Expression and regulation of osteopontin and connective tissue growth factor transcripts in rat anterior pituitary

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Abstract

Cell–cell interactions are important regulatory elements in anterior pituitary (AP) physiology. As model systems to study pituitary cell–cell interactions, AP cells kept either as monolayers or as organotypic reaggregate cultures were analyzed by differential display PCR. We identified six cDNA fragments (osteopontin (Opn), connective tissue growth factor (CTGF), α_v -integrin, cathepsin H, lysozyme and O-acetyl GD₃ ganglioside synthase) that showed elevated expression in monolayers compared with reaggregate cultures and the AP. The adenohypophyseal mRNA expression of Opn and CTGF, two secreted signaling substances, was studied in more detail.

In situ hybridization histochemistry revealed that Opn mRNA expression is restricted to a subpopulation of

gonadotropes whereas CTGF hybridization signals could not be ascribed to any known cell type. Opn transcript levels were downregulated in the APs of lactating rats and decreased when rats received s.c. injections of 17 β -estradiol for 5 days. The mRNA expression was higher in male than in female rats and increased after gonadectomy. CTGF transcript levels were higher in male compared with female rats and were increased in pregnant rats and in rats treated for 5 days with triiodothyronine or dexamethasone.

These results indicate that Opn and CTGF may be of physiological importance as local communication factors in the AP.

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Introduction

The anterior pituitary (AP), the master gland of the endocrine system, is dynamically controlled by a multitude of regulatory mechanisms which have to be integrated at this central structure according to the physiological conditions and the needs of the body. The development, maintenance and function of the highly differentiated cell types of the AP is determined not only by peripheral and hypothalamic signals, including their corresponding receptors, but also by a multitude of intrapituitary chemical mediators that act via paracrine, autocrine or juxtacrine communication mechanisms (for review see Denef 1994, Renner *et al.* 1996, Ray & Melmed 1997).

Meanwhile, a vast array of signaling molecules (e.g. bioactive peptides, cytokines, growth and differentiation factors as well as extracellular membrane components) have been identified and for some of them we are beginning to understand partly their diverse functions. Nevertheless, for the delicate balance of pituitary homeostasis many more intrapituitary communication factors might be required.

As a model system to search for new factors that are involved in pituitary cell–cell interactions we kept dispersed AP cells either as monolayer cultures or in the form of the organotypic reaggregation cell culture system developed by the group of Denef (Denef *et al.* 1989). In the latter system, pituitary cells do not associate randomly but form three-dimensional tissue-like structures (Vanderschueren *et al.* 1982) with intimate cell–cell contacts and interactions.

To identify genes that are regulated by cell–cell interactions and which may be involved in intrapituitary cell–cell communication, we compared the gene expression of the differently cultured pituitary cells by the differential display PCR (DD-PCR) technique. Using only a limited set of arbitrary primers, we detected several DD-PCR amplification products. After identification of their nucleotide sequences, the differential expression of most DD-PCR amplification products could be verified by Northern blot analysis. Two of these products, namely osteopontin (Opn) and connective tissue growth factor (CTGF), are known secreted growth factors that are expressed by a variety of tissues and organs. Surprisingly,

neither peptide has been detected in the AP as yet. Since Opn (for review see Denhardt & Noda 1998, Rittling & Denhardt 1999) and CTGF (for review see Brigstock 1999, Lau & Lam 1999) have been recognized as chemical mediators that are especially important during development and tissue remodeling processes, we were interested in identifying the cell types synthesizing these regulatory peptides in the AP and in studying their expression under various experimental conditions.

Materials and Methods

Animals

Sprague–Dawley rats were used. The animals, maintained according to the guidelines of the Animal Welfare Committee of the Medizinische Hochschule Hannover, had free access to water and standard laboratory chow. An ambient temperature of 22 °C and alternating 12 h light:12 h darkness cycles were controlled automatically.

