Human proximal tubular epithelial cells express somatostatin: regulation by growth factors and cAMP

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Turman, Martin A., and Courtney A. Apple. Human proximal tubular epithelial cells express somatostatin: regulation by growth factors and cAMP. Am. J. Physiol. 274 (Renal Physiol. 43): F1095–F1101, 1998.—Somatostatin modulates several renal tubular cell functions, including gluconeogenesis and proliferation. In this study, we demonstrate that cultured human proximal tubular epithelial cells (PTEC) express somatostatin. We also demonstrate positive and negative regulation of PTEC somatostatin production. We found that PTEC derived from 14 different human donors consistently expressed somatostatin mRNA and/or peptide as detected by RT-PCR and enzyme-linked immunosassay. Furthermore, Northern blot analysis revealed that PTEC express the same size mRNA transcript (750 nucleotides) as human thyroid carcinoma (TT) cells. The PTEC mitogens, epidermal growth factor (EGF) and hydrocortisone, inhibit PTEC somatostatin secretion, whereas forskolin (a direct stimulator of adenylate cyclase) and fetal bovine serum stimulate secretion. These findings raise the possibility that renal-derived somatostatin modulates tubular cell function in an autocrine/paracrine manner. Herein we demonstrate that cultured human proximal tubular epithelial cells (PTEC) express somatostatin mRNA and peptide. We also demonstrate that fetal bovine serum (FBS) and cAMP stimulate, whereas epidermal growth factor (EGF) and hydrocortisone inhibit, secretion of somatostatin peptide.

METHODS

Cell culture. Primary cultures of human PTEC were generated from normal human cadaveric kidneys unsuitable for renal transplantation as previously described (8, 39). Tissue was provided by the Ohio State University and Children's Hospital Cooperative Human Tissue Network. PTEC growth medium consisted of Gibco MEMα (Life Technologies) with 10% heat-inactivated FBS, 5 µg/ml hydrocortisone (Collaborative Research, Bedford, MA), 10 ng/ml recombinant human EGF (GIBCO), 100 U/ml penicillin/streptomycin, and ITS (Collaborative Research). ITS consists of 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenium acid, and 1.25 mg/ml bovine serum albumin. We have previously (25) characterized these tubular cells as being uniformly positive for cytokeratin, γ-glutamyl transferase, alkaline phosphatase, and URO-3 (Signet Laboratories, Dedham, MA), indicating that these cells are of proximal tubular origin and retain proteins expressed by PTEC in vivo (2, 8). Cells were plated at ~1 × 10^5 cells/ml and split 1:3 or 1:4 when confluent. Cells used for experiments were between passages 3 and 8.

The human neuroblastoma cell line, SKNSH, and the human thyroid medullary carcinoma cell line, TT, were purchased from the American Type Culture Collection (Manassas, VA). SKNSH cells were cultured and maintained as previously described (20) in MEM with L-glutamine, Earle's salts, nonessential amino acids, and 15% FBS. TT cultures were maintained in RPMI 1640 with 15% FBS and L-glutamine, as recommended by the American Type Culture Collection.

Reverse transcription and polymerase chain reaction. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with previously described oligonucleotide primers (40). These primers were designed from published gene and cDNA sequences for somatostatin (35) (accession no. J00306; the constitutively expressed protooncogene, c- abl (36) (no. M14752); human β-actin (19) (no. M10277); neuropeptide Y (NPY) (18) (no. M14752); and vasoactive intestinal peptide (VIP) (38) (no. M11552). Each primer pair amplifies across an intron/exon splice site such that products derived from mRNA can be easily differentiated from products derived from genomic DNA. We have previously used the somatostatin primers to detect somatostatin mRNA in cadaveric kidney specimens and cultured human mesangial cells (40) and human neuroblastoma tumor (1). The VIP and NPY primers have been used to detect VIP and NPY mRNA in SKNSH...
human neuroblastoma cells (40) and VIP mRNA in megakaryocytes (24).

Total RNA was isolated from PTEC using the RNAzol method as described by manufacturer (Cinna/Biotex, Friendswood, TX). Total RNA (1 µg) was reverse transcribed with random hexamer primers followed by amplification of cDNA by polymerase chain reaction (GeneAmp kit; Perkin-Elmer Cetus, Norwalk, CT). Reaction mixtures were subjected to 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min for 33 cycles followed by 72°C for 9 min. RT-PCR products were resolved by electrophoresis at 100 V through 1.75% agarose in 1X TAE (0.04 M Tris acetate, 0.001 M EDTA, pH 8.0) and visualized with ethidium bromide.

