Expression of the gene for tear lipocalin/von Ebner's gland protein in human prostate

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Abstract Northern analysis of human multiple tissue blots containing poly A^+ RNA from spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes revealed that a prostate specific transcript hybridizes to a tear lipocalin/von Ebner's gland protein (TL/VEGP) gene probe. To characterize this transcript, the corresponding cDNA was amplified by reverse transcription (RT)-PCR. Cloning and sequence analysis showed that it was identical to the tear lipocalin cDNA isolated from human lachrymal glands. Immunohistochemical analysis on thin layer sections of human prostate using a tear lipocalin specific antiserum confirmed the expression of this cDNA in prostate. Thus, our results clearly argue against a unique function of TL/VEGP in human tear fluid or saliva. The human cDNA was expressed in *E. coli* using the pQE system yielding a recombinant protein which shows biochemical properties identical to the native TL/VEGP.

Key words: Tear lipocalin; Lingual von Ebner's gland protein; Northern analysis; RT-PCR; Recombinant protein; Prostate

1. Introduction

Tear lipocalin (also called tear prealbumin or human von Ebner's gland protein, VEGP) is an intrinsic member of the lipophilic-ligand-carrier superfamily (lipocalins), abundantly produced by the human lachrymal [1] and lingual von Ebner's glands [2]. Closely related proteins have also been isolated from rat [3] and pig [4]. Within the divergent lipocalin protein family the TLs/VEGPs share high amino acid sequence similarity with a group of molecules including odorant-binding protein II of rat [5] and two putative murine pheromone-binding proteins [6]. Therefore, it was suggested that the protein produced by the von Ebner's glands might be involved in taste transduction, most likely of bitter substances [2,3]. However, no in vitro binding of these compounds to the purified rat VEGP could be detected [7], although transgenic mice expressing the rat gene showed reduced sensitivity to the bitter compound denatonium benzoate [8]. In contrast, the protein purified from human tear fluid showed binding of retinol in vitro [1] and it was demonstrated recently that a number of fatty acids, including stearic and palmitic acids and cholesterol are native ligands of TL/VEGP [9]. In addition, an inherent antimicrobial activity of this protein was described [10].

In order to provide further data on the expression of the human gene in different organs which might provide new hints as to the biological function of TL/VEGP, we have used multiple tissue Northern blot analysis. Here we report the cloning and sequencing of a TL/VEGP cDNA from human prostate. The cDNA obtained could be expressed in *E. coli* as a non-fusion protein and was active in in vitro binding of retinol.

2. Materials and methods

2.1. RNA preparation and Northern blot analysis

Total RNA from various human tissues was extracted by RNazolB (Cinna/Biotecx) which is based on the method developed by Chomczynski and Sacchi [11]. RNA was examined on 1.2% agarose-formaldehyde gels. Commercially available multiple tissue RNA blots (Clontech) were used for Northern blot analysis. Blots were hybridized for 18 h at 42°C in 5×SSPE, 10×Denhardt's solution, 2% SDS, and 100 μg/ml salmon sperm DNA with a ^{32}P-labeled TL/VEGP cDNA probe [1]. Blots were washed in 0.1×SSC, 0.1% SDS, at 50°C for 1 h.

2.2. Reverse transcription, RT-PCR analysis and molecular cloning

5 μg of total RNA was reverse transcribed using 0.5 μg of $(dT)_{12-18}$ as primer together with 200 units of Superscript II reverse transcriptase, according to the manufacturer's specifications (Gibco BRL).

An aliquot (10%) of the cDNAs was amplified by PCR using the primers 5′-TCAGACGAGGAGATTCAGGATG-3′ and 5′-ATGGGTGTCACCGATTCCAG-3′ corresponding to nucleotides 130–151 and 211–230 of the lachrymal gland specific TL cDNA sequence [1]. PCR amplification was performed by the following reaction conditions (final concentrations): 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.01% Gelatine, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM dNTPs, 50 pmol of primers in a total volume of 50 μl. Each cycle entailed denaturation at 95°C for 60 s, annealing at 55°C for 60 s and primer extension at 72°C for 60 s. PCR products were analyzed on 2% agarose gels stained with ethidium bromide. As a control the experiment was performed using RNA without reverse transcription. For cloning of the entire coding region of the prostate specific TL/VEGP cDNA RT-PCR was performed using primers 5′-GTGGACTCAGACTCCGGAG-3′ and 5′-GAGGAGCCAAGGTGTCCCC-3′.

The resulting PCR fragments were purified by electrophoresis in a 2% agarose gel and ligated into the TA cloning vector pGEM (Promega Corp., USA) using the manufacturer's protocol. Both strains of the double-stranded DNA were sequenced by the dideoxy-termination method [12] using Sequenase (USB, USA).

