Localization of vascular endothelial growth factor (VEGF) receptors in normal and adenomatous pituitaries: detection of a non-endothelial function of VEGF in pituitary tumours

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Abstract

As for any solid tumour, pituitary adenoma expansion is dependent on neovascularization through angiogenesis. In this process, vascular endothelial growth factor (VEGF) and its receptors VEGFR-1, VEGFR-2 and neuropilin-1 (NRP-1) may play an outstanding role. The intention of this work was to study the expression/localization and possible function of VEGF receptors in pituitary adenomas. VEGF receptor mRNA and protein expression was studied by *in situ* hybridization, immunohistochemistry and RT-PCR in 6 normal human pituitaries, 39 human pituitary adenomas and 4 rodent pituitary adenoma cell lines. VEGFR-1 expressing somatotroph MtT-S cells were used as a model to study the role of VEGF on cell proliferation and to elucidate the underlying mechanism of action. In normal pituitaries, VEGFR-1 was detected in endocrine cells, whereas VEGFR-2 and NRP-1 were exclusively expressed in

endothelial cells. In pituitary tumours, a heterogeneous VEGFR expression pattern was observed by IHC. VEGFR-1, VEGFR-2 and NRP-1 were detected in 24, 18 and 17 adenomas respectively. In the adenomas, VEGFR-1 was expressed in epithelial tumour cells and VEGFR-2/NRP-1 in vessel endothelial cells. Functional studies in VEGFR-1-positive MtT-S cells showed that the ligands of VEGFR-1 significantly stimulated cell proliferation. This effect was mediated through the phosphatidylinositol-3-kinase-signalling pathway and involves induction of cyclin D1 and Bcl-2. Based on our results, we speculate that the ligands of VEGF receptors, such as VEGF-A and placenta growth factor, not only play a role in angiogenesis in pituitary adenomas, but also affect the growth of pituitary tumour cells through VEGFR-1.

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Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is known to be a fundamental process in the expansion of solid tumours (Carmeliet 2003, Ferrara 2004) and is also essential for pituitary adenoma progression (Gorczyca & Hardy 1988, Turner *et al.* 2000, 2003, Vidal *et al.* 2001). The intratumoral vessel system is necessary to supply tumour cells with nutrients and oxygen and to remove waste products. Sprouting of vessels into an expanding tumour is a complex process involving endothelial cell proliferation, vascular tube formation as well as tumour matrix degradation and remodelling during vessel invasion (Carmeliet 2003, Ferrara 2004). Many different angiogenic factors are involved in neovascularization, such as fibroblast growth factor-2, platelet-derived growth factors, transforming growth factor- β

and members of the vascular endothelial growth factor (VEGF) family (Carmeliet 2003, Ferrara 2004).

VEGF-A is one of the most important angiogenic factors stimulating endothelial cell proliferation, motility and permeability (Ferrara 2004, Tammela *et al.* 2005). The VEGF protein family further comprises VEGF-B, -C, -D, -E and placenta growth factor (PlGF) (Ferrara 2004, Tammela *et al.* 2005). VEGF-A binds to different tyrosine kinase receptors, such as VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are mainly expressed in the endothelial cells of blood vessels (Ferrara 2004, Tammela *et al.* 2005). These cells also express neuropilin-1 (NRP-1), a VEGFR-2 co-receptor lacking intracellular signalling tyrosine kinase domain and enhancing only VEGF-A binding to VEGFR-2 (Soker *et al.* 1998). Mitogenesis, chemotaxis and changes in endothelial cell morphology are mainly mediated through VEGFR-2

phosphorylation, subsequent to VEGF binding (Waltenberger *et al.* 1994), while VEGFR-1 seems to be less important in these processes. The original assumption that the members of the VEGF family are specific regulators of the growth and function of endothelial cells had to be revised when VEGF receptors were detected on a number of non-endothelial cells as various tumour cell types (Jackson *et al.* 2002, Fan *et al.* 2005). Since their growth was affected by VEGF-A, this factor is now thought to support progression of some tumours by concomitantly stimulating tumour cell growth and tumour neovascularization.

In normal pituitary, both folliculostellate (FS) cells and endocrine cells were identified as sources of VEGF (Gospodarowicz *et al.* 1989, Jabbour *et al.* 1997, Fan & Iseki 1998, Gloddek *et al.* 1999, Renner *et al.* 2002, Cristina *et al.* 2005, Nakakura *et al.* 2006). In human pituitary adenomas, tumour cells synthesize and release VEGF-A (Lloyd *et al.* 1999, Iuchi *et al.* 2000, Lohrer *et al.* 2001, McCabe *et al.* 2002, Fukui *et al.* 2003, Viacava *et al.* 2003, Onofri *et al.* 2004). Human adenomas are mostly less well vascularized than the normal anterior pituitary (Gorczyca & Hardy 1988, Turner *et al.* 2000, Vidal *et al.* 2001, De la Torre *et al.* 2005) and no enhanced VEGF-A expression was found in human pituitary tumours, in comparison with the normal gland, with the exception of the study of McCabe *et al.* (2002). Only in rapidly growing oestradiol-induced experimental rat prolactinomas with a vessel density higher than in the normal rat pituitary, was an over-expression of VEGF-A was observed (Banerjee *et al.* 1997, 2000).

So far, little information is available about the VEGF receptor expression in normal and tumoral pituitary. Studies in normal animal pituitaries, oestradiol-induced rat prolactinomas and rat pituitary tumour cell lines have led to controversial results (Banerjee *et al.* 1997, 2000, Jabbour *et al.* 1997, Vidal *et al.* 2002). In a first study in humans, VEGFR-2 mRNA and protein over-expressions were shown in the extracts of pituitary tumour tissue in comparison with the normal human anterior pituitary tissue (McCabe *et al.* 2002).