Southern hybridization of somatostatin RT-PCR products. Southern analysis with a 42-residue dinucleotide complementary to somatostatin cDNA was used to confirm that somatostatin RT-PCR products contained somatostatin-specific sequence as previously described (40). This probe (5’ CTGGGACAGATCTTCAGTGCCGACATCTTCTCCTCGT 3’) is complementary to a region of somatostatin cDNA nested in between the binding sites for somatostatin RT-PCR primers. The RT-PCR products were transferred to a nylon membrane and hybridized with the 42-nucleotide somatostatin probe, which was 32P-labeled with T4 polynucleotide kinase. Hybridization was performed in 50% formamide at 42°C overnight. The membranes were washed sequentially with 2× SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) at room temperature, 2× SSC with 1% sodium dodecyl sulfate at 65°C, and then 0.1× SSC at room temperature. Bound 32P-labeled probe was detected by autoradiography. To control for nonspecific hybridization, c-abl RT-PCR products were also included on all blots.

DNA sequencing of PTEC somatostatin RT-PCR products. To confirm that PTEC-derived somatostatin RT-PCR products were identical to the published somatostatin cDNA sequence (35), we sequenced RT-PCR products after cloning into pGEM-T (Promega, Madison, WI). pGEM-T contains a 3’ thymidine residue to facilitate ligation of PCR products. The cloning site was nested in between the binding sites for somatostatin RT-PCR primers. After incubation for 2 h at room temperature, unbound material was removed, and the plates were washed. Wells were then incubated with streptavidin-horseradish peroxidase conjugate followed by substrate solution (hydrogen peroxide and 3,3’,5,5’-tetramethylbenzidine dihydrochloride). After stopping the reaction with 2 N HCl, absorbance at 450 nm was measured with an ELISA reader.

According to the manufacturer, the somatostatin antisera used for this assay does not cross-react with substance P, NPY, VIP, insulin, glucagon, or amylin amine. The linear range for the somatostatin ELISA is 10 to 2,000 pg/ml. The intra-assay and interassay variation is <5% and <14%, respectively.

Radioimmunoassay for NPY and VIP. RIAs for NPY and VIP were performed by the Core Peptide Laboratory of the General Clinical Research Center (RR-34) at the Ohio State University using assays subjected to rigorous quality control as previously published (5, 21, 22, 27). Characteristics of the antisera used for these assays have been published previously (21, 22, 40). The lower limit of sensitivity of the VIP RIA is 5 pg/ml (5) and 20 pg/ml for the NPY RIA (personal communication, T. M. O’Dorisio, Ohio State University). Samples were prepared for RIA as previously described (40).

Measurement of cellular cAMP content. PTEC were exposed to MEM alpha or MEM alpha with 10 µM forskolin (Sigma, St. Louis, MO) and/or 1 mM IBMX (Sigma). After incubation at 37°C for 20 min, cells were extracted as described (41) for 2 h at 4°C with ice-cold acid-ethanol (100% ethanol brought to pH 3.0 with hydrochloric acid). Extracts were dried at 37°C under nitrogen and then assessed for cAMP content with the TiterFluor Dual Range cAMP Fluorescence Immunoassay (PerSeptive Biosoystems, Framingham, MA) as described by manufacturer.

Data analysis. For immunoreasays, data are expressed as means ± SE. Results of independent experiments were pooled and groups were compared by one-way ANOVA or t-test, as appropriate. For ANOVA, post hoc analysis between groups was performed using the Student-Newman-Keuls test. Significance was defined as P < 0.05.

RESULTS

Proximal tubular cells express somatostatin mRNA. RT-PCR of PTEC RNA with somatostatin-specific primers resulted in a single product ~356 bp in size (Fig. 1). This corresponds to the expected size of an RT-PCR product.
product derived from somatostatin mRNA, whereas amplification of the somatostatin gene from genomic DNA results in a 1,233-bp fragment. Somatostatin RT-PCR products were detected with PTEC RNA derived from six of six different donors. No products were obtained when reverse transcriptase was omitted from the reaction mixture (Fig. 1).

