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In Vitro Characterization of Somatostatin Receptors in the Human Thymus and Effects of Somatostatin and Octreotide on Cultured Thymic Epithelial Cells

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ABSTRACT

Somatostatin (SS) and its analogs exert inhibitory effects on secretive and proliferative processes of various cells *via* high affinity SS receptors (SS-R). SS analogs bind with different affinity to the five cloned SS-R subtypes. Octreotide, an octapeptide SS analog, binds with high affinity to the SS-R subtype 2 (sst_2). SS-R have been demonstrated *in vivo* and *in vitro* on cells from endocrine and immune systems. Among the lymphatic tissues, the thymus has been shown to contain the highest amount of SS, suggesting a local functional role of the peptide.

We investigated the SS distribution and SS-R expression pattern in the normal human thymus using autoradiography, membrane homogenate binding studies, and RT-PCR. In addition, the effect of SS and octreotide on growth of cultured thymic epithelial cells (TEC) was studied.

By autoradiography, binding of [^{125}I-Tyr^0]-SS-28 and [^{125}I-Tyr^3]-octreotide was detected in all seven thymuses studied. Specific [^{125}I-Tyr^3]-octreotide binding was shown on membrane preparations from thymuses, while not from cultured thymocytes. RT-PCR showed the expression of sst_1, sst_{2A} and sst_3 messenger RNA (mRNA) in the thymic tissue, whereas sst_1 and sst_{2A} mRNAs were found in isolated TEC. SS mRNA was present in thymic tissue and in isolated TEC. SS and octreotide significantly inhibited ^{3}H-thymidine incorporation in 3 of 3 and 6 of 6 TEC cultures, respectively. The percent inhibition ranged from 38.8 to 66.8% for SS and from 19.1 to 59.5% for octreotide.

In conclusion, SS mRNA and sst_1, sst_{2A}, and sst_3 mRNAs are expressed in the normal human thymus. Cultured TEC selectively express sst_1 and sst_{2A} mRNA and respond *in vitro* to SS and octreotide administration with an inhibition of cell proliferation. These data suggest a paracrine/autocrine role of SS and its receptors in the regulation of cell growth in thymic microenvironment. (*Endocrinology* **140:** 373–380, 1999)

SOMATOSTATIN (SS), a peptide hormone originally isolated from the hypothalamus as a GH-releasing inhibiting factor, has been found throughout the central nervous system where it predominantly acts as a neurotransmitter, as well as in widely distributed endocrine cells in other tissues (1–3). It has diverse biological effects on cellular function, including inhibitory effects on secretive and proliferative processes and modulatory actions on the response of various cells to endocrine stimulation (2–4). The various actions of SS are mediated through five specific high affinity membrane receptors (SS-R) (5). These receptors have been demonstrated *in vitro* in normal and tumoral tissues by classical binding techniques, *in situ* hybridization and RT-PCR (4, 6–8). In addition, using radiolabeled SS analogs, several scintigraphic studies have shown the *in vivo* localization of SS-R-positive tumors (9, 10). SS analogs bind with different affinity to the five different SS-R subtypes (sst_{1-5}). Octreotide, the most extensively studied SS analog, binds with high affinity to sst_2 and with lower affinity to sst_3 and sst_5 (5, 8). Among the five subtypes, sst_2 seems the most important in mediating the antisecretive effect of octreotide (11).

Like other regulatory peptides found in the brain, SS has also been localized in lymphatic tissue (12). Among the lymphatic organs, the thymus from different species including the human has been shown to contain the highest amount of SS, suggesting a modulatory role of the peptide in this organ (13–15). Furthermore, SS-R have been demonstrated *in vitro* in various lymphatic tissues (16). Recently, a high *in vivo* uptake of ^{111}In-DTPA-D-Phe^1-octreotide was shown in patients bearing thymomas and thymic carcinoids (17, 18), and a successful treatment with octreotide was reported in one patient with thymoma (19). The presence of SS and SS-R in the thymus suggests their involvement in controlling the immuno- and/or neuroendocrine functions in this organ. Moreover, SS analog treatment of thymic tumors and related paraneoplastic diseases might represent a new therapeutic approach to these disorders.

The present study was designed to investigate the expression and role of SS and SS-R subtypes in the normal human thymus. The receptor expression pattern in the thymuses was studied *in vitro* by SS-R autoradiography, membrane homogenate binding studies, and RT-PCR to identify SS-R subtypes. In addition, the *in vitro* effect of SS, octreotide, and the

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growth factors insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF) on cell proliferation was investigated in primary human thymic epithelial cell (TEC) cultures. Because fibroblasts often contaminate cultures of epithelial cells, we used a specific antibody to human fibroblasts (mAb ASO2) coupled with magnetic beads for the selective removal of fibroblasts from these cultures. This new monoclonal antibody recognizes a human fibroblast-specific antigen located on the cell surface (20).

