PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ROLE OF SOMATOSTATIN RECEPTORS IN THE HUMAN THYMUS

Ferone Diego

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FYSIOLOGISCHE EN PATHOFYSIOLOGISCHE ROL VAN SOMATOSTATINE RECEPTOREN IN DE MENSELIJKE THYMUS

THESIS

to obtain the degree of doctor from
the Erasmus University Rotterdam
on the authority of the Rector Magnificus
Prof. dr. P.W.C. Akkermans M.A. and
according to the decision of the Doctorate Board.
The public defence shall be held on
Monday, 8th May 2000 at 15.45 hrs

by

Diego Ferone

born in Capri (Italy)

DOCTORAL COMMITTEE

Supervisor:	Prof. dr. S.W.J. Lamberts
Assistant supervisor:	Dr. L.J. Hofland
Other members:	Prof. dr. R. Benner Prof. dr. E.P. Krenning Prof. dr. I.P. Touw

Paranimfen: Wouter W. de Herder, Rosario Pivonello

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
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en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
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door

Diego Ferone

geboren te Capri (Italië)

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Paranimfen: Wouter W. de Herder, Rosario Pivonello

Dit proefschrift is tot stand gekomen binnen de afdeling Inwendige Geneeskunde
van de Erasmus Universiteit Rotterdam.

In de drukkosten van dit proefschrift werd bijgedragen door Novartis Farma, Milano, Italië.

ai miei genitori

per Annamaria

to my parents

for Annamaria

"All our knowledge brings us to our ignorance"

T.S. Elliot, "The Rock"

PUBLICATIONS BASED ON THE STUDIES DESCRIBED IN THE THESIS

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Ferone D, van Hagen PM, Colao A, Lombardi G, Lamberts SWJ, Hofland LJ. Distribution, role and function of somatostatin receptors in immune cells. In: Lamberts, S.W.J. (ed): Octreotide: The Next Decade. BioScientifica Ltd, Bristol 1999: pp 259-275.

Chapter II

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Ferone D, Pivonello R, Lichtenauer-Kaligis EGR, van Hagen PM, Waaijers M, van Koetsveld PM, Mooy DM, Colao A, Lamberts SWJ, Hofland LJ 2000 Quantitative and functional distribution of somatostatin receptor subtypes in human thymocytes. *Submitted*

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Chapter III

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CONTENTS

ABBREVIATIONS

ACTH	adrenocorticotropin
α-MSH	alpha-melanocyte-stimulating hormone
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosin monophosphate
Ci	Curie(s)
cpm	counts per minute
CRF	corticotropin releasing factor
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EDTA	ethylenediamine tetra-acetate
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
g	gram(s)
GH	growth hormone
GHRH	Growth hormone-releasing hormone
h	hour(s)
HBSS	Hanks' balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
HLA	human leukocyte antigen
HPRT	hypoxanthine guanine phosphoribosyltransferase
HSA	human serum albumin
IFN-γ	interferon gamma
Ig	immunoglobulin
IGF-I	insulin-like growth factor 1
IL	interleukin

LH	Luteinizing hormone
m	milli (10^{-3})
M	molar (mole per liter)
max	maximum
MEM	minimal essential medium
mg	milligram
MG	myasthenia gravis
MHC	major histocompatibility complex
min	minute(s)
ml	milliliter
μ	micro (10^{-6})
μg	microgram
μl	microliter
mRNA	messenger ribonucleic acid
n	nano (10^{-9})
N	normal (concentration)
no.	number
P	probability
PAP	peroxidase anti peroxidase
PBS	phosphate-buffered saline
PE	phycoerythrin
PHA	phytohemagglutinin
POMC	proopiomelanocortin
PRCA	pure red cell aplasia
PRL	prolactin
PTPase	protein tyrosine phosphatase
ROI	region of interest
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SP	substance P
SPECT	single photon emission computed tomography
SRS	somatostatin receptor scintigraphy
SS	somatostatin

SSR	somatostatin receptor(s)
sst_{1-5}	somatostatin receptor subtypes 1-5
TCR	T-cell receptor
TEC	thymic epithelial cells
TNC	thymic nurse cells
TNF-α	tumor necrosis factor-alpha
T/NS	total/non specific
Tris	tris(hydroxymethyl)-aminomethane
TRH	thyrotropin-releasing hormone
TSH	thyrotropin
T-to-B	tumor to background
VIP	vasoactive intestinal peptide
WHO	World Health Organization
yr	year(s)

CHAPTER I

SOMATOSTATIN RECEPTORS IN THE IMMUNE SYSTEM

INTRODUCTION

During the first half of the previous century, the evidence that the endocrine and nervous systems integrate and regulate different functions in the human body coincided with the new findings supporting the concept that immune mechanisms may be influenced by these systems as well. In the past decades, many studies were carried out to understand the molecular and cellular basis of interactions between the immune system and neuroendocrine activities.

The interactions between the immune, endocrine and nervous systems are very complex. Although the accumulated information is enormous, many reciprocal interactions are poorly understood, several mechanisms are still difficult to interpret, and in some cases the results are even contradictory. Nevertheless, increasing numbers of researchers and many laboratories are nowadays focused on understanding how hormones, neuropeptides, neurotransmitters and specific products of immune cells exert their regulatory functions and act in integrating such different systems.

How immune and neuroendocrine mechanisms may affect each other is summarized in Figure 1. Firstly, immune, endocrine and neural cells can express receptors for cytokines, hormones and neuropeptides. Secondly, immune and neuroendocrine products may coexist

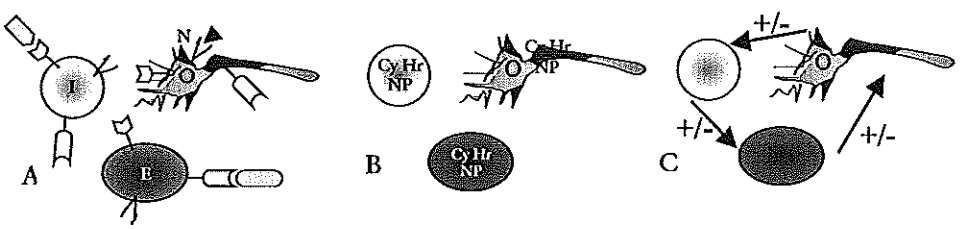

FIGURE 1. Representation of the different ways of communication between immune, endocrine and nervous systems. A) Reciprocal receptors for cytokines, hormones and neuropeptides expressed on immune, endocrine and neural cells. B) Immune and neuroendocrine products coexist in lymphoid, endocrine and neural tissues. C) Specific mediators of one of the three systems can affect the others.
I, immune cell; E, endocrine cell; N, neural cell; Cy, cytokines; Hr, hormones; NP, neuropeptides.

in lymphoid, endocrine and neural tissues and may be produced by all this type of cells (1). Thirdly, specific mediators for one of the three systems can affect the other two.

The functional interactions between immune, endocrine and neural cells occur at different levels, which underline their relevance in controlling physiological processes, any disturbances herein may be potentially involved in certain pathological conditions (1-2). Studies in animal models have demonstrated that abnormalities in the immune-neuro-endocrine communications may start or contribute to the development of chronic autoimmune diseases. In analogy, similar questions have been raised for humans.

Direct cell-to-cell communication is one of the mechanisms involved in immuno-neuro-endocrine interaction and neuroendocrine and immune systems share a number of ligands and related receptors (3). It has been hypothesised that the majority of these signalling molecules are highly conserved, and that *via* these products the systems in many species are able to establish a much more sophisticated physiological intra- and intersystem communication circuit (4). The bi-directional neuroendocrine-immune interactions may constitute an important homeostatic mechanism and more recent studies addressed the question of the role of neuropeptides in regulating immune functions, starting from the developmental phases of the immune system (5).

Specific receptors for neuroendocrine regulatory peptides have been detected on cells of lymphoid tissues, and the pathway of interactions suggests a close topographical link between the various systems. In addition, lymphoid tissues are directly and extensively innervated, the nerve fibers being in direct contact with lymphocytes or their precursors and macrophages, performing their neuro-effector functions.

This evidence, together with the co-localization of neuroendocrine and immune cells and their locally released products, point for a pivotal role of neuropeptides in the modulation of the immune functions. Abnormalities in the cross talk between immune, endocrine and nervous systems could have crucial importance in the development of severe diseases. A clearer understanding of these interactions may have important therapeutic applications.

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NEUROPEPTIDES AND THEIR RECEPTORS IN THE IMMUNE SYSTEM

Neuropeptides are classically produced and secreted by neuroendocrine cells and neurons, and act as neurotransmitters and/or mediators of well-defined hormonal activities in specific tissues and cells. Communication through soluble effector molecules is not confined to the endocrine and nervous systems, but it is very important in the immune system as well. Moreover, various peptide hormones are involved in the three systems. The presence of peptide neurotransmitter and hormone receptors on immune cells suggests that the neuropeptides regulating the central and peripheral nervous system and the endocrine system could be considered as endogenous immunomodulatory substances, as well as bi-directional communicators between the immune and neuroendocrine systems.
Several neuropeptides and their respective receptors have been detected on cells of the immune system. Many studies described their potential role in regulating immune functions and the reciprocal immuno-neuro-endocrine interactions. This chapter gives a brief overview on the most well characterized effector systems, focusing particularly on neuropeptides and their potential role in the diagnosis and treatment of immune mediated diseases.

NEUROPEPTIDES IN THE IMMUNE SYSTEM

In their physiological environment, immune cells are exposed to agents such as hormones and neuropeptides, which do not strictly belong to the immune system. Moreover, immune cells can produce hormones and neuropeptides themselves. The first evidence was that of propiomelanocortin (POMC)-derived peptides, such as adrenocorticotrophic hormone (ACTH) and β-endorphins. ACTH-like immunoreactivity was demonstrated in leukocytes (1). It is intriguing that corticotrophin releasing factor (CRF), which is the natural stimulator of pituitary ACTH secretion, is also produced by immune cells (2), and that its production is clearly increased during inflammation (3). Moreover, POMC-derived alpha-melanocyte-stimulating hormone (α-MSH) is produced in the spleen (4), whereas, α-MSH can regulate several activities of immune cells (5). Growth hormone (GH) and prolactin (PRL) are produced by distinct population of immune cells and may play a role in immunoregulation by

exerting paracrine and autocrine actions (6-8). GH induces insulin-like growth factor 1 (IGF-I) production by the liver, as well as in a number of other tissues and organs. IGF-I mediates GH action and is a peptide structurally related to insulin. The production and paracrine action of both IGF-I and insulin has been described in immune system as well (9). The presence of neuropeptides such as vasoactive intestinal peptide (VIP), substance P (SP) and somatostatin (SS) has been demonstrated at either protein or messenger ribonucleic acid (mRNA) levels in immune tissues and cells (10-12). Apart from regulating neuroendocrine functions, these substances are present in the innervated immune tissues as well, and may play a major role in immunoregulation. VIP containing nerves are distributed in many lymphoid organs. Moreover, VIP is produced and secreted by thymocytes (13), and may modulate immune functions within the thymus. In fact, through direct binding to receptors on thymocytes, VIP affects three important functions of these cells, namely cytokine production, mobility and apoptosis (13).

SP belongs to a family of tachykinin peptides that share a common C terminal amino acid sequence (14). The mammalian tachykinins include SP, neurokinin A, neurokinin B, and two N-terminally extended forms of neurokinin A, i.e. neuropeptide K and neuropeptide Y. These molecules are involved in several aspects of neuroimmunomodulation (15). SP has been demonstrated in a subset of lymphocytes and monocytes/macrophages at mRNA and protein levels (16-18). Sequence analysis of these products confirmed that the structure was identical to that found in neuronal cells. Moreover, SP has been detected in the outer cortex of the thymus and in the peptidergic neurones innervating this organ (19-22). SP is the major mediator of neurogenic inflammation. It induces degranulation of both mucosal and intestinal type of mast cells. In addition, SP affects the functions of lymphocytes, macrophages, eosinophils and neutrophils (9).

A number of other mediators, including vasopressin, oxytocin, bombesin, enkephalins neurophysin are shared between the neuroendocrine and immune systems as well (9). Their localization and role are under investigation in order to understand for each mediator the pathways through which it does participate in the immunoregulatory network. This is crucial for the assessment of their potential pathophysiological significance, and for the application of this knowledge in clinical setting.

Finally, SS is also present in lymphoid organs, and its localization and actions will be discussed in paragraph I.4 of the present chapter.

In Table 1 the principal neuropeptides involved in controlling the immune system are indicated together with their effects on immune function. Several reports agree that

TABLE 1. Effects of neuropeptides on immune functions.

Neuropeptide	Main Effect			
	immunoglobulin production	cytokine secretion	cell-mediated immunity	inflammation
Adrenocorticotrophic hormone (25)	↓	↑↓	↓	-
Corticotrophin releasing hormone (25)	↓	↑↓	↓	↓
Vasopressin (9,24)	-	-	-	↑
β-endorphin (9,24)	↑	↑↓	↑	↑
α-malanocyte stimulating hormone (25)	-	↓	↓	↓
Enkefalin (9,24)	↑	↑	↑	↑
Calcitonin gene-related peptide (9)	-	↓	-	↓
Nerve growth factor (9)	↑	-	-	↑↓
Somatostatin (29)	↓	↑↓	-	↑↓
Substance P (29)	↑↓	↑	-	↑
Vasoactive intestinal peptide (13,29)	↑↓	↓	↑↓	-

↑, *increased response/activity*; ↓, *decreased response/activity*; ↑↓, *variable response*

administration of hormones and neuropeptides may lead to stimulate or depress immune/inflammatory response, depending on the dose and the timing of their administration. Moreover, gender differences in the response of the immune system to neuroendocrine agents have been documented as well. In general, in females the immune response is stronger and the serum immunoglobulin (Ig) concentration is higher (23). However, females have a higher incidence of certain autoimmune disease (24).

While ACTH, glucocorticoids and sex steroids in general depress the immune response *in vivo*, insulin, GH, PRL and thyroid hormones may increase it (23-28). Opioid peptides are reported to either stimulate or inhibit the immune response, depending on the process evaluated, from the cell type and from the experimental condition (24). SP is generally considered as a stimulator of the immune system, while VIP and neuropeptide Y as inhibitors (29).

Neuropeptides may affect other immune mechanisms like the activity of lymphocytes after recognition of an antigen as well as negative and positive selection during their ontogeny. The first paragraph of the next chapter will focus in part on this latter aspect, describing the presence and influence of neuropeptides within the thymus, which is the organ where T cell repertoire is generated.

NEUROPEPTIDE AND HORMONE RECEPTORS IN THE IMMUNE SYSTEM

Immune cells may bind different hormones and neuropeptides. Receptors for regulatory products of the hypothalamus-pituitary-adrenal axis are widely represented along the immune system. CRF, ACTH and POMC-related peptides, as well as glucocorticoid receptors have been described in lymphoid and accessory cells (2, 30-33). In clinical practice, ACTH and glucocorticoids are still among the most effective agents for the treatment of various inflammatory conditions and diseases with underlying immune abnormalities.

Receptors for estrogen and testosterone have been described on immune and accessory cells with special emphasis for their role in the thymic microenvironment on developing immune cells (34-36). GH and PRL receptors have common features with some cytokine receptors and their distribution on immune cells received particular attention because of the well-established actions of both pituitary hormones within the immune systems, as well as for their local production in immune organs (7,37).

Two VIP_1 and VIP_2 receptors have been cloned recently, and VIP receptor distribution on immune cells has been extensively investigated (38). Although the immunoregulatory properties of VIP have been not fully understood, an example of the importance of this neuropeptide has been demonstrated in the thymus where both receptor isoforms are expressed on developing thymocytes (13).

Thymocytes as well as T- and B-lymphocytes and macrophages express specific receptors for SP (16,17). In addition, very high numbers of SP receptors are expressed in the germinal centre of lymph nodes of patients with autoimmune diseases (39,40). The distribution of SP receptors in immune tissues suggested that after the labeling of SP with radioactive Indium, this radiopharmaceutical might be used to visualize SP receptor-positive tissues. Recently [^{111}In-DTPA-Arg^1]-SP has been used to successfully visualize the thymus and inflammatory sites of disease in patients with autoimmune diseases during *in vivo* SP receptor scintigraphy (41).

SS receptor distribution on immune cells will be discussed in paragraph I.4, together with the respective peptide distribution in immune cells.

The distribution of neuropeptide receptors has some common characteristics. Their expression pattern on different types of immune cells is heterogeneous, suggesting that the signal mediated by these agents can preferentially target different types of immune responses. Another important aspect is that the number or the activity of neuropeptide receptors on

immunocytes may change during the specific and aspecific activation of these cells, suggesting that signals mediated by one of this effectors could be predominantly perceived by antigen-activated cells (42). This evidence may partially explain the rather common occurrence of conflicting observations that neuroendocrine-mediated activities exert on immune cells.

CONCLUSIONS

Current evidence indicates that the neuroendocrine system regulates immune reactions and *viceversa* the immune system signals towards the neuroendocrine system. These interactions can be identified either as a long loop, where the activation leads to the release of mediators affecting distant structures of the network, or as a short loop in which local interactions occur *via* specific agents exerting their reciprocal effects within the tissue or organ where they have been released. This integrated mechanism is essential in normal physiology and seems to be of particular importance for immune homeostasis. Moreover, abnormalities of neuroimmunomodulation may contribute to the aetiology of autoimmune diseases, immunodeficiency and chronic inflammatory diseases. For example, neuropeptide receptors on target cells can be up- or down-regulated by chronic exposure to the ligand, whereas changes in level or activity of local peptidases could alter the concentration of bioactive ligand (43,44). Such changes could severely affect the balance of neuropeptides locally present within the tissue, potentially leading to induction of an inappropriate immune response, and consequently disease. Whereas a malfunction of this circuit may lead to certain stages of specific disease, the blocking of the altered processes might offer an efficient way to abolish deleterious immune-mediated neuroendocrine and metabolic derangement.
Importantly, the introduction of agonists and antagonists of neuropeptides involved in immunomodulation is starting to become of interest in the treatment of patients with immune-mediated diseases. Finally, the receptor status in these patients, which can be studied with modern nuclear medicine techniques using peptide-receptor scintigraphy (chapter III), may be related to the disease and may be predictive for the efficacy of therapy.

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SOMATOSTATIN AND SOMATOSTATIN RECEPTOR SUBTYPES

As introduced in the previous paragraph, many recent studies pointed to the presence and functional roles of several neuropeptides such as SS, SP and VIP and their receptors on cells of the immune system. Generally, SP may enhance immunoglobulin (Ig) M and IgA production from B cells, and seems to produce mainly a stimulatory effect (1). Conversely, SS and VIP seem to have a predominant inhibitory action on immune functions (1-3). In view of new recent insights, primary and secondary lymphoid organs can be considered as preferential sites of immune-neuro-endocrine interactions.

Emerging data regarding the potential role of SS and its receptors in the regulation of immune functions, both in animals and humans, have projected this ubiquitous peptide among the classical regulatory peptides with a proved immunomodulatory function. This section will introduce and overview the principal characteristics of SS and its 5 receptor subtypes.

SOMATOSTATIN

SS is a peptide hormone initially isolated from the hypothalamus and characterized as a growth hormone-releasing inhibiting factor. SS was successively found to be widely distributed throughout the human body. Outside the central nervous system, the peptide is present in a variety of endocrine and non-endocrine tissues, and it appears to have many functions. Since its discovery in 1973 by Guillemin *et al.*, (4) knowledge of the functional role of SS in regulating neurotransmission in the brain, as well as in the regulation of secretion processes in the anterior pituitary gland, the pancreas and the gastrointestinal tract, has increased considerably.

Proteolytic processing of larger precursor molecules, e.g. prepro-SS and pro-SS, results in the formation of SS. In mammals, after cleavage of the pro-SS molecule two biologically active forms of SS consisting of 14 (SS-14) or 28 (SS-28) amino acids are generated (5).

In the central nervous system SS acts as a true neurohormone and neurotrasmitter, with a generally inhibitory action in many regions (cerebral cortex, limbic system, brain stem,

spinal cord) (5,6). In the peripheral nervous system it is found in sympathetic and sensory neurones (7,8) and may have a down-modulatory effect on nociception, i.e. the perception of harmful and/or painful stimuli (9).

However, the additional biological functions of this peptide, in a variety of endocrine and non-endocrine tissues, include inhibitory effects on secretive and proliferative processes and modulatory actions on the response of various cells to endocrine stimulation (7,8,10,11). SS exerts an inhibitory activity on the secretion of different pituitary and gastrointestinal hormones. When produced in the hypothalamus, SS reaches through the portal circulation the anterior pituitary, where it inhibits the secretion of GH but also of other pituitary hormones such as thyroid-stimulating hormone (TSH) and PRL. SS is produced in specialised cells (D cells) in the gastrointestinal tract and the pancreas (12,13). In peripheral tissues the effect of SS is also mainly inhibitory, regulating amongst others the secretion of calcitonin, gastrin, insulin, glucagon and VIP. In addition, it reduces the exocrine secretion of pancreatic enzymes and gastric acid and can inhibit the contractility of stomach, small gut and gallbladder (10,11,14). Finally, an antiproliferative action has been observed in many cell types *in vitro* (15).

SOMATOSTATIN ANALOGS

SS has a plasma half-life shorter than three minutes. For this reason, metabolically stable analogs with a longer half-life have been synthesized for clinical application. Structure-function analysis of native SS and peptide analogues has shown that the amino acid residues Phe^7, Trp^8, Lys^9 and Thr^{10} are necessary for receptor binding. These residues are at the apex of the loop formed by the disulphide bridge connecting the two cysteine residues in native SS and have been shown to form a β-turn (16). Trp^8 and Lys^9 are essential for biological activity whereas the flanking residues tolerate minor substitutions (17). As further discussed below, the development of SS receptor (SSR) subtype-selective analogs has played a critical role in understanding many mechanisms of receptor activities. These compounds are a shortened version of the native peptide and more resistant to proteolytic degradation. Most of them are cyclic octapeptides, however the smallest SS analogues that are capable of receptor binding are hexapeptides (17). Three octapeptide SS analogs, octreotide, lanreotide and vapreotide have been already introduced in the clinical practice (Figure 1); all of them bind to sst_2, sst_3 and sst_5, but not sst_1 and sst_4. None of these analogs

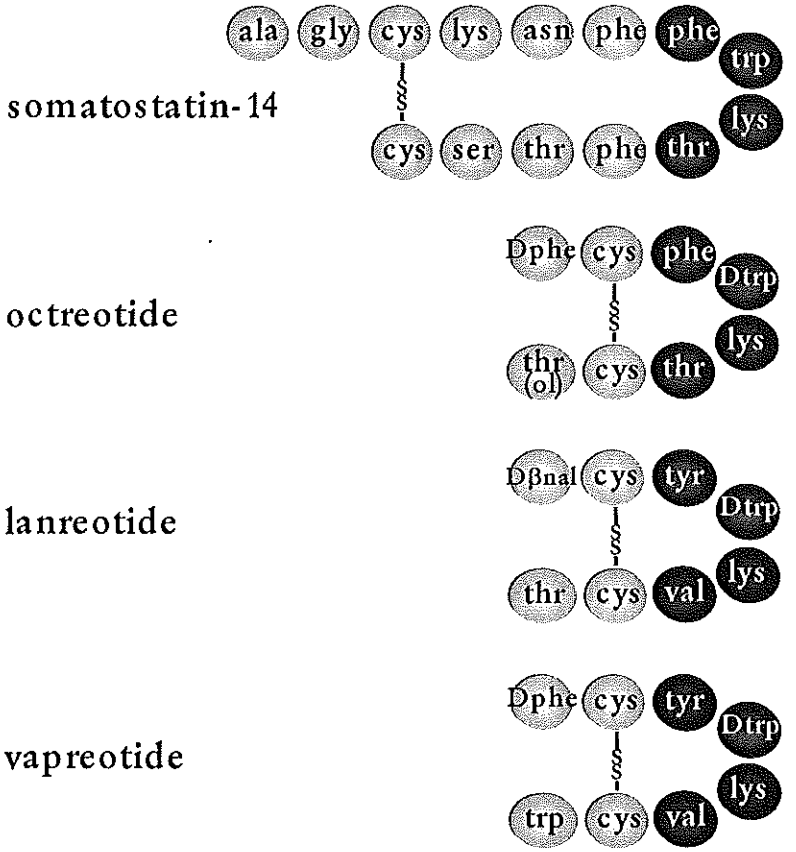

FIGURE 1. Amino acid sequences of native somatostatin-14 and synthetic stable octapeptide analogs octreotide (SMS 201-995), lanreotide (BIM23014) and vapreotide (RC-160). The dark grey colour indicates the amino acids essential for the binding with the receptor.

bind to only one of the subtypes. However, recently new non-peptide subtype-selective agonists have been developed and preliminary evidence showed encouraging results on the potential role of these compounds in defining the physiological functions of each SSR subtype (18).

SOMATOSTATIN RECEPTOR SUBTYPES

Between 1992 and 1994, five different SSR genes have been cloned and identified (17,19,20). They originate multiple SSR subtypes (sst_{1-5}), which are identical in 42 to 60% of their amino acid sequence (19). Although the genes encoding the five SSR subtypes have a high degree of sequence homology, they are localized on different chromosomes, which allows a tissue-specific regulation of their expression (21) and suggests diverse functions of the receptor subtypes in different organs. All the genes are intronless except for the sst_2 gene, which contains a cryptic intron sequence and encodes for two receptor proteins, sst_{2A} and sst_{2B}, originating from alternative splicing and differing in the length of their cytoplasmic tail (17).

Structure of somatostatin receptors

SSR belong to a superfamily of receptors with seven transmembrane-spanning α-helical

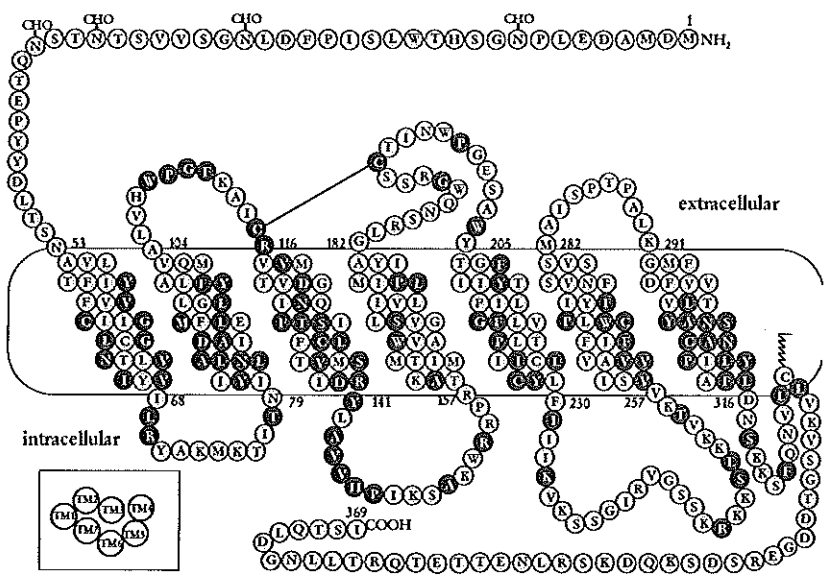

FIGURE 2. Structure and orientation of the somatostatin receptors (SSR) within the plasma membrane. Sequence of the human SSR subtype 2, with the 7 membrane spanning α-helical segments. The invariant amino acids are shown in dark gray. CHO indicates the potential site of glycosylation in the extracellular NH_2-terminal domain area. Disulfide bond is indicated between two cysteine residues. The inset shows the possible arrangement on the 7-membrane segments. Redrafted from reference 20.

domains connected by short loops, having an N-terminal extracellular domain and a C-terminal intracellular domain (Figure 2). Hydrophobic and charged amino acids within the transmembrane domains 3, 6 and 7 are important for the interaction with the ligand (22,23). However, the extracellular loop 2, between domains 4 and 5, may also be involved (24-26).

Distribution and signal transduction pathways

Multiple SSR can be expressed in the same SS-target tissue and this makes difficult to establish their individual functional role. The expression of SSR subtypes has been studied firstly at mRNA level by *in situ* hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) (21). Recently, using polyclonal antibodies specific for individual subtypes, SSR have been investigated at protein level by immunohistochemistry in rat (27,28) and human tissues (29-31). The distribution of SSR subtypes has been extensively characterized in the rat, where mRNA for all the subtypes has been detected in brain and pituitary, while sst_2 is the subtype apparently predominant in the pancreas and adrenal

TABLE 1. Characteristics of the five somatostatin receptor subtypes.

	Somatostatin receptor subtypes				
Property	sst_1	sst_{2A}	sst_3	sst_4	sst_5
Chromosomal localization	14	17	22	20	16
Number of amino acids	391	369	418	388	363
Second messenger coupling					
⇒ Adenylyl cyclase	↓	↓	↓	↓	↓
⇒ Ca channels	↓	↓			
⇒ K channels		↓			
⇒ Na/H exchange	↓	↔↑		↑	
⇒ tyrosine phosphatase	↔↑	↑	↑	↑	↔
⇒ MAP kinase	↑	↓	↑↓	↑	↓
Ligand binding (IC_{50} nM)					
⇒ somatostatin-14	2.3	0.2	1.4	1.8	0.9
⇒ octreotide	>1000	0.6	34.5	>1000	7.0
Receptor internalization*	+/-	+	+	+	+
Receptor desensitization*	±	+	+	-	+
Receptor phosphorylation	n.d.	+	+	-	n.d.
Receptor regulation by agonists	↑	↑↓	↑	↑	↑

*, The extent of receptor internalization and receptor desensitization may change depending on the receptor subtypes and on the experimental conditions in cells lines and primary cultures of tumor cells.
n.d., not determined; +/-, contradictory results.
Data derived from references: 21,45,46,49-59.

tissues. Sst_3 mRNA has been detected in spleen, lymph nodes and liver, whereas sst_4 in the lung and sst_5 in small gut and adrenal gland (32). In human brain all five SSR subtype mRNAs are expressed in a characteristic, specific pattern (21). Sst_1, sst_2, sst_3, and sst_5, while sst_4 mRNA is not expressed in the adult pituitary (33) and all five SSR subtype proteins are variably expressed in normal human pancreatic islets (34). Generally, tumors originating from SS-target tissues express a high density of SSR (35,36), and in most cases express multiple SSR subtypes as well (21,37). The five SSR subtypes couple differentially to the second messenger systems known to be activated upon SS binding to its receptor. Studies on signal transduction pathways have demonstrated that all five subtypes are linked to adenylyl cyclase *via* G-proteins (for reviews see 38,39). Moreover, SSR subtypes are associated with other signal transduction mechanisms, such as Ca^{2+}- and K^+-ion channels, as well as activation of tyrosine phosphatase (PTP) or MAP kinase activity, phospholipase A2 and others (20,38,40). While the inhibitory effects on adenylate cyclase activity and on the influx of Ca^{2+} are linked to inhibition of secretion processes, the activation of PTP or MAP-kinase activity may play a role in the regulatory effects that SS may exert on cell proliferation (15,41,42). The principal characteristics of SSR subtypes are summarized in Table 1

Receptor binding

Each SSR subtype seems to serve a distinct biological function and the signalling mechanisms triggered by specific subtypes seem to differ among the cells in which they are expressed (43). Moreover, the expression and the activity of SSR can be regulated by their own ligand.

The five SSR subtypes bind SS-14 and SS-28 with high affinity but can be further divided into two subclasses on their ability to bind structural octapeptide SS analogs. Sst_1 and sst_4 receptors do not bind octapeptide analogs, whereas sst_{2A}, sst_3 and sst_5 receptors display a high, low, and moderate affinity, respectively, towards octapeptide SS analogs such as the clinically used octreotide and lanreotide (Table 1).

Receptor internalization, desensitization and regulation

Receptor-mediated internalization of ligands after binding to their receptors has been described for several neuropeptides, including SS. Internalization of the hormone-receptor complex may play a role in regulation of receptor number of cell surface as well as in

hormone degradation and transport. Internalization of ligand-receptor complexes is a mechanism to induce desensitization of responsiveness to SS in order to keep hormone levels within the physiological range (44). Agonist-induced internalization of the SS-SSR complex may play a role in intracellular signalling as well. Moreover, phosphorylation of SSR may be an important mechanism involved in the internalization and desensitization of the receptors (Table 1) (45,46). Desensitization of the inhibitory effect of SS on hormone release *in vitro* suggests that agonist-induced tachyphylaxis occurs directly at the level of the SSR expressing target cell and tachyphylaxis to the inhibitory effect of SS (-analogs) may occur in many different organ systems. SSR subtypes are differentially sensitive to undergo agonist-induced desensitization.

Treatment with SS-analogs may induce either down- or up-regulation of SSR. Ligand-induced up-regulation of membrane-expression of SSR subtypes has been observed in cell lines and primary cells cultures (47-49). In cell lines expressing SSR, it has been shown that prolonged agonist treatment resulted in up-regulation of the membrane expression of sst_1, sst_2 and sst_4 (49).

Receptor regulation may play a relevant role in determining cellular responsiveness to SS as well as in investigating SSR-expressing tissues by the application of modern techniques based on receptor detection. Therefore, molecular mechanisms by which these phenomenon occur provide important new areas for future investigations.

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DISTRIBUTION, ROLE AND FUNCTION OF SOMATOSTATIN RECEPTORS IN IMMUNE CELLS IN HEALTH AND DISEASE

In the previous sections it has been discussed how the functional interactions between immune, neural and endocrine cells may play a relevant role in many physiological and pathological processes. Receptors for signalling molecules are shared between the three systems. Among these molecules, SS appeared to be one of the most representatives (Figure 1). In the present paragraph we will focus on the distribution and the functional role of SS and SSR on immune cells.

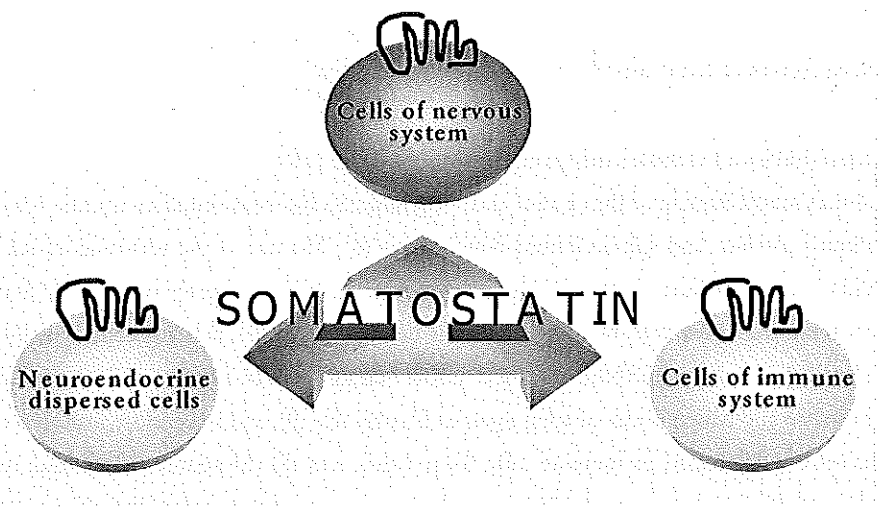

FIGURE 1. Schematic representation of how nervous, endocrine and immune systems can affect each other. Neural, endocrine and immune cells express receptors for neuropeptides, such as somatostatin. Neuroendocrine products coexist in neural, endocrine and lymphoid tissue.

Since Bhathena and co-workers in 1981 firstly identified SS-binding sites on human mononuclear leukocytes (1), a number of studies pointed to the presence of SS and its receptors on immune cells and to the significance of this neuropeptide as an intersystem signalling molecule. The first line of evidence indicated that SS down-modulates a number of immune functions, like lymphocyte proliferation, Ig production and the release of pro-inflammatory cytokines such as interferon gamma (IFN-γ) (for a review

see 2). Moreover, lymphoid organs are highly innervated by neurones containing peptide neurotransmitters and the nervous system can influence cells of lymphoid organs directly with release of its products from nerve endings or by regulating the local blood flow. Neuropeptides in the gastrointestinal tract may regulate in a paracrine fashion the function of cells of the gut-associated lymphoid tissue and other immune cells present in this compartment (3,4). Signals mediated by hormones and neuropeptides may influence different types of immune responses and the number and the functionality of receptors may increase during the activation of immune cells, indicating that antigen-activated cells might be more susceptible to neuroendocrine signals (5). These and other peculiar aspects of this interactive system should be taken into consideration when evaluating the large number of *in vivo and in vitro* studies on the effects of SS and on the presence and significance of specific SSR in lymphoid tissues.

SOMATOSTATIN RECEPTORS IN THE IMMUNE SYSTEM

Distribution of somatostatin receptors on immune cells

Animal models Binding of fluorescent and ^{125}I labelled SS has been found on murine B and T Peyer's patches- and spleen-derived lymphocytes (6). The use of the universal ligand, SS, did not allow the identification of the specific receptor subtypes involved in the SS binding on these cells. However, by using molecular biology techniques, it has become possible to identify SSR subtype mRNAs in rat and mouse immune cells (7-10). Sst_3 mRNA was found in spleen and lymph nodes of adult Sprague-Dawley rats (9), and sst_3 and sst_4 mRNAs were selectively expressed in immune cells from Lewis rats (8). Moreover, a previous study showed the presence of sst_2 mRNA in rat thymocytes, and demonstrated that the activation of cells with phytohemagglutinin and interleukin 1 up-regulates the expression of sst_1 mRNA (10). Murine T lymphocytes selectively expressed sst_2 mRNA (7). This difference between rats and mice underlines the possibility of species variability in the distribution of SSR in immune cells and, as it will discussed in detail below, suggests that the rat may not be a suitable model to study the role of SS in human immune diseases. However, the different experimental conditions and the rapid turnover of neuropeptide receptor expression on immune cells may account for these conflicting results as well.

Humans In the past decades, much evidence has been accumulated on the presence of SSR on cells of the human immune and hematopoietic systems (for a review see 2). Bhathena

and co-workers showed SS binding-sites on human immune cells *in vitro*, and demonstrated specific, low affinity binding of iodinated SS on enriched preparations of human monocytes and lymphocytes; the monocyte fraction showed a higher number of receptors compared with lymphocytes (1). Subsequently, using fluorescent SS, a single class of low affinity SSR was demonstrated on human mitogen-activated peripheral blood lymphocytes and two classes, one of low affinity and a second with higher affinity were detected on lymphoblastic leukaemia cells (11). No distinct predilection of SSR positive cells among peripheral lymphocytes was detected, whereas in this study resting lymphocytes, granulocytes and red blood cells did not show SS binding. A Jurkat line of human leukaemic T cells and U-266 IgE producing myeloma cells also showed high and low affinity binding sites for fluorescent and radiolabelled SS (12). In contrast with this last observation, Nakamura and co-workers did not find SSR on the Jurkat cell line. However, they were able to detect a large number of SS binding sites on the human adult T leukaemic cell line MT-2 and the human T cell line Molt-4F, while a lower number on the Epstein-Barr virus transformed B cell line Isk (13). The K_d value in the nanomolar range of SS binding on leukaemic cells and the greater proportion of SSR-positive cells in Peyer's patches (14,15) suggest that the receptor expression on immune cells might be related to the activation and/or proliferation state of these cells.

By RT-PCR, a number of lymphoid cell lines of different origin (T cell, B cell, myeloma- and leukaemic origin) were shown to variably express sst_2, sst_3 sst_4 and sst_5 mRNAs, while sst_1 was absent (16). Moreover, sst_2 mRNA expression in normal human peripheral blood mononuclear cells was very low compared with the expression of this SSR subtype in cell lines and in peripheral blood mononuclear cells from leukaemic patients. In addition, sst_2 mRNA expression in normal peripheral blood mononuclear cells increased after activation with phytohemagglutinin, supporting the concept that the receptor expression pattern in human lymphoid cells may be dependent upon their state of activation. (16). In addition, it has recently been demonstrated that the Jurkat T-cell line selectively expresses sst_3 mRNA, suggesting the involvement of this SSR subtype in the regulation of T-cell function (17). Finally, sst_2 mRNA expression in a number of cell lines of the human T- and B cell lineage has been found as well (18). Some caution should be taken, however, with the extrapolation of data derived from immortalized cell lines, which may have characteristics different from primary lymphoid cells (19).

Neuropeptide receptor expression for vasoactive intestinal peptide, substance P and SS has been found in different organs of the human immune system, such as lymph nodes, tonsils, Peyer's patches, spleen and thymus (14,15). The binding of the sst_2-preferring ligand, [^{125}I-Tyr^3]-octreotide, was found mainly localized in the medullary region in the thymus, but diffusely in the red pulp of the spleen and in the germinal centres of lymphoid follicles. The distribution and potential role of SSR in the human thymus will be discussed in the next chapters; however, it is anticipated that the architecture of the network between SS and its receptors in this lymphoid organ is rather complex. SSR subtypes are heterogeneously expressed on different cell subsets, and this is suggestive for a specific and important activity of SS in thymic microenvironment.

Lymphomas and thymic neoplasms

Most of the information on SSR expression on human immune cells derives from *in vivo* SSR scintigraphy after injection of radiolabeled SS analogs. This technique has become of routinely use in the localization of neuroendocrine tumors and their metastases, and is discussed in detail in chapter III.1. However, in recent years SSR scintigraphy has been employed in visualizing other non-neuroendocrine tumors expressing SSR, as well as granulomatous and autoimmune diseases (20-22). SSR have been detected *in vivo* and *in vitro* in both T and B non-Hodgkin's lymphoma and Hodgkin's disease and their metastase.s (23-26). The *in vivo* imaging technique using [^{111}In-DTPA-D-Phe^1]-octreotide has contributed to optimize the staging procedures in patients with malignant lymphomas. *In vitro* autoradiography demonstrated the presence of SSR predominantly in the lymphoblastic areas of lymphomas, which represents the active part of these tumors. Moreover, the expression of sst_2 and sst_3 mRNA in non-Hodgkin's lymphomas and sst_2 mRNA in Hodgkin's disease has been detected (27). Somatostatin binding sites have been detected by *in vitro* autoradiography using [^{125}I-Tyr^3]-octreotide on cells from patients with acute lymphoblastic leukaemia and acute myeloid leukaemia (28). Moreover, somatostatin and octreotide inhibited spontaneous leukaemic cell growth in approximately 33% of cases (28). Preliminary data demonstrated a partial remission in 36% of patients with low-grade non-Hodgkin's lymphomas treated with octreotide (29). The accurate evaluation of SSR subtype status in lymphoproliferative diseases might become of value in the medical treatment of these patients when new subtype selective analogs will be available for clinical application and/or for radiotherapy with β-emitting radionuclide-coupled SS analogs.

