SOMATOSTATIN RECEPTORS AND THEIR LIGANDS IN THE HUMAN IMMUNE SYSTEM

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SOMATOSTATINE RECEPTOREN EN LIGANDEN IN HET HUMANE IMMUUNSYSTEEM

PROEFSCHRIFT

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Chapter II

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Dalm VA, van Hagen PM, de Krijger RR, Kros JM, van Koetsveld PM, van der Lely AJ, Lamberts SW and Hofland LJ. 2003 Distribution pattern of somatostatin and cortistatin mRNA in human central and peripheral tissues. *Submitted*

Lichtenauer-Kaligis EG, Dalm VA, Oomen SP, Mooij DM, van Hagen PM, Lamberts SW and Hofland LJ. 2003 Differential expression of somatostatin receptor subtypes, somatostatin and cortistatin in human peripheral blood mononuclear cell subsets. *Submitted*

Dalm VA, van Hagen PM, van Koetsveld PM, Achilefu S, Houtsmuller AB, Pols DH, van der Lely AJ, Lamberts SW and Hofland LJ. 2003 Expression of somatostatin, cortistatin and somatostatin receptors in human monocytes, macrophages and dendritic cells. Am J Physiol Endocrinol Metab 285(2):E344-353

Chapter III

Ferone D, Pivonello R, van Hagen PM, Dalm VA, Lichtenauer-Kaligis EG, Waaijers M, van Koetsveld PM, Mooij DM, Colao A, Minuto F, Lamberts SW and Hofland LJ. 2002 Quantitative and functional expression of somatostatin receptor subtypes in human thymocytes. Am J Physiol Endocrinol Metab 283(5):E1056-1066

Chapter IV

Dalm VA, van Hagen PM and Krenning EP. 2003 The role of octreotide scintigraphy in rheumatoid arthritis and sarcoidosis. Q J Nucl Med *in press*

Dalm VA, Hofland LJ, Mooy CM, Waaijers M, van Koetsveld PM, Langerak AW, Staal FT, van der Lely AJ, Lamberts SW and van Hagen PM. 2003 Somatostatin receptors in malignant lymphomas: targets for radiotherapy? *Submitted*

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Chapter I.1

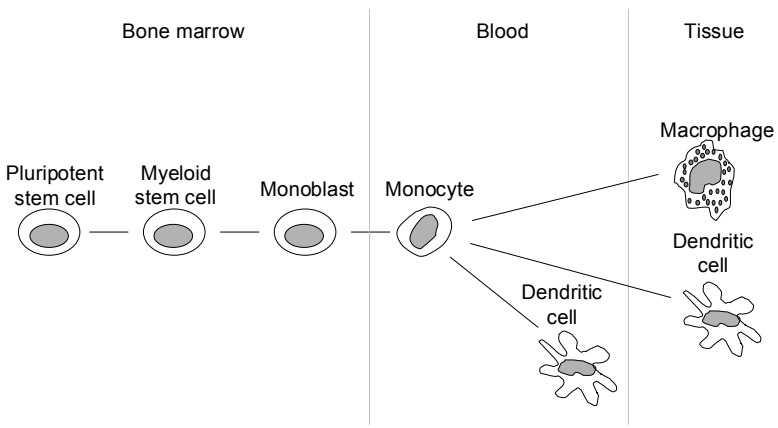
GENERAL INTRODUCTION

Maintenance of homeostasis is essential for survival of the mammalian organism. For a long time it was believed that the different systems in the human body act independently from each other to achieve this goal. However, during the last decades it has become more evident that the different systems in the human body integrate and regulate different functions in close interaction. Numerous studies have provided evidence that the immune, endocrine and neural systems interact to maintain homeostasis. The first suggestion that neuropeptides and neurohormones might play regulatory roles in the immune system came with the detection of corticotropin (ACTH), which is a peptide hormone, in the immune system (1,2). Since then, expression of various neuroendocrine hormones, neuropeptides and their receptors has been described in cells of the immune system (for a review see: (3-5)). In addition, lymphoid organs are extensively innervated and neuropeptides can reach and might act on lymphocytes and macrophages via this route. On the other hand, expression of cytokines and its receptors, which were thought to be restricted to the immune system, has been described in the neuroendocrine system (3). These findings point to the existence of bi-directional regulatory pathways between the immune and neuroendocrine systems and have led to an increasing number of studies, evaluating neuroendocrine-immune interactions. These interactions are very complex. Many reciprocal interactions are still poorly understood and many mechanisms are still difficult to interpret. However, it has become more evident that the neuroendocrine-immune interactions are very important in maintaining homeostasis and that disturbances in these links may be involved in pathological conditions. For instance, infections are regarded by the neuroendocrine system as stressors and the neuroendocrine system has to maintain homeostasis of the body. Activation of the immune system by an agent not only evokes potential dangers to the agent, but potentially also to the integrity of the host, because overly vigorous responses may kill the host in the process of controlling an infection. Therefore, the neuroendocrine system has to constantly monitor and, if necessary, regulate the immune cell functions. On the other hand, the immune system needs the neuroendocrine system to help determining how to respond in case of an invading agent (6). Any disturbances in the cross-talks between both systems could be very important in the development or maintenance of disease and a clearer understanding of these interactions may have important therapeutical applications.

Cells of the monocyte lineage: an important cellular component of the immune system

In the previous paragraph, the existence of interactions between the neuroendocrine and immune system has been introduced. An important cellular component of the immune system, which is involved in the initial activation (innate immunity), are the cells of the monocyte lineage, i.e. monocytes, macrophages and dendritic cells. These cells play important regulatory roles in human host defense by phagocytosis, cytokine production and antigen presentation. Macrophages were already identified in 1882 as cells, present in loose connective tissue, that were able to phagocytose foreign material (7). In 1972 the mononuclear phagocyte system (MPS) was postulated based on similarities in morphology, function and hematopoietic origin (8). The different tissue macrophages, dendritic cells, the peripheral blood monocytes (which are the common precursors of macrophages and dendritic cells) and their bone marrow precursors are now considered to belong to the MPS (9). Figure 1 shows schematically the development of the different cells from the pluripotent bone marrow stem cells.

Figure 1: Development of cells of the monocyte lineage



This figure shows the development of monocytes and its functionally derived cells, macrophages and dendritic cells, from the pluripotent stem cells, originating in the bone marrow.

Monocytes that circulate in blood can leave the bloodstream and differentiate in the organs (10). These migrated and differentiated monocytes are called macrophages. In the different tissues they can enter, these cells have various important functions in host defence, but also in organ homeostasis. As mentioned before, an important function of macrophages is phagocytosis. In physiological state, macrophages remove erythrocytes in the spleen, liver and bone marrow and platelets from peripheral blood. On the other hand, macrophages are able to phagocytose and destroy microorganisms (11). The process of

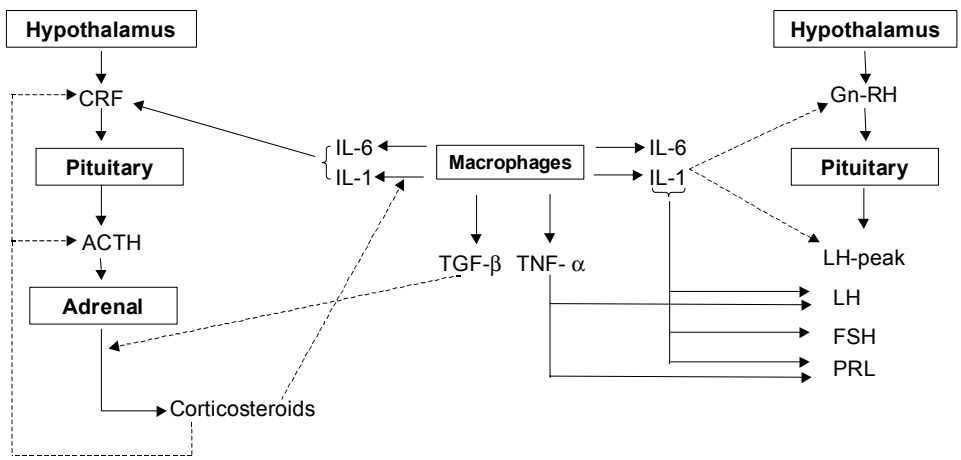
phagocytosis is mediated via recognition of extracellular pathogens by different receptors, which are expressed on monocytes and macrophages. Receptors that are involved in this process include Fc-receptors (12), complement receptors (13), carbohydrate receptors, like the macrophage mannose receptor (14), and receptors that recognize pathogen-associated molecular patterns (PAMPs) of microorganisms (15). These properties make macrophages an important component in removing foreign material in host defense (16). In contrast to the macrophages, dendritic cells do not play a very important role in phagocytosis of foreign material. However, these cells are nowadays known as very important antigen-presenting cells (for an extensive review see: (17)). Antigen-presenting cells are able to endocytose proteins from extracellular pathogens and degrade them to small peptides. After endocytosis and processing of the antigenic protein, the degraded peptides can be presented on the cell surface of dendritic cells in association with a major histocompatibility complex class II (MHC) molecule, forming a complex of MHC and peptide antigen. The MHC molecules are essential for recognition of the specific antigen by T-cells (18). Immature dendritic cells, residing in peripheral tissues, avidly ingest and process foreign antigens, but upon activation dendritic cells are triggered to maturity and this enhances their phagocytosis capacity (19). A very important factor in dendritic cell activation is the upregulation of the chemokine receptor CCR7 (20). Expression of CCR7 allows dendritic cells, which have now matured and lost their capacity to uptake antigen (21), to migrate into the secondary lymphoid organs, where the antigen-MHC complex can interact with T-cells and in turn activates T-cells. The subsequent activation of T-helper cells (22,23) and generation of cytotoxic T-cells (19) is essential for elimination of intracellular pathogens. Like dendritic cells, macrophages are as well able to present antigenic material to T-lymphocytes (24), although it has been shown that macrophages can only interact with specific T-cell subsets (25,26). Finally, cells of the monocyte lineage can regulate the immune response via secretion of biologically active molecules, such as cytokines (27). Macrophages are not only capable of producing various cytokines, but they can also respond to cytokines secreted by themselves or by other cells via specific receptors on their cell surface.
As macrophages are important regulatory cells in inflammatory processes, they can either produce pro-inflammatory, like interleukin (IL)-1, IL-7, tumour necrosis factor α (TNF-α) and interferon- γ (IFN-γ) (28,29) or anti-inflammatory, like IL-4, IL-10, IL-13 (30,31) cytokines. Generally, pro-inflammatory cytokines upregulate the production of other pro-inflammatory mediators, whereas the anti-inflammatory cytokines can repress the production of mediators. Because of the production of both pro- and anti-inflammatory cytokines, macrophages play a very important role in inflammation, especially in controlling the immune response, keeping the inflammatory reaction in balance. Disturbances in this equilibrium may have important consequences in developing disease.

Neuroendocrine-immune interactions

In the first paragraph, the neuroendocrine-immune interactions were discussed. It has been shown that macrophages are involved in these interactions. Macrophages, by secretion of IL-1, IL-6, transforming growth factor β (TGF-β) and TNF-α, regulate various secretion

functions in the endocrine system (for a review see (32)). Interestingly, it was demonstrated that IL-1 (33) and IL-6 (34), which were produced by macrophages, stimulated corticotrophin releasing factor (CRF) secretion at the hypothalamic level. CRF stimulates the secretion of ACTH from the pituitary and, consequently, the secretion of glucocorticoids by the adrenal. In turn, high glucocorticoid levels inhibit the secretion of CRF at the hypothalamic level (35), but also the secretion of IL-1 by macrophages. This negative feedback-loop clearly demonstrates the interaction between the two systems. TGF- β, which is also a macrophage product, has been shown to inhibit steroidogenesis in the adrenal by decreasing the enzymatic activity of 17α-hydroxylase/20,17-lyase (36,37). Cytokines have also been shown to play a regulatory role in the hypothalamic-pituitary-ovary axis (HPO). IL-1 inhibits gonadotropin-releasing hormone (GnRH) secretion by the hypothalamus and the preovulatory luteinizing hormone (LH)-peak (38). TNF-α stimulates the secretion of LH and prolactin (PRL) from the pituitary (39), while in animal models it has been demonstrated that IL-1 and IL-6 induce significant release of follicle-stimulating hormone (FSH), LH and PRL (40). Figure 2 shows schematically the described interactions between macrophages and the endocrine system.

Figure 2: Macrophages involved in neuroendocrine-immune interactions



Endocrine-immune interactions focussing on interactions between macrophages and the hypothalamic-pituitary-adrenal axis (HPA, left) or the hypothalamic-pituitary-ovary axis (HPO, right). CRF=corticotropin releasing factor, ACTH=adenocorticotropic hormone, IL-1, IL-6=interleukin 1,6, TGF-β=transforming growth factor β, TNF-α=tumor necrosis factor α, Gn-RH=gonadotropin-releasing hormone, LH=luteinizing hormone, FSH=follicle stimulating hormone, PRL=prolactine, (——) represents stimulation and (- - - ·) represents inhibition.

Numerous studies have reported on a differential expression of receptors for hormones and neuropeptides, and their ligands, on cells of the monocyte lineage (for a review see: (5)). Table 1 summarizes the distribution pattern of the receptors for different hormones and neuropeptides, as well as their ligands, in cells of the human monocyte lineage, in order to demonstrate the wide expression of these compounds in immune cells.

Table 1: Expression of different hormones and neuropeptides in cells of the human monocyte lineage

Hormone/ neuropeptide	Expression of hormone/neuropeptide	Expression of receptors for hormone/neuropeptide	Reference
CRF	+	+	(41,42)
ACTH	n.i.	+	(43)
α-MSH	+	+	(44)
GH	+	+	(45,46)
PRL	+	+	(47,48)
LH/FSH	n.i.	+	(49)
TSH	+	+	(50)
VIP	n.i.	+	(51)
Substance P	+	+	(52)
Vasopressin	n.i.	+	(53)
Bombesin	+	+	(54)
Somatostatin	n.i.	+	(55)

α-MSH = alpha-melanocyte stimulating hormone, GH = growth hormone, TSH = thyroid stimulating hormone and VIP = vasoactive intestinal peptide.
+ indicates the proven expression of the hormone, neuropeptide or its receptors in cells of the monocyte lineage, n.i. = not investigated

This broad and differential expression of hormones, neuropeptides or their receptors in cells of the monocyte lineage points to a neuroendocrine control of immune functions of these cells. Taking into consideration the important functions of both macrophages and dendritic cells in host defense and the previously discussed neuroendocrine-immune interactions, it is challenging to further evaluate the regulatory mechanisms of the neuroendocrine system on immune cell function.

