at micromolar concentrations (data not shown). Scatchard analysis revealed a single-class of high-affinity binding sites with a K_d of $0{\cdot}32 \pm 0{\cdot}1$ nmol/l and a receptor concentration of $2{\cdot}43 \pm 1{\cdot}2$ nmol/kg protein (Fig. 3). In comparison with normal adrenomedullary tissue, membrane preparations from the pheochromocytoma tissues showed a significantly higher specific ^{125}I-IGF-I binding of $7{\cdot}0 \pm 1{\cdot}1\%$, as well as an elevated mean IGF-IR concentration of $5{\cdot}8 \pm 0{\cdot}5$ nmol/kg protein ($P<0{\cdot}05$). The mean IGF-IR concentration was about 2·4-fold higher in pheochromocytomas as compared with normal adrenomedullary tissue. However, IGF-I was equally potent in displacing the labeled ligand from pheochromocytoma membranes (ED_{50} $5{\cdot}6 \pm 1{\cdot}0$ ng/ml), and the Scatchard analysis showed a single class of high affinity binding sites with

Table 1 IGF-I-receptor mRNA levels and clinical characteristics of evaluated normal adrenomedullary tissue and pheochromocytomas

			Catecholamine secretion (µg/24 h)		
	Diagnosis		Adrenaline (Norm: 4–20 µg/d)	Noradrenaline (Norm: 20–105 µg/d)	**IGF-IR-mRNA ×10³ molecules/µg RNA**
Patient sex/age					
♂/58	Normal adrenal medulla	—	—	—	513
♀/72	Normal adrenal medulla	—	—	—	625
♂/72	Normal adrenal medulla	—	—	—	798
♀/61	Normal adrenal medulla	—	—	—	2622
♀/70	Normal adrenal medulla	—	—	—	2623
♀/55	Normal adrenal medulla	—	—	—	2620
♂/58	Normal adrenal medulla	—	—	—	2554
♂/51	Normal adrenal medulla	—	—	—	2953
♂/68	Normal adrenal medulla	—	—	—	2964
♂/66	Normal adrenal medulla	—	—	—	3648
♀/64	Normal adrenal medulla	—	—	—	3283
♀/71	Normal adrenal medulla	—	—	—	3762
♀/69	Normal adrenal medulla	—	—	—	7200
♂/49	Pheochromocytoma	Sporadic/benign	246·2	688·1	2303
♂/51	Pheochromocytoma	Sporadic/benign	187·2	543·6	2850
♂/33	Pheochromocytoma	MEN IIa/benign	155·5	2557·4	4446
♂/29	Pheochromocytoma	VHL/benign	187·6	1455·6	5130
♀/34	Pheochromocytoma	MEN IIa/benign	88·3	1866·8	5244
♂/41	Pheochromocytoma	Sporadic/benign	46·8	546·7	5700
♂/38	Pheochromocytoma	Sporadic/benign	286·4	1433·1	6042
♀/48	Pheochromocytoma	Sporadic/benign	345·9	685·9	6384
♂/52	Pheochromocytoma	Sporadic/benign	172·5	571·6	6384
♀/44	Pheochromocytoma	Sporadic/benign	285·6	1479·1	6452
♀/56	Pheochromocytoma	Sporadic/benign	455·6	1885·3	7068
♂/36	Pheochromocytoma	Sporadic/benign	155·4	894·3	9120
♀/30	Pheochromocytoma	VHL/benign	187·2	3655·6	9918
♀/35	Pheochromocytoma	VHL/benign	245·6	1465·7	12540
♀/62	Pheochromocytoma	Sporadic/benign	99·4	1006·4	14250
♀/58	Pheochromocytoma	Sporadic/malignant	286·5	3148·6	20520

MEN IIa, multiple endocrine neoplasia IIa; VHL, von Hippel-Lindau.

