Lactotrophs: The new and major source for VEGF secretion and the influence of ECM on rat pituitary function in vitro

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Abstract. Vascular endothelial growth factor (VEGF) plays a pivotal role in pituitary endocrine function by influencing fenestration and blood vessel growth. Folliculostellate (FS) cells, which represent only a small number of pituitary cells, are recognized to produce VEGF. Tissue sections and primary pituitary cell cultures from rat pituitary glands were performed to co-localize VEGF and pituitary lactotrophs, which represents nearly 50\% of all pituitary cells, by immunofluorescence. VEGF is co-localized with prolactin-producing cells in vivo and in vitro. FS cells are present infrequently in vivo (1.6\%) and in vitro (2.4\%). Culture supernatants were analyzed for the presence of VEGF by ELISA. VEGF levels are always significantly lower in supernatants from the cells that are seeded on Matrigel extracellular matrix (ECM) compared to the cells grown on plastic. Lower VEGF concentrations in supernatants from the pituitary cells cultured on ECM may reflect a more adequate cell environment compared to culture on plastic. These results demonstrate for the first time, that VEGF is expressed by lactotrophs, which outnumber FS cells. These results are of potential clinical relevance especially in oncology for the interpretation of studies investigating anti-angiogenic treatment of pituitary tumors.

Introduction

Folliculostellate (FS) cells are the first cell type in which vascular endothelial growth factor (VEGF) production was detected. Until now, FS cells are believed to be the only cell population within the pituitary gland to produce VEGF (1,2). S-100 is a reliable marker for FS cells (3). Vankelecom \textit{et al} (4) described that ~7.5\% of cultured mouse pituitary cells were stained for the presence of S-100. It has been suggested that FS cells perform several supportive functions. They are involved in the regulation of phagocytosis (5), and produce various growth factors (6,7) and cytokines (4,8). Recent studies suggest that they represent pluripotent adult stem cells (9). \textit{In situ} hybridization has demonstrated homogeneous signals of VEGF mRNA in rat pituitary, with an upregulation of VEGF expression in estrogen-treated animals (10). Western blot analysis and RT-PCR of anterior pituitary tissues showed the presence of VEGF164 and VEGF120 (10). Jabbour \textit{et al} (11) described that only scattered positive FS cells are present within the sheep pituitary. They identified 20\% that were double-stained cells for VEGF and S-100; ~80\% of cells that were stained only positive for VEGF, but were unidentified.

It is well known that extracellular matrix (ECM) plays an important role in studying cellular function in vitro. In the physiological environment, like ECM, cytokines and hormones, are absent under culture conditions of the pituitary cells. Laminine and type IV collagen are typical ECM constituents in both the epithelial and vascular basement membrane of the pituitary gland (12,13). The ECM components are able to influence the morphological appearance, growth behavior and migratory activity of epithelial cells. Both, tumor and normal cells of epithelial origin (lactotrophs), are more likely to reflect their \textit{in vivo} counterparts when maintained on ECM, instead of uncoated plastic (14). ECM components also influence pituitary function \textit{in vivo} (15,16).

The present study was designed to identify VEGF producing cells, other than FS cells, in the anterior pituitary gland of rats \textit{in vivo} and \textit{in vitro} and to investigate the influence of ECM (Matrigel) on VEGF production in primary pituitary cell cultures.

Materials and methods

\textit{Ethics statement}. The present study was performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the local Ethics Committee for animal experiments of RWTH University of Aachen, School of Medicine (permit no. 1077 A4; 2001). All the experiments were performed without suffering of the animals.

\textit{Animals}. Adult female Sprague Dawley rats of 250 g body weight were used, bred from a colony at the Institute for animal research (RWTH University of Aachen, Germany). Rats were housed in groups of four per cage in a quiet and light-regulated environment.

\textit{Vascular endothelial growth factor (VEGF) production was detected. Until now, FS cells are believed to be the only cell population within the pituitary gland to produce VEGF (1,2). S-100 is a reliable marker for FS cells (3). Vankelecom \textit{et al} (4) described that ~7.5\% of cultured mouse pituitary cells were stained for the presence of S-100. It has been suggested that FS cells perform several supportive functions. They are involved in the regulation of phagocytosis (5), and produce various growth factors (6,7) and cytokines (4,8). Recent studies suggest that they represent pluripotent adult stem cells (9). \textit{In situ} hybridization has demonstrated homogeneous signals of VEGF mRNA in rat pituitary, with an upregulation of VEGF expression in estrogen-treated animals (10). Western blot analysis and RT-PCR of anterior pituitary tissues showed the presence of VEGF164 and VEGF120 (10). Jabbour \textit{et al} (11) described that only scattered positive FS cells are present within the sheep pituitary. They identified 20\% that were double-stained cells for VEGF and S-100; ~80\% of cells that were stained only positive for VEGF, but were unidentified.