To study the influence of peripheral hormones on the expression of CTGF and Opn, adult (4- to 6-month-old) male and female rats were gonadectomized and killed 2 weeks after surgery. Alternatively, adult male rats received a single s.c. injection of dexamethasone (100 µg/100 g body weight (BW)), testosterone (200 µg/100 g BW), triiodothyronine (T₃) (10 µg/100 g BW), 17β-estradiol (0.5 µg/100 g BW), aldosterone (5 µg/100 g BW) or vehicle (sesame oil) and were killed after 6 h, or they received the hormone injections twice-daily (0800 and 2000 h) for 5 days. All hormones were purchased from Sigma Chemical Co. (Deisenhofen, Germany). After decapitation, APs were removed quickly and frozen in liquid nitrogen. APs were also collected from pregnant and lactating animals.

Rat AP cell culture

Immediately after decapitation, APs were removed aseptically and minced with a razor blade. Tissue blocks were treated enzymatically using trypsin and were finally dispersed mechanically to single cells, as described by Denef *et al.* (1989). Cells were either plated on 35 mm Petri dishes (coated with poly-D-lysine, plating density ~1250 cells/mm²) or were allowed to reassociate into three-dimensional cell reagggregates by continuous gyratory shaking. AP cells were cultured for up to 21 days in a humidified CO₂ incubator at 37 °C in chemically defined medium containing 10% heat-inactivated fetal calf serum (FCS) (CC Pro, Neustadt, Germany) or for 7 days without FCS in the same medium consisting of DMEM/Ham's F12 1:1 medium, supplemented with 1 g/l NaHCO₃, BSA, transferrin, insulin, ethanolamine, sodium selenite, ethanol, catalase, penicillin and streptomycin (Denef *et al.* 1989). As indicated, heat-inactivated

FCS (10% v/v) was added to the culture medium at day 2 of culture. Every second day, half of the medium was replaced by fresh medium.

DD-PCR

DD-PCR was performed using the RNA image kit (Gen Hunter, Brookline, MA, USA) with minor modifications. Briefly, total RNA was isolated using the RNeasy system (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was treated with desoxyribonuclease (Boehringer Mannheim, Mannheim, Germany) to remove contaminating chromosomal DNA. RNA (0.2 µg) was reverse transcribed with the arbitrary primers HT₁₁-A, HT₁₁-C or HT₁₁-G. The cDNAs were amplified by PCR in the presence of [α -³⁵S]dATP (Hartmann Analytik, Braunschweig, Germany) using a Perkin-Elmer (Norwalk, CT, USA) thermal cycler. Parameters for the PCR reaction were as follows: initial denaturation at 95 °C for 5 min, annealing at 40 °C for 1 min followed by addition of 1.25 U AmpliTag Polymerase (Perkin-Elmer) at 72 °C (hot start modus) prior to denaturation at 95 °C for 30 s, annealing at 40 °C for 2 min and 30 s extension at 72 °C for 40 cycles followed by a final extension at 72 °C for 7 min.

The amplification products were visualized by autoradiography after denaturing PAGE. Amplification products showing differential expression were excised, reamplified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Plasmid DNA was isolated and the nucleotide sequence was determined from both ends of the insert using oligonucleotide primers flanking the multiple cloning site of the vector. The derived sequences were submitted to GenBank for comparison with known sequences using the BLAST algorithm (Altschul *et al.* 1997).

Northern blot analysis

APs (from five animals) or AP cells (approximately 10 × 10⁶ cells) were homogenized in 4 ml buffer consisting of 0.1 M Tris, 0.5 M LiCl, 10 mM EDTA, 5 mM dithiothreitol, 1% SDS, pH 8. Poly(A)⁺-enriched RNA was isolated using magnetic dT₂₅ polystyrene beads (Deutsche Dynal, Hamburg, Germany) following the manufacturer's instructions. Poly(A)⁺-enriched RNA (5–15 µg) was size fractionated by electrophoresis in a denaturing formaldehyde/agarose gel (2.2 M formaldehyde and 1.5% agarose). The RNA was capillary transferred to a nylon membrane (Nytran NY 12 N; Schleicher and Schuell, Dassel, Germany) and cross-linked by UV irradiation.