Somatostatin and somatostatin-like peptides

Somatostatin

Somatostatin (SS) is a neuropeptide, which was initially isolated from the ovine hypothalamus gland and characterized as a GH release inhibiting factor (GRIF) (56). GRIF was later called SS because of its supposed specific function, i.e. the inhibition of somatotropin or growth hormone release (57). In mammals, proteolytic processing of a 116 amino acid prepro-SS and subsequent 92 amino acid pro-SS results in the formation of 2 biologically active forms of SS, consisting of 14 (figure 3) or 28 amino acids, SS-14 and SS-28, respectively (58). Structure function analysis of native SS has demonstrated that the amino acid residues Phe^{7}, Trp^{8}, Lys^{9} and Thr^{10} are essential for binding of SS to its receptors (59). These residues are at the apex of the loop formed by the disulphide bridge connecting the two cysteine residues and have been demonstrated to form a β-turn (60). Trp^{8} and Lys^{9} are essential for the biological activity of SS whereas the flanking residues tolerate minor substitutions (61).
In the central nervous system SS acts as a neurotransmitter, with generally inhibitory actions in many regions, i.e. cerebral cortex, limbic system, spinal cord and brain stem (62,63). In the peripheral nervous system, SS is expressed in sympathetic and sensory neurons (64,65), and it has been demonstrated that SS may have an inhibitory effect on nociception, i.e. perception of harmful and/or painful stimuli (66,67). SS is also widely distributed in other issues throughout the human body. The gastro-intestinal tract and endocrine glands are the major sites of production (64,65). SS mainly plays an important inhibitory role in different secretion processes. SS produced in the hypothalamus reaches the anterior pituitary through the portal circulation and inhibits the secretion of GH but also of other pituitary hormones like TSH and PRL. In peripheral tissues SS regulates the secretion of saliva, calcitonin, renin, insulin and glucagon (64). In addition, it reduces the exocrine secretion of pancreatic enzymes and gastric acid and can inhibit the contractility of stomach, gut and gallbladder (68-70). Finally, SS was shown to have antiproliferative effects on many cell types in vitro (71).

Cortistatin

Recently, de Lecea et al. isolated a DNA clone encoding a novel neuropeptide from rat brain. Because of its localization in the cerebral cortex, this neuropeptide was called cortistatin (CST) (72). Comparable to SS-14 and SS-28, proteolytical processing of the procortistatin in rat results in the production of two different isoforms, CST-14 and CST-29. Interestingly, CST-14 shows high structural resemblance to SS-14. 11 of 14 amino acid residues are identical in both peptides, including two cysteines that may cause the peptide to cyclize as well as the 4 amino acids, which are known to be essential for SS to bind to its receptors. The human peptide, when compared to the rat CST, is predicted to carry an N-terminal extension by three amino acids, yielding a 17 residue sequence as the active peptide (figure 3) (73,74). Despite their structural similarities, SS and CST are

products of two distinct genes. The gene for CST was mapped on chromosome 1 (75), whereas SS is encoded on chromosome 3 (76). Because of its structural similarity to SS, i.e. the expression of the 4 amino acids essential for SS to bind to the somatostatin receptors (sst), it has been suggested that CST could be an alternative ligand to the sst. Indeed, it has been shown that CST binds with high affinity to all 5 sst subtypes (74,77). Until now, most of the actions of CST have been designated to regulation of behaviour, sleep and memory mechanisms localized in the brain region. Both overlapping as well as differential effects of CST, when compared to SS, have been described on the various mechanisms. Overlapping effects for CST and SS have been described on the neuronal electrical activity in the hippocampus (72). Both compounds have been shown to depress the neuronal activity. SS and CST both reduce development of seizures, and thus have an anticonvulsive effect (78) and they both can deteriorate memory consolidation (79). On the other hand, many differences in effects between SS and CST have been described. For example, CST-treated rats showed clear hypoactive behaviour and in these animals, the electroencephalogram (EEG) showed a dramatic increase in cortical slow waves (72). Subsequently, after administration of CST, rats spent more time in slow-wave sleep and less time in Rapid Eye Movement (REM) sleep, while administration of SS results in sleep periods dominated by REM sleep, without significantly affecting the other phases of sleep (72,74). Moreover, it has been demonstrated that CST expression is upregulated during sleep deprivation in rats (80). During sleep deprivation also upregulation of SS has been demonstrated in the brain (81,82). This suggests that SS and CST both have sleep regulatory functions and probably interplay in regulation of sleep, both in their own specific way. Previous studies demonstrated that acetylcholine (ACh) is at low concentration in the cortex during slow-wave sleep and higher concentrations of ACh are associated with wakefulness and REM sleep (83). Therefore, the question was addressed whether CST produces its sleep promoting effects by modulating ACh activity. It has been shown that CST antagonizes the effects of ACh (72), while SS is known to enhance the effects of ACh (84). Because of the distinct actions of SS and CST it is hypothesized that, instead of both peptides acting on the same sst, a specific receptor for CST might be present (85,86). Until now, a specific receptor for CST has not been demonstrated and therefore it is hypothesized that CST acts via the sst as well. Table 2 summarizes the effects and focuses on the differences in functions of both SS and CST on various central mechanisms.

Octreotide

Because of its inhibitory effect on hormone production, it was suggested that SS could play a role in therapy of diseases caused by overproduction of hormones by endocrine tumours (87). However, SS has a very short half-life (88) and turned out to be unsuitable for use in therapeutical implications. Therefore more stable SS analogues have been developed, in which D amino acids have been introduced to decrease enzymatic degradation.

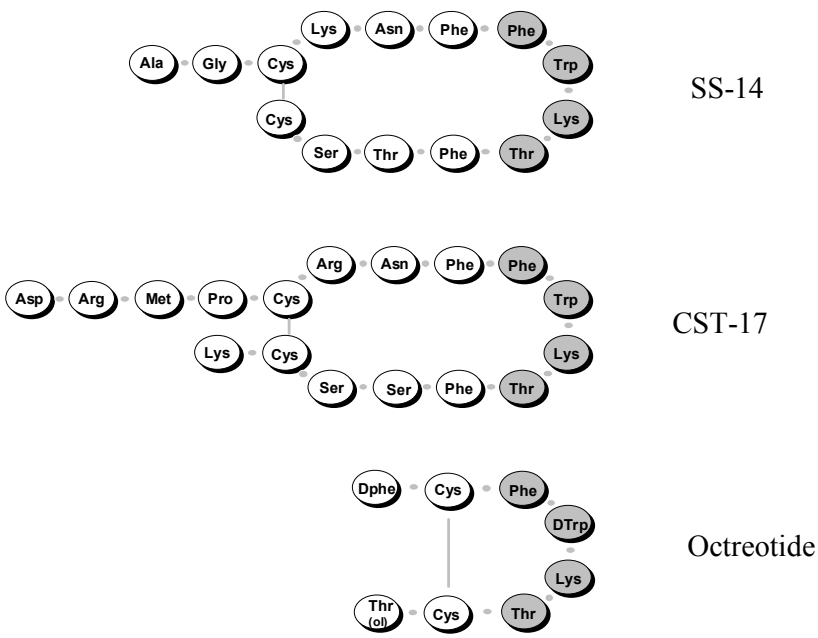
Table 2: Effects of somatostatin (SS) and cortistatin (CST) on different central mechanisms

Mechanism	SS	CST
Neuronal activity hippocampal neurones	↓	↓
Development of seizures	↓	↓
Memory consolidation	-	↓
Locomotor activity	↑	↓
Effect on acetylcholine	↑	↓
Time spent in REM sleep	↑	↓
Time spent in slow wave sleep	-	↑

↑ = upregulation, ↓=downregulation and - = no known effect of either SS or CST, after (72,77-79,84,89)

An example of a SS analogue that is nowadays widely used in the treatment of endocrine active tumours like GH-secreting pituitary adenomas and gastroenteropancreatic tumours (71), is octreotide (figure 3), which is an eight amino acid containing peptide. The group of 4 amino acids, which have been demonstrated to be essential for binding to the sst, forms the basis for the activity of octreotide.

Figure 3: Structures of somatostatin-14, octreotide and cortistatin-17

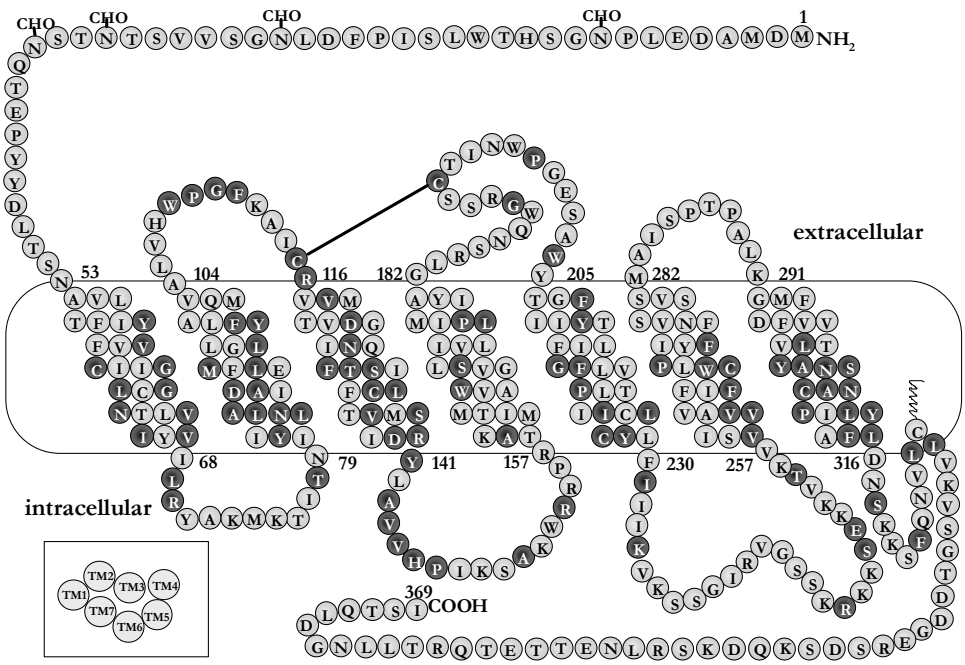


Somatostatin receptor subtypes

Structure

Sst belong to the superfamily of G-protein coupled seven transmembrane receptors, having an N-terminal extracellular domain and a C-terminal intracellular domain (figure 4).

Figure 4: Structure of the somatostatin receptor



Structure and orientation of the somatostatin receptor (sst) within the plasma membrane. Sequence of the human sst_2. CHO indicates potential sites of glycosylation in the extracellular NH_2-terminal domain area. A disulfide bond is indicated between two cysteine residues. The inset demonstrates the possible arrangement of the 7-membrane segments.

Hydrophobic and charged amino acids within the transmembrane domains 3, 6 and 7 are important for the interaction with the ligand. However, the extracellular loop 2, between domains 4 and 5 may also be involved (90-94). Until now, 5 different sst have been described, named sst_{1-5} (95). The 5 sst range in size from 363 to 418 amino acids and there

is high homology (between 45 and 61 %) in the amino acid sequences (96). When the sequences of the human sst subtypes are compared to rodent sst, also high homology is found. Identity between the different subtypes varies from 81 % for sst_5 and 97 % for sst_1 (61,97). Whereas the sst_1, sst_3, sst_4 and sst_5 genes give rise to a single receptor protein, alternative splicing of the sst_2 gene generates two receptor protein isoforms, sst_{2A} and sst_{2B}, which only differ in length and amino acid sequence in their cytoplasmic tails (98,99).
The sst are most closely related to the opioid receptor family. There is approximately 30 % identity between the opioid receptors and sst (100,101).
Although the sst show high homology, their encoding genes are localized on different chromosomes, which allows a tissue-specific regulation of their expression (102) and suggests divers functions of the different subtypes in different organs.

Receptor binding

The 5 known sst subtypes (sst_{1-5}) show a specific distribution in different organs and the natural ligands SS-14 and SS-28 both have high binding affinity to all five receptor subtypes (95). The sst family can be further divided in two subclasses, the first consisting of sst_2, sst_3 and sst_5 and the second consisting of sst_1 and sst_4, based on differential binding affinities of the various ligands (96). Conformationally restricted octapeptide analogues of SS, like octreotide, bind with low affinity to sst_1 and sst_4, whereas these analogues bind with higher affinity to sst_2, sst_3 and sst_5 receptors (96,103). CST-17 also binds with high affinity to all 5 sst (74). Recently, different studies reported on the development of novel sst analogues that can bind to more sst subtypes than the sst_2 preferring ligand, octreotide. A promising example is SOM230, which shows high binding affinity to all sst, but sst_4. SOM230 may have new therapeutical implications, when compared to the more selective octreotide (104). Another novel SS analogue, BIM-23244, shows high binding affinity to sst_2 and sst_5 selectively and can therefore play a role in treatment of GH-secreting tumours, which are known to express both sst_2 and sst_5 (105). Of high interest at this moment are the so-called chimeric molecules that can bind to different receptors. BIM-23A387 is a chimeric molecule that binds with high affinity to sst_2 and to the dopamine receptor D2 (D2DR) (106). Previous studies demonstrated that this molecule showed enhanced potency with respect to inhibition of GH and PRL release from acromegalic patients when compared to selective sst_2 or D2DR agonists (106). Table 3 shows the different binding affinities of SS, CST, octreotide, SOM230, BIM-23244 and BIM-23A387 to each of the 5 sst.

Sst distribution patterns

Expression of specific sst was first described in the pituitary GH_4C_1 cell line, back in 1978 (107). Subsequent studies, using a variety of techniques, showed sst expression in different densities in brain, kidney, gut, pancreas, pituitary, adrenals and thyroid gland (for reviews see (61,97,102)). The expression of sst has been extensively studied in the

Table 3: Binding affinities of somatostatin-14, octreotide, cortistatin-17 and several novel SS analogues to the 5 somatostatin receptor subtypes.

	sst_1	sst_2	sst_3	sst_4	sst_5
somatostatin-14	1	0.05	0.1	0.2	0.2
cortistatin-17	7	0.6	0.6	0.5	0.4
octreotide	>1000	0.6	34.5	>1000	7
SOM230	9.3	1	1.5	>100	0.16
BIM-23244	>1000	0.3	133	>1000	0.7
BIM-23A387	22	293	0.1	77	ni

ni = not investigated, values represent IC_{50} in nM. After (74,77,104-106)

rat, where mRNA for all 5 subtypes has been demonstrated in brain and pituitary, while sst_2 is the predominantly expressed subtype in the pancreas and adrenal tissues. Sst_3 mRNA has been detected in spleen, lymph nodes and liver, sst_4 in the lung and sst_5 in small gut and adrenal gland (108). In human, sst are widely expressed as well. In the human brain all five sst subtypes are expressed in a characteristic, specific pattern (102). All sst, except sst_4 are expressed in the human pituitary (109) and all 5 sst are differentially expressed in the normal pancreatic islets (110). Human adrenal preferentially expresses sst_2 (111), normal thyroid gland predominantly expresses sst_3 and sst_5 (112), whereas all 5 subtypes have been described in the stomach (113). Apart from the expression of sst in healthy tissues, sst have been detected in most tumours originating from physiological sst positive tissues, i.e. pituitary tumours, gastroenteropancreatic tumours (114,115) and brain tumours (116). Sst have also been found to be expressed in tumours originating from various other organs, like breast carcinomas (117) and lung tumours (118). Most cases express multiple sst subtypes (102), although the sst_2 is the receptor which is predominantly expressed.