More recently, a high *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide has been detected in patients bearing a thymoma or a thymic carcinoid (30-32). Although the functional and clinical significance of the *in vivo* uptake the radiolabelled SS analog in these tumors is unclear so far, the characterization of tumor SSR pattern might help to explain the successful treatment with the SS analog octreotide reported in two thymoma patients (32,33). In chapter III preliminary data on the characterization and potential significance of SS and specific SSR subtypes in thymic tumors will be illustrated, with special emphasis on the role played by different subtypes in determining the uptake of radiopharmaceutical SS analogs.

Autoimmune and granulomatous diseases

A number of autoimmune and granulomatous diseases (Table 1) have been shown to express SSR *in vivo* and *in vitro* (34-36). Affected joints of patients with rheumatoid arthritis have been clearly visualized during *in vivo* SSR scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide, and *in vitro* autoradiographic studies have shown SSR mainly associated to the vascular compartment of the inflamed synovium (35,37). Recently the presence of SSR (i.e. sst_{2A}) has been confirmed by immunohistochemistry using sst_{2A} specific antibodies. Sst_{2A} immunoreactivity in the synovium of affected joints in patients with rheumatoid arthritis was observed not only on the endothelial cells of venules, but also on cells belonging to monocyte/macrophage lineage, as it was demonstrated by the co-localization of sst_{2A} with specific markers, such as CD14 (38). Conversely sst_{2A} immunoreactivity did not co-localized with CD3, which is a specific marker for T lymphocytes (38).

TABLE 1. Autoimmune and granulomatous diseases expressing somatostatin receptors.

Autoimmune diseases	Granulomatous diseases
Arthritis	Sarcoidosis
Rheumatoid arthritis	Tuberculosis
Systemic lupus erythematodes	Wegener's granulomatosis
Sarcoidosis	Crohn's disease
Henoch-Schönlein purpura	Aspergillosis
Sjogren's syndrome	
Ophthalmic Graves'disease	
Autoimmune uveitis	
Ulcerative colitis	

[^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy has been employed to visualize granulomatous lesions in patients with sarcoidosis, aspergillosis and Wegener's disease. Moreover, the uptake index of the radiotracer in patients with sarcoidosis has been reported to be lower or absent in patients scanned after successful treatment with

corticosteroids (34). These data suggest that SSR scintigraphy may contribute to the staging and the prediction of corticosteroid therapy in these patients and may optimize the choice of biopsy site in sarcoidosis (34). Another argument in favour of the significance of SSR scintigraphy in predicting and monitoring the outcome in patients undergoing corticosteroid therapy has been recently discussed for the treatment of Graves' ophtalmopathy with methylprednisolone (39). In fact, in patients showing the highest score at SSR scintigraphy, a significant improvement occurred in orbital indexes during treatment with the corticosteroid (39). This preliminary finding suggests that [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy may be considered a useful approach to select the patients for proper treatment with corticosteroids.

SSR have been localized by *in vitro* autoradiography using [^{125}I-Tyr^3]-octreotide in biopsies of lesions from patients with sarcoidosis and inflamed bowel disease(s) (34,40). In sarcoidosis the granulomas were specifically labelled in the region of epithelioid and giant cells, while in inflammatory bowel disease(s) mainly the endothelium of venules expressed somatostatin-binding sites (34,40). The presence of SSR in inflamed lesions of granulomatous and autoimmune diseases suggests an active role of the peptide in the pathophysiology of inflammation. The cellular localisation of sst_{2A} protein in tissue biopsies from human immune-mediated diseases has been recently investigated using a polyclonal antiserum directed against a conserved epitope in the intracellular C-terminal domain of the sst_{2A} receptor. In biopsies from patients suffering from granulomatous diseases such as sarcoidosis and Wegener's granulomatosis, sst_{2A} expression was associated with cells of the mononuclear phagocyte lineage, including epithelioid cells and multinucleated giant cells within the granulomas (41). In none of the biopsies, sst_{2A} expression was observed on T lymphocytes.

On the basis of classical ligand-binding studies and RT-PCR studies it had previously been assumed that lymphocytes were the major cell types expressing sst_2 in human immune-mediated diseases (2,14). Based on more recent studies it seems that expression of sst_2 in the human immune system is mainly confined to cell subsets belonging to the monocyte-macrophage lineage.

FUNCTIONAL ROLE OF SOMATOSTATIN RECEPTORS ON IMMUNE CELLS

The presence of SSR on immune cells implies that they are able to show a functional response to the natural ligand. There is increasing evidence that SS can influence the functions of immune cells, both in health and disease (for a review see 2). SS has been demonstrated to inhibit T lymphocyte proliferation and colony formation (42-44), although studies have shown a stimulatory effect on proliferation (2) and secretion of IL-2 by a human T cell line as well (15). In addition, SS may display an inhibitory action on natural killer cell activity (45), as well as on the β_1-integrin-mediated adhesion of resting T-cells to fibronectin (46).

In B cells, SS may have a modulatory activity on immunoglobulin secretion by plasma cells. SS inhibits IgE and IgG4 production, while the SS analog octreotide was shown to reduce the number of plaque-forming cells in mitogen-stimulated peripheral blood mononuclear cells (47,48). SS also possesses a modulatory effect on human monocyte function. In enriched monocyte fractions obtained from peripheral blood mononuclear cells, the peptide has been shown to inhibit tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β) and IL-6 secretion, as well as the chemotactic response to neutrophils, in parallel with a down-regulation of human leukocyte antigen-DR (HLA-DR) expression (49). Finally, short-term incubation with octreotide stimulates phagocytosis of latex particles by monocytes (50).

SS may be involved in the regulation of immune cell functions via different pathways. Primary and secondary lymphoid organs contain nerve endings, which may release SS, strongly supporting a neuroendocrine modulation of immune functions by the neuropeptide via an alternative pathway as well (2,51). Moreover, like other classical regulatory peptides, SS immunoreactivity has been detected in lymphoid tissues, lymphocytes and monocyte/macrophage of different species, including humans (52-54). Although SS mRNA has been demonstrated only in the thymus and the spleen so far, these findings support the potential autocrine/paracrine regulatory role of the neuropeptide (55,56). In order to define such autocrine/paracrine actions better, the following chapter will focus on the *in vitro* effects of SS, and of the SS analog octreotide, on proliferation of primary cultured thymic cells, as well as on the disturbances in the endogenous production of this neuropeptide. This latter event may be related to the pathogenesis of autoimmune and neoplastic diseases involving the thymus in humans.

Until now, few data are available on the cellular signalling mechanisms coupled to SSR activation in immune cells. In peripheral blood mononuclear cells from healthy subjects and from patients with acute leukaemia, SS inhibited adenylyl cyclase activity, however, only at very high, non-physiological concentrations (57). In a recent study, Cardoso and co-workers showed that SS inhibits adenylate cyclase activity in mitogen-activated human peripheral blood mononuclear cells and Jurkat T cells in a dose-dependent fashion at nanomolar concentration (15). In Jurkat T cells SS stimulated cell proliferation and IL-2 secretion. Considering that sst_3 was the only SSR subtype found to be expressed in this cell line, this effect may be mediated via a functional sst_3 receptor negatively coupled to the adenylyl cyclase pathway. In the majority of the studies, the effects of SS on immune cell function, either on secretion, proliferation or on other functions, is "biphasic". This

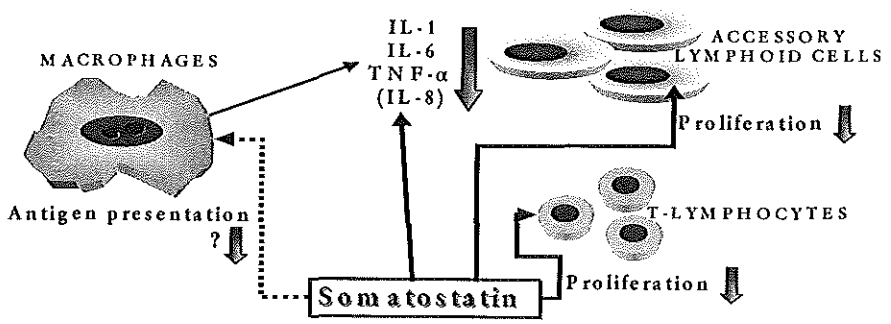

FIGURE 2. Potential mechanisms of action of somatostatin on immune cells. Somatostatin may inhibit cell proliferation and secretion. Somatostatin might play a role in regulating antigen presentation properties by cells of the monocyte-macrophage lineage, presumably in an inhibitory manner.

phenomenon displays in a very narrow dose-range, with a maximal inhibitory effect at nanomolar concentrations, while a lower or absent effects is observed at higher (micromolar) concentration (17). This points to a very rapid desensitization of the receptors. The role of this "biphasic" response of immune cells to SS is still unclear, but may involve receptor internalization with a subsequent down-regulation, an uncoupling from second messenger activation, or even the activation of different intracellular second messenger pathways via distinct SSR subtypes.

Conclusions

In conclusion, SSR subtypes are expressed on cell-specific subsets in the immune system. The expression of neuropeptide receptors on immune cells seems to be dynamically regulated and may depend on the traffic of these cells through and within lymphoid structure and homing in tissues. SS may regulate immune cell functions via different pathways. A suggestive and hypothetical pathway is proposed in Figure 2. However, further studies are needed to clarify the role of the individual SSR subtypes. The synthesis and availability of new selective SS analogs (58) and antagonists may be of much help in this respect.

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AIMS OF THE THESIS

From the brief overview presented in the previous paragraphs it can be concluded that SS potentially represents an important effector molecule in the complex network of interactions between the neuroendocrine and the immune systems. However, although SS is a highly conserved neuropeptide, which is widely distributed in the human body, a number of its functions are still poorly understood. Furthermore, the heterogeneous expression of the five SSR subtypes requires new knowledge not only about the significance of its distribution pattern on specific cell subsets, but on their mechanisms of action as well.

In the last decades the functional significance of SS and its receptors in the endocrine system has been extensively investigated, while recent studies focused on the functions of this neuropeptide in the cross-talk between the endocrine and the immune systems. The thymus is an organ in which this intersystem communication circuit is examplary represented. In this main lymphoid organ, precursors of T cells undergo a process of maturation and selection that ends with the constitution of the final T-cell repertoire. Alterations in this highly complex mechanism may result in disturbances of the immune homeostasis.

At present, few and contradictory data regarding the presence of SSR in the normal and neoplastic thymus are available. Moreover, most of this information derives from studies in animals, whereas data in humans are lacking. On this basis, the following questions were raised:

A) First, which are the SSR subtypes expressed in the normal human thymus? Secondly, which impact might SS and its receptors have in the physiology of this primary lymphoid organ and in the maintainance of the homeostasis between the different cell components?

Taking into consideration some recent new insights, the studies carried out for this thesis primarily focus on the natural ligand and its receptor subtype distribution, as well as on their functional role in this complex organ. The cloning of the genes for the five SSR subtypes, together with the recent availability of antibodies raised against SSR subtypes

allow to study their cell-type specific distribution. Herein, *a)* the distribution of SS and its receptors is investigated in the normal human thymus. The presence of SS and SSR subtypes will be investigated on the three main cell populations in the normal human thymus, namely thymic epithelial cells, thymocytes and macrophages using different techniques. Since SS acts *via* different receptor subtypes, *b)* the functional significance of specific SSRs expressed in the tissues, and in isolated and enriched cell preparations will be evaluated. The experiments mainly focus on the control of cell proliferation.

In the microenvironmental and lymphoid compartments within the human thymus the neuroendocrine activities are considered of crucial importance for the homeostasis of the immune system. Since the thymus drives the generation of the T-cell repertoire, *c)* the intrathymic circuit involving SS, SSR subtypes and thymocyte maturation will be investigated and discussed.

The thymus is an organ that undergoes a physiological age-dependent involution. The mechanisms regulating this phenomenon are poorly understood. Therefore, it is evaluated whether potential links exist between the presence and activity of SS and SSR within the human thymus and the physiological involutive process of this organ. At this purpose, *d)* the expression of SSR will be studied with two different techniques and will be correlated to the chronological age in a series of normal human thymus.

B) The results of studies evaluating the role of SS and its receptors in thymus physiology raise a second series of questions related to their role in the pathological conditions of this organ, as well as to the consequences for diagnosis and therapy using targeted SS analogs.

Considering the potential important functional role of neuropeptides in the regulation of the function of thymic cells, disturbances in their expression or in the expression of neuropeptide receptors might be involved in thymic diseases. Thymic tumors can be visualized by SSR scintigraphy using radiolabeled SS analogs. Since conflicting data are present in the literature on the expression of SSR in thymic tumors, an additional aim is to evaluate the presence and functional significance of SS and its receptor subtypes in thymic epithelial tumors. For this, *a)* a paradigmatic case of thymoma, studied both *in vivo* and *in vitro*, is firstly presented. Thereafter, *b)* the results of *in vivo* SSR scintigraphy on a larger series of thymomas will be correlated with the distribution of SSR subtypes in these tumors evaluated *in vitro*. Additionally, using immunohistochemistry and quantitative techniques, *c)* the heterogeneity of distribution as well as the level of SSR expression will be further

investigated in normal and abnormal human lymphoid tissues, including the normal spleen and thymus as well as hyperplastic and neoplastic thymic tissues. The data from these studies might be potentially important to elucidate some of the conflicting data which exist in the literature on the role played by distinct receptor subtypes in determining the uptake of radiolabeled SS analogs in lymphoid tissues.

These issues are important arguments to open the general discussion on the significance of SS and SSR subtypes in the physiology of the human thymus, as well as in pathological conditions involving this organ. Moreover, this latter aspect will include some considerations concerning the use of SS analogs in autoimmune as well as neoplastic diseases involving the immune system.

SS analogs are currently used in clinical practice. Imaging techniques and medical therapy with available SS analogs are based on the selective affinity of these analogs for distinct receptor subtypes. However, most of the data regarding thymic cells, and in general regarding the immune system, are derived from studies in transfected cells or from primary experimental cultures from animal models. In the general discussion, in the light of results from studies using human tissues or cells, these studies will be revisited and discussed.

CHAPTER II

SOMATOSTATIN RECEPTORS IN THE NORMAL HUMAN THYMUS

THE THYMUS, GENERAL INTRODUCTION

In this chapter, the results of investigations on the significance of SS and its receptor subtypes in the human thymus are presented. The essential role of the thymus in the development of the immune system was demonstrated approximately 40 years ago. Nowadays, we should consider this organ as an immune-neuro-endocrine network from development to aging, and the interdependence of the neuroendocrine and immune systems and their communication circuit as well.

STRUCTURE OF THE THYMUS

The thymus is a central lymphoid organ in mammals, which plays a vital role in the control of the immune system. The presence of the thymus and its functionality are required for a full establishment of immunocompetence.

The thymus is a bilobed gland derived from the third and fourth pharyngeal pouches. From its origin the organ descends to the anterior-superior mediastinum, where it normally lies in the prevascular space (1). Ectopic locations can be the cervical region as well as the posterior mediastinum (2).

After birth, the thymus grows during the first year of life and normally reaches its peak size in early childhood. The organ has a typical arrow-head configuration with the two lobes cojoined along the midline. The thymus begins an involution process with age that exhibits a constant velocity during the first decade, then the velocity of involution decreases progressively (3). The most impressive change in the human thymus during aging is its nearly complete lipomatous atrophy (3). Although it is the most evident age-related change, the atrophy represents only a final state of the involution. It is preceded by several morphological events, which may have important immunological implications (3). It is important to underline that during adulthood most of the thymic tissue is replaced by connective and adipose tissues, although some functional tissue remains throughout life (3,4). Figure 1 shows a schematic and histologic representation of the age-related changes in the human thymus. Many stimuli (such as overexposure to radiation and some

chemicals) can cause accidental involution, and in these cases the organ is usually capable of regeneration if the offending stimulus is removed (4).

The thymus has a typical morphology, is covered by a thin connective tissue capsule and lobulated by septa originating from it (Figure 2). The capsule contains blood and nervous supply. Septa, carrying vessels and nervous fibres, penetrate from the capsule into the central part of the organ designing the structure of cortex and medulla (Figure 2). These two regions can be distinguished because of the different density of lymphoid cells. The cell types are the same but their distribution within the lobules is not uniform. The septation of the thymus is not complete. Serial sectioning demonstrates that the different lobes and lobules are continuous with each other through bridges of parenchymal material (Figure 3).

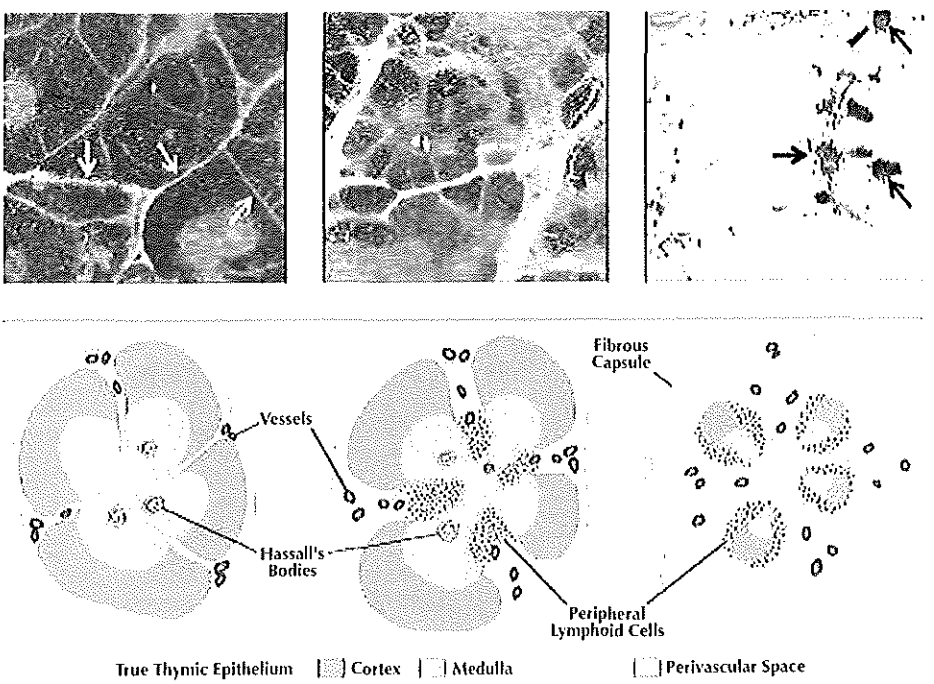

FIGURE 1. Thymus histology at three different stages of physiological thymic involution. From the left to the right: 6 days old, 15 years old and 63 years old thymus. Top, haematoxylin/eosin stained sections showing the sharp distinction between the dark-stained cortex and light-stained medulla (white and black arrows). Bottom, the scheme shows the division in true thymic epithelium, the site of activities for thymocytes maturation, and perivascular space, the site from where the involution starts. (From: http//www.var.vet.uga.edu).

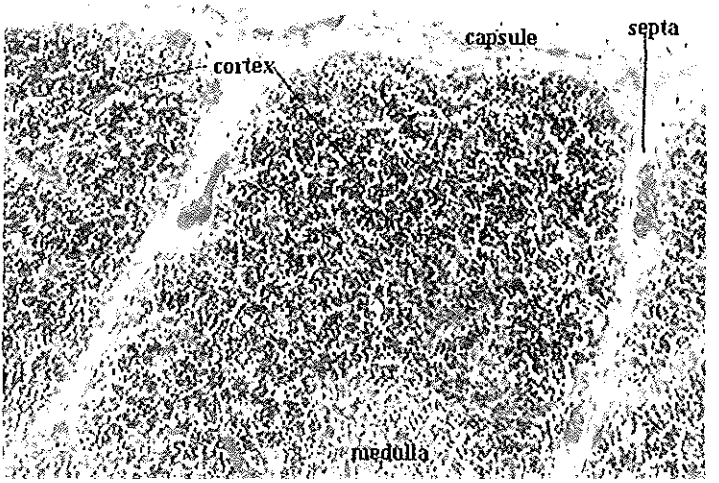

FIGURE 2. Hematoxylin/eosin stained section of human thymus clearly showing the structure of thymic lobules. The capsule is evident on the top of the figure as well as the septa containing blood vessels. The dark-stained cortex and light-stained medulla are evident as well. The diverse intensity of the coloring in the two regions is due to the different density of lymphoid cells, which are significantly more prevalent in the cortex. (From: http//www.var.vet.uga.edu).

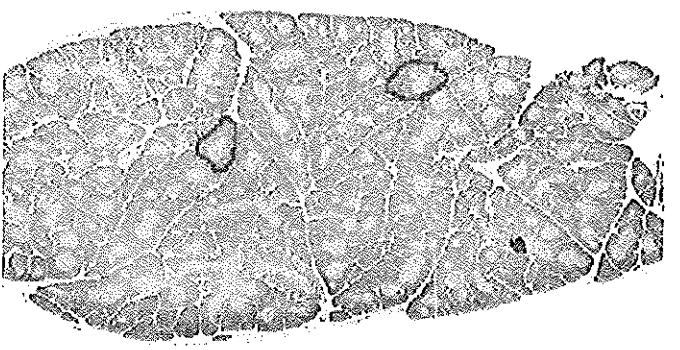

FIGURE 3. Hematoxylin/eosin stained section of human thymus at lower magnification compared to Figure 1. The lobules are easily visible and some cuts of the lobules (in red) appear like round lymphatic nodules. However, looking carefully at the whole section, many lobules (in green) are long and have extended pale medullary areas. These are characteristic for the thymus. The difference in shape of the lobules is due only to the sectioning area. (From: http//www.var.vet.uga.edu).

Blood vessels enter the thymus through the capsule, and travel along septa to the corticomedullary border (Figure 4A), at which point they enter the parenchyma. Arterioles entering the thymus send capillaries to the cortex, which branch at the periphery and return. At the corticomedullary junction, postcapillary venules are found, which represent specialized sites of transit of matured lymphocytes into the blood. The vessels are surrounded by the perivascular space.

The outer cortex houses many large lymphoid elements and blasts, while the inner cortex contains smaller lymphoid cells and a lower number of large lymphoid blasts (Figure 4B). The medulla exhibits small and medium-sized lymphoid cells, as well as its typical Hassall's corpuscles (Figure 5A and B).

CELL TYPES IN THE THYMUS

The thymus has four principal cell types: thymocytes, the so called "lymphocytes of the thymus", which are the precursors of peripheral lymphocytes; few macrophages and dendritic cells, which are all cells of bone marrow origin. The fourth type is a heterogeneous population of specialized thymic epithelial cells (TEC), which provide a supporting framework (the *stroma*) for the parenchyma.

Thymocytes, dendritic cells and macrophages

Most of the "lymphocytes in the thymus" are T-cells. In order to acquire immunocompetence, these cells must reside in the thymus for a period of time. Macrophages are interspersed among them, and can be identified as larger cells with vacuolar nuclei. Frequently coarse granules of undigested material may be present in the cytoplasm of macrophages. The bone marrow derived cells are differentially distributed between the thymic cortex and medulla. The cortex contains only immature thymocytes and scattered macrophages, whereas mature thymocytes, along with another small subset of thymic cells, the dendritic cells, and most of macrophages, are found in the medulla. This reflects the different developmental events occurring in the two main compartments of the human thymus.

TEC

TEC are almost impossible to demonstrate in simple haematoxylin/eosin preparations, being obscured by thymocytes that cover them. Hence, it may be possible to miss them in currently used specimens as such, even though they are indeed present (Figure 4).

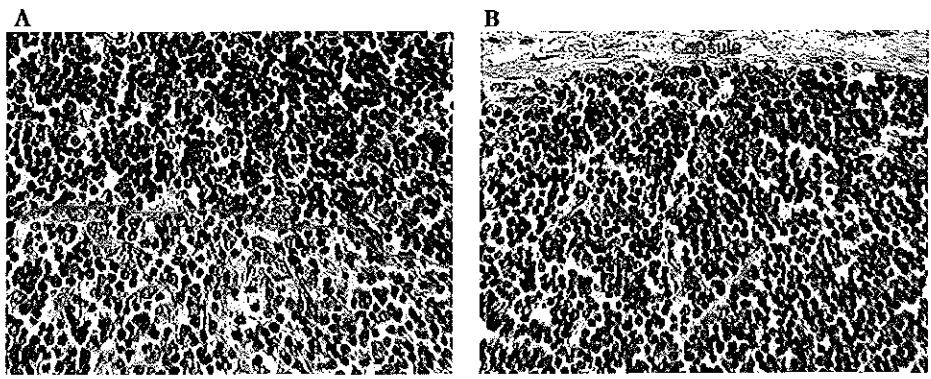

FIGURE 4. Hematoxylin/eosin stained sections of human thymus. A) The red line runs roughly along the junction of the cortex with the medulla. Many mature thymocytes that emerge from the cortex enter the blood stream at this junctional area. The nuclei of epithelial cells are much more numerous in the medulla and visible in the medulla. B) The cortex of the thymus has many capillaries (C) but they are "tight" capillaries that are part of a blood-thymus barrier. At this low magnification the nucleus of an epithelial (R) cell is hardly visible, while there are thousands of small heterochromatic nuclei of developing thymocytes that give the thymus cortex its dark color. (From: http//www.var.vet.uga.edu).

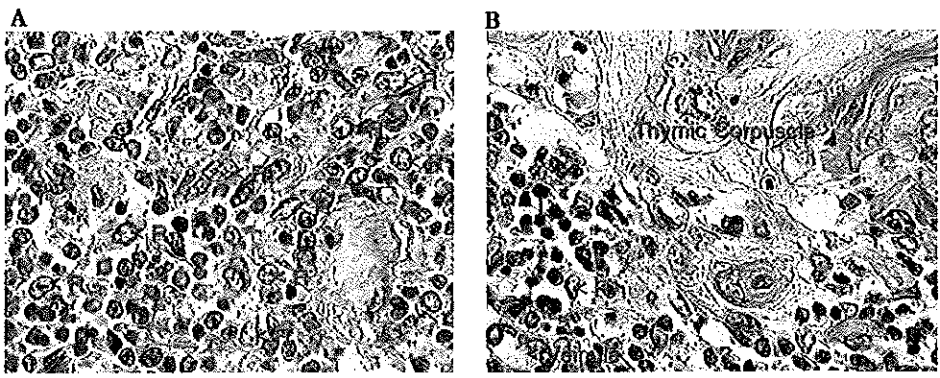

FIGURE 5. Hematoxylin/eosin stained sections of human thymus. A) View of the medulla of the thymus with epithelial cell nuclei labelled (R). It appears evident that many more of these cells are in the medulla. B) View of a thymic corpuscle (Hassal's corpuscle) in the medulla. In the background of lymphocytes (L) and epithelial cell nuclei (R). (From: http//www.var.vet.uga.edu).

The thymic *stroma* is not fibrillar in nature, as is the stroma of other lymphatic organs. It is mainly cellular. TEC are branching and tree-like in shape, and put forth processes, supported by intracellular cytoskeletal elements. These processes come into contact with each other, and are held together at the points of contact with desmosomes. Most importantly to normal thymic function, these cells form a physical, cellular boundary between the parenchymal thymocytes and the rest of the body. At the periphery of the cortex, subjacent to the capsule, and around all blood vessels, the processes form a

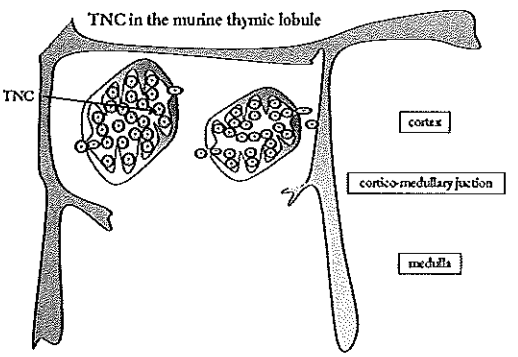

FIGURE 6. Thymic nurse cell (TNC) in murine thymus is a particular lymphoepithelial complex, where one epithelial cell is closely associated with a variable number of thymocytes. The TNC are located in the cortex.

continuous cellular layer constituting the blood-thymus barrier, to limit exposure of the lymphocytes to blood-borne antigens. The reason for this arrangement is that the thymus has to be maintained as an immunologically protected site. Thymocytes here are undergoing maturation and specialization. TEC, together with macrophages and dendritic cells drive the maturation and differentiation of thymocytes into mature lymphocytes, through cell-to-cell contacts and via soluble products. Thymic epithelium is a heterogeneous tissue, and cells in different locations within the lobules may influence specific steps in thymocytes maturation. At least three phenotypically different types of epithelial cells are present in the thymus, the cortical TEC, medullary TEC, and the subcapsular epithelium. However, it seems that more subsets may be distinguished and identified on the basis of different content and type of cytokeratin (5). Peculiar lymphoepithelial complexes have been identified in murine thymic cortex and isolated *in vivo* (6). These are multicellular structures formed by one TEC that can contain many thymocytes, the complex has been called thymic nurse cell (TNC) (Figure 6). TNC can

create special conditions for thymocyte development and form a unique microenvironment where sophisticated interactions may occur. They offer an excellent model for the *in vitro* study of the intrathymic thymocyte migration mechanisms (7). Recent insights underline the important participation of extracellular matrix components, such as laminin and fibronectin and their respective integrin receptors VLA-4/VLA-5 and VLA-6, to the complex cascade of events leading in the maturation and selection of T lymphocytes (8-10). TEC may undergo degeneration, and organize themselves into concentric eosinophilic whorls of material, called thymic corpuscles or Hassall's corpuscles (Figure 5). These are of unknown function, and appear very early in life.

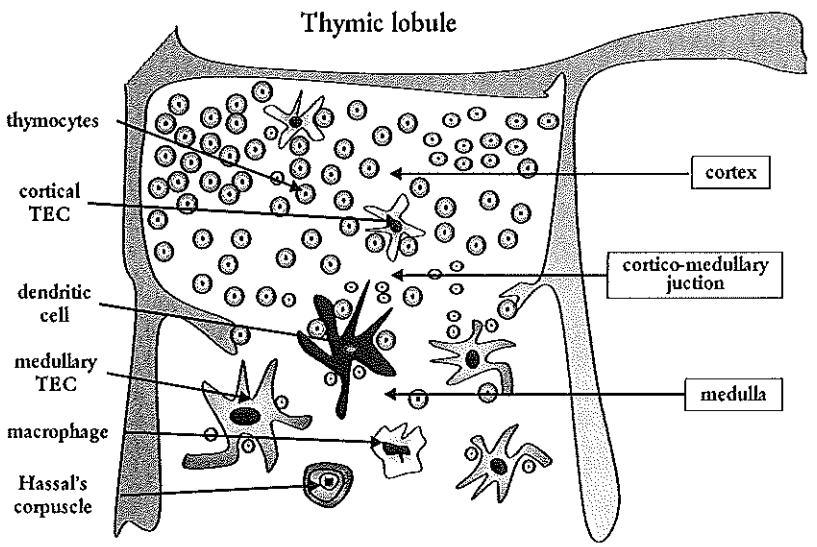

FIGURE 7. Schematic reproduction of a thymic lobule. Thymocytes during maturation are driven from the cortex to the medulla having in the mean time many contacts with a heterogeneous cellular network, including thymic epithelial cells (TEC), dendritic cells and macrophages. TEC represent a heterogeneous group with distinct subset of cells in the cortex and in the medulla.

The schematic structure of a human thymic lobule is shown in Figure 7.

FUNCTION OF THE THYMUS

Although both B and T cells derive from bone marrow stem cells, T cells do not differentiate in the bone marrow, but rather migrate at very early stage to the thymus. The thymus provides the specialized microenvironment where receptor gene rearrangement and

maturation of T cells occur (11). Because T cells recognize foreign antigen only in the form of peptide fragments bound to molecules encoded by the major histocompatibility complex (MHC), only those T cells able to identify the body's own MHC molecules will contribute to adaptive immune responses. Thus, it is mandatory that each individual T cell is able to recognize foreign antigens bound to MHC molecules (self restriction). However, T cells should be unable to recognize self peptides (self tolerance). T cells are selected to fulfil these properties during their maturation in the thymus. Here, they undergo the two selective processes, the positive selection, in which they are screened for self MHC restriction, and the negative selection, which eliminates those cells specific for self antigens. The differentiation involves sequential expression of a variety of membrane markers and rearrangements of the T-cell receptor (TCR) genes (Figure 8).

In the thymus, the immature T cells, thymocytes, proliferate and differentiate. The positively selected cells migrate to the T-cell dependent areas of peripheral lymphoid organs, such as spleen, lymph nodes and tonsil (11). This process persists throughout life, however, the number of developing elements decays with ageing. Together with differentiation, precursors of T cells interact with various components of the thymic microenvironment (11). During migration thymocytes follow a predisposed direction and are driven from the cortex to the medulla. In the first step, after a very active proliferative phase, thymocytes with high avidity are negatively selected and deleted by apoptosis, while those with intermediate avidity for recognition of MHC-self peptide are rescued and positively selected. Positive selection seems mainly mediated by TEC, whereas bone marrow-derived cells (macrophages and dendritic cells) essentially exert negative selection (11,12).

The differentiation process involves sequential expression and down-regulation of membrane proteins such as the TCR (coupled on the cell membrane with the CD3 complex) and more molecules, including CD4 and CD8, which further define distinct stages of maturation (13). Most immature and intermediate thymocytes, bearing the phenotypes $CD3^-CD4^-CD8^-$ and $CD3^+CD4^+CD8^+$, respectively, are located in the cortex, whereas mature $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ can be found in the medulla (Figure 8). Thymic microenvironmental cells influence thymocyte differentiation and proliferation *via* soluble factors, such as cytokines and thymic hormones (14-16). However, thymocyte-TEC interactions are bi-directional, products of thymocytes can modulate TEC functions as well (17,18).

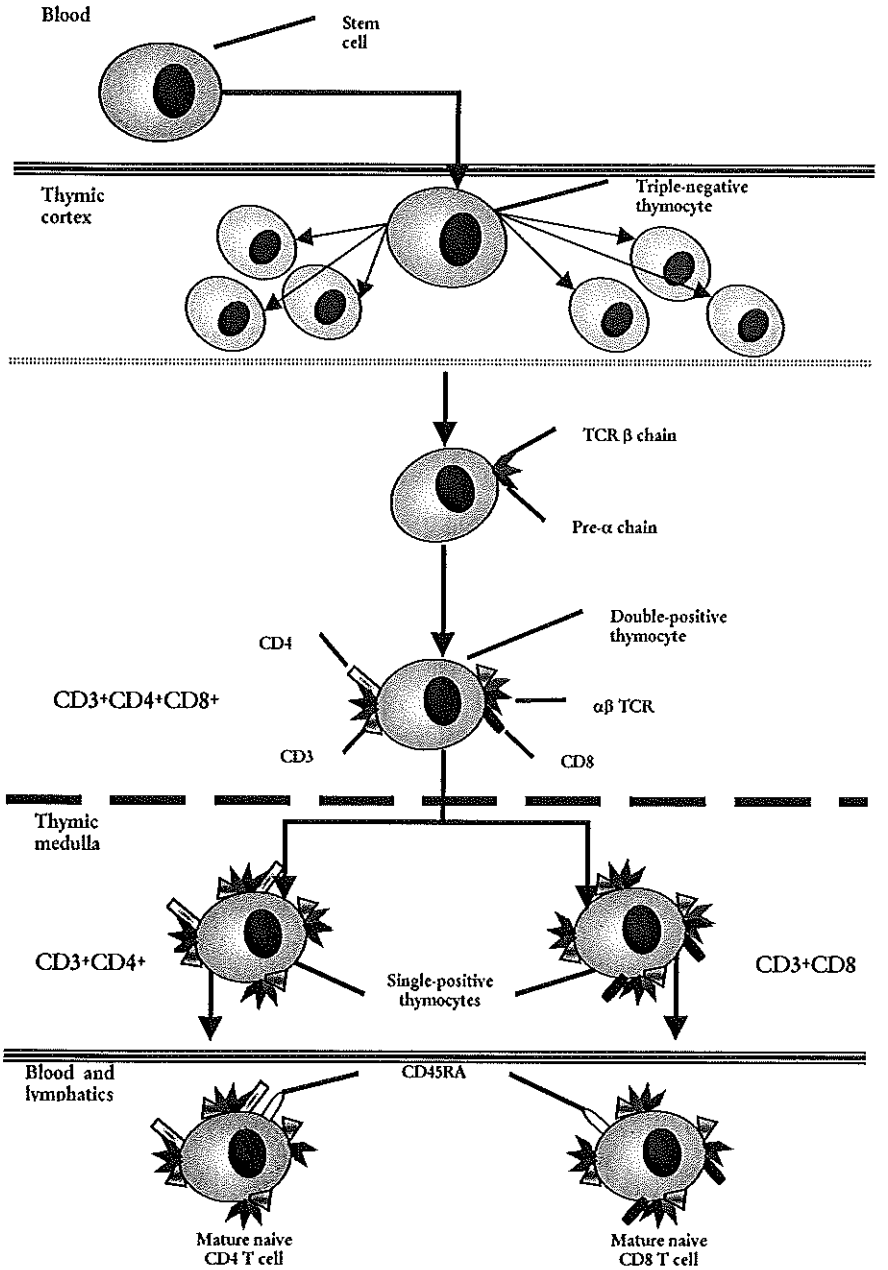

FIGURE 8. Thymocyte differentiation through thymic miroenvironement. Thymocytes migrate during differentiation from the cortex to the medulla. Bone marrow-derived thymocytes enter the thymus and undergo active proliferation. The cells start the process of maturation as triple negative ($CD3^-CD4^-CD8^-$), then they become double positive ($CD3^+CD4^+CD8^+$) and are finally committed in single positive ($CD3^+CD4^+$ $CD8^-$ or $CD3^+CD4^-CD8^+$) thymocytes. The cell antigen receptor (TCR) is an heterodimer most commonly formed by α- and β-chains. The mature T cell has the phenotype expressing a marker called CD45RA, which characterizes T cells that have never been activated by antigen.

Macrophages, which are mainly located in the medulla and at the cortico-medullary junction, can also interact with differentiating thymocytes via membrane proteins and cytokines (11). These aspects emphasize the notion that several paracrine circuits involve the various cell networks within the thymic microenvironment.

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THE THYMUS, AN INTERFACE BETWEEN THE NEUROENDOCRINE AND THE IMMUNE SYSTEMS

Considering the role of immunomodulator attributed to several neuroendocrine factors and the bi-directional neuro-endocrine-immuno interactions via endocrine, paracrine and autocrine pathways, the various intrathymic cellular interactions described in the previous paragraph, as well as the development of thymocytes itself can be targets for neuropeptide activities. This concept is rather old, however, evidence for this concept was provided only in the last decades.

The frequent association of acromegaly with thymic hyperplasia was established by pathologists at the beginning of the last century. In 1921, Hammar showed that the thymus often involutes under the influence of various environmental or emotional factors, and pointed out that thymic hyperplasia is frequently associated with hormonal changes (e.g. castration, Graves' disease, Addison's disease, and acromegaly). Furthermore, the role of the pituitary in thymic growth was observed by Smith in 1930.

Several hormones, such as GH and its peripheral mediator IGF-I, PRL, ACTH and thyroid hormones as well as neuropeptides modulate the production of thymic hormones (1). These factors seem also to enhance the expression of extracellular matrix ligands and receptors, influencing the level of TEC-thymocytes adhesion (1). These findings have been confirmed by the recent characterization of receptors for many of the above mentioned ligands on thymic cells (2-7). Special attention has been dedicated to intrathymic circuits involving the local production of GH and IGF-I and their activities on both the lymphoid and stromal compartments (4,5). Moreover, all these factors, as well as glucocorticoids, appear also to regulate epithelial cell growth in the thymus (8). More recently, glucocorticoids have been supposed to regulate the expression of MHC in the thymic microenvironment (9). Pituitary hormones, thyroid hormones, steroids, neuropeptides, such as VIP, SP, SS, and more recently melatonin, can indirectly and directly modulate thymocyte development, conveying positive and negative signals for thymocyte proliferation (1,10). Moreover, consistent evidence that cytokine production by thymocytes may be under neuroendocrine control has been provided (11-13).

One of mechanisms much more studied is the control of intrathymic cell death mediated by steroids. In general glucocorticoids and, to lesser extend, sex steroids promote apoptosis of thymocytes, playing then a role in the selection of the T-cell repertoire (14-19). Interestingly, protection from apoptosis may be under neuroendocrine control as well. In fact, dihydroepiandrosterone may be able to rescue thymocytes from apoptosis induced by pharmacological doses of glucocorticoids *in vivo* (20,21). Also VIP, as well as melatonin has been proposed as "protectors" of thymocytes from apoptosis induced by dexamethasone *in vivo* (22,23).

Thymocyte traffic, in terms of entrance of precursors, driving processes within the thymus as well as exit from the organ, are apparently under neuroendocrine control (24-28). This appears of particular importance taking into consideration that an imbalance in this important process may result in abnormal traffic of immature or wrongly selected cells towards peripheral lymphoid organs, which may lead to the development of autoimmune diseases.

The intrathymic production of many of these factors which we have previously discussed, as well as the expression of their specific receptors have been characterized during recent years (Table 1). Thus, in addition to the classical endocrine pathways, paracrine and autocrine mechanisms are implicated in the influence of hormones and neurpeptides on the thymus (1). This strongly suggests that normal circulating levels as well as controlled local production of these factors are necessary to preserve the homeostasis and the various biological functions related to both the microenvironmental and lymphoid cells of this important organ.

However, the neuroendocrine control of the thymus is a much more complex phenomenon with a wealth of sophisticated mechanisms. In particular, the intrathymic biological circuit involving *in situ* production of mediators appears to be very relevant in various aspects, including the influence of neuropeptides. Independent of which pathway is triggered, the neuroendocrine control of the thymus is articulated pleiotropically, with modulation in the expression of several genes in different cell types.

Considerable research has now been focused on SS and its receptors as clinical consequences of their local activity in the cross-talk between specific subsets of thymic cells in both normal and pathological events in the human thymus.

