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Oxytocin Synthesis and Oxytocin Receptor Expression by Cell Lines of Human Small Cell Carcinoma of the Lung Stimulate Tumor Growth through Autocrine/Paracrine Signaling[1]

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ABSTRACT

The objective of the present work was to investigate the existence of an oxytocin (OT)-mediated autocrine/paracrine signaling upon small cell carcinoma of the lung (SCCL) cell growth. In that view, OT receptor (OTR) expression, concomitant with OT synthesis and secretion, was evidenced on three different SCCL cell lines (DMS79, H146, and H345) and related to the vasopressin (VP) system. Specific *OT*, *VP*, *OTR*, *V1a VP receptor* (*V1aR*), and *V1b/V3 VP receptor* (*V1bR/V3R*) transcripts were identified by reverse transcription-PCR in all cell lines studied. Binding of ^{125}I-(d(CH$_2$)$_5^1$,Tyr(Me)^2,Thr^4,Orn^8,Tyr^9-NH$_2$)-vasotocin (OVTA) was observed on all SCCL cell lines, with a K_d (dissociation constant) ranging from 0.025–0.089 nM, depending on the cell line and the analytical method. Selectivity of ^{125}I-OVTA binding was confirmed by displacement curves obtained with various OTR and VP receptor agonists and antagonists (OT, OVTA, L-371,257, VP, F180). Immunocytochemistry identified cellular OT and VP, and peptide secretion was measured in supernatants of SCCL cultures. [^{3}H]Thymidine incorporations, applied on H345 cells, demonstrated a dose-dependent mitogenic effect of exogenous OT (1 and 100 nM) that was abolished by the OTR antagonist OVTA. A decrease of proliferation was also observed with OVTA alone, showing a functional mitogenic effect of tumor-derived OT. Taken together, these observations demonstrate the existence of a functional OT-mediated autocrine/paracrine signaling actively implicated in growth and development of SCCL tumors. Furthermore, these findings point to the potential of OT antagonists for development as therapeutic agents for the treatment of SCCL.

INTRODUCTION

Of the various types of human lung cancer, SCCL,[3] also called "oat-cell" carcinoma, is one of the most aggressive. In the United States, SCCL accounts for 20% of all primary lung carcinoma. The overall cumulative 5-year survival rate is <1%, compared with 12–25% for other types of lung cancer (1). SCCL is most often associated with overexpression of *VP*, a neurohypophysial gene (2). *VP* overexpression leads, in many cases, to an ectopic secretion of VP that induces a paraneoplastic syndrome with water intoxication, hyponatremia, and hypernatriuria known as Schwartz-Bartter syndrome (3). High plasma concentrations of VP and associated NP (VP-NP), a highly conserved 10-kDa domain present in VP precursor (4–10), are biological hallmarks of this syndrome. However, all neurohypophysial gene-expressing tumors do not secrete peptides. A NP-related protein is targeted to the outer surface of cell membrane as a NP-related cell surface antigen (8, 11). *In vitro*, VP has been shown to exert trophic and mitogenic effects on various human SCCL cell lines. This mitogenic effect seems to be mediated by the V1aR (12–15). The expression by SCCL cell lines of all known VP receptor subtypes [V1aR, V1bR (also called V3R), and V2R] has been demonstrated by North *et al.* (16, 17), suggesting a multifaceted autocrine role of VP on SCCL growth.

High NP plasma levels are reported in approximately two of three cases of SCCL (8). The evidence of ectopic secretion is not restricted only to VP and related NP. High levels of the other neurohypophyseal hormone OT, together with OT-NP, were measured in plasma of 30–40% of these cases (6, 8, 18), as well as in acetone extracts of SCCL tumors (5). Nevertheless, up to now, very little is known about the pathophysiological consequences of *OT* overexpression and OT secretion by SCCL on tumor development and patient survival. The expression of OTR has been demonstrated in breast cancer, in neuroblastomas and glial tumors, and in endometrial adenocarcinomas. In these cases, OT exhibits an OTR-mediated antiproliferative effect on cancer cells (19–29). On the contrary, an increase of cell proliferation through an OTR-mediated effect has been demonstrated in human trophoblast and choriocarcinoma cell lines (30).

Therefore, there was a high interest in assessing the biological role of OT on SCCL cells. The objective of the present work was to determine the existence of an OT-mediated autocrine/paracrine signaling upon SCCL cell growth. OTR expression, OT synthesis, and secretion were investigated on three different SCCL cell lines (DMS79, H146, and H345). Then, the implication of exogenous and endogenous OT was assessed on cell proliferation. To characterize the whole neurohypophyseal peptide system of SCCL cell lines, VP and VPR expression and VP or VP-NP synthesis and secretion were also investigated.