Somatostatin receptor signalling

As described above, SS exerts its effects via its specific G-protein coupled seven transmembrane receptors, to which all 5 sst are coupled (119). The intracellular G-proteins are linked to multiple second messenger systems, including adenylyl cyclase (119,120), Ca^{2+} and K^+ ion channels (121-123), tyrosine kinase (PTP) (124-128), MAP kinase (MAPK) (129-132), phospholipase A2 (133) and C (134), guanylate cyclase (135) and Na^+/H^+ antiporter (136). The working mechanisms of the key signalling pathways will be discussed briefly. Activation of the receptor coupled to adenylyl cyclase will inhibit adenylyl cyclase and subsequently reduce the intracellular levels of cyclic AMP (cAMP) (119). Activation of ion-channel coupled sst results in a decrease in influx of Ca^{2+}, either directly through inhibition of Ca^{2+}-channels (137) or indirectly by stimulating K^+ efflux

(138,139). Inhibition of both cAMP and Ca^{2+} mediates for a large part the blockade of secretion caused by sst. Stimulation of tyrosine phosphatase activity (126) and inhibition of MAPK activity (130) by sst are thought to be involved in anti-proliferative effects of SS. Less prominent signalling pathways include the phospholipase A2 and C pathways, the guanylate cyclase pathway, which is thought to have an inhibitory effect on cell growth (140), and the Na^{+}/H+ antiporter (141). Of many mechanisms it has been elucidated which sst subtypes are involved. However, for the guanylate cyclase pathway it is only known that SS can biphasically stimulate or inhibit guanylate cyclase activity (135), but the sst subtypes involved have not been characterized until now. Table 4 summarizes sst characteristics and their involvement in the different effector coupling mechanisms.

Table 4: Characteristics of the cloned somatostatin receptor subtypes

	sst_1	sst_2	sst_3	sst_4	sst_5	Reference
Chromosomal localization	14	17	22	20	16	(61,97,102)
Number of amino acids	391	369	418	388	363	(95)
G-protein coupling	+	+	+	+	+	(119)
Effector coupling:						
• Adenylate cyclase	↓	↓	↓	↓	↓	(119,120)
• Ca^{2+} channels	↓	↓				(121,122)
• K^{+} channels		↓				(123)
• Tyrosine phosphatase	↑	↑	↑	↑	↑	(124-128)
• MAP kinase	↑	↓	↑↓	↑	↓	(129-132)
• Phospholipase A2				↑		(133)
• Phospholipase C		↑			↑	(134)
• Na^{+}/H^{+} exchange	↓					(136)

Homo- and heterodimerization of sst

Recent studies have demonstrated that different G protein-coupled receptors might form homo- or heterodimers, instead of acting as monomers, with different functional properties (142-144)**.** In vitro studies on dimerization of sst have shown that sst are able to form both homo- and heterodimers as well (145-147). Sst_5 was found to form either homodimers, which were induced dose-dependently when activated by a ligand, or heterodimers with sst_1, but not with sst_4**.** Moreover, sst_1 was found to be internalized only

when hetero-dimerized with sst_5 and not when expressed alone (145). In another study (147), it was demonstrated that sst_2 and sst_3 were both able to form homodimers as well as heterodimers. However, the sst_3 in the sst_2-sst_3 heterodimers became functionally inactive and the sst_2-sst_3 dimer showed a greater resistance to agonist-dependent desensitization. Both studies demonstrated that homo- and heterodimerization of the sst can alter the functional properties of the receptor with respect to ligand binding, desensitization, internalization and upregulation. As described previously, opioid receptors resemble a receptor family closely related to the sst family (100,101). It was demonstrated that sst could also form heterodimers with the μ-opioid receptor (MOR1) (146). Formation of these heterodimers cross-modulated desensitization, phosphorylation and internalization. For example, treatment of the cells expressing the sst_2-MOR1 dimer with a sst_2-selective agonist induced internalization of the MOR1 together with the sst_2, whereas cells expressing MOR1 selectively did not internalize MOR1 in response to incubation with the sst_2 selective ligand. The functional significance of the formation of homo- and heterodimers in vivo is under current investigation.

Somatostatin receptors and somatostatin receptor subtype expression in the immune system

As described before, the neuroendocrine and immune systems are in close relationship with each other and different neuropeptides, like VIP and substance P (SP), and their receptors are expressed in the immune system and play important roles in immune cell function (148,149). VIP is expressed in neurones, which innervate many lymphoid organs, but VIP is also expressed and secreted by thymocytes (150). Binding of VIP to VIP-receptors on thymocytes affects cytokine production, mobility and apoptosis (150). SP belongs to the family of tachykinin peptides (SP, neurokinin A, neurokin B, neuropeptide K and neuropeptide Y) (151). These molecules are involved in different aspects of neuroimmunomodulation (152). Expression of SP has been demonstrated on lymphocytes (149), monocytes, macrophages (52), outer cortex of the thymus and nerves innervating the thymus (153,154). SP plays a role in the regulation of immunoglobulin production and cytokine secretion (155). VIP and SP exert their effects via specific receptors expressed on different immune cell subsets. VIP-1 and VIP-2 receptors have been described to be widely expressed in the immune system (156). Receptors for SP have been detected on thymocytes, T- and B-lymphocytes and macrophages (52,149). A number of other neuropeptides, like bombesin, oxytocin and vasopressin, are expressed in cells and tissues of the immune system as well (5). Their localization and functional significance in the immunoregulatory network are under investigation.
SS is a neuropeptide with a broad spectrum of biological functions as well. The expression of other neuropeptides in tissues and cells of the immune system suggests that SS and its receptors as well might be expressed and play a role in the immune system. Indeed, apart from the expression of sst in the classical target organs of the peptide SS, sst have also been demonstrated in the immune systems of different species. Below, the expression and expression patterns of the different sst subtypes are presented in more detail for the murine, rat and human immune system, respectively.

Murine immune system

Initial studies demonstrated a selective expression of sst_2 in both fetal and adult murine thymus (157,158). Binding sites for fluorescent SS were demonstrated on splenic B- and T-lymphocytes (159) and RT-PCR studies revealed that the sst_2 was the sst subtype selectively expressed in murine splenocytes (160). Isolated murine T- and B-lymphocytes and macrophages selectively express sst_2 mRNA as well (158). Although sst_2 mRNA seemed to be the sst predominantly expressed in the murine immune system, one group reported the expression of sst_2, sst_3 and sst_5 on murine monocytes (161), while expression of sst_5 mRNA has also been described in the murine spleen (162). We ourselves detected a broad expression of sst_4 mRNA as well and found the expression of both sst_2 and sst_4 mRNA in murine bone marrow, thymus, spleen and PBMC (163) (table 5).

Rat immune system

Expression of sst subtypes has not been extensively studied in the rat. In comparison to the expression pattern of the sst in the murine immune system, different subtypes have been described to be expressed in the rat immune system. Studies by our group demonstrated that sst_3 and sst_4 mRNA were the main subtypes expressed in the rat thymus (164). However, studies by the group of Sedqi et al. (165) demonstrated that isolated and cultured rat thymocytes express sst_1 and sst_2 mRNA. In the spleen, expression of sst_3 and sst_4 (164) mRNA or sst_3 and sst_5 (108) mRNA has been described. Rat microglial cells, which are the resident macrophages of the central nervous system, expressed the mRNA for sst_2, sst_3 and sst_4 (166). Expression of sst in peripheral lymphocytes, monocytes or macrophages has not been studied in the rat.

Human immune system

Back in 1981, the group of Bhathena and co-workers first identified expression of sst on human mononuclear leukocytes (167). They found low-affinity binding sites on resting monocytes and lymphocytes. Subsequently, using fluorescent SS, sst were identified on mitogen-activated human peripheral lymphocytes (168). Resting peripheral blood lymphocytes, granulocytes and red blood cells did not express sst. Various cell lines of different origin (T cell, B cell, myeloma and leukemia) have been shown to express sst as well (169,170). By in vitro receptor autoradiography using the sst_2-preferring ligand [^{125}I-Tyr^3]-octreotide, SS binding sites have been demonstrated in lymphoid organs in human. Human gut-associated lymphoid tissues (palatine tonsils, ileal Peyer's patches, vermiform appendix, and colonic solitary lymphatic follicles) (171), thymic tissue and spleen (172,173) also clearly showed sst expression.
Using reverse transcriptase polymerase chain reaction (RT-PCR), the sst subtype expression in different cells and tissues of the human immune system has been studied (170,174-182). In human bone marrow cells, which are the common progenitors of the mature immune cells, a selective expression of sst_2 mRNA has been detected (174).
Human thymic tissue expresses sst_1, sst_2 and sst_3 mRNA (175). It was shown that the thymic epithelial cells selectively expressed sst_1 and sst_2, while the immature thymocytes predominantly express sst_2 mRNA and mature thymocytes express sst_3 mRNA (176). In the red pulp of human spleen, diffuse binding of [^{125}I-Tyr^3]octreotide was found (111). Interestingly, expression of sst_2 mRNA in normal peripheral blood mononuclear cells was very low compared to expression of sst_2 mRNA in peripheral blood mononuclear cells from leukaemia patients or in these cells after stimulation with PHA (170). Normal T-lymphocytes (177) and the Jurkat T-cell line (178,179) selectively express the sst_3 mRNA. However, there are also studies showing expression of all 5 subtypes on human T-lymphocytes (180). Moreover, it has been demonstrated that T-and B-cell lines can express sst_2 (181), but caution should be taken, because characteristics of these immortalized cells may differ from primary lymphoid cells. B lymphoblasts were shown to express sst_2 (182), but not much is know about expression of sst in mature B-lymphocytes, monocytes, macrophages and dendritic cells. Table 5 summarizes the

distribution pattern of the different sst subtypes in the immune systems of mouse, rat and human, respectively.

Table 5: expression of sst subtypes in cells of the murine, rat and human immune system.

5a: sst subtype expression in the mouse

Tissue	sst_1	sst_2	sst_3	sst_4	sst_5	After reference(s)
Bone marrow		+		+		(163)
Thymus		+		+		(157,158,163)
Spleen		+		+	+	(160,162,163)
PBMC		+		+		(158,163)
Macrophages		+				(158)
Monocytes		+	+		+	(161)

5b: sst subtype expression in the rat

Tissue	sst_1	sst_2	sst_3	sst_4	sst_5	After reference(s)
Thymus	+	+	+	+		(164,165)
Spleen			+	+	+	(108,164)
Macrophages		+	+	+		(166)

5c: sst subtype expression in human

Tissue	sst_1	sst_2	sst_3	sst_4	sst_5	After reference(s)
Bone marrow		+				(174)
Thymus	+	+	+			(175,176)
Spleen		+				(111)
PBMC		+				(170)
T-lymphocytes			+			(177)
T- and B-cell lines		+	+			(181)

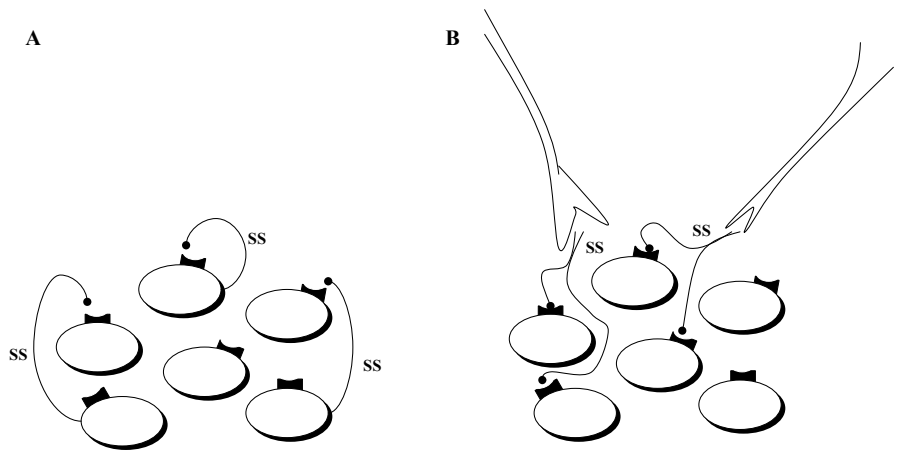
In conclusion, this paragraph demonstrated that sst are widely expressed in the immune cells and tissues of human, mouse and rat. However, many inter-species differences with respect to sst subtype expression patterns exist. Therefore, the question is raised whether results of studies using animal models can be compared to results in human studies, because different sst subtypes can be involved. Moreover, it should be questioned whether animal models can be used to investigate the role of sst in the human immune system, as

these models do not seem to fully represent the situation in the human immune system. In order to better understand the role of sst in the human immune system, both in vitro and in vivo studies should be performed selectively using cells and tissues from the human immune system.

Expression of ligands for sst in the immune system

The differential expression of sst in cells and tissues of the different immune systems suggests that these receptors play regulatory roles in immune cell function. Because SS itself has a very short half-life and thus is rapidly degraded (88), it is likely that SS is expressed in the vicinity of its receptors. Lymphoid tissues are highly innervated (183) and SS might reach the sst in the immune cells via these neurones. SS might also be expressed in the immune cells itself and act in a paracrine/autocrine way to modulate immune-cell function (figure 5).

Figure 5: sst ligands in the human immune system



A schematic view on how neuropeptides, for instance somatostatin (SS), may exert their effects on immune cells, either produced by the immune cells itself acting in a pracarine/autocrine way (A) or produced by the nerve endings, which innervate immune tissues (B)

On the other hand, it might be possible that other SS-like peptides, like CST, which binds to the various sst (74), may play a role in immune cell function as well. In this paragraph, the data on expression of ligands for the sst will be discussed.

Mouse immune system

SS expression is found in the murine thymus, where it is shown to enhance thymocyte development (157). SS was found to increase the number of thymocytes, induced migration and enhanced progression of the thymocytes to a more adult stage, with respect

to CD-marker patterns. To a greater extent, the expression of SS has been studied in granulomas formed following Schistosoma Mansoni infection in mice. These granulomas consist of eosinophils, macrophages and T- and B-lymphocytes (184). It has been demonstrated that macrophages and B-lymphocytes in these granulomas are the source of SS production (158) and SS was shown to decrease the production of IFN-γ by T-lymphocytes (185). Interestingly, expression of SS in splenic macrophages can be detected after stimulation with LPS and IFN-γ, whereas unstimulated splenic macrophages do not express SS (186). The upregulation influenced by inflammatory mediators points to a regulatory role of SS in the T-cell response (158). In conclusion, we can say that SS in the murine immune system is widely expressed and different studies have demonstrated effects of SS in this system.

Rat immune system

As well as in the murine thymus, SS is expressed in thymic tissue of the rat (187,188). It is suggested that SS acts in a paracrine mode to modulate the T-lymphocyte development. Moreover, SS has also been detected in rat spleen (188). Isolated lymphocytes express SS and SS was shown to act in a paracrine/autocrine way to inhibit lymphocyte proliferation (189). As well as in mice, SS is expressed in the rat immune system, with distinct functional properties.