Here, for the first time, the localization of VEGFR-1, VEGFR-2 and NRP-1 in normal human pituitary and pituitary tumours at mRNA and, in particular, at protein level has been studied. Since VEGFR-1 was detected in endocrine cells of both normal and tumoral pituitary, functional aspects related to this receptor have also been investigated.

Materials and Methods

Human tissues

This study was performed after approval of the ethics committee of the Max Planck Institute and in the case of pituitary adenomas, informed written consent was received from each patient. During autopsy, six normal human pituitaries were obtained from carefully selected patients with unknown endocrine disturbances. The post-mortem delay was < 12 h. According to clinical, biochemical, radiological and surgical

findings as well as by routine immunohistochemistry (IHC), 39 pituitary adenomas (Table 1) were diagnosed and classified as somatotrophinomas (11 cases), corticotrophinomas (3 cases), clinically non-functioning adenomas (17 cases), prolactinomas (6 cases) and thyrotrophinomas (2 cases). All patients with somatotrophinomas and prolactinomas were pre-treated with somatostatin analogues and dopamine agonists respectively. Since medication was stopped at least 1 week prior to surgery and the proposed anti-angiogenic action of these drugs has not been observed in pituitary adenomas (Lohrer *et al.* 2001, Vidal *et al.* 2001), we also included tissues from these tumours in our study. All the tumours were benign and graded by a modified Hardy's classification (Table 1, Bates *et al.* 1997). Tissue fragments of normal pituitaries and pituitary adenomas were shock-frozen and stored at -80 °C until use.

IHC

For immunohistochemical detection, 8 µm sections of shock-frozen tumour tissue were thaw-mounted onto Super Frost Plus slides (Menzel-Glaser, Hamburg, Germany), fixed in 4% paraformaldehyde in PBS and stored in 96% ethanol, at 4 °C until use.

Different antibodies were used to detect intratumoral VEGF receptors (anti VEGFR-1, -2 and NRP-1) and VEGF-A (anti VEGF-A) expression, vessel density (anti CD-31) and proliferation index (PI) (anti Ki-67) (Table 2). Slides were first incubated for 30 min in serum (diluted in a ratio of 1:10 in Tris-base saline buffer, pH 7.6) of the same animal in which the specific biotinylated secondary antibody was raised. Subsequently, the slides were incubated overnight at 4 °C with different primary antibodies at the corresponding dilution (Table 2). After washing thrice in Tris-base saline buffer, the corresponding biotinylated secondary antibody, diluted in a ratio of 1:300, was added at room temperature for 30 min. The slides were again rinsed thrice in Tris-base saline buffer and incubated for 30 min with the avidin-biotin-peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) at room temperature. Colour development was performed using 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) with 0.01% hydrogen peroxide, applied for the corresponding incubation time listed in Table 2. Finally, the slides were counterstained in toluidine blue, fixed in Roti-Histol (Roth, Karlsruhe, Germany) and coverslipped using Roti-Histokitt (Roth). Negative controls were performed omitting the different primary antibodies and no staining was detected in negative control sections.

Double immunohistochemistry

After immunohistochemical staining for VEGFR-1 with DAB, sections of human normal adenohypophysis were incubated for 2 h at room temperature with monoclonal antibodies against adrenocorticotrophic hormone (ACTH) (Dako Cytomation, Glostrup, Denmark), luteinizing hormone (LH) (Immunotech, Marseille, France) and prolactin (PRL) (Immunotech) diluted

Table 1 Vessel density, VEGF-A and VEGF receptor expression in normal and tumoral pituitary

	Sex	Age (years)	Grade	PI (%)	Vessel count (CD31 +)	VEGF-A, IHC	VEGFR-1		VEGFR-2		NRP-1, IHC
							IHC	ISH	IHC	ISH	
Tissue											
NP1	M	67	—	0.3	21–30	+++	++	+	++++	+	+++
NP2	M	46	—	0.6	>30	+++	++	n.d.	++++	n.d.	+++
NP3	F	37	—	0.6	>30	+++	++	n.d.	+++	n.d.	+++
NP4	F	73	—	0.6	>30	+++	++	n.d.	+++	n.d.	++++
NP5	F	59	—	0.3	>30	+++	++	n.d.	+++	n.d.	+++
NP6	M	67	—	0.6	>30	+++	++	n.d.	++++	n.d.	++++
S1	M	29	III	1.3	21–30	+++	++	n.d.	—	—	—
S2	F	28	II	0	>30	++++	++++	n.d.	—	n.d.	++
S3	M	41	II	0	10–20	++	+	+	—	—	+
S4	F	52	III	2	10–20	—	++++	n.d.	—	n.d.	—
S5	M	70	III	7.6	<10	—	—	n.d.	—	n.d.	—
S6	F	51	II	4.6	10–20	++	+	n.d.	+++	n.d.	—
S7	F	77	III	0	>30	—	++	+	—	n.d.	++
S8	F	60	III	0.6	>30	++	++++	n.d.	++	n.d.	++
S9	M	44	II	0	21–30	+++	—	n.d.	—	n.d.	++
S10	F	47	I	0	>30	++++	—	n.d.	—	n.d.	—
S11	F	50	II	0	10–20	++++	—	n.d.	—	n.d.	—
C1	F	46	III	2.6	<10	++++	++++	+	++++	+	++++
C2	F	52	II	1	10–20	++++	—	n.d.	+	n.d.	—
C3	F	29	III	0	<10	+++	++	n.d.	—	n.d.	—
NF1	M	48	III	1	10–20	+++	++++	n.d.	++++	n.d.	+++
NF2	F	49	III	2	<10	++	++++	n.d.	—	n.d.	—
NF3	F	64	III	0.5	21–30	++++	++++	+	—	—	—
NF4	M	35	III	0	<10	++++	—	n.d.	++++	n.d.	—
NF5	M	68	III	0.5	>30	++++	—	n.d.	—	n.d.	—
NF6	M	53	III	0	>30	+++	+	n.d.	+++	n.d.	—
NF7	M	39	III	0	<10	++++	++++	+	—	—	—
NF8	M	61	III	0	21–30	+++	+	+	—	—	—
NF9	F	59	III	0	<10	++++	—	n.d.	++++	n.d.	—
NF10	F	55	III	2.3	>30	++++	—	—	+	+	—
NF11	F	52	II	1.3	>30	—	—	—	+++	+	+
NF12	F	30	III	2	21–30	+++	++	n.d.	++++	n.d.	++++
NF13	F	66	III	2.6	10–20	++++	+	n.d.	++++	n.d.	+++
NF14	M	61	II	0	10–20	—	++++	n.d.	—	n.d.	—
NF15	F	48	III	9	>30	++++	—	n.d.	++++	n.d.	+++
NF16	M	75	II	0	21–30	++++	+	n.d.	++++	n.d.	+++
NF17	F	32	II	1	10–20	+++	—	n.d.	++++	n.d.	+
L1	F	37	III	0.6	<10	++++	+	n.d.	++++	n.d.	—
L2	M	28	III	0	21–30	++++	+	+	—	—	—
L3	M	28	III	4.3	10–20	++++	++++	n.d.	++++	+	+++
L4	M	43	II	1.6	>30	+++	—	—	—	—	+
L5	F	26	I	0	>30	+	—	—	—	—	—
L6	M	43	III	10.6	10–20	++	—	n.d.	++++	+	+++
T1	F	29	III	1.3	<10	++++	+	n.d.	—	n.d.	—
T2	M	32	III	0.7	10–20	++++	++++	+	—	—	+++