Materials and Methods

Samples

Thymic tissues were removed routinely from 7 patients (age range, 1 month to 16 yr) to allow adequate exposure of the heart during cardiovascular surgery. Samples from these thymuses were used in the present study. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation, informed consent was obtained from patients or their parents. All samples were histopathologically normal and were taken fresh at the operation, quickly frozen on dry ice, and stored at −80 C for autoradiography and RT-PCR studies. From six thymic tissues, specimens were also used for the establishment of primary TEC cultures.

SS receptor autoradiography

Receptor autoradiography was carried out as described by Reubi *et al.* (21). Briefly, 10-micrometer thick cryostat (Jung CM3000, Leica Corp., Germany) sections of the tissue samples were mounted onto precleaned gelatin-coated microscope glass slides and stored at −80 C for at least 3 days before the experiment to improve the adhesion of the tissue to the slide. As radioligands, the SS analogs [^{125}I-Tyr^3]-octreotide and [^{125}I-Tyr^0]-SS-28 (ANAWA Laboratories, Wangen, Switzerland) were used. Specific activities of the radioligands amounted approximately 2000 Ci/mmol. To wash out endogenous SS, the sections were preincubated at room temperature for 10 min in 170 mM Tris-HCl (pH 7.4). Thereafter, the sections were incubated for 60 min at room temperature in 170 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1% BSA, 40 μg/ml bacitracin with [^{125}I-Tyr^3]-octreotide (final concentration approximately 80–160 pmol/liter) or [^{125}I-Tyr^0]-SS-28 (approximately 80–160 pmol/liter). Nonspecific binding was determined in a sequential section in the presence of excess unlabeled Tyr^3-octreotide (1 μM) or SS-28 (1 μM), respectively. The incubated sections were washed twice for 5 min in incubation buffer containing 0.25% BSA and once in incubation buffer without BSA. After a short wash with distilled water to remove salts, the sections were air dried and exposed to Kodak X-OMAT AR or Hyperfilm-^{3}H (Amersham, Houten, The Netherlands) for 3–7 days in x-ray cassettes. Histology was performed on hematoxylin-eosin stained sequential cryosections.

RT-PCR

Poly A$^+$ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from cell pellets containing approximately 10^6 cells or from approximately 50 mg frozen powdered tissue that was grinded in a metal homogenizer cooled by liquid nitrogen. The cells were lysed during 2 min on ice in a buffer containing 100 mM Tris-HCl (pH 8), 500 mM LiCl, 10 mM EDTA (pH 8), 1% LiDS, 5 mM DTT and 5 U/100 μl RNAsin (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. To the supernatant 100 μl prewashed Dynabeads Oligo $(dT)_{25}$ were added, and the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with 10 mM Tris-HCl (pH 8), 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, and once with a similar buffer from which LiDS was omitted. Poly A$^+$ mRNA was eluted from the beads in 50 μl of a 2 mM EDTA solution (pH 8) during 2 min at 65 C. To avoid contamination by genomic DNA, the isolated polyA$^+$ RNA was subjected to a second purification by capturing the RNA on a fresh aliquot of prewashed Dynabeads Oligo $(dT)_{25}$ and washing the captured RNA as above. Finally, the captured RNA was washed once with buffer used for the reverse transcriptase reaction.

Complementary DNA (cDNA) was synthesized using the poly A$^+$ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ in a buffer containing 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM DTT, 10 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 10 U RNAsin, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 20 μl. This mixture was incubated for 1 h at 41 C.

One-tenth from each cDNA library immobilized on the paramagnetic beads was used for each amplification. The amplification reaction mixtures contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd., Cambridge, UK), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd., Cambridge, UK), 5 pmol of each of a pair of oligonucleotide primers specific for the human sst_{1-5} receptor subtypes, the β-actin gene or the human SS gene (see Table 1) in a buffer of 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 in a final volume of 50 μl. The sequences of the primers for sst_{1-5} are derived and/or adapted from Kubota *et al.* (8) and Wulfsen *et al.* (22). The PCR reaction was carried out in a DNA thermal cycler with heated lid (Perkin Elmer Cetus Instruments, Gouda, The Netherlands). After an initial denaturation at 94 C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 C for 1 min, annealing for 2 min at 59 C, and extension for 1 min at 72 C. After a final extension for 7 min at 72 C, 10 μl aliquots of the resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A$^+$ mRNA preparation (because the SS-R subtypes genes are intron-less), the cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair. Amplification of the cDNA samples with the β-actin specific primers served as positive control for the quality of the cDNA. To exclude contamination of the PCR reaction