Human immune system

In contrast to the rodent immune systems, not much is known about the expression of SS in the human immune system. Thymic tissue is known to express SS, but the expression is selectively localized in the thymic epithelial cells of the thymus and not in the lymphoid component (175). It is not known whether SS is expressed in the other components of the human immune system. However, when we take into consideration the differential expression of the sst subtypes throughout immune cells and tissues and the relative short half-life of the peptide, it is likely that a ligand is expressed, probably by the sst-expressing cells itself. In this prospect, the detection of CST is of high interest. It was first found to be selectively expressed in rat cortex (72), but other studies reported the expression in human leukocytes as well (75). However, a clear description of the expression of SS or CST in the different human immune cells and tissues lacks until now. A better insight in the expression pattern of either SS or CST may contribute to understanding the functional significance of sst on immune cells.

Functional significance of sst in the human immune system

The expression of different sst subtypes on immune cells implies that they can functionally respond to their natural ligand(s). Indeed, SS exerts multiple effects in the immune system, of which the most prominent will be introduced below.

Effects on cell proliferation

With respect to cell proliferation it is known that sst_1, sst_2, sst_4 and sst_5 are able to arrest cell growth and thus may contribute to an anti-proliferative effect of SS (124,127). On the other hand, SS can also induce apoptosis. Sst_3 is involved in apoptosis by inducing p53 and BAX (190,191). Equivocal studies have reported on the effects of SS on T-lymphocyte proliferation. Both inhibitory (192-194) and stimulatory (195,196) effects have been described. In B-lymphocytes only one study reported the inhibition of proliferation in a leukemia B-cell line (197)**.** Currently no consensus exists with respect to the actions of SS on lymphocyte proliferation. The varying results among the studies described might be explained by the different experimental conditions described and secondly, by the involvement of different sst subtypes and multiplicity of underlying transduction mechanisms.
Both are related to the possibility that, even after purification, heterogeneous cell populations exist.

Effects on secretion processes

The most studied effects of SS on T-lymphocytes concern the regulation of IL-2 secretion. Again both stimulatory (198) and inhibitory (177) effects of SS were found. It should be taken into consideration that in these studies the T-lymphocytes originated from different sites and the action of SS might depend on cell origin. SS was also found to have an inhibitory effect on IFN-γ production by PBMC (199). Interestingly, it was demonstrated that an anti-sst_2 antibody could block the SS-inhibited IFN-γ production in mice, pointing to the direct actions of SS on the production of this cytokine (158).
In human B-lymphocytes, immunoglobulin (IgA, IgE, IgG and IgM) secretion is decreased by SS (200,201) and SS inhibits IgE-stimulated histamine and leukotriene-D4 release from basophils (202).
Activated monocytes and macrophages secrete the inflammatory cytokines TNF-α, IL-1β and IL-6. This secretion could be decreased by SS (203). However, another study reported stimulation of IL-6 secretion by activated monocytes (204). Both SS and octreotide inhibited the respiratory burst in human monocytes dose-dependently (205). In summary, we can conclude that SS has a predominant inhibitory effect on secretion processes in immune cells. However, this conclusion is mostly based on in vitro studies and the functional significance of these results for modulation of the immune system by SS in vivo, e.g. with respect to secretion processes remains largely unknown.

Migratory effects

SS might as well mediate the regulation of migration of different cells, for example towards sites of inflammation. SS has been shown to control adhesion of T-lymphocytes to fibronectin, which is an important glycoprotein of extracellulair matrix (206). More recently, it has been demonstrated that SS might act as chemo-attractant to human primitive hematopoietic cells (207). These studies suggest that SS may have migratory effects on immune cells and therefore can either play a role in inflammatory processes by directing immune cells toward the affected sites or may play a role in developmental pathways of immature progenitor cells.

In summary, we can conclude that the role of SS in the human immune system needs to be further elucidated. Many equivocal results have been described in the in vitro experiments and not much is known yet about the exact expression pattern of SS or SS-like ligands in the immune system. However, the expression of different sst subtypes in immune cells and the expression of SS in human thymic tissue (176) together with the innervating neurones, which can "deliver" SS at sites of sst expression (183) suggests that the SS-sst system may play an important role in modulating immune cell function.

Sst expression in diseases affecting the human immune system

Apart from the expression of sst in immune cells and tissues in physiological conditions, sst have been demonstrated in different inflammatory diseases and malignancies affecting the immune system. Most of the data on sst expression in immune diseases are obtained by [^{111}In-DTPA-D-Phe^{1}]-octreotide (^{111}In-pentetreotide) scintigraphy studies. Sst scintigraphy using radioiodinated SS analogues ([^{123}I-Tyr^{3}]octreotide) was initially introduced successfully for visualization of neuroendocrine tumours, which are known to express high levels of sst (114,208). After intravenous injection of radionuclide-labeled SS analogues, primary tumours and metastases can be visualized using a gamma camera (208,209). However, scintigraphy using [^{123}I-Tyr^{3}]octreotide showed to have several drawbacks. Some of its major drawbacks were the short half-life, rapid clearance from the blood, and the accumulation of radioactivity in the intestines (210). Therefore, a new radioloabeled SS analogue, ^{111}In-pentetreotide, was developed (211,212). ^{111}In-pentetreotide scintigraphy is nowadays widely used in diagnosis of neuroendocrine tumours (208,212-216). However, in recent years, sst scintigraphy has been employed in visualization of other non-neuroendocrine tumours and granulomatous or autoimmune diseases as well. In this paragraph the expression of sst and possible functional implications of SS in human immune diseases will be introduced.

Lymphomas

Using sst scintigraphy, sst have been detected in both T and B non-Hodgkin's lymphomas, as well as in Hodgkin's lymphomas (217-220). In vitro autoradiography studies have demonstrated that sst are mainly expressed in the lymphoblastic areas of the lymphomas, which are the most active parts of these tumours. In non-Hodgkin's lymphomas, sst were detected in vitro in 92% of low-grade malignancies, 100% of intermediate-grade malignancies and 70% of high-grade malignancies. Sst were expressed in about 100% of the Hodgkin's lymphomas (221). Moreover, it has recently been shown that Hodgkin's lymphomas and non-Hodgkin's lymphomas expressed sst_2 and sst_3 mRNA selectively (222). Different in vivo sst scintigraphy studies have demonstrated that a very high sensitivity of scintigraphy to detect Hodgkin's lymphomas was obtained, varying from around 90 to 100 % (217,218,223-225). Sensitivity to detect non-Hodgkin's lymphomas by sst scintigraphy has also been shown to be high, between 80 and 100 % (217,218,223,224,226-228). Moreover, it was demonstrated that sst scintigraphy revealed affected sites, which were not previously detected by conventional techniques (227,228). These findings have contributed to the use of sst scintigraphy in diagnosis and staging of both non-Hodgkin's and Hodgkin's lymphomas. The expression of sst in lymphomas might be of value, apart from their role in diagnosis and staging, in treatment of these diseases as well. Preliminary data showed that patients with non-Hodgkin's lymphomas, which were treated with octreotide, showed a partial remission in 36 % of the cases (229). In literature it has been hypothesized that SS or one of its analogues might play a future

role in treatment of malignant lymphomas, either as 'cold' peptides (230) or as β-emitting radionuclide coupled peptides (231).

Rheumatoid arthritis

The most characteristic manifestations of rheumatoid arthritis (RA)- joint pain, joint swelling and reduced mobility- are the result of synovitis, an inflammatory process in the synovial tissue. Joint structure destruction, which results in consequent deformation and loss of function, is caused by this inflammation. RA is characterized by persistent immunological activity, in which diffuse lymphocytic infiltration (predominantly CD4-positive lymphocytes) and/or macrophages are present in most cases (232). It was hypothesized that these cells, which are in activated state in synovitis, might express sst, which might allow the detection of the inflammatory joints by sst scintigraphy. In an initial study expression of sst was demonstrated in all patients suffering from RA (233). Immunohistochemistry studies revealed that sst_2 was the sst subtype expressed in affected joints of RA patients (234). Macrophages were the source of sst_2 expression in these joints, while T-lymphocytes did not express sst_2. In vitro studies showed that incubation of synovial cells, which expressed sst_1 and sst_2, with SS resulted in inhibition of the synovial cell proliferation (235). Moreover, in a clinical trial, using the SS-analogue octreotide, significant improvement was found in disease state (236). These findings indicate that SS and its receptors may have an important role in treatment of autoimmune disease, like RA. Moreover, β-emitting radionuclide coupled SS analogues may play a therapeutic role in refractory RA patients, which do not respond to conventional therapies, by possibly targeting the macrophages. Uptake of radioactivity by these macrophages may then result in the so-called “bystander effect”, which means that these cells generate significant toxicity to the surrounding infiltrating cells. On the other hand, development of radionuclide-coupled sst_3-preferring ligands may be useful in targeting the T-lymphocytes, which express sst_3 (177), present in the inflammatory processes.

Sarcoidosis

Sarcoidosis can be characterized as a granulomatous disorder of unknown origin. Granulomas formed in sarcoidosis consist of modified macrophages and lymphocytes. Activated CD4-positive lymphocytes, which are present in the lung, secrete IL-2 (237). IL-18 has also been reported to play a role in granuloma formation (238). Production of these cytokines leads to IFN-γ production and macrophage activation and finally, granuloma formation (238). In 60% of the patients the granulomatous response resolves after 2-5 years. CD8-positive T lymphocytes (239) and IL-10, which suppresses the inflammatory response (240) play an important role in granuloma resolution. Aggregated granulomas may also result in pseudotumour lesions and in advanced stages they may become enclosed by fibrous tissue and can eventually be replaced by hyaline scars. IL-8, IL-12 and TNF are the cytokines mainly involved in chronic disease (241,242). The increase of IL-8 is associated with TGF-β, a cytokine that promotes fibrosis. Fibrosis in

the lung is correlated with poor pulmonary function and a poor prognosis with increased morbidity and mortality (243). Therefore, prevention of fibrosis in sarcoidosis is of major importance. Many groups have studied therapeutical compounds for sarcoidosis. However, until now, no good medicines to treat sarcoidosis have been described (244). Sst have been localized by in vitro receptor autoradiography using [^{125}I-Tyr^{3}]octreotide in biopsies of lesions of patients suffering from sarcoidosis (245). Binding of [^{125}I-Tyr^{3}]octreotide was mainly found in the region of epithelioid cells and giant cells. In further detail, sst_2 expression was demonstrated in granulomas formed in sarcoidosis in man (246). The expression of sst_2 was associated with cells of the mononuclear phagocyte lineage, including epithelioid cells and multinucleated giant cells. In vivo sst scintigraphy studies showed visualization of disease-affected sites in 97-100% of sarcoidosis patients (245,247). Moreover, previously undetected involved sites of inflammation were detected by sst scintigraphy and it was demonstrated that uptake of the radionuclide-labeled SS analogue was decreased after successful treatment with corticosteroids (247). Because of the low number of studies describing in vivo visualization of sarcoid granulomas, we can not yet conclude whether this might have clinical importance. However, it seems that sst scintigraphy may have implications in diagnosis of sarcoidosis and possibly in treatment follow-up. On the other hand, because of the expression of sst in sarcoid granulomas, SS analogues may have therapeutical implications, possibly via inhibiting secretion of pro-inflammatory cytokines.

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Chapter I.2

AIM OF THE THESIS

As presented in the overview in the previous chapter, it has become more evident during the last decades that different systems in man are not working independently from each other to maintain homeostasis, which is necessary for survival, but more likely they interact in order to reach this goal. In recent years it has been demonstrated that the neuroendocrine and immune systems interact as well. The functional significance of somatostatin has been extensively studied in the neuroendocrine system, but as with some other neuropeptides, it has been suggested that somatostatin plays a regulatory role in the immune system as well. The first step in evaluating the functional significance of somatostatin and somatostatin receptors in cells and tissues of the human immune system in physiology and pathophysiology is elucidation of the expression pattern of somatostatin and its receptors in the various components of the immune system. Receptors for somatostatin have been described in the immune systems of both rodents and man, although the expression patterns of somatostatin receptor subtypes differ between species. Although the expression pattern of somatostatin receptor subtypes has been elucidated partially in man, expression patterns are still controversial in T- and B-lymphocytes and remain unclear in cells of the monocyte lineage, i.e. monocytes, macrophages and dendritic cells. Moreover, it is not known whether the natural ligand, somatostatin, itself is expressed in the immune system. This brings us to the following questions:

Is somatostatin expressed in cells and tissues of the human immune system? Secondly, which somatostatin receptor subtypes are expressed in human lymphocytes and cells of the monocyte lineage?

Cloning of the genes for somatostatin receptors and somatostatin allows to study their expression patterns in different immune cells.
With respect to the first question, the expression of somatostatin in immune cells, like monocytes, macrophages, dendritic cells and lymphocytes and in immune tissues like thymus, spleen and bone marrow will first be investigated by reverse transcriptase polymerase chain reaction (RT-PCR). Recently, a novel somatostatin-like peptide has been characterized, named cortistatin. Therefore, the expression of cortistatin in cells, in both basal and activated state, and tissues of the immune system as a possible ligand for somatostatin receptors will be evaluated by RT-PCR as well. By autoradiography we will investigate whether cortistatin is able to bind, and thus act as possible ligand, to somatostatin receptors in the immune system. Finally, expression of somatostatin and cortistatin will be evaluated in different tissues throughout the human body by quantitative PCR, in order to determine the place of the immune system in the human body, with respect to expression of these somatostatin receptor ligands.
Subsequently, in detail, the somatostatin receptor expression pattern will be investigated in T- and B-lymphocytes and monocytes from healthy donor blood and in macrophages and dendritic cells, which are obtained by in vitro cultures of monocytes. Somatostatin receptor expression patterns will be studied using the reverse transcriptase-polymerase chain reaction technique. In order to get more insight in the expression pattern of somatostatin receptors in mature and/or stimulated immune cells, quantitative polymerase chain reaction assays will be developed to investigate regulation of somatostatin receptor expression patterns during differentation of monocytes into mature immune cells, i.e.

macrophages and dendritic cells. Moreover, expression of somatostatin receptors will be investigated in in vitro cultured and stimulated immune cells, i.e. monocytes, macrophages, dendritic cells, T- and B-lymphocytes. Cells will be stimulated with lipopolysaccharide, interferon-γ, phytohemagglutinin and glucocorticoids (dexamethasone) to evaluate the regulation of somatostatin receptor mRNA levels by stimulation of the different immune cells. In vitro cultured and stimulated cells will be studied, as models for in vivo activated cells, like for instance in infections. Evaluating the expression patterns of somatostatin, cortistatin and somatostatin receptor expression in cells and tissues of the human immune system automatically evokes the next question:

Which functional effects do somatostatin receptors and their ligands have in the immune system?