TABLE 1. Hormones, neuropeptides and their receptor expression within the thymus.

Effector	Intrathymic production	Receptor expression
"Classic" hypothalamic peptides		
⇒ GnRH	+	n.d.
⇒ GHRH	+	+
⇒ CRH	+	n.d.
⇒ TRH	+	+
⇒ SS	+	+
"Classic" anterior pituitary hormones		
⇒ ACTH	+	+
⇒ GH	+	+
⇒ PRL	+	+
⇒ LH	+	n.d.
"Classic" posterior pituitary hormones		
⇒ Vasopressin	+	+
⇒ Oxytocin	+	+
Steroids and thyroid hormones		
⇒ Cortisol	+	+
⇒ Estradiol	n.d.	+
⇒ Progesterone	n.d.	+
⇒ Testosterone	n.d.	+
⇒ Triiodothyronin/thyroxin	n.d.	+
Miscellaneous		
⇒ Insulin	+	+/-
⇒ Enkephalin	+	+
⇒ β-endorphin	+	+
⇒ VIP	+	+
⇒ PACAP	n.d.	+
⇒ CGRP	+	+

GnRH, gonadotropin releasing hormone; GHRH, growth hormone releasing hormone; CRH, corticotropin releasing hormone; TRH, thyrotropin releasing hormone; SS, somatostatin; ACTH adrenocorticotropin hormone; GH, growth hormone, PRL, prolactin; LH, luteinizing hormone; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; CGRP, calcitonin gene-related peptide; n.d. not detected.

Finally, many of the concepts related to the immunoneuroendocrine interactions occurring within the thymus may be extrapolated to the periphery and can be helpful to understand other still obscure mechanisms involved in the communication between these systems (Figure 1).

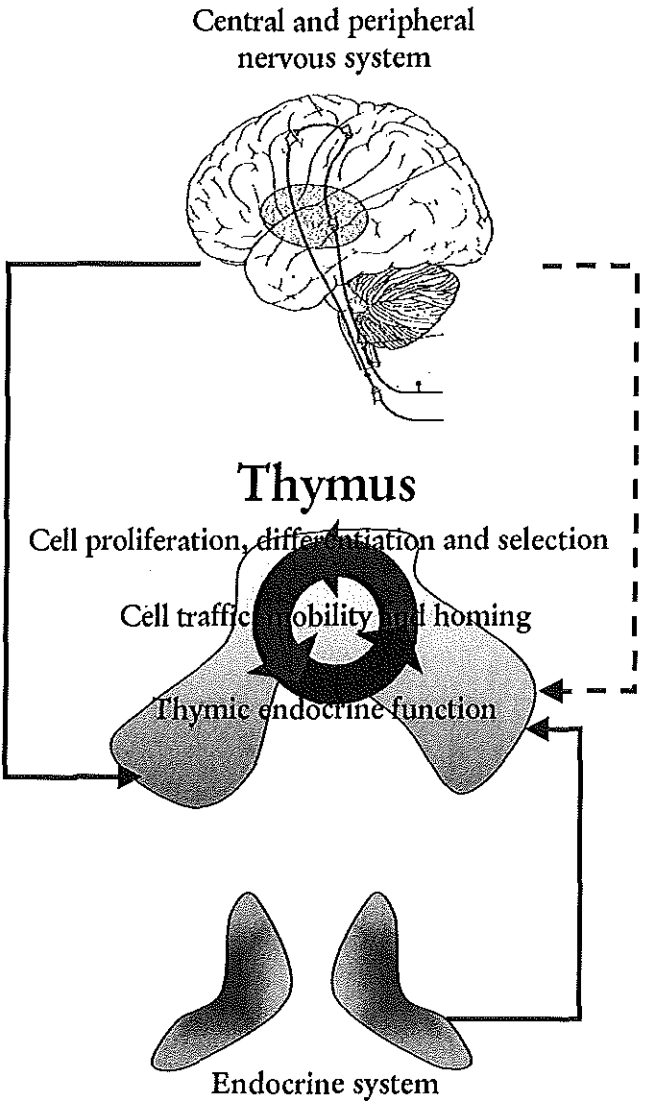

FIGURE 1. Neuroendocrine control of the thymus. The central and peripheral nervous system interact with the thymus *via* specific products and through the innervation; the endocrine system influences many thymic functions *via* its specific products (long loop circuits). However, the immuno-neuro-endocrine interaction in the thymus is based on the exchange of local paracrine signals as well (arrow circle).

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SOMATOSTATIN RECEPTORS IN THE THYMUS

INTRODUCTION

A discussed before, thymic hormones and cytokines play a critical role in the proliferation, differentiation and selection of precursor cells along the T-cell lineage (1). Among the locally produced hormones and neuropeptides that may participate in an autocrine and paracrine manner to thymic functions, some have well-characterized actions, whereas SS has been identified, but not investigated in detail (2-8). SS-binding sites have been demonstrated in the thymus of adults and children *in vitro* (9,10). Moreover, *in vivo* the presence of SSR has been shown in thymic epithelial tumors (11-13). However, the precise localization of SSR, as well as the distribution of the different subtypes has never been investigated within the human thymus so far.

Although, experimental models in animals have suggested the significant participation of SS and other neuropeptides in the activities of the thymus, the exact role of SS in this contest is still obscure. The following sections will briefly illustrate the emerging data regarding the potential role of SS and its receptors in the regulation of thymic functions in animal models. In addition, the final paragraph will introduce preliminary observations in humans.

SOMATOSTATIN AND SOMATOSTATIN RECEPTORS IN THE THYMUS

Classical regulatory peptides, such as SS, can be produced and may be involved in the regulation of cell functions in the thymus. These factors act directly on TEC and thymocytes, suggesting the existence of specific receptors on these cells.

The presence of SS mRNA or immunoreactivity for SS has been demonstrated in the chicken, rat and murine thymus (7,14,15). In both chicken and rat thymus the concentration of SS was higher compared with the spleen, although 50 times less than that found in the periventricular region of the hypothalamus (15). SS-positive cells were found within the medulla, around the cortico-medullary junction and in a small subset of

thymocytes in the thymic medulla as well (15). Moreover, SS has been shown to exert an antiproliferative effect on concanavalin A-activated rat thymocytes (16), while not on human phythoemagglutinin (PHA)-activated thymocytes (17). A recent study demonstrated the expression of pro-SS in murine thymic macrophages, whereas no expression was found in thymocytes, dendritic cells and TEC (7). Furthermore, other pancreatic hormones such as preproinsulin, propancreatic polypeptide and proglucagon were found in other cell subsets in the thymus, indicating that these hormones are differentially expressed in mice (7).

Sst_2 mRNA has been detected in murine resting thymocytes (18). This seems to contrast with the expression in rat, where thymocytes selectively express sst_3 and sst_4 mRNAs (19). However, another study showed the presence of sst_2 mRNA in rat thymocytes, and demonstrated that the activation of cells with PHA and IL-1 upregulates the expression of sst_1 (20). Although the significance of the presence of specific SSR subtypes remains to be clarified, it seems that the activation of immune cells may result in modification of their SSR expression pattern. Moreover, the involvement of SSR in modulation of thymocyte proliferation and differentiation is arguable.

In humans SS inhibits hormone secretion in virtually all neuroendocrine cell types, however, its biological significance in the thymus is still unknown. Intrathymic production of SS has been demonstrated (21,22). Endogenously synthesized regulatory peptides are supposed to be present in the vicinity of the receptor-expressing target cells. Reubi *et al.* first described the presence of SS-binding sites in the human thymus of both newborn and adult by autoradiography (9). The binding was mainly localized in the thymic medulla, where TEC are the predominant cell type. However, many cells in this area contain thymic hormones, suggesting their neuroendocrine characteristic (23), and the diffuse and homogeneous labeling pattern within the thymic medulla could mean that other types of cells might contain SSR as well.

Thymic cells are under the control of hormones and neuropeptides through specific receptors. In the human thymus, cortex and medulla display a low density of VIP receptors presumably associated with T cells, while it seems that epithelial cells in the medulla express SSR. In addition, blood vessels express substance P receptors (10). These findings demonstrate a strong compartmentalization of neuropeptide receptors in lymphoid tissues and support the complex mode of action of neuropeptides in the immune system.

Recently, modern nuclear medicine imaging techniques have been used to visualize *in vivo* thymic neoplasms (11-13). Localization studies of SSR on specific cell types within SSR positive tissues have demonstrated heterogeneity of receptor distribution. However, in many cases the precise cellular localization of the different subtypes remains difficult to establish. This limit appears especially evident in tissues formed by several different cellular components, like the lymphatic ones. Moreover, the expression of neuropeptide receptors on immune cells seems to be dynamically regulated and may depend on the traffic of these cells through and within lymphoid structure and homing in tissues. The recent availability of antibodies raised against selective peptide sequences of different SSR subtypes has allowed to document the distribution of the receptor proteins at the cellular level in transfected cells, animal tissues as well as in human pancreas and tumors by immunohistochemistry (24-30). With this new approach lymphoid tissues have been recently investigated for the expression of SSR subtypes as well (31-33). The main advantage of this technique is the possibility to localize the receptor proteins at cellular levels.

In conclusion, multiple SSR subtypes are expressed on cell-specific subsets in the rat and murine thymus. No data are available on SSR subtype expression in the human thymus. Moreover, SS is locally produced in the thymus. SS, in a way similar to its well-known activity on almost all neuroendocrine cells, might modulate the functions of thymic cells, participating to the maturation of T lymphocytes.

In this chapter, the results of investigations on the expression and function of SS and SSR subtypes in the different cell subsets of the human thymus are presented.

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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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IN VITRO CHARACTERIZATION OF SOMATOSTATIN RECEPTORS IN THE HUMAN THYMUS AND EFFECTS OF SOMATOSTATIN AND OCTREOTIDE ON CULTURED THYMIC EPITHELIAL CELLS

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 5 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 the reverse transcriptase polymerase chain reaction (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 7 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 mRNA in the thymic tissue, while 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.

INTRODUCTION

Somatostatin (SS), a peptide hormone originally isolated from the hypothalamus as a growth hormone-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 5 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 reverse transcriptase polymerase chain reaction (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 5 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 5 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 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. Since 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).

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 6 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, 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 (1μM) or SS-28 (1μM), 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) 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.

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 units RNAsin and 2 units AMV Super Reverse Transcriptase (HT Biotechnology Ltd., Cambridge, UK) in a final volume of 20 µl. This mixture was incubated for 1 hr 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 units 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 (since 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

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.

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

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/L), fungizone (0.5 mg/L), 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. Lewis; 2 mg/ml) for 1-2 h at 37°C. The dispersed cells were precultured for 5-7 days in 15 mg/cm² collagen type I (Collagen S, type I, Boehringer Mannheim B.V., Mannheim, Germany) precoated 75-cm² flasks (Costar, Cambridge, MA) at a density of $5x10^6$ 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) supplemented with 10% FCS, penicillin (10^5 U/L), fungizone (0.5 mg/ml), L-glutamine (2 mmol/L). 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 anti mouse IgG coated magnetic beads and a ASO2 anti-human 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), were treated once again with the coated beads. These remaining suspension containing isolated TEC were 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, Houten, The Netherlands) 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.

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, 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, 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.

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 in order 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

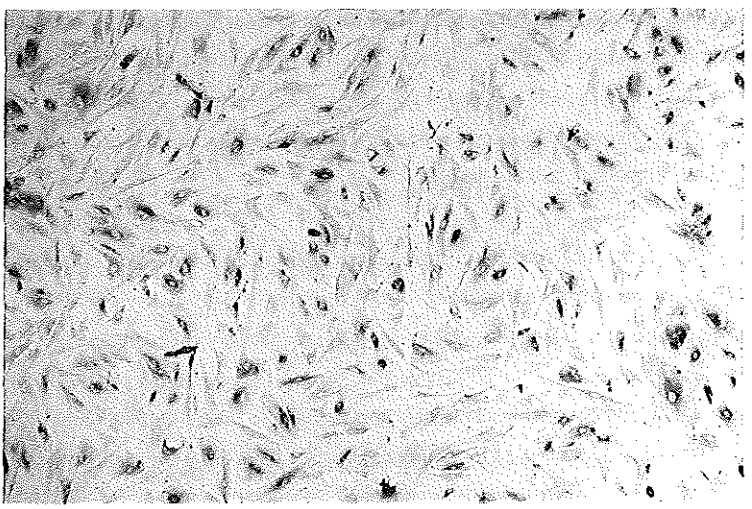

FIGURE 1. Staining for keratin of thymic epithelial cells (TEC) cultured on coverslips for the same period as TEC used for the experiments. No fibroblasts (keratin-negative) are present.

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 7 normal thymuses. Binding of the sst_2 subtype selective ligand [^{125}I-Tyr^{3}]-octreotide was in parallel positive in all these 7 cases (Table 2), and in agreement with a

TABLE 2. Somatostatin and somatostatin receptor subtype expression in human thymic tissue as determine 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.

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, while 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 Figure 3.

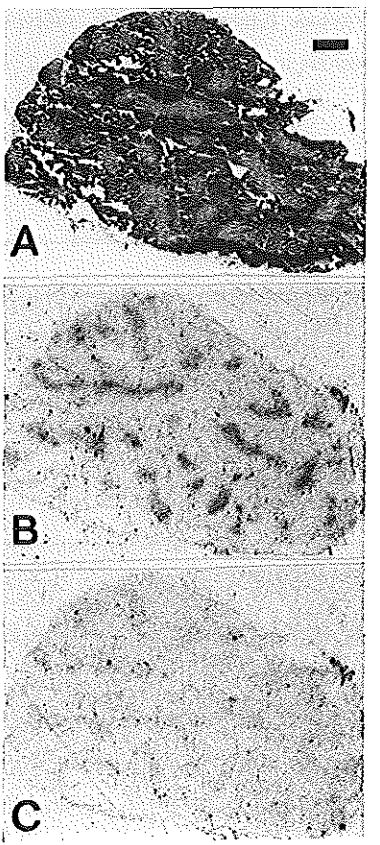

FIGURE 2. Expression of SS-R in human thymus. Photomicrograph of SS-R-autoradiography. *A)* hematoxylin-eosin stained section; *B)* autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide; *C)* autoradiogram showing nonspecific binding (in the presence of 1 μM of Tyr^3-octreotide). *Bar*, 1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

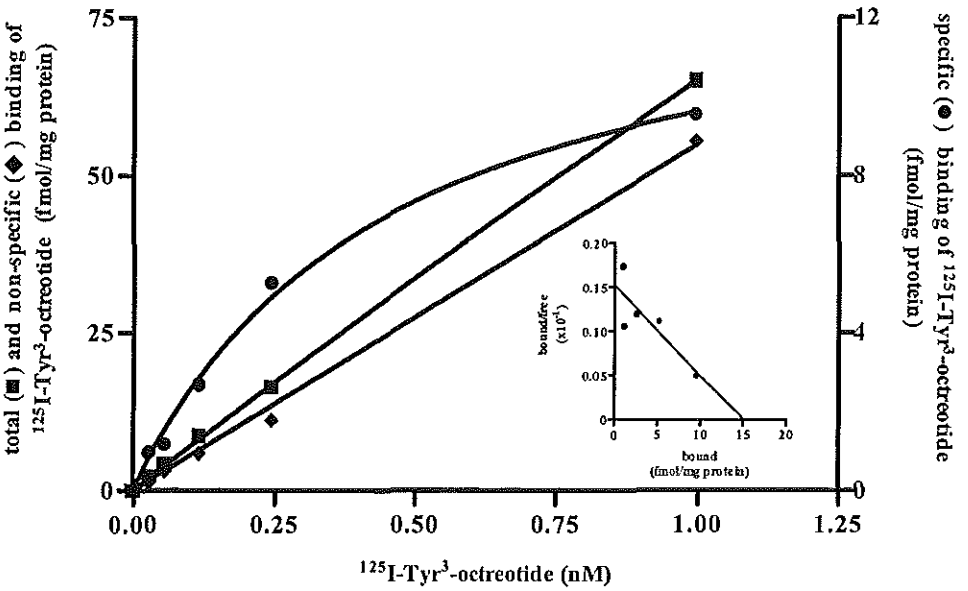

FIGURE 3. Binding of [^{125}I-Tyr^3]-octreotide to a membrane homogenate preparation of a human thymus. ■, total binding; ◆, non-specific binding in presence of 1 μM of Tyr^3-octreotide; ●, specific binding (total minus non-specific binding). Inset: Scatchard analysis of the binding data (K_d, 0.5 nM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

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 indipendent experiments.

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).

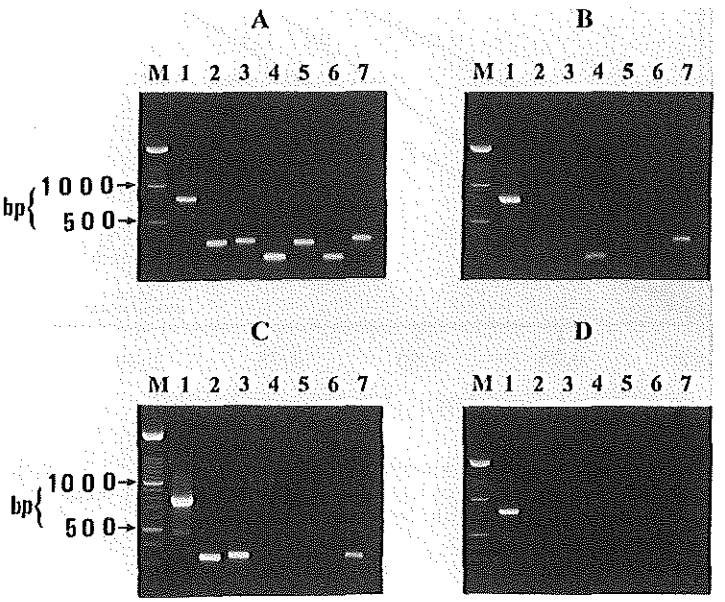

FIGURE 4. Heterogeneous expression of β-actin, sst_1 sst_{2A}, sst_3 and SS mRNAs in the human thymus. Poly A⁺ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. *1*, β -actin; *2*, sst_1; 3, sst_{2A}; *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.

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.

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 3 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 3 cultures (Fig. 5A), and by 19.1, 48.2, and 59.5% in 3 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 2 out of 3 cultures and EGF significantly stimulated ^{3}H-Thymidine incorporation in all the 3 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).

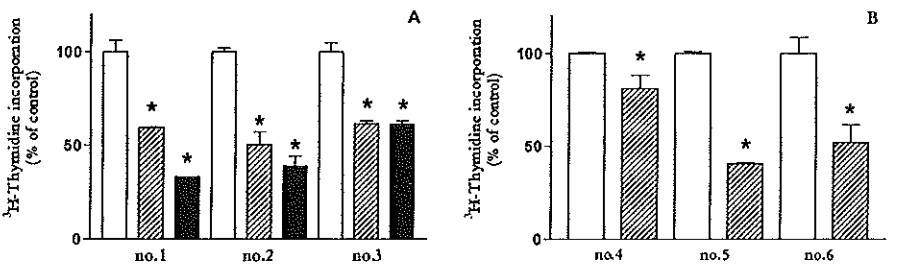

FIGURE 5. *A*) effects of SS and octreotide on ^{3}H-Thymidine incorporation in TEC cultures of 3 different thymuses (nos. 1-3, Table 2). *B*) effects of octreotide on ^{3}H-Thymidine incorporation in TEC cultures of 3 other thymuses (nos. 4-6, Table 2). Thymic epithelial cells were incubated in MEM + 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.

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*	974.6±25.8*
5.	1347.3±14.6	1624.8±75.3	3326.8±57*
6.	756.3±64	993.5±89.3*	4125.7±270.3*

The data represent the Mean±SEM of 4 wells per treatment group. *, $p<0.01$ *vs.* control.

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 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, while no binding was found on cultured thymocytes. Interestingly, RT-PCR showed the selective expression of sst_1 and sst_{2A} on the cultured TEC, while 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 to 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. Furthermore, TEC seems 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 4 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, while 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 mainly 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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Quantitative and functional distribution of somatostatin receptor subtypes in human thymocytes.
2000 *Submitted*

QUANTITATIVE AND FUNCTIONAL EXPRESSION OF SOMATOSTATIN RECEPTOR SUBTYPES IN HUMAN THYMOCYTES

ABSTRACT

We recently demonstrated the expression of SS and SS receptor (SSR) subtype 1 (sst_1), sst_{2A} and sst_3 in normal human thymic tissue and of sst_1 and sst_{2A} on thymic epithelial cells (TEC). We also found an inhibitory effect of SS and octreotide on TEC proliferation. In the current study we further investigated the presence and function of SSR in freshly isolated human thymocytes, a heterogeneous cell population displaying different levels of maturation. We firstly demonstrate specific high affinity [^{125}I-Tyr^{11}]-SS-14 binding on thymocyte membrane homogenates, although the number of binding sites is relatively low. By RT-PCR, sst_{2A} and sst_3 mRNA expression was detected. After separation of thymocytes into subpopulations, we found by quantitative RT-PCR that sst_{2A} and sst_3 are differentially regulated in intermediate/mature and immature thymocytes. The expression of sst_3 mRNA is higher in the intermediate/mature $CD3^+$ fraction compared with the immature $CD2^+CD3^-$, while sst_{2A} mRNA is less abundant in the intermediate/mature $CD3^+$ thymocytes. In cultured thymocytes no SSR subtype mRNA was detectable. Since sst_3 is mainly expressed on intermediate/mature thymocytes and most of these cells generally die by apoptosis, it might be that sst_3 is involved in this process. SS-14 inhibited ^{3}H-Thymidine incorporation in thymocyte cultures, indicating the presence of functional receptors. The heterogeneous expression of SSR within the human thymus and the endogenous production of SS emphasize their potential role in the bi-directional interaction pathway between the two main cell components of the thymus involved in intrathymic T-cell maturation.

INTRODUCTION

The immune and neuroendocrine systems cross talk by sharing ligands and receptors. Neurohormones modulate the function of lymphoid organs and are produced by immune cells as well, thereby exerting a paracrine/autocrine action in immunoregulation (1). Receptors for different neurohormones, such as hypothalamic-pituitary and gastrointestinal hormones, are expressed by immune and lymphoid accessory cells (1,2). These neuroendocrine circuits seem to exert a pleiotropic control on the physiology of the thymus, the main lymphoid organ (3). Particularly, the intrathymic production of classical neurohormones suggests that paracrine and autocrine interactions, mediated by these compounds and their respective receptors, influence both thymic lymphoid and stromal compartments (3,4).

Somatostatin (SS) is one of the neuropeptides implicated in neuro-immuno-endocrine interaction (5). The wide spectrum of actions of SS and the presence of SS receptor (SSR) expression in lymphoid organs implies a broad functional role of this peptide in the immune system (2,5).

We have recently demonstrated the expression of SS and of three different SSR subtypes (sst) within the human thymus (6). Messenger RNAs encoding for sst_1, sst_{2A} and sst_3 receptors were found in a series of normal thymic tissues. Sst_1 and sst_{2A} were selectively expressed on cultured thymic epithelial cells (TEC) and both SS and its analog octreotide inhibited *in vitro* TEC proliferation. No SSR subtype mRNA was detectable in 7-14 day-cultured thymocytes (6), while recently, our preliminary data have demonstrated a low number of SS-binding sites on freshly isolated human thymocytes (7). On the other hand, SSR are expressed on thymocytes of different animal species (8-10), and in humans SS is known to modulate different functions of T lymphocytes, which directly derive from thymocytes (5). In fact, sst_3 mRNA has been recently demonstrated constitutively expressed in human resting peripheral T lymphocytes, while the mitogenic activation of these cells seems to induce the expression of sst_5 mRNA (11). Thymocytes are a heterogeneous cell population. In fact when progenitors enter the thymus from bone marrow they lack most of the specific T-cell markers. Most immature human thymocytes are characterized by the presence of CD34 antigen (12). This antigen is highly expressed on the pluripotent stem cell in bone marrow and the expression diminishes through lineage

commitment (13). The interactions with thymic microenvironment trigger the expression of T-cell specific surface molecules. Firstly CD2 is the marker of immature thymocytes when they do not express the TCR-CD3 complex or the coreceptors CD8 and CD4 (14,15). These cells are called "double negative" thymocytes and are a highly heterogeneous pool of cells that include several early stages in T-cell development (14,15). Thus, thymocytes undergo maturation through a series of stages that can be distinguish by the differential expression of the TCR-CD3 complex, CD8 and CD4. $CD3^-CD4^+CD8^-$ represents an intermediate thymocyte subset before the "double positive" $CD4^+CD8^+$ thymocytes stages (16). Finally the $CD3^+CD4^+CD8^+$ subset further differentiates into mature $CD4^+$ or $CD8^+$ single positive thymocytes (14-16).

The current study was designed to investigate the presence and potential role of SSR in human thymocytes. The receptor expression pattern was evaluated *in vitro* in freshly isolated thymocytes by SSR binding studies on membrane homogenate and by RT-PCR to identify and quantify SSR subtypes on different thymocyte subsets. In addition, the *in vitro* effect of SS and octreotide on cell proliferation was investigated in isolated human thymocytes.

MATERIALS AND METHODS

Samples. Thymic tissues were removed from 8 patients (age range between 3 months and 5 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 and informed consent was obtained from the patients or their parents. All samples were histopathologically normal and were taken fresh at the operation.

Protocol of the study. Thymocytes were freshly isolated from the 8 samples and used for binding studies on membrane homogenates with iodinated SS-14 and octreotide (nos. 1-8, Table 1), while thymocytes derived from 4 samples were separated in subpopulations for RT-PCR studies (nos. nos. 5-8, Table 1; see below). Thymocytes from 3 samples of the same series (nos. 5-7, Table 1) were used for the *in vitro* primary cell cultures.

Cell dispersion, cell separation and cell culture. Thymocytes were collected using a filter chamber (NPBI, Emmer-Compascuum, The Netherlands) and placed in RPMI-1640 (Gibco BRL, Life Technologies LTD, Paisley, Scotland) supplemented with 10% heat-inactivated FCS, penicillin (10^5 U/L) and fungizone (0.5 mg/L). The pH of the medium was adjusted to 7.4. Cell viability was determined before each study and was more than 95%. These thymic cell suspensions generally contain more

than 95% thymocytes, as it has been demonstrated by flow cytometry (FACS®) and anti-CD2 antibodies, which selectively bind to thymocytes, in a series of normal pediatric thymuses (11). To confirm this we performed FACS® analysis in one case (no. 5, Table 1) using FACScan cytometer (Beckton Dickinson & Co., Erembodegem, Belgium) and anti-CD2 antibodies (Beckton Dickinson & Co.). Cytometry and additional fluo-conjugated antibodies were used to determine the proportion of the different thymocyte subsets and the monocyte-macrophage fraction (anti- CD3, CD34, and CD14, all from Beckton Dickinson & Co.). Thymic cells (10^6) were sorted by setting appropriate electronic gates with the dual-laser FACS® system (Beckton Dickinson & Co.) For RT-PCR analysis, thymic cells were first depleted from the monocyte fraction (see below) and subsequently separated into subpopulations by using magnetic beads coated with specific antibodies (Dynal AS, Oslo, Norway). The cells were suspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), and incubated with the coated beads in plastic tubes kept on ice for 30 min. By continuous rotation of the tubes, the cells and beads were kept in suspension. The tubes were then placed in a magnetic rack to separate the supernatant from the beads-captured cells. The non-selected cells in the supernatant were used for the subsequent rounds of selection with appropriate antibody-coated beads. The beads-captured cells were washed 5 times with PBS containing 0.5% BSA, counted and evaluated for specificity by determining the percentage of cells rosetted by the beads, which was higher than 98% in all the cases. The thymocyte suspension was depleted from the monocyte fraction by using beads coated with CD14 antibodies ($CD14^+$). In order to isolate intermediate/mature thymocytes ($CD3^+$), anti-CD3-coated beads were used. The remaining cells (after a second round of depletion with anti-CD3-coated beads) were further incubated with anti-CD2-coated beads to obtain the immature thymocyte fraction ($CD2^+CD3^-$). Very early thymocytes were isolated using anti-CD34-coated beads. Additional freshly isolated thymocytes, which did not undergo beads separation, (5×10^6 cells per well) were seeded in 1 ml culture medium in 24-well plates (Costar, Cambridge, MA). Then, test substances were added, and the cells were incubated for 24 h for functional experiments. Cell viability was constantly tested during the separation procedure as well as before and after functional studies, and was satisfactorily.

SSR membrane binding studies. The method of membrane isolation and the reaction conditions were previously described (6). [^{125}I-Tyr^{11}]-SS-14 (Amersham, Houten, The Netherlands) and [^{125}I-Tyr^3]-octreotide (Novartis Pharma, Basel, Switzerland) binding to the thymocyte membranes was analyzed. Briefly, membrane preparations (corresponding to 30-50 µg protein) of freshly dispersed cells were incubated in a total volume of 100 µl at room temperature for 30 min with increasing concentration of [^{125}I-Tyr^{11}]-SS-14 or for 60 min with increasing concentrations of [^{125}I-Tyr^3]-octreotide, with and without excess (1 µM) of unlabeled SS-14 or octreotide, respectively, in

HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/L bacitracin, 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 an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a γ-counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled SS-14 or octreotide.

Functional studies. In all experiments SS-14 (Bachem Inc., Hannover, Germany) and octreotide (Novartis Pharma) were used at a concentration of 10^{-13}, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M. After 24 h, proliferation was measured by adding 1 μCi of [methyl-^{3}H]-Thymidine (91 Ci/mmol; Amersham) for the last 6 h in each well of the 24-well plates. Thereafter, the cell suspension was transferred to 5-ml tubes and precipitated with 10% trichloroacetic acid and the pellet was washed once again in trichloroacetic acid. After solubilization in 1 M NaOH, the cells were transferred to scintillation counting vials 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).

RT-PCR studies. RT-PCR was performed as previously described (6). Briefly, poly A+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from cell pellets containing 0.5-1x10^6 cells per sample. cDNA was synthesized using the poly A+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ as solid phase and first strand primer. One-tenth of the cDNA was used for each amplification by PCR using primer sets specific for human $sst_{1\text{-}5}$, SS and hypoxantine guanine phosphoribosyltransferase (HPRT). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A+ mRNA preparation (since the SSR subtype 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 HPRT specific primers served as positive control for the quality of the cDNA. To exclude contamination of the PCR reaction 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 SSR receptor subtypes, 0.001 to 0.1 μg of human genomic DNA, representing approximately 300 to 30.000 copies of sst-template was amplified in parallel with the cDNA samples. As a positive control for the PCR of the HPRT and SS cDNA aliquots of a cDNA sample known to contain SS and HPRT mRNA were amplified, because these primer-pairs did enclose introns in the genomic DNA. In the thymocyte cell preparations only sst_2 and sst_3 mRNAs were detectable. In order to quantify sst_2 and sst_3 mRNAs a quantitative RT-PCR was performed by

TaqMan® Gold nuclease assay (The Perkin-Elmer Corporation, Foster City, CA) and the ABI PRISM® 7700 Sequence Detection System (The Perkin-Elmer Corporation) for real-time amplification, according to the manufacturers instructions. The specific primer sequences that were used included:

sst_{2A} forward 5'-ATGCCAAGATGAAGACCATCAC-3',

sst_{2A} reverse 5'-TGAACTGATTGATGCCATCCA-3'

sst_3 forward 5'-CTGGGTAACTCGCTGGTCATCTA-3'

sst_3 reverse 5'-AGCGCCAGGTTGAGGATGRA-3'

HPRT forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3'

HPRT reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'.

The probe sequences that were used included:

sst_{2A} 5'-FAM-TGGCTCTGGTCCACTGGCCCTTTG-TAMRA-3'

sst_3 5'-FAM-CGGCCAGCCCTTCAGTCACCAAC-TAMRA-3'

HPRT 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'.

The amount of sst_2 and sst_3 mRNA was determined by means of a standard curve generated in each experiment from known amounts of human genomic DNA. For the determination of HPRT mRNA amount, the standard curve was obtained by including dilutions of a pool of cDNAs known to contain HPRT. The amount of sst_2 and sst_3 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Statistical analysis. Data are expressed as Mean±SEM, n = 4 wells per treatment group. All data were analyzed by the 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. SSR binding data were analyzed by the method of Scatchard. Receptor binding studies and RT-PCR experiments were performed at least twice.

RESULTS

SSR binding study Using membrane homogenate binding, specific [^{125}I-Tyr^{11}]-SS-14 binding was demonstrated on freshly isolated thymocytes in all cases examined (n=6). Binding of [^{125}I-Tyr^{11}]-SS-14 could be displaced with excess unlabeled SS-14. Scatchard analysis of the binding data revealed a single class of high affinity binding sites with an apparent K_d ranging between 0.4±0.1 and 3.1±1.0 nM and a low maximum binding capacity (B_{max}) ranging between 10±1.9 and 59±5.5 fmoles/mg membrane protein (Table 1). As a control for binding, rat brain cortex membranes were used. An example of saturation binding data

with Scatchard analysis is shown in Figure 1. Using [^{125}I-Tyr^{3}]-ocreotide, no specific binding was detected on membrane preparations of thymocytes (Table 1).

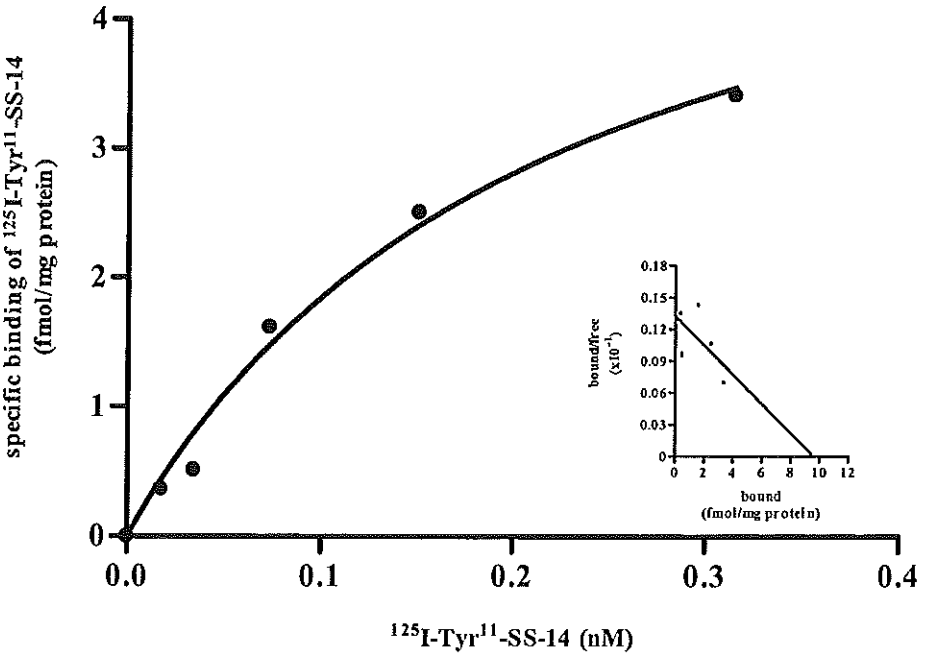

FIGURE 1. Binding of [^{125}I-Tyr^{11}]-SS-14 to a membrane homogenate preparation of human thymocytes. •, Specific binding (total minus non-specific binding in presence of 1 μM of SS-14). Inset: Scatchard analysis of the binding data (K_d, 0.43 nM and B_{max}, 10 fmol/mg membrane protein; no. 5, Table 1).

TABLE 1. Somatostatin receptor expression in human thymocytes determined by Scatchard analysis of [^{125}I-Tyr^{11}]-somatostatin-14 and [^{125}I-Tyr^{3}]-octreotide binding on membrane homogenates.

Samples	[^{125}I-Tyr^{11}]-SS-14 binding		[^{125}I-Tyr^{3}]-octreotide binding	
	K_d (nM)	B_{max} (fmoles/mg protein)	K_d (nM)	B_{max} (fmoles/mg protein)
1.	3.1±1.0	59±5.5	-	-
2.	0.7±0.05	11±1.5	-	-
3.	1.2±0.6	31±6.1	n.p.	n.p.
4.	n.p.	n.p.	-	-
5.	0.4±0.1	10±1.9	-	-
6.	n.p.	n.p.	-	-
7.	0.8±0.2	29±3.4	-	-
8.	2.1±0.8	47±3.3	-	-
rat brain	1.4±0.2	469.3±89.1	0.8±1.1	496.5±41.5

SS-14, somatostatin-14; K_d, dissociation constant; B_{max}, maximum binding capacity; -, undetectable; n.p., not performed. The data represent the Mean±SEM of at least two independent experiments.

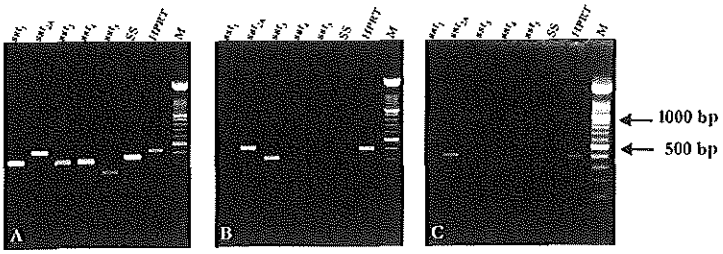

FIGURE 2. Heterogeneous expression of SS and sst_{1-5} mRNAs in human thymocytes. Poly A$^+$ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. M, 100-bp ladder; *A)* control; *B)* freshly isolated thymocytes; *C)* CD14$^+$ cells; (no. 5, Table 1). RT-PCR analysis of each tissue was performed at least twice with identical results.

TABLE 2. Heterogeneity of somatostatin and somatostatin receptor subtype mRNA expression in different fractions of human thymocytes and CD14$^+$ thymic cells as determined by RT-PCR in 4 cases (nos. 5-8 Table 1).

Cell subset	RT-PCR						
	sst_1	sst_{2A}	sst_3	sst_4	sst_5	SS	HPRT
Freshly isolated thymocytes	-	+	+	-	-	-	+
CD3$^+$ cells	-	+	+	-	-	-	+
CD2$^+$CD3$^-$ cells	-	+	+	-	-	-	+
Cultured thymocytes	-	-	-	-	-	-	+
CD14$^+$ cells	-	+	-	-	-	-	+

SS, somatostatin. Each case was evaluated at least two times in independent experiments and yielded identical results.

SS and SSR subtype expression: By RT-PCR, sst_{2A} and sst_3 mRNA expression was detected in freshly isolated thymocytes from 4 cases (nos. 5-8, Table 1), whereas mRNA encoding for SS, sst_1, sst_4 and sst_5 was absent (Fig.2B). No mRNA encoding for any SSR subtype was detectable in thymocytes after 7-14 days culture (data not shown), confirming our previous observation (6). In $CD14^+$ cells the presence of only sst_{2A} mRNA was detected (Fig.2C). RT-PCR of thymocytes after separation into immature $CD2^+CD3^-$ and intermediate/mature $CD3^+$ fractions revealed sst_{2A} and sst_3 mRNA expression in both subsets. Table 2 summarizes the results of RT-PCR analysis and an example is shown in Figure 2 (no. 5, Table 1). Quantitative RT-PCR analysis revealed a higher number of sst_3 mRNA copies in the intermediate/mature $CD3^+$ thymocyte fraction compared to the immature $CD2^+CD3^-$ one (Fig.3A). Conversely, the number of sst_{2A} mRNA copies was higher in the immature $CD2^+CD3^-$ fraction compared to the mature $CD3^+$ thymocytes in 3 of 4 cases (Fig.3B). The sst_3/sst_{2A} ratio increased with the level of thymocyte maturation (Fig.3C). Moreover, in one case (no. 8, Table 1) we isolated the very early $CD34^+$ thymocytes and we found that sst_{2A} mRNA expression was higher (5.1 *vs.* 2.6 sst_{2A}/HPRT), while sst_3 expression was lower (0.5 *vs.* 12.1 sst_3/HPRT) compared to the immature $CD2^+CD3^-$ fraction. The method of beads separation showed that thymocytes were more than 95%, while $CD14^+$ cells were less than 5% among the filtered thymic cells, in all the cases examined. In order to confirm this finding we performed FACS® analysis in one of these cases (no. 5, Table 1) and the results are shown in Figure 4. Among these cells, thymocytes at different levels of maturation yielded 95.8% (Fig.4A), $CD14^+$ cells yielded 3.4% and very early CD34 thymocytes yielded 1% (Fig.4B).

These data are in agreement with other authors, which performed this evaluation on a larger series of age- and sex-matched pediatric thymuses (11).

Effect of SS and octreotide on ^{3}H-Thymidine incorporation in thymocytes SS-14 significantly inhibited ^{3}H-Thymidine incorporation in all the 3 cultures (nos. 5-7, Table 1) of freshly isolated thymocytes in a dose dependent manner.

The inhibition was statistically significant at a concentration of 10^{-6} (ranging between 45 and 57%) and 10^{-8} M (ranging between 27 and 41%) in all 3 cases, at a concentration of 10^{-10} M in 2 of 3 (21 and 41%, respectively), and at a concentration of 10^{-12} M in 1 of 3 (26%) (Fig. 5A-C).

The SS analog octreotide significantly inhibited ^{3}H-Thymidine incorporation only in 1 culture of thymocytes at concentrations of 10^{-6} (43%) and 10^{-8} M (23%) (Fig. 5D-F).