TABLE 1. Primers used for RT-PCR analysis

	Sequence (5′- 3′)[a]	Position[b]	Size of PCR product
Sst_1 (forward)	ATGGTGGCCCTCAAGGCCGG	754	318 bp
Sst_1 (reverse)	CGCGGTGGCGTAATAGTCAA	1071	
Sst_{2A} (forward)	TCCTCTGGAATCCGAGTGGG	709	332 bp
Sst_{2A} (reverse)	TTGTCCTGCTTACTGTCACT	1040	
Sst_3 (forward)	TCATCTGCCTCTGCTACCTG	662	221 bp
Sst_3 (reverse)	GAGCCCAAAGAAGGCAGGCT	882	
Sst_4 (forward)	ATCTTCGCAGACACCAGACC	547	323 bp
Sst_4 (reverse)	ATCAAGGCTGGTCACGACGA	869	
Sst_5 (forward)	CCGTCTTCATCATCTACACGG	596	223 bp
Sst_5 (reverse)	GGCCAGGTTGACGATGTTGA	819	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	1	349 bp
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	348	
β-actin (forward)	ATC CTCACCCTGAAGTACCC	190	762 bp
β-actin (reverse)	GATCTCCTTCTGCATCCTGT	951	

[a] The sequences of the primers for sst_{1-5} are derived and/or adapted from Kubota *et al.* (8) and Wulfsen *et al.* (22).
[b] The position is given of the 5′ nucleotide of the primer relative to the first nucleotide of the coding region in the cDNA sequence.

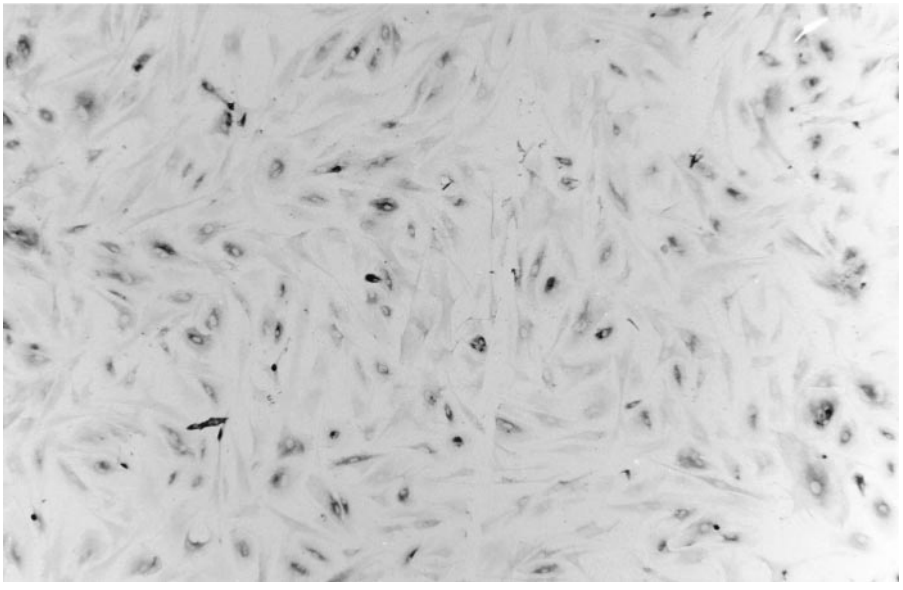

FIG. 1. Staining for keratin of thymic epithelial cells (TEC) cultured on coverslips for the same period as TEC used for the experiments. No fibroblasts (keratin-negative) are present.

mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the SS-R receptor subtypes, 0.01 μg of human genomic DNA was amplified in parallel with the cDNA samples. As a positive control for the PCR of the β-actin and SS cDNA aliquots of a cDNA sample known to contain SS (and β-actin) mRNA were amplified, because these primer-pairs did enclose introns in the genomic DNA.

Cell dispersion and cell culture

After the specimens were placed in HBSS supplemented with HSA 5% (Cealb, CLB, Amsterdam, The Netherlands), penicillin (10^5 U/liter), fungizone (0.5 mg/liter), blood clots, and fibrous capsules were carefully removed and the specimens were dissected and washed several times with the HBSS + HSA. The minced tissues were enzymatically dissociated with collagenase (Sigma Chemical Co., St. Louis, MO; 2 mg/ml) for 1–2 h at 37 C. The dispersed cells were precultured for 5–7 days in 15 mg/cm^2 collagen type I (Collagen S, type I, Boehringer Mannheim, Mannheim, Germany) precoated 75-cm^2 flasks (Costar, Cambridge, MA) at a density of 5×10^6 cells/flask in 10 ml culture medium. The culture medium, the same used during the experiments, was MEM D-valine (Gibco BRL, Life Technologies Ltd., Paisley, Scotland, UK) supplemented with 10% FCS, penicillin (10^5 U/liter), fungizone (0.5 mg/ml), L-glutamine (2 mmol/liter). The pH of the medium was adjusted to 7.4. The cells were cultured at 37 C in a humid CO_2-incubator. After this period, cell viability was detected by trypan blue exclusion and varied between 65 and 90%. The cells, which had not attached to the flasks, mainly thymocytes, were harvested.