MATERIALS AND METHODS

Reagents and Cells

DPBS (17-513F), FCS, and RPMI 1640 were purchased from BioWhittaker. TriPure isolation reagent RNase-free DNase I, complete protease inhibitor mixture tablet, the Expand Reverse Transcriptase System, and the Expand Long Template PCR System were purchased from Roche. Other reagents were Triton X-100 (ICN); Tissue-Tek (Sakura); [^{3}H]dThd (Amersham Pharmacia Biotech); scintillation liquid (Packard Bioscience); primers, Moloney murine leukemia virus reverse transcriptase, Taq polymerase, ethidium bromide, and agarose (Life Technologies, Inc.); and the QIAquick Gel Extraction Kit (Qiagen). OT, VP, insulin-transferrin-selenium solution (I-1884), and BSA (BSA fraction V, A-4503) were obtained from Sigma. OTR ligand was ^{125}I-OVTA

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[3] The abbreviations used are: SCCL, small cell carcinoma of the lung; VP, vasopressin; OT, oxytocin; OTR, OT receptor; V1aR, V1a VP receptor; V1bR/V3R, V1b/V3 VP receptor; V2R, V2 VP receptor; NP, neurophysin; OVTA, (d(CH$_2$)$_5^1$,Tyr(Me)^2, Thr^4,Orn^8,Tyr^9-NH$_2$)-vasotocin; EGF, epidermal growth factor; CK, cytokeratin; LDH, lactate dehydrogenase; dThd, thymidine; DPBS, Dulbecco's PBS; B/Bo, percentage of radioactive ligand binding displacement; mAb, monoclonal antibody; IR, immunoreactive; RT-PCR, reverse transcription-PCR; Ab, antibody; ATCC, American Type Culture Collection; NSB, nonspecific binding.

(New England Nuclear; Ref. 31). OTR antagonists were OVTA (Bachem; Ref. 32) and L-371,257 (kindly provided by L. Koch; Merck Research Laboratories; Ref. 33). The V1aR agonist F180 was kindly provided by P. Riviere (Ferring Research; Refs. 34 and 35). Glycerol, paraformaldehyde, and methanol were obtained from Merck Eurolabo. Normal goat serum and antihuman CK mAb MNF116 were obtained from Dako. Abs directed to neurohypophysial peptides were anti-OT mAb O33 (36), rabbit anti-OT serum (O4; Ref. 37), anti-VP mAb C2.23 (kindly given by A. Burlet; Ref. 38), and rabbit anti-OT serum (AOS) purchased from Phoenix Pharmaceuticals, Inc. Human recombinant EGF (E-9644), mouse isotype controls IgG1 (M-5284), IgM (M-5909), and rabbit immunoglobulin (I-5006) were purchased from Sigma. Secondary Abs were rhodamine (TRITC)-conjugated Affinipure goat antirabbit IgG (secondary Ab B), FITC-conjugated Affinipure goat antimouse IgG (secondary Ab C), and FITC-conjugated Affinipure goat antimouse IgM (secondary Ab M). All conjugated Abs were obtained from Jackson.

SCCL cell lines DMS79, H146, and H345 were purchased from ATCC.

Cell Culture and Human Tissues

SCCL cell lines were maintained in RPMI 1640 + 10% FCS, sometimes supplemented as recommended by ATCC, in a humidified atmosphere of 5% CO_2 and 95% by air at 37°C. Human hypothalamic tissue, frozen in liquid nitrogen or Tissue-Tek embedded on dry ice, was obtained surgically within 10 min after death.

RNA Isolation

Total RNA was isolated from SCCL cell lines, human hypothalamus, and Chinese hamster ovary cells using TriPure isolation reagent. To remove genomic DNA contamination, RNA samples were treated with RNase-free DNase.

OT and *VP* RT-PCR and Sequencing

Total RNA (1–5 μg) from SCCL cell lines and human hypothalamus (positive control) was reverse-transcribed in a total volume of 50 μl by the Expand Reverse Transcriptase System using oligo(dT) primer. Reverse transcription products (1:50) were used directly for PCR. PCR was performed with the Expand Long Template PCR System according to the manufacturer's instructions in a UNO II rapid thermocycler (Biometra). After an initial denaturation step at 94°C for 5 s, reaction mixtures were submitted to 35 cycles comprising 94°C for 45 s (denaturation), 66°C for 45 s (annealing), and 72°C for 45 s (elongation). After amplification, an additional extension step at 72°C for 10 min was performed. The sequences of primers are presented in Table 1. After migration in 2% agarose gels and staining with ethidium bromide, RT-PCR transcripts were excised, purified with the QIAquick Gel Extraction Kit, and sequenced directly by a DNA sequencer (Eurogentec). Reverse primers used for PCR served as sequencing primers (Table 1).

OTR and *VPR* RT-PCR and Sequencing

cDNAs were synthesized from total RNA (3 μg) of SCCL cell lines and Chinese hamster ovary cells expressing human V1aR, V1bR/V3R, V2R, and OTR from transfected plasmids (positive control). The reaction was performed using Moloney murine leukemia virus reverse transcriptase (200 units) and random primers as described previously (39). The first-strand synthesis reaction (1:6) was amplified for 30 cycles using Taq polymerase (1 unit) and 100 pmol of each forward and reverse primer (Table 1). Cycling parameters were 94°C for 90 s, 70°C for 90 s, and 72°C for 120 s. Negative control RT-PCR reactions were performed by omitting reverse transcriptase or RNA from the reaction mixture. PCR products were separated on a 2% agarose gel and revealed by ethidium bromide staining. PCR products were subcloned using pGEM-T easy vector systems (Promega) and sequenced.

Table 1 *Synthetic primers*

Synthetic primers selected for RT-PCR and sequencing of OT (OTf, OTr), VP (VPf, VPr), OTR (OTRf, OTRr), V1aR (V1af, V1ar), V1bR/V3R (V1bf, V1br), and V2R (V2f, V2r).