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concentration of 300,000 cells/well. Cells were incubated into 24-well plates (tpp tissue culture plates, sigma) at a

pellets were incubated for 5 min with a trypsin inhibitor (type I-s, 5 mg/5 ml medium) was added for 1 min. After centrifugation at 500 g for 2 min, supernatants were removed and cell pellets were washed 3 times and then cut into small pieces.

Materials. Cell culture materials and reagents were obtained from Greiner Labortecnik (Sologen, Germany), Falcon (Heidelberg, Germany), Seromed (Berlin, Germany) and Sigma (Deisenhofen, Germany). Matrigel was obtained from Becton-Dickinson (Heidelberg, Germany).

Cell culture. Rat pituitary monolayer cell cultures were generated following a modified protocol (17). In brief, the pituitaries were washed and then cut into small pieces.

Cell dissociation was performed with trypsin-type III (25 mg/5 ml medium) for 15 min at 37°C. DNase type V solution (10 mg/5 ml medium) was added for 1 min. After centrifugation at 500 g for 2 min, supernatants were removed and cell pellets were incubated for 5 min with a trypsin inhibitor (type I-S, 5 mg/5 ml medium) and again centrifuged for 2 min. The cells were then incubated in EDTA solution, 2×5 min in 2 mM EDTA and 2×5 min in 1 mM EDTA. The digested pieces of the pituitary were dispersed and cell viability was determined by trypan blue exclusion. After counting, the cells were seeded into 24-well plates (TPP tissue culture plates, Sigma) at a concentration of 300,000 cells/well. Cells were incubated at 37°C and 5% CO₂ in medium 199 with 2.2 g/l NaHCO₃ supplemented with 10% FCS (inactivated for 1 h at 56°C), 2 mM glutamine and penicillin/streptomycin (10⁵ U/10⁵ µg/ml). After 2 days the medium was changed. On day 4, monolayers were washed with PBS and serum-free culture medium was added for 24 h to dilute the serum. On day 5 serum free media were replaced for another 48-h incubation period.

Culture on Matrigel and plastic. Accordingly, cells were cultivated on Matrigel (Becton-Dickinson). Matrigel was diluted 1:1 with a serum-free medium. Culture plates were covered with Matrigel solutions and incubated for 1 h at 37°C for polymerization. Cells were seeded on top of the gel. Supernatants from Matrigel-coated wells as well as from plastic, were pooled (12-wells/culture), centrifuged and stored at -20°C for VEGF quantification.

Immunohistochemistry. Immunohistochemistry was performed on formalin fixed (fixation with buffered formalin, pH 6.5-7.2) paraffin-embedded pituitary gland sections (4 µm) and cryostat sections of resected pituitary glands (6 µm). A streptavidin-biotin-peroxidase method was employed. Paraffin sections were deparaffinized and rehydrated in PBS. Cryosections were fixed in acetone (4°C for 10 min). Cell cultures were fixed in methanol (4°C for 10 min). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 30 min. Paraffin sections were incubated with trypsin (Dako, Hamburg, Germany) for 15 min. For negative controls, phosphate-buffered saline (PBS, Dulbecco) diluent, without Ca²⁺ and Mg²⁺, containing 1.5% bovine serum albumin (BSA) replaced the primary antibody. In addition, rabbit immunoglobulin G (IgG; Dako), goat IgG (Dianova, Hamburg, Germany) and normal mouse IgG (Dianova) were used at the identical concentration as the primary antibody. Human endometrium of mid-luteal phase, from a patient of proven fertility, served as a positive control for VEGF staining. Endometrial tissue block was used with the informed consent of the participant patient (18).

Primary antibodies (Table I) were applied overnight at 4°C for paraffin embedded sections, whereas cryosections, cytospin-preparations of pituitary cell suspension (detection of lactotroph and Fs cells) and cultured cells were incubated for 4 h. We used the LSAB2-kit (kit for use on rat specimens, Dako) for primary mouse and rabbit antibodies. Visualization of specific antigens was performed by peroxidase-catalyzing substrate and converting with chromogene aminoethyl carbazole (AEC; Zymed Laboratories Inc., San Francisco, CA, USA) to a red-colored deposit.