Immunostaining intensity for VEGF-A and VEGFR-1 was assessed according to an arbitrary scale: — no immunoreactivity, + <10%, ++ 10–30%, +++ 31–60%, ++++ 61–100% immunopositive cells. Immunoreactivity for VEGFR-2 and neuropilin-1: — no immunoreactivity, + <10%, ++ 10–30%, +++ 31–60%, ++++ 61–100% of CD31-positive vessels which show immunostaining for VEGFR-2 and neuropilin-1. The results of ISH were scored as positive (+) or negative (—) only; n.d., not determined. NP, normal pituitary; S, somatotroph adenomas; C, corticotroph adenomas; NF, non-functioning adenomas; L, lactotroph adenomas; T, thyrotroph adenomas; M, male; F, female; PI, proliferation index; IHC, immunohistochemistry; ISH, *in situ* hybridization. According to the modified Hardy's classification: grade I tumours were microadenomas (<10 mm in diameter); grade II tumours were non-invasive macroadenomas (>10 mm in diameter); and grade III tumours were locally invasive macroadenomas.

in a ratio of 1:1000. Anti-thyroid-stimulating hormone (TSH) (Immunotech), anti-follicle-stimulating hormone (FSH) (Immunotech) and anti-growth hormone (GH) (gift from Dr C J Strasburger, Berlin, Germany) antibodies were diluted in a ratio of 1:800. Staining was detected using the

avidin–biotin–alkaline phosphatase complex (Vector Laboratories Inc.) and Vector Red (Vector Laboratories, Inc.) as a chromogen. After processing, the slides were counterstained with toluidine blue, fixed in Roti-Histol (Roth) and cover-slipped using Roti-Histokitt (Roth).

Table 2 Overview of the antibodies used in IHC (upper panel) and Western immunoblotting (WIB, lower panel)

	Primary antibodies	Secondary Ab	Incubation in chromogen
Antigens			
VEGF-A	Goat anti-human (1:200) ^a	Horse anti-goat	5 min
VEGFR-1	Rabbit anti-human (1:100) ^a	Goat anti-rabbit	2 min 30 s
VEGFR-2	Rabbit anti-human (1:100) ^a	Goat anti-rabbit	4 min
NRP-1	Goat anti-human (1:500) ^a	Horse anti-goat	6 min
CD31	Mouse anti-human (1:500) ^b	Horse anti-mouse	45 s
Ki-67	Mouse anti-human (1:100) ^b	Horse anti-mouse	1 min
ACTH	Mouse anti-human (1:1000) ^b	Goat anti-mouse	30 min
LH	Mouse anti-human (1:1000) ^c	Goat anti-mouse	30 min
PRL	Mouse anti-human (1:1000) ^c	Goat anti-mouse	30 min
TSH	Mouse anti-human (1:800) ^c	Goat anti-mouse	30 min
FSH	Mouse anti-human (1:800) ^c	Goat anti-mouse	30 min
GH	Mouse anti-human (1:800) ^d	Goat anti-mouse	30 min
P-PDK1	Rabbit anti-rat (1:1000) ^e	Donkey anti-rabbit	–
P-PTEN	Rabbit anti-rat (1:1000) ^e	Donkey anti-rabbit	–
P-Akt(Thr308)	Rabbit anti-rat (1:1000) ^e	Donkey anti-rabbit	–
P-Akt(Ser473)	Rabbit anti-rat (1:1000) ^e	Donkey anti-rabbit	–
P-GSK-3β	Rabbit anti-rat (1:1000) ^e	Donkey anti-rabbit	–
Cyclin D1	Mouse anti-human (1:1000) ^f	Donkey anti-mouse	–

Primary antibodies for IHC (dilution in brackets) were from ^aSanta Cruz Biotechnology, Santa Cruz, CA, USA, ^bDako Cytomation, Glostrup, Denmark, ^cImmunotech, Marseille, France and ^dProf. C. J Strasburger from the University of Berlin, Germany. Primary antibodies for WIB were from ^eCell Signalling Technologies, Beverly, MA, USA and ^fBD Biosciences, San Diego, CA, USA. All secondary antibodies for IHC were biotinylated and were from Vector Laboratories Inc., Burlingame, CA, USA. All secondary antibodies for WIB were horseradish peroxidase conjugated and were from Amersham Biosciences, Bucks, UK.