Primer	Nucleotide	Sequence	Strand
Forward			
OTf	515–537	5′-CGC-CGG-ACC-TCG-ACG-TGC-GCA-A-3′	+
VPf	1870–1893	5′-CGG-CGT-TTG-CTG-CAA-CGA-CGA-GA-3′	+
OTRf	756–780	5′-GGG-CGC-GTG-GCC-CTG-GCG-CGT-GTC-A-3′	+
V1af	756–782	5′-GTC-GCG-CCA-GAG-CAA-GGG-TGC-AGA-GC-3′	+
V1bf	792–819	5′-CGG-GTC-AGC-AGC-ATC-AAC-ACC-ATC-TCA-3′	+
V2f	653–679	5′-CCC-TGG-GTA-TCG-CCG-CCT-GCC-AGG-TG-3′	+
Reverse			
OTr	1162–1187	5′-CAT-CAA-AGT-TTC-AGC-GCT-GGG-AGA-A-3′	−
VPr	2297–2321	5′-CGG-AGG-TTT-ATT-GTC-CGT-GCT-GCA-3′	−
OTRr	1137–1164	5′-CGT-GGA-TGG-CTG-GGA-GCA-GCT-CCT-CTG-3′	−
V1ar	1198–1224	5′-GAA-GAT-TTA-GGC-GAG-TCC-TTC-CAC-AT-3′	−
V1br	1020–1046	5′-GGG-CCG-CGG-TAA-CAG-GTG-GCT-GTT-GA-3′	−
V2r	1059–1090	5′-GGA-GCT-GGC-GGT-GGT-GCA-GGA-CTC-ATC-TTG-G-3′	−

OT Binding Assays

Saturation Binding. Incubation was carried out with 2×10^6 cells/sample in a final volume of 500 μl of assay buffer [DPBS + 1% BSA (pH 7.5)] in a 30°C water bath for 1 h. ^{125}I-OVTA (0.005–0.32 nM) was added either without or with 0.5 μM OT, assumed to reflect NSB. The reaction was stopped on an ice bath, and 2 ml of assay buffer (4°C) were added to each tube before centrifugation (700 × *g*, 4°C, 10 min). Supernatants were discarded, and pellets were washed with 10 ml of assay buffer (4°C), followed by an additional centrifugation step. After removal of the supernatants, pellets were homogenized with assay buffer (200 μl). Each sample was performed in duplicate; NSB and total binding (B_T) values were measured by automatic gamma counter (Wallac; Perkin-Elmer).

Competitive Displacements. Selectivity of ^{125}I-OVTA binding was assessed by competition studies with various neurohypophysial receptor ligands. Competitive binding curves were generated with cold OT, OVTA, L-371,257 (OTR antagonist), VP, and F180 (V1aR agonist). Incubation was carried out with 2×10^6 cells/sample in a final volume of 500 μl of assay buffer in a 30°C water bath for 1 h. Cold ligand (0–200 nM) was added with ^{125}I-OVTA (0.08 nM). The procedure was ended as reported above.

OT and VP Immunocytochemistry

SCCL cells (0.1×10^6) were transferred on microscope slides by centrifugation in a cytospin tube (100 × *g*, 10 min.). Cells were fixed successively by paraformaldehyde (4°C, 20 min) and methanol (−20°C, 7 min) and then incubated for 30 min at room temperature with 10% normal goat serum to prevent NSB of goat secondary Ab. Single immunostaining was performed with the following reagents: (*a*) antihuman CK mAb MNF116 (1:40) followed by secondary Ab C (FITC-conjugated goat antimouse IgG; 1:40); (*b*) anti-OT mAb O33 (1:50) followed by secondary Ab M (FITC-conjugated goat antimouse IgM; 1:40); (*c*) anti-OT O4 (1:1600) followed by secondary Ab B (TRITC-conjugated goat antirabbit IgG; 1:50); (*d*) anti-OT AOS (1:100) followed by secondary Ab B (TRITC-conjugated goat antirabbit IgG; 1:50); and (*e*) anti-VP mAb C2.23 (1:50) followed by secondary Ab C (FITC-goat antimouse IgG; 1:40). After the final wash, slides were mounted with glycerol/gelatin medium. Nonspecific staining of secondary Abs (Ab B, C, or M) was assessed by incubation with buffer [50 mM Tris-HCl and 0.01% NaN_3 (pH 7.6)] instead of the first Ab. Nonspecific staining of first Abs was assessed by replacing the first Ab with equivalent amounts of respective immunoglobulin isotype controls (mouse IgG1 instead of anti-CK MNF116 and anti-VP C2.23, mouse IgM instead of anti-OT O33, and rabbit IgG instead of anti-OT O4 and AOS).

