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Expression of functional active Toll like receptor 4 in normal and adenomatous pituitary cells

Dissertation

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To my Mother and in memory of my Father

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CONTENTS

ABBREVATIONS

Abbreviation	Definition
ACTH	Adrenocortocotrophic hormone
BMP-4	Bone morphogenetic protein
bp	Base pair
cAMP	Cyclic adenosine mono-phosphate
ECM	Extracellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FS	Folliculostellate cells
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	Growth hormone
IHC	Immunohistochemistry
IGF-I	insulin-like growth factor
ir	Immunoreactivity
IL-6	Interleukin 6
ISH	In situ hybridization
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccaride
MAPk	Mitogen activated protein kinase
NFκB	Nuclear factor kappa-B
NGF	Nerve growth factor
POMC	Proopiomelanocortin
PCR	Polymerase chain reaction
PRL	Prolactin
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
TGF	Tumour growth factor
Tlr4	Toll like receptor 4
TSH	Thyroid stimulating hormone

1. INTRODUCTION

1.1 The pituitary gland

The pituitary gland controls various homeostatic functions, such as metabolism, growth and reproduction, through consolidating the signals from the brain to different endocrine organs. The pituitary is located under the brain in the sella turcica and consists of the adenohypophysis and the neurohypophysis. Subdivided into the anterior and intermediate lobes the adenohypophysis consists of six adenohypophysal cell types, which secrete hormones, namely corticotrophs, somatothrophs, lactotrophs, mammosomatotrophs, thyrotrophs and gonadotrophs. Also present in the pituitary are folliculostellate cells (FS), an astrocyte-like cell type of unknown origin and poorly understood function. Corticotrophs secret adrenal corticotropic hormone (ACTH), that stimulates the secretion of glucocorticoids in the adrenal. Somatothrophs produce growth hormone (GH), which stimulates bone and muscle growth directly through GH receptors or indirectly through the stimulation of an insulin-like growth factor (IGF-1). Lactotrophs release prolactin (PRL), which stimulates lactation and inhibits gonadal functions. Mammosomatotrophs synthesise both GH and PRL and differentiate into somatothrophs during growth phases and into lactotrophs during pregnancy (Asa and Ezzat, 2002). Thyrotrophs secret thyroid stimulating hormone (TSH), which stimulates the production of thyroid hormones by the thyroid gland. Gonadotrophs synthesise follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are in charge of germ-cell development and sex steroids production in the gonads. FS cells do not produce any hormones, but a number of different growth factors and cytokines, which act by paracrine mechanisms on hypophyseal endocrine cells.

1.2 Pituitary adenomas

Human pituitary adenomas develop typically from cells of the adenohypophysis and are usually non-metastasising tumours with a significant minority of cases, exhibiting a more aggressive behaviour (Pardo et al., 2001). Pituitary tumours comprise 10-15% of intracranial tumours at surgery and 6 to 23 % of intracranial tumours at autopsy (Kovacs and Horvath, 1987). Patients with pituitary neoplasm could have symptoms of a mass lesion (headache, visual field disturbances, ophthalmoplegia, facial pain from compression of the first or second branch of the trigeminal nerve, anterior pituitary insufficiency) or endocrine abnormality (hyper-or hypofunction, or combination of the two), or both.

1.2.1. Classification

The classification of pituitary adenomas can be functional, anatomic/ radiological, histological, immunohistochemical, ultrastuructural, clinicopathologic.

1.2.1.1. Functional classification

Depending on hormone excess and clinical symptoms, pituitary tumours can be hormonally inactive (HI) or hormonally active. The latter can be subdivided into prolactinomas, somatotrophinomas, corticotrophinomas, gonadotrophinomas and thyreotrophinomas (table 1).

Table 1. *Epidemiology of clinical manifest pituitary adenomas (adapted from Jockenhövel 2002)*

Adenoma	Prevalence per100 000	Incidence per 100 000/year	Percentage of adenomas
Prolactinoma	30-50	2-5	55%
Hormone inactive	6-10	1	30%
Somatotrophinoma	5-7	<1	10%
Corticotrophinoma	1-3	<1	5%
Thyreotrophinoma	1	<1	1%
Gonadotrophinoma	1	<1	1%
All adenomas	40-70	6-8	100%

Somatotrophinomas cause gigantism before the end of puberty and acromegaly in adults by hypersecretion of GH, resulting in an elevated level of circulating IGF-1. Acromegaly is an insidious, chronic disease, characterised by bony and soft tissue overgrowth. Common clinical symptoms include headache, excessive perspiration, fatigue, paresthesiae, weakness, joint pain, and weight gain. Patients may also present with osteoarthritis, carpal tunnel syndrome, visual abnormalities, sleep apnoea, or reproductive disorders (Nabarro, 1987). Transsphenoidal surgical resection is the therapy of the first choice. The treatment with somatostatin analogues can supress GH secretion and cause tumour volume reduction, but to reveal the role of somatostatin analogues as primary therapy of acromegaly need further studies.

Prolactinomas are associated with hyperprolactinaemia. Most patients experience a high prolactin level, which usually parallel the size of tumour, hypogonadism with subsequent infertility, galactorrhoea, sexual dysfunction and osteoporosis. Most prolactinomas show good response to dopamine agonists, although there are few cases with resistance to dopamine-like drugs. Reduction of dopamine - binding sites or even an absence of dopamine D2 receptors has been demonstrated in

prolactinomas from patients, which were resistant to dopamine treatment (Caccavelli et al., 1994).

Corticotrophinomas cause Morbus Cushing or Nelson syndrome, usually present with endocrinological manifestation rather than a mass effect, since they are mostly microadenomas. ACTH overproduction leads to glucorticoid excess from adrenal, resulting in symptoms of Cushing's syndrome, such as weight gain, truncal obesity, plethoric "moon face", the "buffalo hump", thinning extremities with muscle atrophy, thin skin, hypertension and psychological disturbances. Nelson syndrome is a syndrome characterised by increased skin pigmentation, visual defects secondary to compression of the optic chiasm, and elevated serum levels of ACTH following adrenalectomy for Cushing's syndrome. The cause of this syndrome is the expansion of an underlying ACTH-secreting pituitary adenoma. Transsphenoidal surgery is the treatment of choice in corticotrophinomas.

TSH- secreting pituitary tumours are associated with signs and symptoms of either hyperthyroidism or mass effects. Transsphenoidal surgery is the treatment of first choice although octreotide treatment can normalise TSH levels and induce tumour shrinkage (Beck-Peccoz et al., 1996).

Hormone inactive pituitary adenomas produce no hormones and, therefore, have no typical hormone excess-related presentation. At diagnosis, patients already have very large tumours, presenting ocular or neurological symptoms rather than endocrinological disturbances. Transsphenoidal surgery is the best way to treat these tumours. In the case of incomplete resection radiation therapy should follow. It has further been shown, that the risk of adenoma recurrence could be reduced with postoperative radiotherapy (Snyder, 1995). However, the treatment with either dopamine or somatostatin agonists may be advantageous to prevent tumour regrowth in patients who have already received conventional therapies.

1.2.1.2. Radiological/ surgical classification

According to Hardy's classification adenomas are classified in five grades and in 6 stages (Hardy, 1979): *Grade I* refers to microadenomas, i.e. <10mm in diameter; *Grade II* refers to macroadenomas (>10 mm in diameter); *Grade III* adenomas with local perforation of sellar floor ; *Grade IV* diffuse perforation of cellar floor, distant spread. *Grade V* spread by CSF/blood. Tumour stage 0: None, A: suprasellar extension, B: third ventricle recess obliterated, C: parasellar, III ventricle grossly displaced, D: intracranial/intradural, E: cavernous sinus/extradural.

1.2.1.3. Histological classification

By cytoplasmatic staining, immunohistochemical staining for hormones and by electron microscopy, hormone active and inactive adenomas can be subdivided in different subtypes (table 2).

1.2.2. Pituitary tumourigenesis

In spite of intensive study during the last decades the pathogenetic mechanism of pituitary adenomas still remains unclear.

Pituitary adenomas are monoclonal in origin, which means that tumours originate from a single transformed cell (Herman et al., 1990), but the cause of the tumour transformation is still unknown.

Two hypotheses have been discussed with respect to pituitary adenoma formation. Pituitary cells are regulated by hypothalamic factors, peripheral hormones and growth factors; thus dysregulation of these signalling pathways could be a cause of tumour development. On the other hand the intrinsic pituitary events, such as an

activating mutations protoncogene or an inhibiting mutations in the tumour suppresser gene, could promote of a clonal expansion and result in adenoma formation (Fig.1).

Table 2. *Histological classifications of pituitary adenomas.*

Adenoma Type	Staining	Immunohistology	Blood hormone levels	Clinical symptoms
Corticotrophinomas *densily granulated* *sparsely granulated*	 B C	ACTH, MSH Endorphins	ACTH	Morbus Cushing or Nelson syndrome
Silent corticotrophinomas *subtype I* *subtype II*	 B/C C	ACTH, MSH Endorphins	None	Mass symptoms
Somatotrophinomas *densily granulated* *sparsely granulated*	 A C/A	GH α-subunit	GH	Acromegaly or gigantism
GH and PRL-producing *mixed GH-PRL* *mammosomaotrophinoma* *acidophilic stem cells*	 A/C A C	GH, PRL α-subunit	GH,PRL	 Acromegaly or gigantism ± hyperprolactinaemia Acromegaly ± hyperprolactinaemia hyperprolactinaemia or occasional acromegaly
Prolactinomas *densily granulated* *sparsely granulated*	 A C	PRL	PRL	Hyperprolactinaemia
Thyreotrophinomas	C/B	TSH α-subunit	± TSH	Hyperthyroidism
Gonadotrophinomas	C/B	FSH, LH α-subunit	None	Mass symptoms
Null cell adenomas *non-oncocytic* *oncocytic*	 C A	None	None	Mass symptoms

Abbreviations: A, acidophilic; B, basophilic, C, chromophobic

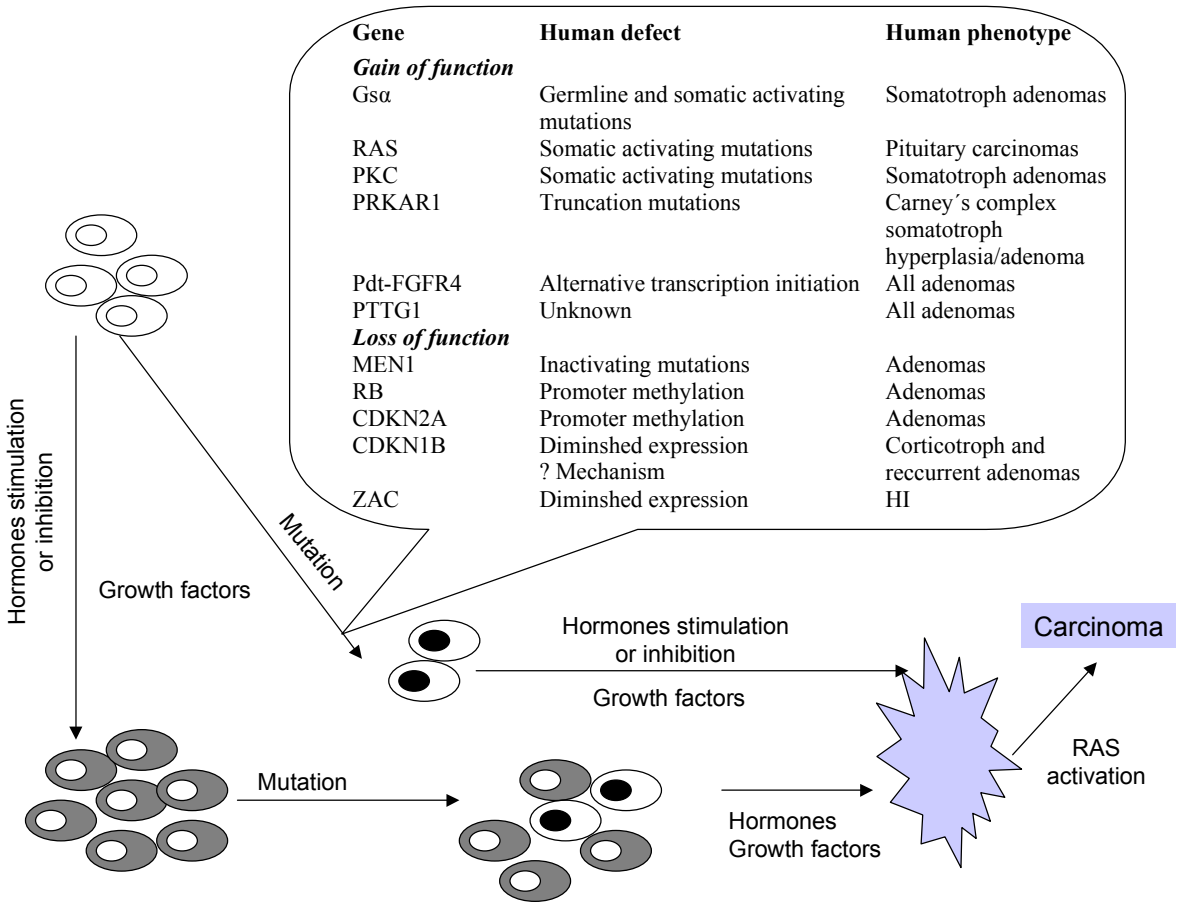

Fig. 1. *Proposed model of pituitary tumourigenesis, showing the concept of the hormonal stimulation theory (left pathway) vs. the intrinsic pituitary defect theory (right pathway). In animal models and patients, hypophysiotrophic hormone excess or growth factor overexpression induce hyperplasia, which may predispose the cells to mutation and may lead to adenoma formation. However, most human pituitary adenomas are unassociated with hyperplasia and may therefore originate from genetic events in a cell that becomes more responsive to hormones and growth factors, resulting in clonal expansion. In the box are shown pituitary gain- and loss- of function events that could be responsible for transforming mutation.*

1.2.2.1. Growth factors

Pituitary cells have been shown to produce various growth factors and cytokines and express their corresponding receptors (Tab. 3). These factors may regulate pituitary cell proliferation and hormone secretion in an autocrine/ paracrine fashion (Renner et al., 1996; Ray and Melmed, 1997)

Table 3. *Overview of the expression of the most relevant growth factors and cytokines in normal pituitary and pituitary tumours .*

Growth factors and cytokines	Expression in normal pituitary	Expression in pituitary adenomas
TGF-β1	L, FS	reduced expression in rat prolactinomas[a]
TGF-β3	L, FS	increased expression in rat prolactinomas[a]
Activin	G	expressed in human inactive and gonadotroph adenomas
Inhibin	G	expressed in human inactive and gonadotroph adenomas
Follistatin	FS	reduced expression in human gonadotroph adenomas
BMP-4	n.k.	over-expression in mice prolactinomas[b] and human prolactinomas
EGF	G,S, C, T	no over-expression in human pituitary adenomas
TGF-α	G,S, C, T	over-expression in rat prolactinomas[a]
FGF-2	FS,L	over-expression in rat prolactinomas[a]
NGF	L	reduced expression in dopamine resistant human prolactinomas
IL-6	FS	tumour cell-derived production in the majority of human pituitary adenomas
LIF	C,FS	reduced expression in human prolactinomas

a) Estradiol-induced prolactinomas in Fischer 344 rats. b) Prolactinomas in dopamine D2 receptor knock-out mice. Abbreviations: C, corticotroph cells; FS, folliculostellate cells; G, gonadotroph cells; L, lactotroph cells; S, somatotroph cells; T, thyreotroph cells, n.k. , not known. For abbreviations of growth factors see front page.