The isolation of TEC was indirectly performed using sheep antimouse IgG coated magnetic beads and a ASO2 antihuman fibroblast primary antibody kit (Dianova GmbH, Hamburg, Germany). This mouse monoclonal antibody reacts specifically with membrane-bound protein of human fibroblasts of different origin (20). The magnetic beads were coated with the specific primary antibody and mixed with the target cell suspension to form bead rosetted cells. Rosetted cells (fibroblasts) were collected at the tube wall and isolated using a magnetic separator rack. For a complete negative selection, the supernatant, containing the cells which were not bound to the beads (TEC), was treated once again with the coated beads. The remaining suspension containing isolated TEC was used for the experiments.

TEC (10,000–20,000 cells per well) were seeded in 1 ml culture medium in 24-well collagen type I precoated plates (Costar) and allowed to attach for 24 h. Then, tests substances were added, and the cells were incubated for 72 h. Proliferation was measured by adding 1 μCi of [methyl-^{3}H]-thymidine (91 Ci/mmol; Amersham) for the last 24 h in each well. Thereafter, the medium was removed and the cells were washed twice with ice cold 0.9% NaCl solution and harvested using a 0.05% NH_3 solution. These samples were exposed overnight to 10% trichloroacetic acid followed by another wash with 0.9% NaCl solution. The cells were transferred to scintillation counting vials after solubilization in 1 M NaOH and incorporated radioactivity was measured, after neutralization with HCl and the addition of scintillation fluid, in a liquid scintillation counter (Betamatic, Packard, Downers Grove, IL). For RT-PCR studies, TEC were seeded in collagen-coated flasks (see above) and grown to confluence. Thereafter, the cells were harvested and mRNA was isolated as described above.

TABLE 2. Somatostatin and somatostatin receptor subtype expression in human thymic tissue as determined by somatostatin receptor-autoradiography and RT-PCR

Tissues	Somatostatin receptor-autoradiography		RT-PCR					
	[^{125}I-Tyr^0]-SS-28	[^{125}I-Tyr^3]-octreotide	sst_1	sst_{2A}	sst_3	sst_4	sst_5	SS
1	+	+	+	+	+	−	−	+
2	+	+	+	+	+	−	−	+
3	+	+	+	+	+	−	−	+
4	+	+	+	+	+	−	−	+
5	+	+	+	+	+	−	−	+
6	+	+	+	+	+	−	−	+
7	+	+	+	+	+	−	−	+

SS, Somatostatin. The experiments were performed at least twice with identical results.

Immunocytochemical detection of keratin on cultured TEC

For keratin staining, TEC were cultured on collagen-coated glass coverslips. The cells were fixed for 10 min with methanol at the end of the incubation period. Cytokeratin staining was performed with a PAP Kit System (code K518; Dako Corp., Glostrup, Denmark). Staining for keratin was performed after each experiment in all TEC cultures studied.

SS receptor binding studies

The method of membrane isolation and the reaction conditions were the same as described by Reubi (23). Briefly, membrane preparations (corresponding to 30–50 μg protein) of tissue samples, freshly dispersed cells or cultured cells were incubated in a total volume of 100 μl at room temperature for 60 min with increasing concentrations of [^{125}I-Tyr^3]-octreotide with and without excess (1 μM) of unlabeled Tyr^3-octreotide in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/liter bacitracin,

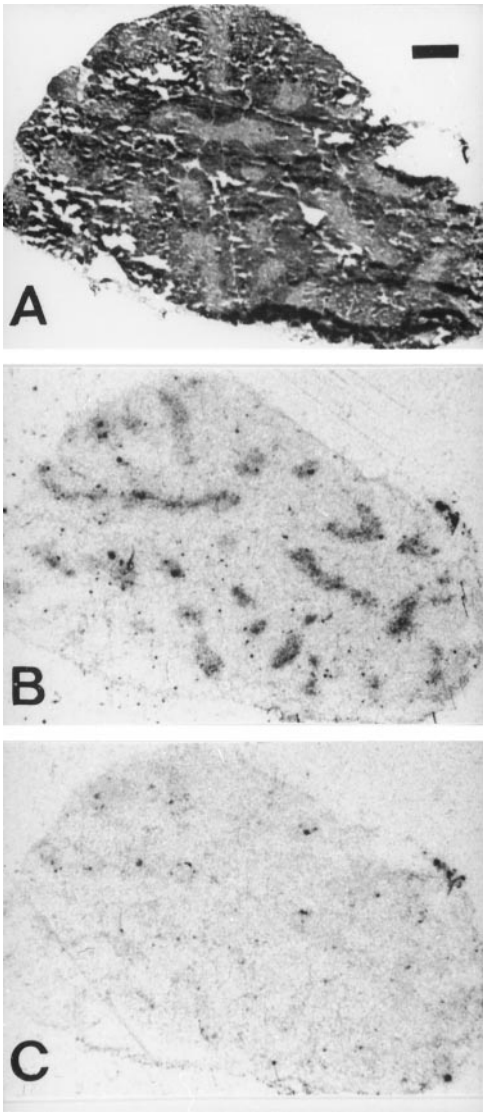

FIG. 2. Expression of SS-R in human thymus. Photomicrograph of SS-R-autoradiography. A, Hematoxylin-eosin stained section; B, autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide; C, autoradiogram showing nonspecific binding (in the presence of 1 μM of Tyr^3-octreotide). *Bar*, 1 mm. Identical expression patterns of SS-R were found in all cases that were studied.

pH 7.6) containing 0.2% BSA. After the incubation, 1 ml ice-cold HEPES buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 rpm in a Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled Tyr^3-octreotide.