2.3. Immunohistochemistry

Immunohistochemistry was performed on 5 μm thick sections taken from formalin-fixed and paraffin-embedded tissues of 5 suprapubic prostatectomy specimens of patients with benign prostatic hyperplasia. Sections were deparaffinized in xylene, rehydrated in graded ethanol solutions and brought into 3% H_2O_2 in methanol to block endogenous peroxidase activity. After rinsing in TBS the sections were incubated with a polyclonal sheep anti TL/VEGP antibody [1] appropriately diluted in TBS/1% BSA/1% human serum. After rinsing in TBS, a peroxidase labeled goat-anti-sheep antibody (Sigma, München,

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; MBP, maltose-binding protein; NTA, nitrilotriacetic acid; TBS, Tris-buffered saline; TL/VEGP, tear lipocalin/von Ebner's gland protein

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Germany) was applied as a secondary reagent. The immunoreaction was developed with diaminobenzidine (DAB, Sigma). After counterstaining with haemalaun the sections were dehydrated in ethanol, cleared in xylene, mounted with coverslips and analyzed by light microscopy.

2.4. Bacterial expression of the TL/VEGP cDNA

For bacterial expression the region of the prostate specific TL/VEGP cDNA which encodes the mature protein was selectively amplified by PCR using the primers TPN: 5′-GATCGCATGCACCACCTCCTGGCCTCAGA-3′ and TPC: 5′-GAAGGATCCATCGCT CCCTGGAGAGCAGGT-3′. Primer TPN incorporates sequences for a unique *Sph*I site and an initiating methionine, TPC has a unique *Bam*HI site. The 494 bp PCR product was cloned into the *Sph*I-*Bam*HI site of pQE-70 (Qiagen Inc., USA) and *E. coli* M15 [13] was used as a host. For purification of the recombinant protein, *E. coli* cultures were centrifuged and the pellet was resuspended in 50 mM Na_2HPO_4 (pH 8.0)/300 mM NaCl at 2–5 vols./g wet weight, and passed twice through a French pressure cell (at 97 MPa). The cell extract was centrifuged at 9000×*g* for 20 min and the recombinant protein present in the supernatant was purified by affinity chromatography on Ni-NTA resin (Qiagen Inc., USA) exactly as described by the supplier. The first 15 amino acids were determined by a modified Edman-degradation protocol [14] on a gas/liquid-phase sequenator.

2.5. Retinol-binding assay

Purified TL/VEGP (1 μg/tube) was incubated with the appropriate amount of [15-^{3}H]retinol (Du Pont-New England Nuclear) in 0.1 ml of 50 mM Tris-HCl (pH 7.7) for 60 min at 4°C. After incubation, bound retinol was separated from free retinol by vacuum filtration over glass fiber filters (Schleicher and Schüll, Germany) pretreated with 0.3% polyethylenimine. Radioactivity was determined by a liquid scintillation counter. Non-specific binding was measured under the same conditions but in the presence of 0.1 mM of unlabeled retinol (Sigma, Germany).

3. Results

A cDNA probe spanning the entire coding region of the TL/VEGP encoding gene [1] was used to analyze the distribution of TL/VEGP specific mRNA in normal tissues including spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocytes. Northern blot analysis (Fig. 1) indicated a single RNA species of 0.7 kb that hybridized with the TL/VEGP gene probe only in poly(A) enriched RNA from prostate. The length of the transcript corresponded exactly to that found in the lachrymal gland [1]. No other signal could be obtained after prolonged (10 days) exposure time. RT-PCR from RNA samples isolated from the individual tissues/glands confirmed this result.

To characterize further the entire prostate specific transcript, poly(A) RNA from human prostate was reverse-transcribed and used as a template for PCR with oligonucleotide primers derived from the 5′- and 3′-non-translated regions of the TL/VEGP cDNA [1]. The resulting 570 bp DNA fragment was ligated into a TA-pGEM cloning vector and 2 positive clones from independent PCR reactions were subjected to nucleotide sequencing. The nucleotide sequence of the cloned fragments (Fig. 2) was 100% identical to the lachrymal gland specific TL/VEGP cDNA sequence [1,15].