For probe synthesis, approximately 50 ng of the cDNA fragments were randomly labeled with [³²P]dCTP (Hartmann Analytik) to high specific activity (>10⁹ c.p.m./µg) using the random primed labeling kit

(Stratagene, La Jolla, CA, USA). Hybridizations were performed at 42 °C in 50% formamide containing 0.5% SDS, 100 µg/ml salmon sperm DNA, 0.5 M NaCl, 12 mM EDTA and 0.09 M sodium phosphate, pH 7.4. After washing under high stringency conditions (59 °C, 0.3% SDS, 0.2 × SSPE) (1 × SSPE: 75 mM NaCl; 5 mM NaH₂PO₄, 0.5 mM EDTA, pH 7.4) membranes were exposed to X-ray films (XOMAT; Kodak, Stuttgart, Germany).

The following cDNA fragments were used: the complete coding region of rat Opn (Oldberg *et al.* 1986) (M14656); a 837 bp fragment corresponding to nucleotide (nt) 1501–2337 of the cDNA encoding rat CTGF (Brauer 1997, Xu *et al.* 2000) (AF120275); a 3269 bp fragment corresponding to nt 449–3717 of the cDNA encoding mouse α_v -integrin subunit (Wada *et al.* 1996) (U14135); a 489 bp fragment corresponding to nt 848–1336 of the cDNA encoding rat cathepsin H (Qian *et al.* 1990) (M38135); a 443 bp fragment corresponding to nt 6249–6691 of the rat lysozyme gene (Yeh *et al.* 1993) (L12459); a 76 bp fragment corresponding to nt 1885–1960 of the cDNA encoding rat O-acetyl-ganglioside synthase (Ogura *et al.* 1996) (D84068); the complete coding region of rat cyclophilin (Danielson *et al.* 1988) (M19533); and the complete coding region of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso *et al.* 1985) (M17701).

In situ hybridization histochemistry (ISH)

Immediately after decapitation, APs were removed and frozen in isopentane cooled (–30 °C to –50 °C) on dry ice. Sections (16 µm thick) were cut on a cryostat, thaw mounted on silane treated slides and stored at –80 °C until further processing.

The following cDNA fragments were used as templates for *in vitro* transcription: a 198 bp fragment corresponding to nt 248–445 of the cDNA encoding rat growth hormone (GH) (Rohn & Weigent 1995) (U62779) in a modified Bluescript KSII+ plasmid (pLS) (Schomburg 1994); a 254 bp fragment corresponding to nt 190–443 of the cDNA encoding rat β -thyrotropin (β -TSH) (Chin *et al.* 1985) (M10902) in pLS; the complete coding region of rat β -follicle-stimulating hormone (β -FSH) (Maurer 1987) in pLS (M36804); a 255 bp fragment corresponding to nt 1566–1820 of the rat prolactin (PRL) gene (Gubbins *et al.* 1980) in pLS (J00769); a 471 bp fragment corresponding to nt 56–526 of exon 3 of the rat pro-opiomelanocortin (POMC) gene (Drouin *et al.* 1985) (J00759) in pGEM4; the complete coding region of rat Opn (953 bp) (Oldberg *et al.* 1986) in pBluescript KSII+ (M14656); and a 827 bp fragment corresponding to nt 1501–2337 of the cDNA encoding rat CTGF (Brauer 1997, Xu *et al.* 2000) in pGEM-T (AF120275).

To generate the RNA probes for Opn and CTGF, *in vitro* transcription was carried out according to the methods described by Heuer *et al.* (1998) using

[α -³⁵S]UTP and [α -³⁵S]CTP as radiolabeled nucleotides. All other RNA probes were generated by use of an RNA transcription kit (Boehringer Mannheim) and digoxigenin-11-UTP as the labeled nucleotide. Following synthesis, the probes for Opn, CTGF, β -FSH and POMC were subjected to mild alkaline hydrolysis to reduce the probe length to approximately 250 nucleotides.