This has led to the hypothesis that in pituitary adenomas, excessive hormone production and the loss of growth control is a consequence of altered expression of cytokines/growth factors and /or their receptors resulting in disturbances of auto-paracrine regulation. Interleukin-6 is supposed to be one of these factors.

1.3. Interleukin-6 expression and action in pituitary.

IL-6 is produced in the normal pituitary by folliculostellate cells (Renner et al., 1998), whereas in the majority of pituitary adenomas, in which FS cells are rare or absent, tumour cells are the source of IL-6 (Jones et al., 1994). It is a potent stimulator of secretion for nearly all the hormones in the normal pituitary and contributes to excessive ACTH production in corticotroph adenomas (Arzt et al., 1999; Renner et al., 1996; Pereda et al., 2000). Interestingly, IL-6 inhibits the growth of normal pituitary cells (Arzt et al., 1993) but differently regulates c-fos expression in pituitary adenomas (Pereda et al., 1996) and stimulates pituitary tumour cell proliferation (Arzt et al., 1993). IL-6 is linked through gp130 to different signalling pathways such as the JAK/STAT pathway or the MAP kinase (MAPk) pathway, however, gp130 also induces cytokine-signalling inhibitors like SOCS-3 (Arzt, 2001). Although the underlying mechanism for the opposing growth effects of IL-6 in normal and adenomatous pituitary cells has not yet been studied, differences in the induction of activating signal pathways or stimulation of cytokine-signalling inhibitor production by the IL-6/gp130 complex may be responsible for the different mitogenic effects of IL-6 in the normal and adenomatous pituitary. The intratumoral production of tumour cell growth-stimulating IL-6 in the majority of pituitary adenomas, makes this cytokine an attractive candidate as an auto-/paracrine stimulator of adenoma progression. Bacterial endotoxin (LPS), IL-1, tumour necrosis factor α (TNF- α), pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal peptide (VIP) and

calcitonin all stimulate IL-6 production in pituitary (Arzt et al., 1999; Lohrer et al., 2000; Kiriyama et al., 2001). In addition, glucorticoid receptors expressed in FS cells (Ozawa et al., 1999) induce the potent inhibition of glucorticoids on IL-6 production (Lohrer et al., 2000; Vankelecom et al., 1989).

Understanding of the mechanism of IL-6 action to alter pituitary function was made possible by using lipopolysaccharide (LPS), a component of the outer layer of the cell wall of gram-negative bacteria (Raetz, 1990), a potent activator of the immune system (Rietschel and Brade, 1992). LPS induces the production of tumour necrosis factor (TNF)-α, IL-1 and IL-6 from macrophages and monocytes (van Deventer et al., 1990) and coupling to LPS binding protein (LBP) enhances its stimulatory potency (Hailman et al., 1994). Free LPS or LPS/LBP complex bind to the membrane-bound CD 14 (mCD14) in macrophages and monocytes (Ulevitch and Tobias, 1995). The LPS/LBP/CD14 complex in turn interact with the toll-like receptor-4 (Tlr4), a member of the toll receptor family (Medzhitov et al., 1997; Poltorak et al., 1998).

We have recently shown that pituitary folliculostellate (FS) cells expressed both CD14 and Tlr4. LPS could stimulate IL-6 production in these cells through the p38 MAPk/NF-kB pathway (Lohrer et al., 2000). Moreover, LPS-stimulated, FS cell-derived IL-6 was shown to enhance the ACTH production in three-dimensional pituitary re-aggregate cell culture in paracrine manner (Gloddek et al., 2001).

1.4. Toll-like receptors

Members of the Toll-like receptor (TLR) family expressed on immune cells are critically involved in the innate immune response interacting differently and in part specifically with cell wall components or DNA of gram-negative and gram-positive bacteria or with viral components. More than 10 members of the TLR family can be found in a search of human and mouse public data basis and ten members have been reported (Akira et al., 2001). Tlr4 and Tlr2 activate immune cells in response to components of the cell gram-negative (predominantly LPS) and gram-positive (predominantly lipoproteins) bacteria, respectively. Tlr9 is activated by unmethylated CpG motifs of bacterial DNA.

TLR have not only been found on immune cells, but in other cells as well ,e.g. Tlr4 expression relatively high in the heart (Frantz et al., 1999). It was found that the expression of Tlr4 and Tlr2 increased in fetal mouse lung with age (Harju et al., 2001). TLR expression was also observed in vascular endothelial cells (Hijiya et al., 2002), adipocytes (Lin et al., 2000), intestinal (Abreu et al., 2002) and renal (Tsuboi et al., 2002) epithelial cells, microglia cells (Bsibsi et al., 2002).

Taxol (Paclitaxel) is a potent anti-cancer drug and the most effective compound in the treatment of mammacarcinomas, tumours of female reproductive organs and a number of other human neoplasias. In mice it has been shown that taxol mimics the effect of LPS and acts anti-proliferative and pro-apoptotic through Tlr4 (Byrd-Leifer et al., 2001); whether this is also the case in humans is still controversially discussed.

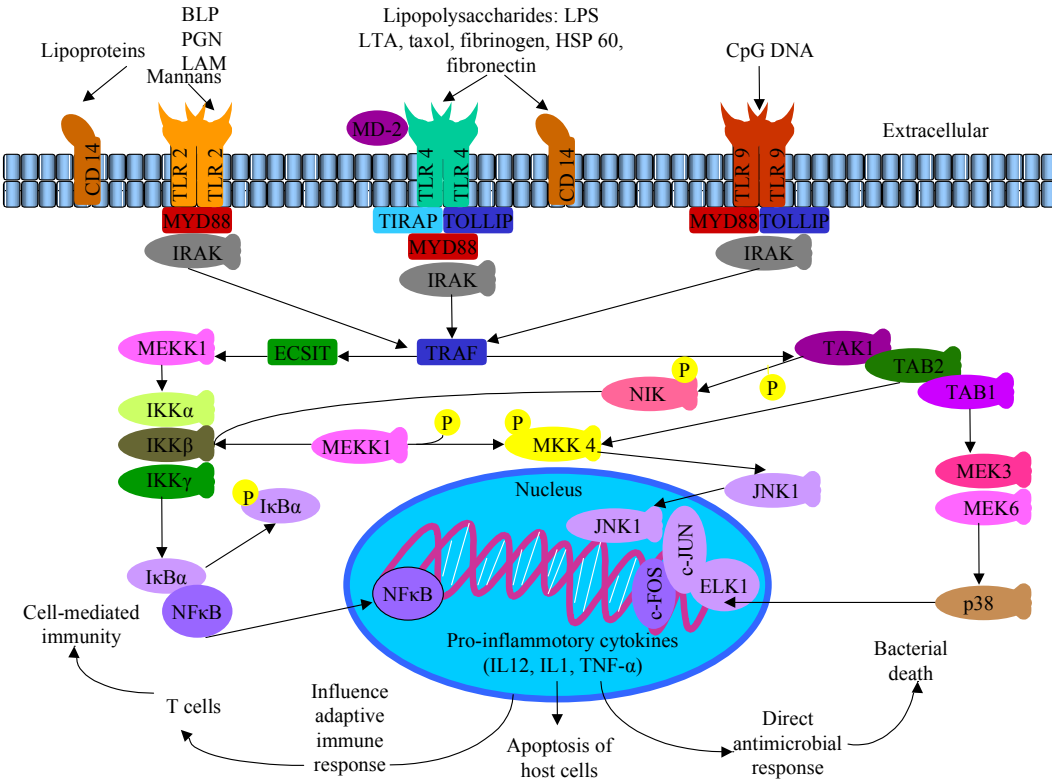

Fig.2. *Toll receptor signalling pathway. Human Tolls contains extracellular leucine-rich repeat domains of the IL-1 receptor (IL-1R), which are designated as Toll/IL-1R (TIR) domains. Mammalian Toll-like receptors signal through the adapter protein MyD88, which, in turn, associates with the serine threonine proteine kinase IL-1R-associated kinase (IRAK). Recruitment of IRAK leads to it activation and association with another adapter protein, tumour necrosis factor receptor-associated factor (TRAF 6), that activates downstream kinases. Oligomerization of TRAF6 results in activation of IkB kinases, followed by phosphorilation and degradation of IkB. On IkB degradation, NFkB factors translocate into the nucleus, where they induce transcription of target genes. In addition, to NFkB activation, TLRs can also initiate mitogen-activated protein kinase signalling cascades, which activate multiple transcription factors, including activator protein1 (cJUN) and ELK1 (Akira, 2003).*

1.5. Aim of the study

The study described herein aims to investigate the expression of receptors for LPS (Tlr4) in endocrine pituitary cells, pituitary tumour cell lines and human pituitary adenoma cells, and to study the functionality of Tlr4 in pituitary adenoma cell cultures and to clarify, whether LPS could act as a pituitary tumour progression factor either directly or by stimulating intratumoral IL-6 production.

2. MATERIAL AND METHODS

2.1. Reagents

Product	Company
[α-^{33}P]dATP	NEN (Cologne, Germany)
ABC blocking kit	Vector Laboratories (Burlingane, CA, USA)
Acetic acid	MERCK (Darmstadt, Germany)
Acridine orange	Sigma (St. Louis. MO, USA)
AG 1478	Calbiochem (Darmstadt, Germany)
Agar	Life Technologies (Paisley, UK)
Ammonium persulfate	Sigma (St. Louis. MO, USA)
Amphotericin B	Biochrom (Berlin, Germany)
Ampuwa water (for ISH)	Frisenius (Germany)
5-Aza-2´-deoxycytidine	Sigma (St. Louis. MO, USA)
Beta-mercaptoethanol	MERCK (Darmstadt, Germany)
Boric acid	Roth (Karlsruhe, Germany)
Bovine serum albumin (for cell culture)	Invitrogen Corp (Paisley, UK)
Chloroform	Sigma (St. Louis. MO, USA)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
DATP	Roche (Mannheim, Germany)
DCTP	Roche (Mannheim, Germany)
DGTP	Roche (Mannheim, Germany)
Diaminobenzidine	Sigma (St. Louis. MO, USA)
Diethyl-dicarbonate	Sigma (St. Louis. MO, USA)
Dimethyl sulfoxide	Sigma (St. Louis. MO, USA)
Dithiothreitol	Sigma (St. Louis. MO, USA)
DNAse	Invitrogen Corp (Paisley, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithouania)
DTTP	Roche (Mannheim, Germany)
Dulbecco's modified Eagle medium (DMEM)	Invitrogen Corp (Paisley, UK)
Dulbecco's modified Eagle medium (DMEM) without Phenol Red	Sigma (St. Louis. MO, USA)
EDTA	MERCK (Darmstadt, Germany)
Entellan	MERCK (Darmstadt, Germany)
Epidermal growth factor	Sigma (St. Louis. MO, USA)
Ethidium bromide	Sigma (St. Louis. MO, USA)
Fetal calf serum	Gibco (Karlsruhe, Germany)
Formamide	Sigma (St. Louis. MO, USA)
Forskolin	Sigma (St. Louis. MO, USA)
Glucose	MERCK (Darmstadt, Germany)
Guanidine thiocyanate	Fluka Chemie AG (Buchs, Switzerland)
Hepes	Sigma (St. Louis. MO, USA)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hyaluronidase	Sigma (St. Louis. MO, USA)
Hydrochloric acid	MERCK (Darmstadt, Germany)

Hydrogen peroxide	Roth (Karlsruhe, Germany)
Isoamylalcohol	MERCK (Darmstadt, Germany)
Isopropanol	Sigma (St. Louis. MO, USA)
Isopropanol	Sigma (St. Louis, Mo, USA)
KH2PO4	MERCK (Darmstadt, Germany)
Levamisole	Sigma (St. Louis. MO, USA)
L-Glutamine	Biochrom AG (Berlin, Germany)
Lipofectamine	Invitrogen Corp (Paisley, UK)
Luciferin	Roche (Mannheim, Germany)
Magnesium chloride	MERCK (Darmstadt, Germany)
Marker 1kbPlus	Life Technologies (Paisley, UK)
OPTIMEM 1	Invitrogen Corp (Paisley, UK)
Paclitaxel	Sigma (St. Lois, Mo, USA)
Paraformaldehyde	MERCK (Darmstadt, Germany)
PD 98059	Calbiochem (Darmstadt, Germany)
Penicillin+Streptavidine mix	Biochrom AG (Berlin, Germany)
Peptone	ICN Pharmaceuticals (Aurora, OH, USA)
Phenol	Roth (Karlsruhe, Germany)
Phosphate based buffer PBS (for cell culture)	Life Technologies (Paisley, UK)
Photoemulsion	Ilford K5 (Eppelheim, Germany)
Plasmid Preparation Kit	QIAGEN (Hilden, Germany)
Polyacrylamide	Invitrogen Corp (Paisle, UK)
poly-L-lysine-coated microscope slides (SuperFrost® Plus)	Menzel-Gläser (Braunschweig, Germany)
Potassium chloride (KCl)	MERCK (Darmstadt, Germany)
Reisin	Bio-Rad (Hercules, CA, USA)
Reporter lysis buffer	Promega GmbH (Mannheim, Germany)
Retinoic acid	Sigma (St. Louis, Mo, USA)
Reverse transcriptase (SuperScript II ™)	Invitrogen (Carlsbad, CA, USA)
Sarcosine, N-Lawryl-	Sigma (St. Louis, Mo, USA)
SB 203580	Calbiochem (San Diego, CA, USA)
Sodium acetate dihydrate	MERCK (Darmstadt, Germany)
Sodium acetate trihydrate	MERCK (Darmstadt, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium dihydrogen phosphate mono-hydrate (NaH2PO4-H2O)	MERCK (Darmstadt, Germany)
Sodium hydrogen phosphate dihydrate (Na2HPO4.2H2O)	MERCK (Darmstadt, Germany)
Sodium peroxide (NaOH)	MERCK (Darmstadt, Germany)
Streptavidin	R&D systems (Minneapolis, MN, USA)
Taq DNA polymerase	MBI Fermentas
TEMED	Sigma (St. Louis, Mo, USA)
Terminal transferase	Roche (Mannheim, Germany)
Tissue-Tek®	Sakura Finetek Europe (Zoeterwoude, The Nederlands)

Toluidin Blue	Sigma (St. Louis, Mo, USA)
Triethanolamine	Sigma (St. Louis, Mo, USA)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Trypsin	Sigma (St. Louis, Mo, USA)
Vector Red	Vector Laboratories (Burlingane, CA, USA)
WST-1 assay	Roche (Mannheim, Germany)
X-ray film	Kodak (New Haven, CT, USA)
Xylol	Roth (Karlsruhe, Germany)
Yeast extract powder	ICN Pharmaceuticals (Aurora, OH, USA)

2.2. Solutions

Collagenase Mix	1000 U/ml Collagenase : 400 mg/ 100ml solution Trypsin inhibitor: 10 mg/ 100ml solution Hyaluronidase : 100 mg/ 100ml solution BSA : 400 mg/ 100ml solution Dnase : 500 µl/ 100ml solution
Citric acid monohydrate (for antigen retrieval)	10mM Citric acid monohydrate: 2.1 g/L Adjust to pH 6.0
Formamide deionized	Add Reisin: 5ml/ 50ml Formamide
Formamide/4xSSC buffer	Formamide deionized: 50ml/ 100ml solution SSC 20x sterile : 20ml/ 100ml solution DEPC water : 30ml/ 100 ml solution
HDB buffer	Glucose : 18 mg/ 100ml solution Penicillin/Streptavidin : 1 ml/ 100ml solution Amphotericin B : 1 ml/ 100ml solution
LB medium	Peptone :10 g/L Yeast extract: 5 g/L NaCl : 5 g/L NaOH 1M : 2 ml/L Adjust to pH 7.0
Luciferase-assay buffer	Tris-HCl 1M pH 7.8 : 7.5 ml/ 100ml solution $MgCl_2$ 1M : 2.5 ml/ 100ml solution Before use add 4 µl/ml ATP 0.1 M
Paraformaldehyde (PFA)	4% paraformaldehyde :4 g/100 ml Sodium phosphate buffer : 20 ml/100ml solution Ampuwa water : 80 ml Add 1M NaOH to pH 7.4 Heat at 56°C to dissolve Filter and cool before usage Store at +4°C for maximum 2 days
Phosphate based buffer (PBS)	1x NaCl : 8 g/L KCl : 0.2 g/L $Na_2HPO_4.2H_2O$: 1.44 g/L KH_2PO_4 : 0.2 g/L Adjust to pH 7.4
Sodium acetate	2M Sodium acetate trihydrate: 27.2 g/ 100ml DEPC : 20 µl Add acetic acid to pH 4.0 Leave at room temperature overnight
Sodium phosphate buffer	50mM $Na_2HPO_4.2H_2O$: 7.06 g/L

	NaH_2PO_4. H_2O : 1.32 g/L Adjust to pH 7.4
Solution D	4M Guanidium thiocyanate 25 mM Sodium citrate pH 7.0 0.5% Sarcosyl dissolved in DEPC To complete the medium add: 180µl beta-mercaptoethanol/25ml solution
SSC	20x NaCl : 175 g/L Sodium citrate dihydrate: 88.23 g/L Adjust to pH 7.0 Filter and autoclave before use
Tris borate EDTA buffer (TBE)	10x Boric acid (H_3BO_3) : 61.83 g/L EDTA : 37.2 g/L Tris pure : 30.03 g/L Adjust to pH 8.0
Tris buffer	Tris pure: 12.114 g/L Adjust to pH 7.6
Tris-based buffer (TBS)	1x Tris pure : 2.42 g/L NaCl : 8 g/L Adjust to pH 7.6
Tris-HCl	1M Tris pure: 121.14 g/L Add 25% HCl to a pH 8.2

2.3 Patients

The present work was approved by the ethics committee of the Max-Planck-Institute of Psychiatry (Munich, Germany).