Immunoreactivity evaluation

The proportion of cells immunopositive for Ki-67, VEGF-A and VEGFR-1 was determined by counting the number of positive cells out of 100 cells in three different areas of each tissue section. Vessel density was determined as previously described (Perez Castro *et al.* 2003) by counting CD31 immunopositive single cells or cell clusters inside an area delimited by an eyepiece grid 12·5×12·5 mm divided in 10×10 squares (Zeiss, Munich, Germany) at a 200× magnification (20× objective and 10× ocular). VEGFR-2 and NRP-1 immunoreactivities were found only in vessel endothelial cells and therefore, the proportion of VEGFR-2 and NRP-1 immunopositive vessels is indicated in Table 1 as a percentage of the CD31 immunopositive vessels.

In situ hybridization

VEGFR-1 cDNA (920 bp fragment, GenBank accession AF063657, nucleotides 617–1537) and VEGFR-2 cDNA (1031 bp fragment, GenBank accession AF035121, nucleotides 1406–2437) were generated by RT-PCR from total human normal pituitary RNA using the primers listed in Table 3. Both the fragments were then cloned into pGEM-T Easy Vector (Promega). For linearization and generation of each riboprobe, the following restriction enzymes (New England Biolabs, Beverly, MA, USA) and RNA polymerases (Roche) were used: VEGFR-1 sense: SacI, T7; VEGFR-1 antisense: SacII, SP6; VEGFR-2 sense: SacII, SP6; VEGFR-2 antisense: SacI, T7. Riboprobes were then labelled with

³⁵S and single *in situ* hybridization was performed as previously described (Cota *et al.* 2003).

Cell culture of rodent tumour cell lines

Somatotroph MtT-S rat pituitary tumour cells (Inoue *et al.* 1990), lactosomatotroph GH3 rat cells, corticotroph AtT20 mouse cells and folliculostellate TtT/GF mouse cells were grown in DMEM supplemented with 10% foetal calf serum, 2·2 g/l NaHCO₃, 10 mmol/l HEPES, 2 mmol/l glutamine, 10 ml/l non-essential amino acids, 10 ml/l MEM vitamins, 5 mg/l insulin, 5 mg/l transferrin, 2·5 mg/l amphotericin-B, 10⁵ U/l penicillin/streptomycin, 20 µg/l sodium selenite and 30 pmol/l triiodothyronine (T3; Henning, Berlin, Germany). Cell culture materials and reagents were obtained from Invitrogen, Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany), Flow (Meckenheim, Germany) and Sigma. Tumour cell lines were grown to confluence and then used for RNA extraction or, in the case of MtT-S cells, also for proliferation studies.

RNA isolation and reverse transcription-PCR (RT-PCR)

VEGFR-1, VEGFR-2 and NRP-1 mRNA expressions were assessed by RT-PCR in all pituitary tumour cell lines and normal rat anterior pituitaries. The latter were obtained after decapitation of 8-week-old male rats and after the removal of neurohypophysis. RNA was extracted and isolated by the guanidium isothiocyanate–phenol–chloroform method (Chomczynski & Sacchi 1987). All RNA samples were

Table 3 List of primers used in the present work

	Sequence (5'-3')	Ta (°C)	Amplified fragment (bp)
Primers			
GAPDH ^a rat/mouse	ATGGTGAAGGTCGGTGTGAACG sense GTTGTCATGGATGACCTTGGC antisense	60	495
VEGFR-1 ^a rat/mouse	CCCGGTTTGCTGAACCTTGTTG sense GGCATTGGTGAAAGCTCCTC antisense	60	271
VEGFR-2 ^a rat/mouse	GCCAATGAAGGGGAAGTGAAGAC sense TCTGACTGCTGGTGATGCTGTC antisense	60	537
NRP-1 ^a rat/mouse	GGCTGCCGTTGCTGTGCGCCA sense ATAGCGGATGGAAAACCTGC antisense	60	383
VEGFR-1 ^b human	CTGTGAAGCAACAGTCAATGG sense CTATTATGCCATGCGCTGAG antisense	58	920
VEGFR-2 ^b human	GAATACCCCTTGAGTCCAATC sense CTGAGTCTTCTACAAGGTCT antisense	58	1031

Ta, annealing temperature; bp, base pairs. For each couple of primers, 35-cycle PCR amplification reaction was performed, with each cycle consisting of denaturation at 94 °C for 1 min, annealing of the primers at 60 °C and chain extension at 72 °C for 1 min.

^aUsed for RT-PCR.

^bUsed in RNA probe generation for *in situ* hybridization.

subjected to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR to confirm that the extracts are not contaminated with DNA. Following the manufacturer's instructions, 1 µg RNA was reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen). PCR-amplified products were visualized on a 1.5% agarose ethidium bromide gel. PCR primers for the detection of both mouse and rat VEGFR-1, VEGFR-2 and NRP-1 (Table 3) were selected from published sequences. The integrity of cDNA obtained from RT reaction was confirmed by amplification of GAPDH. In negative controls, cDNAs were omitted.

Proliferation assay

The proliferation of MtT-S cells in response to VEGF-A, VEGFR-1-specific ligand, PlGF, or VEGFR-2-specific ligand, VEGF-E, was measured by [³H]-thymidine incorporation as previously described (Renner *et al.* 1998). With various concentrations (0.1–100 ng/ml) of VEGF-A, PlGF or VEGF-E (all from R&D Systems, Wiesbaden, Germany), 20 000 cells were plated per well and treated for different periods (up to 96 h) in serum-free medium. A neutralizing anti-VEGF-A antibody (Upstate, Lake Placid, NY, USA) was used to block the growth effects of VEGF-A. For experiments with the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002, cells were pre-treated with 30 µM inhibitor for 1 h in serum-free medium before stimulation with PlGF. During the last 3 h of the various stimulation periods, 0.5 µCi/ml [³H]thymidine was added to the cells.