Considering the potential important role of neuropeptides in the development of thymocytes, the effects of somatostatin, octreotide and cortistatin on isolated human thymocytes will be studied. The experiments mainly focus on the effects on cell proliferation. Effects on cell proliferation will be determined by [methyl-^{3}H]-thymidine incorporation experiments.
In order to evaluate the general effects of somatostatin analogues on gene expression in macrophages, we will perform a micro-array experiment, in which we will focus on the effects of octreotide on gene expression in these cells. This experiment will provide a better insight in the pathways in macrophages that are influenced by somatostatin analogues.
Subsequently, a model will be set up to evaluate the effects of somatostatin and its analogues on macrophage-induced fibroblast proliferation, as an in vitro model for sarcoidosis. Sarcoidosis is an inflammatory disease that may result in destructive fibrosis, a process to which until now no successful medication exists. Inhibition of fibrosis would contribute to a better prognosis and lower morbidity. Macrophages are a key component in sarcoidosis and therefore, the effects of macrophages on fibroblast proliferation will be evaluated. The factors that are responsible for fibroblast proliferation will be determined by enzyme-linked immunosorbent assay and the effects of somatostatin and its analogues, octreotide and cortistatin, on secretion of these compounds will be studied. Moreover, the direct effects of macrophages on fibroblast proliferation will be studied in co-cultures. The results of these studies may help to provide an answer to the question whether somatostatin analogues may play a future role in the treatment of inflammatory disease to diminish fibrosis.
Investigating the possible therapeutical implications of somatostatin analogues in diseases affecting the human immune system raises the question

whether radionuclide labeled somatostatin analogues may play a role in treatment of diseases affecting the human immune system.

As radionuclide labeled somatostatin analogues are successfully used in visualization (diagnosis) and therapy of neuroendocrine tumours, the possible role of radionuclide labeled somatostatin analogues in the treatment of human lymphoid malignancies will be studied. In vitro studies will be performed to determine somatostatin receptor subtype

expression patterns, somatostatin receptor expression levels and ability of B lymphoid and myeloid cell lines to internalize the radionuclide labeled somatostatin analogue. On basis of these experiments the role of peptide radionuclide targeted therapy in lymphoid malignancies will be discussed.
Finally, in the general discussion the implications of the findings of the studies presented in this thesis will be discussed.

Chapter II

SOMATOSTATIN RECEPTORS AND THEIR LIGANDS IN HUMAN IMMUNE CELLS

II.1

CORTISTATIN RATHER THAN SOMATOSTATIN AS A POTENTIAL ENDOGENOUS LIGAND FOR SOMATOSTATIN RECEPTORS IN THE HUMAN IMMUNE SYSTEM

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Abstract

Cells of the human immune system have been shown to express somatostatin receptors (sst). The expression of sst suggests a functional role of the peptide somatostatin (SS). However, SS expression has not been demonstrated yet in different human immune tissues. Therefore, we investigated by RT-PCR the expression of both SS and cortistatin (CST), a SS-like peptide, in various human lymphoid tissues and immune cells. We detected SS mRNA expression in the human thymus only, while not in thymocytes. CST mRNA was clearly expressed in the immune cells, lymphoid tissues, and bone marrow. Using quantitative RT-PCR, significant differences in expression levels between tissues were demonstrated. Expression of CST mRNA was upregulated during differentiation of monocytes into macrophages and dendritic cells and could be upregulated by lipopolysaccharide stimulation. Two differently sized cDNA fragments of CST were detected in the majority of cells and tissues. However, whereas both fragments were detected in nearly all T-cell lines (7 out of 8), most of the B-cell lines expressed the short fragment only (8/10). Using autoradiography we showed that CST displaced [^{125}I-Tyr^{3}]octreotide binding with relative high affinity on human thymic tissue and sst_2-expressing cells. This is the first extensive study demonstrating that human lymphoid tissues and immune cells express different levels of CST mRNA and that its expression can be regulated. On the basis of these observations we hypothesize a role for CST as an endogenous ligand of at least the sst_2 receptor in the human immune system, rather than SS itself.

Introduction

Somatostatin (SS) is a neuropeptide widely distributed throughout the human body. The central nervous system, gastro-intestinal tract and endocrine glands are the major sites of production (1,2). SS has a predominantly inhibitory action, especially with regard to the release of mediators, such as hormones (3,4). The action of SS is mediated through G-protein coupled seven transmembrane receptors of which 5 different receptors were cloned, named sst_{1-5} (5). The receptor subtypes show a specific distribution in different organs and the natural ligands SS-14 and SS-28 both have high binding affinity to all five receptor subtypes (5). The sst family can be divided in two subclasses, the first consisting of sst_2, sst_3 and sst_5 and the second consisting of sst_1 and sst_4, based on differential binding affinities of the various ligands (6). Conformationally restricted octapeptide analogues of SS bind with low affinity to sst_1 and sst_4, whereas these analogues bind with higher affinity to sst_2, sst_3 and sst_5 receptors. Recently, a peptide bearing strong structural resemblance to SS was discovered. This peptide, named cortistatin (CST) has an identical receptor binding domain (7) and binds with high affinity to all five human sst subtypes (8). CST mRNA was first found to be expressed in the human brain, but in further studies in other tissues like stomach, kidney and leukocytes as well (9). Until now, no specific receptor for CST has been found. Expression studies by RT-PCR showed that with different primer sets, two differently sized cDNA fragments could be detected, a 317-bp fragment in the human brain, kidney, stomach and leukocytes, and a 701-bp fragment selectively expressed in the human brain (9).
In previous studies a differential expression of sst in human immune cells and tissues has been observed. This differential expression of sst on immune cells suggests that these receptors play a role in the human immune system (10). Little is known however, with respect to the expression of SS in the human immune system. In the human thymus SS mRNA was found, synthesized in the epithelial and not in the lymphoid component (11). In order to explore the expression of SS and its related compound CST, we investigated mRNA expression of SS and CST (iso-forms) by RT-PCR in different immune tissues like thymus, spleen and bone marrow. In the human thymus, we distinguished thymic epithelial cells (TEC) from thymocytes to investigate whether differences in expression of CST and SS mRNA exist between these cells in this organ. Moreover, a number of human immune cell subsets, i.e. peripheral blood leukocytes (PBMC), monocytes, macrophages, dendritic cells and B- and T-lymphocytes were studied as well. Finally, SS and CST mRNA expression was evaluated in human T- and B-cell lines of different maturation levels. Using quantitative RT-PCR (Q-PCR), differences in expression levels of CST mRNA between immune cells and tissues were investigated. Monocytes and monocyte-derived macrophages, both unstimulated and lipopolysaccharide (LPS)-stimulated, were evaluated to get insight into the regulation of CST mRNA expression during differentiation of monocytes as well as in cells in activated state. In addition to the mRNA studies we performed receptor autoradiographic studies using [^{125}I-Tyr^3]octreotide on human thymic tissue and sst_2 transfected cells in order to evaluate whether CST is able to bind to sst receptors in human immune tissue.

Material and Methods

Isolation of blood mononuclear cells
PBMC were isolated from buffy coats by Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) density gradient centrifugation. Subsequently, B- and T-lymphocytes were separated from PBMC using CD-19- and CD3-antibody coated magnetic beads, respectively, as described previously in detail (11). Monocytes were isolated from the PBMC fraction using a Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation as described before (12). Isolated cells were frozen in 10% DMSO-medium and stored in -80° C until use.

Cell-culture
Monocytes were cultured and allowed to differentiate into macrophages and dendritic cells as described before (13). FACS-analysis (data not shown) confirmed phenotypes of the cultured cells. Monocytes and macrophages were stimulated for 24 hours with LPS (Sigma Aldrich bv, Zwijndrecht, The Netherlands) at a final concentration of 2 μg/ml. Thereafter, cells were collected and stored at -80 ˚ C.

T-and B-cell lines
The human cell lines we used included $CD3^-$ (HSB-2, Molt-3, ALL-1, MT-1), TCRγδ (Peer) or TCRαβ (Molt-16, HuT-78, HPB-ALL) T-cell lines and precursor (RS4;11a, Nalm-1, BV-173, Nalm-6), mature (JY, TMM), Burkitt (Raji, Daudi) and plasma (LP-1, L-363) B-cell lines. Total RNA preparations from these cells were isolated as described before (14).

Samples
Normal thymic tissues were obtained from children during cardiovascular surgery. Thymic epithelial cells were collected as described in detail previously (11). Thymocytes were collected using a filter chamber (NPBI, Emmer-Compascuum, The Netherlands). Splenic tissue was obtained from patients suffering from splenic rupture. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained. Samples were taken directly at operation, quickly frozen on dry ice and stored at -80˚C. Tissue was squeezed and lysed before use as described below. Human bone marrow cells were obtained from healthy donors following informed consent.

RT-PCR studies
RT-PCR was performed as described previously (11). Briefly, poly A^+ mRNA from tissue samples and cells was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). cDNA was synthesized from the poly A^+ mRNA, which was eluted from the beads in H_2O for 10 minutes at 65 °C, using Oligo $(dT)_{12\text{-}18}$ Primer (Life Technologies). 20 μl of the cDNA was used for each PCR-amplification using primer sets for human SS, CST and HPRT (as a control) (Table 1). Two different primer sets were used, cortistatin A and B to detect expected cDNA fragments of 173 bp and 701 bp respectively (figure 1, ref 9). cDNA of human brain RNA (Invitrogen, Groningen, The Netherlands) was used as a

positive control. The PCR-reaction was performed as described before (11). Identities of the products were confirmed by direct sequencing using an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems, The Netherlands) according to manufacturers protocol.

Table 1. Primers used for RT-PCR analysis

Primer	Sequence (5' - 3')	Expected Size
HPRT (forward)	-CAGGACTGAACGTCTTGCTC-	413
HPRT (reverse)	-CAAATCCAACAAAGTCTGGC-	
Somatostatin (forward)	-GATGCTGTCCTGCCGCCTCCAG-	349
Somatostatin (reverse)	-ACAGGATGTGAAAGTCTTCCA-	
Cortistatin A (forward)	-GCAAATTCGCTCTAAACACAGGA-	173
Cortistatin A (reverse)	-TTGGGAAGGAGGAGAGGAAAGAT-	
Cortistatin B (forward)	-CTCCAGTCAGCCCACAAGAT-	701
Cortistatin B (reverse)	-CAAGCGAGGAAAGTCAGGAG-	

Primer sets for cortistatin were derived and/or adapted from Ejeskar et al. (9).

Q-PCR studies

Total RNA was isolated using either a High Pure RNA Tissue Kit or High Pure RNA Isolation Kit for tissue- and cell samples resp. (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturers protocol. cDNA was synthesized in an RT-reaction as described previously (11), using 2000 ng total RNA per reaction in a total volume of 40 µl. Q-PCR was performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, The Netherlands) , 300 nM forward primer , 300 nM reverse primer , 200 nM probe and 1 µl cDNA template, corresponding to 50 ng total RNA in the RT-reaction, in a total reaction volume of 25 µl. The reactions were carried out in an ABI 7700 Sequence Detector (Perkin Elmer, The Netherlands). After an initial heating at 95° C for 8 minutes, samples were subjected to 40 cycles of denaturation at 95° C for 15 seconds and annealing for 1 minute at 60° C. The primer sequences we used included: CST forward 5'-GGAGAGAAGCTCCAGTCAGC-3' and CST reverse 5'-GGTCCACTCAAACCACCAA-3'. The probe sequence for CST mRNA was: 5'-FAM-TATGCTCGCTGTCTCGGCCG-TAMRA-3'. The expression levels of CST mRNA were determined relatively by means of a standard curve generated in each experiment from cDNA isolated from a RAJI cell-line and is given in arbitrary units.

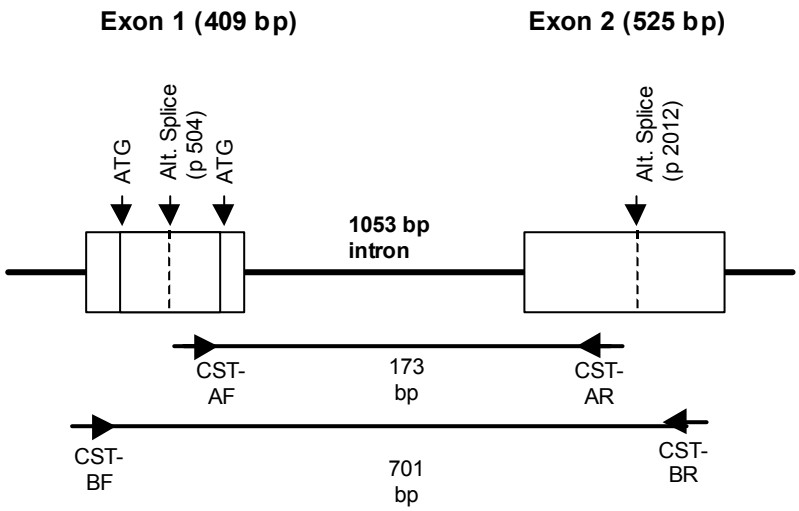

Figure 1: Overview of the genomic organisation of preprocortistatin. Exons are boxed. Alternative splicing sites (indicated by p504 and p2012) and primers used for expression studies are indicated. Sequences of different primer sets are listed in table 1. Overview and primers are adapted from Ejeskar et al. (9). CST-AF: forward primer for CST A mRNA, CST-AR: reverse primer for CST A mRNA, CST-BF: forward primer for CST B mRNA, CST-BR: reverse primer for CST B mRNA.

Somatostatin receptor autoradiography

Binding of CST to sst in the human thymus was investigated by autoradiography on unfixed cryosections using [^{125}I-Tyr^{3}]octreotide, which was prepared as described previously (15). Thymic tissue was collected at operation, immediately frozen and small parts were embedded in TissueTek (Miles Inc., Elkhart, USA) and processed for cryosectioning. 20 µm thick sections were mounted on gelatin-coated glass slides and stored at -80° C for 3 days to improve adhesion of tissue to the slides. Autoradioghraphy was performed as described previously (16) on cryostat sections of human thymic tissue and cell pellets of stably sst_2-transfected CC531 colon adenocarcinoma cells, serving as a control for binding of the different compounds to sst_2 specifically. CC531 cells were originally established from an adenocarcinoma and maintained by serial passage after trypsinization in culture medium (17). The human sst_2 cDNA in pBluescript (pBS) was a kind gift of Dr. G.I. Bell (Howard Hughes Medical Institute, Chicago, IL, USA). This sst_2 cDNA was excised from pBS and inserted into the Nhe-I and Sal-I cloning sites of the retroviral vector pCI-neo (Promega, Madison WI, USA). After exposure to Kodak Biomax MS-film the number of pixels was quantified automatically using an AlphaImager^{TM} 1220 analysis imaging system (Alpha Innotech Corporation, San Leandro CA, USA). Values are expressed as the percentage of specific binding on control sections without unlabeled peptide.

Results

By RT-PCR studies we investigated the expression of both SS and CST mRNAs in various tissues and cells of the human immune system. As shown in panel 2 of figure 2, representing SS mRNA expression, SS was only detected in whole thymic tissue and TEC, while not in thymocytes, pointing to an expression of SS only in the epithelial compartment of the human thymus and not in the lymphoid component, as demonstrated previously (11). In addition, no SS mRNA was expressed in human spleen and bone marrow. CST mRNA, however, was clearly expressed in all tissues tested (panel 3 and 4). The expected 173 bp cDNA fragment of CST is detected in all tissues in which also the expected 701 bp cDNA fragment was found, because the set of primers encoding the short fragment is in between the two primers encoding the longer fragment (figure 1). When both cDNA fragments are detected, no statement can be made, whether there might be another iso-form of the CST, because the shorter fragment could be part of the longer fragment. However, compared to human brain cDNA, the 701 bp fragment was expressed relatively weak in the tested samples. In human spleen, we only detected the short, 173 bp, cDNA fragment, whereas both fragments were detected in the other three samples, i.e. the thymus, thymocytes and bone marrow. Next to the expression of SS mRNA in human TEC, expression of CST mRNA was detected in these cells as well. As shown in panel 2, SS mRNA was not detected in monocytes, macrophages, dendritic cells and PBMC, whereas in all these cells CST mRNA was clearly detectable. In monocytes only the short, 173 bp, fragment, could be detected. The results of RT-PCR for the long, 701 bp, fragment showed a very weak signal, compared to human brain mRNA, and might even be below the detection limit in some cell-samples due to its very weak expression.