Tumour tissue was obtained by transsphenoidal surgery from 67 patients (23 female, 44 male; aged 14 to 80 years) with hormone-producing (n=42) or hormone-inactive (n=25) pituitary adenomas. Elevated serum levels of ACTH/cortisol, GH/IGF-I, PRL and TSH as well as corresponding clinical symptoms were observed in patients with endocrine-active tumours (10 corticotroph, 18 somatotroph, 12 lactotroph, 2 thyreotroph adenomas). Diagnosis of hormone-active adenomas was further confirmed by functional endocrine testing. Nuclear magnetic resonance imaging or computerised tomography showed that all corticotroph tumours were microadenomas, whereas the remaining hormone-active and all nonfunctioning tumours were macroadenomas. By routine immunohistochemistry, corresponding hormone expression was observed in endocrine-active tumour tissue. Immunohistochemical investigations showed that the group of clinically hormone-inactive tumours consisted of 4 gonadotropin-immunopositive tumours and 21 null cell adenomas or oncocytomas. Most of the patients with somatotroph adenomas had been pre-treated with somatostatin analogues. All of the patients with prolactinomas had to be operated on due to a resistance to dopamine agonist therapy.

Normal human pituitary (n=3; 2 female, 1 male; aged 30 to 49), kindly provided by Prof. W. Eisenmenger from the Institute of Forensic Medicine of the University of Munich, were obtained during an autopsy. The post mortem delay was less than 16 hours.

Human tonsils obtained from the Department of Otolaryngology/Head & Neck Surgery of the Technical University of Munich were used as positive controls in Tlr4 mRNA and protein expression studies.

2.4 Tissue preparation and cell culture

Where possible, human pituitary tumour tissue was devided into three pieces: one part was used for RNA extraction, another fragment was shock-frozen for in-situ hybridization (ISH) or immunohistochemistry (IHC), and the third piece was enzymatically dispersed to establish primary adenoma cell cultures. Normal human pituitaries were only divided for RNA extraction and ISH/IHC.

For primary pituitary Tudor cell culture, adenoma tissue, obtained at transsphenoidal microsurgery, were washed several times with HDB buffer. Sliced tissue fragments were dispersed mechanically and enzymatically in collagenase-mix (Renner et al., 1994). The cells were washed by repetitive centrifugation and resuspension, and were finally resuspended in DMEM culture medium (pH 7.3) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2.2 g/l $NaHCO_3$, 10 mM HEPES, 2 mM 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, 10^5 U/l penicillin/streptomycin, 20 µg/l sodium selenit, and 30 pM T_3 (Henning, Germany). Cell viability was consistently over 90 % as assessed by acridine orange/ethidium bromide staining. Cells were plated in 48 well plates (100,000 cells/well in 0.5 ml culture medium) and incubated for 2 to 3 days in a 5% CO_2 atmosphere at 37°C. After the cells had attached to the plates, stimulation experiments were performed as described below. In most cases, the small amount of tissue available limited the number of experiments that could be undertaken with each tumour.

Rodent and human cell lines were grown routinely in the same medium and under the same conditions as described above. Tlr4 expression was studied in corticotroph mouse AtT20 cells, gonadotroph-like mouse αT3-1 cells, lactosomatotroph rat GH3 cells, FS-like mouse TtT/GF cells, FS-like human PDFS cells and hormoninactive-like HP75 cells. Studies on hormone production and growth were conducted in AtT20 and GH3 cells.

2.5. Cell proliferation and viability

Cell proliferation was measured using the WST-1 proliferation assay (Roche Molecular Biochemicals, Penzberg, Germany). This assay measures the activity of the mitochondrial succinate-tetrazolium reductase system. In living cells, the WST-1 substance is converted to a dye which can be detected by an ELISA reader. For this assay, cells were seeded in a 96-well plate (1000 cells/ well), and after 3-4 days they were stimulated. After 24 hours, the WST-1 reagent was added to a final dilution 1:10, and after 2 hours incubation, the plate was measured with the ELISA reader at 450 nm.

Cell viability was determined by acridine orange/ ethidium bromide staining. Acridine orange, but not ethidium bromide, can be incorporated into living cells with intact cell membranes and the cell nucleus will stain green. If the cell is dead, ethidium bromide will reach the nucleus bypassing the destroyed cell membrane, intercalate into the DNA and stain the cell nucleus red. A mix of equal volumes of cell suspension and 1:1 acridine orange/ethidium bromide was placed on a Neubauer chamber. Examination was conducted under UV light. The cell viability was determined as the percentage of green (= live cells) in the total number of cells seen on each chamber area.

2.6. Gene expression studies

2.6.1. RNA extraction

Tissue fragments of normal or adenomatous pituitary was embedded in 800 µl Solution D and homogenized using the Ultra-TURRAX T8 (IKA Labortechnik) homogenizer. Solution D was composed by guanidine thiocyanate and beta-mercaptoethanol which by inhibiting the RNases prevents a possible RNA degradation. To the homogenized tissue, 80 µl (1/10 of the initial Solution D volume) sodium acetate was added to precipitate the genomic DNA, followed by 800 µl phenol and 160 µl Chloroform: Isoamylalcohol (49:1). After 15 min incubation in ice, the solution was centrifuged for 30 min at maximum speed (12000 rpm) at 4°C. At this stage, due to the phenol, two phases are clearly visible, separated by a thin zone containing the precipitated genomic DNA. The lower phase contains the phenol-soluble proteins and cell rests. The upper phase, containing the RNA, was carefully collected, taking special care not to be mixed with the genomic DNA- containing the intermediate zone, and was transferred to a new Eppendorf tube. Isopropanol was added in a volume equal to the upper phase, and the solution was incubated at –20°C for 2-12 hours to allow the complete RNA precipitation. After 30 min centrifugation, the supernatant was discarded, the pellet was washed with ice-cold 70% ethanol, and the solution was centrifuged for another 30 min. Next the ethanol was carefully discarded, the pellet was let to completely air dry, before being dissolved in 20-100 µl water supplemented with 0.1% (v/v) DEPC. DEPC inactivates RNases, thus protecting RNA against degradation.

RNA from cell culture was extracted as described above. After removing the cell culture medium and washing it with PBS, an appropriate amount of solution D was added in the petri-dish or well (usually it is 1 ml solution D per petri-dish, 800 µl per well in a 6-well plate and 200 µl per well in a 48-well plate).

When enough material was obtained, each RNA sample was aliquoted, keeping one aliquot in -20°C for current use, and the remaining at -80°C for long term storage.

The concentration and cleanliness of each RNA sample was determined using a spectrophotometer (Ultrospec II, Pharmacia). Nucleic acids have an absorption maximum at 260 nm, while for proteins the value reaches 280 nm. The ratio A_{260}/A_{280} gives information about the quality of the RNA preparation, i.e. if it is free from proteins and phenol rests. The optimal value for a very clean RNA solution lies between 1.9 and 2.0. RNA concentration is calculated using the following formula: $A_{260} \cdot 40 \cdot 60 = \chi$ µg/µl, where 40 is the concentration in µg/µl of RNA giving A_{260} value equal to 1, and 60 is the dilution in which the RNA is measured (1µl RNA solution + 59µl DEPC water)

2.6.2. Reverse transcriptase – polymerase chain reaction (RT-PCR)

1 µg of RNA was incubated with 2mM dNTP mix, 2µl random primers (Hexanucleotide Mix), 10mM dithiothreitol (DTT) and 200 U reverse transcriptase (SuperScript II) all diluted in 1x First Strand buffer, at 45°C for 1 hour. The reaction is inactivated by boiling at 95°C for 5 min. The cDNA produced was stored at -20°C.

For the PCR reaction 2 µl cDNA were added in a reaction mixture containing:

3 µl 10x PCR buffer

1.8 µl 25mM $MgCl_2$

3 µl 2mM dNTP Mix

1 µl amplification primer 1; 10 pmol/µl

1 µl amplification primer 2; 10 pmol/µl

0.3 µl *Thermus aquaticus* (*Taq*) DNA polymerase

17.9 µl autoclaved, distilled water

The PCR reaction parameters were: denaturation at 95°C for 1 min, annealing at 55-65°C for 1 min, polymerization at 72°C for 1 min, for 35-40 cycles. In every PCR reaction, cDNA from normal human pituitary was used as positive control and reaction in absence of template as negative control. To ensure that the RNA samples were not contaminated by genomic DNA, PCR reaction was carried for a housekeeping gene (beta-actin or GAPDH) using as template RNA sample which had not been reverse-transcribed.

After each reaction, the products were separated in a 1.2-1.5% agarose gel depending on the size of the product (1.2% for 400-1000 bp, 1.5% for 200-400 bp) and visualized by ethidium bromide under UV light. Electrophoresis took place in 1x TBE buffer for 15-20 min at 80 V. The 1kb Plus DNA marker was used to determine the fragment size.

The primers used are listed in Table 4, with their sequences, annealing temperature and predicted product size. The sequence of each was checked using the NCBI BLAST program, to exclude the possibility of a sequence similarity with genes other than the one under investigation. All primers were synthesised by MWG Biotech, were reconstituted with autoclaved distilled water to reach a concentration of 100 μM, and were stored at -20°C. The annealing temperature for each pair of primers was determined by PCR in a range of 55, 60, and 65°C using cDNA from normal human pituitary as template. The optimum temperature was the one that yielded an intense signal with no secondary amplification fragments.

Table. 4. *List of primers used.*

Name	**Sequence (5'-3')**	**Tm**[a)]	**Fragment (bp)** [b)]
beta-actin-s human	ACG GGG TCA CCC ACA CTG TGC	60	660
beta-actin-a human	CTA GAA GCA TTT GCG GTG GAC GAT G		
GAPDH-s rat	ATG GTG AAG GTC GGT GTG AAC G	60	495
GAPDH-a rat	GTT GTC ATG GAT GAC CTT GGC		
SF1-s	GCA TCT TGG GCT GCC TGC AG	71	230
SF1-a	CCT TGC CGT GCT GGA CCT GG		
hTlr 4-s	ACA GAA GCT GGT GGC TGT G	59	290
hTlr 4-a	TCT TTA AAT GCA CCT GGT TGG		
mTlr 4-s	GGG TCA AGG AAC AGA AGC AG	60	264
mTlr 4-a	GCT CAT TTC TCA CCC AGT CC		
Pit-s	AGT GCT GCC GAG TGT CTA CCA	59	560
Pit-a	TTT CTT TTC CTT TCA TTT GCT		

a) annealing temperature; b) size of PCR product.

2.6.3. In situ hybridization

2.6.3.1. Principle.

In situ hybridization is a method of localising and detecting specific mRNA sequences in morphologically preserved tissues sections or cell preparations by hybridizing the complimentary strand of a nucleotide probe to the sequence of interest.

The first step of *in situ* hybridization is the selection of a probe type. Several different probes can be used, such as double strand DNA (dsDNA), single stranded DNA (ssDNA), single stranded complimentary RNA (sscRNA), and synthetic oligonucleotides.

In order to observe where the probe had hybridized (bound) within tissue section or within cells and thus to determine where target mRNA is localised, an easily detectable substance or "label" has to be attached to the probe before hybridization.

Classically oligonucleotide probes are either 5' or 3' end-labeled or 3' tailed, with modified nucleotides that have a "label" attached that can be detected after the probe has hybridized to its target. With end-labeling a single modified ddNTP (that incorporates the label) is added to either the 5' or the 3' end of the molecule enzymatically or during probe synthesis. 3' tailing involves addition of a tail (on average 5-50 nucleotides long of modified dNTPs depending on the method used) using the enzyme terminal transferase (TdT).

Traditionally oligonucleotide probes have been radiolabeled. ^{3}H, ^{33}P or ^{35}S are the most commonly used radioisotopes. Radiolabeled probes are visualized by exposure of the tissue section or cells (to which the labeled oligonucleotide has be hybridized) against photographic film which is then developed

Instead of using photographic film to detect the probe within the tissue section, the slide containing the section of interest may also be dipped into a photographic emulsion which is allowed to dry. The slide is stored in the dark at -80C to allow the slide emulsion to become exposed. After the incubation period, the slides are then developed in the same way as normal photographic film. Thus you view the section or cells of interest through the developed photographic emulsion and the black silver grains indicate the sites of the labeled transcripts. This is particularly useful for investigating gene expression on a cell by cell level.

It is important that the probe reaches the target, which is the mRNA of the target gene located in sample. With a tissue section this may not be much of a problem , but in whole cells or even in whole organisms then there are cell membranes which have to be crossed. The act of fixation results in cross-linking of proteins, which once again may present an obstacle to good infiltration of the probe, and finally mRNA sequences are often surrounded by proteins which may mask the target sequence.

Three common reagents used to permeabilize tissue are HCl, detergents (Triton or SDS) and Proteinase K.

The composition of the hybridization solution is critical in controlling the efficiency of the hybridization process. Hybridization depends on the ability of the oligonucleotide to anneal to a complementary mRNA strand just below its melting point (Tm). The value of the Tm is the temperature at which half of the oligonucleotide is present in a single stranded form.

The factors that influence the hybridization of the oligonucleotide probe to the target mRNA are: temperature, pH, monovalent cation concentration, presence of organic solvents. The following is a typical hybridization solution with a hybridization temperature of around 37 C and an overnight incubation period- Dextran sulphate, formamide and DTT (dithiothreitol), SSC (NaCl + Sodium citrate) and EDTA.