Double staining of VEGF and prolactin. Staining was performed on 24-well culture plates. After removing culture media, cells were fixed with methanol (4°C for 10 min), rehydrated with PBS and blocked with a donkey serum (dilution 1:20 in PBS), followed by an overnight incubation with prolactin antibodies (dilution 1:30 in PBS/2% rat serum, Santa Cruz, CA, USA) at 4°C. The secondary antibody (TRITC, donkey anti-goat, Dianova) was diluted 1:100 in PBS (pH 8.2) containing a 2% rat serum and was incubated for 30 min. After three rinses with PBS, the cells were blocked with goat

| Antibody | Antigen | Dilution | Source |
|----------|---------|----------|--------|
| Monoclonal antibodies | S-100 | S-100 protein | 1:100 | BioGenex, Hamburg, Germany |
| Polyclonal antibodies | Prolactin (rabbit) | Prolactin | 1:1000 | Biotrend, Köln, Germany |
| | VEGF (147) (rabbit) | VEGF | 1:30 | Santa Cruz Biotechnology, Heidelberg, Germany |
| | S-100 (rabbit) | S-100 protein | 1:300 | Dako, Hamburg, Germany |
| | Prolactin (M-19) (goat) | Prolactin | 1:30 | Santa Cruz Biotechnology, Heidelberg, Germany |
serum (dilution 1:20 in PBS, 10 min), followed by incubation with an anti-VEGF-A antibody (dilution 1:30 in PBS/2% rat serum, Santa Cruz) overnight at 4°C. The secondary antibody (FITC, goat anti rabbit, Linaris) was diluted 1:50 in PBS (pH 8.2+2% rat serum) for 30 min. The cells were incubated with KCl (200 mmol) for 5 min and then with DAPI (0.2 µg/ml PBS pH 7.0, Sigma) for 1 h and finally mounted with medium for fluorescence analysis (Vector Laboratories, Burlingame, CA, USA).

For negative controls phosphate-buffered saline (PBS, Dulbecco, w/o Ca++, w/o Mg++) containing 2% rat serum replaced the primary antibody. Rabbit IgG (Dako) and goat IgG (Dianova) were applied at equal concentrations as the primary antibody.

**VEGF detection within culture medium.** VEGF was quantified by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany; test validations according to the provider's specification), which is able to identify the VEGF-164 and VEGF-120 isoform of the mouse. Cross-reactivity (>95%) to the corresponding rat VEGF enables the application of this ELISA. Protein detection is done by the Lowry-test.

**Identification of FS.** FS cells, identified by monoclonal and polyclonal antibodies against S-100 protein (Table I), were counted on cytospin-preparations of dispersed pituitary cells, after seven days of culture and on cryostat sections as well as on formalin-fixed and paraffin-embedded sections of the pituitary gland.

**Estimation of cell type distribution.** For semi-quantification analysis of the different cell types 10 high-power fields were analyzed.

**Statistical analysis.** Experiments were repeated six times. The Mann-Whitney U test was used for statistics. Data are shown as means ± standard deviation.

Illustrations were performed with Axiovert 135 microscope (Zeiss, Göttingen, Germany). We performed quadruple overlay of immunohistochemical analysis as follows: i) immunocytochemical detection of prolactin; ii) immunocytochemical detection of VEGF; iii) DAPI counterstaining; iv) light microscopic picture.

**Results**

**FS in vivo and in vitro.** FS cells are identified by two different antibodies against S-100 protein. Both antibodies, monoclonal (BioGenex, Hamburg, Germany) and polyclonal (Dako), showed similar results. S-100 positive FS cells are infrequently detected in cryostat (Fig. 1A) and paraffin sections (data not shown) of the anterior pituitary gland. Approximately 1.6% of the pituitary cells stain positive for S-100. We also performed a cytospin-preparation of dispersed pituitary cells (before seeding into culture wells) to identify S-100 positive cells by immunocytochemistry. Only few FS were identified (Fig. 1B), even after seven days of culture only the scattered stained cells were visible (2.4% S-100 positive cells; Fig. 1C).
**VEGF and prolactin in vivo and in vitro.** Results demonstrate a uniform and nearly identical staining pattern for VEGF and prolactin on paraffin serial sections of pituitary cells (Fig. 2A and B). Detection of lactotroph cells on cytospin-preparations was performed in pituitary cell culture (Fig. 1D). Approximately 50% of the cells stained were positive for prolactin. After seven days of culture, the cells with small nuclei (functional pituitary cells) were positive for VEGF, as well as for prolactin (Fig. 2C and D). Cells with large nuclei (fibroblasts, positive for vimentin, data not shown) were negative for VEGF and prolactin. The staining demonstrate that groups of cells are positive for both prolactin and VEGF (Fig. 2E on Matrigel and F on plastic). Few cells were stained only for VEGF. Nearly 21% of the cells were completely negative for both proteins. The staining pattern of cells cultured on plastic, did not differ from those cultured on Matrigel, although the staining intensity for VEGF was appreciably less in the cells cultured on ECM. Negative control...
VEGF production in primary rat pituitary cell culture. ELISA results reveal less statistically significant VEGF production (P<0.0022) of cells cultured on Matrigel (724±243 pg/mg protein) than uncoated wells (Fig. 4). Values are means ± standard deviation. VEGF, vascular endothelial growth factor; ECM, extracellular matrix.