ISH was carried out as described previously (Schäfer & Day 1995, Heuer *et al.* 1998). Briefly, frozen sections were fixed in 4% buffered paraformaldehyde, treated with 0.1% Triton X-100 in PBS, acetylated, dehydrated and dried. The sections were hybridized overnight at 58 °C in a humidified chamber using 5×10^4 d.p.m./µl ³⁵S-labeled sense or antisense cRNA probes for Opn and CTGF respectively, either alone or in combination with 50–200 ng digoxigenin-labeled cRNA probes (β -TSH, β -FSH, PRL, GH and POMC). The probes were diluted in hybridization buffer consisting of 50% formamide, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris-HCl pH 7.4, 1 × Denhardt's solution (Denhardt 1966), 100 µg/ml sonicated salmon sperm DNA, 1 mM EDTA and 10 mM dithiothreitol. The sections were washed in 2 × SSC (1 × SSC: 150 mM NaCl; 15 mM sodium citrate, pH 7.2) and treated with RNaseA and RNaseT1. Successive washes followed at room temperature (1 ×, 0.5 ×, 0.2 × SSC) and at 65 °C (0.2 × SSC). For the detection of Opn and CTGF mRNA, the tissues were dehydrated, dipped in Kodak NTB2 nuclear emulsion and exposed for 2 weeks. Autoradiograms were developed in Kodak D19 for 4 min and fixed in Rapid Fix (Kodak) for 4 min.

For the combined detection of the radiolabeled (CTGF or Opn) and the digoxigenin-labeled signals, the sections were exposed to alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim, diluted 1:500) overnight at 4 °C, followed by 3 h incubation in chromogen solution containing nitroblue tetrazolium (0.41 mM) and 5-bromo-4-chloro-3-indolylphosphate (0.38 mM). The color reaction was stopped by rinsing the slides overnight in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). The tissue was dehydrated, dipped in Ilford K5D nuclear emulsion (Dreieich, Germany) and exposed for 2 weeks.

Detection of CD68 or complement C_{3b} receptor by immunocytochemistry (ICC) and detection of CTGF mRNA by ISH

Cryostat sections were fixed in 3.7% formalin for 5 min, followed by successive washings in 50, 100 and 50% acetone for 2 min each. After washing in Tris-buffered saline (TBS, 0.05 M Tris-HCl, 150 mM NaCl, pH 7.5), the sections were incubated in blocking buffer consisting of TBS, 3% BSA and 5 mg/ml heparin (Gibco BRL, Eggenstein, Germany) for 30 min. The primary antibodies (ED-1 or OX-42, both mouse anti-rat supplied by Serotec, Wiesbaden, Germany), both diluted 1:500 in

blocking buffer, were applied separately for 1 h at room temperature. After three washes in TBS, the sections were incubated for 30 min at room temperature with biotinylated secondary antibody (rabbit anti-mouse; Dako, Hamburg, Germany) diluted 1:300 in blocking buffer. After washing in TBS (three times) the avidin-biotinylated peroxidase complex (Dako) was applied for 30 min at room temperature, following the recommendations of the supplier. Diaminobenzidine (Sigma fast; Sigma) was used as chromogen. After washing in H₂O the sections were postfixed in 4% buffered paraformaldehyde for 10 min and subsequently subjected to ISH for CTGF as described above.

Detection of CTGF mRNA by ISH and S-100 protein by ICC

Cryostat sections of APs were prepared from animals that were transcardially perfused with 4% buffered paraformaldehyde. The sections were postfixed in 4% buffered paraformaldehyde for 30 min at room temperature and subjected to ISH using digoxigenin-labeled Opn mRNA probes as described above. Following the color reaction, the sections were incubated in TE buffer overnight, followed by three washes in TBS containing 0.1% Triton X-100 (TBS-Triton). The primary antibody (S-100, polyclonal antiserum, supplied prediluted by Immunotec, Hamburg, Germany) was applied for 1 h at room temperature. After washing in TBS-Triton (three times), the sections were incubated for 30 min at room temperature with Cy3-conjugated mouse anti-rabbit antibody (dilution 1:300; Dianova, Hamburg, Germany), rinsed with TBS, covered with mounting medium (Citifluor; Plano, Marburg, Germany) to retard fading and examined under a fluorescence microscope (Olympus BH3, New York, NY, USA).