VEGF-A determination

Production of VEGF-A by MtT/S cells was measured in the cell culture supernatant by specific ELISA according to the instruction of the manufacturer (R&D Systems, Wiesbaden, Germany) and as previously described (Lohrer *et al.* 2001).

Western immunoblotting

MtT-S cells were stimulated with 50 ng/ml PlGF for 30 min, 1, 3 and 6 h to study the phosphorylation of components of the PI3K signal transduction pathway, such as phosphoinositide-dependent kinase-1 (PDK1), phosphatase and tensin homologues (PTEN), Akt (Thr308 and Ser473) and glycogen synthase kinase-3 subunit β (GSK3-β). For the detection of Bcl-2 and cyclin D1, the cells were treated with 50 ng/ml PlGF for 24–96 h. To suppress the PI3K pathway, MtT-S cells were pre-treated for 1 h with 30 µM LY294002 and then stimulated with 50 ng/ml PlGF for 30 min. Proteins were extracted in a commercially available proteases inhibitor cocktail (Sigma). According to the manufacturer's instructions, 50 µg protein mixture obtained from each sample were separated using a pre-cast Tris-glycine 10% gel (Anamed, Darmstadt, Germany) in an Invitrogen electrophoresis apparatus (Invitrogen), and then transferred on a nitrocellulose membrane (Hybond ECL). After blocking for 2 h at room temperature in a 1× TBS/0.1% Tween solution (TBS-T) containing 5% milk powder, the membrane was then incubated overnight at 4 °C with the primary antibody diluted (see Table 2) in 2.5% milk powder/TBS-T. Subsequently, the membrane was washed and incubated with HRP-conjugated secondary antibody diluted in a ratio of 1:1000 in 2.5% milk powder/TBS-T for 1 h at room temperature. The membrane was then washed and protein bands were visualized using a commercially available chemiluminescence kit (Roche) according to the manufacturer's instructions.

Statistical analysis

The Fisher exact test was used for statistical analysis of the relationship between VEGF/VEGF receptor expression and tumour grade, proliferation index or vessel density. Cell proliferation studies were performed in quadruplicate wells and the results are expressed as the mean ± S.E.M. Statistics of

Figure 1 Expression and localization of VEGFR-1, VEGFR-2 and NRP-1 in normal human anterior pituitary. By single IHC (A)–(C), VEGFR-1 was found in endocrine cells throughout the anterior pituitary (A), whereas the expression of VEGFR-2 and NRP-1 was restricted to vascular structures (B) and (C). Inserts show negative controls in which the primary antibody has been omitted. (D–I) Double IHC shows VEGFR-1 (brown staining) co-localization with cells immunopositive for ACTH, GH, PRL, LH, FSH (red staining); no receptor was found in TSH-expressing cells. Thin arrows mark representative cells, which are positive for VEGFR-1; open arrows show examples of hormone-positive cells; and black arrows point to representative cells in which hormones and VEGFR-1 are co-localized. Magnification $\times 200$.

the growth and secretion studies were performed by ANOVA in combination with Scheffe's test. Statistical significance was considered at $P < 0.05$.

Results

Vessel count, proliferation index and tumour grade in normal and adenomatous pituitaries

Five out of six normal human adenohypophyses analysed showed a vessel density higher than 30 vessels per field and in

one case the vessel number was within 21–30 vessels per counting area. In all the normal pituitaries studied, the proliferation index was between 0.3 and 0.6% (Table 1).

Two pituitary adenomas were identified as grade I tumours, 11 as grade II and 26 as grade III tumours. No expression of the proliferation marker Ki67 could be detected in tissue sections of 16 adenomas. In five adenomas, the proliferation index was lower than 1%, in ten cases between 1 and 2% and in eight cases $> 2\%$. In 9 tumours the number of vessels was lower than 10, in 12 cases between 10

Figure 2 Expression and localization of VEGFR-1, VEGFR-2 and NRP-1 in human pituitary adenomas. A heterogeneous expression of VEGFR-1 (brown) was observed in the tumour cells in different types of pituitary adenomas (A), (D) and (G). In contrast, VEGFR-2 (B), (E) and (H) and NRP-1 (C), (F) and (I) were found only in vessel cells of those pituitary adenomas that were immunopositive for these two receptors. Tumour types and numbers are indicated in the left upper corner of the pictures. Inserts show negative controls in which the primary antibody has been omitted. Magnification $\times 200$. NF, non-functioning adenomas; L, lactotroph adenomas; S, somatotroph adenomas.

and 20, in 7 adenomas between 21 and 30 and in 11 cases >30 (Table 1).

VEGF-A expression in normal and adenomatous human pituitary

All the six normal pituitaries expressed VEGF-A to a similar extent in 31–60% of all cells, confirming previous reports that not only FS cells (5–10% of all pituitary cells), but also endocrine cells produce VEGF-A (Jabbour

et al. 1997, Fan & Iseki 1998, Cristina *et al.* 2005). In pituitary adenomas, VEGF-A was not detectable in 5 out of 39 cases (13%), which is in agreement with a previous study in which 8% of the adenomas studied did not secrete VEGF-A *in vitro* (Lohrer *et al.* 2001; Table 1). In 1 adenoma, VEGF-A immunoreactivity was found in $<10\%$ of all cells, in 5 cases in 10–30% of all adenoma cells, in 9 adenomas in 31–60% of the tumour cells and in 19 adenomas in more than 61% of the cells (Table 1).