To confirm the local expression of this cDNA immunohistochemical analysis was performed on thin paraffin sections of prostate using a TL/VEGP specific antiserum [1]. Staining with this antiserum was detectable predominantly in acini of the peripheral and transitional zone of the prostatic glands and was confined to the secretory epithelium exhibiting a polarized reactivity within the cytoplasm and the luminal surface

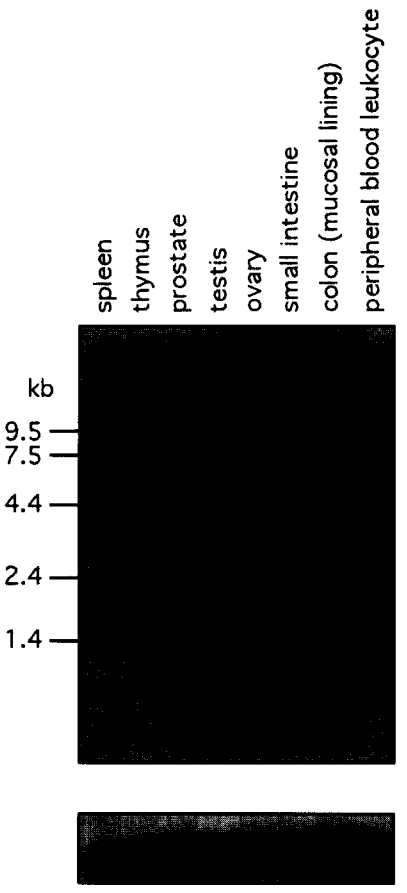

Fig. 1. Human multiple tissue Northern blot hybridized with a TL/VEGP cDNA probe. Each lane contains approx. 2 μg of poly A$^+$ RNA isolated from the tissues indicated. After stripping the blot was rehybridized with a human β-actin cDNA for a loading control (below).

of the cells (Fig. 3). No specific staining was detectable in the basal layer of the epithelium or in stromal cells.

In order to produce high amounts of TL/VEGP for further structural and functional studies the prostate specific TL/VEGP encoding cDNA was expressed in *E. coli*. In a previous study we have used the pMal system for prokaryotic expression of the lachrymal gland specific TL/VEGP cDNA. However, this approach resulted in a TL/VEGP-MBP fusion protein which did not bind to the affinity column and therefore could not be easily purified and obtained in large quantities [15]. In the present study we therefore used the pQE vector system [16] which allows production of a mature protein. For facile purification the recombinant protein was produced with a 6×His tag attached to the C-terminus. In the recombinant *E. coli* strain M15 the production of TL/VEGP was efficiently induced by IPTG and the protein was maintained soluble in the cytoplasma. SDS-PAGE analysis of cell extracts after induction of heterologous gene expression (Fig. 4) indicated the presence of an additional protein band with an apparent molecular mass of 20 kDa, which is in accordance with that calculated from the deduced amino acid sequence (including the 6×His tag). A TL/VEGP antiserum specifically reacted with this protein band (data not shown). After one-step purification by affinity chromatography using an Ni-NTA resin [17], the protein was subjected to N-terminal amino acid sequence analysis and the sequence found was: MHHLLAS-

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                                    gtggactcagactccggag   -1
ATGAAGCCCCTGCTCCTGGCCGTCAGCCTTGGCCTCATTGCTGCC    45
CTGCGGCCCACCACCTCCTGGCCTCAGACGAGGAGATTCAGGATG    90
TGTCAGGGACGTGGTATCTGAAGGCCATGACGGTGGACAGGGAGT   135
TCCCTGAGATGAATCTGGAATCGGTGACACCCATGACCCTCACGA   180
CCCTGGAAGGGGGCAACCTGGAAGCCAAGGTCACCATGCTGATAA   225
GTGGCCGGTGCCAGGAGGTGAAGGCCGTCCTGGAGAAAACTGACG   270
AGCCGGGAAAATACACGGCCGACGGGGGCAAGCACGTGGCATACA   315
TCATCAGGTCGCACGTGAAGGACCACTACATCTTTTACTGTGAGG   360
GCGAGCTGCACGGGAAGCCGGTCCGAGGGGTGAAGCTCGTGGGCA   405
GAGACCCCAAGAACAACCTGGAAGCCTTGGAGGACTTTGAGAAAG   450
CCGCAGGAGCCCGCGGACTCAGCACGGAGAGCATCCTCATCCCCA   495
GGCAGAGCGAAACCTGCTCTCCAGGGAGCGATTAGggggacacct   540
tggctcctc                                        549
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Fig. 2. Nucleotide sequence of the human prostate specific TL/VEGP cDNA. The primers used for RT-PCR span the entire coding region of the cDNA and are shown in lower-case letters.

DEEIQDVS. With the exception of the N-terminal methionine, which is a product of the ATG codon of the expression vector, this sequence is identical to the deduced amino acid sequence and that of the native protein [1]. The amount of purified recombinant protein obtained was about 15 mg/l *E. coli* culture.