Other components are added to decrease the chance of nonspecific binding of the oligonucleotide probe and include: ssDNA, tRNA acts as a carrier RNA, polyA, Denhardts solution.

Following hybridization the material is washed to remove unbound probe or probe which has loosely bound to imperfectly matched sequences. Washing should be carried out at or close to the stringency condition at which the hybridization takes place with a final low stringency wash.

2.6.3.2. Protocol for cryostat sections

ISH was performed with shock-frozen pituitary and adenoma, which was cut into 8-µm sections were thaw-mounted onto sterile poly-L-lysine-coated slides, fixed in 4% phosphate-buffered paraformaldehyde and stored in 96% ethanol at 4°C until use. hTlr4 oligodeoxynucleotides pos. 308 (ODN; MWG Biotech, Ebersberg, Germany) complementary to coding parts of Tlr4 mRNA were 3'-end labeled with [α^{33}P]-dATP (NEN Life Science Inc., Boston, MA) by terminal transferase (Roche Molecular

Diagnostics, Mannheim, Germany). The sequences are 5’ AAG CTC AGG TCC AGG TTC TTG GTT GAG AAG GGG AGG TGG TCG GGG 3’. After rehydratation in PBS and a passage into 0,25% acetic anhydarate in 0,1 M triethanolamine-HCl pH 8,0/ 0,9% NaCl for 10 min, sections were dehydrate in ethanol , 2 min 70 % ethanol, 2 min 96% ethanol and finally air dried. Each section was hybridizate with a solution containing 50,000-200,000 cpm of radioactive probe, 50 % deionized formamide, 4x SSC, 10% dextrane sulfate, 100 µg/ml polyadenylic acid, 5X Denhardts, 25 mM sodium phosphate, pH 7.0 0,1 mM. After washing in 1xSSC for 10 min at room temperature (rt), 1xSCC 30 min at 55 C, 1xSCC, 0,1xSCC, for 1 min each at room temperature and dehydratation in ethanol sections were dipped in Ilford K5 photoemulsion (Ilford, Dreieich, Germany) and developed after 28 days and counterstained with toluidine blue. For negative control, a 100-fold excess of non-labeled ODN was added to the radioactive probe and applied to the adjacent section on the same slide. Human tonsils served as positive control.

2.7. Protein studies

2.7.1. Immunohistochemistry

2.7.1.1. Principle

In order to visualise the protein distribution and intensity of expression in the normal cellular and tissue environment, the method of immunohistochemistry was employed. Specific antibodies (*primary antibodies*) are used to identify the protein of interest. This antigen-antibody binding is followed by a series of immunological interactions, which lead to the enzymatic reaction that will allow the visualisation of the signal. After the primary antibody has attached to its epitope, it is linked to a secondary antibody, that can recognise and therefore bind to the epitopes of the host animal in which the primary antibody was raised; i.e. when the primary antibody is mouse monoclonal the secondary antibody is anti-mouse and so on. The secondary antibody is then linked to the enzymatic complex that will catalyze the chromogen used and visualise the signal. One system used here is the avidin-biotin system, in which the secondary antibody is conjugated to biotin beads, which can bind to the avidin-enzyme complex. The enzyme can be peroxidase or alkaline phosphatase.

2.7.1.2. Primary antibodies

The primary antibodies and dilutions used are given in Table 5. Each antibody was tested and optimised on control tissue, which was usually human normal tonsil. The standard testing dilution range was 1:50 to 1:2000.

Table.5. *List of the primary antibodies used in this group of studies.*

Antibody	Source	Host	Dilution
Tlr4	Zymed Laboratories Inc., San Francisco, CA	rabbit [a]	1:200
Human TSH	Immunotech (Marseille, France)	mouse [b]	1:500-800
Human FSH	Immunotech (Marseille, France)	mouse [b]	1:500-800
Human LH	Immunotech (Marseille, France)	mouse [b]	1:500-800
Human Alpha-Subunit	Immunotech (Marseille, France)	mouse [b]	1:500
Human PRL	Immunotech (Marseille, France)	mouse [b]	1:400
Human ACTH	DAKO (Glostrup, Denmark)	mouse [b]	1:100
Human GH	Gift from Dr C. J. Strasburger (Medizinische Klinik, Munich, Germany)	mouse [b]	1:800

a) polyclonal, b) monoclonal

2.7.1.3. Protocol for cryostat sections

Frozen normal and adenomatous pituitary tissues were cut in a cryostat (Leica CM3050 S), in 8 μm thick sections. The slides were fixed in 4% freshly prepared ice cold PFA, dehydrated and stored in 96% ethanol, at +4°C. At the time of the experiments, sections were briefly incubated in TBS for 5 min, followed by 30 min blocking. Blocking was performed using serum from the same animal species in which the secondary antibody was generated; in our case blocking solutions were consisting of horse or goat serum diluted 1:10 in TBS. After blocking the endogenous peroxidase activity with 1% H_2O_2 diluted in TBS for 15 min, sections were incubated overnight with the primary antibody at 4°C. Then, after washing three times with TBS, 5 min each, sections were incubated for 30 min with the secondary antibody at room temperature, and after 3 more washings, with the ABC complex for another 30 min. The ABC was prepared in saline-free tris-based buffer (Tris buffer) at least 30 min

prior to use, to allow complex formation. After washing three times in TBS, slides were immersed in freshly prepared DAB (1 mg/ml) supplemented with 0.01% hydrogen peroxide. Because DAB is light-sensitive, the reaction was carried on in semi-darkness, and the reagent was covered with aluminum foil during the experiment. The time of incubation in DAB varied and was determined for each primary antibody separately. The optimal time was the one which gave the strongest expected signal with the lowest possible background.

A variation of the method made use of ABC linked to alkaline phosphatase (ABC-AP) and in that case, colour was developed using the Vector Red kit. Levamisole (10 mM) was added to the reaction mixture to block the endogenous alkaline phosphatase activity. This method was used for immunohistochemical determine action of hormones in each tumour.

After the development of a signal, slides were washed 3 times in TBS and counterstained with toluidine-blue, which stains the cell nuclei pale blue, affording a good view of the tissue organisation. Excess colour was removed by immersing the slides in 70% ethanol supplemented with acetic acid, and sections were dehydrated, fixed in xylol and coverslipped using Entellan. Evaluation of immunohistochemistry was performed using the Axioscop II microscope (Zeiss).

Double IHC for Tlr4 and hormone co-localization was conducted by performing first IHC for Tlr4; visualising immunoreactivity with DAB, and then IHC for the hormone, visualising immunoreactivity with Vector Red (Vector). All the buffers used were phosphate-free. Endogenous alkaline phosphatase activity was blocked by adding 10 mmol/L levamisole (Sigma, St. Louis, MO, USA) to the Vector Red substrate.

2.8 Transfection studies

2.8.1. Plasmid preparation

The plasmid used in this study was a vector expressing the luciferase gene under the control of 770 bp of the rat POMC promoter (Liu et al., 1992).

Competent bacteria (i.e. bacteria pre-treated in such way that they posses pores and can therefore incorporate a plasmid easily), were transformed, left on agar containing ampicillin at 37°C to grow overnight, and stored at +4°C. A colony was selected from each plate and left in 250ml LB medium containing 50µg/µl ampicillin overnight at 37°C. Plasmid preparation was performed using the QIAGEN plasmid purification system.

2.8.2. Cell transfection

Cell transfection was performed on confluent AtT-20 cells grown in a 24-well plate, using Lipofectamine as follows: 500ng of POMC-Luc was mixed with 2,5µl/well of Lipofectamine in OPTIMEM1 medium, and incubated at room temperature for 45 min. During this time, the Lipofectamine neutralises the plasmid DNA and surrounds it by lipid molecules. Cells were washed twice with OPTIMEM1, and transfected with plasmid for 6 hours. At this point, the lipid molecules of the lipofectamine will fuse with the cell membrane, emptying the plasmid content into the cytoplasm. Next, the transfected cells were supplemented with fresh DMEM containing 10% FCS and left for 18 hours to recover before stimulating with LPS for 6, 12, 18 or 24 hours.

2.8.3. Reporter assay

To determine the levels of POMC driven luciferase expression after stimulation with LPS, a luciferase reporter assay was employed. Luciferase is an enzyme found in the *Photinus pyrallis*, the common firefly. Luciferase emits light in the presence of luciferin and ATP, which can be detected at 562nm wavelength.

After washing once with PBS the cells were lysed using the reporter lysis buffer and scraped. The lysates were transferred into Eppendorf tubes and centrifuged briefly. The supernatants were collected to be assayed as follows: 50µl supernatant was mixed with equal amount of ATP-containing Luciferase-assay buffer, and luciferase activity was measured in a Berthold luminometer, using 4 mg/ml luciferin as substrate. The experiments were carried out 6 times.

2.9. Stimulation and measurement of IL-6 in pituitary adenoma cell cultures

Before the measurement of basal or stimulated IL-6 production in human pituitary adenoma cell cultures, an initial culture medium was removed and the cells were washed twice with PBS. Adenoma cell cultures were then cultivated for an additional 24 hrs in serum-reduced (2% FCS) culture medium with and without (basal IL-6 production) the different stimuli as indicated. Human IL-6 was measured in the cell culture supernatants by ELISA (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions and as previously described (Pereda et al., 2000).

2.10. Hormone secretion studies

Hormones were measured by direct radioimmunassay (RIA). RIA is an method for measuring the quantity of indicator molecules, labelled with a radioisotope by counting radioactive decay events in a scintillation counter. A fixed quantity of antibody is attached to a solid support. The immobilised antibody will bind a finite

portion of added radiolabeled indicator antigen. How much antigen binds depends on the antigen concentration and the affinity of the antibody for the antigen. In the assay, the test solution of unknown antigen concentrations of unlabeled antigen for their ability to inhibit competitively the binding of the radiolabeled indicator antigen (tracer) to the immobilised antibody. The greater the content of competing antigen in the test or standard solution, the less a radiolabeled indicator antigen is bound. The results for the standard solutions of a known antigen concentration are used to derive an inhibition curve as function of antigen concentration, from which the concentration in the test sample can be calculated.

ACTH in medium was directly measured with the use of standards diluted in DMEM: A N-terminal-specific antibody was generated as previously described (Stalla et al., 1986; Stalla et al., 1989).

Rat (r) and rPRL in the medium were directly measured with reagents kindly provided by the National Hormone and Pituitary Program (Baltimore, MD).

2.11. Statistics

Statistics to determine the significance of LPS treatment on POMC promoter activity, on cell proliferation and on IL-6 expression, were performed using a t-test.

3. RESULTS

3.1. Expression of Tlr4 in pituitary cell lines

In clonal mouse, rat and human pituitary tumour cell lines Tlr4 synthesis was studied using RT-PCR with primers specific for mouse/rat or human Tlr4. Murine and human Tlr4 mRNA was detected in RNA preparations from a mouse spleen and human tonsils, which were used as positive controls (figure 3A, 3B). In rodent cell lines, apart from FS-like TtT/GF cells, Tlr4 synthesis was found in corticotroph mouse AtT20 cells, but not in gonadotroph mouse αT3-1 and lactosomatotroph rat GH3 cells. In human cell lines, production of Tlr4 mRNA was detected in FS-like PDFS cells, in hormone-inactive-like HP75 cells and breast cancer cell line MCF 7. The results show that not only FS cell lines but also some, but not all, epithelial pituitary tumour cell lines synthesize Tlr4. Expression of Tlr4 in MCF7 cells indicates, that this receptor may also has relevance in other endocrine tumour tissues.

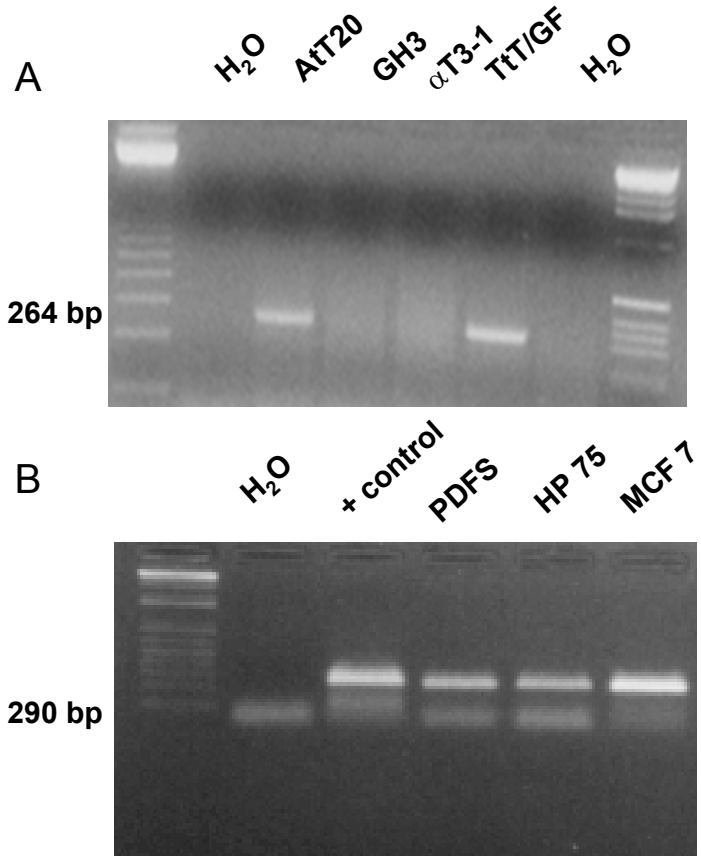

Fig. 3. *Tlr4 gene expression in mouse/rat as determined by RT-PCR (A) and human pituitary epithelial cell lines and breast cancer epithelial cell line (B). Human tonsils was used as positive control in B. The predicted amplifications products (264bp for murine Tlr4 and 290 bp for human Tlr4) are not present in negative control. As negative control was used water instead of template.*

3.2. Expression of Tlr4 in normal and tumoural human pituitary

In 3 normal human pituitaries and in 67 pituitary adenomas (table 6), Tlr4 mRNA expression was determined by ISH and protein expression was studied by IHC. In addition, in 3 of 3 normal pituitaries and in 25 of 67 adenomas, Tlr4 mRNA expression was investigated by RT-PCR.

Tlr4 mRNA expression was found in normal pituitaries by RT-PCR (figure 4) and by ISH (pictures not shown).

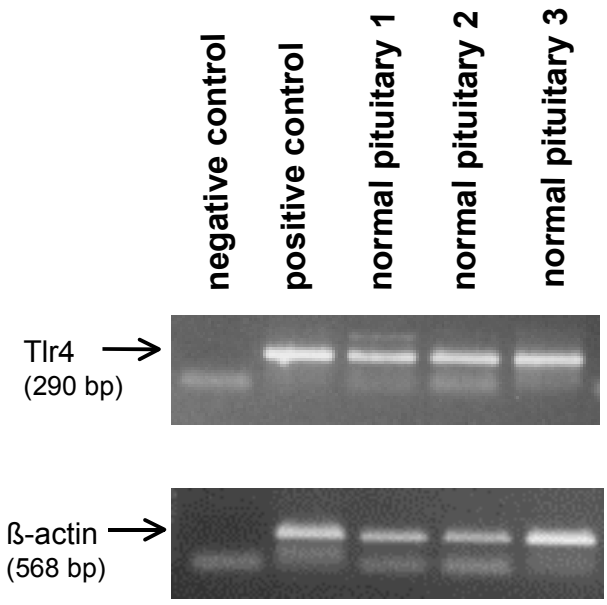

Fig. 4. *Tlr 4 gene expression in 3 normal pituitaries by RT-PCR (first panel). Positive control was a human tonsil. The predicted amplifications product 290 bp for Tlr4 is not present in negative control. As negative control was used water instead of template. B-actin in the same set of pituitaries (second panel).*

By ICH in all 3 normal pituitaries, scattered expression of Tlr4 protein was observed in the anterior pituitary and highly expressed in posterior lobe (figure 5 A, B). Double-immunocytochemical studies revealed Tlr4 expression in all types of endocrine cells, but only very few hormone-producing cells were co-expressing Tlr4 (figures 5C, D; 6 A, B, C, D).