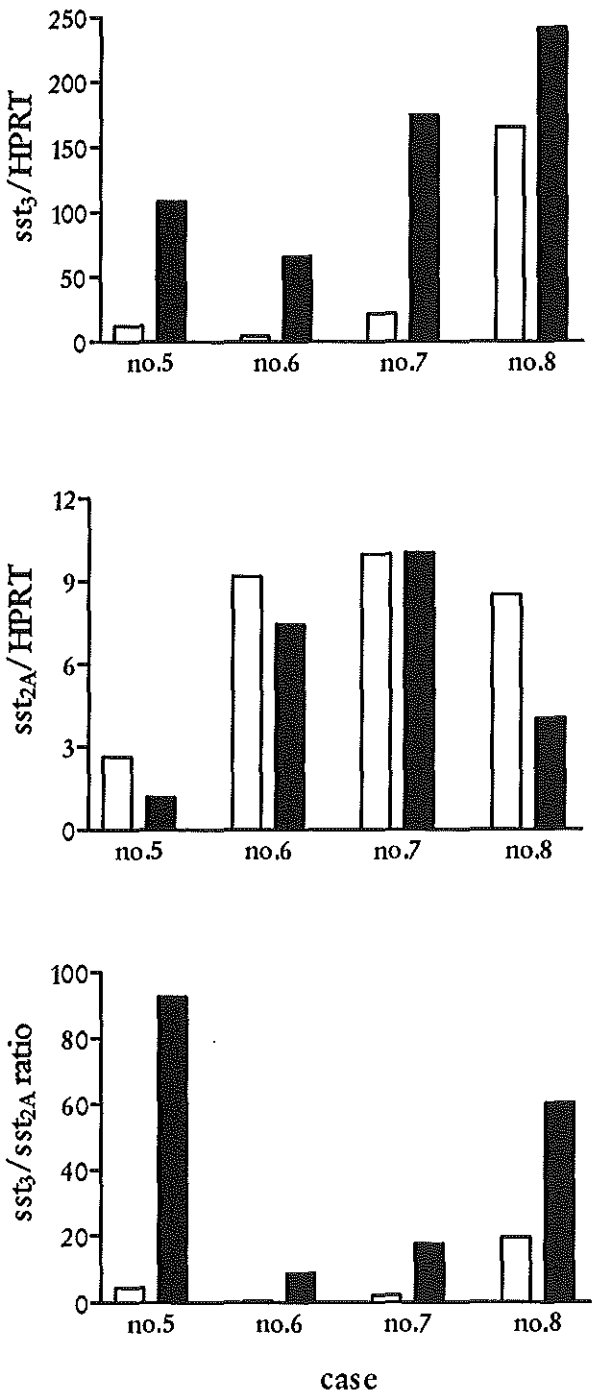

FIGURE 3. Quantitative RT-PCR in human thymocytes. Quantitative analysis of RT-PCR data showed a different amount of sst_{2A} and sst_3 mRNA in immature $CD2^+CD3^-$, and intermediate/mature $CD3^+$ thymocytes calculated relative to the amount of HPRT and given in arbitrary units. *A)* sst_3/HPRT mRNA ratio; *B)* sst_{2A}/HPRT mRNA ratio; *C)* sst_3/sst_{2A} mRNA ratio.
Data derived from 4 different thymuses (nos. 5-8, Table 1). Immature $CD2^+CD3^-$ □, mature $CD3^+$ ■ thymocytes.

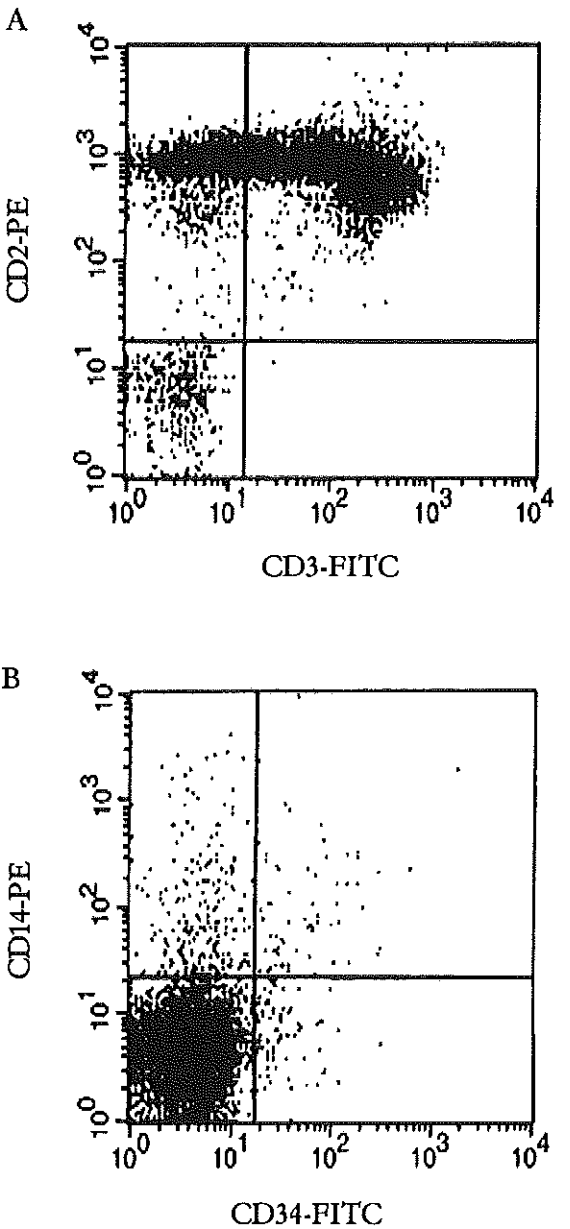

FIGURE 4. Flow cytometric analysis of thymic squeezed cells. 10^6 cells were labelled with fluorescein isothiocyanate- (FITC) and phycoerythrin- (PE) conjugated antibodies *A)* $CD2^+$ and $CD3^+$ cells were sorted after setting of the gates as indicated in the figure and 95.8% of cells were identified as thymocytes *B)* $CD14^+$ and $CD34^+$ cells were sorted after setting of the gates as indicated in the figure and 3.4% of cells were identified as monocytes ($CD14^+$), while 1% as early thymocytes ($CD34^+$).

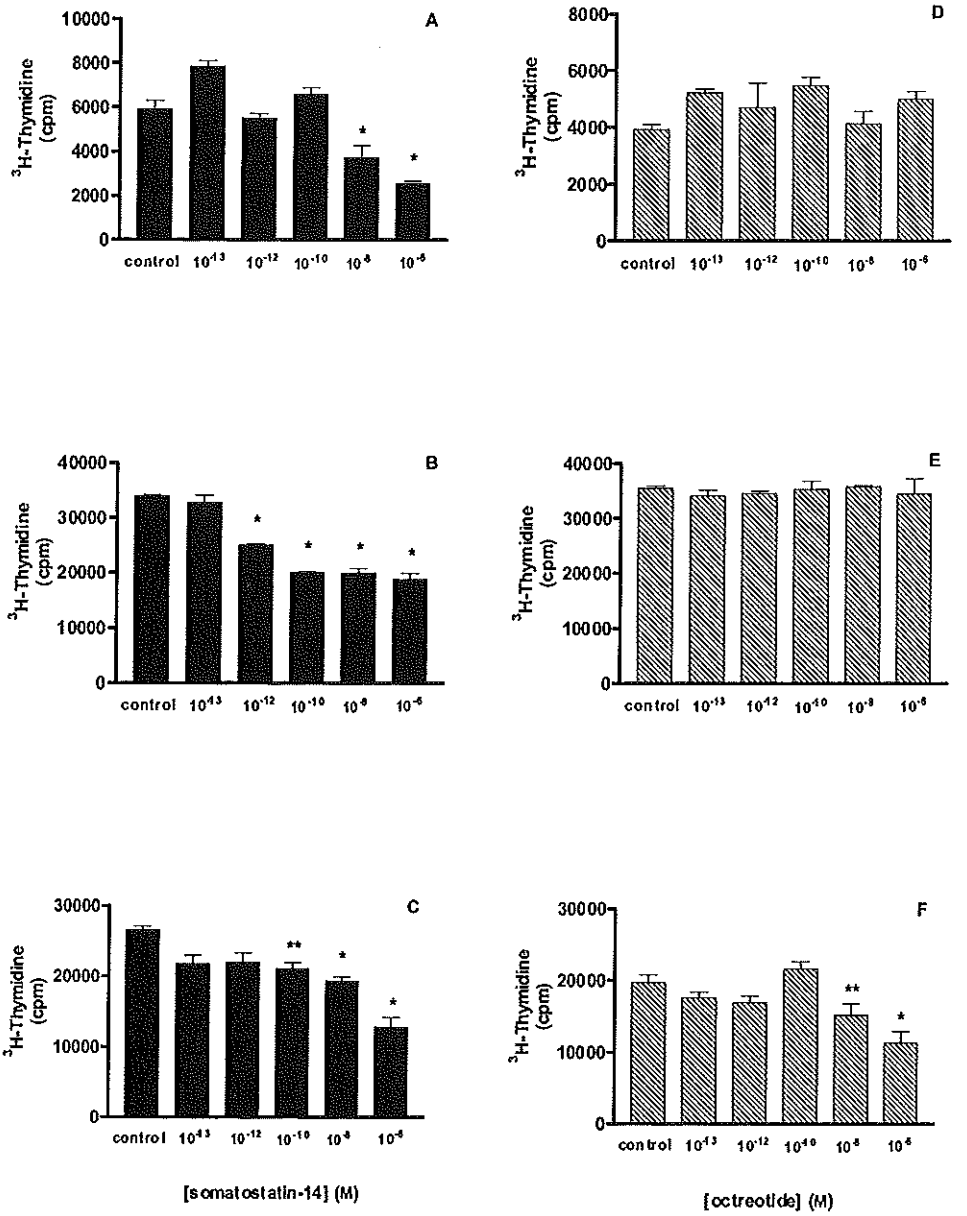

FIGURE 5. Effects of somatostatin (*A-C*) and octreotide (*D-F*) on ^{3}H-Thymidine incorporation in thymocyte cultures from 3 different thymuses (nos. 5-7, Table 1). Thymocytes were incubated in RPMI-1640 supplemented with 10% heat-inactivated FCS, penicillin and fungizone during 24 h in quadruplicate without or with the drugs indicated at the concentrations of 10^{-13}, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M. Values are expressed as counts per minute (cpm) and are Mean±SEM (n=4 well per treatment group).
*, $p<0.01$ *vs.* control; **, $p<0.05$ *vs.* control. SS-14 ■ and octreotide ▨.

DISCUSSION

The thymus is responsible for promoting the differentiation and maturation of lymphoid precursor cells into mature T lymphocytes. The developing T cells are embedded in an epithelial network known as the thymic stroma. Other cells of haematopoietic origin participate in constituting the complex architecture of this organ; these cells include dendritic cells and macrophages (17,18). Interactions between thymic stromal cells and thymocytes are mediated by direct contact and *via* soluble factors, and play a crucial role in T cell development (19,20). Among the soluble factors, neuropeptides have been demonstrated to be deeply involved in the regulation of thymic functions. The intrathymic production of classical hormones suggests that in addition to the endocrine circuits, paracrine/autocrine interactions may exist in the thymus, influencing both the lymphoid and stromal compartment of the organ (3).

SS is a representative neuropeptide with a wide spectrum of actions (21). The biological effects of SS are mediated *via* five specific, high-affinity, G-protein coupled receptors (22). The presence of the neuropeptide and its receptors has been demonstrated in the human thymus (6,23,24). We have recently described the expression of sst_1, sst_{2A} and sst_3 mRNAs in human thymic tissue. Cultured TEC selectively expressed sst_1 and sst_{2A} mRNA (6). Conversely, in long-term cultured thymocytes SSR mRNA was undetectable (6), while SS-binding sites were found on freshly isolated thymocytes (7). Sst_2 mRNA was detected in murine resting thymocytes (8), in contrast with the expression in the rat, where these cells selectively express sst_3 and sst_4 mRNAs (9). These differences pointed to species-specific expression of SSR subtypes in immune cells. Moreover, another study showed the presence of sst_2 mRNA in fresh rat thymocytes, and demonstrated that the activation of these cells upregulates the expression of sst_1 (10). It should be mentioned that many extrinsic factors and changes in the microenviromental conditions might regulate the expression of SSR (25,26). SS itself could be involved in the regulation of receptor expression. SS induced an increase in its receptor binding and SSR subtype mRNA expression in GH3 pituitary cell line (27).

In the present study, using freshly isolated thymocytes, we firstly demonstrated specific [^{125}I-Tyr^{11}]-SS-14 binding on cell membrane homogenates, while [^{125}I-Tyr^3]-octreotide binding was undetectable. The number of SS-binding sites was very low. These thymic cells are a heterogeneous population mainly formed by intermediate/mature thymocytes (11-15).

Subsequently, we characterized the SSR subtype expression by RT-PCR. In freshly isolated thymocytes sst_{2A} and sst_3 mRNA expression was detected, while in thymocytes after 7-14 days culture, no mRNA encoding for SSR subtypes was detectable. Since in freshly isolated thymocytes are present cells at different level of maturation, we investigated whether sst_{2A} and sst_3 mRNA could be differentially regulated and expressed in the different stages of maturation of human thymocytes. We separated whole thymocytes into intermediate/mature $CD3^+$ and immature $CD2^+CD3^-$ fractions, and by RT-PCR, we detected sst_{2A} and sst_3 mRNA in both thymocyte subpopulations. However, by quantitative RT-PCR analysis we demonstrated the predominant expression of sst_3 mRNA in $CD3^+$ thymocytes. These cells represent the subset of thymocytes, which have reached a higher level of maturation during the complex cascade of events occurring in the thymic network (28). Interestingly, sst_3 mRNA has been found constitutively expressed in peripheral T lymphocytes which directly derive from mature thymocytes (11). Conversely, a predominant expression of sst_{2A} mRNA was found in the $CD2^+CD3^-$ thymocytes, which are the immature fraction and, very interestingly, sst_{2A} mRNA was also the predominant receptor in the very early $CD34^+$ thymocytes, which belong to the most immature subset of lymphoid cells in human thymus. In fact, when progenitor cells enter the thymus from bone marrow, they lack most of the surface antigens characteristic of mature T cells. Thereafter the interactions with the thymic stroma trigger rapid proliferation and the expression of the first T-cell specific surface molecule CD2. The $CD2^+CD3^-$ thymocytes form in the developed thymus a small but highly heterogeneous pool of cells (29). The low proportion of this latter subset might explain the lack of binding of the sst_2-preferring ligand [^{125}I-Tyr^3]-octreotide at the study on thymocyte membrane homogenates. In addition, it should be considered that the number of SS-binding sites was very low, in agreement with the presence of a low number of SS-binding sites on circulating human immune cells (11). On the other hand, the binding of the universal ligand [^{125}I-Tyr^{11}]-SS-14, which binds all 5 SSR subtypes, is likely due to the binding of this ligand to the sst_3 subtype, which is mainly expressed on the intermediate/mature cells, representing the major proportion of thymocytes. Most of these cells are destined to die as a consequence of failing selection (30). Cell death in the thymus occurs by a process known as programmed cell death or apoptosis, which is a common feature in many developmental pathways (30). The expression of the sst_3 on these cells is intriguing, since this SSR subtype is involved in agonist-mediated apoptosis (31). Conversely, since the immature $CD2^+CD3^-$

thymocytes are intensively proliferating cells undergoing a rearrangement process, the predominant presence of the sst_{2A} on this very small subset suggests the involvement of this SSR subtype in the early phase of thymocyte development within the thymus. These data provide the evidence for a strong compartmentalization of neuroendocrine peptide receptors in lymphoid tissue (2), as it is also shown by the expression of vasoactive intestinal peptide (VIP) receptors on murine and rat thymocytes (32). In fact, the two VIP receptors display a distinct distribution in different thymocyte subsets, suggesting that the expression of neuropeptide receptors could be developmentally regulated and *viceversa* (32). Finally, it is also noteworthy to mention that in $CD14^+$ cells, which are cells belonging to the monocyte-macrophage lineage, the presence of only sst_{2A} mRNA was detected. This finding is in agreement with our previous reports on the selective expression of this SSR subtype on human macrophages and monocytes (33,34, Lichtenauer-Kaligis EGR *et al.*, unpublished observations).

We have previously demonstrated that SS mRNA is present in human TEC (6). In the present study we found that SS mRNA was not detectable in thymocytes. SS has been shown to exert an antiproliferative effect on concanavalin A-activated rat thymocytes (35), while SS was inactive on human phytohaemagglutinin-activated thymocytes (36). In the present study, we found an inhibition of the ^{3}H-Thymidine incorporation in early cultures of thymocytes during the administration of SS-14, supporting the presence of functional receptors on these cells. The inhibition of ^{3}H-Thymidine incorporation by octreotide was statistically significant in only one out of the three cases, and only at high concentrations. This SS analog binds with high affinity sst_{2A} and with lower affinity sst_3 and sst_5 (22). The possibility that at high concentration, octreotide acted *via* the sst_3, or that a higher number of sst_{2A}-expressing cells were present only in the culture showing the inhibitory effect of the SS analog can not be ruled out. All together, these data suggest that SS produced by TEC may affect the thymocyte population in a paracrine manner.

The heterogeneous expression of SSR within the human thymus, together with the endogenous production of SS and other neurohormones, emphasizes once more the role played by neuropeptide hormones in the human thymus. Finally, the maturation and selection of the T cell repertoire is one of the most intriguing processes and involves a number of factors. SS, likely produced by TEC (6), seems to affect both the lymphoid and microenviromental compartments of the thymus. TEC are known to drive the most important phases of T cell maturation and differentiation, however, thymocytes might

affect TEC functions as well (37). Thus, a bi-directional interaction pathway exists between the two main cell components of the thymus, and SS might be part of this complex circuit. Moreover, SS is known to affect the production of immunoglobulins and interleukins (38,39), which are well recognized factors participating at the sophisticated and elegant process leading to the maturation of cellular immunity. In this light SS may represent an important molecule involved in the chain of events, resulting in the generation of T cell repertoire.

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AGE-RELATED DECREASE OF SOMATOSTATIN RECEPTOR NUMBER IN THE NORMAL HUMAN THYMUS

ABSTRACT

The thymus exhibits a pattern of ageing orientated towards a physiologic involution. The structural changes start with a steadily decrease of thymocytes, while no major variations occur in the number of thymic epithelial cells (TEC). The data concerning the role of hormones and neuropeptides in thymic involution are equivocal. We recently demonstrated the presence of somatostatin (SS) and three different SS receptors (SSR) subtypes in the human thymus. TEC selectively expressed SSR subtype 1 (sst_1) and sst_{2A}. In the present study we investigated whether SSR number is age-related in the thymus. Binding of the sst_2-preferring ligand [^{125}I-Tyr^3]-ocreotide was evaluated in a large series of normal human thymuses of different age by SSR autoradiography and ligand binding on tissue homogenates. The score at autoradiography, as well as the number of SSR at membrane homogenate binding (B_{max}) were inversely correlated with the thymus age ($r=-0.84$, $P<0.001$; $r=-0.82$, $P<0.001$, respectively). The autoradiographic score was positively correlated with the B_{max} values ($r=0.74$, $P<0.001$). Since TEC number, in the age range considered, remains unchanged, the decrease of octreotide binding sites might be due to a reduction of sst_{2A} receptor number on TEC. The age-related expression of a receptor mainly involved in controlling secretive processes is in line with the evidence that the major changes occurring in TEC with ageing are related to their capabilities in producing thymic hormones. In conclusion, SS and SSR might play a role in the involution of the human thymus. These findings underline the links between the neuroendocrine and the immune systems and support the concept that neuropeptides participate in development of cellular immunity in humans.

INTRODUCTION

The thymus, the primary lymphoid organ responsible for differentiation and maturation of the specific T cell repertoire, exhibits an ageing behaviour, which is unique because of its irreversible physiologic involution (1). This phenomenon is characterized by a progressive structural change of the gland, starting at an early stage of life. Lipomatous atrophy is the most evident age-related change in the thymus, although it represents the final state of the involution (2). The early stages of this process are essentially characterized by a steady decrease of the number of thymocytes, the lymphoid cellular component, and of thymic dendritic cells, while no major changes are found in the number of thymic epithelial cells (TEC), which represent the most relevant component of the thymic stroma (3). However, the human thymic epithelium is capable of undergoing sequential stages of maturation in the postnatal thymus (4). The factors regulating the involution process of the thymus have not been completely clarified yet. Particularly, contradicting hypotheses have been raised concerning the potential role of hormones and neuropeptides in this process. For instance, thymic involution is considered either dependent or independent from puberty (5,6). Because several neuropeptides are localized in lymphoid tissues, and because somatostatin (SS) may influence cells of the immune system, we have recently searched for the presence of SS and SS receptors (SSR) in the normal human thymus (7). SS and three different SSR subtypes (sst), sst_1, sst_{2A} and sst_3, were expressed in the human thymic tissue, while sst_1 and sst_{2A} were selectively expressed on cultured TEC (7). Moreover, SS and octreotide administration induced an *in vitro* inhibition of TEC proliferation, which is presumably mediated by receptors of the sst_{2A} subtype (7). These data support the concept of a modulatory action of SS on cell functions within the thymus. In addition, a functional role of SS and SSR in the involution process of the thymus can be hypothesized as well. In order to evaluate whether the SSR pattern shows an age-related change, we studied the binding of the sst_2-preferring ligand [^{125}I-Tyr^3]-ocreotide in a large series of normal human thymuses of different age. SSR density was determined both by SSR autoradiography and by ligand binding studies on tissue homogenates. The results were correlated with the chronological age of the thymuses.

METHODS

Samples Thymic tissues were removed from 30 patients (15 males and 15 females, age ranging between 15 days and 21 years) 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 and 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 ligand-binding on cryostat sections and membrane homogenates. The 30 thymic tissue samples were divided in 5 different groups on the basis of an arbitrary age range: Group 1 (10 cases), 0-12 months; Group 2 (5 cases), 13-24 months; Group 3 (5 cases), 25-72 months; Group 4 (5 cases), 73-120 months; Group 5 (5 cases), > 120 months.

SS receptor binding on cryostat sections Receptor autoradiography was carried out as described by Visser-Wisselaar *et al.*, (8). Briefly, 10-micrometer thick cryostat (Jung CM3000, Leica, Germany) sections of the tissue samples were mounted onto precleaned gelatine-coated microscope glass slides and stored at -80°C for at least 3 days before the experiment, in order to improve the adhesion of the tissue to the slide. As radioligand, the SS analog [^{125}I-Tyr^3]-octreotide (Novartis Pharma, Basel, Switzerland) was used. Specific activities of the radioligand 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 binding buffer (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 pmoles/liter). Non-specific binding was determined in a sequential section in the presence of excess unlabeled Tyr^3-octreotide (1 μM). The incubated sections were washed twice for 5 min in binding buffer containing 0.25% BSA and once in binding buffer without BSA. After a short wash in distilled water to remove salts, the sections were air dried and exposed to Kodak X-OMAT AR or Hyperfilm-^{3}H (Amersham) for 3-7 days in X-ray cassettes. Histology was performed on hematoxylin-eosin stained sequential cryosections. A sample was considered positive for [^{125}I-Tyr^3]-octreotide binding when the signal obtained in a control section was displaced by an excess of unlabeled octreotide by more than 50% (9). The binding signals obtained were analyzed densitometrically using a computer-assisted image processing system and quantified by calculating the ratios between the regions of interest delineated on the total (T) and non-specific (NS) binding sections. Using the T/NS ratios, the amount of binding in every section was graded as negative [0], for T/NS ranging from 0 to 1.9, positive [1], for T/NS ranging from 2 to 3, and strongly positive [2], for T/NS greater than 3.

SS receptor binding on membrane homogenates The method of membrane isolation and the reaction conditions were the same as described by Reubi (10). Briefly, membrane preparations (corresponding to 30-50 μg protein) of tissue samples were incubated in a total volume of 100 μl at room temperature for 60 min with increasing concentrations of [^{125}I-Tyr^3]-octreotide without and with excess (1 μM) of unlabeled octreotide in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/liter bacitracin, 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 an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a γ-counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled octreotide.

Statistical analysis Data are expressed as Mean±SEM. All data were analyzed by ANOVA to determine overall differences between groups. When significant differences were found, a comparison between groups was made using the Newman-Keuls test. The comparison between categorical data among the groups was analysed using the Fisher exact test. The correlation study was performed by using non-linear or linear analysis calculating the Spearman or Pearson coefficients respectively, where appropriate. SSR binding data were analyzed by the method of Scatchard. Receptor binding studies were performed at least twice.

RESULTS

SSR binding on cryostat sections Figure 1 shows an exemplary case for each age group of the specific binding of the sst_2 subtype preferring ligand [^{125}I-Tyr^3]-octreotide on cryostat sections of human thymus. At autoradiography, the binding was not homogeneously distributed but mainly localized in the medullary region of the thymuses (Fig. 1). Using a three-point-score, the amount of binding was graded as strongly positive (2) in 8/10 cases (80%) of group 1, in 1/5 cases (20%) of groups 2 and 3. The binding was graded as positive (1) in 2/10 (20%) of group 1, in 4/5 (80%) of groups 2 and 3, in 1/5 (20%) of group 4. The binding was graded as faint or negative (0) in 4/5 (80%) of group 4 and in 5/5 (100%) of group 5 (Table 1). The percentage of cases with a grade 2 and 1 was significantly higher in the group 1 compared with groups 4 and 5 ($P<0.005$) and in groups 2 and 3 compared to groups 4 and 5 ($P<0.05$). The mean values of total-to-non specific ratio (T/NS) displayed a progressive decrease with the increasing age range in the 5 groups (Fig. 3A). The mean T/NS values were significantly higher in group 1 than in group 4 and

5 ($P<0.05$). The decrease in the T/NS values with the increasing age of the cases in the 5

TABLE 1. Somatostatin receptors in human thymic tissues as determined by somatostatin receptor-autoradiography using [^{125}I-Tyr^3]-octreotide.

Age range groups (months)	Score number of cases (%)		
	0[a]	1[a]	2[a]
1. (0-12)	0/10 (0%)	2/10 (20%)	8/10 (80%)
2. (13-24)	0/5 (0%)	4/5 (80%)	1/5 (20%)
3. (25-72)	0/5 (0%)	4/5 (80%)	1/5 (20%)
4. (73-102)	4/5 (80%)	1/5 (20%)	0/5 (0%)
5. (>102)	5/5 (100%)	0/5 (0%)	0/5 (0%)

a) 0, negative (T/NS ranging from 0 to 1.9); 1, positive (T/NS ranging from 1.9 to 3); 2, strongly positive (T/NS greater than 3).

different groups shows an exponential rather than a linear trend (Fig. 3A). Histology was normal in all the samples and no major structural differences were found between the different groups.

SSR binding on membrane homogenates Using [^{125}I-Tyr^3]-octreotide, specific binding was detectable on membrane preparations of all thymic tissues, except in 4 cases of group 4 and 3 cases of group 5. Scatchard analysis of the binding data revealed a single class of high affinity binding sites with an average apparent K_d of 0.6 ± 0.1 nM. The maximum binding capacity (B_{max}) was low with an average of 18.5 ± 3.6 fmoles/mg membrane protein, in the cases with detectable [^{125}I-Tyr^3]-octreotide binding. An example for each group of saturation curve with Scatchard analysis of the binding data is shown in Fig. 2. The mean values of B_{max} displayed a progressive decrease with the increasing age range in the 5 groups (Fig. 3B). The mean B_{max} values were significantly higher in group 1 than in group 4 and 5 ($P<0.001$). The decrease in the B_{max} values with the increasing age of the cases in the 5 different groups shows an exponential rather than a linear trend (Fig. 3B). As a positive control for ligand binding, SSR-positive mouse AtT-20 pituitary tumor cell membranes were used (K_d of 0.19 ± 0.03 nM; B_{max} 705 ± 64 fmoles/mg membrane protein). No specific binding was detectable on a proven SSR negative cell line and tissue (8).

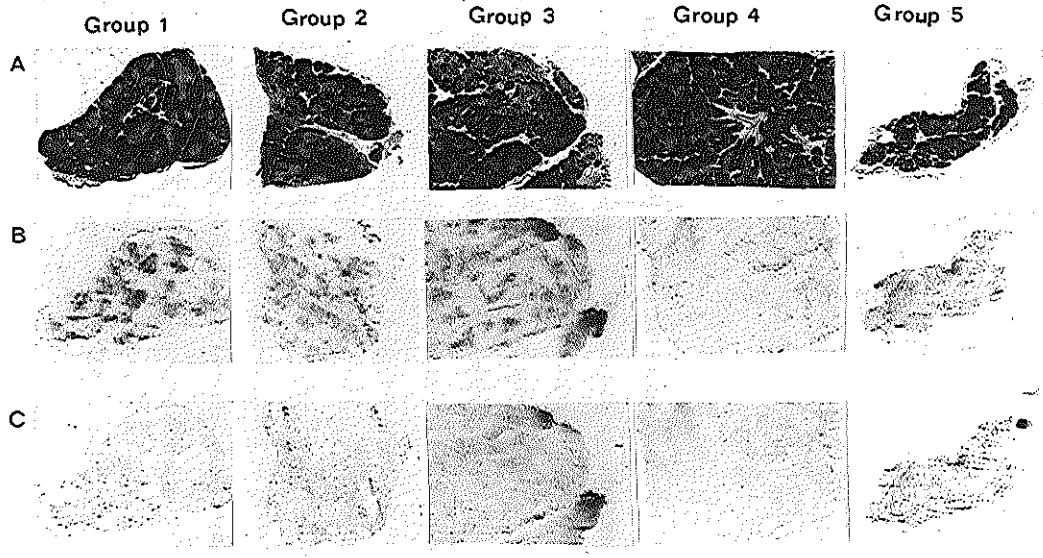

FIGURE 1. SSR expression in human thymuses of different age. Exemplary cases. Photomicrograph of SSR-autoradiography: Group 1 (age range 1-12 months); Group 2 (age range 13-24 months); Group 3 (age range 25-72 months); Group 4 (age range 73-120 months); Group 5 (age range >120 months). *A)* hematoxylin-eosin stained section; *B)* autoradiogram showing total binding of [^{125}I-Tyr^{3}]-octreotide; *C)* autoradiogram showing non-specific binding (in the presence of 1 uM of Tyr^{3}-octreotide). *Bar*, 2 mm.

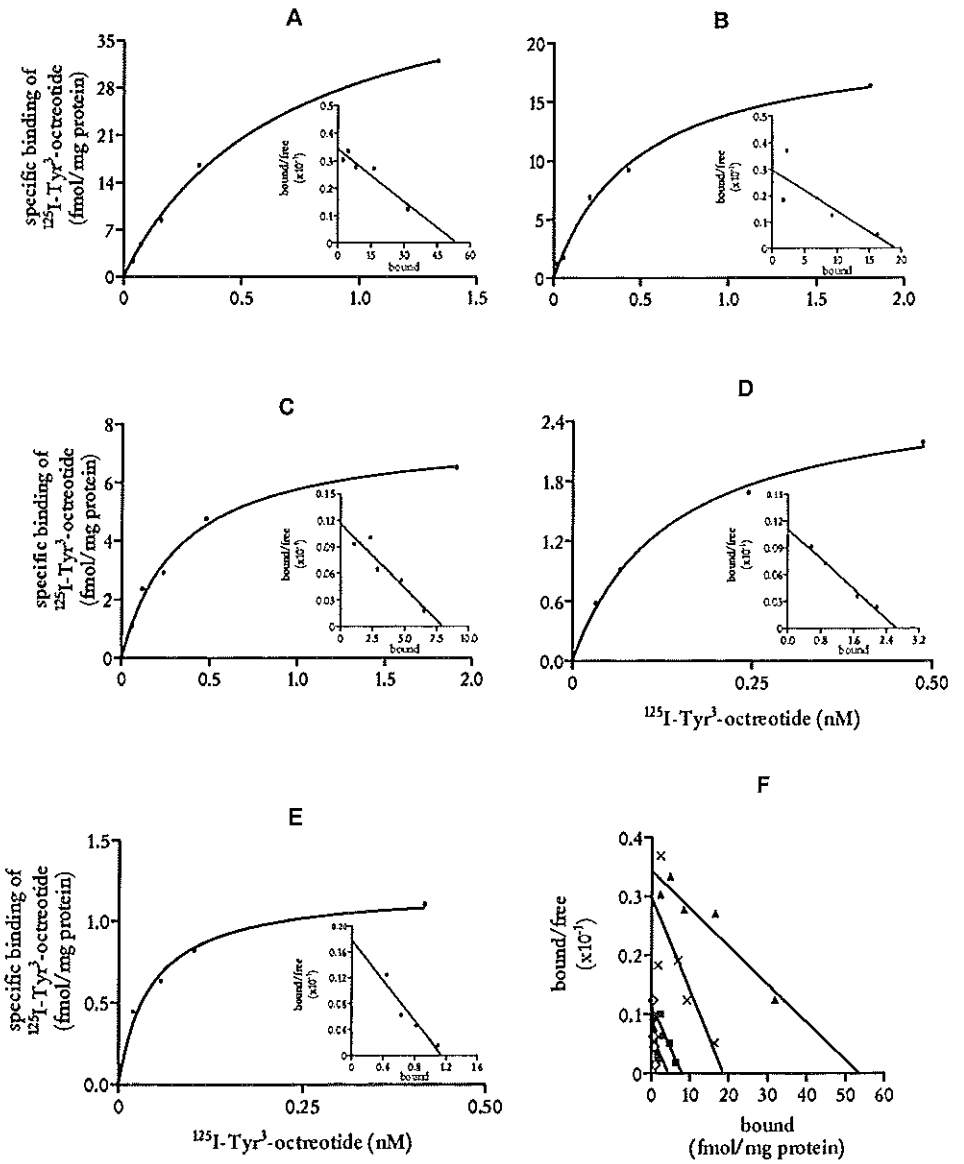

FIGURE 2. SSR expression in human thymuses of different age. Exemplary cases. Binding of [^{125}I-Tyr^3]-octreotide to a membrane homogenate preparation of human thymuses: *A)* Group 1 (age, 3 months; B_{max}, 54; K_d, 0.8); *B)* Group 2 (age, 14 months; B_{max}, 18; K_d, 0.3); *C)* Group 3 (age, 35 months; B_{max}, 8; K_d, 0.3); *D)* Group 4 (age, 97 months; B_{max}, 2.7; K_d, 0.1); *E)* Group 5 (age, 144 months; B_{max}, 1.1; K_d, 0.1). Saturation curves indicate the specific binding (total minus non-specific binding in presence of 1 µM of Tyr^3-octreotide); *Insets*: Scatchard analysis of the binding data; *F)* Summary of the Scatchard analysis data. ▲, Group 1; ×, Group 2; ■, Group 3; ●, Group 4; ◊, Group 5.

Correlations A significant correlation was found between ligand binding studies on cryostat sections or on membrane homogenates and age of the thymus. In detail, the T/NS ratios at autoradiography ($r=-0.84$; $P<0.001$) and the B_{max} values at membrane homogenate binding study ($r=-0.82$; $P<0.001$) were inversely correlated with the age of the thymus (Fig. 4 A and B). In addition, the T/NS ratios at autoradiography were positively correlated with the B_{max} values at membrane homogenate binding study ($r=0.74$; $P<0.001$) (Fig. 4 C). Conversely no correlation was found between the estimated K_d values and the age of the thymuses and between gender and both T/NS and B_{max} values (data not shown).

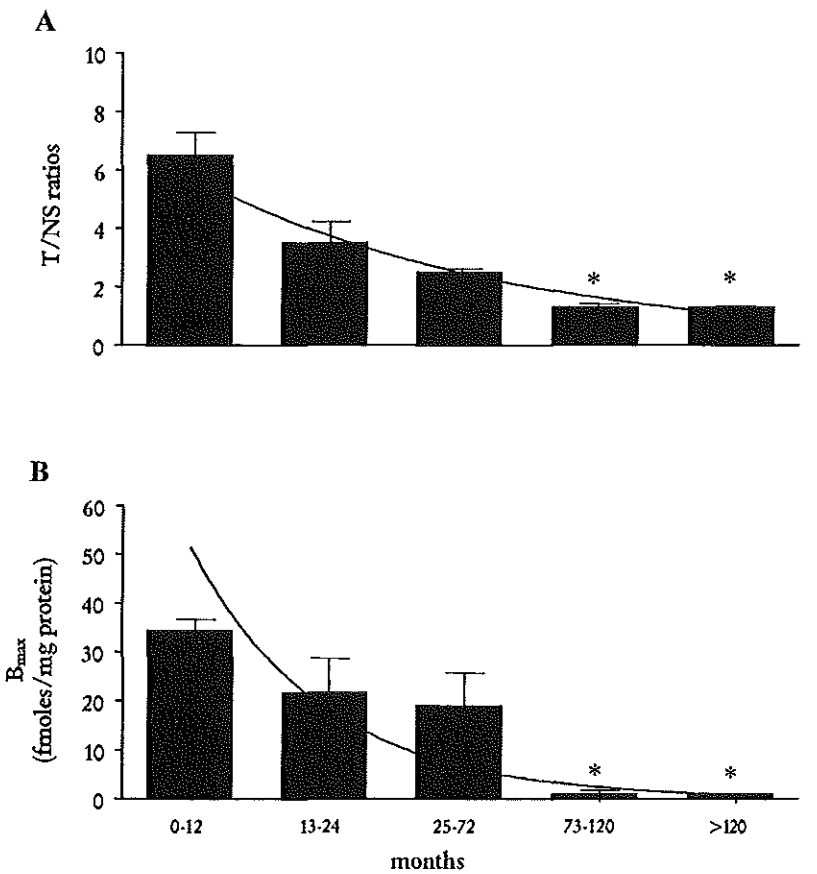

FIGURE 3. SSR expression in human thymuses of different age. *A*) Total-to-non-specific (T/NS) binding ratios values calculated at autoradiographic binding study on cryostat sections; *B*) B_{max} values detected at binding studies on membrane homogenates of thymic tissues in the 5 groups. Bars represent the value of T/NS ratios and B_{max} (fmoles/mg protein) and are expressed as Mean±SEM; *, $P<0.05$ and $P<0.001$ *vs.* Group 1, respectively. Lines represent the exponential trend of the changes in T/NS ratios and B_{max} with the increasing age in the 5 different groups of thymuses.

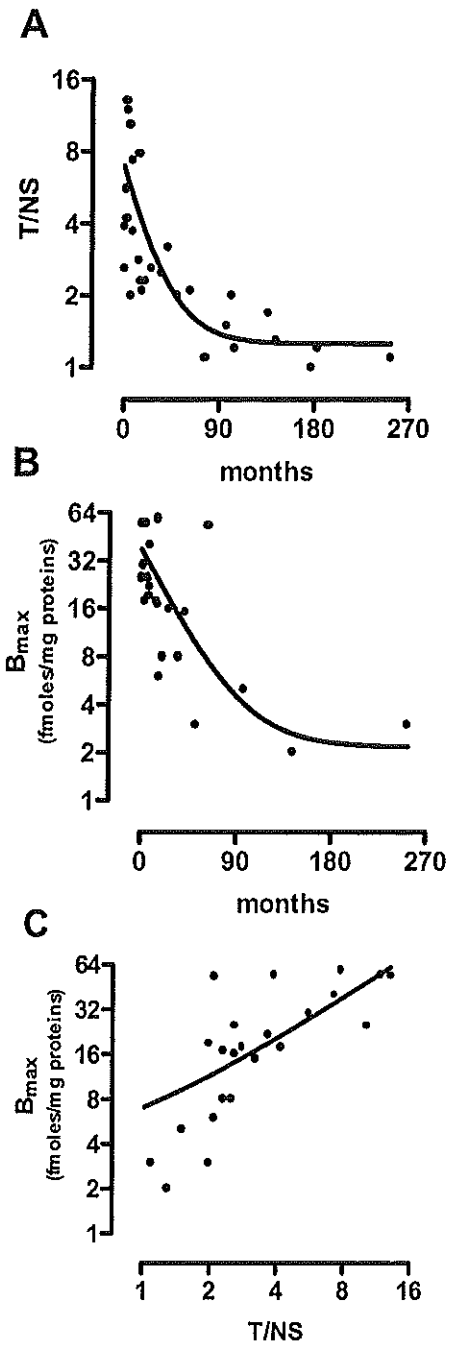

FIGURE 4. SSR expression in human thymuses of different age. *A)* Correlation between age and total-to-non-specific ratios values (T/NS). *B)* Correlation between age and B_{max} values; C) Correlation between T/NS and B_{max} values. T/NS ratios and B_{max} (fmoles/mg protein) values detected at binding studies on cryostat sections and membrane homogenates of thymic tissues of the 30 cases distributed *per* age (months).

DISCUSSION

The involution of the human thymus with age is a complex phenomenon and remains poorly understood. The human thymus interacts with products of endocrine glands throughout life and although conflicting results have been reported, thymic involution seems in part dependent to age-related alterations in the interaction between the neuroendocrine activity and the thymus itself (5,6). Moreover, the intrathymic production of hormones and neuropeptides and the presence of specific receptors represent an autocrine/paracrine pathway, which, in addition to classical endocrine circuits, might modulate the activities of both the lymphoid and stromal components of this organ (11). In fact, TEC produce thymic peptides and other factors known to modulate the main function of the gland, namely the development of the T cell repertoire, and many hormones and neuropeptides may participate to this function by interacting *via* specific receptors with both developing immune cells and with TEC (12). However, the role of neuropeptides and their receptors in thymic involution is still debated.

SS is a well-characterized neuropeptide with a wide spectrum of action and recent insights have strongly suggested that the thymus might belong to the list of its target organs (7,13-15). The five different SSR subtypes recently cloned and characterized (16,17), show a tissue specific distribution. The majority of SS-target tissues express multiple SSR, making it difficult to understand the functional role(s) of the individual SSR subtypes. The most well known SS analog, the octapeptide octreotide, binds with high affinity to the sst_2 subtype (18). In the endocrine system, where they have been better characterized, SSR subtypes are involved in the control of hormone secretion and cell proliferation, exerting mainly inhibitory effects *via* distinct mechanisms (19,20). The sst_2 subtype plays a major role in this system. While in the endocrine system SSR activation leads to inhibitory effects, in the immune system both stimulatory and inhibitory effects have been reported (21). Moreover, very little is know with the respect to the cellular signalling mechanisms coupled to SSR activation in immune cells (22).

In normal human thymus, we recently demonstrated the presence of three different SSR subtypes, sst_1, sst_{2A} and sst_3 (7). In TEC, sst_1 and sst_{2A} were selectively expressed, and TEC seemed to produce SS, since SS mRNA was present in these cells (7). All together, these findings pointed towards an important role of the sst_{2A} receptor in the human thymus,

which seems to be confirmed by the heterogeneity of the expression of this subtype among thymic cells (7).