VEGFR-1 expression in normal and adenomatous human pituitary

VEGFR-1 was found in all the six normal human adenohypophyses studied. Interestingly, the VEGFR-1 signal was not found in endothelial cells but was detectable in approximately 30% of the endocrine cells (Fig. 1A). VEGFR-1 immunoreactivity was observed in subsets of ACTH-, FSH-, GH-, PRL- and LH- secreting cells (Fig. 1D–H). In pituitary adenomas, VEGFR-1 expression was variable and could not be detected in 15 tumours. In VEGFR-1 immunopositive adenomas, the receptor was exclusively expressed in tumour cells but not in vessel endothelial cells (Fig. 2A, D and G). VEGFR-1 immunoreactivity in more than 61% of the cells was found in 11 pituitary tumours, in 10–30% of the cells in four adenomas and in <10% of the cells in nine cases (Table 1). *In situ* hybridization (ISH) analysis, in a subgroup of pituitary specimens studied (Table 1), showed a similar localization pattern of VEGFR-1 mRNA in tumour cells and confirmed receptor synthesis in normal pituitary and pituitary adenomas (Fig. 3A).

VEGFR-2 expression in normal and adenomatous human pituitary

Immunohistochemistry (IHC) analysis for VEGFR-2 showed that this receptor was expressed in vessel endothelial cells in normal (Fig. 1B) and adenomatous (Fig. 2B, E and H) pituitaries. In three normal pituitaries examined, more than 61% of all CD31-positive vessels expressed VEGFR-2 also, whereas in three cases, the percentage of VEGFR-2-positive vessels was between 31 and 60%. In pituitary tumours, no VEGFR-2 immunostaining could be detected in 21 out of 39 cases. In 12 tumours, more than 61% of all vessels expressed VEGFR-2. In three adenomas, the percentage of VEGFR-2-positive vessels was between 31 and 60%, in one case between 10 and 30% and in two cases lower than 10% (Table 1). ISH analysis, in a subgroup of the pituitary specimens studied (Table 1), confirmed the VEGFR-2 synthesis in vessel endothelial cells (Fig. 3B).

NRP-1 expression in normal and adenomatous human pituitary

Endothelial cells in normal and adenomatous pituitaries were found to be positive for the VEGFR-2 co-receptor NRP-1 also (Figs 1C and 2C, F and I). In four out of six normal anterior pituitaries, the number of NRP-1 expressing vessels was within a range of 31–60% of all CD31-positive vessels, while in two cases it was higher than 61%. No NRP-1 expression could be detected in 22 out of 39 pituitary adenomas and in the remaining tumours, NRP-1 expression was heterogeneous. In four adenomas, <10% of all CD31-positive vessels expressed NRP-1, and in four tumours, NRP-1 was found in 10–30% of all vessels. In seven adenomas, NRP-1 was found in 31–60% of CD31-positive vessels, and in two tumours, in more than 61% (Table 1).

Relationship between VEGF-A/VEGF receptors expression and tumour grade, PI or vessel number in pituitary adenomas

All normal pituitaries studied showed concomitant VEGF-A and VEGFR-1, VEGFR-2 and NRP-1 expression. The same was observed only in 7 out of 39 adenomas, whereas in the vast majority of the tumours, 1 (11 adenomas) or 2 (16 tumours) of the VEGF receptors could not be detected. The absence of all VEGF receptors was found in five adenomas, and in a somatotrophinoma, neither VEGF-A nor any of the receptors was detectable by IHC (Table 1).

We confirmed previous observations that VEGF-A expression or release did not correlate, in the majority of the cases, with pituitary adenoma type or size, vessel density and proliferative index (Lohrer *et al.* 2001, Borg *et al.* 2005; Table 4). Concerning VEGF receptors, only a significant correlation was found between low expression of VEGFR-2 and low PI values as well as between low expression of NRP-1 and low PI values (Table 4). There was also no relationship between the expression of VEGF-A and its different receptors (not shown).

VEGF-A and VEGF receptor expression in rodent pituitary tumour cell lines

The degree of VEGF-A release by MtT-S cells (1.65 ± 0.22 pg/1000 cells per 24 h) was similar as in the other pituitary tumour cell lines, in which VEGF-A secretion had already been studied (Gloddek *et al.* 1999, Lohrer *et al.* 2001).

VEGFR-1, VEGFR-2 and NRP-1 mRNA were detected in normal rat anterior pituitary by RT-PCR. None of the rodent tumour cell lines synthesized VEGFR-2, whereas all of them expressed NRP-1 mRNA although the function of the latter is unclear in the absence of VEGFR-2. VEGFR-1 mRNA expression was found only in MtT-S cells (Fig. 4) and therefore, this cell line was used as a model to explore the function of VEGFR-1 in pituitary adenoma cells.

Growth studies in MtT-S cells

VEGF-A dose-dependently stimulated the [³H]thymidine incorporation in VEGFR-1-expressing MtT-S cells (Fig. 5A). In the presence of a 100-fold excess of a neutralizing VEGF-A antibody (10 µg/ml), the growth-stimulating effect of VEGF-A (100 ng/ml) was completely reversed (data not shown). The VEGF-A antibody itself did not affect the basal growth of MtT-S cells (data not shown). VEGFR-1-specific PlGF also significantly enhanced the [³H]thymidine incorporation in MtT-S cells in a dose-dependent manner (Fig. 5B), whereas VEGFR-2-specific VEGF-E had no effect (Fig. 5C).