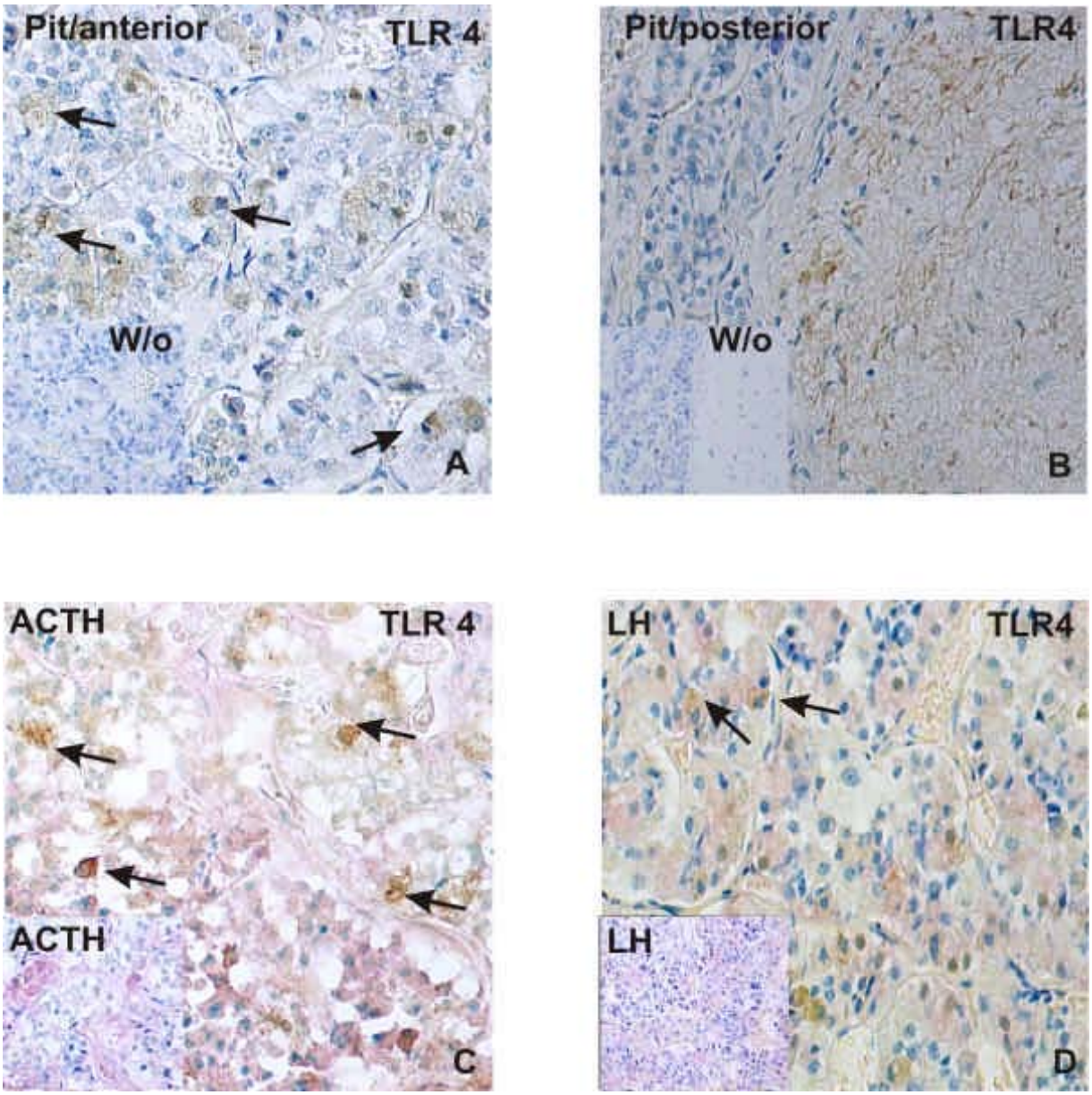

Fig. 5. *Tlr4 protein expression in human pituitary as determined by immunohistochemistry. A. Tlr4 protein expression in anterior pituitary; immunopositive cells pointed with black arrows. B. Strong immunoreactivity for Tlr4 protein (brown) in posterior lobe. Tlr4 colocalization with ACTH (C) and with LH (D) (black arrows) in normal anterior pituitary by double immunohistochemistry. All sections are counterstained with toluidine blue. Insets in pictures A, B- specificity of immunoreactivity using preabsorbed serum, inset in picture (C)-ACTH immunostaining (red) and in (D)-LH (red) immunostaining without antibody for Tlr4.*

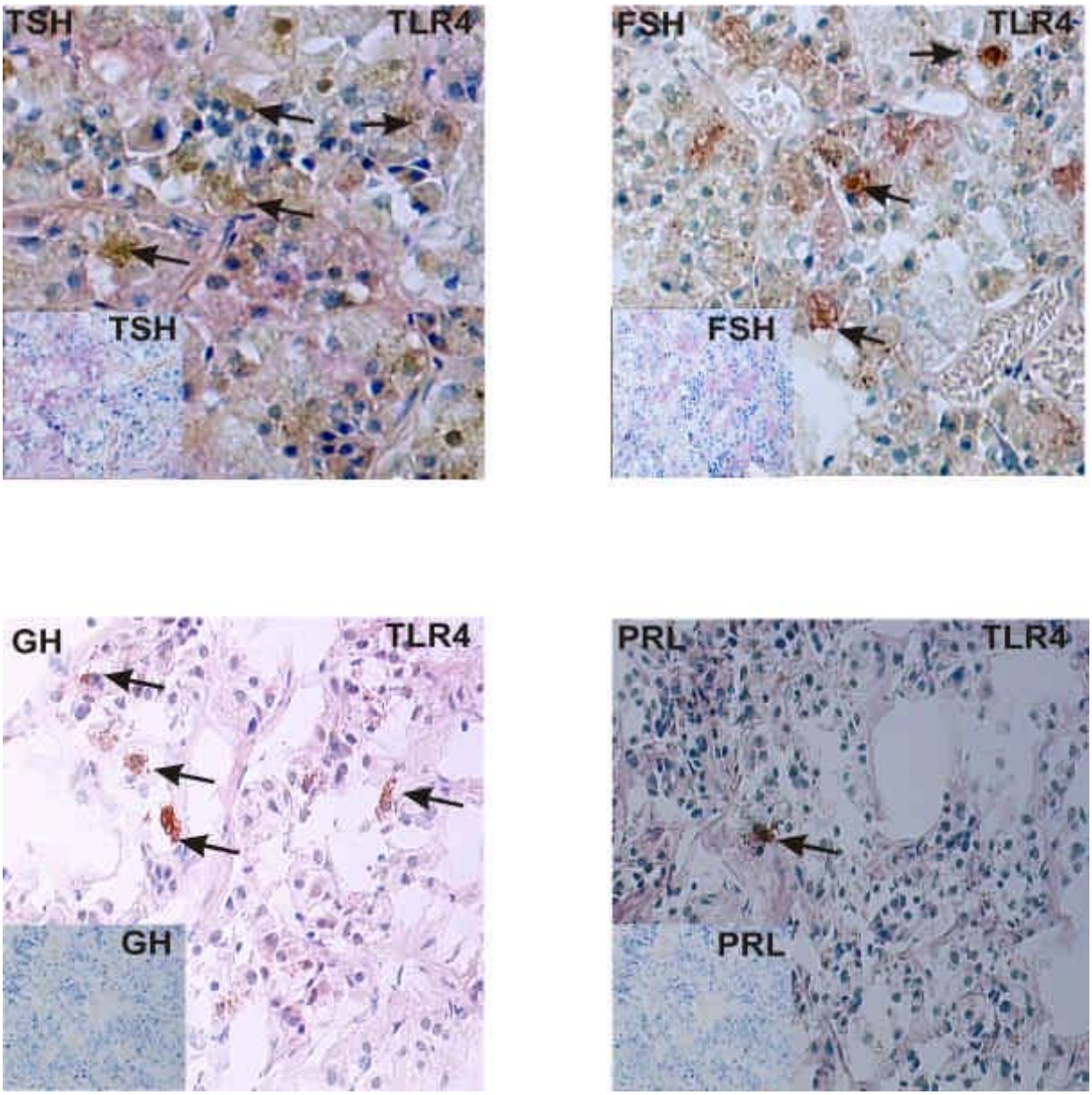

Fig. 6. *Tlr4 protein expression in human anterior pituitary by double immunohistochemistry. Tlr4 colocalization with TSH (A), FSH (B), GH (C) and PRL (D) (black arrows) in normal anterior pituitary. All sections are counterstained with toluidine blue. Inset in (A)-TSH staining (red), (B)-FSH staining (red),(C)-GH staining and (D)- PRL staining without Tlr4 antibody.*

ISH and IHC for Tlr4 were performed in all 67 adenomas (table 6). Adenomas which show Tlr4 protein expression on pituitary tumour cells by IHC were considered to represent Tlr4-positive pituitary tumours since only Tlr4 protein-expressing adenomas would functionally respond to LPS (or other Tlr4 ligands). Tlr4 protein expression was found in 26 out of 67 adenomas (table 6). In the majority of Tlr4-positive adenomas found (21 out of 26 cases), scattered Tlr4 protein expression in less than 5 % of all adenoma cells was observed whereas only a few adenomas showed higher Tlr4 immunopositivity (figures 8C and D). ISH studies on cellular Tlr4 mRNA showed only a very faint expression of Tlr4 transcripts (figures 8A and B) indicating a low synthesis- and turnover-rate of Tlr4. This may explain why only in 15 out of 26 Tlr4-immunopositive adenomas Tlr4 mRNA could be found by ISH. Despite the presence of Tlr4 protein, no Tlr4 mRNA expression could be detected in 11 of 26 adenomas. Interestingly, Tlr4 mRNA could be found in 8 adenomas, which did not express Tlr4 protein indicating disturbances at the level of transcription or protein processing. Considerable discrepancies were also observed between Tlr4 mRNA expression detected by PCR and Tlr4 protein expression. 14 adenomas in which Tlr4 mRNA was found by PCR did not express Tlr4 protein at adenoma cell level, suggesting that by PCR also Tlr4 from white blood cells may have been amplified in adenoma tissue fragments contaminated with coagulated blood.

In summary, Tlr4 protein could be detected in adenoma cells in 26 out of 67 pituitary adenomas (table 6). However, even in Tlr4-positiv adenomas, only a subset of cells represents a possible target of LPS. Tlr4-producing tumours were found in all types of pituitary adenomas (table 7) indicating no prevalent expression of Tlr4 in subgroups of hormone-producing or hormone-inactive pituitary tumours.

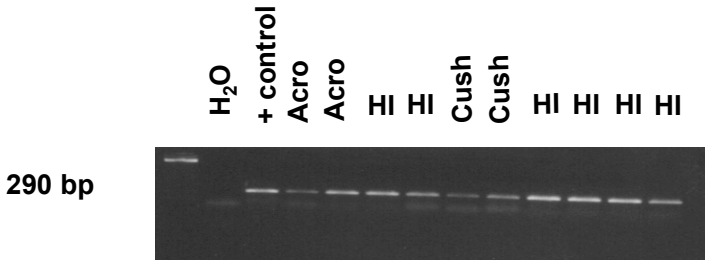

Fig. 7. *Tlr.4 gene expression in 10 representative out of 25 pituitary adenomas. Acro: acromegaly associated adenomas; Cush: Cushing's associated adenomas; HI: hormone inactive adenomas. A human tonsil was used as positive control. As negative control was used water instead of template.*

Tab. 6. *Expression Tlr4 in pituitary adenomas (list of the 67 tumours).*

	Description	***gender***	***age***	**PCR**	**ISH**	**ICH a)**	**evaluation**
1.	Acro	m	27		-	-	-
2.	Acro	f	28		+	+	+
3.	Acro	m	50	+	-	++	+
4.	Acro	m	?	+	-	-	-
5.	Acro	f	37		-	-	-
6.	Acro	f	53		-	-	-
7.	Acro	m	31		+	+	+
8.	Acro	m	29	++	-	-	-
9.	Acro	m	63		+	+	+
10.	Acro	f	71		-	+	+
11.	Acro	f	41	-	+	-	-
12.	Acro	f	38		-	-	-
13.	Acro	m	31	-	-	-	-
14.	Acro	m	30		-	-	-
15.	Acro	f	51		-	+	+
16.	Acro	m	67		-	++	+
17.	Acro	m	42		-	-	-
18.	Acro	f	76	++	-	+	+
19.	Cush	m	23	+	+	+	+
20.	Cush	f	22		+	+	+
21.	Cush	m	32	+	+	+	+
22.	Cush	f	23		+	+	+
23.	Cush	m	44		-	-	-
24.	Cush	m	14	+	-	-	-
25.	Cush	m	31	+	+	-	-
26.	Cush	f	52	++	-	-	-
27.	Cush	m	59		-	+	+
28.	Cush	m	20	++	-	-	-
29.	HI	m	80	+++	+	++	+
30.	HI	f	51		+	++	+
31.	HI	m	48		+	-	-
32.	HI	f	65	+++	-	-	-
33.	HI	m	46		+	+	+
34.	HI	m	49		+	+	+
35.	HI	m	34		-	-	-
36.	HI	m	76		-	-	-
37.	HI	m	39	++	-	-	-
38.	HI	m	47		-	+	+
39.	HI	m	59		-	-	-
40.	HI	f	51		+	++	+
41.	HI	f	30		+	-	-
42.	HI	m	52	++	-	-	-
43.	HI	m	49	+	-	-	-
44.	HI	m	53	-	-	-	-
45.	HI	m	76		-	-	-
46.	HI	m	78	+	-	-	-
47.	HI	m	60		-	+	+
48.	HI	f	61		-	-	-
49.	HI	f	59		-	+	+
50.	HI	m	35	+	-	-	-
51.	HI	f	70		-	-	-
52.	HI	f	52		-	-	-
53.	HI	m	45		-	-	-
54.	PRL	m	34		-	-	-
55.	PRL	f	41	+	-	+	+

56.	PRL	m	42		+	-	-
57.	PRL	m	41	-	-	+	+
58.	PRL	m	43	-	+	-	-
59.	PRL	m	38		+	-	-
60.	PRL	f	18	+	+	-	-
61.	PRL	f	26		-	-	-
62.	PRL	m	30		+	+	+
63.	PRL	m	43		+	+	+
64.	PRL	m	30		-	-	-
65.	PRL	f	27	-	-	-	-
66.	TSH	m	43		+	-	-
67.	TSH	f	56		+	+	+

a) Tlr4 immunoreactivity was taken as standard for the final evaluation and was determined by two independent investigators and categorized in 3 classes: (-) no Tlr4 ir, (+) weak ir (less than 10 % adenoma cells were positive for Tlr4), (++) moderate ir (10 to 30 % were positive).

Tab. 7. *Overview for Tlr4 expression in pituitary adenomas.*

Tumour type	Number of tumours	Tlr4-positive adenomas
HIA	25	8 (32%)
SA	18	8 (44%)
LA	12	4 (33%)
CA	10	5 (50%)
TA	2	1 (50%)
Total	67	26 (39%)

Abbreviations: CA, corticotroph adenomas; HIA, hormone-inactive adenomas; LA, lactotroph adenomas; SA, somatotroph adenomas; TA, thyreotroph adenomas.