Because somatostatin serves as a neuropeptide in the nervous system, we also tested PTEC for expression of other neuropeptides in addition to somatostatin. When PTEC RNA was subjected to RT-PCR with VIP- or NPY-specific oligonucleotides, no RT-PCR products were obtained (Fig. 1). Previously, we demonstrated that, with these same primers, SKNSH neuroblastoma cells express NPY and VIP mRNA, but not somatostatin mRNA (40), confirming the adequacy and specificity of these primers. Thus our current results demonstrate that cultured human PTEC specifically produce somatostatin mRNA but do not produce detectable NPY or VIP transcripts.

Somatostatin RT-PCR products from PTEC RNA derived from two different donors were analyzed by Southern blot analysis to confirm that they contained somatostatin-specific nucleotide sequences. To control for nonspecific hybridization, c-abl RT-PCR products were also included on the blot. $^{32}$P-labeled somatostatin probe hybridized specifically to the somatostatin RT-PCR products but not to c-abl RT-PCR products (Fig. 2). The ability of somatostatin-specific probe to hybridize specifically to somatostatin RT-PCR products confirms that the somatostatin RT-PCR products are not amplification artifacts.

To determine whether the 356-bp somatostatin RT-PCR product from PTEC is identical to the published somatostatin cDNA sequence in its entirety, somatostatin RT-PCR products from three different donors were cloned into pGEM-T, and the insert was sequenced. The sequences of all three inserts from cloned RT-PCR products were identical to the published somatostatin sequences of all three inserts from cloned RT-PCR cloned into pGEM-T, and the insert was sequenced. The tin RT-PCR products from three different donors were somatostatin cDNA sequence in its entirety, somatostatin RT-PCR product from PTEC is identical to the published amplification artifacts.

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Northern blot analysis of PTEC somatostatin mRNA. Poly(A)$^+$ RNA from PTEC and whole cadaveric human renal cortex, as well as total RNA from human thyroid carcinoma (TT) cells, were blotted and hybridized with a $^{32}$P-labeled somatostatin cDNA probe. TT human thyroid carcinoma cells express an abundant 750-nucleotide somatostatin mRNA transcript (33) and were used as a positive control. An ~750-nucleotide somatostatin transcript was detected for all three sources of RNA (Fig. 3). These results confirm that PTEC and whole kidney express somatostatin mRNA and indicate that renal-derived somatostatin mRNA is the same size as in human thyroid carcinoma cells.

PTEC express somatostatin peptide. To determine whether PTEC translate somatostatin mRNA into peptide, PTEC culture supernatants were tested for somatostatin immunoreactivity. Medium that overlaid PTEC cultures for 48 h (conditioned medium) contained significantly greater quantities of somatostatin (1,385 $\pm$ 316 pg/ml, means $\pm$ SE) than untreated control medium that had not been exposed to cells (72 $\pm$ 3 pg/ml) (Fig. 4). FBS contains a small amount of somatostatin, which accounts for the small amount of somatostatin detected in untreated medium. The amount of somatostatin secreted from PTEC derived from different donors varied considerably, ranging from 266 to 4,439 pg/ml. PTEC cellular extracts had no detectable somatostatin. Dilution of somatostatin from PTEC culture supernatants paralleled the dilution curve for synthetic somatostatin standard, indicating that PTEC-derived somatostatin has the same immunodilution properties as synthetic somatostatin standard (14).

To determine whether PTEC secrete neuropeptides other than somatostatin, PTEC cell extracts and conditioned medium were analyzed by RIA for NPY and VIP. Neither PTEC-conditioned medium nor cellular ex-
media were subjected to Sep-Pak C18 hydrophobic chromatography. Conditioned and untreated control media were collected after incubation in MEM.

Fig. 4. PTEC secrete somatostatin. Culture supernatants (conditioned media) were collected after incubation in MEMa with 10% fetal bovine serum (FBS) for 48 h. Conditioned and untreated control media were subjected to Sep-Pak C18 hydrophobic chromatography. Media samples and cell extracts were then tested for somatostatin by enzyme-linked immunoassay (EIA). Reported values were derived from 13 different duplicate samples from 10 experiments with PTEC derived from 10 different donors and are expressed as means ± SE. PTEC derived from 12 of 12 donors expressed somatostatin peptide as detected by EIA of culture supernatants. There was 100% concurrence in five cases in which PTEC derived from a single donor were tested for both somatostatin mRNA and peptide expression. These results indicate that PTEC consistently produce somatostatin.