In addition, no SS mRNA expression was detectable in macrophages activated with LPS during 24 and 48 hours (data not shown). In order to further evaluate the expression of SS and the potential 2 CST iso-forms in lymphocytes, we also performed RT-PCR analysis on B-and T-cells in peripheral blood of healthy volunteers and T- and B-cell lines with different levels of maturation. The results of these studies are summarized in tables 2 and 3.

Table 2: mRNA expression of SS and CST in different peripheral blood cells

Cell type	HPRT	SS	CST A	CST B
PBMC	+	-	+	+
Monocytes	+	-	+	-
B-lymphocytes	+	-	+	+
T-lymphocytes	+	-	+	+

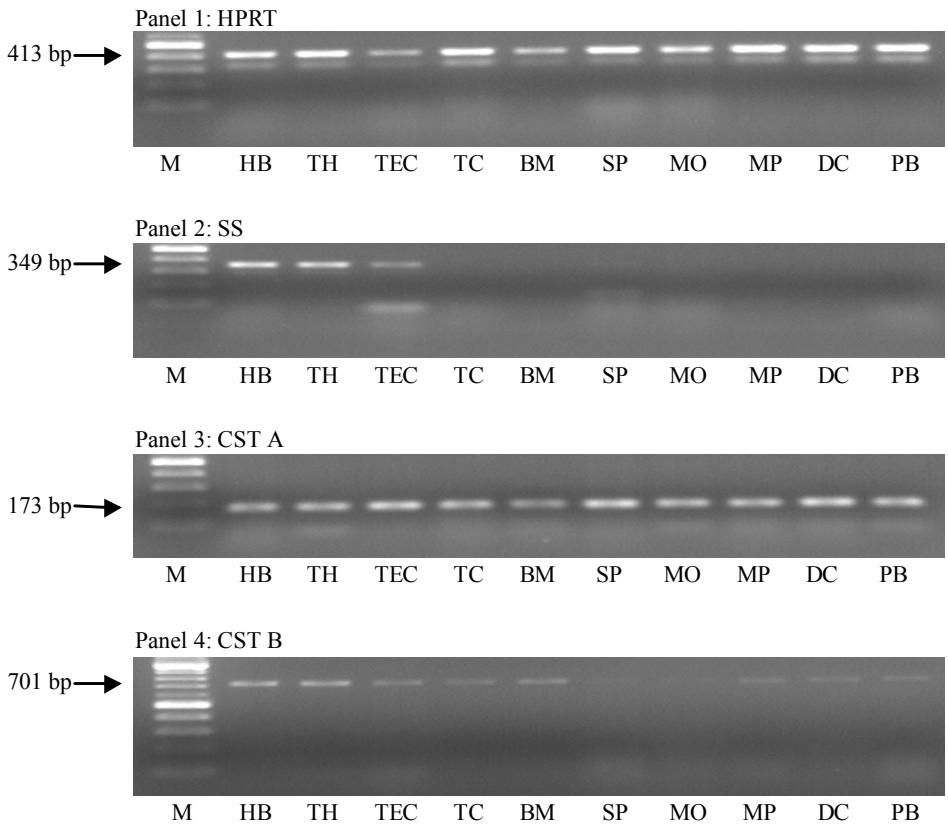

Figure 2: Expression of SS and CST mRNAs in human immune tissues and cells. Poly-A^+ mRNA was prepared from human brain (HB), thymus (TH), thymic epithelial cells (TEC), thymocytes (TC), bone marrow (BM), spleen (SP), monocytes (MO), macrophages (MP), dendritic cells (DC) and peripheral blood mononuclear cells (PB). cDNA was synthesized and amplified using primers specific for HPRT (413 bp fragment; panel 1), SS (349 bp fragment; panel 2) and two isoforms of CST (173 and 701 bp, respectively; panels 3 and 4).

In agreement with the observations in the primary human immune cell types, no expression of SS mRNA could be detected. In B-and T-cells both fragments of CST were detected (table 2). Surprisingly, a clear difference was found in the expression of both forms in B- and T-cell lines. Whereas the 173 bp cDNA CST fragment was found in the majority of the T- (7 out of 8) and B- (9 out of 10) cell lines, the longer fragment was detected in nearly all T-cell lines (7 out of 8), while only 2 out of 10 B-cell lines expressed this iso-form (table 3).

Table 3: mRNA expression in different T- and B-cell lines

	CELL LINE	HPRT	Sst_2	SS	CST A	CST B
T-cell lines	HSB-2	+	+[b]	-	+	+
	Molt-3	+	-	-	+	+
	ALL-1	+	-	-	+	+
	MT-1	+	+[b]	-	+	+
	Peer	+	+[a]	-	+	+
	Molt-16	+	-	-	+	+
	HuT-78	+	-	-	-	-
	HPB-ALL	+	+[a]	-	+	+
B-cell lines	RS4,11	+	-	-	+	-
	Nalm-1	+	-	-	+	+
	BV-173	+	-	-	+	-
	Nalm-6	+	-	-	+	-
	Raji	+	-	-	+	+
	Daudi	+	-	-	+	-
	JY	+	+[b]	-	+	-
	TMM	+	+[b]	-	-	-
	LP-1	+	-	-	+	-
	L-363	+	-	-	+	-

Data of sst_2 expression are derived from [a] Lichtenauer et al. (18) and [b] van Hagen et al. (19).
Total mRNA was isolated from CD3- (HSB-2, Molt-3, ALL-1, MT-1), TCR-γδ (Peer) or TCR-αβ (Molt-16, HuT-78, HPB-ALL) T-cell lines and precursor (RS4;11, Nalm-1, BV-173, Nalm-6), mature (JY, TMM), Burkitt (Raji, Daudi) and plasma (LP-1, L-363) B-cell lines.
On these samples RT-PCR analysis was performed as described in material and methods section. Specific primer sets for SS and CST (173 bp (CST A) and 701 bp fragment (CST B)) were used to detect SS or CST mRNA.

To evaluate differences in expression levels of CST mRNA between the different human immune cells and -tissues, Q-PCR was performed. Results of the Q-PCR experiments are summarized in table 4.

Table 4: CST mRNA expression levels in different human immune cells and tissues

Sample	n	Relative amount of CST mRNA	% relative to thymus
Thymus	3	22 ± 12	71
TEC	3	31± 8	100
Thymocytes	2	4.6 ± 0.5	15
Spleen	2	2.8 ± 0.9	9
Bone marrow	2	< detection limit	-
PBMC	3	0.8 ± 0.2	2.5
Monocytes	4	0.15 ± 0.05	0.5
Macrophages	4	9.6 ± 1.4	31
Dendritic cells	4	1.7 ± 0.3	5.5

Table represents the quantified expression levels of CST mRNA in different human immune cells and -tissues. Values are presented as arbitrary units, relative to a standard curve generated from a RAJI cell-line and are results from quantitative RT-PCR experiments using 50 ng/µl total RNA for the RT-reaction per sample. **n** represents the number of samples investigated and the relative amount of CST mRNA is expressed as the mean ± SD of these samples. In the last column expression of CST mRNA is shown, as a percentage relative to expression of CST mRNA in human thymic epithelial cells (TEC), which is set at 100%.

As shown, significant differences in expression levels of CST mRNA were observed between the different cells and tissues. A relative high level of CST mRNA was measured in the human thymus and TEC, a lower expression was detected in spleen and human thymocytes, whereas in human bone marrow CST mRNA levels were below detection limit for Q-PCR. In the cell samples, a relative low expression level of CST mRNA was detected in PBMC and monocytes, whereas expression of CST mRNA was considerably higher in monocyte-derived macrophages and dendritic cells, approximately 60- and 10-fold respectively compared to monocytes. Regulation of CST mRNA expression was studied in monocytes and monocyte-derived macrophages cultured for 6 days. Results of these experiments are shown in figure 3.

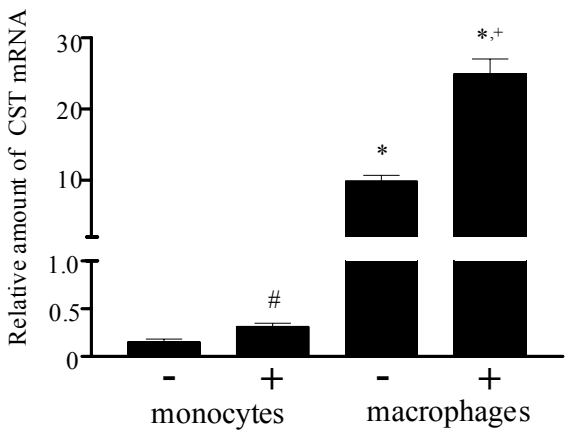

Figure 3: Regulation of CST mRNA expression in monocytes and macrophages. The bars represent expression of CST mRNA in monocytes and macrophages without (-) or with (+) LPS-stimulation. The bars represent means ± SD of 4 independent experiments and represent arbitrary units relative to a standard curve generated from a RAJI-cell line as results of quantitative RT-PCR using 50 ng/µl total RNA in the RT-reaction per sample. Monocytes: 0.15 ±0.05, monocytes + LPS: 0.3 ±0.04, macrophages: 9.6 ±1.4 and macrophages + LPS: 28.9 ±3.2. *P<0.0001 vs monocytes, $^{\#}$P=0.002 and $^{+}$P<0.0001 vs cells without LPS.

As described above, resting monocytes showed low expression of CST mRNA. When monocytes differentiated into mature macrophages in vitro, CST mRNA levels increased approximately 60-fold. LPS stimulation of both monocytes and macrophages resulted in a 2 and 3-fold increase in CST mRNA respectively, compared to these cells in unstimulated conditions.

In order to determine whether CST binds to human endogenously expressed sst we performed displacement studies by autoradiography on tissue sections of human thymus using [^{125}I-Tyr^{3}]-octreotide. [^{125}I-Tyr^{3}]-octreotide binding was mainly localized in the medullary region of the thymus. Unlabeled octreotide, CST-17 and SS were used to investigate displacement of [^{125}I-Tyr^{3}]-octreotide binding from its receptor, which in the human thymus probably is sst_2 as previously described (11). Binding of [^{125}I-Tyr^{3}]-octreotide was displaced in a dose-dependent manner by unlabeled octreotide, SS and CST-17. Figure 4 (left panel) shows that octreotide and SS both displaced [^{125}I-Tyr^{3}]-octreotide binding with high affinity (IC_{50} values of 6.8 x 10^{-10} M and 1.9 x 10^{-9} M, respectively). CST-17 displaced [^{125}I-Tyr^{3}]-octreotide binding as well, however with a slightly lower affinity (IC_{50} value of 2.2 x 10^{-8} M) compared to SS and octreotide. Binding and displacement of [^{125}I-Tyr^{3}]-octreotide to sst_2 receptors was confirmed on human sst_2-transfected cells as shown in figure 4 (right panel). The IC_{50} values for displacement of

[^{125}I-Tyr^3]-octreotide binding to sst_2 receptors were 1.2 x 10^{-9}, 1.7 x 10^{-9} and 4.0 x 10^{-9} M, respectively for SS, octreotide and CST-17.

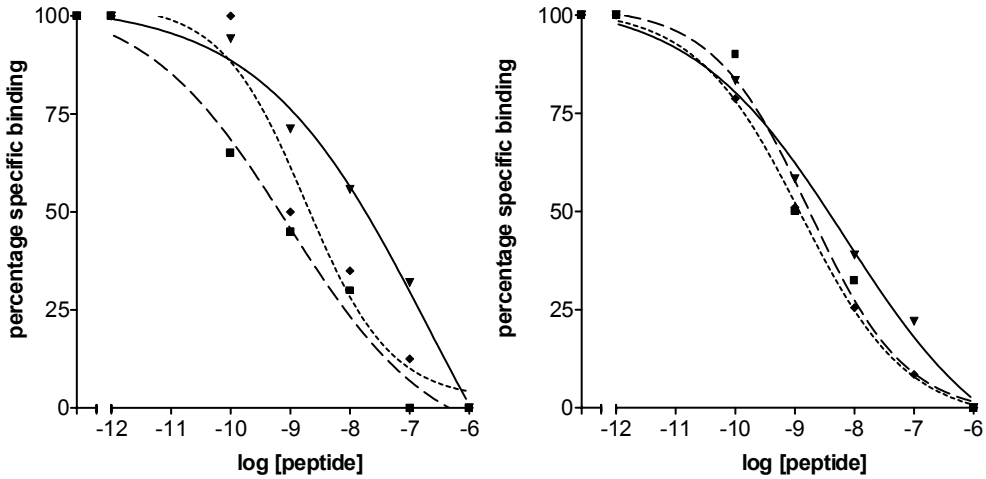

Figure 4: Displacement of [^{125}I-Tyr^3]octreotide binding on cryostat sections of human thymic tissue (left panel) and cell pellets of human sst_2-expressing cells (right panel).
20 and 10 µm sections respectively were incubated with [^{125}I-Tyr^3]octreotide without or with increasing concentrations of unlabeled SS, octreotide or human CST-17. After exposure to Kodak Biomax MS-film the number of pixels was quantified automatically using an AlphaImager^{TM} 1220 analysis imaging system (Alpha Innotech Corporation, San Leandro CA, USA) . Values are expressed as the percentage of specific binding on control sections without unlabeled peptide. Symbols represent displacement by SS (····· ♦ ·····), octreotide (– – ■ – –) or CST-17 (——▼——).