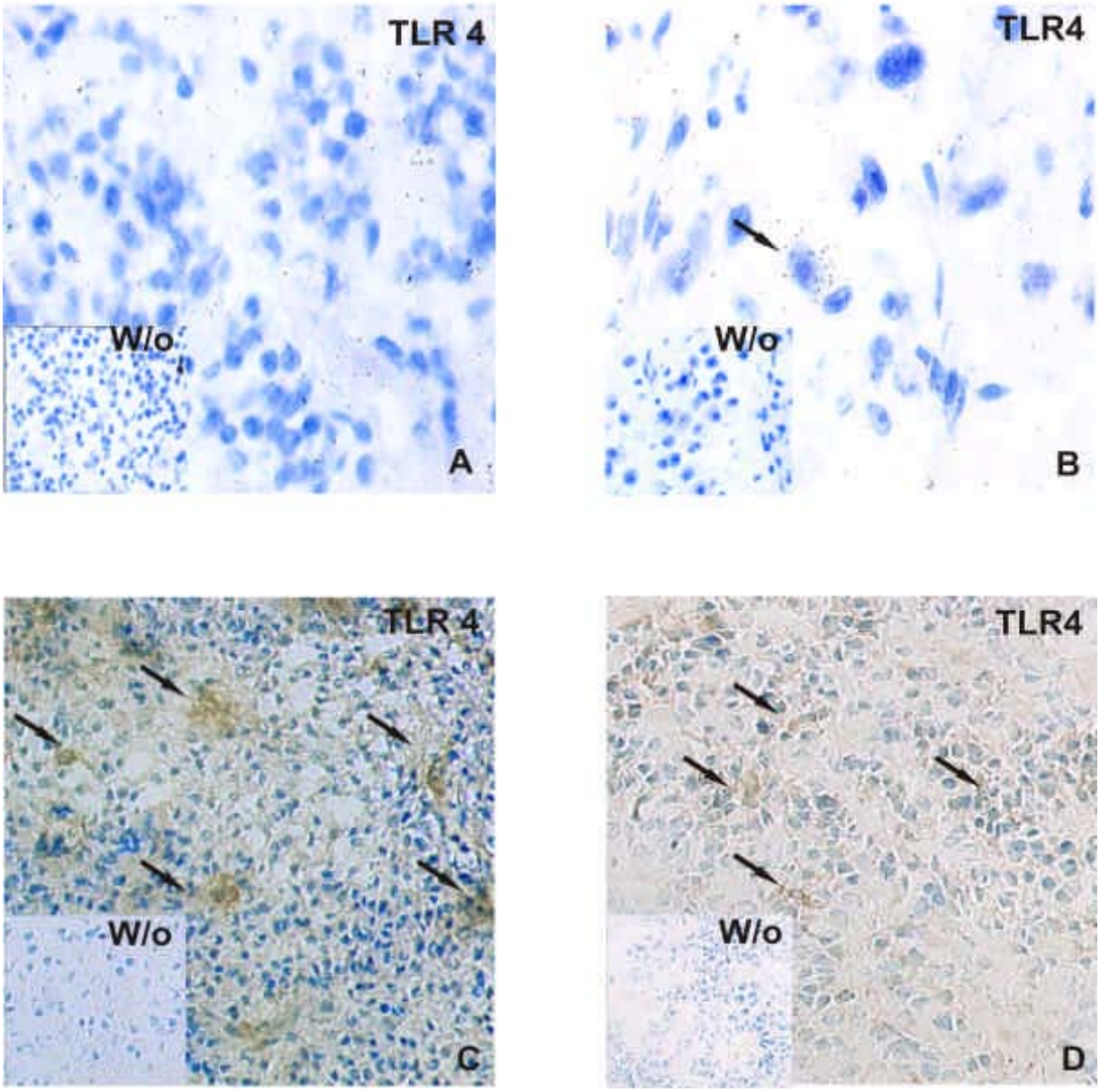

***Fig. 8**. Tlr4 mRNA (determined by in situ hybridization) and protein expression (immunohistochemistry) in human pituitary adenomas. A and B. weak Tlr4 mRNA expression, determined by ISH (silver grains). C. Moderate immunoreactivity for Tlr4 protein (brown) in Cushing tumour. D. Weak immunoreactivity for Tlr4 protein (brown) in a prolactinoma. Insets in A and B control for signal specificity using an excess of unlabeled oligonucleotides. Insets in C and D specificity of immunoreactivity using preabsorbed serum.*

3.3. Effect of LPS on proliferation in AtT20 and GH3 cells

In Tlr4-positive AtT20 cells and in Tlr4-negative GH3 cells direct effects of LPS on cell growth were studied. LPS dose-dependently inhibited the proliferation of AtT20 cells. Significant inhibition was already observed at 100 ng/ml LPS and maximum inhibitory effects were obtained after treatment of AtT20 cells with 1 µg/ml LPS(figure 9). On GH3 cells LPS did not reveal any effect on the cell proliferation (data not shown).

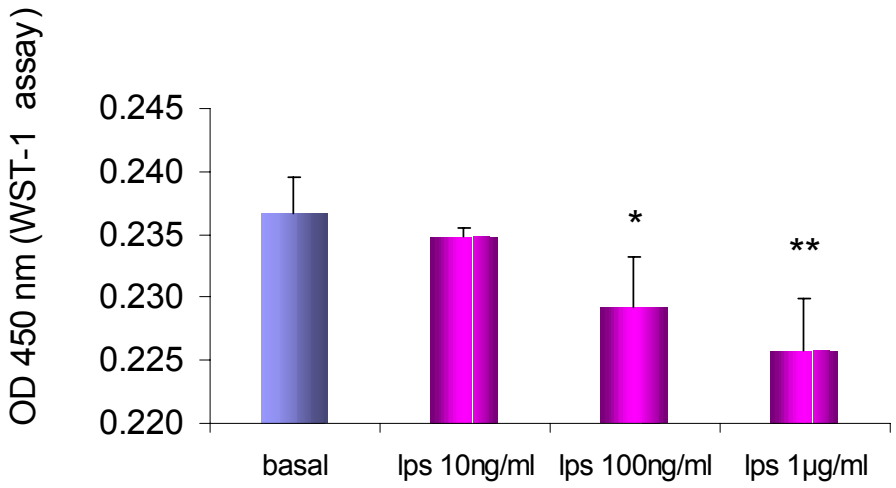

Fig. 9. *LPS inhibits cell proliferation in AtT20 cells. AtT20 cells were treated with LPS for 24 hours. Proliferation was measured by WST-1. **or p=0.003, * p=0,017 in comparison to basal.*

3.4. Effect of LPS on hormone production in AtT20 and GH3 cells

As for proliferation, LPS effects on hormone synthesis and release were comparatively studied in atT20 cells and GH3 cells. In AtT20 cells transiently transfected with a POMC-luciferase (POMC-LUC) reporter plasmid, forskolin significantly enhanced the luciferase activity whereas LPS did not induce POMC transcription (figure 10). Forskolin as a stimulator of adenyl-cyclase activates the cAMP signal trasduction pathway, which, in turn mediates hormonal secretion in AtT20 cells (Lamas et al., 1997). The release of ACTH was also not affected after short (6h)- or long (24h)-term treatment with different doses of LPS. No effect of LPS on GH or PRL secretion was also observed in GH3 cells (data not shown).

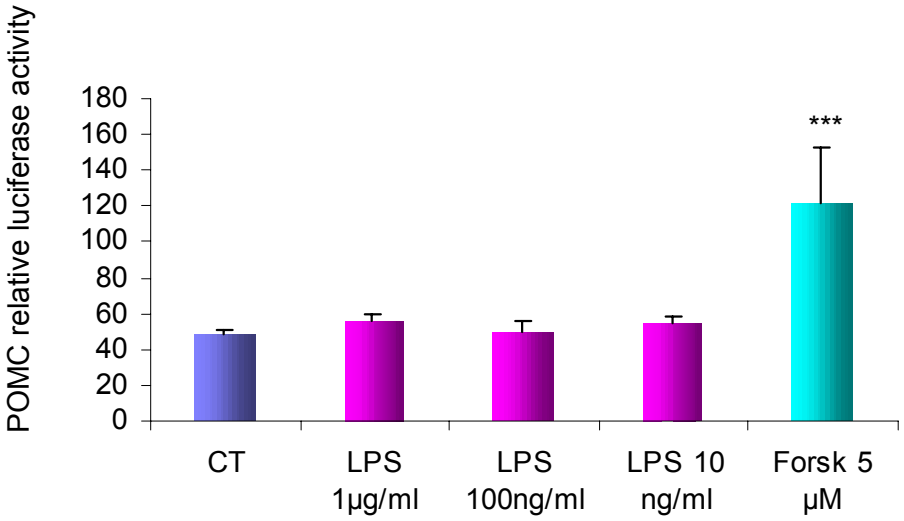

Fig. 10. *In AtT-20 cells transfected with 500 ng POMC-Luc stimulation with LPS 10, 100 ng/ml, 1µg/ml and Forskolin (Forsk) for 6 hours resulted only in increase of forskolin induced luciferase activity. *** p< 0,001 in comparison to basal activity.*

3.5. Effect of LPS on IL-6 secretion by pituitary adenoma cell cultures

The effects of LPS on growth of pituitary tumours are difficult to investigate, since pituitary adenoma cells grow very slowly in culture. Therefore the effect of LPS on IL-6 production was studied in pituitary adenoma cell cultures, since pituitary tumor cells produce IL-6 in the majority of pituitary adenomas. Among the11 pituitary adenomas, from which cell cultures were established, 3 tumours (1 Tlr4-positive and 2 Tlr4-negative adenomas) did not produce detectable amounts of basal IL-6 (table 8).

Tab. 8. *Effect of LPS on IL-6 secretion in Tlr4-positive and -negative pituitary adenomas.*

Tumour type	IL-6 secretion (pg/ml)		Tlr4
	basal	LPS [a]	
HI	n.d.	1409±315[b]	yes
HI	n.d.	n.d.	no
HI	n.d.	n.d.	no
HI	1140±58	7160±1064[b]	yes
SA	15,1±2,0	206±35[b]	yes
HI	18,5±2,3	1447±254[b]	yes
HI	278±41	1519±49[b]	yes
HI	78±12	98±18	no
HI	471±25	537±65	no
HI	211±41	229±30	no
SA	714±28	712±50	no

a) IL-6 values after 24h stimulation with 1 µg/ml LPS; b) p<0.01. n.d., not detectable

Variable basal IL-6 production ranging from 78 to 1140 pg/ml was measured in 8 adenomas, 4 of which were Tlr4-positive. LPS strongly stimulated IL-6 release in all cell cultures from Tlr4-positive adenomas even in the tumour cell culture in which no basal IL-6 secretion was detectable. In contrast, in adenoma cultures of Tlr4-negative adenomas, LPS had no effect on IL-6 secretion in IL-6-producing adenomas and could not induce IL-6 production in adenomas with undetectable basal IL-6. Dose-response studies (1ng/ml to 10 µg/ml LPS) showed that the maximal IL-6 stimulation was achieved at 1 µg/ml LPS (figure 11). Dexamethasone was a potent inhibitor of LPS-induced IL-6 secretion in all LPS-responsive adenomas. In dose-response studies (1 nM to 10 µM), 100 nM dexamethasone completely blocked the IL-6 secretion stimulated by 1 µg/ml LPS (figure 11).

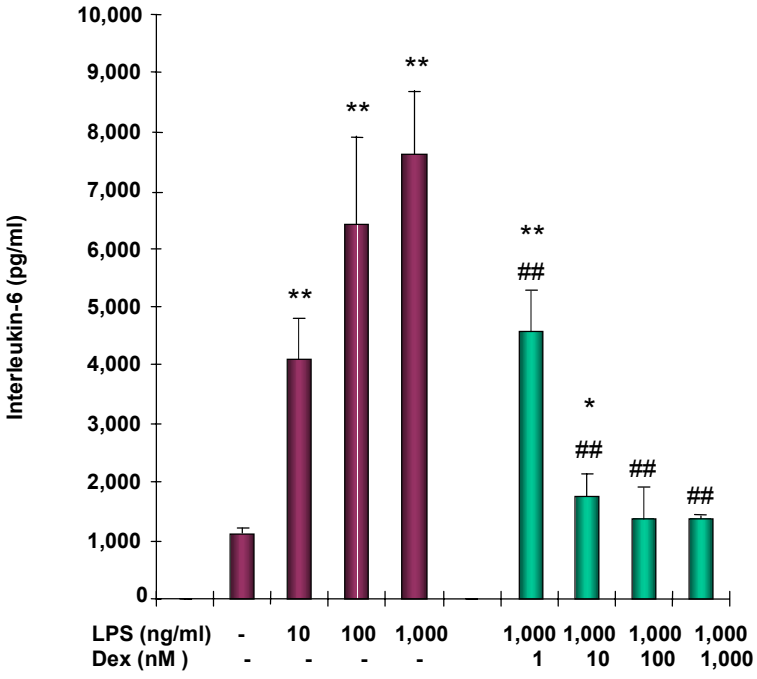

Fig. 11. *LPS-induced IL-6 secretion and its inhibition by dexamethasone in a nonfunctioning pituitary adenoma cell culture. * p<0,05; ** p<0,01 vs. basal IL-6. # p<0,05; ## p<0,01 vs. IL-6 secretion in cells stimulated with 1000ng/ml LPS.*

In 3 adenomas, in which LPS stimulated the IL-6 secretion, the effect of the p38α MAP kinase inhibitor SB203580 (20 µM) was studied. In all tumour cell cultures, SB203580 suppressed the LPS-induced IL-6 production. This suggests that similar to FS pituitary cells, the p38α MAP kinase pathway is involved in LPS-induced IL-6 secretion in pituitary adenomas.

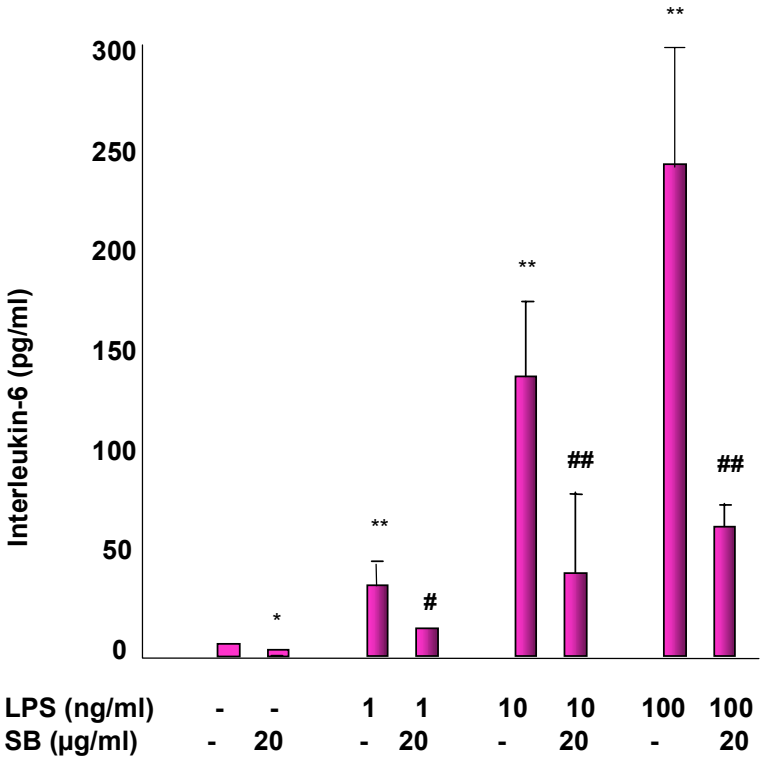

*Fig. 12. Suppression of LPS induced IL-6 secretion by the p38α MAP kinase inhibitor SB 203580 in a somatotorph adenoma cell culture. * p<0,05; ** p<0,01 vs. basal IL-6; # p<0,05; ## p<0,01 vs. corresponding IL-6 secretion by cells without SB treatment.*

3.6. Effect of paclitaxel (taxol) on the cell proliferation and hormone secretion in AtT20 cells.

Not only LPS, but also the chemotherapeutical drug taxol is supposed to act through Tlr4, at least in rodent cells. Paclitaxel (Taxol) inhibited the proliferation of AtT20 cells in dosage-dependent manner. In contrast to LPS, apoptotic changes as cell rounded and detachment were additionally observed after 24 h of treatment with taxol (Fig.13). Short-term (4 h) and long-term (24 h) treatment of AtT20 cells with taxol (10nM to 10 μM) did not significantly alter ACTH secretion (data not shown). The proliferation and cell morphology of Tlr4 negative GH3 cells was not affected by taxol treatment (data not shown).