Intracellular signalling mechanisms induced through VEGFR-1

Since VEGF-A and PlGF were reported to stimulate growth of endothelial cells through the PI3 kinase pathway (Gerber *et al.* 1998, Yu *et al.* 2001), the role of this signalling cascade in MtT-S

Figure 3 Detection of VEGFR-1 and VEGFR-2 mRNA in human pituitary tumours by *in situ* hybridization. (A) Representative figures show the expression of VEGFR-1 mRNA (black spots) in scattered tumour cells (closed arrows) but not in cells along a vessel (open arrows). Synthesis of VEGFR-2 was found in cells of vascular structures as shown in (B) in cells surrounding a vessel lumen. Cell nuclei were stained with toluidine blue. Magnification $\times 400$.

cells was studied. PlGF treatment increased phosphorylation of PDK-1, Akt (both Thr308 and Ser473) and GSK-3 β (Fig. 6A) but did not affect the phosphorylation status of PTEN phosphatase, a negative regulator of the PI3 kinase pathway (Cantley & Neel 1999). Downstream mediators of PlGF actions seem to be the cell cycle progression factor cyclin D1 and the cell survival factor Bcl-2, since their production was stimulated by PlGF (Fig. 6B). The PI3 kinase inhibitor LY294002 inhibited PlGF-induced Akt phosphorylation in Thr308 (Fig. 6C), suppressed cyclin D1 and Bcl-2 production (Fig. 6C) and blocked PlGF-induced growth stimulation of MtT-S cells (Fig. 6D), confirming the involvement of PI3 kinase cascade in these processes.

Discussion

Despite some recent progress, the functions of VEGF-A and its receptors VEGFR-1, VEGFR-2 and NRP-1 in normal pituitary and pituitary adenomas are poorly characterized. In the present paper, we studied, for the first time, the localization of VEGF receptors in normal and adenomatous pituitary and provided the first evidence that VEGF-A may have different functions in pituitary adenomas, through differently localized VEGF receptors.

VEGF-A is known to induce vascularization under physiological and pathophysiological conditions and it is also needed to maintain existing vascular structures (Ferrara 2004, Tammela *et al.* 2005). Moreover, VEGF-A is an important regulator of the permeability of endothelial cell layers (Ferrara 2004, Tammela *et al.* 2005). We observed concomitant expression of VEGF-A and all the three VEGF receptors in all densely vascularized, normal anterior pituitaries. Since there is

Table 4 Comparison of tumour grade, proliferative index (PI) and vessel count (VC) with the degree of VEGF-A, VEGFR-1, VEGFR-2 and NRP-1 expression in human pituitary adenomas (A) and their corresponding *P* values (B)

	VEGF-A		VEGFR-1		VEGFR-2		NRP-1	
	Low	High	Low	High	Low	High	Low	High
A								
Grade								
I/II	5	8	11	2	9	4	12	1
III	6	20	17	9	15	11	18	8
PI								
$\leq 2\%$	8	23	22	9	22	9	27	4
$> 2\%$	3	5	6	2	2	6	3	5
VC								
≤ 20	7	14	13	8	11	10	15	6
> 20	4	14	15	3	13	5	15	3
B	VEGF-A		VEGFR-1		VEGFR-2		NRP-1	
Grade	0.453		0.276		0.728		0.225	
PI	0.663		1.000		0.037		0.009	
VC	0.382		0.569		0.323		0.464	

Low, -, +, ++. High, +++, +++++ (see Table 1). Significant correlations are indicated in bold.

Figure 4 Detection of VEGFR-1, VEGFR-2 and NRP-1 mRNA by RT-PCR in lactosomatotroph GH3 rat cells (lane 1), folliculostellate TtT/GF mouse cells (2), somatotroph MtT-S rat cells (3), corticotroph AtT20 mouse cells (4) and in normal rat pituitary (5). The predicted amplification products for all the receptors were found in extracts of normal rat pituitary (A)–(C). NRP-1 mRNA was detected in all rodent tumour cell lines (C) whereas none of them was positive for VEGFR-2 mRNA (B). Only MtT-S cells expressed VEGFR-1 (A). The integrity of cDNA was confirmed by amplification of GAPDH (D). For each cell line, no bands were detected in negative controls in which the cDNAs were omitted (not shown).

no obvious need for neovascularization in normal pituitary, the VEGF/VEGF receptor system may predominantly play a role in the maintenance of the vascular system and the regulation of endothelial cell permeability. A dense and highly permeable vessel system is needed, in normal anterior pituitary, for an optimal action of hypophysiotropic hormones on endocrine pituitary cells and for the rapid release of hypophyseal hormones into the circulation (Turner *et al.* 2000). The effects of VEGF-A on vessel integrity and permeability in normal human pituitary

Figure 5 Effect of members of the VEGF protein family on the proliferation of somatotroph MtT-S rat pituitary tumour cells. Growth was determined by [3 H]thymidine incorporation. After 96 h, growth was significantly stimulated by (A) VEGF-A and (B) placenta growth factor (PIGF) both acting through VEGFR-1. In contrast, VEGFR-2-specific VEGF-E did not induce MtT-S cell proliferation (C). Values are expressed as percentage of control (basal growth without stimuli corresponds to 100%). * $P < 0.05$; † $P < 0.001$ vs control.

seem to be mediated mainly through VEGFR-2 and its co-receptor NRP-1, which were found to be expressed in vessel endothelial cells. Interestingly, VEGFR-1 was predominantly expressed in nearly all types of endocrine pituitary cells, suggesting additional roles of VEGF-A in normal pituitary that still have to be identified.