In the present study, we have found additional evidence for a functional role of the sst_{2A} receptor in the thymus. This SSR subtype may be involved in the processes linked to the thymic age-related changes, since its expression undergoes significant changes with increasing age. In fact, using two different techniques, we observed an inverse relationship between the number of binding sites for the sst_2-preferring ligand, [^{125}I-Tyr^3]-octreotide, and the age of the thymus, the number of [^{125}I-Tyr^3]-octreotide-binding sites were significantly higher in the younger subjects. This finding is in line with the recently reported evidence of an *in vivo* thymic uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in the 3 youngest patients (ages, 4, 5 and 16 months) out of 11 underwent SSR scintigraphy to evaluate abdominal or pelvic neuroblastoma (23). Conversely, no thymic concentration of [^{111}In-DTPA-D-Phe^1]-octreotide was documented in the relatively older children of the same series and in a series of adult patients with thymic hyperplasia (24). In the present study, the ages of the thymus were inversely correlated with both the results of the autoradiographic studies on thymic cryostat sections and the B_{max} values measured at the ligand binding studies on tissue homogenates of the corresponding cases. Moreover, the score of the autoradiography was positively correlated with the values of the B_{max}. While autoradiography is a semiquantitative method for the evaluation of the ligand binding, the B_{max} value directly indicates the density of binding sites for a specific ligand expressed on cell membranes. Moreover, it should be pointed out that, at the ligand binding on membrane homogenates, the estimated K_d values were not correlated with the thymus age. This suggests that the change in the number of SSR binding sites is not related to changes in receptor affinity. Considering that sst_{2A} is mainly expressed on TEC in the medullary compartment of the human thymus (7,13,15), it might appear that the reduction of the number of binding sites is related to the decrease in the number of sst_{2A}-expressing cells. However, this seems unlikely, since the number of TEC, in the age range considered in our study, remains almost unchanged (2,3). In fact, little or no decrease in the number of keratin-positive cells has been demonstrated, while especially thymocytes, and to a much lesser extent, dendritic cells dramatically decrease in number during the ageing process (3). During postnatal development, the thymic epithelium displays a pathway of differentiation similar to that observed in other epithelial organs throughout the human body, rather than a real change in the number of its cellular elements (4). This observation supports the

concept that the thymic epithelium is capable of undergoing sequential stages of maturation during fetal and postnatal thymic development. Moreover, even considering that at a certain point the number of TEC declines, contributing to the significant reduction of the octreotide-binding sites, we have observed a progressive decrease of the number of octreotide-binding sites starting after the first years of life, when the number of TEC is still unchanged. Since two different techniques gave a comparable result, it is suggested that the decrease in the number of sst_{2A} occurs on TEC. Although in cultured thymocytes no mRNA encoding for SSR subtypes was detectable (7), we have recently demonstrated SSR binding on freshly isolated thymocytes (25). Preliminary RT-PCR data have shown the expression of sst_{2A} and sst_3 mRNAs in resting thymocytes. However, sst_3 seems to be the SSR subtype predominantly expressed in the heterogeneous pool of T lymphoid cell precursors, whereas sst_{2A} mRNA expression is limited to a very small subset of immature cortical thymocytes (unpublished observations). The recent evidence of a selective expression of sst_3 mRNA in peripheral human T lymphocytes (26) is in line with our observation in thymocytes, which are the natural precursors of circulating T cells. Although the immature thymocytes are localized in the cortical region of the human thymus, a contribution of their loss to the decline of octreotide-binding sites can not be fully ruled out. However, it should play a minor role in this phenomenon because, according to the autoradiographic pattern, the decline of octreotide-binding sites occurs mainly in the medullary region of the thymus, where TEC are the predominant cell type displaying sst_{2A}-binding.

sst_{2A} is the SSR subtype involved in controlling secretive processes in SS target cells. Thus, the reduction of sst_{2A} receptor on TEC might be in line with the evidence that the major changes occurring in these cells during ageing are related more to their functional capabilities in producing thymic hormones, rather than to modification in their number (3). The decrease in sst_{2A} receptor numbers might be related to the production of substances modulating the thymus involution, as well as the maturation of T cells. Which is the factor(s) involved in regulating receptor expression needs to be further investigated. However, in the light of studies in which stimulation of neuropeptide receptors by their own ligand was shown to result in receptor internalization (27,28), it is possible that a downregulation of sst_{2A} receptors might occur, as consequence of ligand-induced internalization. In fact, the sst_{2A} receptor has been demonstrated to efficiently internalize bound ligand in many cell systems (29-31). Furthermore, it has been demonstrated in rat

brain that endogenous SS regulates cell surface sst_{2A} receptors (32). The presence of endogenous SS within the human thymus (7,33-37) might account for a regulation of sst_{2A} receptor expression on TEC by this mechanism. Conformational changes and/or chemical alterations of the internalized receptor might explain why the exogenous ligand does not recognize its specific receptor (30). However, the influence of additional factors, like cytokines or other neuropeptides, can not be ruled out either in such a complex organ. It is known that hormones and neuropeptides can modulate TEC physiology exerting a pleiotropic action on thymic stroma. In fact, glucocorticoid, thyroid, and pituitary hormones can modulate extracellular matrix ligands and receptors (11). Moreover, the expression of receptors for neuropeptides, such as vasoactive intestinal polypeptide, appears to be developmentally regulated in several systems, including the thymus (38,39).
In conclusion, the expression of sst_{2A} is inversely correlated with the age of the human thymus. The receptor itself and, obviously SS, might play a role in the involution of the thymus, consequently affecting the main function of this organ. Although further studies are required to clarify this complex, but fascinating network between the neuroendocrine and the immune systems within the human thymus, our findings raise the possibility that neuropeptides may participate in the intrathymic maturation and differentiation of T cell repertoire, which leads to the development of cellular immunity in humans.

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CHAPTER III

SOMATOSTATIN RECEPTORS IN THYMIC TUMORS

SOMATOSTATIN RECEPTOR SCINTIGRAPHY

In the last two decades increasing efforts have been undertaken to introduce peptide receptor scintigraphy for *in vivo* application in patients bearing tumors. The visualization of the tumor occurs after the intravenous injection of a radioactive peptide (ligand), which binds the receptor expressed on the membrane of cells. In general, the higher density of receptors on tumor cells compared with the normal tissues, or the high number of cells expressing the targeted receptor allows to discriminate between positive lesions and surrounding tissue. A gamma camera is used to visualize the accumulated radioisotope. Planar images can already depict a clear localization of the tumor, whereas tridimensional reconstruction of planar acquisitions by single-photon emission computed tomography (SPECT) may provide real maps of radioactivity within the target organ or tumor tissue.

SSR have been demonstrated in a large number of human tumors, mainly neuroendocrine tumors, which may express the receptors over 100-fold compared to the tissue of origin (1-5). Since native SS in unstable and has a short half-life, synthetic analogs have been developed for clinical applications (6). Human SSR positive tumors show a high uptake of [^{111}In-DTPA-D-Phe^1]-octreotide at SSR scintigraphy (1). SSR scintigraphy using [^{111}In-DTPA-D-Phe^1]-octreotide has become a standard procedure in the work-up of patients with neuroendocrine tumors to obtain an accurate estimate of the spread of tumors. Besides that, SSR scintigraphy has been proposed to select candidates responsive to specific peptide treatment (3,7). Physiological accumulation of the radiopharmaceutical occurs in the spleen, kidneys, urinary bladder, liver, thyroid, salivary glands and pituitary gland (5). Of particular interest is the accumulation in normal organs that are known to express SSR, like spleen, pituitary and to a lesser extent thyroid. Later in this chapter, this issue will be further discussed concerning the role of specific SSR subtypes in determining the uptake of the radioligand.

[^{111}In-DTPA-D-Phe^1]-octreotide is considered as a sst_2-preferring ligand, which suggests a crucial role of sst_2 receptor in determining the accumulation of radioactivity in tumor cells following internalization of the radioligand-receptor complex. Radiolabeled octapeptide SS-analogs are internalized in a high amount by SSR-positive mouse and human tumor cells

(8-10). Evidence for a predominant importance of the sst_2 receptor in determining the uptake of the radiopharmaceutical [^{111}In-DTPA-D-Phe^1]-octreotide by SSR positive tumors is presented from studies showing that sst_2-expressing cells internalize SS (11), as well as octreotide (1). However, on the basis of the high SS-internalization rates of the sst_3 and sst_5 subtypes, it cannot be excluded that sst_3 and sst_5 receptors might play a role as well (11).
Apart from [^{111}In-DTPA-D-Phe^1]-octreotide other radiolabeled SS analogs, suitable for diagnostic or therapeutic applications as well, have been synthesized (5,12,13). Moreover, radiotherapy, as well as targeted chemotherapy, using SS analogs is considered as a future effective and feasible approach to treat patients with advanced, metastatic SSR-positive neuroendocrine tumors (3,14,15). SSR scintigraphy has been successfully used to visualize sites of disease in patients with abnormalities of the immune system (16-20). Neoplastic and inflammatory lesions have been clearly visualized in lymphomas, granulomatous diseases and autoimmune diseases. The *in vivo* evidence of SS binding sites using SSR scintigraphy opened a new field of interest for the role of SSR in the immune system. Moreover, the presence of SSR has been demonstrated *in vitro* in tissues belonging to the heterogeneous group of tissues and cells, which are formed the organs of the immune system (21-23). An additional observation is the visualization of thymic epithelial tumors using [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy (24,25). This seems to have relevance for selecting patients, which may benefit from treatment with SS analogs (26). However, as previously discussed for the normal thymus (chapter II), these complex pathologic entities have not been accurately investigated so far. In the coming paragraphs, after an overview on the most recent clinicopathological findings on thymoma (chapter III.2), the results of investigations on the distribution and relevance of SS and its receptors in this rather unexplored category of tumors will be presented. These studies start from the *in vivo* evaluation of SS binding sites in a large series of human thymoma by [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy, followed by in vitro studies evaluating the expression and functional significance of SSRs in these tumors.

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THYMIC EPITHELIAL TUMORS

In the past, various types of neoplasms have been included in the spectrum of thymoma. These include the thymic localization of Hodgkin's disease and other categories of lymphomas, seminoma, thymolipoma, carcinoid and carcinoma (1-3). However, thymomas are a highly heterogeneous category of epithelial thymic tumors. A strict definition of thymoma describes this entity as a tumor composed of cytologically bland, neoplastic thymic epithelial cells accompanied by variable admixture of benign lymphocytes.

CLASSIFICATION

In the beginning of the sixties, thymomas were histologically divided in 4 categories, based on the predominance and shape of the the specific thymic cell type, as well as on the basis of the lymphocyte/epithelial cell ratio. These four categories comprise spindle cell, lymphocytic thymoma, predominantly lymphocytic and predominantly mixed lymphocytic and epithelial thymoma. However, these criteria generated considerable confusion. Therefore, thymic epithelial tumors have been recently classified by the World Health Organization (WHO) (4). Since several controversies occurred in the past concerning the classification and nomenclature of thymic epithelial tumors, the WHO based its own classification on a combination of letters and numbers (Table 1). However, it is useful to mention two other world-wide classifications that are still in use, i.e. the Levine & Rosai classification, and the more recent Marino, Müller-Hermelink, Kirchner & coworkers classification (5-8). The three classifications of thymic epithelial tumor are summarized in Table 1. According to Levine and Rosai classification, thymic tumors were grouped considering their invasiveness (stage) and the occurrence of cytological atypia. This first step was helpful for a better clinical prognostic relevance of the system (5). In 1985, Marino, Müller-Hermelink, Kirchner & coworkers proposed an innovative and interesting classification based on the morphological resemblance of neoplastic epithelial cells to subsets of normal TEC (6-8). The histogenetic classification of Marino, Müller-Hermelink, Kirchner has been shown to be an independent prognostic factor. Medullary and mixed

thymomas were considered benign tumors with no risk of recurrence, even in the presence of capsular invasion. While organoid and cortical thymoma showed intermediate invasiveness and low but significant risk of late relapse, well-differentiated thymic carcinomas were always invasive and displayed the highest risk of relapse and death (9). The WHO classified these tumors following different criteria. The two major categories included tumors depending on whether the cells show spindle or oval shape (type A), and dendritic or epithelioid features (type B). Tumor displaying a combination of these appearances were classified separately (type AB). The type B category is further divided in subgroups according to the increasing epithelial/lymphocyte ratio and occurrence of atypia. Separately were considered the nonorganotypic carcinomas (type C), which included a large group of tumors arising outside the thymus (4).

TABLE 1. The WHO classification of thymic epithelial tumors compared with the clinicopathologcal and to the histologic classifications.

WHO	Clinicopathological classification	Histologic classification
A	Benign thymoma	Medullary thymoma
AB		Mixed thymoma
B1	Malignant thymoma	Predominantly cortical thymoma
B2	Category I	Cortical thymoma
B3		Well differentiated carcinoma
C	Malignant Thymoma	Epidermoid (squamous cell) carcinoma
	Category II	Epidermoid nonkeratinizing carcinoma
		Lymphoepithelioma-like carcinoma
		Sarcomatoid carcinoma
		Clear cell carcinoma
		Basaloid carcinoma
		Mucoepidermoid carcinoma
		Undifferentiated carcinoma

CLINICAL CHARACTERISITCS AND MANAGEMENT

Thymoma is the most common tumor occurring in the anterior-superior mediastinum. In general it is a benign tumor, but occasionally it shows a serious clinical aggressiveness. Although metastases are rare outside the thoracic cavity, these tumors may behave in a local aggressive fashion, infiltrating the lung, pleural and pericardial space. Many studies revealed that the tumor stage is the most relevant prognostic factor (8,10-12), although it

has been shown that histology is another important marker in predicting relapsing behaviour of these tumors (10-12).

Only one third of patients bearing thymoma present symptoms of local compression, such as dyspnoea, dysphagia, cough and *vena cava* superior syndrome. In another third the diagnosis is usually made *via* the accidental detection of a mass at chest X-ray. Thymomas are frequently associated with paraneoplastic syndromes. Among these, *myasthenia gravis* (MG) is the most common one (13). MG occurs as typical complication of WHO type A, AB and B1-3 thymoma, but not of C. MG-associated thymomas are tumors able to produce maturation of immature precursors of T cells (14). Moreover, they display disturbances of lympho-epithelial interactions, but generally do not express autoantigens, which is a common feature in MG not associated with thymoma (13). MG is associated with other thymic pathologies as well, including thymitis and thymic hyperplasia. (15).

Other thymoma-associated syndromes include agammaglobulinemia, pure red cell aplasia, Cushing's syndrome, thyroid autoimmunity and more rarely other autoimmune diseases. The surgical resection of the tumor is unpredictable for the development of the paraneoplastic syndromes.

Surgery is considered the treatment of choice, while it is still discussed whether there is a role for adjuvant radiotherapy in a resectable stage. Radiotherapy may be the primary treatment in unresectable stages. However, there are some arguments in favour of a surgical debulking in unresectable stages as well (16). Moreover, the results of current oncological approaches to inoperable thymomas are rather disappointing. An overall response rate of about 50% with a median survival time of 3-5 years is achieved with aggressive and expensive chemotherapy (16,17).

New insights

Two very new important aspects about these tumors recently emerged. Firstly, an original study demonstrated the presence of neuroendocrine differentiation in non-neuroendocrine thymic epithelial tumors (18). While "classic" neuroendocrine markers or cells are common features in thymic carcinoids, the presence of focal or dispersed neuroendocrine cells in thymic carcinoma and atypical thymoma may reflect multidirectional differentiation within the tumor. The neuroendocrine differentiation may represent an additional marker of these thymic tumors, next to cell atypia, expression of specific epithelial cell clusters and lack of

immature T cell infiltration, (18). Moreover, detection of endocrine products in thymoma is intriguing, particularly in the light of the second new finding regarding this class of neoplasms. In fact, it has recently been shown that thymoma can be visualized by a radionuclide-coupled SS analog, currently in use as diagnostic procedure for several neuroendocrine tumors, as well as experimentally for other pathological entities involving the immune system (19-25). As already discussed in the previous paragraph, [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy allows the visualization *in vivo* of tumors and tissues expressing SSR. Tumor visualization was positive in most thymomas, but not in thymic hyperplasia. This can be considered as an innovative approach for this procedure to distinguish tumoral from non-tumoral thymic mass (19), which was rather difficult with other traditional imaging techniques (26).

In the next paragraphs, studies will be presented on the characterization and significance of SSR in thymic tumors.

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SOMATOSTATIN RECEPTOR SUBTYPES IN HUMAN THYMOMA AND INHIBITION OF CELL PROLIFERATION BY OCTREOTIDE *IN VITRO*

ABSTRACT

Somatostatin (SS) and SS receptor (SSR) subtypes, codenamed sst_{1-5}, are heterogeneously expressed in the normal human thymus. This suggests their involvement in controlling the immune and/or neuroendocrine functions in this organ. Moreover, recently a high *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide has been reported in patients bearing thymoma. The present study characterizes *in vivo* and *in vitro*, functional SS binding sites in a human thymoma. A high uptake of [^{111}In-DTPA-D-Phe^1]-octreotide was observed in the chest of a patient with *myasthenia gravis* due to a cortical thymoma. Specific binding of [^{125}I-Tyr^{11}]-SS-14 was found on a membrane preparation of the surgically removed thymoma. Scatchard analysis showed high affinity binding sites [K_d, 47.5±2.5 pM] with low maximum binding capacity [B_{max}, 23.5±2.5 fmoles/mg membrane protein]. RT-PCR analysis showed the presence of sst_1, sst_{2A} and a predominant sst_3 mRNA expression in the tumor tissue. Primary cultured tumor cells expressed sst_3 mRNA only. In contrast to the normal thymus, SS mRNA was not expressed. By immunohistochemistry, the tumor cells highly expressed sst_3 receptors, weakly expressed sst_1 receptors, and showed no immunostaining for sst_{2A} receptors. sst_{2A} immunoreactivity was found in the stromal compartment of the tumor, particularly on the endothelium of small intratumoral blood vessels. In primary cultured tumor cells, both SS and octreotide (10 nM) significantly inhibited ^{3}H-thymidine incorporation by 40.6 and 43.2%, respectively. The following conclusions were reached. 1) As this tumor displayed a high immunoreactivity for sst_3 and the cultured tumor cells expressed the sst_3 mRNA only, this SSR may be the subtype involved in the inhibition of epithelial tumor cell proliferation by octreotide *in vitro*. 2) A loss of endogenous SS production in this thymoma might be implicated in the uncontrolled cell growth. 3) In this case, the sst_3 may play a role in determining the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide at *in vivo* SS receptor scintigraphy.

INTRODUCTION

The thymus is the primary lymphoid organ where cytokines and thymic hormones participate in regulating the proliferation and differentiation of T cells (1). These factors are produced by the thymic stroma. The preponderant cell type in the stroma is of epithelial origin, consisting of at least two distinct subsets, cortical and medullary thymic epithelial cells (TEC) (2). Recently, attention has been drawn to the presence of neuroendocrine and peripheral hormones, which seem to participate in an autocrine and paracrine manner to the functioning of TEC (3). Some of these hormones have well-characterized actions on immune cell differentiation and proliferation, whereas others, such as somatostatin (SS), have been identified, but not investigated for their function within the thymus (4).

The biological effects of SS, including the inhibition of hormone and exocrine secretion, as well as the modulation of neurotransmission and cell proliferation, are mediated through five different G protein-coupled, high affinity, membrane receptor subtypes (sst_{1-5}) (5,6). SS receptors (SSR) have been demonstrated in normal tissues and in many tumors at the protein and mRNA levels by receptor binding studies on tissue homogenates, by *in vivo* and *in vitro* SSR autoradiography, by *in situ* hybridization as well as by RT-PCR (7,8). Furthermore, SSR subtypes have been localized at the cellular level by immunohistochemistry using a specific anti-peptide antibody in a series of human tumors and in normal human pancreas as well (9-13). Localization studies of SSR on specific cell types within SSR-positive tissues demonstrated heterogeneity of receptor distribution (14).

Octreotide, an octapeptide SS analog, binds with high affinity to sst_2 and with a relatively lower affinity to sst_3 and sst_5 (15). Imaging techniques with [^{111}In-DTPA-D-Phe^1]-octreotide have visualized SSR in various human neoplasms *in vivo*. In many cases, a positive scan predicts a good response to treatment with octreotide (16,17).

We have recently characterized the SS and SSR subtype distribution pattern within the normal human thymus (18). SS, and sst_1, sst_{2A} and sst_3 mRNAs were found in thymic tissue, whereas enriched cultured TEC selectively express sst_1 and sst_{2A}, as well as SS mRNA (18). Moreover, successful treatments with octreotide have been reported in two patients with thymoma (19,20). To unravel the functional significance of SSR in human thymomas, we investigated in the present study the expression and functional role of SS and SSR subtypes in a cortical thymoma. *In vivo* SSR scintigraphy was performed in a patient admitted for *myasthenia gravis*. SSR expression on the tumor tissue was investigated using *in vitro* SSR

binding studies, RT-PCR and immunohistochemistry. Moreover, the effect of SS and octreotide on cell proliferation were evaluated in a primary cell culture of the thymoma.

METHODS

In vivo ***study.*** Scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide was performed as previously reported (21). Briefly, planar and single photon emission tomography (SPECT) images of the chest were obtained 24 h after the injection of 228 Mbq [^{111}In-DTPA-D-Phe^1]-octreotide (Mallinkrodt, Petten, The Netherlands). Imaging studies were performed using a two-headed gamma camera (Picker 2000, Picker Instruments., Cleveland, Ohio, USA) for planar imaging and a three-headed gamma camera (Picker 3000) for SPECT studies, both equipped with a medium-energy-collimator. Acquisition time for planar spot images was 15 min.

Samples. The thymoma was surgically removed from a female patient (age 55 yr). Samples from this tumor and from three normal thymuses (from children undergoing cardiac surgery) were used in the present study. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained from the patients or their parents. Samples were taken directly at the operation, quickly frozen on dry ice and stored at -80°C for autoradiographic and RT-PCR studies, or fixed in 10% paraformadehyde overnight for immunohistochemistry. An additional specimen of the tumor was used for the establishment of a primary culture.

SS receptor autoradiography and RT-PCR. Receptor autoradiography was carried out on 10-micrometer thick cryostat sections using [^{125}I-Tyr^{11}]-SS-14 and [^{125}I-Tyr^3]-octreotide as radioligands (SA ~2000 Ci/mmol) as previously described (18). RT-PCR was performed as previously reported (18). The size of PCR products were 318, 332, 651, 221, 323, 223, 349, and 762 bp, for sst_1, sst_{2A}, sst_{2B}, sst_3, sst_4 and sst_5, SS, and β-actin, respectively.

SS receptor binding studies. The method of membrane isolation and the reaction conditions were described previously (18). [^{125}I-Tyr^{11}]-SS-14 and [^{125}I-Tyr^3]-octreotide were used as radioligands.

Immunohistochemical localization of sst_1, sst_{2A} ***and*** sst_3 ***receptors.*** Paraffin-embedded sections (5 μm) were deparafinized, rehydrated, exposed to microwave heating (in citric acid buffer) at 100°C for 15 min, rinsed in tap water followed by phosphate-buffered saline and subsequently incubated for 15 min in normal goat serum (1:10 dilution in phosphate-buffered saline + 5% BSA). The sections were then incubated overnight at 4°C with the sst_1 (R1-201) and sst_{2A} (R2-88)

antibodies (gift from Dr. A. Schönbrunn) (22,23) in a dilution of 1:500 (12) and with the sst_3 antibodies (Biotrend, Cologne, Germany) in a dilution of 1:3000. Finally, a standard streptavidin-biotinylated alkaline phosphatase or peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used according to the manufacturer's recommendation to visualize the bound antibodies. Negative controls for immunohistochemistry included 1) omission of the primary antibody and 2) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM).

Cell dispersion and cell culture. Normal and neoplastic TEC were isolated as previously described (18). Briefly, the thymoma tissue was enzymatically dissociated with collagenase (Sigma Chemical Co., St. Louis, MO; 2 mg/mL) for 1 h at 37°C. The dispersed cells were precultured for 5-7 days in 15 mg/cm² collagen type I (Collagen S, type I, Roche Molecular, Mannheim, Germany)-precoated 75-cm² flasks (Costar, Cambridge, MA) at a density of $5x10^6$ cells/flask in 10 mL culture medium. The culture medium, the same as that used during the experiments, was MEM D-valine (Gibco BRL, Life Technologies LTD, Paisley, Scotland) supplemented with 10% FCS, penicillin (10^5 U/L), fungizone (0.5 mg/ml), L-glutamine (2 mmol/L). 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 detected by trypan blue exclusion was 90%, and the cells that had not attached to the flasks, mainly thymocytes, were harvested.

The isolation of epithelial cells was indirectly performed using sheep antimouse IgG-coated magnetic beads and an 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. The magnetic beads were coated with the specific primary antibody and mixed with the target cell suspension to form bead-rosetted cells (fibroblasts) which were collected at the tube wall and isolated using a magnetic separator rack. For a complete negative selection, the supernatant, containing the epithelial cells that were not bound to the beads, was treated once again with the coated beads. This remaining suspension containing isolated epithelial cells was used for the experiments. The cells (20,000/well) were seeded in 1 mL culture medium in 24-well collagen type I-precoated plates (Costar), and allowed to attach for 24 h. Thereafter, test substances were added, and the cells were incubated for 72 h. Cell proliferation was measured by adding 1 µCi of [*methyl*-^{3}H]-thymidine (91 Ci/mmol; Amersham, Pharmacia Biotech, Houten, The Netherlands)/well during the last 24 h, as described in detail previously (18). For RT-PCR studies, neoplastic TEC were seeded in collagen-coated flasks (see above) and grown to confluence. Thereafter, the cells were harvested and mRNA was isolated as described previously (18). For keratin staining, cells 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, Glostrup, Denmark). Cultured thymocytes were collected and processed for mRNA analysis by RT-PCR as previously described (18).

Test substances. SS-14 (Bachem Inc., Hannover, Germany) and octreotide (Novartis, Basel, Switzerland) were used at a concentration of 10 nM.

Statistical analysis. Data are expressed as mean ± SEM. Binding experiments were performed at least twice, and the data shown for the displacement study are derived from triplicate values. In functional studies, there were four wells per treatment group. The 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. SSR binding data were analyzed by the method of Scatchard.

RESULTS

Scintigraphy with ^{111}In-DTPA-D-Phe^1-octreotide. A high uptake of [^{111}In-DTPA-D-Phe^1]-octreotide was detected after 24 h in the mediastinum (Fig. 1A-B). Computed tomographic scanning of the chest confirmed the diagnosis of thymic enlargement (Fig. 1C).

SS and SSR (subtype) expression. At autoradiography, no specific binding of [^{125}I-Tyr^{11}]-SS-14 or [^{125}I-Tyr^3]-octreotide was found in the thymoma (data not shown). However, Scatchard analysis of [^{125}I-Tyr^{11}]-SS-14 binding on an enriched- membrane preparation of the thymoma tissue revealed high affinity binding sites with an apparent K_d of 47.5±2.5 pM and a low maximum binding capacity (B_{max}) of 23.5±2.5 fmoles/mg membrane protein. Figure 2A shows a representative experiment. No specific binding was detectable on the tumor-derived cultured thymocytes. In addition, octreotide (100 nM) displaced [^{125}I-Tyr^{11}]-SS-14 binding on membrane preparations of the thymoma tissue significantly less compared with SS-14 (49.5% *vs.* 90.8%, respectively; Fig. 2B). On normal human thymus membranes high affinity binding sites for [^{125}I-Tyr^3]-octreotide were detected [K_d 200±70 pM; B_{max} 4.7±0.3 fmoles/mg membrane protein (Mean±SEM)].

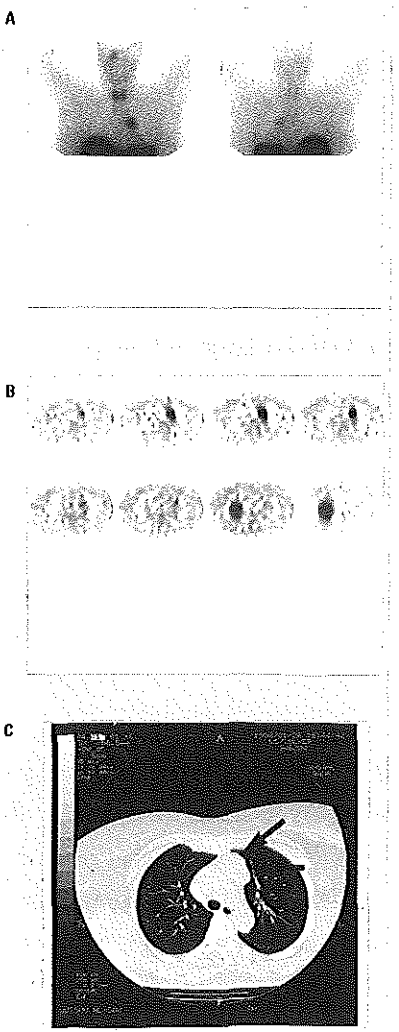

FIGURE 1. A, Planar images of the chest 24 h after injection of [^{111}In-DTPA-D-Phe^1]-octreotide. Anterior (left) and posterior (right) views. There is normal uptake in the thyroid, and some accumulation in the nose region due to common cold. An abnormal uptake in the region of the left hilum is seen. B, Transversal SPECT images of the chest 24 h post-injection. Images are from cranial to caudal. The left side of the images represents the right side of the patient. In the upper row, the accumulation at the site of the thymoma is seen, located left-sided in the anterior mediastinum. The last two images in the lower row show uptake in the liver. C, Computed tomographic scan of the chest. The arrows indicate the extent of the thymic mass.

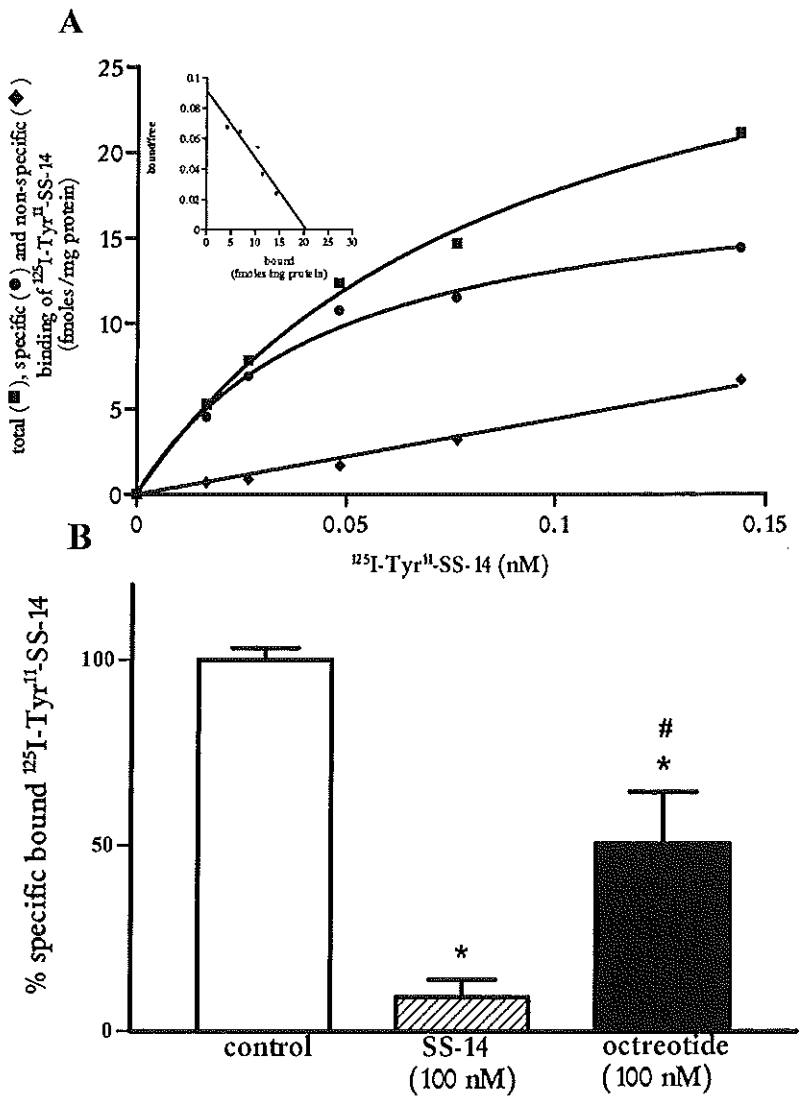

FIGURE 2. Expression of SSR in a membrane preparation of the human thymoma. A, Saturation curves indicate total, (■), non-specific (◆) and specific (●) binding of [^{125}I-Tyr^{11}]-SS-14. *Inset*: Scatchard analysis of [^{125}I-Tyr^{11}]-SS-14 binding to thymoma tissue membranes [K_d, 50 pM; B_{max}, 21 fmoles/mg membrane protein]. B, Displacement of [^{125}I-Tyr^{11}]-SS-14 binding by SS-14 (100 nM) and octreotide (100 nM). *, $p<0.01$ *vs.* control; #, $p<0.01$ *vs.* SS-14. Control, 922.3±3.2 cpm; SS-14, 84.7±4.7 cpm; octreotide, 465.7±14 cpm. Data are derived from triplicate values and are the mean±SEM.

By RT-PCR, sst_1, sst_{2A} and a predominant sst_3 mRNA expression was detected in the thymoma tissue (Table 1). Conversely, mRNAs encoding for SS, sst_{2B}, sst_4 and sst_5 were absent (Table 1). In the cultured neoplastic TEC, RT-PCR analysis showed the presence of sst_3 mRNA only (Table 1). In cultured thymocytes only mRNA for β-actin was detected

(Table 1). As previously described (18), the normal thymus expressed SS, sst_1, sst_{2A}, and sst_3 mRNAs, whereas cultured TEC expressed SS, sst_1, and sst_{2A} mRNAs (Table 1). By immunohistochemistry, a high immunoreactivity for sst_3 receptors and a very weak immunostaining for sst_1, but not for sst_{2A} receptors was observed on the tumor cells (Fig. 3A and C). sst_{2A} was weakly expressed in the stroma, mainly on endothelium of few small intratumoral vessels (Fig. 3B). In all the cases the immunostaining could be completely abolished by preabsorption with 100 nM of the respective antigen peptides (Fig. 3D, E, and F)

TABLE 1. Somatostatin and somatostatin receptor subtype mRNA expression in normal human thymus and in a cortical thymoma as determined by RT-PCR.

Samples	RT-PCR							
	sst_1	sst_{2A}	sst_{2B}	sst_3	sst_4	sst_5[a]	SS[b]	β-actin
Normal human thymus	+	+	-	+	-	-	+	+
Normal TEC[c]	+	+	-	-	-	-	+	+
Thymocytes[d] (normal thymus)	-	-	-	-	-	-	-	+
Thymoma	±	±	-	+	-	-	-	+
Neoplastic TEC	-	-	-	+	-	-	-	+
Thymocytes[d] (thymoma)	-	-	-	-	-	-	-	+

[a] somatostatin receptor subtypes 1-5; [b] somatostatin; [c] thymic epithelial cells; [d] cultured thymocytes.

In vitro ***effect of SS and octreotide on ^{3}H-thymidine incorporation of cultured neoplastic TEC.*** SS-14 (10 nM) and the SS analog octreotide (10 nM) significantly inhibited ^{3}H-thymidine incorporation by 40.6% and 43.2%, respectively, in the cultured tumor cells (Fig. 4). The epithelial origin of the cultured tumor cells was confirmed by immunocytochemical staining for cytokeratin that revealed a 100% pure epithelial cell culture.

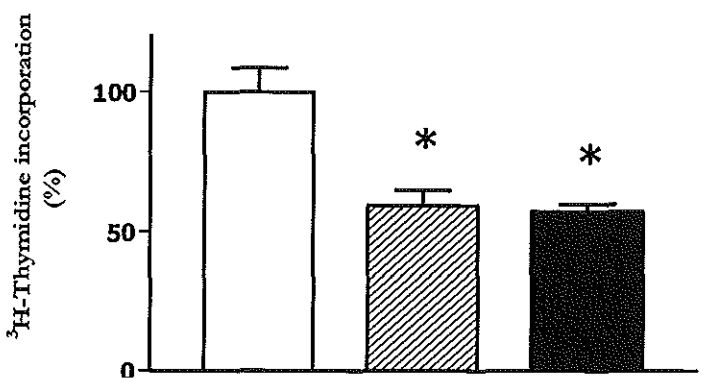

FIGURE 4. Effects of SS and octreotide on ^{3}H-thymidine incorporation in a primary culture of the thymoma. Neoplastic thymic epithelial cells were incubated in MEM and 10% FCS for 72 h in quadruplicate without or with 10 nM of the drugs indicated. Values are expressed as a percentage of ^{3}H-thymidine incorporation by control cells and are the mean±SEM (n = 4 wells). *, $p<0.01$ *vs.* control. ▨ SS-14, ■ octreotide. The control value of ^{3}H-thymidine incorporation was 182.3±15.9 cpm.

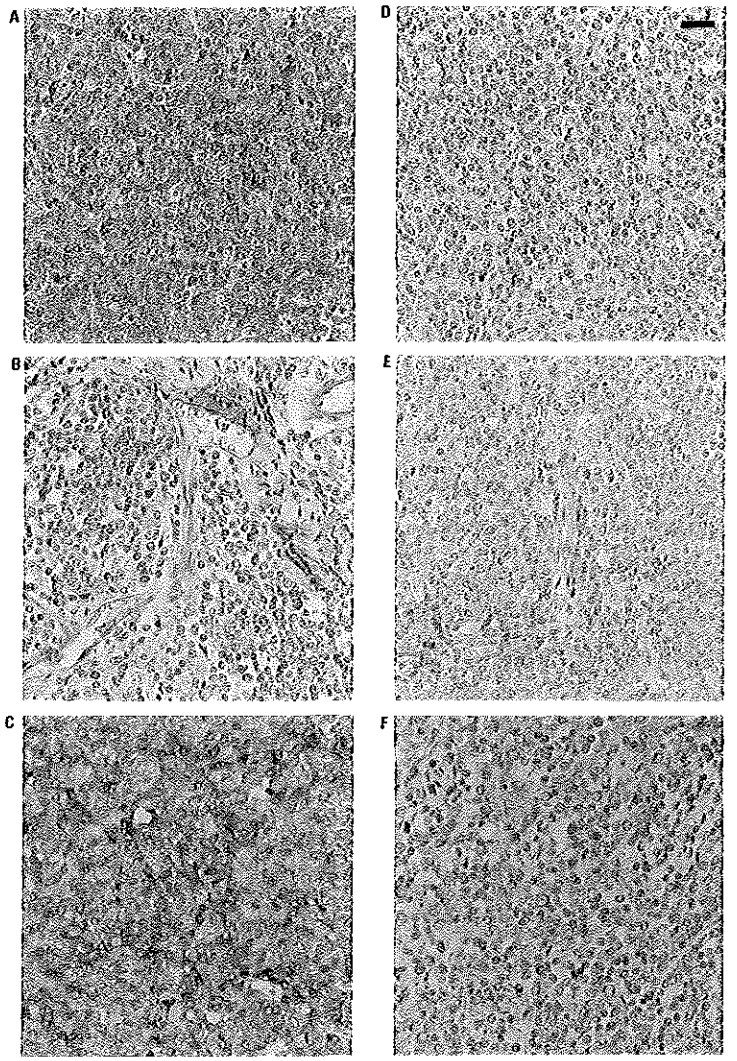

FIGURE 3. Immunohistochemical detection of sst_1, sst_{2A} and sst_3 receptors in the thymoma. *Left panel*: A, Weak diffuse sst_1 immunoreactivity within the tumor. Section developed with 3,3¹ diaminobenzidine; B, sst_{2A} immunoreactivity located on the endothelium of an intratumoral vessel. Section developed with 3,3¹ diaminobenzidine; C, High diffuse sst_3 immunoreactivity within the tumor. Section developed with New Fuchsine/Naphtol AS-MX. *Right panel*: D-F, Adjacent sections showing displacement of immunostaining after the preabsorption of the antibodies with 100 nM of the respective peptide antigens. The sections are slightly counterstained with hematoxylin. Magnification X400; *Bar* 25 μm.

DISCUSSION

Human thymoma is the most common tumor of the anterio-superior mediastinum and is composed of cytologically bland, neoplastic TEC accompanied by a variable admixture of benign lymphocytes (24). This tumor is frequently associated with paraneoplastic phenomena, such as *myasthenia gravis*, and/or other autoimmune-related disorders and hematological abnormalities (24). The microenvironment is abnormal in thymoma and it differs from the normal thymus in the prevalence of cortical areas and the deficiency of medullary ones (25).

A high *in vivo* uptake of [^{111}In-DTPA-D-Phe^{1}]-octreotide has been detected in patients bearing thymoma or thymic carcinoid, whereas the normal or hyperplastic thymus is not visualized (20,26). Moreover, successful treatments with octreotide and prednisone and with octreotide alone have been reported in two patients with thymoma (19,20). In one case the treatment resulted in both tumor size reduction and an improvement of the autoimmune-related disease (19). The precise mechanisms involved in this effect are unclear, however. In a previous study, using *in vitro* SSR autoradiography, Reubi *et al.* did not find SS-binding sites in four thymomas of undefined histological subtype, whereas SSR receptors were detectable in the normal thymus (27). The lack of *in vitro* detection of SSR in human thymoma seems in contrast with the compelling evidence that tumors originating from SSR-positive tissues generally express SSR (8,28).

We have recently characterized SSR subtype expression in the normal human thymus (18). sst_1 and sst_{2A} mRNAs were expressed in the thymic tissue and isolated TEC, whereas sst_3 mRNA was detectable in thymic tissue only and not on enriched cultured TEC or cultured thymocytes (18). As several different subsets of epithelial cells and thymocytes have been described within the human thymus, the sst_3 receptor might be expressed on another cell subset or might undergo rapid downregulation during culturing. In fact, it is well known that the expression of neuropeptide receptors on immune cells is dynamically regulated and depends on the traffic of these cells through and within lymphoid compartments and homing in the tissues (29,30). Moreover, preliminary data show sst_{2A} immunoreactivity on stromal cells in the medullary compartment of normal human thymus (31). All of these findings are in agreement with the evidence of a strong compartmentalization of neuroendocrine peptide receptors in lymphoid tissue (32).