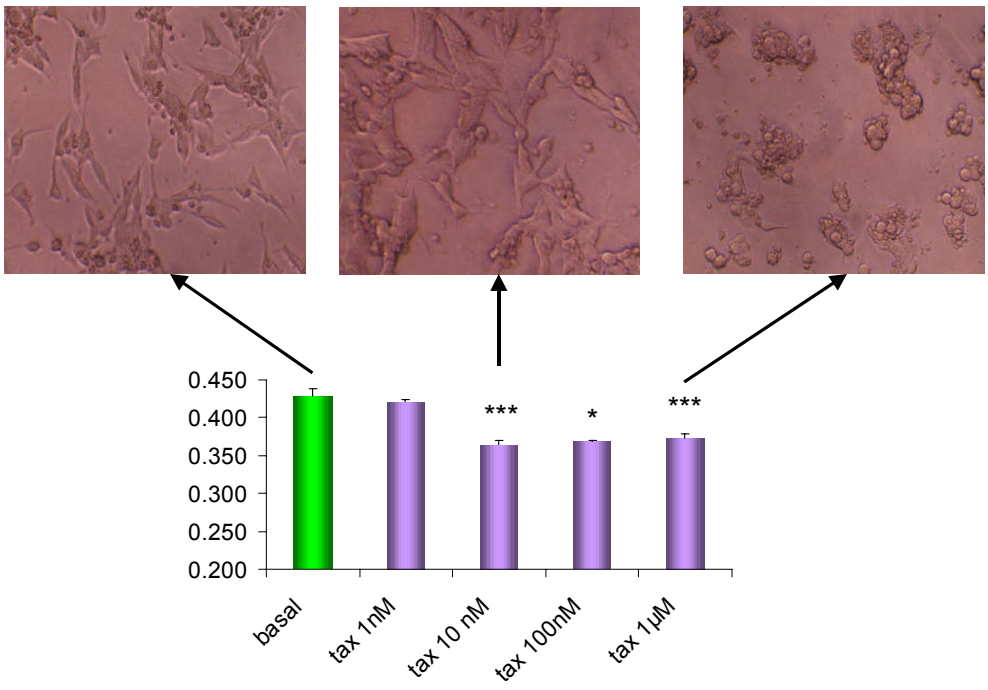

Fig.13. *Effect of paclitaxel (taxol) on the cell proliferation in AtT20. AtT20 cells were treated with taxol for 24 hours. Proliferation was measured by WST-1. *** p=0.001, * p=0,013 in comparison to basal. For basal, 10 nM, 1μM concentration of taxol were presented morphological images, which showed cellular rounding up, induced by 1 μM taxol.*

4. DISCUSSION

In the present work it has been shown for the first time that the Toll-like receptor 4 which represents the major signal transducer of bacterial lipopolysaccharide, is expressed not only in folliculostellate cells of the anterior pituitary but also in small subsets of all endocrine pituitary cells. Moreover, heterogeneous Tlr4 expression was found in 26 out of 67 pituitary tumours studied. It could also be demonstrated that the Tlr4 is functionally active in the subpopulation of Tlr4-positive adenomas and in a Tlr4-expressing pituitary cell line. This suggests that during transient or chronic bacterial infectious and inflammatory processes, circulating LPS could affect the progression of Tlr4-positive pituitary adenomas.

Only few years ago, Tlr4 has been identified as receptor and signal transducer of lipopolysaccharides, the endotoxins of gram-negative bacteria (Medzhitov et al., 1997). Tlr4 belongs to a set of germline-encoded receptors, including also other members of the Toll-like receptor family which are responsible for the innate immunity, representing the first line of host defence against infection and malignant transformation (Asea et al., 2002). Toll-like receptors are abundantly expressed in immune cells and play an outstanding role in the innate immune response to bacterial, viral, fungal and protozoan pathogens. TLR signalling can induce the production of proinflammotory cytokines and upregulate expression of costimulatory molecules, thereby activate consequently innate and adaptive immune system (Kaisho and Akira, 2002).

It was revealed by genetic analysis, that Tlr4 is a critical signal transducer for LPS (Poltorak et al., 1998), but F protein from respiratory sincytial virus is also acting through Tlr4 (Kurt-Jones et al., 2000). Interestingly, host origin fibrinogen (Smiley et al., 2001), the extra domain A of fibronectin (Okamura et al., 2001), heat shock

protein 60 (Hsp60) (Ohashi et al., 2000) and Hsp70 (Lipsker et al., 2002) all represent activators of Tlr4. It was found, that human Hsp60 and Hsp70 can activate NFκB and MAPKs through Tlr2 or Tlr4 (Vabulas et al., 2001). In addition, CD14, which interacts with Tlr4 and is needed for an optimal response of the Tlr4 to LPS, recognises apoptotic cells (Devitt et al., 2003). Thus, Tlr4 and its co-receptor CD14 not only recognise exogenous bacterial or viral pathogens, but also respond to endogenous signals of mammalian eukaryotic cells such as extracellular matrix components or stress-, apoptosis- or necrosis-associated proteins (Matzinger, 2002). Therefore, Tlr4 may belong to the factors that provide a molecular basis for recently developed hypotheses of immune activation, the so-called danger model (Matzinger, 2002) and the surveillance hypothesis (Johnson et al., 2003). In these models it is thought that the immune system does not only discriminate between self- and nonself-cells (or antigens of these). It is also activated in response to antigens that are associated with danger signals released from damaged or stressed cells after pathogen or toxin exposure, mechanical disruption and so on. Transformed cells of tumours at advanced stages also produce danger signals. Initially, many tumour cells do not appear dangerous to immune system, because they grow as healthy cells without showing changes of extracellular matrix composition or Hsp production which could both act as danger signals (Kaisho and Akira, 2002). With increasing size the tumour structure becomes less well organised and disturbances of the extracellular matrix composition occur. Moreover, cells of poorly vascularised areas within the tumour are stressed due to insufficient supply with nutrients and oxygen, resulting in an increased Hsp production. Thus ECM fragments and heat-shock proteins of progressing tumours may probably activate immune cells through Tlr4 to attack the growing tumour cells, however the tumour may have developed too far to be brought under control of the immune system.

In many organs, epithelial cell layers, in particular those with contact to the environment, represent a major side of tumourigenesis (Pardoll, 2003). It was shown that many of these epithelial cells also contain Toll-like receptors, which are thought to play a role in the induction of local inflammation as part of the host-defense mechanisms against microbial pathogens. After activation by pathogenic antigens, epithelial Toll-like receptors like Tlr4 stimulate the production of chemokines and cytokines in infected epithelial cell layers to induce invasion and migration of immune cells to the site of pathogenic attack and to induce the inflammatory response. At present it is not clear, whether Toll receptors like Tlr4 are lost or persist in epithelial cells after tumoural transformation. In epithelial tumour cell lines, among them the MCF7 mamma carcinoma cell line, Tlr4 synthesis was detected in the present work. This indicates that Toll-like receptors may still persist in transformed epithelial cells. After ligand binding, Tlr4 has been shown to activate the MAP-kinase pathways and NF-kB which both represent important intracellular signal pathways affecting both function and proliferation of cells. Thus, the question arises whether increasing levels of circulating bacterial LPS during transient or chronic infectious processes affect epithelial tumour cell proliferation and function. However, expression and potential pathophysiological functions of Toll-like receptors have so far not been studied in epithelial tumours.

In the present work, synthesis and expression of Tlr4 was studied in normal and tumoural pituitary epithelial cells. In normal anterior pituitary, scattered Tlr4 protein expression was found. Some of the cells that are positive for Tlr4 may represent FS cells which have previously been shown to express this receptor (Lohrer et al., 2000). However, by double immunohistochemical studies, Tlr4 protein expression was also detected in all endocrine pituitary cell types of the anterior pituitary but only few

corticotroph, somatotroph, thyreotroph, gonadotroph and lactotroph cells were immunopositive for Tlr4. The function of Tlr4 in these small subsets of different endocrine anterior pituitary cells remains unclear since LPS has so far not been shown to affect directly the function or growth of normal endocrine pituitary cells. In vitro and in vivo effects of LPS on anterior pituitary hormone secretion are predominantly mediated through LPS-induced, immune cell-derived cytokines of the circulation. However, LPS can also enhance through Tlr4 of FS-cells the production of intrapituitary IL-6 which can stimulate hormone release in paracrine manner, as recently demonstrated in a three-dimensional pituitary cell culture model (Gloddek et al., 2001). But apart from this modulatory role of the Tlr4 of FS cells on immune-endocrine interactions, other functions of this FS cell receptor may be thinkable which are based on the danger or the surveillance model. It is known, that the pregnancy-associated enlargement of the lactotroph cell population is reduced by apoptosis in the post-partum or post-lactation period. The cell debris of apoptotic cells is removed by FS cells through phagocytosis. Degraded ECM-products or DNA of apoptotic cells may probably act through the Tlr4 or other Toll-like receptors of FS cells to induce phagocytosis. Moreover, folliculostellate Tlr4 may be involved in hypophysitis, which is characterised by microbial pathogen-induced massive immune cell filtration of the pituitary and excessive local inflammatory processes, which damage or destroy hormone producing pituitary cells. An over-reaction of FS cells in response to pathogens may be responsible for this fatal process. Finally, Tlr4 of FS cells may play a role in the accumulation of FS cells that has been observed at the border between normal and tumoural pituitary tissue. Factors derived from the tumour (e.g. altered components of the extracellular matrix) probably act through Tlr4 to induce migration and/or proliferation of FS cells in the neighbourhood of the tumour. However, the meaning of FS cell accumulation at the tumour border is

unclear since it seems that FS cells are not able to eliminate tumour cells or to stop tumour expansion.

In contrast to anterior pituitary, a strong expression of Tlr4 protein was found in posterior lobe. Studies in the brain have shown that glial cell types like astrocytes or oligodendrocytes, but not neurons express Tlr4 and other Toll-like receptors (Bsibsi et al., 2002). Therefore, in posterior pituitary, Tlr4 may also not be synthesised by the vasopressin- or oxytocin producing neuronal cells but may be expressed in pituicytes, which represent an IL-6-producing, microglia-like cell type. The presence of Tlr4 in pituicytes may well explain the activation of IL-6 in response to the bacterial endotoxin in posterior pituitary (Spangelo et al., 1994; Hansen et al., 1999; Grinevich et al., 2003). It was further demonstrated, that IL-6 stimulates vasopressin release in human and rats (Mastorakos et al., 1994). It needs to be clarified whether during the course of infectious diseases, pathogens like LPS might influence the water homeostasis through a mechanism involving Tlr4-induced IL-6 production and subsequent paracrine vasopressin stimulation within the posterior pituitary.

In pituitary adenomas, a heterogeneous expression of Tlr4 was found. In 39% of the tumours studied, variable expression of Tlr4 was observed, whereas no receptor expression was detected in the remaining proportion of tumours. Since pituitary adenomas are monoclonal in origin, which means that they derive from a single transformed cell, the Tlr4-negative tumours may originate from a neoplastic Tlr4-negative pituitary epithelial cell. In Tlr4-positive adenomas the situation is much more complex, since only small subpopulations of cells express Tlr4. In some tumours 10 to 15% of the cells were positive for Tlr4 protein whereas in the majority of pituitary adenomas, a scattered Tlr4 expression in less than 5% of all tumour cells was observed. Based on the monoclonal concept of tumourigenesis, Tlr4-positive adenomas may have developed from normal Tlr4-negative pituitary epithelial cells

part of which have acquired the ability to express Tlr4. However, development from Tlr4-positive epithelial pituitary cells and partial loss of Tlr4 during tumour progression may also be possible. Since folliculostellate cells are absent (or extremely rare) in pituitary adenomas, this cell type does not represent the Tlr4-positive subset of cells within the tumour. At present, the reason for the different degree of Tlr4 expression or loss of Tlr4 is not yet known. Moreover, Tlr4 absence or presence was not associated with a particular pituitary adenoma phenotype.

The variability in Tlr4 expression is also reflected at the level of epithelial pituitary tumour cell lines. As expected, Tlr4 was present in both folliculostellate cell lines (mouse TtT/GF and human PDFS cells) that have been cloned so far. However, Tlr4 was found only in 2 epithelial tumour cell lines (mouse corticotroph AtT20 cells and human HP75 cells, which have been cloned from a nonfunctioning adenoma) but not in lactosomatotroph rat GH3 and in gonadotroph mouse αT3-1 cells. Although the reason for the different Tlr4 expression in epithelial cell lines is not clear, the different Tlr4 expression profile allows comparative functional studies on the effects of LPS in Tlr4-positive and –negative cells.

To study the functionality of Tlr4, investigations of effects of LPS on hormone secretion are not useful, since it has already been shown that LPS had no direct effects on hormone synthesis and release which has been confirmed in the present work (see below). Studies of LPS effects on adenoma cell proliferation are difficult to perform, because in contrast to rapidly growing pituitary tumour cell lines (see below), the proliferative index is extremely low in primary cell cultures of pituitary tumours. Therefore, the functionality of Tlr4 was examined by studying effects of LPS on the intratumoral IL-6 secretion in primary cell cultures of pituitary tumours. In contrast to normal pituitary, in which FS-cells exclusively produce IL-6, tumour cells are the major source of IL-6 in approximately 70 % of adenomas that basally produce

detectable amounts of IL-6. In the present work, basal production of IL-6 was measured in 8 out of 11 adenoma cell cultures. In Tlr4-positive adenoma cell cultures, which released IL-6 under basal conditions, LPS strongly stimulated IL-6 production. In an adenoma, which basally produced no detectable amounts of IL-6, LPS also induced the release of IL-6. Suppression of LPS-induced IL-6 by the specific p38αMAP kinase inhibitor SB203580 suggests that similar as in FS cells, the p38 α MAP kinase signal pathway is involved in Tlr4-induced IL-6 production in adenoma cells. Moreover, as already observed in FS cells, dexamethasone was a potent inhibitor of LPS-induced IL-6 production by adenoma cells. These data show that LPS can strongly stimulate IL-6 production in the subset of Tlr4-positive pituitary adenomas. IL-6 is an effector of both hormone secretion and growth of pituitary tumour cells. It has been shown that IL-6 could stimulate the secretion of ACTH in human corticotroph adenoma cell cultures and could enhance the release of GH in human somatotroph tumours. Moreover, IL-6 was shown to stimulate the proliferation of pituitary tumour cells and is therefore considered to represent a tumour progression factor. Therefore, by stimulating IL-6 in Tlr4-positive pituitary adenomas, LPS might indirectly contribute to the excessive hormone secretion and may support tumour expansion.