Regulation of PTEC somatostatin expression. PTEC secrete substantial amounts of somatostatin when cultured in the presence of MEMa with 10% FBS (Fig. 4). To determine whether the various components of complete PTEC growth medium influence somatostatin secretion, we assessed the ability of individual medium components to alter PTEC somatostatin production (Fig. 5). To control for variability in somatostatin secretion by PTEC derived from different donors, each donor served as its own control for these experiments. Thus somatostatin secretion is expressed as percent of that obtained with MEMa medium with 10% FBS (M + FBS) and no other supplements. In the presence of unsupplemented MEMa without serum, PTEC secrete only 15% as much somatostatin as in the presence of 10% FBS. Addition of the nonserum supplements of complete growth medium (i.e., EGF, hydrocortisone, and ITS) to MEMa decreased somatostatin secretion to a lower level than that obtained in the presence of MEMa alone. Furthermore, the nonserum growth supplements decreased serum-stimulated somatostatin secretion markedly when added to M + FBS. These results indicate that FBS stimulates, whereas the nonserum growth supplements inhibit, PTEC somatostatin secretion.

To determine which growth supplements inhibit FBS-stimulated somatostatin secretion, PTEC were incubated with individual supplement components in addition to FBS.
Addition of the ITS supplement or insulin alone did not alter somatostatin secretion. However, EGF and hydrocortisone decreased somatostatin secretion to 53 ± 6% and 44 ± 13%, respectively, compared with that obtained in the presence of MEM α with 10% FBS. These results indicate that EGF and hydrocortisone inhibit FBS-stimulated PTEC somatostatin secretion.

The inhibitory effects of EGF and hydrocortisone on somatostatin secretion were dose dependent (Figs. 6A and B, respectively). EGF and hydrocortisone maximally inhibited somatostatin secretion at 10 to 50 ng/ml and 10⁻² to 10⁻⁶ M, respectively.

In other somatostatin-producing cells, factors that increase intracellular cAMP levels would be expected to induce somatostatin expression by PTEC. To increase intracellular cAMP levels, PTEC were exposed to forskolin, which directly activates adenylate cyclase, and IBMX, which decreases degradation of cAMP by inhibiting phosphodiesterase. To determine whether cAMP induction correlates with increased somatostatin secretion, PTEC were incubated with forskolin and/or IBMX for 24 h, and then somatostatin content of culture supernatants was assessed. Incubation with forskolin or with forskolin plus IBMX increased somatostatin secretion 45-fold and 71-fold above basal levels, respectively (Fig. 7). Correspondingly, incubation of PTEC with forskolin or with forskolin plus IBMX increased cAMP content 38-fold and 85-fold above basal levels, respectively (data not shown). These results indicate that PTEC somatostatin secretion is greatly augmented by agents that increase intracellular cAMP.

**DISCUSSION**

We previously demonstrated that somatostatin mRNA is expressed in samples of cadaveric human renal cortex and in cultured human mesangial cells (40). We also demonstrated that mesangial cells secrete somatostatin peptide (40). Because proximal tubular cells express somatostatin receptors (10, 30), we hypothesized that PTEC also express somatostatin, providing an autocrine/paracrine mechanism for somatostatin to regulate PTEC function and growth. In this study we confirm this hypothesis. Several methods were used to verify that PTEC consistently express authentic somatostatin mRNA and peptide. Results of Southern blot analysis and sequencing of PTEC somatostatin RT-PCR products disprove the possibility that somatostatin-specific PTEC RT-PCR products were amplification artifacts. Northern blot analysis further confirms ex-
pression of somatostatin mRNA in PTEC. Northern blot analysis also demonstrates that PTEC somatostatin transcripts are processed to the same size as somatostatin mRNA in other cell types, indicating that PTEC somatostatin transcripts do not undergo alternative splicing.

Authenticity of PTEC somatostatin peptide was confirmed by the ability of PTEC-derived somatostatin to bind to somatostatin-specific antiserum and to parallel the immunodilution curve of synthetic somatostatin standard. Furthermore, cAMP, a known inducer of somatostatin in other cell types, greatly stimulates PTEC somatostatin secretion.