OT and VP-NP Production

DMS79 (1×10^6 cells/ml), H146 (2×10^6 cells/ml), and H345 (4×10^6 cells/ml) cell lines were cultured in 12-well plates. Cells were incubated in 1 ml of RPMI 1640 + 10% FCS, sometimes supplemented as recommended by ATCC, in a humidified atmosphere of 5% CO_2 and 95% by air at 37°C. Media and cells were separated every 24 h until 120 h of culture. Incubation medium aliquots were frozen directly at −20°C, whereas cells were lysed in 500 μl of deionized water and then frozen at −20°C. OT enzyme immunoassay (18) and VP-NP RIA (40) were performed on supernatants and cell contents. RNA was isolated from cell pellets at each incubation step to perform OT and VP RT-PCR. LDH was measured, using a standard clinical chemical procedure, to estimate the rate of living cells at each incubation step. In that view, after separation of supernatants and cells, cell pellets were lysed with Triton 2% in RPMI 1640 (900 μl). Protease inhibitor (40 μl) and glycerol (900 μl) were added to each sample of supernatant and cell solution before storage at −20°C.

Cell Proliferation

Incorporation of [^{3}H]dThd into DNA was measured by liquid scintillation counting. H345 cells in stock culture were washed in DPBS and replated in culture flasks (25 cm^2) in RPMI 1640 added with 5% charcoaled-FCS (charcoal treatment discards small molecules and proteins such as OT and VP) and 1% insulin-transferrin-selenium solution at an initial density of 1×10^5 cells/ml. After 24 h of incubation in a humidified atmosphere of 5% CO_2 and 95% O_2 at 37°C, OT, OVTA, OT + OVTA, VP, or EGF were added to the culture medium at final concentrations ranging from 1–100 nM. Each day, 1 ml of cell suspension was taken and distributed in quadruplicate on 96-well plates (200 μl/well). [^{3}H]dThd (25 μl, 11.5 μCi/ml) was added, and plates were incubated for 4 h in a humidified atmosphere of 5% CO_2 and 95% by air at 37°C. Cellular DNA was harvested from the cells using a Titertek cell harvester (Flow Laboratories). One-min sample counts were obtained in a Beckman liquid scintillation counter (Beckman).

Statistical Analyses

Binding assays were analyzed with GraphPad Prism software, using a one site binding (hyperbola) model or a one site competition model without weighting method.

Proliferation curves were analyzed with GraphPad Prism software, using ANOVA followed by Student-Newmaan-Keuls test.

RESULTS

RT-PCR and Sequencing. Total RNA preparations from SCCL cell lines DMS79, H146, and H345 and hypothalamus (positive control) were used in RT-PCR studies to generate products representing OT mRNA and VP mRNA. In each case, a cDNA product of the expected size (OT, 288 bp; VP, 258 bp) was obtained (Fig. 1*A*).

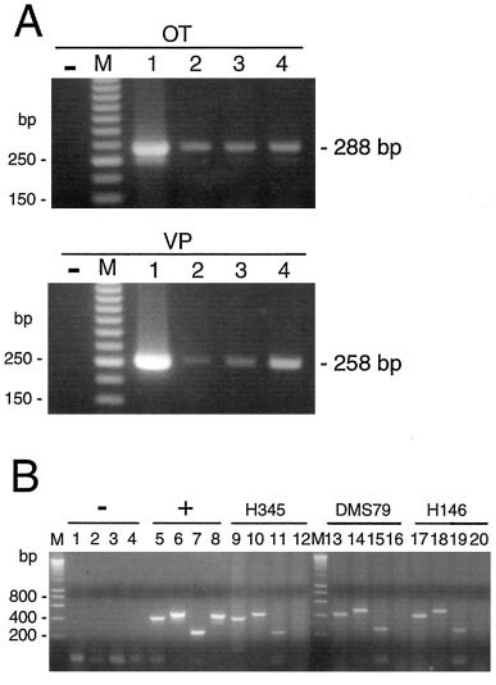

Fig. 1. OT, VP, and receptor RT-PCR. *A*, OT and VP RT-PCR products obtained from total RNA preparation of human hypothalamus (*Lane 1*) and SCCL cell lines DMS79 (*Lane 2*), H146 (*Lane 3*), and H345 (*Lane 4*). H_2O was used as negative control (−), and *Lane M* represents the molecular weight marker. *B*, RT-PCR products of OTR (*Lanes 5, 9, 13*, and *17*), V1aR (*Lanes 6, 10, 14*, and *18*), V1bR/V3R (*Lanes 7, 11, 15*, and *19*), and V2R (*Lanes 8, 12, 16*, and *20*) obtained from total RNA preparation of SCCL cell lines H345, DMS79, and H146 and from CHO cells expressing human V1aR, V1bR/V3R, V2R, and OTR from transfected plasmids (positive control, +). H_2O was used as negative control (−) respectively for OTR (*lane 1*), V1aR (*lane 2*), V1bR/V_3R (*lane 3*) and VeR (*lane 4*). Selected primers yielded cDNAs of 408 (OTR), 468 (V1aR), 254 (V1bR/V3R), and 437 (V2R) bp.