Results

We used the DD-PCR technique to compare the gene expression in AP cells that were kept as monolayer or as reaggregate cultures. Using a limited set of only 12 arbitrary primers we detected about 100 amplification products that appeared to be differentially expressed by this screening method. After subcloning and nucleotide sequencing 36 products could be attributed to known sequences. For selected products the differential gene expression in the culture systems was verified by Northern blot analysis (Fig. 1). In addition to these probes, the mRNA expression in AP and brain was also analyzed. In agreement with the DD-PCR screening results the transcript levels of these products were always elevated in the monolayer compared with the reaggregate cultures kept either for 3 weeks in medium containing 10% FCS (Fig. 1) or for 7 days in chemically defined medium (data

Figure 1 Differential expression of identified DD-PCR products. Poly (A⁺)-enriched RNA was prepared from brain and AP, as well as from AP cells cultured for 3 weeks either as monolayers or as reaggregates in medium containing 10% FCS. Northern blot analysis was carried out as described in Materials and Methods. Cyclophilin signals were used as an internal standard. The positions of the 18S (open arrowhead) and 28S (closed arrowhead) rRNA are indicated.

Osteopontin

CTGF

Figure 2 Analysis of Opn and CTGF mRNA expression in rat pituitary by ISH. Frozen pituitary sections of male rats were hybridized with ^{35}S -labeled riboprobes specific for Opn and CTGF as described in Materials and Methods. Hybridization signals were visualized by darkfield illumination. Staining was not observed when the sense probes for CTGF and Opn were used. Bar=100 μm .

not shown). Among the differentially expressed products we detected two products which are known to act as secreted growth factors in other tissues, namely CTGF and Opn, as well as α_v -integrin subunit, a constituent of the well characterized Opn receptors $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ (Denhardt & Noda 1998).

Localization of CTGF and Opn in the rat AP gland

ISH was performed to analyze the mRNA distribution of Opn and CTGF in the AP. For both peptides hybridization signals were easily detected (Fig. 2). While for Opn the distribution was restricted to the AP, hybridization signals for CTGF were also found in the posterior pituitary, but not in the intermediate lobe.

To identify the cell types synthesizing these growth factors, we analyzed the mRNA distribution of Opn and CTGF with the respective ^{35}S -labeled cRNA probes and used digoxigenin-labeled RNA probes for PRL, GH, β -FSH, β -TSH and POMC to localize the hormone-producing cells of the AP. By this method, strong hybrid-

ization signals for Opn could be detected only in the β -FSH-positive gonadotrophic cells (Fig. 3) but not in the other hormone-producing cells. While all Opn signals colocalized with β -FSH mRNA expressing cells, some of the cells containing β -FSH transcripts did not contain Opn mRNA, indicating that Opn transcripts are exclusively restricted to a subpopulation of gonadotrophic cells.

For CTGF we were unable to identify the cell type expressing the mRNA of this growth factor. As shown in Fig. 4 CTGF transcripts do not colocalize with any cell type expressing mRNA of the AP hormones tested. To identify CTGF mRNA expression in non-hormone-producing cell types we combined the ISH technique for CTGF with the ICC method for the detection of folliculostellate cells, macrophages and dendritic cells using antibodies against the cell marker proteins S-100, CD68 (ED1 antibodies) and complement C_{3b} receptor (OX-42 antibodies). Despite all efforts we were unsuccessful in defining the CTGF mRNA expressing cells in the AP.