Since it is known that native TL/VEGP binds retinol in vitro, we tested the recombinant protein for its ability to bind [^{3}H]retinol. Analysis of binding assays indicated that it binds retinol with an apparent K_D of 0.4 μM, which is almost identical to that found with the native protein purified from human tear fluid [1].

4. Discussion

The data presented in this paper clearly demonstrate that human TL/VEGP is produced by the prostate, thus they are in contrast to previous reports concluding that it is a tear fluid and saliva specific protein [2,9,18]. TL/VEGP is one of the rare examples within the lipocalin superfamily where the protein was isolated bound to its native ligands. Recent work clearly demonstrated that the protein purified from human tear fluid contains fatty acids, cholesterol, fatty alcohols as well as glyco- and phospholipids [9]. Together with our previous results demonstrating in vitro binding of retinol this protein has to be defined as broad substrate binding lipocalin.

There is good evidence that its biological function might also be multifunctional. The significant amino acid sequence similarity to odorant-binding proteins and the high expression by the lingual von Ebner's glands, which are intimately associated with taste buds of circumvallate and foliate papillae, have suggested a function of this protein in taste transduction [2]. Although binding studies with several taste compounds failed to demonstrate a specific role as a tastants carrier, transgenic mice which normally lack the presence of a homologous protein, showed reduced sensitivity to the bitter compound denatonium benzoate when expressing the rat gene [8]. This effect which indeed indicates a certain role in taste transduction may however be due to a more indirect function of TL/VEGP. It was demonstrated recently that a lipoprotein composed of phosphatidic acid and β-lactoglobulin, another lipocalin member with significant amino acid similarity to TL/VEGP, selectively suppressed the taste response to bitter substances in frog and man, obviously by binding of the lipoprotein complex to the hydrophobic sites of the receptor membranes [19]. A similar function might be proposed for TL/VEGP in von Ebner's saliva.

In tear fluid this protein is associated with a number of lipophilic compounds normally present in the tear lipid layer. Therefore, it could promote surface spreading of this lipid film or prevent hydrophobic molecules from contaminating the underlying mucous layer of the ocular surface important for preventing the disruption of the tear film [9].

A biological function of the prostate specific TL/VEGP has yet to be defined. The high amino acid sequence similarity to putative pheromone-binding proteins is noteworthy, but does not necessarily implicate an action as a pheromone carrier.

Fig. 3. Immunohistochemical staining in the peripheral zone of prostatic glands with a TL/VEGP specific antiserum. The reactivity is confined to the cytoplasm and luminal surface of the secretory epithelial cells (×600).

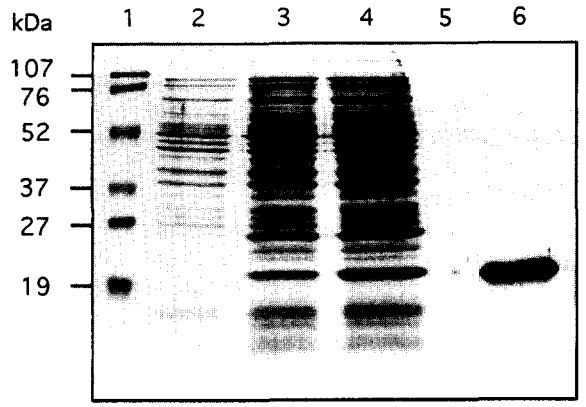

Fig. 4. Production of rTL/VEGP in *E. coli* and purification of the recombinant protein as analyzed by 0.1% SDS-12% PAGE. Lanes: 1, molecular mass markers; 2, *E. coli* cell extract prior to induction; 3,4, *E. coli* cell extracts after induction with IPTG for 3 and 5 h, respectively; 6, 10 μg of recombinant protein after purification by Ni-NTA affinity chromatography. The gel was stained with Coomassie Blue.

There are several other biochemical features of this protein that might be of relevance for a prostate specific function. Thus, both proteins, isolated from humans and from pig, showed binding of retinol in vitro [1,4], therefore an extraplasmatic carrier function for this important biological ligand might be considered. Due to its broad ligand specificity, its abundance and distribution TL/VEGP would also be ideally suited for the clearance of lipophilic, potentially harmful substances and might therefore act as a protection factor of epithelia. On the other hand, there are several indications that this protein exhibits antibacterial activity [10,20]. It is known that fatty acids exert antimicrobial effects and can inactivate enveloped viruses [21,22]. Thus, the antimicrobial activity could be mediated by the lipid ligands rather than the protein itself.

In summary, our present investigations clearly argue against a specific role of TL/VEGP in taste transduction or a unique function in stabilizing of the tear film. The production of large amounts of recombinant protein by the method described should allow a more detailed characterization of this lipocalin member.

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