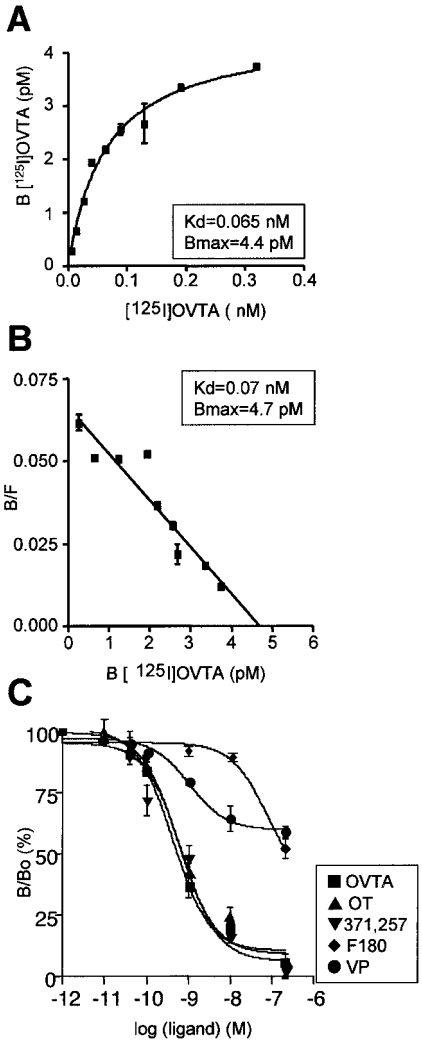

Fig. 2. OTR binding on H345. *A*, hyperbolic analysis of saturation binding with ^{125}I-OVTA. Data are expressed as a function of bound ^{125}I-OVTA (pM) ± SD (B ^{125}I-OVTA = B_T − NSB [concentration of radioactive ligand binding]) *versus* ^{125}I-OVTA (nM) added. *B*, Scatchard analysis of H345 saturation binding with ^{125}I-OVTA. Data are expressed as a function of B/F ratio between radioactive ligand bound and free ligand ± SD *versus* B ^{125}I-OVTA (pM). *C*, competitive displacement of ^{125}I-OVTA with OVTA (OTR antagonist; ■), OT (▲), L-371,257 (OTR antagonist; ▼), F180 (V1aR agonist; ◆), and VP (●). Data are expressed as a function of B/B0 (%) ± SD, *versus* log (ligand) (M, molar).

Transcript sizes were predicted from GenBank sequence HUMOTNPI (G189414) for the human *prepro-oxytocin-NP* gene and GenBank sequence HUMVPNP (G340302) for the human *prepro-8-arginin-NP* gene. Sequencing of OT and VP PCR products confirmed the specificity and selectivity of the OT and VP primers. Each primer set fits with mRNA regions corresponding to minimal sequence similarity between *OT* and *VP*. Indeed, each PCR product presents 90–99% homology compared with their respective GenBank sequence.

Four different primer sets were used to amplify OTR, V1aR, V1bR/V3R, and V2R. In all pairs, the position of the oligonucleotides fitted with a region corresponding to minimal homology between OTR and different VPR. Moreover, the priming sites were separated by an intron, thus distinguishing products from cDNA from those generated through any contaminating genomic DNA (for OTR, V1aR, and V1bR/V3R) or genomic amplification with a different size than the cDNA product (for V2R; data not shown). As shown in Fig. 1*B*, a single product of the expected size (408, 468, and 254 bp) was amplified in each of the SCCL cell lines for OTR, V1aR, and V1bR/V3R, respectively. No amplification of V2R (437 bp) mRNA was

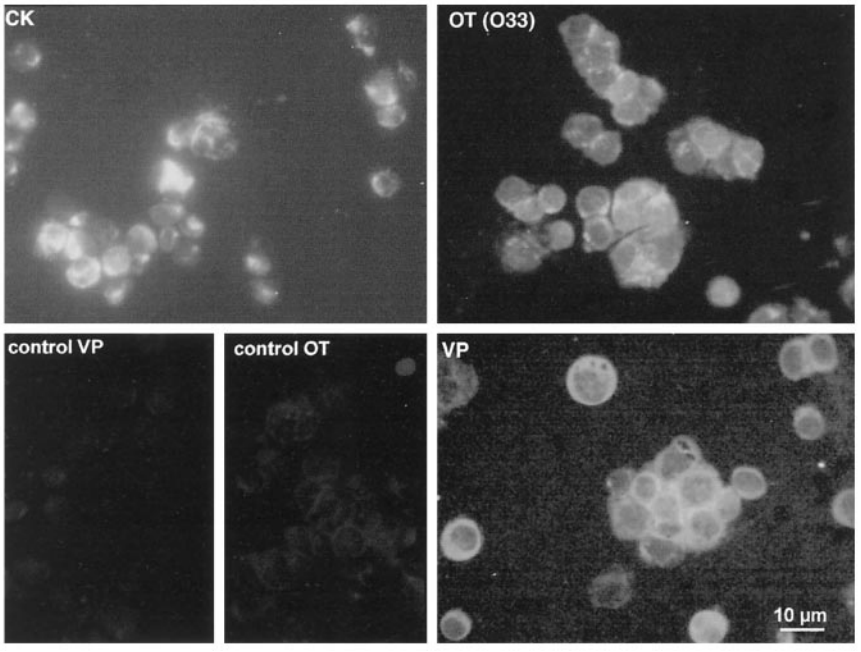

Fig. 3. OT and VP immunocytochemistry. H345 cells are labeled by fluorescence immunostaining with antihuman CK mAb MNF116 (*CK*) and anti-VP mAb C2.23 (*VP*). Immunolabeling with anti-OT mAb O33 [*OT (O33)*] is presented on DMS79. Labeling with anti-OT O4 and AOS exhibits similar staining (data not shown). For all Abs tested, similar results were obtained with the three SCCL cell lines studied (data not shown). Control OT and control VP represent nonspecific staining for anti-OT O33 and anti-VP C2.23, respectively. It is obtained by replacing the first Ab with IgM and IgG1 isotype controls, respectively.

observed for SCCL cell lines, even though a product of 437 bp was amplified in the positive control. Oligonucleotide sequences of each amplified fragment correspond to OTR, V1aR, and V1bR/V3R sequences described by Kimura *et al.* (41), Thibonnier *et al.* (42), and Sugimoto *et al.* (43).