Somatostatin expression was detected in PTEC derived from 14 of 14 different donors, indicating that PTEC consistently express somatostatin. The fact that PTEC do not produce NPY or VIP and that SKNSH cells do not produce somatostatin attests to the cellular specificity of expression of these neuropeptides and the specificity of the assays used to detect these products. Thus our results establish that cultured human PTEC express somatostatin.

PTEC cultured in medium with fetal bovine serum contained, on average, 1.385 pg/ml of somatostatin, corresponding to 0.8 nM (Fig. 4). In the presence of forskolin and IBMX, somatostatin content of medium increased to 56,700 pg/ml (35 nM) (Fig. 7). Most somatostatin receptors have affinity constants for somatostatin in the nanomolar range (26, 29). By radioligand binding assays, the affinity constant for somatostatin receptors on opossum kidney (OK) proximal tubular-like cells is 24.5 nM (10). Thus PTEC produce physiologically significant amounts of somatostatin. In the in vivo microenvironment of the proximal tubule, local concentrations of PTEC-derived somatostatin could reach even higher concentrations than in culture supernatants. Because proximal tubular cells express somatostatin receptors (10, 30) and physiological relevant amounts of somatostatin, we speculate that locally produced somatostatin modulates proximal tubular cell proliferation and function in an autocrine/paracrine manner.

The physiological role of somatostatin in the kidney and for proximal tubules is currently unknown. Somatostatin inhibits proliferation of opossum kidney OK proximal tubular cells (10) and rat mesangial cells (34). In a human pancreatic cell line (MIA PaCa-2), somatostatin inhibits EGF-induced proliferation by activating a phosphotyrosine phosphatase activity that dephosphorylates and inactivates EGF receptor (23). In light of the ability of somatostatin to inhibit EGF-induced proliferation, our observation that EGF inhibits PTEC somatostatin secretion is especially interesting. We speculate that the ability of EGF to inhibit PTEC somatostatin production provides an autocrine/paracrine feedback mechanism for EGF to overcome somatostatin-mediated inhibition of PTEC proliferation.

As with EGF, hydrocortisone is mitogenic for PTEC (2) and inhibits somatostatin secretion (Figs. 5 and 6). In human thyroid medullary carcinoma cells, a low concentration of dexamethasone (10<sup>-8</sup>–10<sup>-10</sup> M) stimulates somatostatin expression; however, higher concentrations (10<sup>-8</sup>–10<sup>-5</sup> M) decrease somatostatin production by accelerating somatostatin transcript degradation (15, 16). The concentration of hydrocortisone in PTEC growth medium (5 µg/ml, or 1.4 × 10<sup>-5</sup> M) corresponds to the glucocorticoid activity of ~5.3 × 10<sup>-7</sup> M dexamethasone, indicating that this concentration inhibits somatostatin secretion by PTEC as for thyroid carcinoma cells. In contrast, we did not detect stimulation of PTEC somatostatin secretion with concentrations of hydrocortisone as low as 10<sup>-11</sup> M (corresponding to 4 × 10<sup>-13</sup> M dexamethasone).

In addition to inhibition of proliferation, PTEC-derived somatostatin may modulate other physiological functions of PTEC. Depending on the somatostatin receptor subtype and G proteins expressed in various cells, binding to somatostatin receptors can lead to inhibition of adenylate cyclase, activation of guanylate cyclase, or to modulation of calcium or potassium flux (7, 23, 26, 32). Because of the diverse secondary signaling mechanisms that somatostatin influences, somatostatin or somatostatin analogs may have many interesting and potentially clinically useful effects on proximal tubule cells. Currently, somatostatin analogs are used for a wide variety of clinical settings, including inhibition of myointimal thickening in chronic allograft rejection (4, 11), inhibition of restenosis after coronary balloon angioplasty (4), visualization and treatment of a wide variety of tumors (12, 31), and for chronic secretory diarrhea and other gastrointestinal disorders (12).

Future studies defining the somatostatin receptor subtypes expressed by PTEC and the intracellular signaling pathways triggered by binding to these receptors will be critical for our understanding of how renal-derived somatostatin may modulate proximal tubular cell growth and function. As such studies unfold, novel methods to manipulate intrarenal somatostatin expression or novel uses for somatostatin analogs in the management of renal disease will become evident.

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