Discussion

Neuropeptide hormones are involved in a pattern of the complex interactions that exist between the human neuroendocrine and immune system. A place in this network might be hypothesized for SS and its receptors as well. Sst have been demonstrated in various endocrine and lymphoid tissues by classical ligand binding studies (20-22). SS mRNA itself has been detected only in the epithelial component of the thymus (11), while not in thymocytes. In addition, it is not known whether the peptide is expressed in other organs of the human hematological and immune systems, like spleen and bone marrow. The presence of ssts, however, in these tissues suggests a regulatory role of these receptors in the human immune system. In contrast to the human immune system, evidence has been provided for the expression and functional role of SS in rat (23) and murine (24) lymphoid tissues. In mice, SS mRNA itself has been detected in splenic macrophages but not in splenic lymphocytes (25), whereas rat B- and T-lymphocytes of spleen and thymus synthesize and release SS (26). In the human immune system, however, it is yet unknown whether SS plays a regulatory role and whether this is comparable to the situation in rodents. Moreover, striking differences between human and rodent species have been demonstrated with respect to the expression of sst subtypes in immune cells. In murine immune cells predominantly sst_2 and sst_4 are expressed, while in human immune cells predominantly sst_2 is expressed and to a lesser extent sst_3 (18). In a previous study we found that SS mRNA was only expressed in the human thymus andTEC, but not in the thymocyte compartment of the thymus (11). Our present study shows that none of the other investigated tissues, nor lymphoid cell samples, expressed SS mRNA. Therefore we analyzed whether a potential novel endogenous ligand for the sst in the human immune system, other than SS itself, i.e. CST, is expressed. CST is known to have high binding affinity to all different human sst subtypes (8), due to its structural resemblance to SS.
We found the expression of both SS mRNA and CST mRNA in human thymus and also in isolated TEC, whereas squeezed thymocytes expressed CST mRNA only. (11)In human spleen and bone marrow no SS mRNA, but only CST mRNA was detected. Moreover, in the different single cell types tested, i.e. human PBMC, T-and B-lymphocytes, monocytes and its functionally derived cells, i.e. macrophages and dendritic cells, no SS mRNA was detected, whereas CST mRNA was clearly expressed in all these cells. These data point to a rather universal expression of CST in immune cells. By Q-PCR significant differences in CST mRNA expression levels were detected between the human immune cells and -tissues. High expression of CST mRNA was detected in thymic tissue and TEC, while we found lower expression in thymocytes and splenic tissue, whereas in bone marrow expression levels were even below detection limit for Q-PCR. The meaning of these differences in expression levels is still unknown, but it might be hypothesized that, although CST mRNA is rather universally expressed in immune cells and tissues, CST plays a more important regulatory role in certain tissues, i.e. the tissues in which CST mRNA is highly expressed. Moreover, we evaluated SS mRNA expression levels in TEC (data not shown) and found very low expression levels of SS, whereas high levels of CST mRNA were detected. This difference might indicate that CST plays a more important role in this cell system than SS. However, this hypothesis should be further studied. Interestingly, differences in CST mRNA expression levels were observed between monocytes and their functionallly derived cells, i.e. macrophages and dendritic cells.

Whereas expression of CST mRNA was relatively low in monocytes, expression in macrophages and dendritic cells was considerably higher (approximately 60- and 10-fold respectively), pointing to an upregulatory mechanism for CST mRNA expression during differentiation and maturation of monocytes into both macrophages and dendritic cells and possibly a more important role for CST in the mature immune system. In concordance with this hypothesis, we observed in our LPS-stimulation experiments, that stimulated monocytes and macrophages expressed approximately 3-fold higher levels of CST mRNA than unstimulated cells did. From these experiments, we hypothesize that CST may have a regulatory role in the human immune system in inflammatory reactions possibly acting via the sst, rather than SS, which is not expressed itself in cells and tissues of the human immune system. However, the exact significance of CST in the human immune system needs to be established.

In the present study we detected two differently sized fragments of CST mRNA, one 173 bp and one 701 bp fragment (figure 1). As one of these fragments (the 701 bp) was very weakly expressed, compared to its expression in the human brain, this might explain why in a previous study this form was not detectable in leukocytes (9). Whereas in our study the majority of primary human immune cells and tissues express both iso-forms, we observed a clear difference in their expression in human T- and B-cell lines. In contrast to the T-cell lines, B-cell lines expressed mainly one iso-form. No clear relationship between the absence and the presence of the expression of the 701 bp fragment and the level of maturation was observed. The exact biological significance of the detection of the two iso-forms is still unknown but suggests a differential regulatory mechanism. According to the literature (9) both sets of primers should detect the sequence coding region for the bio-active form of CST, i.e. CST-17. Although expression of CST mRNA is abundant in cells and tissues of the human immune system and differences in expression levels have been demonstrated, the question still stands whether the CST mRNA is successfully translated into the CST protein in these cells and tissues. Further studies will be necessary to clarify this issue. However, our observations, that CST mRNA expression was upregulated during differentiation and maturation of monocytes into macrophages and dendritic cells and by LPS-stimulation of monocytes and macrophages, suggests that CST itself plays at least a role in these cells.

To investigate whether CST was able to bind to sst in human thymus, sst autoradiography was performed. We found that CST concentration-dependently was able to displace [^{125}I-Tyr^{3}]-octreotide binding in the human thymus. It is known that [^{125}I-Tyr^{3}]-octreotide binds to sst_2 receptors in the human thymus. These receptors are predominantly localized in the medullary region of this organ (22,27). Compared with SS and octreotide, CST has a slightly lower affinity for sst_2 in human thymic tissue (approximately 20-fold). These data demonstrate for the first time that CST is able to displace with a relative high affinity [^{125}I-Tyr^{3}]-octreotide binding from sst endogenously expressed in a human sst expressing organ. As indicated before, previous studies have already shown that CST has a high affinity for the different human sst subtypes in stably transfected cell lines (8). In agreement with these observations we also found that CST-17 displaced [^{125}I-Tyr^{3}]-octreotide binding from sst_2 receptors in a transfected cell model with an affinity comparable with that of SS and octreotide. Because no SS mRNA, but only CST mRNA was detected in the samples tested, we hypothesize on the basis of both the expression of CST and the observation that CST is able to bind to human sst_2 receptors, that CST, rather than SS, may act via the sst in the human immune system in an autocrine and/or paracrine function. With regard to the slightly lower binding affinity of CST to sst the question can

be addressed whether a specific CST receptor, for which CST has a higher binding affinity, is present in lymphoid cells and tissues. In brain, evidence for a specific CST receptor has been proposed on the basis of findings that the effects of CST in human brain can be distinct from those found for SS (28). However, studies in rat and mouse brain showed that CST and SS expression is differently regulated by different stimuli (29) and SS and CST both use different signal transduction pathways to generate their effect (29). These findings might explain the different effects of SS and CST in the brain as well, when both peptides would act via the sst.

Functional aspects of CST in the human immune system are unknown at present. There is however, a striking resemblance between CST/SS and the family of chemokines. The latter are chemotactic cytokines that coordinate development, differentiation, trafficking and effector functions of leukocytes and their progenitors (30). As CST and SS, chemokines are also cyclic peptides that are characterized by their relative position of the cysteine residues. They all induce cell migration and activation by binding to specific G-protein-coupled 7 transmembrane receptors on the target cells (30). SS analogues have a predominantly immunosuppressive effect in the human immune system but exert powerful unique migration-inducing effects on normal and leukemic hematopoietic progenitor cells (31).

In summary: we described for the first time that no SS mRNA was detectable in different human immune and hematological cell types, whereas in all samples tested CST mRNA was found. Significant differences in expression levels were observed between the different immune cells and -tissues investigated. CST mRNA was upregulated during differentiation of monocytes into both macrophages and dendritic cells and by LPS-stimulation of monocytes and macrophages, pointing to a regulatory role of CST in the human immune system. Interestingly, human B-cell lines mainly expressed only one of two cDNA fragments pointing to the existence of 2 CST iso-forms in the human immune system. Considering the important role of dendritic cells and macrophages in autoimmune diseases, and the expression of sst_2 receptors on these cell types (16,32) the functional significance of CST in these cells should be evaluated in further studies. In addition, human T- and B-lymphocytes may express sst, pointing to a potential role of CST in the function of these cells as well.

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II.2

DISTRIBUTION PATTERN OF SOMATOSTATIN AND CORTISTATIN mRNA IN HUMAN CENTRAL AND PERIPHERAL TISSUES

Submitted

Abstract

Somatostatin receptors (sst) and their endogenous ligand, somatostatin (SS), are widely expressed throughout the human body. Recently, the cDNA of a novel SS-like peptide, named cortistatin (CST), has been cloned. This CST was found to be expressed in more restricted areas, like brain cortex, testes, kidney, stomach and leukocytes. Further studies demonstrated a selective expression of CST in tissues and cells of the human immune system, while SS was not expressed. In the present study we investigated the expression pattern of both SS mRNA and CST mRNA in various human central and peripheral tissues by quantitative RT-PCR (Q-PCR), in order to evaluate whether CST is more widely expressed in man than described until now and to investigate the relationship between SS and CST expression levels in various tissues. Previously, 2 different CST mRNA isoforms have been described. Therefore, we investigated the expression of both isoforms by RT-PCR in the different tissues as well. We demonstrate for the first time that CST mRNA is widely expressed in the human body, pointing to a possible broader functional role of CST than assumed until now. Interestingly, a selective expression of CST mRNA and not SS mRNA was only found in cells of the human immune system, whereas other tissues expressed both SS and CST mRNA.

Introduction

Somatostatin (SS) is a 14 amino-acid neuropeptide, which is widely expressed and has many functions throughout the human body. In the central nervous system, SS acts as a neurotransmitter with both inhibitory and excitatory actions (1). In peripheral tissues, the gastro-intestinal tract and endocrine glands are the major sites of production (2). SS mainly plays an important inhibitory role in various secretion processes (2-4). The actions of SS are mediated through five different somatostatin receptor subtypes (sst_{1-5}) (5). These sst are widely and differentially expressed as well. Sst expression has been described in the brain, kidney, gut, pancreas, pituitary, adrenals and thyroid gland (for a review: (6)). Recently, the cDNA of a neuropeptide with strong structural similarities to SS has been cloned (7). This 17-amino acid neuropeptide cortistatin (CST) was found to be selectively expressed in the brain cortex. Like SS, CST contains the 4 amino acids Phe^7, Trp^8, Lys^9 and Thr^{10} (7), which are essential for binding to the 5 sst (8). Previous studies have demonstrated that CST binds with high affinity to all 5 sst subtypes (9). In initial studies, expression of CST has only been described in the cerebral cortex (7,10), but further studies demonstrated expression of CST in human kidney, stomach, testes and leukocytes as well (11). Therefore it may be hypothesized that CST could play a role in peripheral tissues as well, rather than being restricted to the brain region. In a previous study, we investigated the expression of SS and CST mRNA in the human immune system. Interestingly, no SS mRNA was detected in any of the cells and tissues, while we found CST mRNA expression in all samples from the immune system tested, suggesting, that CST may be the endogenous ligand for sst, rather than SS (12).
These findings raised the question whether CST mRNA is expressed in more peripheral tissues and, with respect to our previous study, whether the expression levels of CST mRNA in the immune system, compared to expression levels in other peripheral tissues, may be of significance in terms of possible functionality. Therefore, we investigated in the present study by quantitative RT-PCR (Q-PCR) the expression of CST mRNA as well as SS mRNA in a large series of human tissues. Moreover, the distribution of the 2 previously detected CST mRNA isoforms was evaluated.

Material and Methods

Tissue samples
Different normal tissue samples were kindly provided by the Erasmus MC Tissue Bank. Thymic tissue samples were collected at operations. The tissue samples were taken post-mortem or at operation, directly frozen and stored in –80 ° C until use. The following tissue sections of adult donors were collected for these studies: frontal cortex (n=1), pituitary (n=3), adrenal (n=5), thyroid (n=3), parathroid (n=1), pancreas (n=4), testes (n=2), liver (n=3), stomach (n=3), ileum (n=4), jejunum (n=2), colon (n=4), rectum (n=2), kidney (n=4), lung (n=3) and muscle (n=1). The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained. Following

samples were obtained as previously described (12): monocytes (n=3), macrophages (n=3), dendritic cells (n=3), thymus (n=3), thymocytes (n=3) and spleen (n=3).

RNA isolation

Collected tissues were grinded to powder on dry ice using a mortar and total RNA was isolated using either a High Pure RNA Isolation Kit for cell samples or a High Pure RNA Tissue Kit for tissue samples (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturers protocol.

Quantitative RT-PCR

cDNA was synthesized in an RT-reaction and Q-PCR was performed as described in detail elsewhere (12). Results are presented as number of copies/ 50 ng total RNA in the RT reaction. The primer sequences we used included: CST forward 5'-GGAGAGAAGCTCCAGTCAGC-3' and CST reverse 5'-GGTCCACTCAAACCACCAA-3', SS forward 5'-AAGCAGGAACTGGCCAAGTACT-3' and SS reverse 5'-GGACAGATCTTCAGGTTCCAGG-3'. The probe sequence for CST mRNA: 5'-FAM-TATGCTCGCTGTCTCGGCCG-TAMRA-3' and for SS mRNA: 5'-FAM-CTGAACCCAACCAGACGGAGAATGATG-TAMRA-3'.

RT-PCR

Expression of 2 CST isoforms was investigated as described previously (12). Primer sets are shown in table 1. As positive controls for SS and CST, cDNA of human brain RNA (Invitrogen, Groningen, The Netherlands) was used.

Table 1: Primers used for RT-PCR analysis

Primer	Sequence (5'-3')	Size (bp)
SS (F)	-GATGCTGTCCTGCCGCCTCCAG-	349
SS (R)	-ACAGGATGTGAAAGTCTTCCA-	
CST A (F)	-GCAAATTCGCTCTAAACACAGGA-	173
CST A (R)	-TTGGGAAGGAGGAGAGGAAAGAT-	
CST B (F)	-CTCCAGTCAGCCCACAAGAT-	701
CST B (R)	-CAAGCGAGGAAAGTCAGGAG-	

Primers for CST are derived and/or adapted from (11)

Results

The results of Q-PCR showed significant differences in expression levels of both SS and CST mRNA between the different tissues (figure 1a and 1b). Highest expression levels of SS mRNA were found in human rectum, stomach, pancreas, jejunum and ileum. High to moderate expression was detected in frontal cortex, colon, liver, kidney, parathyroid, pituitary, lung and adrenal, whereas a relatively low expression of SS mRNA was found in testes, thyroid and muscle (figure 1, upper panel). Compared to frontal cortex, hippocampus, pons and brainstem expressed lower levels of SS mRNA (15500, 3378 and 11763 copies/50 ng RNA, respectively (n=1, data not shown)). In concordance with previously described RT-PCR data (12), no SS mRNA could be detected in cells and tissues of the human immune system by Q-PCR as well, except for the thymus and spleen. Expression of CST mRNA in the different tissues, in general, was lower when compared to SS mRNA expression levels and no correlation can be found between SS and CST mRNA levels in the various tissues (figure 1, lower panel). Compared to frontal cortex, hippocampus, pons and brainstem expressed low CST mRNA levels (905, 80 and 20 copies, respectively (n=1, data not shown)). A true selective expression of one of the 2 peptides was only found in cells of the immune system for CST.