OT Binding Assays. Binding of ^{125}I-OVTA is observed in SCCL cell lines H345 (Fig. 2) DMS79, and H146 (data not shown). Depending on the analytical method (hyperbola or Scatchard), K_d ranges from 0.025–0.06 nM for DMS79 cells and from 0.037–0.089 nM for H146 cells. Both analyses give similar K_d values (0.065 and 0.07 nM) for H345 cells. Saturation bindings with ^{125}I-OVTA exhibit K_d values of the same order for the three SCCL cell lines. On the contrary, the estimated number of binding sites per million cells is about 10 times higher for H345 cells than for H146 and DMS79 cells. Indeed, 1.10–1.18 fmol/million cell binding sites (depending on analysis method) are deduced from B_{max} (maximal bindings value) for H345 cells, whereas there is 0.15–0.20 fmol/million DMS79 cells binding sites, and 0.10–0.15 fmol/million H146 cells binding sites. Characterization of the receptor evidenced by saturation binding with ^{125}I-OVTA has been investigated by displacement with various ligands. On H345 cells, OVTA (OTR antagonist), OT, and L-371,257 (OTR antagonist) displace ^{125}I-OVTA in a similar manner with an average EC_{50} of 0.57 ± 0.14 (SD) nM. Displacements observed with VP and F180 (V1aR agonist) do not reach 50% B/Bo, even if ligand concentrations are 200 nM. If ligand is used at 1 nM, the average displacement obtained with OVTA, OT, and L-371,257 is 42.1% B/Bo, whereas it is 90.7% B/Bo with F180 and 79.5% B/Bo with VP. For DMS79 cells, a preponderant displacement is observed with OTR ligands (OVTA > L-371,257 > OT). Displacements with VP and F180 never exceed 40% B/Bo. For H146 cells, similar displacement curves are observed with OVTA, L-371,257, OT, and VP. However, F180 displacement remains over 60% B/Bo even when the ligand concentration used is 200 nM.

OT and VP Immunocytochemistry. Fig. 3 shows the staining obtained with anti-CK (MNF116) and anti-VP (C2.23) on H345, as well as the staining obtained with anti-OT (O33) on DMS79. Similar labeling was observed with other anti-OT Abs (O4 and AOS), and comparable results were obtained for all three different SCCL cell lines studied (data not shown). Staining obtained with anti-CK demonstrates the epithelial phenotype of SCCL cells. Distinct spots are observed with anti-OT mAb O33, whereas a more diffuse labeling is observed with anti-VP mAb C.2.23. No staining is observed in the nucleus of cells; the crown-like staining obtained with anti-neurohypophysial peptide Abs seems to correlate with cytoplasm and cell membrane localization.

OT and VP Production. IR OT and IR VP-NP were measured in cell contents, as well as in supernatants of H345 cells cultured for a total period of 120 h. Expression of VP and OT mRNA and LDH levels were established for each incubation step. Data for IR OT has been presented elsewhere (18). IR OT measurement in supernatants ranged from 5–40 pg/ml, and the relative IR OT cellular fraction ranged from 6.5–14 pg/10^6 H345 cells. Similar results were obtained for DMS79 and H146 cells, but in these SCCL cell lines, the relative IR OT cellular fraction seemed to be significantly higher than the H345 one. Indeed, IR OT measurement in supernatants was in the same range of concentration for the three cell lines, whereas the relative IR OT cellular fraction ranged from 32.5–70 pg/10^6 cells for H146 and 65–140 pg/10^6 cells for DMS79. IR VP-NP levels are presented in Fig. 4*A*, and a maximal secretion of 3.2 ng/ml is measured after 96 h of incubation. Hormone production is concomitant with OT and VP mRNA production (Fig. 4*B*). LDH quantification shows a constant number of living H345 cells ($\pm 4 \times 10^6$ cells) during the whole culture procedure (Fig. 4*C*).

OT Effect on Cell Growth. The effect of OT on H345 cellular proliferation was evaluated by [^{3}H]dThd incorporation assays (Fig. 5). After 48 h of OT treatment, a significant increase in cell proliferation was observed, compared with the control curve, with 1 nM OT (*, $P < 0.01$) and 100 nM OT (**, $P < 0.001$; Fig. 5*A*). The mitogenic effect of exogenous OT was dose dependent and time persistent. Indeed, the incubation with 1 nM OT showed a growth increase ranging from 18–25%, and the effect of 100 nM OT was from 28–58%. When cells were incubated with a mix of OVTA (1 nM) and OT (1 nM), the OT mitogenic effect was completely abolished, and the proliferation profile was maintained in the range of the control curve. A significant reduction of cell growth (**, $P < 0.001$) was detected after 24 h of OVTA (1 nM, OTR antagonist) treatment. This decrease