Regulation of transcript levels

By autoradiography and phosphoimaging (Fujix, BAS 1000; Fuji, Japan) we analyzed the transcript levels of Opn and CTGF (normalized to GAPDH and cyclophilin respectively) in the AP of control animals (injected with sesame oil as vehicle) and in male rats that had received multiple injections of peripheral hormones (Fig. 5). When the animals were treated twice-daily for 5 days, the mRNA levels of CTGF were increased in the AP of rats injected with T_3 (~ 3 -fold) and dexamethasone (~ 3 -fold) but were not altered after injection of aldosterone and the sex steroids testosterone and 17β -estradiol. In contrast, Opn mRNA levels were decreased in 17β -estradiol treated animals (~ 4 -fold) and to some extent also after prolonged treatment with testosterone (~ 2 -fold). When the animals received only a single injection of the hormones 5 h prior to being killed, only the CTGF mRNA levels were increased (~ 6 -fold) in rats treated with dexamethasone whereas all other transcript levels were not affected by the short-term treatment.

Next we analyzed whether the transcript levels of CTGF and Opn were influenced by the gender of the animals or by conditions with altered endocrine status such as gonadectomy, pregnancy or lactation (Fig. 6). Compared with female rats, the mRNA levels of CTGF and Opn were both higher in male rats (~ 1.6 -fold and ~ 12 -fold respectively). In both genders the Opn mRNA levels increased after gonadectomy (~ 11 -fold in females and ~ 3.5 -fold in males), whereas the CTGF transcripts were only marginally affected. The Opn mRNA levels were not altered during pregnancy but decreased (~ 3 -fold) during the lactation period. In contrast, CTGF transcript levels were elevated during pregnancy (~ 2.5 -fold) and returned to basal levels during lactation.

Figure 3 Analysis of Opn and β -FSH mRNA expression in rat pituitary. Frozen pituitary sections of male rats were hybridized with ^{35}S -labeled riboprobes specific for Opn and with digoxigenin-labeled riboprobes specific for β -FSH and analyzed as described in Materials and Methods. (A) High power darkfield micrograph of Opn hybridization signals; (B) combined darkfield and brightfield illustrations of Opn and β -FSH hybridization signals; (C) brightfield micrograph of β -FSH hybridization signals. The arrow indicates the position of a β -FSH-positive cell. Staining was not observed when the sense probes for β -FSH and Opn were used. Bar=50 μm .

Discussion

As shown in the extensive studies by Carl Denef and co-workers (Denef *et al.* 1989, Vanderschueren *et al.* 1982) the organotypic reaggregate cultures can be considered as an ideal model system to study paracrine as well as cell–cell interactions in the AP in contrast to the monolayer cultures in which the cellular communication systems are not preserved. By analyzing AP cells that were kept either as monolayer or as reaggregate cultures, we previously demonstrated that synthesis and secretion of thyrotropin-releasing hormone by pituitary cells is strongly influenced not only by the culture medium but also by cell–cell interactions (Peters *et al.* 1997). As a first step in the search for other cell signaling molecules we were interested in using a more general approach by identifying genes that are differently expressed in the two culture systems using for analysis the DD-PCR technique (Liang & Pardee 1992). With this screening method, which is now well established, we indeed readily detected many ($n=36$) differently expressed amplification products using only a limited set ($n=12$) of arbitrary primers.

For most (70%) of these products their differential expression in the monolayer and reaggregate cultures could be verified by Northern blot analysis. The fact that we identified many products that are derived from genes which are expressed at very low levels underlines the sensitivity of the DD-PCR technique. It may also be interesting to note that in the monolayer cultures most gene products were expressed at considerably higher levels not only as compared with the reaggregates but also as compared with the AP.