Test substances

In all experiments SS-14 (Bachem, Inc., Hannover, Germany) and octreotide (Novartis, Basle, Switzerland) were used at a concentration of 10 nM, IGF-I (Bachem, Inc.) at a concentration of 10 nM and EGF (Bachem, Inc.) at a concentration of 10 ng/ml.

Statistical analysis

Data are expressed as mean ± SEM, n = 4 wells per treatment group. All data were analyzed by ANOVA to determine overall differences between treatment groups. When significant differences were found, a comparison between treatment groups was made using the Newman-Keuls test. SS-R binding data were analyzed by the method of Scatchard. Receptor binding studies and RT-PCR experiments were performed at least twice.

TABLE 3. Somatostatin receptor expression in human thymic tissue determined by Scatchard analysis of [^{125}I-Tyr^3]-octreotide binding on membrane homogenates

Tissues	[^{125}I-Tyr^3]-octreotide binding	
	K_d (nM)	B_{max} (fmol/mg protein)
4	0.7 ± 0.2	15.5 ± 0.5
5	0.03 ± 0.01	3.5 ± 0.5
6	0.7 ± 0.1	18.5 ± 2.5
Rat brain	0.9 ± 1.3	196.5 ± 46.5

K_d, dissociation constant; B_{max}, maximum binding capacity. The data represent the mean ± SEM of two independent experiments.

Results

Fibroblast-epithelial-cell separation and staining for keratin in cultures of TEC

The cell selection system using a mouse monoclonal antibody to human fibroblasts coupled to magnetic beads enabled us to identify and eliminate fibroblasts from our cell cultures to obtain 95–100% pure TEC cultures. This purity was demonstrated by immunocytochemical staining for keratin in the cultured TEC. Figure 1 shows an example of the staining for keratin of a TEC population that was used for the functional study (see below). Virtually no fibroblasts (keratin-negative) were present.

SS and SS-R (subtype) expression

At autoradiography, [^{125}I-Tyr^0]-SS-28 binding was found in the seven normal thymuses. Binding of the sst_2 subtype selective ligand [^{125}I-Tyr^3]-octreotide was in parallel positive in all these seven cases (Table 2), and in agreement with a previous study (16). Binding was not homogeneous and mainly localized in the medulla (Fig. 2).

Using [^{125}I-Tyr^3]-octreotide, specific binding was demonstrated on membrane preparations of thymic tissue, whereas no binding was found on the cultured thymocytes. Binding of [^{125}I-Tyr^3]-octreotide could be displaced with excess unlabeled Tyr^3-octreotide. Scatchard analysis of the binding data revealed a single class of high affinity binding sites with an apparent K_d ranging from 0.03 ± 0.01 to 0.7 ± 0.1 nM and a low maximum binding capacity (B_{max}) ranging from 3.5 ± 0.5 to 18.5 ± 2.5 fmol/mg membrane protein (Table 3). As a control for binding, rat brain cortex membranes were used. An example of saturation binding data with Scatchard analysis is shown in Fig. 3.

By RT-PCR, SS, sst_1, sst_{2A} and sst_3 mRNA expression was detected in all thymuses (example in Fig. 4; no. 5, Table 2). Conversely, mRNA encoding for sst_4 and sst_5 was absent (Table 2; Fig. 4). In cultured TEC, RT-PCR analysis showed the presence of SS, sst_1 and sst_{2A} mRNA, while in cultured thymocytes only mRNA for β-actin was detected (Table 4; Fig. 4).