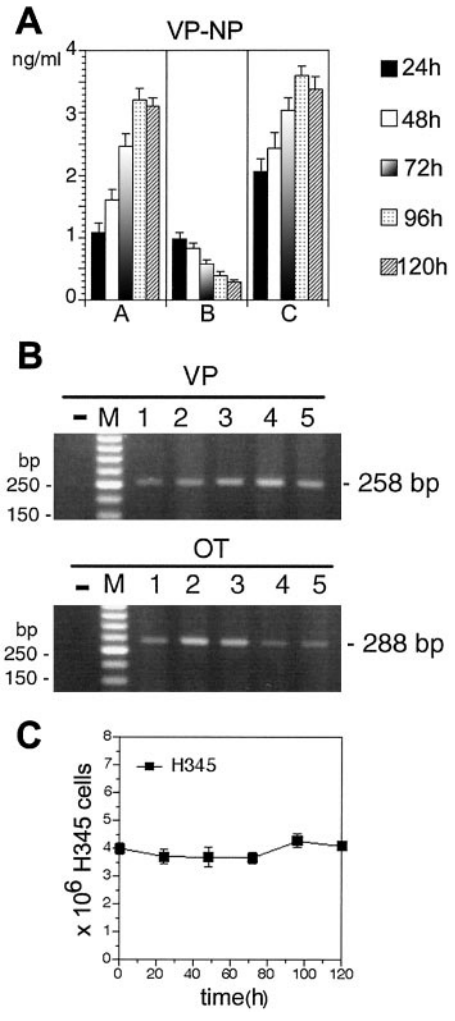

Fig. 4. VP-NP and OT release. *A*, histogram represents IR VP-NP concentrations (expressed in ng/ml ± SD) measured by RIA in supernatants (*A*) and cell contents (*B*) of SCCL cell line H345 at each incubation step: 24 h (■), 48 h (□), 72 h (*gray box*), 96 h (*dotted box*), and 120 h (▨). The total OT and VP-NP production (*C*) was calculated from *A* + *B*. *B*, VP and OT RT-PCR products (258 and 288 bp, respectively) obtained from total RNA preparation of H345 isolated at each incubation step: 24 h (*Lane 1*); 48 h (*Lane 2*); 72 h (*Lane 3*); 96 h (*Lane 4*); and 120 h (*Lane 5*). H_2O was used as negative control (−), and *Lane M* represents the molecular weight marker. *C*, graph represents the number of H345 living cells, expressed as million ± SD, during the whole culture procedure (from 24 h up to 120 h). The number of living cells was evaluated from LDH measurement in supernatants and cell extracts at each incubation step.

of cell proliferation was observed at all time points (**, $P < 0.001$) and ranged from 33–48%. VP (1 nM) and EGF (8 nM) stimulation was used as positive control (Fig. 5*B*). A significant mitotic effect appeared after 48 h of treatment (**, $P < 0.001$), ranging from 24–49%. The increase in cell proliferation induced by exogenous OT is in the same order of magnitude as the one induced by VP or EGF.

DISCUSSION

Although the expression of all known VP receptors (V1aR, V1bR/V3R, and V2R) has already been reported in SCCL (17), the expression of OTR has never been investigated in SCCL cell lines. Our data clearly demonstrate that OTR mRNA is detected in three SCCL cell lines. Binding of ^{125}I-OVTA is observed on all SCCL cell lines studied, with a K_d ranging from 0.025–0.089 nM, depending on the cell line and the analytical method. These values correspond to the characteristics of ^{125}I-OVTA binding to mammary tissue, uterus, and testis (44). The selectivity of ^{125}I-OVTA binding is confirmed by the results of displacements observed with various OTR and VPR agonists and antagonists. For H345 cells, parallel and equal displacements are observed with OVTA, OT, and L-371,257 (OTR antagonist), whereas VP and F180 (V1aR agonist) show a very poor displacement of ^{125}I-OVTA. Indeed, VP and F180 displacement curves are shifted to the right and do not reach 50% B/Bo when OVTA, OT, and L-371,257 curves exhibit a complete displacement. These data identify the neurohypophysial binding site present at the H345 cell surface as true OTR. For DMS79 and H146 cells, the binding site density is about 10 times lower than that for H345 cells. The order of binding affinity for DMS79 was OVTA > L-371,257 > OT ≫ VP ⋙ F180. For H146, similar displacements were observed with OVTA, OT, L-371,257 and VP, whereas only F180 exhibited very low affinity. The variations observed between these displacement profiles could result from various OTR densities or could be due to the concomitant presence of VPR, as shown by RT-PCR and competitive binding analyses. As a matter of fact, V1aR and V1bR/V3R mRNAs were also identified in concordance with previous studies (17). However, with the primers and the experimental conditions used in this study, we were unable to observe V2R mRNA amplification, although