Discussion

This study demonstrates for the first time, since the identification of VEGF in FS (1) that lactotrophs are the main source of VEGF production in the normal pituitary gland. Jabbour et al (11) identified scattered positive FS cells within the sheep pituitary. Approximately 80% of non-FS cells were stained positive for VEGF and remained unidentified. Lactotrophs apparently are the main source of VEGF-production in the pituitary gland and represent a significant portion of the unidentified population.

Here, we demonstrate that FS cells are only infrequently present in vivo (1.6%, paraffin section of rat pituitary), after dispersing cells in cell culture (cytospin-preparation) and in vitro (2.4%), as identified by two different antibodies against S-100 protein. Our results differ from the data of Vankelecom et al (4), where they identified 7.5% FS cells in mouse pituitary using another antibody against the S-100 protein. This can be explained by the fact that the quantity of pituitary FS cells varies between different rat strains such as Fischer 344 and Sprague Dawley rats used in this study. It has been described that Fischer 344 rats have significantly more FS cells (19). Based on studies, that estrogen-induced prolactin secreting tumors in the F344 rat are associated with upregulation of VEGF, it was suggested that lactotrophs may be responsible for VEGF production (20). Our immunohistological detection of widespread VEGF protein signals on cryostat as well as paraffin sections of the anterior pituitary gland confirm the results of Ochoa et al (10). They detected a uniform expression VEGF mRNA by in situ hybridization throughout the anterior lobe of the rat pituitary gland. This uniform mRNA expression indicates that not only scattered FS cells were responsible for the VEGF mRNA production. It was speculated that lactotrophs and FS cells, which together comprise 50-60% of all pituitary cells (21), may be involved in the VEGF secretion. Our investigations show that lactotrophs are the main source of VEGF production under physiological conditions.

Since curcumin suppresses VEGF release in pituitary adenomas, it has been suspected that it may inhibit pituitary adenoma progression not only through anti-proliferative and pro-apoptotic actions, but also by suppressing pituitary tumor neovascularization (22). Our results strongly support the notion that curcumin has an important impact on lactotrophs and their VEGF production. It is also notable that estradiol stimulates VEGF and interleukin-6 in human lactotroph and lactosomatotroph pituitary adenomas (23). Estrogen administration enhances the expression of proangiogenic factors (e.g., VEGF) in pituitary grafts (24). Indeed, prolactinomas show higher VEGF protein expression compared to nonfunctioning or ACTH- and GH-secreting adenomas (25). The overexpression of vascular endothelial growth factor in pituitary adenomas is associated with extracellular growth and recurrence. Therefore VEGF and its receptors (VEGFR’s) may play an important clinical role in targeted tumor therapy of pituitary tumors (26).

Results of in vivo studies cannot exclude the involvement of contributing factors, i.e., cells or ECM molecules. In vitro studies performed to examine the effects of VEGF on endothelial cells show that VEGF in the presence of a basal lamina-type ECM specifically induces fenestrations in endothelial cells (27). Other data suggest that Matrigel is necessary to prevent biased functions of cells in culture. It is well documented that epithelial cells dedifferentiate in vitro when cultured on plastic (28). Our results demonstrate that lactotrophs are positive for pan-cytokeratin (data not shown), which is typical for epithelial cells, implying that these cells also need ECM for adequate function. With quantification of VEGF in the culture medium by ELISA, it was apparent that Matrigel inhibits VEGF secretion from pituitary cells in culture. As demonstrated by several authors, ECM supports pituitary cell function. Matrigel reduces proliferation and increases prolactin expression of GH3 cells (29). Pituitary cells cultured on plastic produce elevated amounts of VEGF, which is explained by the increased cellular stress that is induced by the artificial plastic surface. Consequently, the cell culture of lactotrophs on ECM better reflects the physiologic conditions compared to uncoated plastic due to disturbed cell function.

In the present study, we demonstrated for the first time, that lactotrophs, which represent ~50% of the anterior pituitary gland cells, are the major source of VEGF production in vitro as in vivo. This is important for the interpretation of anti-angiogenic treatment and therapeutic response of pituitary tumors treated with anti-VEGF therapy.

ECM is able to influence VEGF release in primary cell culture. We conclude that in vitro studies with cell lines and
primary cell cultures should be performed on ECM to avoid false results.

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