Among the differentially expressed gene products, we were most interested in studying CTGF and Opn in more detail. In various tissues CTGF and Opn, both secreted soluble proteins, have been recognized as very important regulatory peptides and potent growth factors. Surprisingly, however, in the AP neither CTGF nor Opn has been detected before. In line with our culture models are the reports describing increased expression of both growth factors during development, differentiation and conditions that are associated with the disruption of normal tissue structures. For example, Opn, a secreted phosphoglycoprotein containing the arginine-glycine-aspartate integrin binding motif, seems to be important in tissue remodeling processes such as tumor growth (as reviewed by Denhardt & Noda 1998), wound healing (Liaw *et al.* 1998), bone resorption (Yoshitake *et al.* 1999) and inflammatory processes (Ashkar *et al.* 2000). Via interaction with its receptors (e.g. $\alpha_v\beta_3$ -integrin and CD44) this regulatory peptide is involved in various processes that depend on cell–cell and cell–matrix interactions (for review see Denhardt & Noda 1998, Rittling & Denhardt 1999). In many tissues, CTGF, a member of the family of connective tissue growth factor/cysteine-rich 6 l/nephroblastoma overexpressed proteins (CCN family), is also involved in

Figure 4 Analysis of CTGF mRNA expression in the rat AP. CTGF mRNA expression was analyzed using the radioactive riboprobe and the mRNA expression of the pituitary hormones GH, PRL, β -FSH, β -TSH and POMC was detected using the respective digoxigenin-labeled riboprobes. Antibodies against protein S-100 (folliculostellate cells), CD68 (macrophages; recognized by ED-1 antibodies) and complement C_{3b} receptor (dendritic cells; recognized by OX-42 antibodies) were used to mark non-hormone-producing AP cells. The combined high power darkfield and brightfield micrographs are presented as indicated. The arrow indicates the position of a CTGF-positive cell. Staining was not observed without primary antibodies and also not when the sense probes for CTGF and the pituitary hormones were used. Bar=50 μ m.

Figure 5 Hormonal regulation of adenohypophyseal Opn and CTGF mRNA steady-state levels. Male rats received twice-daily s.c. injections of dexamethasone (100 µg/100 g BW), testosterone (200 µg/100 g BW), T_3 (10 µg/100 g BW), 17 β -estradiol (E_2) (0.5 µg/100 g BW), aldosterone (5 µg/100 g BW) or vehicle (sesame oil) and were killed after 5 days. The APs were prepared and the mRNA levels were analyzed by Northern blot, as described in Materials and Methods. The GAPDH signals were used as an internal standard.

various events that require cell–cell and cell–matrix interactions such as angiogenesis, tumor growth, inflammation and wound repair (for review see Lau & Lam 1999, Brigstock 1999).

In agreement with its localization in gonadotropic cells, the adenohypophyseal Opn mRNA levels were considerably influenced by sex steroids but not by the other steroid

Figure 6 Influence of the endocrine status of the animals on the expression of Opn and CTGF mRNA in rat pituitaries. Northern blot analysis was performed with poly (A^+)-enriched RNA prepared from the APs of male, female, lactating (day 10 of lactation), pregnant (day 18 and 15 of gestation), ovariectomized, orchiectomized and newborn rats.

hormones tested. In male rats receiving high doses of steroid hormones for prolonged periods of time, the Opn mRNA levels were only significantly reduced after injection of 17 β -estradiol and to a lesser extent after testosterone treatment (in part possibly due to the conversion of testosterone to 17 β -estradiol). Correspondingly, after gonadectomy the Opn transcript levels were found to be increased in male and in female rats. In addition, we observed a clear gender difference with ~12-fold higher Opn mRNA levels in male compared with female rats. As known, Opn expression (which usually correlates with Opn secretion) is influenced by many agents acting on many cell types through diverse signaling pathways (for review see Denhardt & Noda 1998). With regard to steroid hormones, Opn expression was found to be upregulated by dexamethasone in rat bone marrow stromal cell cultures (Rickard *et al.* 1994) and in rat bone organ cultures (Chen *et al.* 1996). In the experimental model of kidney stone formation, progesterone and 17 β -estradiol inhibited the glyoxylic acid induced increase of Opn mRNA levels in rat kidney (Umekawa *et al.* 1995) whereas in mouse skin the Opn mRNA levels were enhanced when the combination of these steroids was applied to the skin of non-pregnant females (Craig & Denhardt 1991). Increased Opn mRNA levels were also observed in the skin of pregnant and lactating mice.