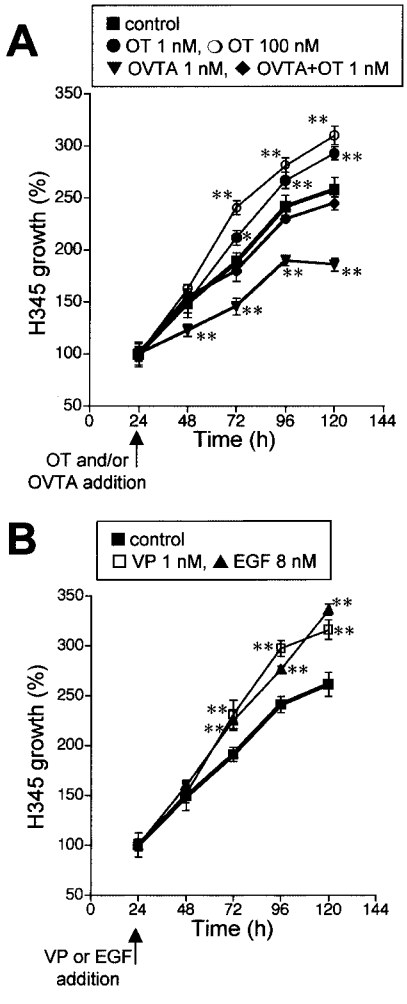

Fig. 5. Effect of OT on H345 cell proliferation. Proliferation of H345 was estimated by [^{3}H]dThd incorporation normalized for each sample at 100% at time = 24 h, before the addition of OT, OVTA, VP, or EGF. Data are expressed as growth percentage ± SD *versus* time (h). In both graphs, * = $P < 0.01$ and ** = $P < 0.001$ (OT, VP, or EGF *versus* control), and the cutoff for significance is 0.05. *A*, H345 proliferation curves under control condition (■), with exogenous 1 nM OT (●) or 100 nM OT (○) treatment, with 1 nM OVTA (▼), or with a mixture (◆) of 1 nM OT and 1 nM OVTA. OT, OVTA, or the mixture is added at time = 24 h. *B*, H345 proliferation curves under control condition (■) and with 1 nM VP (□) or 8 nM EGF (▲) treatment. VP and EGF are added at time = 24 h.

North *et al.* (17) have previously demonstrated the presence of this receptor in SCCL cells. Nevertheless, our observations strongly support the expression of OTR by H345, DMS79, and H146 SCCL cell lines. In particular, the cell line H345 seems to be a very appropriate model for additional studies about OTR expression in SCCL.

The presence of IR OT has been reported previously in acetone extracts of SCCL tumors (5). In the present study, OT synthesis and release by SCCL cells expressing OTR were further investigated. The presence of *OT* transcripts was evidenced by RT-PCR with a novel set of specific primers, designed and controlled on human hypothalamus mRNA. The identification of IR OT was performed using fluorescence immunocytochemistry with three different Abs. Our experiments demonstrate an endogenous OT peptide synthesis. The release of OT has been quantified on H345, DMS79, and H146 cells by OT enzyme immunoassay (18). The variations in total OT levels during the whole culture could reflect a balance between its synthesis/secretion and enzymatic degradation, which could be influenced during autocrine/paracrine signaling. Continuous OT production during the whole culture is confirmed by OT RT-PCR on mRNA cell extracts. The characterization of the neurohypophyseal peptide system in these SCCL cells includes *VP* expression (as shown by RT-PCR) and VP-NP secretion (which reflects the amount of VP released by SCCL cells). The experiments have been performed in such a way that VP and OT synthesis and release could be compared. Our results show that both OT and VP are concomitantly synthesized and released by the three SCCL cell lines investigated, even though in the hypothalamus, these peptides are synthesized by distinct neurons (45). Thus, in the limits of cell line biology, our data clearly demonstrate that, in addition to the VP system, OT and OTR are also implicated in SCCL biology.

In a recent editorial (46), Bussolati and Cassoni emphasized the complexity of the OT/OTR system in physiology and pathophysiology. In breast cancer, *in vitro* effects of OT on cell proliferation vary from antiproliferative to mitogenic (26, 28, 47, 48). In choriocarcinoma, OT promotes cell growth (30), whereas it decreases proliferation of glial tumors, endometrial adenocarcinomas, and neuroblastomas (23, 27). Neurohypophysial peptides were also shown to exert mitogenic effects on freshly isolated immature T cells (thymocytes; Ref. 49). In our experiments, we observed a mitogenic effect of exogenous OT on H345 cell proliferation. This effect, tested at 1 and 100 nM, was dose dependent and time persistent. It was already significant ($P < 0.01$) after a 48-h incubation with 1 nM OT, which is an efficient and physiological value to activate OTR. This mitogenic effect of exogenous OT (1 nM) was abolished by 1 nM OVTA, demonstrating that the increase of proliferation observed was really induced by OT. The effect of endogenous OT was assessed by incubating the cells with 1 nM OVTA alone. The inhibition of cell proliferation obtained under these conditions strongly supports a mitogenic action of the OT secreted by the SCCL cells. Such an effect of OT that mimics a growth factor was also recently suggested to be involved in a study on Kaposi's sarcoma cells (50) as well as when OTR presents a caveolar localization (51).

In conclusion, the present study demonstrates concomitant OT synthesis and secretion, as well as OTR expression, by three distinct human SCCL cell lines. Moreover, we observed an exogenous as well as an endogenous OT-induced increase in SCCL proliferation. The clinical significance of these findings is currently being investigated in human patients with this type of lung cancer. Nevertheless, immunotherapy toward tumor-derived OT or OT-NP already appears as a valuable new perspective not only for the treatment of SCCL patients with poor response to chemotherapy and radiotherapy-resistant disease but also for all patients expressing OTR. Finally, our results suggest that OT antagonists may offer promise as a potential new therapeutic modality for the treatment of SCCL.

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