In the present report, we found a high uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in a patient with cortical thymoma and, in agreement with the study by Reubi *et al.* (27), a lack of SSR-binding sites by autoradiography performed on cryostat sections from the tumor tissue. However, we were able to detect a very low density of specific high affinity binding sites of [^{125}I-Tyr^{11}]-SS-14 on an enriched membrane preparation from the thymoma tissue. By RT-PCR, we found mRNAs encoding for sst_1, sst_{2A} and sst_3 in the thymoma tissue, whereas mRNA encoding for sst_3 was detectable in primary cultured neoplastic cells. Conversely, no binding was detectable and no mRNAs encoding for SSR subtypes were found on the tumor-derived cultured thymocytes. Consonant with the presence of sst_1 mRNA and the low affinity of octreotide for sst_3 receptors, we found that octreotide only partially displaced [^{125}I-Tyr^{11}]-SS-14 binding on cell membrane from this tumor. To further characterize the heterogeneous expression of SSR subtypes in this tumor, we used three recently developed polyclonal SSR antibodies, highly specific for sst_1, sst_{2A} and sst_3 receptors (11,22,23). By immunohistochemistry, we observed a high immunoreactivity for sst_3 receptors and a very weak expression of sst_1 receptors on the tumor tissue. Conversely, the tumor cells did not express sst_{2A} receptors, whereas sst_{2A} immunoreactivity was localized in the stroma, mainly on the endothelium of scattered small intratumoral vessels. These findings confirm the predominant presence of sst_3 in this tumor. In fact, the mRNA encoding for this protein has been detected by RT-PCR on both tumor tissue and cultured tumor cells and our hypothesis is that this cortical thymoma might have arisen from a subset of thymic sst_3-expressing cells. However, the diffuse immunoreactivity for sst_3 in the tumor tissue suggests the presence of this SSR subtype on reactive thymocytes as well. Although mRNA encoding for sst_3 was undetectable in cultured thymocytes from both thymoma and normal thymus, our recent observation showed the presence of SS-binding sites on freshly isolated thymocytes (31). This finding might explain the diffuse immunoreactivity for sst_3 in the thymoma and confirm the evidence that neuropeptide receptor expression in lymphoid tissues might rapidly change in conditions different from those in the natural microenvironment (33).

sst_3 receptors have been shown to display the highest amount of agonist-dependent receptor internalization compared to the other SSR subtypes (34). Although the affinity of octapeptide SS analogs for sst_3 is lower compared with that for sst_2, the high internalization of sst_3 receptors might be the possible mechanism regulating the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide within thymomas. Indeed, the present study demonstrates that

sst_3 is the subtype predominantly expressed in this tumor displaying a significant *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide. Another suggestive hypothesis, which can not be completely excluded, is the presence of a novel unidentified SSR involved in determining the *in vivo* uptake of the radiotracer.

Cell subsets containing neuropeptide hormones are present in the cortical areas of normal thymus as well as in thymoma (35). The identification of SS and its receptors in the normal thymus strongly supports the paracrine actions of the peptide in modulating the immuno- and/or neuroendocrine functions in this organ (18,27). The pattern of distribution of SS-binding sites observed in normal thymus seems to be still preserved in thymic hyperplasia associated with *myasthenia gravis*, but seems to be lost in thymoma (27). Furthermore, in the present case, SS mRNA was not detectable in either thymoma tissue or the primary cultured tumor cells, whereas it was detected in normal human thymic tissue and in normal cultured TEC (18). This finding suggests that the loss of local SS production might be implicated in the uncontrolled cell growth and/or the tumor autoimmune-related diseases and possible tumor pathogenesis. In support of this hypothesis, we found that the *in vivo* administration of SS-14 and the SS analog octreotide at high concentration (10 nM) significantly inhibited ^{3}H-thymidine incorporation in the cultured tumor cells. Both compounds displayed a comparable ability in inhibiting ^{3}H-thymidine incorporation by cultured cells. As SS-14 binds sst_3 with higher affinity than octreotide, a difference in the percentage of inhibition should have been observed. However, taking into consideration that we measured the uptake of ^{3}H-thymidine after 72 h, we might have underestimated the true inhibition degree by SS-14, due to a lower stability of the latter compound. The inhibition of cell proliferation by SS-14 and octreotide, and the predominant expression of sst_3 in the thymoma, are in line with emerging data regarding signal transduction pathways linked to SSR that show a cell cycle-dependent induction of apoptosis by octreotide (36). Octreotide-induced apoptosis seems signaled through the sst_3 and associated with dephosphorylation-dependent conformational change in wild-type p53 (37). Although controversial data about alterations of p53 expression have been reported in thymic epithelial tumors (38,39), SS-induced apoptosis might represent an additional mechanism involved in the regulation of cell proliferation in human thymus. Furthermore, this finding may form the basis for the inhibition of tumor growth in patients with thymoma treated with octreotide. As octreotide binds to sst_{2A}, sst_3 and sst_5, but not to sst_1, and considering

that in the present case sst_{2A} and sst_5 are not expressed in the tumor cells, the *in vitro* effect of SS and octreotide seems to be mediated directly by sst_3.

In conclusion, although further investigations are required, the loss of SS production in combination with a predominant expression of sst_3 receptor in this thymoma might be implicated in uncontrolled cell growth and tumor pathogenesis. These data support the physiological paracrine/autocrine role of SS in the human thymic microenvironment and indicate that a disturbance in this system might be involved in the pathogenesis of thymic neoplasms. Furthermore, SSRs are localized in different complementary cell compartments in the normal and neoplastic human thymus. At least three SSR subtypes seem to control neuroendocrine function as well as the cell growth in this organ. Moreover, the sst_3 subtype may play an important role in determining the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide during *in vivo* SSR scintigraphy. The inhibition of cell proliferation by octreotide through sst_3 may explain the reported successful treatment of thymoma with this SS analog. Finally, SSR scintigraphy and SS analog treatment of thymomas may represent a new diagnostic and therapeutic approach to these tumors.

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Lastoria S, Ferone D, Hofland LJ, De Chiara A, Montella L, van Hagen PM, Muto P, Lamberts SWJ, Palmieri G.
***In vivo* detection and *in vitro* localization of somatostatin receptors in human thymic tumors: preliminary study on 14 cases.**
2000 Submitted

IN VIVO DETECTION AND *IN VITRO* LOCALIZATION OF SOMATOSTATIN RECEPTORS IN HUMAN THYMIC TUMORS: PRELIMINARY STUDY ON 14 CASES

ABSTRACT

We have previously shown that most thymic tumors can be visualized in humans by scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide, and that therapy with somatostatin (SS) analogs exert antineoplastic effects. These findings suggest that SS receptors (SSR) are expressed on these tumors. However, previous studies did not present evidence of the presence of such receptors in tissue specimens. Therefore we have utilized two polyclonal antibodies raised against a synthetic peptide fragment of SSR subtype 2A (sst_{2A}) and sst_3. Immunohistochemical analysis was performed on tissue specimens of 14 surgically removed thymic tumors from patients previously studied by SSR scintigraphy.

By scintigraphy, 13 out of 14 thymic tumors showed detectable uptake of [^{111}In-DTPA-D-Phe^1]-octreotide with tumor to backgound ratios ranging between 1.4 to 6.3 fold. One tumor was not visualized. By immunohistochemistry, 4 tumors demonstrated positive immonostaining for both sst_{2A} and sst_3; 2 were positive for sst_{2A} and 5 for sst_3 only; 3 tumor samples stained completely negative. The staining was highly heterogeneous and sst_{2A} and sst_3 expression was associated with different cell types. In particular, sst_{2A} expression was clearly associated with thymic epithelial cells in 2 cases; sst_3 expression was frequently found associated with thymocytes; sst_3 immunoreactivity was weakly present on tumor cells in most cases, however. The staining was specific in all the cases examined.

This study clearly demonstrates the *in vitro* expression of at least two of five SSR subtypes in a series of human thymic tumors. Furthermore, the discrete concordance of scintigraphic and immunohistochemical results, demonstrates that specific receptor-mediated binding of [^{111}In-DTPA-D-Phe^1]-octreotide occurs in most thymic tumors.

INTRODUCTION

The clinical implications of high peptide receptor expression by human tumors for imaging and therapy have been clearly demonstrated for somatostatin (SS) receptors (SSR) (1-4). Imaging by [^{111}In-DTPA-D-Phe^1]-octreotide as radioligand is a well-established *in vivo* method to visualize SSR-expressing tumors (4). Therapy with SS analogs has been demonstrated to be effective in some SSR positive neuroendocrine tumors (3,5). Both diagnostic and therapeutic applications of SS analogs follow the *in vitro* identification of these tumoral receptors by ligand techniques (6-8).

The development of antibodies specific for different SSR subtypes may be used to study their expression in tissue sections by immunohistochemistry (9,10). Preliminary reports indicated that immunohistochemistry is a reliable method to evaluate the expression of SSR subtypes either in carcinoids or other SSR positive tumors (11-15). Immunohistochemistry is also easier to perform and more practical for clinical use than reverse transcriptase polymerase chain reaction (RT-PCR) or *in situ* hybridization methods. Furthermore, immunohistochemistry can be applied on fixed materials; thus, it is mandatory when frozen material lacks.

We have found that human thymic tumors are detectable by SSR scintigraphy (SRS) using [^{111}In-DTPA-D-Phe^1]-octreotide, showing very high tumor-to-background (T-to-B) ratios (16). It has also been shown that patients with thymic tumors may successfully respond to SS-analog based therapy (17,18). Besides this evidence, SSR expression was not found in thymic tumors by *in vitro* autoradiographic techniques, while a high content was found in thymic carcinoids, as well as in normal young or adult normal thymuses (19). Recently, using combined evaluation by *in vitro* techniques, we demonstrated the expression of sst_1, sst_{2A} and sst_3 in a cortical thymoma, which was detected by SRS (20).

The aim of the present study was to verify the expression of sst_{2A} and sst_3 in a series of 14 thymic tumors, which had been pre-operatively imaged by SRS scintigraphy. This preliminary study was designed in order to evaluate whether the presence of these SSR might be related to the *in vivo* scintigraphic results.

MATERIALS AND METHODS

Patients Fourteen patients (6 females and 8 males, age-range 19-74 yr) with a diagnosis of thymic tumor entered the study. Three patients were untreated, 4 had received only chemotherapy and the remaining patients underwent different combinations of chemotherapy, surgery and radiotherapy. Myasthenia gravis was the most common associated paraneoplastic syndrome observed in the group of patients. Pure red cell aplasia was present in two patients. The patients' profile at study entry is reported in Table 1.

TABLE 1. Patients' profile at study entry.

Case	Sex/age	WHO classification	Previous therapy	Sites of disease	Associated syndrome
1.	F/68	C	CHT	mediastinum, lung	
2.	F/57	B2	CHT	mediastinum, pleural	PRCA
3.	F/67	C	None	mediastinum, pleural	
4.	F/50	B3	None	mediastinum, pleural	
5.	M/26	B2	CHT-S	mediastinum	MG
6.	F/50	C	CHT-RT-S	mediastinum, pleural, lung	
7.	M/58	B2	CHT	mediastinum	PRCA
8.	F/74	B1	S-CHT-RT	mediastinum	MG
9.	F/61	C	None	mediastinum, pericardium	
10.	M/19	C	CHT	mediastinum, cervical	
11.	M/53	B2	S-CHT	mediastinum	
12.	M/52	B2	S-CHT	mediastinum	MG
13.	M/46	B3	S-CHT	pleural	
14.	M/71	B2	S-CHT	mediastinum	

CHT, chemotherapy; S, surgery; RT, radiotherapy; PRCA, pure red cell aplasia; MG, myasthenia gravis.

Tissue samples The samples of 14 histologically proven thymomas were collected and used for immunohistochemical evaluation in this study. All samples were formalin-fixed and paraffin-embedded. Fixation was performed for 24-36 hours. The tumors were classified according to the new WHO classification of thymic epithelial tumors (21) and the results are reported in Table 1.

Scintigraphic studies Scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide (Mallinkrodt, Petten, The Netherlands) was performed as previously reported (16). Briefly, planar and tomographic images were collected 24 h after the i.v. injection of 180-222 MBq of radioligand. Labeling was performed according to the manufacturer instructions; injected preparations had <2% of uncoupled ^{111}Indium. Images were obtained by gamma-camera (Siemens, Erlagen, Germany) equipped with a medium energy collimator (photopeaks were set at 172 and 242 keV, 10% window). T-to-B ratios were obtained by using the regions of interest (ROIs) method. A ROI was manually drawn around the area(s) of radioligand uptake; similar one was placed in chest where no uptake was detectable. In each patient, the T-to-B ratio was obtained in the planar or tomographic images of the chest obtained 24 h p.i., dividing the counts in the thymic tumor by those in the background area.

Immunohistochemical localization of sst_{2A} and sst_3 receptors Paraffin-embedded sections (5 μm of tickness) were deparafinized, rehydrated, and heated in microwave (in citric acid buffer, pH 6.0) at 100°C for 15 min, rinsed in tap water followed by phosphate-buffered saline (PBS) and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% bovine serum albumin, BSA). The sections were then incubated overnight at 4°C with sst_{2A} and sst_3 antibodies (Biotrend, Cologne, Germany) in a dilution of 1:3000. Finally, a standard streptavidin-biotinylated-alkaline phosphatase complex (ABC kit, Biogenix, San Ramon, CA) was used according to the manufacturer recommendations to visualize the bound antibodies. Negative controls for immunohistochemistry included: *a*) omission of the primary antibody; *b*) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM).

RESULTS

Somatostatin receptor scintigraphy results SRS was positive in 13 out of 14 patients included in this study. In 4 cases at least two sites of abnormal radioligand uptake were seen in the chest, while in 9 other patients a single area of uptake was recognized. The degree of [^{111}In-DTPA-D-Phe^1]-octreotide uptake was very heterogeneous, as documented in Figure 1. The values of T-to-B ratios ranged from 1.4 to 6.3 fold. The SRS results and T-to-B ratios are shown, case by case in Table 2.

TABLE 2. Summary of somatostatin receptor scintigraphy results and immunohistochemical localization of sst_{2A} and sst_3 receptors.

Case	Somatostatin receptor scintigraphy		Classification	Immunohistochemistry	
	sites of uptake	T-to-B ratios		sst_{2A}	sst_3
1.	mediastinum, lung	4.2/3.3	C	scattered cells	-
2.	mediastinum	3.1	B2	scattered cells	+
3.	mediastinum	3.4	C	+	n.e.
4.	mediastinum	6.2	B3	-	+
5.	mediastinum	1.4	B2	+	+
6.	mediastinum, lung	6.3	C	+	+
7.	mediastinum, pleural	2.1/1.4	B2	+	+
8.	mediastinum	2.6	B1	-	-
9.	mediastinum	2.1	C	-	-
10.	mediastinum, cervical	1.5/1.5	C	-	+
11.	mediastinum	3.2/1.7	B2	-	+
12.	mediastinum	3.3	B2	-	+
13.	negative	-	B3	-	-
14.	mediastinum	1.4	B2	n.e.	+

n.e., not evaluable.

FIGURE 1. Somatostatin receptor scintigraphy in patient with invasive thymoma. Planar images of the chest 24 h after injection of [^{111}In-DTPA-D-Phe^{1}]-octreotide. Anterior (left) and posterior (right) views. There is abnormal uptake in the mediastinum and in the left lung (no. 1 Table 2). The physiologic uptake or the radiotracer in the thyroid, pituitary, as well as in the liver is also evident.

Immunohistochemical detection of sst_{2A} and sst_3 Expression of sst_{2A} and sst_3 receptors or their co-expression was clearly detectable by immunohistochemistry in 11 out of 14 thymic tumors, while 3 thymomas were completely negative. In 12 biopsies immunohistochemistry was complete for both subtypes. In 2 cases immunohistochemistry results were evaluable for 1 subtype only. These results are summarized in Table 2. The immunostaining was usually weak to moderate. However, for both subtypes a wide heterogeneity of distribution was found. Immunohistochemistry provided a unique opportunity to exactly localize the cellular distribution of SSR within thymic tumors. The expression of sst_{2A} was diffuse within the tumor in 2 cases only, while in the remaining sections immunoreactivity was highly heterogeneous and confined to limited areas in the tissue samples. The expression of sst_3 was found diffuse within the tumor on both cell types, but mainly associated with thymocytes. The specificity of sst_{2A} and sst_3 immunostaining was confirmed in all biopsies. In fact, on adjacent sections, after absorption with specific peptide antigen (100 nM), the immunostaining of these antisera was completely prevented.

In 4 thymic tumor biopsies (nos. 2,5,6,7; Table 2), both sst_{2A} and sst_3 receptors were localized. In details, in 2 cases (nos. 6 and 7; Table 2) sst_{2A} immunoreactivity was present on tumor cells, while in the other 2 samples (nos. 2 and 5; Table 2) immunoreactivity was detected on few scattered cells in one case and on the stroma in the remaining case. In one of the two cases with immunohistochemical co-expression of sst_{2A} and sst_3, the tumor displayed the highest level of [^{111}In-DTPA-D-Phe^1]-octreotide uptake *in vivo*. The diffuse sst_{2A} immunoreactivity in the tumor sample from this case is shown in Figure 2A (no. 6, Table 2). In Figure 2B an example of sst_3 immunoreactivity is shown (no. 5, Table 2).

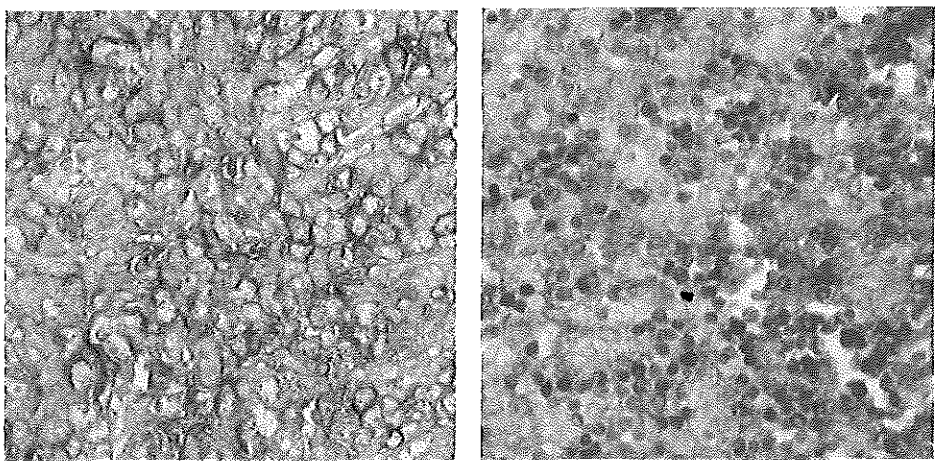
In 5 cases the expression of sst_3 receptor only was detectable (nos. 4,10,11,12,14; Table 2). Co-expression of sst_{2A} receptors was not detectable in 4 biopsies and not evaluable in the remaining one (no. 14; Table 2).

In 2 cases, the expression of only sst_{2A} receptors was demonstrated (nos. 1,3; Table 2). Among these 2 cases sst_3 receptor was undetectable in one (no. 1; Table 2) and not evaluable in the remaining case.

In 3 cases the expression of both sst_{2A} and sst_3 receptors was undetectable (nos. 8,9,13; Table 2).

Preliminary comparative analysis of in vivo *and* in vitro *results* We compared the results of immunohistochemistry for sst_{2A} and sst_3 receptors with T-to-B ratios measured at SRS.

Beside the relatively small number of cases we have analyzed, a positive trend between the T-to-B ratios and immunostaining seems evident. In particular, the expression of both subtypes and sst_{2A} alone has been observed within thymic tumors with higher radioligand concentration (Table 2).



A B

FIGURE 2. Immunohistochemical detection of sst_{2A} and sst_3 receptors in the two different thymomas. *A)* Diffuse moderate sst_{2A} immunoreactivity localized on tumor cells (no. 6, Table 2); *B)* Weak diffuse sst_3 immunoreactivity within the tumor, moderate immunoreactivity localized on reactive thymocytes (no.5, Table 2). Sections developed with New Fucsine/Naphtol AS-MX. The sections are slightly counterstained with hematoxylin. Magnification 400 X; *Bar* 25 μm.

DISCUSSION

In this study we systematically analyzed and compared the scintigraphic results from [^{111}In-DTPA-D-Phe^1]-octreotide scans and the immunohistochemical results on their surgical biopsies in the same patients with thymic tumors. The immunohistochemical identification of sst_{2A} and sst_3 in 11 of 14 thymic tumors confirms that these SSR are expressed in these rare human malignancies. We previously demonstrated that [^{111}In-DTPA-D-Phe^1]-octreotide visualized the majority of thymic tumor deposits greater than 15 mm (16). Moreover, therapy with SS analogs plus prednisone was effective in a patient unresponsive to other conventional treatments (17). Lately, another case of a patient with a thymoma visualized with [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy and successfully treated with

SS analog therapy has been reported (18). However, there was no *in vitro* evidence that these tumors may express SSR, with the exception of one case (19,20). Thus, the possible mechanisms involved which might explain these results remained unclear. Furthermore, by autoradiography Reubi *et al.* demonstrated SSR in normal human thymuses with a lack of binding sites in 4 epithelial thymic tumors (19). This was the only example of tumors originating from SSR positive tissues not expressing such receptors.

Thymomas consist of thymic epithelial cells mixed to benign lymphocytes and stromal cells (21-23). The microenvironment in these thymic tumors differs from the normal adult thymus for the prevalence of cortical areas and deficiency of medullary ones (24). In normal thymus sst_1 and sst_{2A} mRNAs have been found to be expressed in thymic tissues and isolated thymic epithelial cells, while sst_3 mRNA was found in thymic tissue only (25). However, preliminary data have demonstrated the presence of SS-binding sites on normal freshly isolated thymocytes (26).

In thymic tumors we frequently found the expression of sst_3, prevalently associated with thymocytes and sst_{2A} expression confined to malignant epithelial cells or within stromal structures. Such differentiated cellular distribution requires further comments. SSR distribution within the normal human thymus seems to display a strong compartmentalization (19,25). This observation had been already previously made for other neuropeptide receptors within this primary lymphoid organ (27). However, the distribution of receptors for neuropeptides and hormones within the thymus underlines the important role exerted by these substances, which are often locally produced, in modulating the physiologic processes occurring in this organ (28). In the thymus lymphoid or accessory cells at different stages of maturation might differentially express neuropeptide receptors (26). Moreover, a dynamic traffic of cells occurs in thymic lobules and different cell subsets interact with each other (29). The intense cell activity is mainly orientated to drive thymocytes migration and differentiation in order to complete normal T cell maturation, which is the main function of this primary lymphoid organ (30,31). Thymic epithelial cells are supposed to be the major cell type involved in this process (29). Different subsets of epithelial cells are present in the normal thymus. At least three principal phenotypes are recognizable: a subcapsular, cortical and medullary epithelium. Within the thymic microenvironment, these cells are exposed to various substances and stimuli. A tumor originated from the human thymus might derive from each cell type, which belongs to these subsets. Different SSR have been described to be selectively expressed in different

regions and perhaps on specific epithelial cell types in the human thymus (25,26). This might partially explain the considerable heterogeneity of receptor expression between thymomas and within the same tumor. Further studies orientated to identified adjunctive markers within the neoplastic cells are mandatory to better understand whether the distribution of SSR might have a rationale or whether the presence of a specific SSR might act as a marker itself.

The analysis of scintigraphic results compared to the presence of SSR expression at immunohistochemistry indicate that in general the higher T-to-B ratios *in vivo* correspond to the expression of at least one of the two SSR subtypes *in vitro*. In particular, the highest ratio has been detected in one patient with a diffuse co-expression of both receptors in the tumor. Moreover, in one of the three tumors, which were negative at SRS, immunohistochemistry failed to detect SSR in the tumor tissue. However, rather high T-to-B ratios were calculated in the two other patients with tumor lacking SSR subtype expression *in vitro*. Although, the sensitivity of the technique or the procedure of fixation of the samples might account for this discrepancy, the presence of another SSR subtype cannot be ruled out.

[^{111}In-DTPA-D-Phe^1]-octreotide binds with high affinity to sst_2 and with lower affinity to sst_3 and sst_5. Two of these SSR subtypes have been evaluated *in vitro* in the present series of human thymomas, and both these receptors might participate in determining the uptake of the radiopharmaceutical. However, in three cases a high uptake *in vivo* corresponded to the selective expression of sst_3 receptors within the tumor, suggesting that despite the lower affinity for [^{111}In-DTPA-D-Phe^1]-octreotide of this subtype compared with sst_{2A}, it may play an important role in the uptake during SRS. On the other hand, since apart from the affinity, additional biological events related to receptor turnover might play a role in displaying the *in vivo* uptake of radiolabeled SS analogs, sst_3 may account for the occurrence of [^{111}In-DTPA-D-Phe^1]-octreotide uptake in thymomas. In fact, recently a high rate of agonist-dependent internalization has been described in cells transfected with this SSR subtype (32). Moreover, sst_3 has been found to be involved in a cell cycle dependent induction of apoptosis by octreotide (33). Apoptosis signaled through the sst_3 could represent an important mechanism in regulating cell survival in the human thymus, and might be related to the successful therapy with octreotide in patients with thymomas (17,18).

In conclusion, although further studies are required to better characterize these rare but intriguing tumors, two SSR subtypes, i.e. sst_{2A} and sst_3, are heterogeneously expressed in thymomas. Both receptors might be involved in determining the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in patients with thymic tumors.

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Immunohistochemical localization and quantitative expression of somatostatin receptors in normal human spleen, thymus and thymoma.
2000 *Submitted*

IMMUNOHISTOCHEMICAL LOCALIZATION AND QUANTITATIVE EXPRESSION OF SOMATOSTATIN RECEPTORS IN NORMAL HUMAN SPLEEN, THYMUS, AND THYMOMA

ABSTRACT

We have recently characterized somatostatin (SS) receptor (SSR) expression pattern in the normal human thymus. Three different SSR subtypes, sst_1, sst_{2A}, and sst_3 were expressed in thymic tissue. Although, this lymphoid organ is not visualized during *in vivo* SSR scintigraphy, thymic tumors can be often detected. [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy allows the visualization of several types of SSR-expressing tumors, as well as normal SS target tissues. Among these, the spleen is clearly depicted. Since both the human spleen and thymus contain SS-binding sites, we evaluated whether the heterogeneity of distribution, he type of SSR, as well as the amount of the different SSR subtypes might explain the apparent contrasting findings in these lymphoid organs. By membrane homogenate binding studies we found [^{125}I-Tyr^3]-octreotide binding-sites both in the spleen and in the normal and hyperplastic thymus, but not in thymomas. On the other hand, [^{125}I-Tyr^{11}]-SS-14 binding was detectable in all cases. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis demonstrated sst_1, sst_{2A} and sst_3 mRNAs in the thymus and thymoma, while the spleen selectively expressed sst_{2A} and sst_3 mRNAs. Sst_{2B}, sst_4 and sst_5 mRNAs were not detected in any of the tissues. Quantitative evaluation of SSR subtype mRNAs showed a high and almost selective expression of sst_{2A} mRNA in the spleen whereas a higher expression of sst_3 mRNA was present in the thymus. The highest expression of sst_3 mRNA was detected in thymic hyperplasia and in thymomas. The highest density of sst_{2A} receptors in the spleen is in line with the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide, which is considered a sst_2-preferring ligand. However, although octreotide displays a lower affinity for sst_3 receptors, the relatively high expression of sst_3 mRNA in thymic tissues suggests that this SSR subtype might play a role in determining the uptake of the radiopharmaceutical at *in vivo* SSR scintigraphy in patients with thymoma. Apart from the affinity of the radioligand for the receptor, also the efficacy of the

internalization of the radioligand-receptor complex might play an important role in determining the radioactivity uptake during *in vivo* SSR scintigraphy.

Finally, the cellular localization of these two SSR subtypes, determined by immunohistochemistry, showed a preferential expression of sst_{2A} receptors on microenvironmental cells and of sst_3 receptors on lymphoid cells in both these organs. In thymomas sst_3 receptor was mainly expressed on reactive lymphoid cells, and to lesser extent on tumor cells. This distribution pattern might explain the different effects of SS on immune cell functions.

INTRODUCTION

The effects of somatostatin (SS) are mediated *via* a family of high affinity G-protein-coupled membrane receptors (SSR) with five known subtypes, codenamed sst_{1-5} (1,2). In SS target tissues, SSR are expressed in a tissue- and subtype-selective manner (3). Like other modulatory neuropeptides, SS acts on cells of the immune system (4-6). Variable effects of SS and its octapeptide-selective analogs have been reported on immune cell functions, both in animals and man (4,5,10,11). These observations might be related to a heterogeneous expression of SSR on immune cells (7-9). The expression of SSR in lymphoid tissues has been investigated *in vitro* by binding studies, using fluorescent and/or iodinated SS and SS analogs, and by reverse transcriptase polymerase chain reaction (RT-PCR) method (4,5,10,11,12). In addition, using radiolabeled SS analogs, several scintigraphic studies have shown the *in vivo* localization of SSR in lymphoid tissues, either in normal or pathological conditions (5,13-16). In particular, the normal human spleen usually displays a high *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide (14). This strong *in vivo* uptake seems to be due to the presence of specific SS binding sites in this lymphoid organ. In fact, high affinity SSR, preferentially located in the red pulp, have been demonstrated by *in vitro* SS binding studies using [^{125}I-Leu^8, D-Trp^{22}, Tyr^{25}]-SS-28 and [^{125}I-Tyr^3]-octreotide (15). Moreover, the presence of specific SSR in the spleen is supported by the fact that the *in vivo* labeling is significantly reduced in patients treated with the octapeptide SS analog octreotide (15). Conversely, although SSR have been clearly demonstrated *in vitro* in the normal human thymus (15,17), this organ is not visualized during scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide, while a high *in vivo* uptake of the radiotracer has been detected in patients with thymic epithelial tumors (18-21).

In order to investigate whether the heterogeneity of expression and differences in the density of SSR might explain this apparent contrast, we have characterized in the present study SS and SSR subtype expression in the normal human spleen and thymus, in thymic hyperplasia and in thymomas by classical binding studies, by RT-PCR, and by immunohistochemistry. Using a quantitative RT-PCR method, we analyzed the amount of sst_{2A} and sst_3 mRNAs in these lymphoid tissues. Since immunohistochemistry, which allows the localization of the receptor proteins at cellular level (22-25), has been recently successfully employed in human lymphoid tissues (26-28), we used two polyclonal antibodies raised against specific peptide fragments of sst_{2A} and sst_3 receptors to further

investigate the expression pattern of these subtypes on frozen and/or formalin-fixed sections of human spleen, thymus and thymoma.

MATERIALS AND METHODS

Somatostatin receptor scintigraphy. Scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide was performed as previously reported (29). Briefly, planar images were obtained 24 h after the injection of 228 Mbq [^{111}In-DTPA-D-Phe^1]-octreotide (Mallinkrodt, Petten, The Netherlands). Imaging studies were performed using a two-headed gamma camera (Picker 2000, Picker Instruments, Cleveland, Ohio, USA) equipped with a medium-energy-collimator. Acquisition time for planar spot images was 15 min.

Samples. Two samples of splenic tissue were obtained from patients operated for pancreatic disorders (1 female, age 25 yr and 1 male, age 47 yr) and were histologically normal. Normal thymic tissues (n=2; 1 female, age 9 yr and 1 male, age 12 yr) were obtained from children undergoing cardiac surgery. The hyperplastic thymic tissue and the two thymomas (one B2 and one A, according to the WHO classification) were surgically removed from patients with *myasthenia gravis* (2 females, age 55 and 40 yr and 1 male, age 35 yr). The protocol was in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained from the patients or their parents. Samples were taken directly at operation, quickly frozen on dry ice and stored at -80°C for ligand binding and RT-PCR studies and for immunohistochemistry. Additional samples were fixed in 10% paraformaldehyde overnight for immunohistochemistry.

SSR binding studies. Receptor autoradiography was carried out on 10-micrometer thick cryostat sections (Jung CM3000, Leica, Germany) using [^{125}I-Tyr^3]-octreotide and [^{125}I-Tyr^{11}]-SS-14 as radioligands (specific activity approximately 2000 Ci/mmol) as previously described (17). The method of membrane isolation and the reaction conditions were described elsewhere (17). Briefly, membrane preparations (corresponding to 30-50 μg protein) of tissue samples were incubated in a total volume of 100 μl at room temperature for 60 min with increasing concentrations of [^{125}I-Tyr^3]-octreotide or [^{125}I-Tyr^{11}]-SS-14 without and with excess (1 μM) of unlabeled octreotide and SS-14 in HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.02 g/liter bacitracin, pH 7.6] containing 0.2% bovine serum albumin (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 an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer, and the final pellet was counted in a γ-counter (1470 Wizard, Wallac, Turku, Finland]. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled ligands.

RT-PCR studies. RT-PCR was performed as previously described (17). Briefly, poly A+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from tissue samples. cDNA was synthesized using the poly A+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ as solid phase and first strand primer. One-tenth of the cDNA was used for each amplification by PCR using primer sets specific for human $sst_{1\text{-}5}$, SS, and HPRT (21). Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A+ mRNA preparation (since the SSR subtype 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 HPRT-specific primers served as a positive control for the quality of the cDNA. To exclude contamination of the PCR reaction 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 SSR receptor subtypes, 0.001 to 0.1 μg of human genomic DNA, representing approximately 300 to 30.000 copies of sst-template was amplified in parallel with the cDNA samples. As a positive control for the PCR of HPRT and SS, aliquots of a cDNA sample known to contain SS and HPRT mRNA were amplified, because these primer-pairs did enclose introns in the genomic DNA. In order to quantify sst_2 and sst_3 mRNAs a quantitative RT-PCR was performed by TaqMan® Gold nuclease assay (The Perkin-Elmer Corporation, Foster City, CA) and the ABI PRISM® 7700 Sequence Detection System (The Perkin-Elmer Corporation) for real-time amplification, according to the manufacturers instructions. The specific primer sequences that were used for the quantitative RT-PCR included:

sst_{2A} forward 5'-ATGCCAAGATGAAGACCATCAC-3',

sst_{2A} reverse 5'-TGAACTGATTGATGCCATCCA-3'

sst_3 forward 5'-CTGGGTAACTCGCTGGTCATCTA-3'

sst_3 reverse 5'-AGCGCCAGGTTGAGGATGRA-3'

HPRT forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3'

HPRT reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'.

The probe sequences that were used included:

sst_{2A} 5'-FAM-TGGCTCTGGTCCACTGGCCCTTTG-TAMRA-3'

sst_3 5'-FAM-CGGCCAGCCCTTCAGTCACCAAC-TAMRA-3'

HPRT 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'.

The amount of sst_2 and sst_3 mRNA was determined by means of a standard curve generated in each experiment from known amounts of human genomic DNA. For the determination of the amount of HPRT mRNA, the standard curve was obtained by including dilutions of a pool of cDNAs known to contain HPRT. The amount of sst_2 and sst_3 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Immunohistochemical localization of sst_{2A} and sst_3 receptors. Immunohistochemistry was performed on 5-micrometer thick sections cut on a cryostat (Jung CM3000). The sections were fixed for 10 min in 4% paraformaldehyde, washed in tap water and phosphate buffered saline (PBS) and incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% BSA). Then, the sections were incubated with antibodies against sst_{2A} (R2-88, gift from Dr. A. Schönbrunn) and sst_3 (Biotrend, Cologne, Germany) overnight at 4°C. The antibodies were used at a dilution of 1:1000 and 1:3000, respectively in PBS + 5% BSA. A standard streptavidin-biotinylated-alkaline phosphatase complex (ABC kit, Biogenix, San Ramon, CA) was used according to the manufacturers recommendation to visualize the bound antibodies. Finally, the sections were developed with New Fuchsine/Naphtol AS-MX, slightly counterstained with hematoxylin and mounted. Paraffin-embedded sections (5 μm) were deparaffinized, rehydrated, exposed to microwave heating (in citric acid buffer, pH 6.0) at 100°C for 15 min, rinsed in tap water followed by PBS. The subsequent steps were performed exactly as in the protocol for frozen sections. The sst_{2A} and sst_3 antibodies were used at a dilution of 1:500 and 1:2000 in PBS + 5% BSA and the sections were incubated at overnight at 4°C. Negative controls for immunohistochemistry included: *a*) omission of the primary antibody; *b*) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM). A tissue was considered positive when the immunostaining was abolished by pre-absorption of the antibody with the respective peptide antigen.

Statistical analysis. Data are expressed as Mean ± SEM. Binding experiments were performed at least twice. SSR binding data on membrane homogenates were analyzed by the method of Scatchard.

RESULTS

Somatostatin receptor binding studies. At SSR scintigraphy a high uptake of [^{111}In-DTPA-D-Phe^1]-octreotide was detected after 24 h in the mediastinum of the patient with thymoma B2, whereas no uptake was measured in the patient with thymoma A (data not shown). Very high physiologic uptake was detected in the spleen (Fig.1A) of a patient with carcinoid syndrome whom underwent SSR scintigraphy for the localization of the tumor. The diagnosis of carcinoid tumor was confirmed later on. No sites of the disease were present in the spleen and this patient was chosen as exemplary case for studying the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in the spleen. SSR scintigraphy was performed in this latter patient after 6 months during therapy with octreotide (500 μg three times a

day). The uptake of the radiopharmaceutical was virtually abolished in the splenic region, while not in the kidneys (Fig.1B).

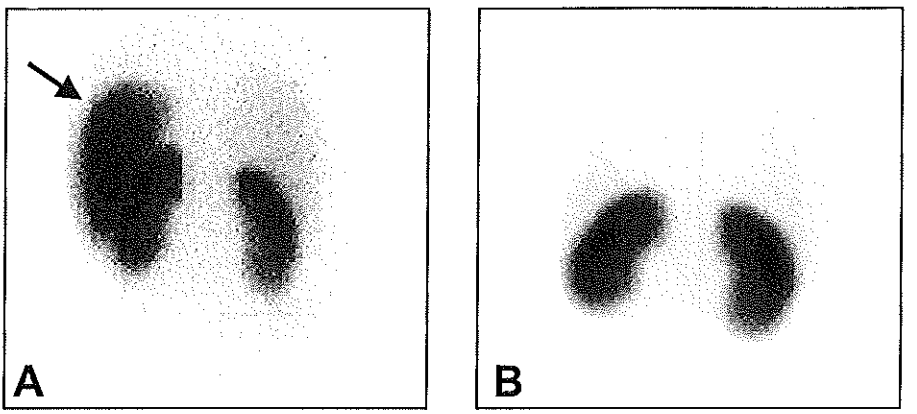

FIGURE 1. SSR scintigraphy in a patient with carcinoid syndrome. Planar images of the abdomen 24 h after injection of [^{111}In-DTPA-D-Phe^1]-octreotide. Poterior-anterior views. *A*) Scan performed before starting octreotide therapy. There is normal uptake in the spleen (arrow) and kidneys. *B*) The same patient 6 months after continuous octreotide therapy. The physiologic uptake of the radiopharmaceutical is abolished in the spleen, while not in the kidneys.

At autoradiography, specific binding of [^{125}I-Tyr^3]-octreotide was found in all splenic and thymic tissues, while no specific binding was detected in the thymomas. The binding was heterogeneous and mainly distributed in the red pulp of the spleen and in the medullary region of the normal thymuses. The binding was specific, since an excess of unlabeled [Tyr^3]-octreotide (1 μM) displaced it. Scatchard analysis of [^{125}I-Tyr^3]-octreotide binding on an enriched membrane preparation of the splenic and thymic tissues revealed high-affinity binding sites in all cases. For the spleen membranes the estimated K_d values were 0.8±0.4 nM, with a maximum binding capacity of B_{max} 163.5±24.5 fmoles/mg membrane protein, for the thymus the values K_d were 0.4±0.1 nM, B_{max} 22±4 fmoles/mg membrane protein (Mean±SEM). Figure 2 shows representative experiments. Although no specific binding of [^{125}I-Tyr^3]-octreotide was found in the thymomas, specific binding of [^{125}I-Tyr^{11}]-SS-14 was found on enriched- membrane preparation of one thymoma tissue (data not shown).

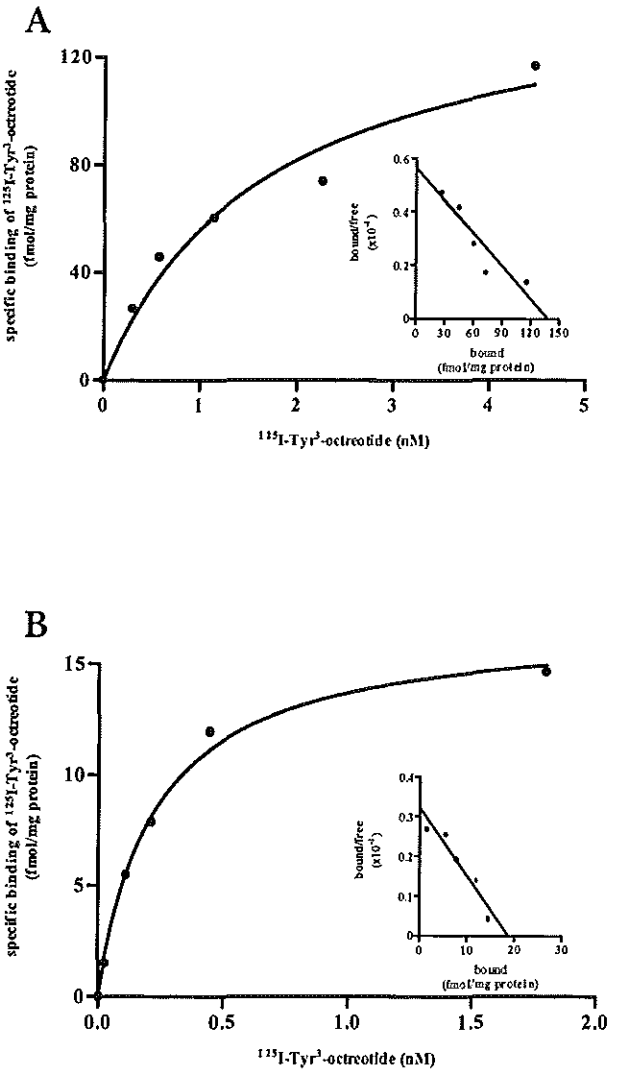

FIGURE 2. SSR expression of in a membrane preparation of human spleen and thymus. Saturation curves indicate specific ● binding of [^{125}I-Tyr^3]-octreotide. *Insets*: Scatchard analysis of [^{125}I-Tyr]-octreotide binding to splenic and thymic membranes *A*) spleen [K_d, 0.4 nM, maximum binding capacity (B_{max}), 139 fmoles/mg membrane protein]; *B*) thymus [K_d, 0.3 nM, B_{max}, 18 fmoles/mg membrane protein].