In contrast, Opn transcript levels in rat AP were considerably decreased during lactation and after prolonged treatment with 17 β -estradiol but were not affected during pregnancy. Thus, as in other tissues, the regulation of Opn expression in the AP seems to be very complex. In search of an explanation one might hypothesize that the effects of 17 β -estradiol, the sex differences and the effects after gonadectomy might possibly be mediated by PRL since PRL synthesis and secretion is generally stimulated by 17 β -estradiol (Lamberts & Macleod 1990). The decreased Opn mRNA levels in lactating rats might also be related to the elevated PRL serum levels induced by suckling. During pregnancy, however, Opn transcript levels were not altered. Again, this might be explained by the fact that in the rat, the PRL serum levels are not increased despite elevated 17 β -estradiol levels (Escalada *et al.* 1996). In this context it may be interesting to note that Opn is expressed in gonadotropic cells, a cell type that is surrounded by clusters of lactotropic cells (Horvath & Kovacs 1988, Allaerts *et al.* 1991) and equipped with PRL receptors (Morel *et al.* 1994, Jin *et al.* 1997, Tortorese *et al.* 1998).

In line with this view are also the reports describing close interactions between these cell types through paracrine factors regulating gonadotrope and lactotrope functions (Denef & Andries 1983, Seuntjens *et al.* 1999). Since lactation and elevated 17 β -estradiol serum levels are both known to affect gonadotropic functions, it is tempting to speculate that Opn may be considered as a locally produced intrapituitary mediator of the

hypothalamic–hypophyseal–gonadal axis. Of course, this hypothesis is rather speculative and has to await further investigation.

For CTGF, despite all efforts we have unfortunately failed to identify the pituitary cell type(s) expressing this growth factor and thus it is difficult to ascribe possible functions for this growth factor in the AP. Among the hormones tested, only dexamethasone significantly increased CTGF transcript levels after long-term treatment and even more impressively within 5 h after a single injection of this steroid (data not shown). A rapid and dramatic induction of CTGF mRNA and protein by dexamethasone has also been observed in cultured fibroblasts and described recently (Dammeier *et al.* 1998). The same authors also reported on dexamethasone induced upregulation of CTGF mRNA levels in heart, kidney and skin of mice that had been injected repeatedly with dexamethasone.

Interestingly, CTGF transcript levels in the AP were also considerably increased after long-term treatment of the animals with T_3 , an effect not described before in any tissue. CTGF mRNA levels were also upregulated (~ 4 -fold) in AP cultures (monolayer and reaggregate cultures) kept for 2 days in medium supplemented with 3 nM T_3 (not shown). However, CTGF mRNA levels were not altered after a single injection of T_3 and thus it is unclear whether T_3 exerts a direct effect on CTGF expression or, and more likely, acts indirectly. As known, in various tissues CTGF expression is influenced by transforming growth factor- β and other growth factors (for review see Brigstock 1999).

On the other hand, however, it has also long been recognized that thyroid hormones induce the expression of various growth-promoting factors (e.g. GH, epidermal growth factor, nerve growth factor) and induce cell growth in various tissues (Ezzat & Melmed 1990, Ray & Melmed 1997). More specifically, with pituitary tumor cells it has been clearly demonstrated that T_3 can induce the production and secretion of a specific pituitary growth factor with selective growth-promoting properties (Hinkle & Kinsella 1986, Ezzat & Melmed 1990).

Taken together, we have provided evidence for the adenohypophyseal synthesis of CTGF and Opn, both growth factors that are known as potent regulatory peptides in various tissues. In the AP, however, we have to await further studies to define the biological functions of these factors in AP physiology and/or pathophysiology and especially within the network of intrapituitary communication.

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