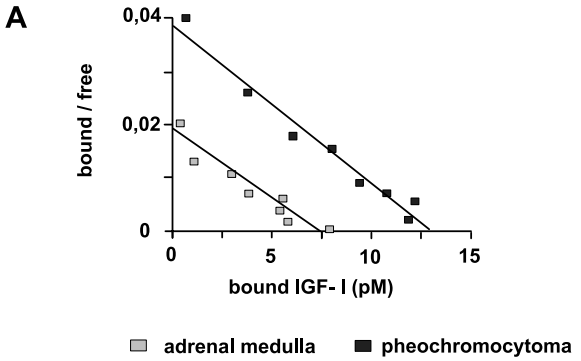
normal binding kinetics in all examined pheochromocytomas (K_d 0·20 ± 0·09 nmol/l), indicating overexpression of normal intact human IGF-IR in human pheochromocytomas. A representative comparison of the Scatchard plots for a pair of normal adrenomedullary and pheochromocytoma tissue samples is shown in Fig. 3.

Discussion

The presence of IGF-I receptors in pheochromocytoma cells has already been described in samples from human adrenal tumors (Kamino *et al.* 1991) as well as in PC12 cells, a cell line derived from rat pheochromocytoma (Dahmer *et al.* 1989). However, information about the IGF-I receptor expression in human pheochromocytoma cells in comparison with normal adrenomedullary tissue has not been available to date.

The present study is the first to report a significant overexpression of the IGF-I receptor in human pheochromocytomas. The analysis of tissue samples from human pheochromocytomas via RT-PCR showed markedly elevated levels of IGF-IR mRNA in pheochromocytoma cells compared with normal adult adrenal medulla. In up to 85% of all pheochromocytomas examined, a more than twofold higher expression of IGF-IR mRNA was observed, with a mean 2·5-fold overexpression. A comparable IGF-IR overexpression was also present at the protein level as confirmed by Scatchard analysis. The binding kinetics of the IGF-I receptors in pheochromocytoma cells were similar to those observed in normal adrenomedullary tissue, suggesting that the abundant IGF-I receptors in pheochromocytomas are functionally intact.

In PC12 cells, IGF-I receptors have been shown to be important for the stimulation of cell replication (Dahmer *et al.* 1989) and the IGFs are potent mitogens stimulating



B

	adrenal medulla	pheochromocytoma
Kd (nmol/l)	0.32 ± 0.1	0.2 ± 0.09
ED_{50} (ng/ml)	6.1 ± 2.0	5.6 ± 1.0
Receptor - number (nmol/kg protein)	2.43 ± 1.2	5.8 ± 0.5*

Figure 3 (A) Scatchard analysis of the competitive ^{125}I-IGF-I binding data to human pheochromocytomas (solid symbols) and normal adrenomedullary tissue (shaded symbols). Each point represents the mean of duplicate determinations of a single representative experiment. (B) Binding characteristics of the IGF-IR in human pheochromocytomas and normal human adrenomedullary tissue. Results are means ±S.E.M. of 5 (normal) and 17 (pheochromocytomas) independently performed binding experiments. *=$P<0{\cdot}05$.

cell proliferation three times over basal. IGF-I was 10 times more potent in stimulating DNA synthesis than IGF-II, suggesting that these effects are mediated by the IGF-IR (Dahmer & Perlman 1988, Nielsen & Gammeltoft 1988). Moreover, binding affinities to the IGF-IR correlate directly with the ability of IGF-I and IGF-II to completely prevent apoptosis in PC12 cells (Forbes *et al.* 2002). In these cells, promotion of cell growth and proliferation by IGF-I is exerted by the ERK pathway (Foncea *et al.* 1997), whereas for prevention of apoptosis the phosphatidylinositol 3-kinase pathway is involved (Kulik *et al.* 1997).