RT-PCR studies. By RT-PCR, mRNAs encoding for sst_{2A} and sst_3 were detectable in the spleen, while not SS, sst_1, sst_4 and sst_5 mRNAs. (Fig.3). In addition, SS, sst_1, sst_{2A} and sst_3 mRNA expression was found in the normal and hyperplastic thymic tissues. In the thymoma tissue sst_1, sst_{2A} and sst_3 mRNA expression was present, whereas sst_4 and sst_5 mRNA was undetectable (Fig.3). SS mRNA was undetectable in the B2 thymoma tissue and sst_{2B} mRNA was undetectable in all the samples of the present series. Quantitative analysis of the sst_{2A} and sst_3 mRNA content showed a much higher expression of sst_{2A} compared to sst_3 mRNA in the spleen (Fig.4A). This is also evident from Figure 3B, which shows the ratio of sst_{2A}/sst_3 mRNA copies. Conversely, the number of sst_3 mRNA copies was higher compared to sst_{2A} in the normal, hyperplastic and in one of the thymomas (thymoma B2; Fig.4A). Moreover, the amount of sst_{2A} mRNA was significantly higher in the spleen compared to the normal thymic tissues and thymoma. The number of sst_3 mRNA copies was higher in the hyperplastic thymus and in the thymoma B2 compared with that in the normal thymic tissue (Fig.4A).

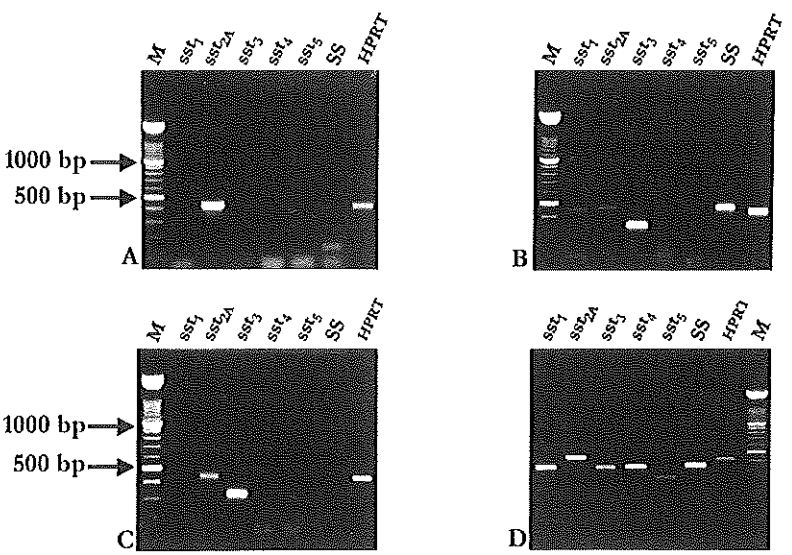

FIGURE 3. Heterogeneous expression of SS and sst_{1-5} mRNAs in normal human spleen, normal thymus and thymoma. Poly A^+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. M, 100-bp ladder; *A)* spleen; *B)* normal thymus; *C)* thymoma; *D)* control. RT-PCR analysis of each tissue was performed at least twice with identical results.

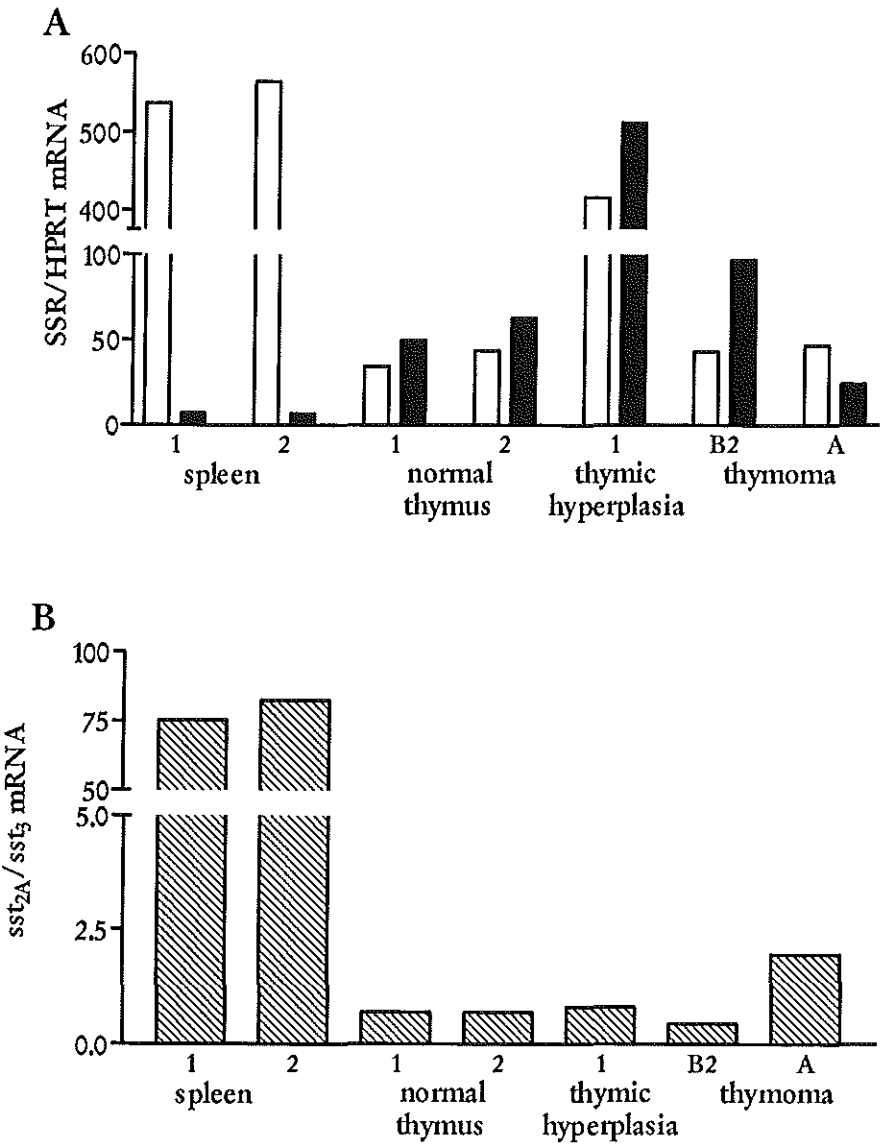

FIGURE 3. Quantitative analysis of RT-PCR data showing the different relative amount of sst_{2A} and sst_3 mRNAs in human spleen, normal and hyperplastic thymus and thymoma tissues calculated relative to the amount of HPRT and given in arbitrary units.
A) sst_{2A}/HPRT mRNA □, sst_3/HPRT mRNA ■. *B)* sst_3/sst_{2A} ratio ▨.

FIGURE 5. Immunohistochemical detection of sst_{2A} and sst_3 receptors in normal spleen, thymus and in thymoma. Upper panel (paraffin-embedded sections of spleen). *A)* sst_{2A}-immunoreactivity localized on microevironmental cells in the marginal zone and in the red pulp. *C)* sst_3-immunoreactivity on lymphoid cells localized in the outer regions of the periarteriolar lymphatic sheaths. *B,D)* adjacent sections showing displacement of immunostaining after preabsorption of the antibodies with 100 nM of the respective peptide antigens. Magnification 200 X; *Bar* 25 μm.Central panel (cryostat sections of thymus). *A)* sst_{2A}-immunoreactivity localized on microevironmental cells in the thymic medulla. *C)* sst_3-immunoreactivity on thymocytes, mainly at the corticomedullary junction. *B,D)* adjacent sections showing displacement of immunostaining after preabsorption of the antibodies with 100 nM of the respective peptide antigens. Magnification 400 X; *Bar* 25 μm. Bottom panel (paraffin-embedded sections of thymoma). *A)* sst_{2A}-immunoreactivity localized on the endothelium of an intratumoral vessel. *C)* sst_3-immunoreactivity within the thymocytes and tumor cells. *B,D)* adjacent sections showing displacement of immunostaining after preabsorption of the antibodies with 100 nM of the respective peptide antigens. Magnification 400 X; *Bar* 25 μm.
Sections developed with New Fuchsine/Naphtol AS-MX. The sections are slightly counterstained with hematoxylin.

Immunohistochemistry. All the samples, except for the thymomas, were histologically normal. In the spleen sst_{2A} immunoreactivity was localized in the marginal zone and diffusely in the red pulp, while sst_3 immunoreactivity was observed in lymphoid-appearing cell clusters in the white pulp, particularly localized in the outer regions of the periarteriolar lymphatic sheaths. No sst_3 immunoreactivity was observed in the red pulp (Fig.5, upper panel). In the thymus, immunoreactivity for sst_{2A} receptors was predominantly localized in the medulla, particularly around Hassal's corpuscles, while sst_3 receptor immunoreactivity was observed mainly at the cortico-medullary junction, but in the medulla as well (Fig.5, central panel). In one of the thymomas, weak sst_{2A} immunoreactivity was found on the endothelium of some small intratumoral vessels, while clear sst_3 immunoreactivity was observed on the tumor cells and reactive thymocytes (Fig.5, bottom panel). In all cases, immunostaining could be completely abolished by pre-absorption with 100 nM of the respective peptide antigens (Fig.4B,D).

DISCUSSION

Based on the higher amount of SSR on tumor cells when compared to normal tissues, *in vivo* SSR scintigraphy using [^{111}In-DTPA-D-Phe^1]-octreotide has become a highly sensitive method in detecting SSR-positive tumors (13,14). However, the visualization of normal organs, namely pituitary gland, thyroid, spleen, liver, and kidney occurs as well. In the spleen it has been demonstrated that the *in vivo* radiolabeled SS analog uptake is due to the presence of SS-binding sites within the tissue (15). Conversely, the normal and hyperplastic thymus, which is known to contain SSR as well, is generally not visualized during SSR scintigraphy (8,15,17). Thymic visualization barely occurred in a few very young children (30), while tumors originating from this organ were successfully localized (18-21). [^{111}In-DTPA-D-Phe^1]-octreotide accumulation in sst_2-expressing cells may be determined by the internalization of the radioligand-receptor complex. Several authors have shown this mechanism, demonstrating the internalization of SS, octreotide and radiolabeled SS analogs into sst_2-expressing cells *in vivo* (31-35). The majority of human SSR-positive tumors, which display a significant *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide, express multiple SSR subtypes (36). It has recently been shown that among several SSR scintigraphy-positive tumors, 3 thyroid tumors lacked the expression of sst_2 receptor (37), suggesting that tissue expression of sst_2, which has the highest affinity for octreotide, is not a prerequisite for

positive imaging. An additional important observation is the absent or only faint expression of sst_2 mRNA in normal thyroid tissue (37,38), since the thyroid gland, as well as the kidneys are constatly visualized during [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy, and the uptake may be slightly decreased but not abolished during continuous treatment with "cold" octreotide. Conversely, the uptake in the spleen is markedly reduced or even abolished during octreotide therapy (39). In agreement with this, in our representative case shown in figure 1 who underwent SSR scintigraphy during long-term treatment with octreotide, the uptake of the radiopharmaceutical was abolished in the splenic region, while not in the kidneys (Fig.1B). Thus, the heterogeneity of SSR subtype expression might play a significant role in determining the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide, accounting for the differential behaviour of SSR-expressing tissue during administration of competitive "cold" octreotide.

In order to evaluate whether the level of different SSR subtypes might be involved in the heterogeneity of *in vivo* [^{111}In-DTPA-D-Phe^1]-octreotide uptake as well, we used quantitative RT-PCR analysis, which allows examining the relative amount of mRNA expression. This technique has been recently successfully employed for the quantification of SSR subtype profiles in human pituitary tumors (40). We found a predominant expression of sst_{2A} mRNA in normal human spleen, while normal or hyperplastic thymus and one of the thymomas expressed relatively higher sst_3 receptor mRNA compared to the spleen. The high and almost selective expression of sst_{2A} mRNA in the human spleen is in line with the evidence of high *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide as well as the decreased accumulation of the radiopharmaceutical occurring during octreotide therapy. In fact, treatment with octreotide may saturate sst_{2A} receptors, which have the highest affinity for this octapeptide SS analog. However, in thymic tissue and especially in the hyperplastic state and in the B2 thymoma, sst_3 mRNA is predominantly expressed. Indeed, this thymoma (B2) was clearly visualized during SSR scintigraphy, suggesting that the involvement of sst_3 receptors in the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide can not be ruled out. *In vitro* studies revealed a low expression of sst_{2A}, the absence of sst_5 and high expression of sst_3 receptor in the B2 thymoma. Considering the highest agonist-dependent internalization rates demonstrated for sst_3 receptor (31), this SSR subtype might be involved in the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide as well. Conversely, in the second tumor, classified thymoma A and not visualized during [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy, the expression of sst_3 mRNA was lower than that in the normal

thymic tissue. Interestingly, the highest amount of sst_{2A} and sst_3 receptors, but with a sst_{2A}/sst_3 ratio comparable to the normal thymus, has been found in the single case of thymic hyperplasia that was studied. Further studies on a larger number of hyperplastic and tumorous thymuses are needed, however, to confirm this observation.

The above-described *in vitro* techniques do not allow to distinguish which cell type expresses a distinct SSR subtype. Moreover, lymphoid tissues are rather complex both in their structure and in the heterogeneity of cell content. In the last few years much progress has been made in the cellular localization of SSR, due to the development of antisera against specific peptide sequences of SSR subtypes. Immunohistochemistry allowed documenting the distribution at the cellular level of the receptor proteins in normal and abnormal tissues (22-25). SSR subtypes have been recently evaluated by immunohistochemistry in lymphoid tissues as well, where the receptor expression is much lower compared to neuroendocrine tissues (26-28). Using two specific antibodies for sst_{2A} and sst_3 receptors, respectively, we found sst_{2A} immunoreactivity mainly located in areas where microenvironmental cells are prevalent, while sst_3 immunoreactivity was preferentially observed on cells that have recognizable lymphoid morphology, in both spleen and thymus. In fact, sst_{2A} immunoreactivity was preferentially located in the medulla of the thymus, where epithelial cells, dendritic cells, and macrophages are predominant cell types, and in the marginal zone and red pulp of the spleen, where monocytes and macrophages are predominant. These data are in agreement with the binding of the sst_2-preferring ligand, [^{125}I-Tyr^3]-octreotide, detected at SSR autoradiography. Conversely, sst_3 immunoreactivity was preferentially observed in the deep cortex of the thymus, as well as in the border areas with the medulla, which are regions containing highly and densely packed developing thymocytes. In the spleen, sst_3 immunoreactivity was detected in the periarteriolar lymphoid sheaths of the white pulp, which are densely packed of T lymphocytes. Finally, immunohistochemistry confirmed the predominant expression of sst_3 in the B2 thymoma tissue, which is a tumor with a predominance of lymphocytes (41). These observations confirm previous data showing a strong compartmentalization of neuropeptide receptors in lymphoid tissues (8). The distribution of SSR in these lymphoid organs suggests that distinct receptor subtypes might be expressed at specific differentiation stages of lymphoid or accessory cells. Moreover, in the spleen the detection of immunoreactive cells near the marginal zone might be related to their antigenic stimulation. The marginal zone is populated by a mixture of macrophages having a CD11b

and CD14 phenotype and many pale endothelioid cells, plus few T and B lymphocytes (42). These findings are in agreement with the recent observation of sst_{2A} immunoreactivity in endothelial cells of venules and capillaries, as well in a subset of cells of the monocyte/macrophage lineage in tissue biopsies from patients with human immune-mediated diseases, such as rheumatoid arthritis (26). Moreover, sst_{2A} immunoreactivity has been observed in biopsies from patients suffering from granulomatous diseases, such as sarcoidosis and Wegener's granulomatosis, again associated with cells of the mononuclear phagocyte lineage, including epithelioid cells and multinucleated giant cells within the granulomas (27). Finally, the predominant immunoreactivity for sst_3 receptor in areas rich of thymocytes in the thymus and in areas rich of T cells in the spleen is in line with the recent data showing the selective expression of sst_3 mRNA in human resting peripheral T lymphocytes (43).

In conclusion, the sst_{2A} receptor determines the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in the spleen. However, since [^{111}In-DTPA-D-Phe^1]-octreotide binds with high affinity to sst_2 receptors, but with lower affinity to sst_3 as well, and because uptake of this radiopharmaceutical is observed in a sst_3-expressing tumor lacking sst_{2A} and sst_5 receptors, the sst_3 may be involved in the *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide. The affinity of the radioligand for the receptor, as well as the efficiency of internalization of the radioligand-receptor complex might play a concomitant role in determining the uptake in receptor-positive tissues during *in vivo* SSR scintigraphy. However, the heterogeneity of expression and the level of SSR subtypes may contribute in determining the uptake as well. Indeed, most of the data on agonist-induced internalization of the 5 SSR subtypes are derived from studies using transfected cell lines. Further data on the internalization of SSR ligands by cells which express SSR subtypes endogenously are mandatory in order to elucidate several remaining questions regarding human SSR-positive cells. Although in lymphoid tissues SSR subtype distribution needs to be further investigated, the present data indicate that specific SSR subtypes are expressed on distinct cell subsets within immune tissues. Such a distribution might explain the heterogeneity of the effects exerted by SS in the immune system.

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CHAPTER IV

GENERAL DISCUSSION

GENERAL DISCUSSION

In the second half of the last century, a large number of studies have demonstrated the existence of tonic control of the immune system by hormones and neuropeptides, as well as the influence of immune-derived products on neuro-endocrine mechanisms (1). Virtually all tissues throughout the human body may contain resident immune cells. During certain physiologic or pathophysiologic conditions, these cells are recruited and are involved in immunological and inflammatory processes. However, resident immune cells and their products coexist with hormones and neuropeptides, which reach the interstitial space or are locally produced by the diffuse neuroendocrine system, or are even generated by immune cells themselves, and interact with them (2,3). The result of this integrated system displays the final responses of the organism in order to preserve its own homeostasis (1-3).

Specialized primary lymphoid organs, apart from being the organs where immune cells develop, may be considered preferential sites where the above-described immune-neuro-endocrine interactions mainly occur. In support of this concept, lymphoid organs are extensively innervated by autonomic and peptidergic fibres. Furthermore, neuropeptides are actively produced in the microenvironment of these organs (1).

SS is a neuropeptide that has been extensively investigated for its well-known inhibitory actions in the nervous and endocrine systems. However, the immunomodulatory role of SS has gained interest since SSR were described in sites of immunological and inflammatory activity, as well as directly on immune cells (4). Although the expression of the five SSR subtypes on endocrine and neural cells has been extensively investigated, the data on immune cells remain contradictory. For example, conflicting results have been obtained in cell lines from different species (animal and human cell lines), as well as primary cultured cells (from animals and humans). This points to a differential expression of SSR on immune cells between animal and human models (5). The situation becomes even more complex as SSR subtype expression in immune cells seems to be dependent on the activation state of cells or on the localization of immune cells (homing in tissues or circulating cells) (1,5). Finally, SS may exert a "biphasic" effect on immune cells, with inhibition at low (nanomolar) concentrations and absence of an effect at higher

(micromolar) concentrations. The reasons for these observations are unclear, but may involve receptor internalization and subsequent down-regulation, uncoupling from second messenger activation, and/or activation of different intracellular second messenger pathways *via* distinct SSR subtypes (6). However, although there are differences between neuroendocrine and immune cells, some basic aspects are comparable between these different cell populations, considering also that these cells closely collaborate and share specific signalling molecules.

The focus of this thesis is the human thymus. This choice was made because this primary lymphoid organ represents a paradigmatic case of integration between the neuroendocrine and immune systems. Complex intrathymic circuits involving the local production of neuropeptides and hormones participate in the primary "lymphoid" function of this organ, namely the development of T-cell repertoire, as well as in the less explored "endocrine function" of the human thymus (7). The main aim of this thesis was to evaluate whether SS and its receptors play a role in this system. Recently, it was demonstrated that thymic epithelial tumors could be visualized *in vivo* by SSR scintigraphy using radiolabeled SS analogs (8,9). Since rather scarce and conflicting data on SSR expression and their significance in the normal and neoplastic thymus were present in the literature, the first step was to characterize the SS and SSR subtype expression in the normal human thymus. Emphasis was given to the specific cellular localization of SSR subtypes and to the potential significance of such distribution (chapter II).

SS and SSR subtypes characterization, distribution and functional role in normal human thymus

Mainly binding studies and RT-PCR were used to characterize SS and SSR subtype expression in normal human thymus. SS and three SSR subtypes, sst_1 and sst_{2A} and sst_3 were found in the tissue (chapter II.4) (10). No expression of the splicing variant sst_{2B} was found in any of the tissues and cells studied. The expression of these different subtypes appeared to be heterogeneous within the tissue and to be localized on different cell subsets. A selective expression of sst_1 and sst_{2A} was found on TEC, in line with the evidence of [^{125}I-Tyr^3]-octreotide binding in the thymic medulla, where TEC is the prevalent cell type (chapter II.4). In fact, [^{125}I-Tyr^3]-octreotide is considered a sst_2-preferring ligand. TEC were found also to be a potential source of SS production within the thymus, since SS mRNA was found to be expressed in these cells (chapter II.4) (10). Conversely, SS mRNA was undetectable in thymocytes and the distribution of SSR subtypes on this heterogeneous cell

population appeared complex (11). In fact, in freshly isolated thymocytes sst_{2A} and sst_3 mRNA expression was detected, while in long-term cultured thymocytes no mRNA encoding for SSR subtypes was detectable (chapter II.5), suggesting a relatively rapid down-regulation of SSRs on thymocytes during culture. Moreover, sst_3 mRNA expression was higher in intermediate/mature thymocytes, whereas sst_{2A} mRNA was highly expressed in the more immature fractions. In addition, sst_{2A} was the predominant receptor in the early $CD34^+$ thymocytes, which belong to the most immature subset of lymphoid cells in human thymus (chapter II.5). The low fraction of the latter cell subsets of the total thymocyte population might explain the lack of binding of the sst_2-preferring ligand [^{125}I-Tyr^3]-octreotide in the study of thymocyte membrane homogenates (chapter II.5). Therefore, the binding of the universal ligand [^{125}I-Tyr^{11}]-SS-14, which binds to all 5 SSR subtypes with high affinity, was likely due to the binding of this ligand to sst_3 receptors on the intermediate/mature cells. These cells represent the major population of thymocytes. In general, the number of SS-binding sites was low, in agreement with the presence of a low number of SS-binding sites on circulating human T lymphocytes (12), which derive from thymocytes. Finally, on macrophages, the third thymic cell component examined, the presence of only sst_{2A} mRNA was detected (chapter II.5). This finding is in agreement with previous reports showing the selective expression of this SSR subtype on human macrophages and monocytes (13,14, Lichtenauer-Kaligis EGR *et al.*, unpublished observations). The presence of functional SSR subtypes on both TEC and thymocytes was demonstrated by *in vitro* studies on isolated cell cultures using SS and the SS analog octreotide (chapters II.4 and II.5).

The first consideration is that the expression pattern of SSR subtypes in the thymus shows a species-specific distribution. There are substantial differences between man (chapter II.5) and the two more extensively studied animal models, i.e. the rat and mouse (15-18). These differences make both these two models rather unsuitable for studying the effects of SS on human thymic cells. Also the local production of SS seems to have a different source in mouse and human. In fact SS mRNA was found in human TEC (chapter II.4), but not in thymocytes or monocyte/macrophage lineage cells, whereas in mouse the peptide is mainly present in macrophages (10,19).

Taking into consideration the peculiar human thymic microenvironment, its architecture and the specific pattern of SSR distribution, the existence of specialized areas where SS and its receptors might exert differential activities may be hypothesized.

On microenvironmental cells, sst_{2A} seems to be the receptor predominantly expressed and an autocrine or paracrine activity of SS could be involved in controlling the different functions of these cells. A potential role for the neuropeptide in controlling TEC growth is suggested by the inhibitory effect of both SS and its octapeptide analog octreotide on unstimulated and growth factor-stimulated proliferation of cultured TEC (10) (chapter II.4). Interestingly, pituitary hormones, which are under hypothalamic SS control, such as GH and PRL, as well as IGF-I enhance TEC proliferation (20-23). However, since sst_{2A} is the SSR subtype mainly involved in controlling secretion processes by neuroendocrine cells, further studies should investigate this possibility for thymic cells as well. In fact, TEC and macrophages produce a significant number of thymic hormones and cytokines, and these thymic secretory substances have previously been demonstrated to be influenced by other neuroendocrine factors (7). Both TEC and macrophages drive the maturation of thymocytes and are actively implicated in the positive and negative selection of the future T lymphocytes. In addition, macrophages are classical antigen-presenting cells and may play this role in the thymus as well. The presence of specific SSR on these cells suggests the involvement of the neuropeptide in regulating such highly specialized cell activities in the thymus. On the other hand, SS and other neuropeptides have been reported to effectively modulate proliferation, cytokine production and antigen presentation by macrophages in the skin under physiological and pathophysiological conditions (24).

However, sst_{2A} receptors are expressed on a subset of human thymocytes as well (chapter II.5). The pattern of SSR subtype expression on thymocytes seems to follow a predisposed order related to the different stage of maturation of the lymphoid cells (chapter II.5). The most immature cortical thymocytes preferentially express sst_{2A}, while moving towards the medulla, where thymocytes are more mature, sst_3 becomes the SSR subtype predominantly expressed on these cells (chapter II.5). This pattern of expression is particularly interesting considering that sst_{2A} has been indicated as the main SSR subtype expressed by progenitor cells in murine bone marrow (Oomen S, unpublished observations). The higher expression of this subtype on $CD34^+$ early thymocytes (chapter II.5) seems in line with this evidence. Precursors from bone marrow reaching the thymic cortex might keep the expression of sst_{2A} until a certain phase of their intrathymic maturation. It is important to underline that immature thymocytes are actively proliferating cells, undergoing a rearrangement process of surface antigen molecules, which will constitute the future phenotype and commitment of the developing thymocyte. The presence of sst_{2A} receptors in this phase might be related to

the necessity of a modulatory action on these processes, which may be exerted by SS *via* this receptor subtype.

Conversely, sst_3 is expressed mainly on thymocytes that have reached a higher level of maturation during the complex cascade of events occurring in the thymic network (chapter II.5). Interestingly, this SSR subtype seems to be selectively expressed on peripheral resting T lymphocytes, which directly derive from mature thymocytes (12). In the thymus, the majority of thymocytes are destined to die as a consequence of failing selection (25). Since cell death in the thymus occurs by apoptosis, the expression of the sst_3 on these cells is intriguing. In fact, this SSR subtype may be involved in agonist-mediated apoptosis (26). The presence of a receptor involved in this sophisticated process is highly suggestive for a modulatory activity of SS in thymocyte deletion. However, further studies should elucidate whether SS might have an inductive or protective effect on programmed cell death in the human thymus. Recently, potent inducers of apoptosis in thymocytes, such as glucocorticoids, have also been shown to be implicated in the rescue of thymocytes from activation-induced cell death (27).

Emerging data have shown that adhesion molecules, extracellular matrix ligands and receptors drive thymocyte traffic, in terms of entrance of precursors, migration within the thymus, as well as exit of mature cells from the organ. This process seems to be influenced by neuroendocrine products as well (28-32). Since SS and other neuropeptides are involved in regulating the migration of immune cells in other compartments throughout the human body (33-35), and considering the peculiar distribution of SSR within the thymus, SS probably plays a role in the modulation of this mechanism. This modulation may be important considering that a disturbance in this process may favour traffic of immature or wrongly selected cells towards peripheral lymphoid organs, enabling the development of autoimmune diseases.

The recent availability of the antibodies specific for sst_{2A} and sst_3 receptors made it possible to further investigate the cellular localization of these receptor subtypes by immunohistochemistry in human lymphoid tissues (chapter III.5). Apart from the thymus, the normal human spleen has been investigated as well. As already discussed, these tissues are highly complex both in their structure and in the heterogeneity of cell content. However, in general, sst_{2A} immunoreactivity was mainly located in areas where stromal, non-lymphoid cells were prevalent, while sst_3 immunoreactivity occurred preferentially on cells with lymphoid morphology, both in the spleen and in the thymus (chapter III.5). The

sst_{2A} immunoreactivity, preferentially located in the medulla of the thymus and in the marginal zone and red pulp of the spleen, is in agreement with the binding of the sst_2-preferring ligand, [^{125}I-Tyr^3]-octreotide, detected by SSR autoradiography, and with the expression of sst_{2A} mRNA in isolated TEC (10). Conversely, sst_3 immunoreactivity preferentially observed in the deep cortex of the thymus as well as in the border areas of cortex and medulla, and in the periarteriolar lymphoid sheaths of the white pulp of the spleen, points to a clear lymphoid cell localization of this SSR subtype. Again, these results were in line with those of ligand-binding studies and RT-PCR. Thus, with this technique, it might be concluded that distinct SSR subtypes are expressed at specific differentiation stages or states of lymphoid and accessory cells.

A novel finding in the spleen is the detection of sst_{2A}-immunoreactive cells near the marginal zone, which might be related to the antigenic stimulation of these cells as well as their activity as antigen-presenting cells (chapter III.5). In general, SSR distribution within these lymphoid tissues might offer an explanation for the variety of effects exerted by stimulation with SS of heterogeneous populations of immune cells.

A final, but not less important, potential activity of SS within the thymus might be its involvement in the control of the physiologic involutive process which occurs in this lymphoid organ with ageing (chapter II.6). The human thymus undergoes involution and decreases its activity in producing mature T cells. However, recent evidence points at the maintenance of an "endocrine" activity of this organ, which might need a differential regulation during ageing (36). Further studies are necessary to clarify whether the decrease of SSR in the human thymus during ageing is a programmed step during the involution or whether it is a consequence of the process in itself.

The local production of SS in the human thymus seems to be extremely relevant since the neuropeptide might act at different levels, on different thymic cells and *via* different receptor subtypes. SS within the human thymus might be considered as a potential modulator of the homeostasis of various cell components as well as a regulator of their specialized activities. Moreover, SS seems to directly participate in the main thymic function, namely the maturation of the human T cell repertoire. The hypothesis of a multiple role of SS in the thymus is in line with the similar observation for another well-known neuropeptide, VIP. In fact, both SS and VIP are produced within this organ and a strong compartmentalization of their respective receptors has been shown on human thymic cells (37). Like SSR, the two VIP receptor subtypes display a distinct distribution in

different thymocyte subsets, at least on murine and rat thymus, confirming that the expression of neuropeptide receptors could be differentially regulated during development (38). Finally, VIP may also affect multiple aspects of thymic cell functions (37,38). An integrated role for these two neuropeptides, possibly together with SP, has been already hypothesized (39). However, the exact parameters regulating a hypothetical intrathymic circuit between these three substances have not been established yet. In general, in different pathophysiological conditions SS and VIP seem to "counteract" the effect of SP on immune cells (40-42). However, in other situations, SS and VIP may have opposite effects on specific cell subsets, while acting synergistically on others (42).

A summarized and suggestive scheme of SSR distribution and SS activities within the normal human thymus in reported in Figure 1.

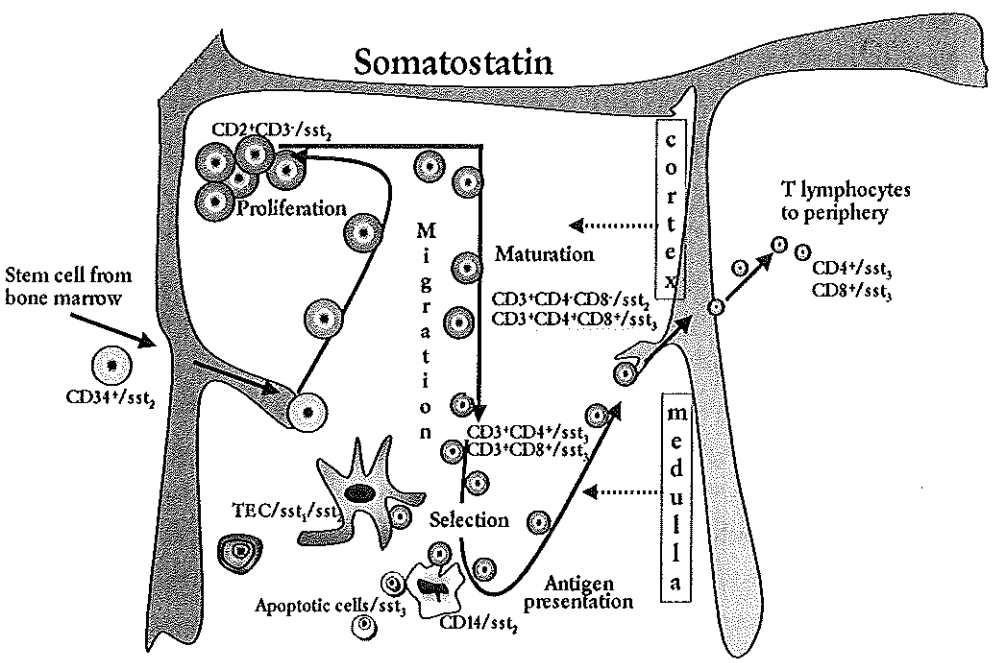

FIGURE 1. Hypothetical scheme for the principal actions of SS within the normal human thymus. SS is locally produced and multiple receptor subtypes are expressed on different cell subsets or are differentially expressed on the same cell type. SS might act at different levels, modulating several processes within the organ. Since SSR are expressed on TEC and thymocytes, cell proliferation and perhaps production of specific factors may be controlled by the neuropeptide. Traffic, maturation, selection and deletion of thymocytes might be influenced by SS as well. Finally, the activities of cells belonging to the monocyte/macrophage lineage might also be targets of SS.

The hypothesis on the relevance of SS and SSR in thymus physiology discussed above form the basis for a better understanding of some mechanisms, which may occur in pathological conditions of this organ.

Distribution and significance of SS and SSR in human thymic tumors

Like other tumors originating from SS-target tissues, human thymic epithelial tumors may express SSR. These neoplasms are visualized *in vivo* during SSR scintigraphy using [^{111}In-DTPA-D-Phe^1]-octreotide (8,9) (chapter III.4). Although preliminary ligand-binding studies failed to detect SSR on cryostat sections of human thymomas (39), at least three SSR subtypes have been detected by immunohistochemistry using polyclonal antibodies specific for sst_1, sst_{2A} and sst_3 receptors (chapters III.3 and III.4). Moreover, in one case the expression of SSR subtypes was confirmed at mRNA level by RT-PCR analysis (43) (chapter III.3). Since the number of receptors in normal thymic tissue as well as in thymoma is rather low, a different sensitivity of the two methods might in part explain this discrepancy. Sst_{2A} and sst_3 receptor expression was found *in vivo* in a series of human thymic tumors that were visualized by *in vivo* SSR scintigraphy as well. Considerable heterogeneity of SSR immunoreactivity was found within and among the tumors (chapter III.4). Although both receptors were expressed within the tumor tissue, overall sst_3 showed higher expression compared to sst_{2A} and displayed a preferential localization on reactive thymocytes. However, human thymomas are a heterogeneous class of tumors (44). Moreover, sst_{2A} and sst_3 receptors are expressed in the normal thymus as well, whereas this organ can not be visualized by SSR scintigraphy. Therefore, the density and the heterogeneity of such distribution have been further investigated (chapter III.5). The normal human spleen was also included in this study, as a paradigmatic case of a normal lymphoid organ, which is in fact visualized during SSR scintigraphy (chapter III.5). The study conducted in the human spleen, the normal and hyperplastic thymus as well as various thymomas revealed a peculiar density and distribution of sst_{2A} and sst_3 receptors in these lymphoid tissues (chapter III.5). Both receptor subtypes were heterogeneously expressed in all tissues, but with a predominant, almost selective expression of sst_{2A} receptors in the spleen, and a prevalent expression of sst_3 receptors in the normal and hyperplastic thymus as well as in one thymoma. Analyzed in detail, these results were useful to clarify some apparently equivocal data. Firstly, the heterogeneity of SSR expression and the density of different receptor subtypes might play a crucial role in determining the *in vivo*

uptake of radiolabeled SS analogs, at least in lymphoid tissues. The data from this study suggested that the presence of sst_2 receptors is not an essential prerequisite for the visualization of SSR-positive tissues during [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy. The accumulation of this radiopharmaceutical in sst_{2A}-expressing cells is determined by the internalization of the radioligand-receptor complex. The high and almost selective expression of sst_{2A} receptors in the human spleen is in line with the evidence of high *in vivo* uptake of [^{111}In-DTPA-D-Phe^1]-octreotide as well as with the decreased accumulation of the radiotracer occurring during "cold" octreotide therapy. In fact, treatment with octreotide may saturate sst_{2A} receptors, which have the highest affinity for this octapeptide SS analog. In one of the two thymomas, sst_3 mRNA was predominantly expressed, suggesting that the involvement of sst_3 receptors in the uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in thymomas cannot be ruled out (chapter III.5). The high agonist-dependent internalization rates that were demonstrated for sst_3 receptors may support this hypothesis (45). Further investigations are necessary to establish if this concept might be valid for other SSR-expressing tissues as well. The possibility that other factors may influence the mechanisms involved in regulating the *in vivo* uptake of radiolabeled SS analogs in lymphoid tissues cannot be ruled out. Receptor turnover and binding properties might be regulated differently in lymphoid cells compared to neuroendocrine cells. In fact, the activation of SSR subtypes on lymphoid cells may induce opposite responses compared with endocrine cells (4-6). Taking into consideration that *in vivo* several factors may interact and influence each other, the target cell system might play its own part in determining the amount and the quality of the radiotracer uptake as well. It has been demonstrated that thymocytes, which are the major cell population in the human thymus, can rapidly down-regulate the expression of their SSR when cultured *in vitro* (chapter II.5). Apparently, thymocytes are cells with a wide plasticity in terms of modulation and expression of surface molecules. From this point of view it would be interesting to investigate what occurs in reactive thymocytes in thymomas. Perhaps *in vivo* SSR expression on these cells may be rapidly regulated and the affinity of receptors for their own natural and synthetic ligands might be differently modulated, depending on factors intrinsic to the tumor as well.

In general, in light of these new findings it may be hypothesized that, on the basis of its binding properties and receptor affinity, [^{125}I-Tyr^3]-octreotide may be considered as a clear sst_2-preferring ligand for *in vivo* binding studies. *In vivo* several exogenous factors may influence these properties and may play a role in determining the uptake of [^{111}In-DTPA-D-

Phe[1]]-octreotide within SSR-expressing tissues and tumors as well. This is a point that may have important consequences for future applications of the currently available SS analog for systemic radiotherapy as well as for the development and use of novel subtype-selective analogs and antagonists. In addition, this knowledge may open new perspectives in the clinical application of SS and its analogs in autoimmune and neoplastic diseases involving the immune system. Literature data point to a certain efficacy of treatment with SS analogs in this respect. Of particular interest is the reported successful treatment of patients bearing thymomas associated with paraneoplastic syndromes with the SS analog octreotide or with the combination of octreotide and corticosteroids (9,46). In one of these patients, a clear reduction of tumor size was observed after long-term treatment with octreotide and prednisone. However, a significant improvement was observed in the thymoma-associated red cell aplasia as well (46). As far as the effect on the autoimmune disease is concerned, further studies should investigate whether the effect of therapy with SS analogs may have a rationale in the treatment of the severe autoimmune diseases which are frequently associated with thymic disturbances.

As for their role in determining the *in vivo* uptake of labeled SS analogs, SSR expression in these tumors is with high probability connected to the effects on the tumor mass exerted by therapy with SS analogs (chapter III.4). A point in favour of this may be extrapolated from the analysis of one paradigmatic case displaying a significant *in vivo* uptake of [^{111}In-DTPA-D-Phe[1]]-octreotide and a preferential expression of sst_3 receptors *in vitro* (chapter III.3). *In vitro*, this cortical thymoma showed a significant inhibition of cell proliferation when SS and octreotide were administered. The two intriguing findings from this tumor were the selective expression of sst_3 on cultured neoplastic cells, which candidate this receptor as a mediator of the *in vivo* inhibitory effects of SS and octreotide, and the lack of endogenous SS in tumor tissue (chapter III.3) (44). As already discussed in the first section, SS is endogenously produced in the normal thymus and a wide spectrum of activities has been suggested for this neuroeptide on the thymic microenvironment, especially with regard to T cell maturation and more general, on cell homeostasis (chapter II). Although very preliminary, the loss of SS in a tumor arising from this organ might be involved in the pathogenesis of the neoplastic transformation as well as in the pathogenesis of the autoimmune diseases often associated with these complex tumors. A disturbance in the network of SS and its receptors in the thymus may be suggestive for its implication in the occurrence of human autoimmune diseases. In this respect it would be interesting to

further investigate the mechanisms of the significant up-regulation of SSR observed in one case of thymic hyperplasia associated with *myasthenia gravis* (chapter III.5). However, as previously underlined, thymomas represent a highly heterogeneous class of tumors and further investigations are mandatory to elucidate the significance of the specific SSR subtype expression in these tumors.

SSR on target cells can be actively up- or down-regulated by the exposure to several heterologous agents as well as to its own ligand. In general, changes in the density, quality and activity of local regulatory factors might alter the distribution and concentration of neuropeptide receptors and their ligands. This may also apply to the human thymus, where the global activity of the organ is based on the balance of several regulating factors locally produced. Disturbances in this delicate equilibrium may lead to the induction of inappropriate immune responses and to the occurrence of diverse diseases.

FUTURE PERSPECTIVES

The data presented and discussed in this thesis indicate that SS is involved in the complex neuroendocrine control of human thymus physiology. The presence of specific and distinct SSRs on both lymphoid and microenvironmental cells suggests that the neuropeptide might be necessary to maintain various biological functions of this organ. Further studies should be designed to establish the functional significance of such a receptor distribution pattern and to elucidate the role of SS in the interactions occurring between thymocytes and microenviromental cells.