In pituitary adenomas, the VEGF/VEGF receptor system may primarily be involved in tumour neovascularization, rather than in permeability regulation. As in the normal anterior pituitary, VEGFR-2 and NRP-1 were localized in vessel endothelial cells, whereas VEGFR-1 was detected in tumour cells. In contrast to normal pituitary, VEGF receptor expression was highly variable in pituitary tumours. Concomitant expression of all the three receptors was rare and in some adenomas none of the receptors could be detected. However, no correlation was observed between the

degree of expression of VEGF and its receptors and tumour progression parameters, such as grading, proliferative index (PI) and vessel density. The reason for this observation is not yet clear. We speculate that most very slowly expanding pituitary tumours undergo temporal changes between phases of growth ($PI > 0$) and growth arrest ($PI = 0$) and VEGF receptor-mediated angiogenesis follows after a delay. In such a scenario, pituitary adenomas would shift among various situations during their development: growth associated with not yet started angiogenesis, growth associated with active angiogenesis, growth arrest associated with angiogenesis not yet finished and growth arrest accompanied with no need of angiogenesis. Since *ex vivo* studies in human adenomas, like

IHC, allow an insight only at a certain time point in the many years time span of pituitary tumour development, only one of the above-mentioned conditions would randomly be observed.

We confirmed previous reports that pituitary adenomas are mostly less well vascularized than the normal pituitary (Gorczyca & Hardy 1988, Turner *et al.* 2000, Vidal *et al.* 2001, De la Torre *et al.* 2005). In agreement with this finding, we observed mainly absence or reduced levels of vascular VEGFR-2 in pituitary adenomas, both at mRNA (by ISH) and protein level (by IHC). However, in a previous study, McCabe *et al.* (2002) reported a strong over-expression of VEGFR-2 mRNA (average 14-fold, maximum 233-fold) in more than 100 human pituitary tumours using quantitative PCR. In the same study, enhanced VEGFR-2 protein expression was also shown (by Western blotting) in few of the adenomas investigated (McCabe *et al.* 2002). Strong over-expression of the VEGF/VEGF receptor system has been reported in rapidly growing and densely vascularized types of solid tumours (Carmeliet 2003, Ferrara 2004). In the case of the pituitary, only oestrogen-induced prolactinomas in Fischer 344 rats exhibit such tumour characteristics and over-expression of both VEGFR-2 and NRP-1 was found in this tumour type (Banerjee *et al.* 1997, 2000). Since the characteristics of human pituitary adenomas are completely different, strong over-expression of VEGFR-2 would not fit to these slowly growing and poorly vascularized tumours. Nevertheless, the discrepant findings on VEGFR-2 expression in human adenomas need to be clarified in future studies.

Our observation of differently localized VEGF receptors suggests that angiogenic effects of the members of the VEGF protein family are mediated through endothelial cell-associated VEGFR-2/NRP-1, whereas additional, non-endothelial

Figure 6 Preliminary insights into intracellular mechanisms of VEGFR-1-mediated growth effects in MtT-S cells. Stimulation of VEGFR-1 expressing MtT-S cells with 50 ng/ml PIGF induced a time-dependent phosphorylation of components of the PI3K signalling pathway. Phosphorylation status of PDK-1 and Akt (Thr308 and Ser473) was enhanced after 30 min, whereas an increase in phosphorylation of GSK-3 β was observed only after 1 h. (A) The phosphorylation status of the intrinsic PI3K inhibitor PTEN remained unchanged. (B) Through this signalling pathway, the production of the cell cycle regulator cyclin D1 and anti-apoptotic-acting Bcl-2 was transiently enhanced. Therefore, the PI3K inhibitor LY294002 not only suppresses phosphorylation of Akt (Thr 308) after 1 h, but also production of cyclin D1 and Bcl-2 after 48 h (C). (D) The PI3K signalling pathway also mediated the growth stimulatory effects of PIGF in MtT-S cells since LY294002 significantly suppressed proliferation after a 96 h stimulation period. It should be noted that LY294002 not only blocked PIGF-induced growth, but also the basal growth rate of MtT-S cells (D), suggesting an important role of the PI3K pathway in the basic growth regulation in this cell line. Values of [3 H]thymidine incorporation (see Fig. 5) are expressed as percentage of control (= 100%). * $P < 0.05$ vs control; † $P < 0.001$ vs PIGF stimulated. CT, control; PI, placenta growth factor; LY, LY294002. Loading controls for the blots were performed incubating the membranes with anti β -actin antibody (data not shown).

functions of VEGF may be induced by VEGFR-1 expressed in pituitary adenoma cells. In addition to non-functioning-like human HP75 pituitary tumour cells (Horiguchi *et al.* 2004), we detected VEGFR-1 in somatotroph MtT-S cells and found that VEGF-A and PlGF stimulated the growth of this cell line. This was mediated through the PI3 kinase pathway, which seems to transduce in this cell line not only growth effects of VEGF-A and PlGF, but also of other growth factors, since the basal PlGF-independent growth of these cells was also strongly suppressed. VEGF-A/PlGF-induced proliferation involved downstream activation of cell cycle promoter cyclin D1, probably through GSK-3 β (Diehl *et al.* 1998) and induction of survival factor Bcl-2 through Akt/cAMP-response element-binding protein interaction (Pug-azhenth *et al.* 2000). Even though we and other groups (Borg *et al.* 2005) could not observe any correlation between VEGF expression and tumour behaviour parameters (grade, PI values), we speculate that the growth of VEGFR-1 expressing human pituitary tumour cells will be stimulated by VEGF-A and PlGF in a similar manner, although this issue still needs to be confirmed in future studies.

In summary, herein it was shown for the first time that VEGF receptors are differently expressed in the endocrine and endothelial cells of both normal human anterior pituitary and pituitary adenomas. VEGFR-2 and NRP-1 were found in vessel endothelial cells and may mediate the angiogenic effects of VEGF-A in paracrine manner. In addition, VEGF-A and PlGF probably might contribute to the complex and multifactorial growth regulation of pituitary adenomas, in autocrine loops, through tumour cell-associated VEGFR-1. Therefore, VEGF-A and other members of the VEGF protein family may support tumour progression, by acting through their differently expressed receptors, concomitantly stimulating the proliferation and the neovascularization of human pituitary adenomas.

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