Overexpression of the IGF-I receptor is not a phenomenon confined to adrenal tumors. In common malignant tumors such as colorectal and gastric cancer as well as in prostate and breast cancer, a strong overexpression of the IGF-IR has been observed. It promotes ligand-dependent neoplastic transformation and there is a quantitative relationship between tumorigenesis and IGF-IR levels, while the absence of IGF-IR prevents malignant growth and transformation (Moschos & Mantzoros 2002). Additionally, in these tumors a positive correlation between IGF-IR overexpression and malignant phenotype has been observed. The mechanisms responsible for enhanced IGF-IR expression in pheochromocytomas and other malignancies are still unclear. However, expression of the IGF-IR is regulated by a variety of factors, including tumor suppressor genes, transcription factors and other growth factors. In several different IGF-IR overexpressing malignant cell systems such as colorectal, gastric, and adrenocortical cancer as well as in osteosarcoma and hematopoetic cells, alterations of tumor suppressor genes and transcription factors important for IGF-IR regulation, such as p53 and Sp1, have been demonstrated (Werner *et al.* 2000, Baserga *et al.* 2003). In normal cells, expression of wild-type p53 was shown to inhibit IGF-IR gene expression, whereas mutant p53 upregulates IGF-IR gene expression in several different tumors. In adrenocortical carcinomas, mutations within the conserved regions of p53 have been found in approximately 30% of malignant adrenocortical tumors, whereas mutations are rarely found in benign adrenocortical adenomas (Fottner *et al.* 2004). Similar results have been found in colorectal-cancer and osteosarcoma cells, suggesting a role for p53 in upregulating IGF-IR expression (Ohlsen *et al.* 1998, Durai *et al.* 2005). Additionally, the expression of the IGF-IR has been shown to be regulated by the transcription factor, Sp1 via specific binding sites within the IGF-IR promoter region. In human gastric cancer, overexpression of the IGF-IR strongly correlated with Sp1 expression and with an advanced tumor stage, increased lymph node metastasis and predicted a poor survival, whereas enforced down-regulation of Sp1 and IGF-IR expression suppressed growth and metastasis of gastric cancer in animal models (Wang *et al.* 2003, Jiang *et al.* 2004). Therefore, altered expression of the IGF-IR, one of the down-stream effectors of Sp1, may play an important role in cancer growth and metastasis. Furthermore, the frequently observed elevated IGF-II concentration in malignancies overexpressing the IGF-IR might additionally contribute to the overexpression of IGF-I receptors in these tumors. In CaCo-2 human colon carcinoma cells, it has been shown that stable overexpression of IGF-II resulted in increased IGF-IR expression with increased proliferation and anchorage-independent growth (Hoeflich *et al.* 1996), and in colorectal carcinomas a positive correlation between the expression of IGF-II and IGF-I receptors has been reported (Weber *et al.* 2002).

The molecular mechanisms by which overexpression of the IGF-IR is induced in pheochromocytoma-associated hereditary syndromes like MEN or von Hippel-Lindau disease are still unclear. However, in

renal carcinoma cells (RCC) wild-type von Hippel-Lindau gene (VHL) has been shown to block protein kinase C-delta (PKC-delta), an important downstream signaling molecule of IGF-IR-mediated cell proliferation and transformation. In mutated VHL, this tumor suppressor function gets potentially lost. VHL has also been shown to regulate the protein expression levels of IGF-IR (Li *et al.* 1998, Datta *et al.* 2000). It is therefore tempting to speculate if alterations in PKC-delta-mediated pathways are involved in the increased expression of IGF-IR in human pheochromocytoma cells.