In epithelial pituitary cell lines, corticotroph AtT20 and nonfunctioning HP75 cells synthesised Tlr4, whereas lactosomatotroph GH3 and gonadotroph αT3-1cells were devoid of Tlr4. Tlr4-positive corticotroph AtT20 cells and Tlr4-negative lactosomatotroph GH3 cells were used to test direct effects of LPS. Both cell lines do not produce IL-6 under basal conditions or in response to LPS (unpublished observations) and, therefore, the effects of LPS will not be disturbed by autocrine acting IL-6. As expected, prolactin and GH secretion were not influenced by LPS in Tlr4-negative GH3 cells. In Tlr4-positive AtT20 cells, LPS had also no effect on

POMC mRNA synthesis and ACTH secretion. This confirms previous results from pituitary monolayer cell cultures in which LPS was not able to affect hormone release directly. In GH3 cells, proliferation was not affected by LPS but interestingly, growth of AtT20 cells was inhibited by LPS. It seems that the suppressive effect of LPS on AtT20 cell proliferation is mediated through a similar, MAP kinase-associated mechanisms as involved in LPS-induced IL-6 secretion, but this needs to be clarified in future studies.

The present data indicate that LPS plays different roles in Tlr4-expressing pituitary adenomas during inflammatory or infectious processes induced by gram-negative bacteria. On the one hand, circulating LPS could directly act anti-proliferative on Tlr4-positive pituitary tumour cells, but on the other hand, LPS could support pituitary adenoma development by stimulating intratumoral IL-6, which will then enhance growth and hormone secretion of pituitary tumour cells. However, only a small subpopulation of adenoma cells express Tlr4 in receptor-positive adenomas and only in this subset of cells LPS would inhibit growth but not influence hormone production. In contrast, in those Tlr4-positive tumours in which IL-6 is produced and strongly stimulated by LPS, both hormone production and proliferation of much more cells would be stimulated by IL-6, since not only Tlr4-positive cells but also other pituitary adenoma cells in the neighbourhood, would be affected by paracrine-acting IL-6. Therefore, the stimulatory effects of LPS-induced IL-6 on proliferation and hormone secretion may dominate upon direct inhibitory growth effects of LPS during gram-negative bacteria-induced infectious or inflammatory processes.

The situation becomes more complex if it is taken into account that during inflammation/infection, anti-inflammatory mechanisms are induced to prevent over-reactions of the activated immune system. In these processes, a rise of anti-inflammatory-acting glucocorticoids in the circulation is critically involved. Since we

observed that the synthetic glucocorticoid dexamethasone was counteracting the effects of LPS on IL-6 secretion in pituitary adenomas, the tumour-promoting activities of LPS through stimulation of intratumoral IL-6 may be reduced when glucocorticoid levels increase. Due to the divergent modes of action of LPS and the counteracting effects of glucocorticoids, at present it remains unclear to which extend LPS will affect development and pathophysiology of Tlr4-positive pituitary adenomas in vivo during gram-negative bacteria-induced infection/inflammation.

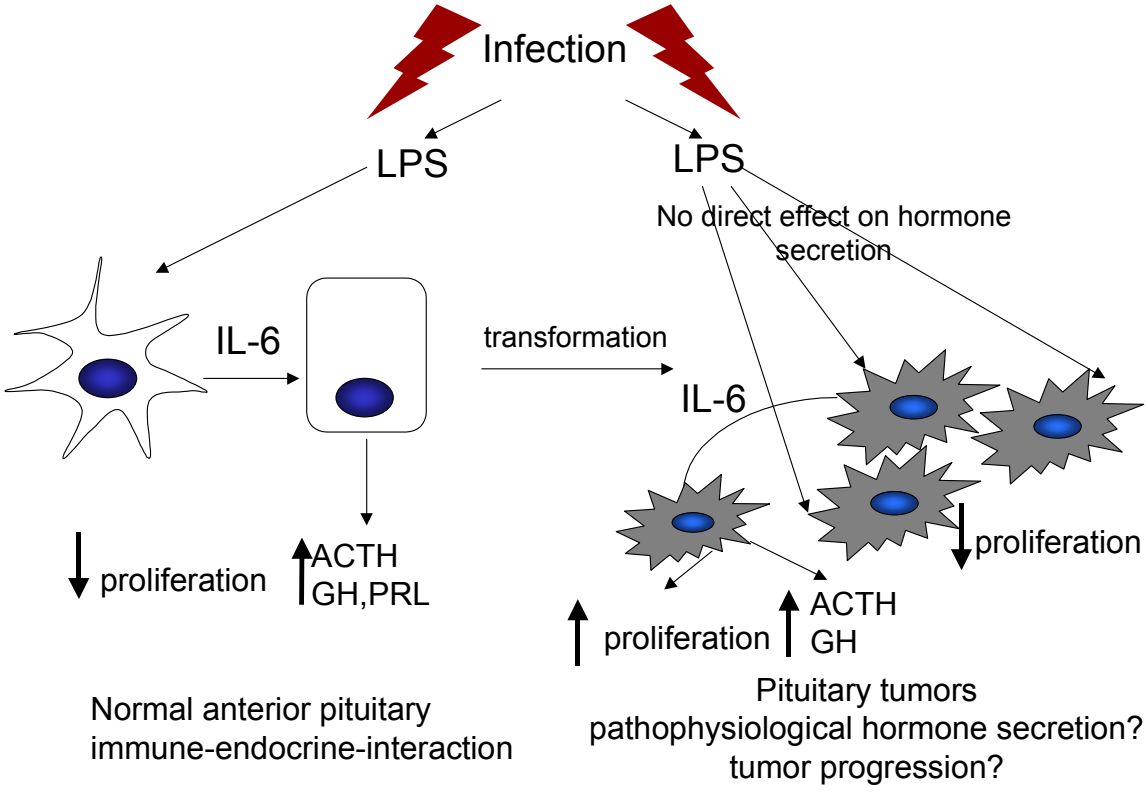

Fig. 14 *Overview about Tlr4-mediated effects of LPS in normal and tumoural pituitary cells (details described in the text).*

Tlr4 is not only mediating effects of LPS but has recently been shown to induce cellular effects in response to compounds associated with ECM degradation. It has previously been shown that pituitary adenoma development is associated with alterations of matrix-metalloproteinases (MMP-2, MMP-4) which are extracellular matrix (ECM) degrading enzymes (Paez et al., 2000). Changes in the ECM composition of pituitary tumours in comparison to normal pituitary have also been found. It needs to be clarified whether Tlr4 plays a role in the effects of ECM components on FS cells and whether alterations of the ECM composition affect IL-6 secretion by similar mechanisms in Tlr4-positive pituitary adenomas.

The Tlr4 is not only a target of microbial pathogens or “danger” signals of mammalian cells, but may also induce signals in response to pharmacological substances such as taxol. This plant product (and its derivatives) is one of the most commonly used and most effective anti-cancer drugs (Crown and O'Leary, 2000). It induces its anti-tumourigenic effect by stabilising cellular microtubules, which results in an arrest of the cell cycle at G2/M phase (Schiff and Horwitz, 1980; Horwitz et al., 1986). Moreover, taxol directly induces apoptosis in several tumour cell lines through different mechanisms, such as phosphorylation of bcl-2, activation of caspase 8 and MAPK signal transduction (Lieu et al., 1998; Chadebech et al., 1999; Yamamoto et al., 1999; Goncalves et al., 2000). In spite of the fact, that the structure of taxol is quite different from LPS (Wani et al., 1971), it shows immunostimulatory features (Byrd-Leifer et al., 2001) and it posses many LPS-like activities, such as induction of LPS-inducible gene expression (Manthey et al., 1992) and activation of NFkB (Perera et al., 1996). After taxol treatment, a significant inhibition of growth in Tlr4-positive AtT20 cells similar to the effects of LPS was observed. However, in contrast to LPS, which did not affect the morphology of mouse AtT20 cells, considerable and dose-dependent morphological changes were found after taxol treatment. This indicates

that the mechanism of LPS and taxol is partially different, probably at level of downstream signalling. Whether Tlr4 is a mediator of effects of taxol also in humans, is still a matter of debate, since from studies in immune cells of mice it seems that taxol is not directly interacting with Tlr4 but with MD-2, an adaptor protein of the active Tlr4 signal complex. In human immune cells, MD-2 is not involved in Tlr4 signal induction. Therefore, in future studies the role of Tlr4 in the mediation of taxol effects needs to be determined by comparatively stimulating Tlr4-positive and –negative mouse and human pituitary tumour cell lines with taxol.

In brief, the data shown in the present work extend previous findings on the immune-endocrine modulatory role of Tlr4 in FS cells of the normal pituitary, and indicate that Tlr4 might be involved in the pathophysiology and progression of Tlr4-positive pituitary adenomas through different mechanisms.

5. SUMMARY

In the present work, the expression and role of Toll-like receptor 4 (Tlr4) in normal and tumoural epithelial pituitary cells was studied for the first time. Tlr4 is the receptor for bacterial lipopolysaccharides (LPS) and therefore is involved in mediating the innate immune response during gram-negative bacteria-induced infection or inflammation. In addition, Tlr4 may play a role in tumourigenesis, since tumour-associated components of the extracellular matrix and heat-shock proteins are also activating ligands of this receptor.

In normal pituitary, Tlr4 is mainly expressed in folliculostellate (FS) cells and plays a role in immune-endocrine interactions, as previously shown. In the present work, sporadic expression of Tlr4 was also found in all types of endocrine anterior pituitary cells. Moreover, a heterogeneous expression of Tlr4 was also found in 26 out of 67 pituitary adenomas studied. In Tlr4-positive adenomas, in most cases a scattered expression of this receptor in less than 5% of all adenoma cells was observed. The Tlr4 expression was not linked to a particular adenoma type (hormone-active or –inactive) or phenotype (micro- or macroadenoma). The tumoural Tlr4 was functional active, since in IL-6-producing, Tlr4-positive adenomas LPS strongly enhanced the IL-6 secretion in a dose-dependent manner. The synthetic glucocorticoid dexamethasone and the p38α MAP kinase inhibitor SB203580 were potent inhibitors of LPS-induced IL-6 secretion. A heterogeneous Tlr4-expression was also observed in endocrine epithelial pituitary tumour cell lines some of which were positive (corticotroph AtT20 cells, inactive human HP75 cells) for Tlr4 whereas no expression was found in lactosomatotroph GH3 or gonadotroph αT3-1 cells. LPS did not affect hormone secretion in AtT20 and GH3 cells. In contrast, LPS suppressed growth in AtT20 cells but had no effect in GH3 cells indicating that LPS can directly inhibit proliferation of Tlr4-positive cells.

Taken together, these results suggest that during transient or chronic infection/inflammation by gram-negative bacteria, LPS may affect tumour growth of Tlr4-positive pituitary adenomas. Whether LPS inhibits or stimulates pituitary adenoma development in vivo depends on the co-expression and stimulation of IL-6 (a tumour progression factor for pituitary adenomas) by LPS in Tlr4-positive adenomas and on the onset and degree of rise of anti-inflammatory-acting glucocorticoids.

Not only LPS, but also taxol, a drug used in chemotherapy of cancer and supposed to act through Tlr4, suppressed growth of AtT20 cells. This indicates that in Tlr4-positive pituitary adenomas various ligands of Tlr4 such as bacterial or viral components, pharmacological substances or intratumoral fragments of the extracellular matrix may affect tumour development. This needs to be clarified in future studies as well as the question whether in general Tlr4 plays a role in the tumourigenesis of other types of Tlr4-positive epithelial tumours.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde erstmals die Expression und Bedeutung des Toll-like Rezeptors 4 (Tlr4) in normalen und adenomatösen epithelialen Hypophysenzellen untersucht. Tlr4 ist der Rezeptor für bakterielle Lipopolysaccharide und ist daher an der Induktion der angeborenen Immunantwort bei Infektions- oder Entzündungsprozessen beteiligt, die durch gram-negative Bakterien verursacht werden. Zusätzlich könnte der Tlr4 eine Rolle bei der Tumorgenese spielen, da Tumor-assoziierte Komponenten der extrazellulären Matrix oder Heat-Shock Proteine ebenfalls aktivierende Liganden des Tlr4 repräsentieren.

In vorhergehenden Arbeiten wurde gezeigt, dass in der normalen Hypophyse der Tlr4 vorwiegend in follikulostellaren (FS) Zellen exprimiert wird und für immun-endokrine Interaktionen von Bedeutung ist. In der vorliegenden Doktorarbeit wurde eine sporadische Expression des Tlr4 auch in allen endokrinen Vorderlappenzellen nachgewiesen. Außerdem wurde eine heterogene Expression des Tlr 4 auch in 26 von 67 untersuchten Adenomen gezeigt. In den meisten Fällen wurde in Tlr4-positiven Adenomen eine verstreute Expression in weniger als 5% aller Adenomzellen beobachtet. Die Tlr4 Expression korrelierte nicht mit einem speziellen Adenomtyp (hormonaktiv oder –inaktiv) oder Phänotyp (Mikro- oder Makroadenom). Der adenomatöse Tlr4 war funktionell aktiv, da LPS in Tlr4-positiven, IL-6-sezernierenden Adenomen die IL-6 Sekretion dosisabhängig stark stimulierte. Das synthetische Glukokortikoid Dexamethason und der p38α MAP Kinase Inhibitor SB 203580 waren potente Inhibitoren der LPS-induzierten IL-6 Sekretion. Eine heterogen Tlr4 Expresion wurde auch in endokrinen Hypophysenzelllinien gefunden, von denen manche Tlr4-positiv waren (kortikotrope AtT20 und inaktive humane HP75 Zellen), während laktosomatotrope GH3 und gonadotrope αT3-1 Zellen keinen Tlr4

exprimierten. LPS hatte in AtT20 und GH3 Zellen keinen Einfluß auf die Hormonsekretion. Im Gegensatz dazu supprimiert LPS das Wachstum von AtT20 Zellen, nicht aber von GH3 Zellen, was darauf hinweist, daß LPS direkt das Wachstum von Tlr4-positiven Zellen inhibiert.

Zusammengefasst weisen diese Egebnisse darauf hin, daß während transienter oder chronischer infektiöser bzw. inflammatorischer Prozesse, die durch gram-negative Bakterien induziert worden sind, LPS da Wachstum von Hypophysentumoren beeinflussen könnte. Ob LPS in vivo die Hypophysentumor-Entwicklung inhibiert oder stimuliert, hängt von der Co-Expression und Stimulation von IL-6 (ein Progressionsfaktor für Hypophysentumoren) durch LPS in Tlr4-positiven Adenomen ab und vom Einsetzen und dem Ausmaß des Anstiegs von anti-inflammatorisch wirksamen Glukokortikoiden.

Nicht nur LPS, sondern auch das bei Krebserkrankungen eingesetzte Chemotherapeutikum Taxol, von dem man annimmt, dass es durch Tlr4 wirksam ist, inhibierte das Wachstum von AtT20 Zellen. Das läßt vermuten, dass in Tlr4-positiven Adenomen auch noch weitere Liganden des Tlr4 wie z.B. andere bakterielle oder virale Bestandteile, Pharmaka oder intratumorale Fragmente der extrazellulären Matrix die Entwicklung von Hypophysentumoren beeinflussen. Dies muss in zukünftigen Studien noch geklärt werden, ebenso wie die Frage, ob der Tlr4 allgemein eine Rolle für die Tumorgenese in anderen Tlr4-positiven epithelialen Tumoren spielt.

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2. Co-author by Therapielexikon Endokrinologie und Stoffechselkrankheiten. Book in preparation.

Publications

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