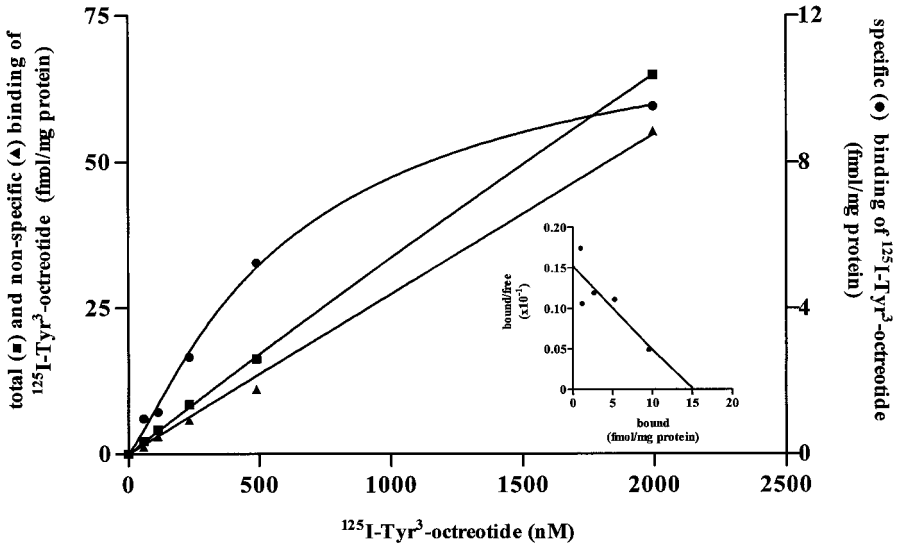

FIG. 3. Binding of [^{125}I-Tyr^3]-octreotide to a membrane homogenate preparation of a human thymus. ■, total binding; ▲, nonspecific binding in presence of 1 μM of Tyr^3-octreotide; ●, specific binding (total minus nonspecific binding). Inset: Scatchard analysis of the binding data (K_d, 0.5 nM and B_{max}, 15 fmol/mg membrane protein; no. 4, Table 3).

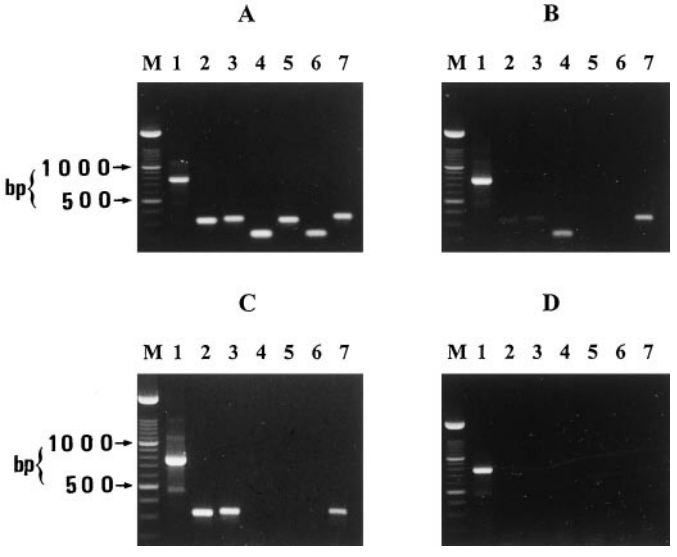

FIG. 4. Heterogeneous expression of β-actin, sst_1 sst_{2A}, sst_3, and SS mRNAs in the human thymus. Poly A^+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. 1, β-actin; 2, sst_1; 3, sst_{2A}; 4, sst_3; 5, sst_4; 6, sst_5; 7, SS. M, 100-bp ladder; A, control; B, thymic tissue; C, thymic epithelial cells; D, thymocytes; (no. 5, Table 2). RT-PCR analysis of each tissue was performed at least twice with identical results.

In vitro effect of SS, octreotide, IGF-I and EGF on ^{3}H-thymidine incorporation in cultured human TEC

SS (10 nM) significantly inhibited ^{3}H-thymidine incorporation by 38.8, 61.1, and 66.8% respectively, in cultures of TEC derived from three different normal thymuses (Fig. 5A). The SS analog octreotide (10 nM) significantly inhibited ^{3}H-thymidine incorporation by 38.7, 49.8, and 40.6%, respectively, in these three cultures (Fig. 5A), and by 19.1, 48.2, and 59.5% in three other additional cultures of TEC (Fig. 5B) derived from the series of thymuses which showed [^{125}I-Tyr^3]-octreotide binding at autoradiographic and membrane binding studies. IGF-I significantly stimulated ^{3}H-thymidine incorporation in two out of three cultures and EGF significantly stimulated ^{3}H-thymidine incorporation in all the three cultures in which were tested (Table 5). Moreover, octreotide significantly inhibited EGF-stimulated ^{3}H-thymidine incorporation by 25 ± 5% in one culture in which it was tested (data not shown).

TABLE 4. Heterogeneity of somatostatin and somatostatin receptor subtype mRNA expression in cultured human thymic epithelial cells and thymocytes as determined by RT-PCR in 5 cases

Cell subset	RT-PCR						
	sst_1	sst_{2A}	sst_3	sst_4	sst_5	SS	β-actin
TEC	+	+	−	−	−	+	+
Thymocytes	−	−	−	−	−	−	+

TEC, Thymic epithelial cells; SS, somatostatin. Each case was evaluated at least two times in independent experiments and yielded identical results.