Previous studies by our own group and by others have shown a critical role of the IGF system in either normal adrenocortical cells or in adrenocortical tumors (Weber *et al.* 1997, Boulle *et al.* 1998, Fottner *et al.* 1998, 2001). Several authors report the effects of high amounts of IGF-II in human adrenal pheochromocytomas on protein and mRNA levels (Hasselbacher *et al.* 1987, Gelato & Vassalotti 1990) despite unaltered levels of IGF-I. Compared with normal adrenomedullary tissue, 20 times more immunoreactive IGF-II per gram of tissue was measured in samples from human pheochromocytomas. IGF-II seems to be secreted by pheochromocytoma cells in an autocrine or paracrine manner, supporting tumor growth locally, while the IGF-II serum levels remain unaltered (Gelato & Vassalotti 1990). We speculate that the marked overexpression of the IGF receptor type I and IGF-II in human pheochromocytoma cells results in a state of constitutive growth stimulation *in vivo*. In malignant adrenocortical carcinomas, overexpression of IGF peptides (mainly IGF-II), receptors (IGF-IR) and binding proteins (IGFBP-2) has been observed. In contrast, expression of IGF-I peptides and receptors appears to be unaltered in adrenocortical hyperplasia and adenomas. Adrenocortical carcinoma, a rare, highly malignant subtype of cancer, showed a 3- to 4-fold increase in IGF-IR expression and a 10- to 100-fold increase in IGF-II expression (Liu 1995, Weber *et al.* 1997). Functionally, an autocrine stimulatory loop contributing to adrenocortical tumorigenesis may underlie this specific expression pattern. A similar pattern of high IGF-II and concomitant IGF-IR overexpression has previously been reported for neuroblastoma cells (Leventhal *et al.* 1990) and more recently by our group in human colon carcinomas (Weber *et al.* 2002). The exact role of the frequently observed overexpression of IGF-binding proteins in parallel with the overexpression of the IGF-I receptor and IGF ligands is still unclear and although high concentrations, especially of IGFBP-2, are a frequent finding in a variety of malignant tumors such as adrenocortical, prostate, breast and colonic cancer, the functional significance remains unclear. Since IGFBPs modulate cellular bioavailability of IGFs and, in addition, have been shown to directly regulate tumor growth and invasion (Hoeflich *et al.* 2001), it is likely that overxpression of IGFBPs in cancer is not merely an epiphenomenon. Similar to these results, one study also reports a higher expression of IGFBP-2 in human pheochromocytomas in comparison with normal adrenal glands (Ilvesmäki *et al.* 1998) and recently published data show that IGFBP-2 plays a critical role in neuroblastoma cell proliferation, migration and invasion, thus pointing to an important role of IGFBP-2 in chromaffin cell tumors (Russo *et al.* 2005). However, additional studies are necessary to further characterize the role of IGFBPs in human pheochromocytomas.

Since overexpression of IGF-IR promotes neoplastic growth (Kaleko *et al.* 1990) and absence of the IGF-IR has been shown to prevent malignant transformation (Rubin & Baserga 1995), it is tempting to speculate about a possible role of IGF-IR in malignant transformation of human pheochromocytoma cells. It would be interesting to elucidate if the degree of IGF-IR expression in pheochromocytomas correlates with the tumor size and a more malignant phenotype, as has previously been reported for other malignant tumors such as colorectal, gastric and mammary cancers and in adrenocortical carcinomas (Fottner *et al.* 2004, Foulstone *et al.* 2005). In the present study, no correlation between clinical characteristics, such as catecholamine secretion or tumor size could be found (Table 1). In contrast, in the subsequent studies, one malignant pheochromocytoma (characterized by the presence of distant metastases) has been examined, and this showed the strongest overexpression of IGF-IR of all investigated pheochromocytomas. This could support the hypothesis mentioned above. However, due to the small number of tumors examined in this study, at this point there is no clear evidence for a correlation between the degree of IGF-IR overexpression and other clinical characteristics and a more malignant phenotype.

Further investigation is needed to clarify if the observed overexpression of IGF-IR is part of a functionally relevant mechanism promoting tumor growth in human pheochromocytoma and possibly promoting malignant transformation of these cells. If so, the IGF system might be an interesting focus for new therapeutic approaches.

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