The use of foetal thymus organ cultures and *in vivo* experiments will certainly be useful to define the extent and the importance of the autocrine and paracrine circuits involving SS and other mediators within the human thymus, including their role in thymic development. Moreover, the use of different knockout mice, as well as genetically engineered animals in which the receptor gene can be activated may represent additional experimental models highly useful in better determining the relative significance of a given receptor and the respective ligand in such a complex system. However, it should be emphasized that caution must be taken in extrapolating data from animal models, considering the apparent differences existing in neuropeptide receptor distribution between different species.

Taking into account the peculiar distribution of SSR receptor subtypes within the human thymus, multiple targets for the activity of SS may be identify in this primary lymphoid

organ. Several hormones and neuropeptides are known to exert a control on the secretion of different intrathymic factors, such as thymic hormones and cytokines, as well as other "classical" hormones and neuropeptides produced by either stromal or lymphoid elements. This is a rather unexplored field, especially concerning the role played by SS. Furthermore, the presence of different SSR subtypes on antigen-presenting cells and on specific thymocyte subsets warrant further investigations. In fact, the potential involvement of SS in controlling specialized activities of these cells, as well as the traffic of lymphoid cells within the thymus might be important to develop new therapeutic strategies based on the selection and manipulation of lymphoid cells during their intrathymic differentiation. This point might become extremely relevant in light of the new advances in transplantation of postnatal cultured thymic tissue in patients with severe immunodeficiency syndrome (47). Finally, a further *in vitro* characterization and more controlled *in vivo* studies including a larger number of cases are required to better understand the functional and clinical significance of SSR in human thymic neoplasms and to clarify the mechanism of action of SS analogs in the treatment of these tumors.

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SUMMARY

Specialized primary lymphoid organs are the preferential sites of immune-neuro-endocrine interactions. SS exerts mainly inhibitory actions in the nervous and endocrine systems. However, it has an immunomodulatory role as well, and the study of its activities in the immune system has opened a new stimulating area of research. Dta on the expression of the five SSR subtypes on immune cells is still conflicting. Results obtained in cell lines as well as primary cultured cells point to a differential expression of SSR on immune cells in animal and human models. In contrast to neuroendocrine cells, SS exerts a "biphasic" effect on immune cells, *via* unclear mechanisms. Although there are differences between neuroendocrine and immune cells, some basic aspects are comparable.

In this thesis the attention has been focused on the human thymus, since this lymphoid organ represents a paradigmatic case of integration between the neuroendocrine and immune systems, where the role of SS and SSR have been poorly explored so far.

Two main questions have been addressed. First, the identification of the SSR subtypes expressed in the normal human thymus and the impact of the system SS/SSR in the physiology of this primary lymphoid organ; secondly their role in the pathological conditions of this organ was investigated. The answers to these questions might have important consequences for the diagnosis and therapy of disturbances involving the immune system.

Chapter I gives a comprehensive introduction to the most recent knowledge on the significance of neuropeptides and their receptors within the immune system, with special emphasis to SS and SSR. In **chapter II,** the first two paragraphs introduce the structure and function of the normal human thymus and its neuroendocrine circuits, followed by the characterization of SS and SSR subtype expression, with special attention to the specific cellular localization of SSR subtypes and to the potential significance of such distribution in thymus physiology and pathophysiology (**chapter II.3, 4 and 5**).

SS, sst_1 and sst_{2A} and sst_3 were found heterogeneously expressed within the tissue and in different cell subsets. A selective expression of sst_1 and sst_{2A} was found on TEC, which also revealed to be a potential source of endogenous SS (**chapter II.4**). Conversely, SS was

undetectable in freshly isolated thymocytes. These cells expressed sst_{2A} and sst_3 receptors as well. However, differently from TEC, after long-term culture thymocytes rapidly down-regulated their SSR expression (**chapter II.5**). While sst_{2A} was progressively higher expressed in the most immature fractions, sst_3 expression was higher in intermediate/mature thymocytes. The number of SS-binding sites was low, in agreement with the same finding on thymocyte-derived circulating T lymphocytes. On thymic macrophages, the third component examined, only the presence of sst_{2A} was found (**chapter II.5**). The species-specific distribution of SSR and the different source of SS in humans makes it difficult to find a model for studying the activity of SS on thymic cells. The presence of functional SSR subtypes on both TEC and thymocytes was demonstrated by *in vitro* studies on primary cultured human thymic cells, using SS and octreotide (**chapters II.4 and II.5**).

Taking into consideration the peculiar microenvironment, the architecture and the specific pattern of SSR distribution in the human thymus, specialized areas were found where SS and its receptors exert differential activities.

Sst_{2A} is the receptor predominantly expressed on microenvironmental cells, where it could be involved in mediating autocrine or paracrine activity of SS. The neuropeptide may control unstimulated and growth factor-stimulated TEC growth (**chapter II.4**). However, TEC and macrophage production of thymic hormones and cytokines might be under influence of SS as well. In addition, the presence of sst_{2A} on macrophages suggests the involvement of SS in the regulation of the antigen presentation activity by these cells.

SSR receptors were expressed on human thymocytes, apparently linked to the level of maturation of the lymphoid cells (**chapter II.5**). The most immature cortical thymocytes preferentially express sst_{2A}, while more mature thymocytes express sst_3. The expression of sst_{2A} on immature early thymocytes is in line with the presence of this subtype in their progenitors in the bone marrow. This receptor might modulate the high proliferation rate of immature thymocytes. Sst_3 is mainly expressed on thymocytes at a higher level of maturation, as well as on peripheral T lymphocytes, which directly derive from mature thymocytes. Many of these cells are destined to die by apoptosis as a consequence of the selection process in the thymus. Sst_3 is involved in agonist-mediated apoptosis and its presence is highly suggestive for a modulatory activity of SS in thymocyte deletion (**chapter II.5**). Neuroendocrine products influence thymocyte traffic. Considering the peculiar distribution of SSR, SS might be involved in modulating this process as well.

The availability of antibodies specific for sst_{2A} and sst_3 receptors made it possible to further investigate the cellular localization of these receptor subtypes by immunohistochemistry in human lymphoid tissues (**chapter III.5**). Sst_{2A} immunoreactivity was mainly located in areas with prevalent microenvironmental cells, while sst_3 immunoreactivity occurred preferentially on cells with lymphoid morphology, both in the spleen and the thymus (**chapter III.5**). The localization of SSR immunoreactivity was is in agreement with the findings by binding studies and RT-PCR. Thus, it was concluded that distinct SSR subtypes are expressed on particular cell types and at specific differentiation stages or state of both lymphoid and accessory cells. In general, SSR distribution within these lymphoid tissues offered an explanation for the variety of effects exerted by SS.

Finally, the potential involvement of SS in controlling the physiologic involutive process occurring in the thymus with ageing is discussed in **chapter II.6**. However, further studies are necessary to clarify whether the decrease of SSR in this lymphoid organ with ageing is a programmed step during the involution, or whether it is a consequence of this process.

In the human thymus, SS seems to act at different levels, on different thymic cells and *via* different receptor subtypes, and might be considered as a potential modulator of the homeostasis of various cell components as well as a regulator of their specialized activities including the maturation of the human T cell repertoire. This is in line with similar observations for other neuropetides, such as VIP and SP. Although not yet established, an intrathymic circuit between these three substances might exist.

Like other tumors originating from SS-target tissues, human thymic epithelial tumors may express SSR. Thymomas are visualized *in vivo* during SSR scintigraphy using [^{111}In-DTPA-D-Phe^1]-octreotide (**chapter III.4**), and at least three SSR subtypes have been detected by immunohistochemistry in these tumors (**chapters III.3 and III.4**). Expression of sst_{2A} and sst_3 receptors was found *in vitro* with considerable heterogeneity in a series of human thymic tumors that were visualized by SSR scintigraphy *in vivo* (**chapter III.4**). Overall sst_3 showed a higher expression compared to sst_{2A} and displayed a preferential localization on reactive thymocytes. Since, sst_{2A} and sst_3 receptors are also expressed in the normal thymus, while this organ is not visualized during SSR scintigraphy, the spleen, the normal and hyperplastic thymus, as well as thymomas were further investigated in order to find out whether the density and the heterogeneity of receptor distribution may play a role (**chapter III.5**). The data suggested that the presence of sst_2 receptors is not an essential prerequisite for the visualization of SSR-positive tissues during [^{111}In-DTPA-D-Phe^1]-octreotide

scintigraphy, and that sst_3 receptors might be involved in determining the uptake, at least in some lymphoid tissues. In light of these new findings, it seems that [^{125}I-Tyr^3]-octreotide may be considered as a clear sst_2-preferring ligand for *in vitro* binding studies, while *in vivo* [^{111}In-DTPA-D-Phe^1]-octreotide might be less selective in this respect. This may have important consequences for the applications of SS analogs for systemic radiotherapy. The introduction of new subtype-selective analogs and antagonists may shed more light on this issue. New potential clinical applications of SS and its analogs in autoimmune and neoplastic diseases involving the immune system are under investigation. Of particular interest is the treatment with octreotide of patients bearing thymomas associated with paraneoplastic syndromes. Although further studies should investigate the mechanisms underlying these effects, therapy with SS analogs may have a rationale in the treatment of autoimmune diseases, whether or not associated wit thymic tumor associated or not to to thymic tumors. Moreover, in one thymoma studied both *in vivo* and *in vitro*, SS and octreotide inhibited the growth of the tumor cells *in vitro*. Interestingly, sst_3 was selectively expressed on cultured neoplastic cells (**chapter III.3**). In addition, the lack of endogenous SS mRNA in tumor tissue and in tumor cell was found. Although very preliminary, this might be involved in the pathogenesis of neoplastic transformation, as well as in the occurrence of autoimmune diseases often associated with these tumors (**chapter III.3**). In this respect it will be interesting to further investigate the mechanisms of the significant up-regulation of SSR observed in one case of thymic hyperplasia associated with *myasthenia gravis* (**chapter III.5**).

We concluded that SS and a set of SSR are expressed in the human thymus in both microenvironmental and lymphoid cells, but apparently selective. A highly specialized intrathymic circuit seems to integrate SS in the main function of this organ as well as in other activities, which needs further investigations. However, these preliminary results point out the important role of this neuropeptide and its receptors in thymic homeostasis. The overall activity of the thymus is based on the balance of several regulating factors, which are produced locally. Disturbances in this equilibrium may lead to the induction of umbalanced immune responses. This might be associated with thymic tumors and autoimmune diseases. Alterations in the circuit involving SS might be implicated in the pathogenesis of these diseases, and therapy with SS analogs might have an application in particular immune disorders.

SAMENVATTING

Lymfoide organen vormen een belangrijke plaats van immuno-neuro-endocriene interacties. Somatostatine (SS) heeft een voornamelijk remmende werking in het centrale zenuwstelsel en het endocriene systeem. Daarnaast heeft het peptide tevens een immunomodulerende rol. Studies met betrekking tot de activiteit van SS in het immuunsysteem vormen een belangrijk nieuw onderzoeksterrein. Het is nog niet opgehelderd welke van de vijf op dit moment bekende somatostatine receptor (SSR) subtypen tot expressie komen in immuuncellen. Resultaten verkregen door middel van studies in cellijnen en primaire kweken van immuuncellen laten duidelijke verschillen zien tussen modelsystemen van dier en mens. In tegenstelling tot neuroendocriene cellen, heeft SS op de funktie van immuuncellen een bifasisch effekt. Het mechanisme hierachter is nog niet opgehelderd. Naast een aantal duidelijke verschillen tussen neuroendocrienecellen en immuuncellen wat betreft de funktie van SS en SSR, zijn er ook overeenkomsten tussen beide celtypen.

Het in dit proefschrift beschreven onderzoek heeft zich met name gericht op de thymus van de mens, aangezien dit lymfoide orgaan een uitgesproken voorbeeld is van de integratie tussen het neuroendocriene systeem en het immuunsysteem. Bovendien is er nog vrijwel niets bekend wat betreft de expressie en rol van SS en SSR in dit orgaan.

Twee belangrijke aspekten zijn in dit proefschrift onderzocht. Ten eerste is de expressie van SSR en van het peptide SS zelf in de normale menselijke thymus vastgesteld en is de functionele betekenis van het SS/SSR systeem in dit primaire lymfoide orgaan onderzocht en bediscussieerd. Ten tweede is de rol van het SS/SSR systeem in de pathologie van de thymus onderzocht. De resultaten en conclusies van deze studies kunnen belangrijke consequenties hebben voor diagnose en therapeutische mogelijkheden bij stoornissen van het immuunsysteem.

Hoofdstuk I geeft een uitgebreide inleiding omtrent de huidige kennis van de rol van neuropeptiden en hun receptoren in het immuunsysteem. Speciale aandacht wordt besteed aan SS en SSR. De eerste twee paragrafen van **hoofdstuk II** geven een introduktie in de struktuur en funktie van de normale menselijke thymus en de neuroendocriene netwerken.

Vervolgens wordt de expressie van SS en SSR in de thymus beschreven, waarbij speciale aandacht is besteed aan de specifieke cellulaire lokalisatie van SSR subtypen en aan de potentiële betekenis van de celspecifieke distributie in de fysiologie en pathofysiologie van de thymus (**hoofdstuk II.3, 4 en 5**).

SS, sst_1, sst_{2A} en sst_3 vertonen een heterogene expressie in de thymus en komen differentieel tot expressie in de verschillende celtypen. Sst_1 en sst_{2A} komen selektief tot expressie in de epitheliale cellen van de thymus (TEC). Bovendien blijken deze cellen zelf de endogene bron van SS produktie te zijn (**hoofdstuk II.4**). Daarentegen blijken thymocyten niet in staat te zijn SS te producren. Thymocyten brengen sst_{2A} en sst_3 receptoren tot expressie. In tegenstelling tot TEC vertoont de expressie van sst_{2A} en sst_3 receptoren op thymocyten een snelle downregulatie wanneer zij in kweek gebracht worden (**hoofdstuk II.5**). Tevens worden verschillen in de expressie van sst_{2A} en sst_3 receptoren tussen verschillende thymocytpopulaties aangetoond. Sst_{2A} receptoren komen met name tot expressie in de immature populatie, terwijl sst_3 receptor expressie hoger is in de meer mature thymocyten. Het aantal SSR bleek erg laag te zijn, hetgeen in overeenstemming is met bevindingen in T-lymfocyten. Deze cellen zijn afkomstig van thymocyten. Op een andere thymuscelpopulatie, de macrofaag, werd alleen expressie gevonden van de sst_{2A} receptor (**hoofdstuk II.5**). De soort-specifieke verdeling van SSR in immuuncellen, alsmede de verschillen in de endogene bron van SS produktie tussen mens en dier, maakt het moeilijk om een geschikt modelsysteem te vinden waarbij het mogelijk is om de effekten van SS op thymuscellen te bestuderen. Het voorkomen van functionele SSR subtypen op zowel menselijke TEC als thymocyten is onderzocht middels *in vitro* studies waarbij remmende effekten van SS en het SS analogon octreotide op de celproliferatie van primaire kweken van deze cellen werden gevonden (**hoofdstuk II.4 en II.5**).

Sst_{2A} is het receptor subtype dat met name tot expressie komt in de thymocyt-omringende cellen, waarbij het betrokken kan zijn bij de autocriene en paracriene aktiviteit van SS. Het neuropeptide remt zowel de ongestimuleerde als de groeifactor gestimuleerde groei van TEC (**hoofdstuk II.4**). De productie en secretie van thymushormonen en cytokinen kan mogelijk eveneens door SS worden beïnvloed. Daarnaast bestaat de mogelijkheid dat de op de macrofaag aanwezige sst_{2A} receptor betrokken is bij de regulatie van de antigeen-presenterende funktie van deze cellen.

Zoals hierboven genoemd, lijkt de expressie van SSR op thymocyten gekoppeld te zijn aan de mate van maturatie van deze cellen (**hoofdstuk II.5**). De meest immature corticale

thymocyten brengen met name sst_{2A} receptoren tot expressie, terwijl de meer mature thymocyten preferentieel de sst_3 receptor expresseren. De expressie van sst_{2A} receptoren op de zeer vroege thymocyt lijkt in overeenstemming te zijn met de aanwezigheid van dit receptor subtype op de voorlopercellen in beenmerg. Dit receptor subtype zou betrokken kunnen zijn bij de modulatie van de hoge proliferatiesnelheid van immature thymocyten. De sst_3 receptor komt met name voor op de meer mature thymocyten, evenals op in het bloed circulerende T-cellen, die direkt afkomstig zijn van de mature thymocyt. De meeste mature thymocyten zijn gedoemd te sterven via een proces van geprogrammeerde celdood (apoptose) ten gevolge van het selektie proces in de thymus. De sst_3 receptor lijkt betrokken te zijn bij agonist-geïnduceerde apoptose. De aanwezigheid van dit receptor subtype suggereert de betrokkenheid van SS bij het proces van deletie van thymocyten (**hoofdstuk II.5**). Produkten van neuroendocriene cellen beïnvloeden de migratie van thymocyten. De karakteristieke distributie van SSR in de thymus beschouwend, zou tevens verondersteld kunnen worden dat SS een regulerende rol speelt in dit proces.

De beschikbaarheid van antilichamen specifiek voor sst_{2A} en sst_3 receptoren maakte het mogelijk om tevens de cellulaire lokalisatie van deze receptor subtypen in menselijke lymfoide weefsels in detail te bestuderen met behulp van immunohistochemische technieken (**hoofdtsuk III.5**). Sst_{2A} immunoreaktiviteit bleek met name gelokaliseerd te zijn in cellen die lymfoide cellen omgeven, terwijl sst_3 receptoren immunohistochemisch werden aangetoond op met name cellen met een lymfoide morfologie, in zowel de thymus als de milt (**hoofdstuk III.5**). Deze lokalisatie van SSR is in overeenstemming met bevindingen op basis van ligand-bindingsstudies en RT-PCR. Wij concluderen daarom dat bepaalde SSR subtypen selektief tot expressie komen op bepaalde celtypen, afhankelijk van de differentiatiestadia van deze cellen. De verdeling van SSRs in deze lymfoide weefsels kan een verklaring zijn voor de verschillende effekten die door SS geïnduceerd kunnen worden.

Tenslotte wordt in **hoofdstuk II.6** de potentiële betrokkenheid van SS in de regulatie van de thymusinvolutie beschreven en bediscussieerd. Deze involutie treedt normaliter op bij het ouder worden. Er zijn echter aanvullende studies nodig om vast te stellen of de gevonden afname van het aantal SSR in de menselijke thymus bij het ouder worden een rol speelt bij dit proces van involutie, of dat het simpelweg een gevolg is van het proces.

In de menselijke thymus lijkt SS een effekt te kunnen hebben op verschillende niveaus, op verschillende celtypen van de thymus en via verschillende receptor subtypen. Het peptide kan daarom beschouwd worden als een mogelijke modulator van de homeostase van de

verschillende celtypen en tevens als een regulator van hun gespecialiseerde aktiviteiten, waaronder de maturatie van het menselijke T-cel repertoire. Een dergelijke hypothese is in overeenstemming met de funktie van andere neuropeptiden, zoals VIP en SP. Hoewel dit nog niet definitief is vastgesteld, zouden deze drie neuropeptiden elkaar in de thymus onderling kunnen beïnvloeden.

Evenals andere tumoren die hun oorsprong hebben in SS-doelwit weefsels, kunnen menselijke epitheliale tumoren van de thymus SSR receptoren bevatten. Deze thymomen worden gevisualiseerd met behulp van SSR scintigrafie, gebruik makend van het radioligand [^{111}In-DTPA-D-Phe^1]-octreotide (**hoofdstuk III.4**). Er konden drie SSR subtypen (sst_1, sst_{2A} and sst_3) door middel van immunohistochemie worden aangetoond in dit type tumor (**hoofdstuk III.3 en III.4**). *In vivo* werd een sterk heterogene expressie gevonden van sst_{2A} en sst_3 receptoren in een serie thymomen. Deze receptoren kunnen tevens gevisualiseerd worden met behulp van SSR scintigrafie *in vivo* (**hoofdstuk III.4**). In het algemeen was de expressie van sst_3 receptoren hoger dan die van sst_{2A} receptoren. Sst_3 receptoren bleken met name voor te komen op reaktieve thymocyten. Aangezien sst_{2A} en sst_3 receptoren tevens aanwezig zijn in de normale thymus, en omdat dit orgaan niet gevisualiseerd kan worden met SSR scintigrafie, terwijl de milt door SSR scintigrafie wel duidelijk zichtbaar kan worden gemaakt, is er verder onderzoek gedaan naar de mate van expressie en de distributie van SSR in de normale milt, de normale en hyperplastische thymus en in thymomen (**hoofdstuk III.5**). De resultaten van deze studies suggereren dat de expressie van sst_{2A} receptoren niet altijd bepalend is voor de mogelijkheid om SSR positieve weefsels te visualiseren met behulp van SSR scintigrafie. Sst_3 receptoren lijken tevens een rol te kunnen spelen in de opname van [^{111}In-DTPA-D-Phe^1]-octreotide in lymfoide weefsels. In het licht van deze nieuwe bevindingen moet vastgesteld worden dat [^{125}I-Tyr^3]octreotide een duidelijk sst_2-selektief ligand is voor *in vivo* bindingsstudies, terwijl [^{111}In-DTPA-D-Phe^1]-octreotide *in vivo* in dit opzicht minder selektief lijkt te zijn. Dit gegeven kan belangrijke consequenties hebben voor het gebruik van radioaktieve SS-analogen bij systemische radiotherapie. De ontwikkeling van nieuwe SSR subtype-selektieve analoga is van groot belang voor verder onderzoek op dit gebied.

De mogelijkheid van het gebruik van SS en SS-analoga bij de behandeling van autoimmuunziekten en tumoren van het immuunsyteem wordt gesuggereerd in de literatuur. Met name van belang is de behandeling met octreotide van patiënten met een thymoom en een gerelateerd paraneoplastisch syndroom. Hoewel aanvullende studies

noodzakelijk zijn om het werkingsmechanisme te onderzoeken, lijkt het erop dat octreotide therapie een plaats kan hebben bij de behandeling van autoimmuunziekten, al dan niet geassocieerd met thymustumoren. Bovendien is er in een bepaald thymoom dat wij zowel *in vivo* en *in vitro* hebben bestudeerd, gevonden dat de groei van de tumorcellen *in vitro* geremd kan worden met SS of octreotide. In dit bepaalde thymoom bleken de tumorcellen alleen sst_3 receptoren te bevatten (**hoofdstuk III.3**). Een andere belangrijke bevinding is dat dit thymoom niet in staat was tot endogene SS produktie. Hoewel preliminair, kan gesuggereerd worden dat het verlies van de mogelijkheid om SS te produceren een rol speelt bij de pathogenese van dit type tumoren, alsmede in het optreden van autoimmuunziekten die vaak geassocieerd zijn met deze tumoren (**hoofdstuk III.3**). In dit kader is het van belang om het mechanisme van de gevonden verhoogde SSR expressie bij thymushyperplasie geassocieerd met myastenia gravis, (**hoofdstuk III.5**) nader te bestuderen.

RIASSUNTO

Gli organi linfoidi primari possono essere considerati i distretti dell'organismo dove hanno luogo importanti interazioni immuno-neuro-endocrine preferenzialmente. Si tratta di organi piuttosto complessi sia per l'eterogeneità dei gruppi cellulari che li compongono sia per la presenza nel loro contesto di svariate sostanze che hanno la prerogativa di appartenere ai tre suddetti sistemi. Il sistema immune ed i sistemi nervoso ed endocrino possono interagire tra loro anche, e specialmente, mediante comuni mediatori. Tra queste sostanze, i neuropeptidi sembrano svolgere un ruolo estremamente rilevante.

La somatostatina (SS) ha un'azione prevalentemente inibitoria sia nel sistema nervoso che nel sistema endocrino. Tuttavia, negli ultimi anni é diventato sempre più evidente il ruolo di immunomodulatore di questo neuropeptide ubiquitario. Infatti, i numerosi studi che hanno valutato l'azione della SS sul sistema immune hanno aperto una nuova e stimolante area di ricerca. La SS esercita i suoi effetti legandosi a specifici recettori di membrana. In quest'ultima decade sono stati identificati 5 diversi sottotipi recettoriali per la SS (SSR) e ne sono stati clonati e geni. I dati relativi all'espressione dei 5 diversi sottotipi di SSR su cellule del sistema immune sono piuttosto conflittuali. Inoltre, i primi risultati ottenuti da studi effettuati su linee cellulari e su colture primarie hanno evidenziato come primo dato importante un'espressione dei recettori diversificata sui sistemi cellulari derivanti da animali e su cellule umane. Un altro aspetto che merita di essere sottolineato riguarda gli effetti esercitati dalla SS su cellule del sistema immunitario, che sembra differenziarsi dai più noti effetti del neuropeptide sulle cellule nervose e sulle cellule endocrine. Infatti *in vitro* la SS esercita un effetto definito "bifasico", ovvero inibitorio a concentrazioni nanomolari, ed un assenza di effetto a concentrazioni micromolari. Tuttavia, sebbene vi siano alcune differenze tra le interazioni della SS con cellule neuroendocrine e con cellule immunitarie, molti altri aspetti sono paragonabili.

In questa tesi l'attenzione é stata focalizzata principalmente sul timo umano, poiché quest'organo linfoide primario rappresenta un caso paradigmatico delle interazioni esistenti tra il sistema neuroendocrino e il sistema immunitario. Nel timo si svolgono complessi processi biologici che portano alla maturazione delle cellule immunocompetenti della linea

T, gli effettori periferici della risposta immunitaria cellulo-mediata. Diversi studi hanno dimostrato che una considerevole parte di questi complessi meccanismi che portano alla maturazione ed alla selezione dei linfociti T sono sotto un sofisticato controllo da parte di molecole classicamente appartenenti al sistema neuroendocrino. E proprio il ruolo della SS e dei suoi diversi sottotipi di recettore é stato finora molto poco esplorato nel timo umano.
All'inizio degli studi contenuti nella presente tesi, due sono state le principali problematiche affrontate. La prima ha riguardato soprattutto l'identificazione dei SSR espressi nel timo umano normale e l'impatto del sistema SS/SSR nei principali processi fisiologici che avvengono all'interno di quest'organo. Successivamente é stato affrontato il potenziale ruolo di questo sistema nelle condizioni patologiche primitive o associate a disfunzioni del timo umano. Le scoperte sugli aspetti riguardanti le possibili relazioni tra le azioni della SS, l'espressione dei suoi recettori e le funzioni del timo umano potrebbero avere importanti conseguenze e fornire nuovi suggerimenti per le problematiche diagnostiche e terapeutiche di alcune malattie del sistema immunitario.
Il **capitolo I** contiene un'introduzione approfondita alle più recenti nozioni sull'importanza di alcuni ormoni, dei neuropeptidi e dei loro recettori nell'ambito del sistema immunitario, con enfasi particolare al significato ed al ruolo della SS e dei suoi recettori. All'inizio del **secondo capitolo**, i primi due paragrafi, invece, sono di introduzione alla struttura ed alla funzione del timo umano normale, con una sezione speciale dedicata al controllo esercitato su queste funzioni dal sistema neuroendocrino. Seguono poi gli studi sulla distribuzione e caratterizzazione della SS e dei suoi recettori, con particolare attenzione alla localizzazione cellulare dei diversi sottotipi di recettore ed al potenziale significato di tale distribuzione nell'ambito della fisio-patologia del timo umano. (**capitoli II.3, 4 e 5**).
I risultati emersi dai primi studi hanno fornito una mappa della localizzazione della SS e dell'espressione di tre diversi sottotipi di recettore, il tipo 1 (sst_1), sst_{2A} e sst_3, eterogeneamente distribuiti nel tessuto e su differenti sottopopolazioni di cellule timiche. Infatti le cellule epiteliali timiche (TEC) esprimono selettivamente i recettori del tipo sst_1 e sst_{2A}: inoltre questo tipo di cellule può essere considerato una potenziale fonte di produzione endogena di SS, poiché l' mRNA codificante per il neuropeptide é stato isolato proprio dalle TEC (**capitolo II.4**). Al contrario, la SS non risultava rilevabile nei timociti isolati da tessuto fresco. Tuttavia su queste cellule erano espressi i recettori sst_{2A} e sst_3. Differentemente dalle TEC, nei timociti in coltura si assisteva ad una "down-regulation" dell'espressione di questi recettori (**capitolo II.5**). I timociti nel timo umano rappresentano

una popolazione cellulare molto eterogenea, formata da cellule in differenti stadi della loro linea maturativa. Un dato molto interessante riguarda il particolare assetto del "pattern" recettoriale che si é andato evidenziando man mano che venivano studiate le diverse sottopopolazioni di timociti. Mentre il recettore di tipo sst_{2A} risultava progressivamente espresso in maniera predominante sulle frazioni di timociti più immature, il recettore di tipo sst_3 veniva riscontrato maggiormente espresso su cellule in fase di maturazione più avanzata. Il numero dei siti di legame per la SS sui timociti risultava essere comunque molto basso, in linea con lo stesso tipo di riscontro ottenuto sui linfociti T periferici che derivano proprio dai timociti. Il timo umano contiene anche un discreto numero di macrofagi, e su questo terzo componente cellulare si é riscontrata l'espressione selettiva del recettore sst_{2A}. Questo dato é in accordo con lo stesso tipo di riscontro su cellule appartenenti alla stessa linea, ma in altri distretti dell'organismo ed in diverse condizioni (**capitolo II.5**). Queste cellule non sembrano contenere SS, che é stata invece evidenziata nei macrofagi timici del topo. Quest'aspetto evidenzia ulteriormente che la distribuzione della SS e dei suoi recettori é specie-specifica, cosa che rende difficoltoso il reperimento di un modello animale adatto a studiare gli effetti del neuropeptide sulle cellule timiche e del sistema immunitario in generale. Per questo motivo la funzionalità dei recettori della SS é stata studiata su colture primarie di TEC e timociti umani, utilizzando sia la SS nativa che un suo analogo sintetico octapeptidico, l'octreotide. Entrambi i composti hanno sortito un effetto inibitorio *in vitro* sulle culture primarie umane di entrambe le popolazioni cellulari timiche (**capitoli II.4 e II.5**).

Considerando il peculiare microambiente timico, la sua architettura e lo specifico "pattern" di distribuzione dei recettori della SS, si può concludere che nel timo umano sembrano esistere aree specializzate dove la SS tramite i diversi sottotipi di recettore é in grado di esercitare un controllo pleiotropico su diverse funzioni di quest'organo.

Il recettore sst_{2A} é il sottotipo prevalentemente espresso sulle cellule del cosiddetto "stroma" timico, dove sembra essere coinvolto nel mediare le attività autocrine o paracrine della SS nell'ambito del microambiente timico. Per esempio, é stato dimostrato che la SS é in grado di inibire la proliferazione delle TEC basale e stimolata da fattori di crescita. (**capitolo II.4**). Non si può tuttavia escludere che essa sia coinvolta anche nella modulazione della produzione di sostanze specifiche da parte delle cellule dello stroma timico. Infatti sia le TEC che i macrofagi producono ormoni timici e citochine. La presenza su queste cellule di un recettore, il sst_{2A}, che nel sistema endocrino é coinvolto

principalmente nel mediare gli effetti inibitori della SS sulla produzione di vari ormoni, fa pensare al suo coinvolgimento in tale tipo di modulazione anche nel sistema immune Un ulteriore aspetto interessante riguarda i macrofagi. Questi svolgono la funzione di cellule presentanti l'antigene, e poiché in altri siti é stata dimostrata un'influenza della SS su questo meccanismo, il suo coinvolgimento nel timo in tale attività é fortemente probabile.

L'espressione dei recettori della SS sui timociti sembra essere correlata al livello di maturazione delle cellule nell'ambito del timo (**capitolo II.5**). I timociti corticali più immaturi esprimevano preferenzialmente il recettore sst_{2A}, mentre invece le sottopopolazioni più avanzate nella linea maturativa mostravano la prevalente espressione del sst_3. L'espressione del recettore sst_{2A} sui timociti che hanno appena colonizzato il timo trova un riscontro nell'evidenza che questo sottotipo di SSR é espresso in cellule progenitrici nel midollo osseo. La funzione di questo recettore sui timociti immaturi potrebbe essere quella di modulare l'alta attività proliferativa di queste cellule. Invece il recettore sst_3 é espresso come, abbiamo visto, sui timociti più maturi e sui linfociti T periferici. Molti timociti in maturazione all'interno del timo sono destinati ad essere selezionati negativamente e soppressi mediante apoptosi. Recentemente é stato dimostrato che proprio il recettore sst_3 sembra essere coinvolto nel mediare l'apoptosi cellulare indotta da agonisti della SS. La presenza di questo specifico recettore sui timociti é altamente suggestivo per un coinvolgimento della SS nella selezione dei timociti o perlomeno nella modulazione di questo processo all'interno del timo umano (**capitolo II.5**). A questo punto é utile ricordare che altri ormoni e classici peptidi neuroendocrini sono in grado di influenzare il traffico e la migrazione dei timociti all'interno del timo. Alla luce della particolare distribuzione e compartimentalizzazione dei SSR nel timo, appare probabile un ruolo di questo neuropeptide in questi processi. Infatti, la SS é in grado di influenzare la migrazione e la chemiotassi di cellule immunocompetenti in altri distretti del corpo umano.

La disponibilità di anticorpi specifici per i SSR ha reso possibile studiare più dettagliatamente l'esatta localizzazione cellulare di queste proteine/recettori mediante immunoistochimica in diversi tessuti e tumori umani. Risultati incoraggianti sono stati recentemente ottenuti anche in tessuti più complessi come il tessuto linfoide. Nel **capitolo III.5** questa tecnica é stata applicata su materiale timico e splenico. Una chiara immunoreattività per il recettore sst_{2A} é stata riscontrata quasi esclusivamente in aree dove le cellule stromali sono prevalenti, mentre il recettore sst_3 risultava localizzato prevalentemente sulle cellule linfoidi sia nel timo che nella milza umani (**capitolo III.5**).

Questi risultati sul "pattern" di distribuzione dei recettori hanno confermato ciò che si era già delineato con gli studi di legame e con RT-PCR. Da questo tipo di studi si giunge alla conclusione che i diversi sottotipi di recettore della SS sono espressi su specifiche sottopopolazioni cellulari ed in specifici stadi della differenziazione sia delle cellule linfoidi che di cellule accessorie presenti nei principali organi del sistema immune. In generale, questo tipo di distribuzione dei recettori nell'ambito degli organi linfoidi potrebbe in parte spiegare la variabilità degli effetti della SS sulle cellule immunitarie.

Il timo é un organo che va incontro ad un fisiologico processo di involuzione con l'età. Il potenziale coinvolgimento della SS in questo processo é stato indagato nel **capitolo II.6.** Tuttavia ulteriori studi sono necessari per chiarire se il decremento dell'espressione dei SSR nel timo con l'aumentare dell'età cronologica entra a far parte attiva del processo stesso oppure se é una conseguenza del processo involutivo.

Nel timo umano la SS sembra agire a differenti livelli, su diverse popolazioni cellulari, e attraverso diversi sottotipi di recettore. Da questo punto di vista, il neuropeptide potrebbe essere considerato un importante modulatore dell'omeostasi tra i vari componenti cellulari timici, ed allo stesso tempo entrare a far parte della regolazione delle varie funzioni di queste cellule, comprese le attività che sono alla base della maturazione del corredo cellulare T nel sistema immunitario umano. Un ruolo simile é stato ipotizzato anche per altri neuropeptidi come il *vasoactive intestinal peptide* (VIP) e la sostanza P (SP). Inoltre, anche se non ancora ben stabilito, é stata proposta l'esistenza di un circuito intra-timico che vede coinvolti e correlati proprio questi tre neuropeptidi in azioni sinergistiche o contrastanti.

Così come per la maggior parte dei tumori che originano da tessuti bersaglio della SS, anche le neoplasie timiche possono esprimere SSR. I timomi, una eterogenea classe di tumori epiteliali del timo, possono essere visualizzati *in vivo* durante scintigrafia con [^{111}In-DTPA-D-Phe^1]-octreotide (**capitolo III.4**). Questo tracciante radiomarcato viene regolarmente usato per la localizzazione di lesioni che esprimono SSR. Inoltre almeno tre diversi sottotipi di recettore della SS sono stati riscontrati in questi tumori mediante immunoistochimica. (**capitoli III.3 e III.4**). In particolare, i recettori sst_{2A} e sst_3 sono stati trovati espressi *in vitro* in modo considerevolmente eterogeneo in una serie di timomi precedentemente visualizzati *in vivo* tramite scintigrafia con l'analogo marcato della SS (**capitolo III.4**). In grandi linee, il recettore sst_3 era maggiormente espresso in confronto al recettore sst_{2A}, mostrando una preferenziale ma non esclusiva localizzazione sui timociti reattivi all'interno del tessuto neoplastico. I recettori sst_{2A} e sst_3 sono espressi anche nel timo umano normale,

tuttavia quest'organo non viene generalmente visualizzato durante la scintigrafia con analoghi della SS marcati. Per questo motivo la milza, il timo normale, iperplastico e neoplastico sono stati ancora più approfonditamente studiati, per cercare di capire se l'eterogeneità e la densità di distribuzione dei SSR potesse essere alla base di questa apparente discrepanza. (**capitolo III.5**). I risultati ottenuti hanno suggerito che la presenza del recettore sst_2 non é un prerequisito essenziale per la visualizzazione di un organo o tessuto durante scintigrafia con [^{111}In-DTPA-D-Phe^1]-octreotide, e che inoltre non si può escludere il coinvolgimento del recettore sst_3 dai possibili meccanismi di captazione del tracciante, perlomeno in alcuni tessuti linfoidi. Alla luce di queste nuove nozioni si é potuto concludere che mentre l'analogo della SS usato per studi di legame *in vitro*, il [^{125}I-Tyr^3]-octreotide, può essere considerato altamente selettivo per il recettore sst_2, quello utilizzato *in vivo*, il [^{111}In-DTPA-D-Phe^1]-octreotide, potrebbe esserlo in grado minore. Questo dato ha un importante impatto per la terapia radiometabolica con analoghi radiomarcati della SS. La disponibilità di nuovi analoghi selettivi ed antagonisti dei sottotipi recettoriali della SS potrà chiarire ancora maggiormente alcuni punti oscuri sul ruolo dei diversi sottotipi nei meccanismi di captazione di radiotraccianti *in vivo*.

Sono già in fase di studio nuove potenziali applicazioni degli analoghi della SS nelle patologie autoimmuni ed in patologie neoplastiche riguardanti il sistema immunitario. Particolare interesse é stato destato dal trattamento con octreotide di pazienti con timoma e relativa sindrome paraneoplastica associata. Sebbene ulteriori studi sono necessari per la chiara comprensione dei meccanismi alla base degli effetti degli analoghi della SS su queste neoplasie, tale terapia potrebbe rappresentare una importante innovazione nel trattamento delle patologie autoimmuni associate o meno a neoplasie timiche. In uno di questi casi di timoma studiati sia *in vivo* che *in vitro*, l'octreotide si é dimostrato efficace nell'inibire la proliferazione delle cellule tumorali *in vitro*. Inoltre, un dato da sottolineare é che le cellule tumorali isolate dal suddetto timoma esprimevano selettivamente il recettore sst_3 (**capitolo III.3**), ed ancora più interessante é risultata la perdita della capacità di produzione di SS endogena in questo timoma. Anche se ancora molto preliminare, quest'aspetto andrebbe tenuto presente per un suo probabile coinvolgimento nella patogenesi di alcuni tumori del timo o delle sindromi paraneoplastiche su base autoimmune così frequentemente associate a questo tipo di neoplasia (**capitolo III.3**). A questo proposito potrebbe essere molto utile studiare più approfonditamente il significato della "up-regulation" dei SSR riscontrata in un caso di iperplasia timica associata a *myasthenia gravis* (**capitolo III.5**).

In conclusione, la SS e alcuni suoi sottotipi di recettore sono espressi nel timo umano sia sulle cellule linfoidi che stromali, ma con un apparente criterio selettivo. Un circuito intratimico altamente specializzato sembra integrare la SS nella funzione principale del timo, ma anche in altre sue funzioni ancora inesplorate. Pur tuttavia, questi risultati evidenziano il ruolo importante di questo ubiquitario neuropeptide e dei suoi recettori nell'omeostasi del timo. La funzione globale del timo é basata sull'equilibrio di molteplici fattori di regolazione, la maggior parte dei quali viene prodotta a livello locale. Alterazioni in questo equilibrio potrebbero indurre risposte anomale del sistema immune, come potrebbe essere il caso delle patologie autoimmuni associate o meno a neoplasie timiche. Se disturbi del circuito che vede coinvolta la SS sono implicati nell'insorgenza di tali disordini, la terapia con analoghi della SS potrebbe avere un razionale in alcune di queste severe malattie.

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CURRICULUM VITAE

The author of this thesis was born on the 11th of February 1962 in Capri, Italy. In 1981 he graduated from High School (Classic Lyceum) at the Liceo Classico Publio Virgilio Marone in Capri. Thereafter, he attended medical school at the "Federico II" University in Napoli, Italy (1982-1992). During his medical studies, he attended as pre-graduate fellow the Department of Molecular & Clinical Endocrinology and Oncology of the above mentioned university (chair Prof. Dr. Gaetano Lombardi), where he was involved in studies on pathogenesis, diagnosis and therapy of pituitary diseases (1988-1992). In 1992 he obtained the degree in Medicine (110/110), as well as the licence to practice as medical doctor in Napoli (90/90). He continued his attendance as post-graduate fellow at the Department of Molecular & Clinical Endocrinology and Oncology and started his training in Endocrinology and Metabolic Diseases in 1992 till the post-graduate degree magna cum laude (50/50) granted in Napoli in 1997. In the same year he was registered as Specialist in Endocrinology and Metabolic Diseases.

Since February 1997 he is research fellow at the Department of Internal Medicine of the Erasmus University Hospital Rotterdam Dijkzigt (chair: Prof. Dr. Steven W.J. Lamberts). In January 1998 he was admitted after open competition as fellow at the School of Specialization in Pharmacology of the Institute of Neuroscience at "Federico II" University in Napoli (chair: Prof. Dr. Lucio Annunziato).

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