Discussion

Neuropeptide hormones produced in the brain and gastrointestinal tract have been found in the thymus and are involved in the complex pattern of interactions existing between the neuroendocrine and the immune systems in this organ (24). In fact, epithelial cell and thymocyte functions are influenced by different hypothalamic and pituitary hormones. A network between these hormones, even locally produced, and the related receptors has been shown (25–28). A place in this network might be hypothesized for SS and SS-R as well. SS-R have been demonstrated in various endocrine and lymphatic tissues by classical biochemical binding studies (4, 6, 7, 16). In addition, SS has been demonstrated in the thymus from different species including humans (12–15), where it seems to be involved in the main function of the gland (12, 13). However, the influence of SS and SS-R on the regulation of TEC function had not been studied so far.

In the present report, we studied the distribution and the expression of SS-R subtypes and SS in 7 normal human thymuses. We first demonstrated specific [^{125}I-Tyr^3]-octreotide binding on cryostat sections from thymic tissue, mainly in the medulla, which is the thymic compartment

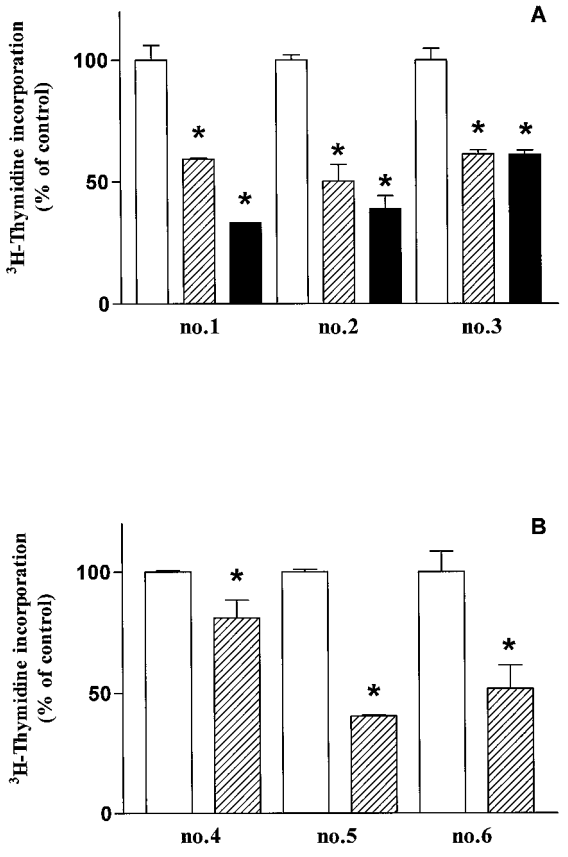

FIG. 5. A, Effects of SS and octreotide on ^{3}H-thymidine incorporation in TEC cultures of three different thymuses (nos. 1–3, Table 2). B, Effects of octreotide on ^{3}H-thymidine incorporation in TEC cultures of three other thymuses (nos. 4–6, Table 2). Thymic epithelial cells were incubated in MEM + 10% FCS during 72 h in quadruplicate without or with 10 nM of the drugs indicated. Values are expressed as percentage of ^{3}H-thymidine incorporation by control cells and are mean ± SEM; *, $P < 0.01$ *vs.* control. ▨, octreotide; ■, somatostatin-14. Control values of ^{3}H-thymidine incorporation were: 3743.8 ± 221.9 (no. 1), 897.1 ± 18.6 (no. 2), 492.0 ± 23.0 (no. 3), 410.5 ± 2.1 (no. 4), 1347.3 ± 14.6 (no. 5), 756.3 ± 64 (no. 6) cpm.

where the TEC is the predominant cell type, as well as in thymic tissue homogenates. Furthermore, we characterized the SS-R subtype expression in the same thymuses that were used for receptor binding studies. In human thymic tissue, we found by RT-PCR the presence of mRNA encoding for sst_1 and sst_{2A} and sst_3. The expression of the subtypes appeared heterogeneous on the different cell subsets. In fact, we found a specific binding of [^{125}I-Tyr^3]-octreotide on membrane preparations from thymic tissue, whereas no binding was found on cultured thymocytes. Interestingly, RT-PCR showed the selective expression of sst_1 and sst_{2A} on the cultured TEC, whereas neither SS-R subtype was found on cultured thymocytes with this technique. Sst_1 and sst_{2A} seem stronger expressed in the cultured TEC compared with the thymic tissue, because with the selection method used to establish primary TEC cultures, an enrichment of SS-R-expressing TEC was obtained. Again, it should be emphasized that the cultured thymocytes, which represent the major cellular component of the thymus, did not express any SS-R subtype. Moreover, the finding that SS-R are expressed on only a subset of the cells in the thymus also explains the relatively low SS-R levels measured by receptor binding studies on membrane homogenates of the whole thymic tissue. Finally, this heterogeneous SS-R expression pattern in the human thymus may also explain the apparent discrepancy between the SS-R levels in the thymic tissues and the maximal inhibitory effect of SS and octreotide on TEC proliferation.

TABLE 5. Effects of IGF-I and EGF on ^{3}H-Thymidine incorporation in cultured thymic epithelial cells

Cell culture	^{3}H-Thymidine incorporation (cpm)		
	Control	IGF-I	EGF
4	410.5 ± 2.1	562.2 ± 24.4[a]	974.6 ± 25.8[a]
5	1347.3 ± 14.6	1624.8 ± 75.3	3326.8 ± 57[a]
6	756.3 ± 64	993.5 ± 89.3[a]	4125.7 ± 270.3[a]

The data represent the mean ± SEM of four wells per treatment group.
[a] $P < 0.01$ *vs.* control.

Furthermore, TEC seem to be the major site of SS production in the normal human thymus. In fact, by RT-PCR we demonstrated the presence of mRNA for SS in isolated TEC from all the thymuses of the present series. Because our study showed the existence of a specific SS-R subtype distribution pattern as well as evidence for endogenous SS production in the human thymus, we also studied the effects of SS and the SS analog octreotide on TEC proliferation. At this purpose, we established pure epithelial cell cultures, demonstrated by staining for cytokeratin that showed 95–100% cytokeratin-positive cells in all the cultures. In these human TEC cultures, we observed a significant inhibition of cell proliferation by SS and octreotide in all cases.

The heterogeneity of distribution of SS-R subtypes on specific cells, in combination with the endogenous production of SS, suggest an important and dynamic regulatory role of this peptide in the human thymus. The data of the present study indicate that SS may exert a paracrine/autocrine inhibitory action. This effect seems to be mediated by the sst_{2A} and/or sst_1, which are selectively expressed on cultured TEC. The binding of SS or its analogs to these receptor subtypes is associated with an activation of intracellular tyrosine phosphatase activity (29, 30). Moreover, sst_{2A} is also linked to cell membrane potassium and calcium channels in a manner that ligand binding influences the intracellular concentration of these ions and the cell membrane polarization (31).

Although we did not investigate the expression of SS-R and the effect of SS analogs on thymic tumoral cells, it can be suggested that a disturbance in this pathway may play a role in the pathogenesis of neoplastic and related autoimmune diseases in the human thymus. Recently, a high *in vivo* uptake of ^{111}In-DTPA-D-Phe^1-octreotide was reported in patients bearing thymic neoplasms (17, 18) and a successful treatment with octreotide was reported in one patient with thymoma (19). This effect might be mediated by SS-R. Although in a previous study no *in vitro* evidence of SS-R has been found in four thymomas, it is well known that there is a considerable heterogeneity between and within differentiated tumors with respect to the density and the specificity of SS-R binding sites (32).

We also demonstrated the expression of sst_3 in the normal

human thymic tissue, whereas sst_3 mRNA was undetectable in cultured TEC. This may be explained by the fact that this receptor subtype is expressed by a different epithelial cell subset or on macrophages. Emerging data regarding signal transduction pathways linked to SS-R showed a cell cycle-dependent induction of apoptosis by octreotide (33). Apoptosis seems signaled through the sst_3 and associated with dephosphorylation-dependent conformational change in wild-type p53 (34). Although controversial data about alterations of p53 expression have been reported in thymic epithelial tumors (35, 36), octreotide-induced apoptosis might represent an additional mechanism involved in the regulation of cell proliferation in human thymus.

Another important aspect involves the local production of growth factors and cytokines by TEC and thymocytes (27, 28, 37). In our study, IGF-I and EGF significantly stimulated *in vitro* TEC proliferation. Moreover, in a preliminary experiment, octreotide significantly inhibited EGF-stimulated cell proliferation. Although an inhibition of the production of these factors might represent an indirect mechanism of action of SS and its analogs, a direct mechanism involving the phosphoprotein phosphatase activity associated to sst_2 could not be ruled out (38). Further studies are required to investigate the precise mechanism of action of SS in inhibiting growth factors-stimulated TEC proliferation.

These findings suggest that the SS-R-mediated effects of SS and octreotide on TEC growth may act *via* not mutually exclusive different mechanisms, supporting the physiological paracrine/autocrine role of SS in controlling cell growth in the human thymic microenvironment. A further conceptual aspect might be the implication of the role of the intrathymic production of SS in the main function of the thymus, namely the maturation and differentiation of T-lymphocytes. Moreover, a disturbance in these loops may be important in the pathogenesis of autoimmune and neoplastic diseases involving this organ. In this respect, a better understanding of the functional significance of the presence of SS and SS-R subtypes in the human thymus might lead to new approaches in the medical management of these disorders.

In conclusion, we have demonstrated a heterogeneous expression of SS-R subtypes within the human thymus. Furthermore, TEC respond *in vitro* to SS and octreotide administration with an inhibition of cell proliferation. These data, together with the evidence of other peptide hormones, cytokines and their receptors in the thymus, support the concept of the thymus as the organ where the interactions between the endocrine and the immune systems are mostly represented. Future studies should investigate the influence of SS and its analogs on local cytokine production, which is known to represent the other arm of the immuno-endocrine pathway.

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