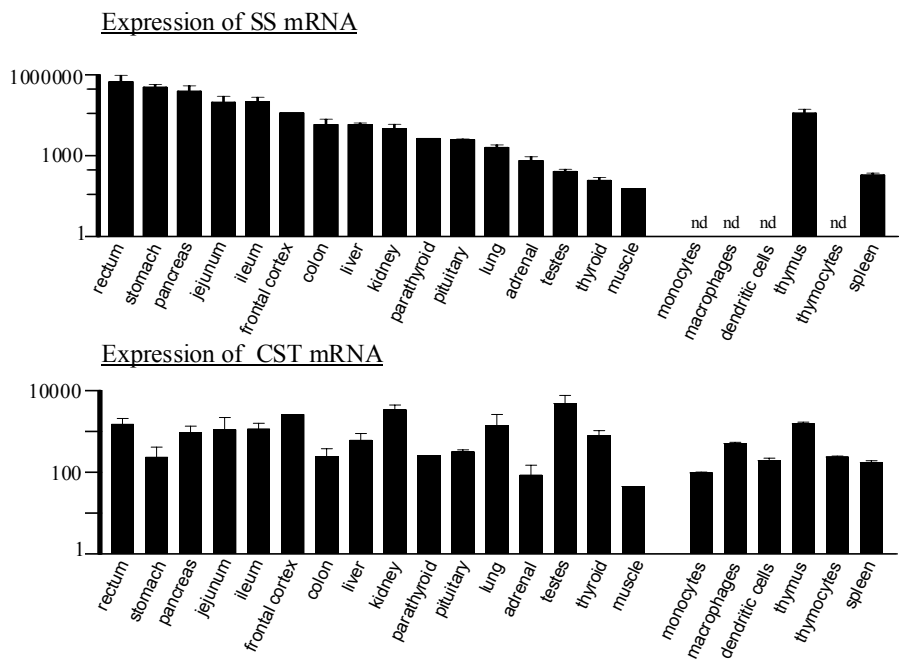

Figure 1: Expression of somatostatin (SS) and cortistatin (CST) mRNA in different tissues. Figure 1a shows the number of copies of SS mRNA/50 ng total RNA in descending order. Figure 1b represents CST mRNA expression levels in the same tissues. nd = not detectable.

However, both testes and thyroid tissue show very low expression levels of SS mRNA, but moderate to high expression of CST mRNA. Finally, we examined the expression pattern of the 2 isoforms of CST in adult tissues, of which an example is shown in figure 2. Pancreatic tissue showed a very weak expression of the 701 bp fragment, whereas all other tissues which were also studied by Q-PCR clearly expressed both isoforms.

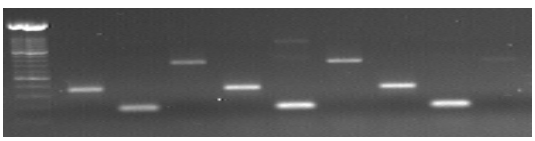

Figure 2: Expression of 2 cortistatin (CST) isoforms**.** This figure shows the expression of SS and 2 CST mRNA isoforms, 173 bp and 701 bp respectively. All tissues expressed SS and 2 CST isoforms. Expression of the short fragment in pancreas was very weak. SS=somatostatin mRNA, A= cortistatin A, 173 bp fragment and B= cortistatin B, 701 bp fragment.

Discussion

In the present study we investigated the expression of both SS and CST mRNA in a series of different human tissues. It should be mentioned that post-mortem tissues were used, which in theory could effect the RNA quality. However, focussing on the expression of SS mRNA, we found the highest SS mRNA expression in tissues of the gastro-intestinal tract and pancreas. These tissues are known to be the major producers of SS (2,13). Moreover, it has been demonstrated that removing and freezing the tissues as soon as possible after death can minimize loss of RNA quality (14). SS mRNA was expressed in all tissues tested, except for cells and tissues of the human immune system. In this respect it should be taken into account, that the cells of the immune system were very homogeneous, whereas tissue sections contain more heterogeneous cell populations. Expression of SS mRNA in these tissues may therefore be the result of expression in specific cell subsets. In contrast to the major SS producing organs, it may be hypothesized that the relatively lower SS mRNA expression found in tissues like adrenal, lung, parathyroid, kidney and, especially the low SS mRNA levels expressing tissues like muscle, thyroid, testes is due to innervating nerve-endings. CST was previously believed to be more selectively expressed than SS (9-11) in tissues throughout the human body. Presence of CST was demonstrated by Northern blotting in brain cortex, kidney and testes (7,11). We indeed found the highest expression levels of CST mRNA in these tissues, but, probably by using a more sensitive technique, we were also able to demonstrate CST mRNA in almost all other tissues tested, indicating a much broader expression pattern than previously assumed. Interestingly, in isolated cells of the immune system (monocytes, macrophages, dendritic cells and thymocytes), of all tissue tested, we found a selective absence of SS mRNA, while CST mRNA was expressed. These immune cells, therefore, seem to be the only cells in the human body, which selectively express one of the 2 known sst-ligands. Of particular interest are the expression levels of CST mRNA in these cells, relative to the other tissues. CST mRNA levels in the immune system are of moderate height and therefore presumably, when translation into CST protein occurs, CST might play a role in a CST-sst immunoregulatory circuit. However, expression of CST protein has not been demonstrated until now and further studies will be necessary to elucidate this issue. In particular, the development of CST antibodies will be of great importance in this respect. Moreover, the functional significance of CST in peripheral tissues is under current investigation. Finally, we found the expression of 2 CST mRNA isoforms in all tissues but the pancreas, which is a classical endocrine organ. The significance of the 2 CST isoforms as well as the selective expression of the short isoform in the pancreas is still unknown.

In conclusion, we showed for the first time a broad expression pattern of both SS and CST mRNA in tissues throughout the human body. CST mRNA is expressed broader than previously assumed and thus may have more regulatory functions than expected from its known restricted distribution pattern. The human immune system seems to be the only system that selectively expresses CST mRNA.

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II.3

DIFFERENTIAL EXPRESSION OF SOMATOSTATIN RECEPTOR SUBTYPES, SOMATOSTATIN AND CORTISTATIN IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELL SUBSETS

Submitted

Abstract

Previous studies demonstrated the presence of somatostatin (SS) binding sites in human lymphoid tissues and peripheral blood cells. However, the role of SS in the human immune system is still unknown. In addition, not much is known about the SS receptor subtype (sst) expression pattern and the expression of SS itself in the immune system, as reports in the literature on both issues have been indeterminate.
The aim of this study was to evaluate the mRNA expression of the five known sst (sst_{1-5}) in peripheral blood mononuclear cells (PBMC) (sub)populations. Moreover, we investigated the expression of the mRNAs encoding the natural ligand SS and the recently described SS-like peptide cortistatin (CST) in immune cell subsets.
Both RT-PCR and quantitative PCR were performed using specific primer sets to evaluate sst, SS and CST mRNA expression in cells under basal conditions or in activated state. FACS analysis using fluorescent SS was performed in order to visualize sst protein on cell membranes.
Peripheral blood B- and T-lymphocytes selectively expressed sst_3 mRNA. Sst_3 expression in B-lymphocytes was significantly lower and neglectible compared with T-lymphocytes. Unstimulated, freshly isolated monocytes did not express any sst mRNA. Upon activation monocytes selectively expressed sst_2 mRNA, whereas T-lymphocyte activation stimulated sst_3 expression. Expression of sst_2 mRNA on monocytes was confirmed by FACS analysis. Sst_3 was found to be preferentially expressed in mature lymphocytes, while immature and leukemic cells predominantly express sst_{2A} mRNA. No expression of SS mRNA could be found in any of the cell types studied. On the other hand, B- and T-lymphocytes did express CST mRNA.
We demonstrate for the first time unequivocally that human peripheral blood B- and T-lymphocytes selectively express sst_3, whereas monocytes do not express at all sst. However, upon activation, monocytes, but not lymphocytes, are induced to express sst_{2A}. No expression of SS mRNA was detected in any cell type, whereas all cell types expressed CST mRNA. The differential expression of sst and CST mRNA in lymphocytes and monocytes suggest a functional role for the CST-sst interaction in immune cells, but further studies should be performed to evaluate the significance of sst and CST in these cells.

Introduction

Somatostatin (SS) is a small neuropeptide that is widely distributed throughout the body and has numerous functions. In the endocrine system it was originally characterized as an inhibitor of growth hormone secretion by the pituitary (1). SS also inhibits hormone secretion from the gastrointestinal tract (2,3), whereas in the nervous system it functions as a neurotransmitter (4). A growing body of evidence suggests a role for SS in the immune system as well (5,6). SS acts on its target cells via specific G-protein coupled transmembrane receptors. Initially, these receptors were identified as binding sites for radiolabeled ligands (7), usually SS or the more stable synthetic analogue octreotide. Later on, the genes of five different subtypes were identified. These receptor subtypes are being characterized with respect to binding affinities for SS analogues, coupling to effector systems for signal transduction and tissue distribution of mRNA expression (8).

Although there are numerous studies demonstrating the presence of SS binding sites or effects of SS in lymphoid tissues, immune cells or lymphoid cell-lines, the data obtained so far do not present a clear view on the function of SS in the immune system. A first step towards understanding should be the elucidation of the expression patterns of SS and its receptor subtypes in the immune system. In situ autoradiography and in vivo scintigraphy repeatedly demonstrated the presence of SS binding sites in human lymphoid tissues (6,9-13), however, it is not yet evident which receptor subtypes are expressed. It is also not clear how receptor expression is regulated in the immune tissues and in immune cells in the peripheral blood.

Reports in the literature on this issue have been quite equivocal. SS binding sites in peripheral blood mononuclear cells (PBMC) have first been described in 1981 by Bathena et al. (14). However, Hiruma et al. (15) were unable to detect SS binding sites on resting PBMC, only after mitogen-activation such binding sites were detected. Similarly, mRNA for somatostatin receptor subtype 2 (sst_2) could barely be detected in unstimulated human PBMC, but was readily detected after mitogen-stimulation (16). In contrast, sst_5 was reported to be responsible for growth-inhibition of PMBC by SS analogues (17). Additionally, several human lymphoid cell lines were shown to bear SS binding sites (18). Such cell lines can also express mRNA for several sst (16,19) and functionally respond to SS or SS analogs (19-21). These data, however, yield by no means a conclusive notion of the expression pattern of sst in human immune cells *in vivo*. Comparison with data obtained with PBMC and other lymphoid tissues from rat (22) or mouse (23) demonstrate species-related differences and add to the confusion as to which sst are expressed in human immune cells.

Therefore, in the present study we set out to clarify the issue whether or not human PBMC subsets in circulation express sst and its natural ligand SS. Moreover, we investigated the expression of the mRNA encoding a recently discovered SS-like peptide, cortistatin (CST), which we previously found to be widely expressed in cells and tissues from the human immune system (24). CST shows high structural resemblance to SS (25) and binds with high affinity to all 5 sst (26). Accordingly, we isolated human PBMC and determined by RT-PCR whether SS, CST or sst were expressed, and whether the expression pattern is altered by mitogen activation. Moreover, we separated the human PBMC cell-fraction into

subpopulations and determined the expression patterns in T-lymphocytes, B-lymphocytes and monocytes. By quantitative PCR we subsequently studied the regulation of the sst and CST mRNA expression in PBMC and monocytes. To gain more insight in the cell type specific expression of SS and its receptors in the human immune system, a comparison was made with the expression levels of sst in several other cell lines and primary human immune cell types.

Material and Methods

Cells, media and reagents

PBMC were isolated from 20-40 ml blood from 8 different healthy donors using standard Ficoll density gradient separation immediately after blood-withdrawal. After Ficoll separation, PBMC were kept on ice to prevent changes in mRNA expression during further treatment of the cells. PBMC or isolated subpopulations were either used directly for mRNA analysis or were cultured for 24 hours at 37°C in a 5% CO_2 incubator in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Life Technologies), 10^5 U/liter penicillin and 1.25 mg/liter fungizone. As mitogenic stimuli were added either 2 µg/ml phytohemagglutinin (PHA), 3 µg/ml concavalin A (ConA), 1 µg/ml lipopolysaccharide (LPS) or 10 µg/ml pokeweed mitogen (PWM). After 24 hours the cultured cells were harvested for mRNA isolation as described below.

For fluorescent ligand-binding experiments (see below) cell populations enriched for lymphocytes or for monocytes were used. Enrichment for lymphocytes was achieved by allowing the monocytes among the PBMC to adhere to culture dishes and subsequently collecting the non-adherent lymphocyte fraction for further culturing. Monocytes were isolated by performing a Percoll density gradient following the standard Ficoll density gradient as described previously (27).

Separation of PBMC into subpopulations

Subpopulations of PBMC were isolated by means of magnetic beads coated with specific antibodies (Dynal AS, Oslo, Norway). For T-lymphocyte isolation, beads coated with CD2 or CD3 antibody were used, while CD19- and CD14 antibody-coated beads were used for B lymphocyte and monocyte isolation, respectively. To distinguish between T-helper cells and cytotoxic T-cells, beads coated with respectively CD4 or CD8 antibodies were used. Isolation of cells was performed following the manufacturers instructions. After Ficoll gradient separation, PBMC were washed twice and resuspended in PBS with 0.5% BSA. Best results were obtained by first depleting the monocytes from PBMC in two rounds of capturing with CD14 antibody-coated beads, in order to prevent non-specific binding of monocytes to other beads. From the remaining PBMC T-lymphocytes were isolated using either CD2 or CD3 antibody-coated beads and at last the CD19 positive B-lymphocytes were isolated. During the 30 minutes attachment to antibody-coated beads the cells were kept on ice. By constant rotation of the tubes the cells and beads were kept in suspension. After attachment the tubes were placed in a magnetic rack to separate the supernatant from the beads. The non-selected cells in the supernatant were

used for subsequent rounds of selection with appropriate antibody-coated beads. The beads with the captured cells were washed five times with PBS/0.5% BSA. Subsequently, cells were counted and evaluated for specificity by determining the percentage of cells rosetted by beads. Cells were thereafter used for RT-PCR analysis or cultured for 24 hours as described above.

RT-PCR

PolyA$^+$ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from approximately 10^6 cells per sample. The cells were lysed during 2 min on ice in 500 µl of a buffer containing 100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA pH 8, 1% LiDS, 5 mM DTT and 10 units RNAsin. To the lysate 30 µl prewashed Dynabeads Oligo $(dT)_{25}$ were added and the mixture was incubated for 5 min on ice. Next, 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. Finally the captured RNA was washed once with buffer used for the reverse transcriptase reaction.

cDNA was synthesized using the polyA$^+$ 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 40 µl. This mixture was incubated for 1 hr at 41°C. Thereafter the reaction was terminated by heating the reaction mixture for 10 minutes to 65°C, capturing of the beads containing the immobilized cDNA library and resuspension of the beads in 200 µl of water.

One-twentieth 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, 5 pmol of each of a pair of oligonucleotide primers specific for human sst_1-$_5$, hypoxanthine guanine phosphoribosyltransferase (HPRT) ,SS or CST (see Table 1a) in a buffer of 10 mM Tris-HCl pH 9, 50 mM KCl, 2.0 mM MgCl2, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 and a final volume of 50 µl. The PCR reaction was carried out in a DNA thermal cycler with heated lid. 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 1.5-2% agarose gels stained with ethidium bromide. The identity of the PCR products has been confirmed by sequencing of the PCR products (not shown).

Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the polyA$^+$ mRNA preparation (since the sst 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 sst receptor subtypes, 0.1 to 0.001 µg of human genomic DNA, representing approximately 30.000 to 300 copies of sst-template, was amplified in parallel

with the cDNA samples. As a positive control for the PCR of the hprt, SS and CST cDNA, aliquots of a cDNA sample known to contain SS, CST and hprt mRNA were amplified, because these primer-pairs did enclose introns in the genomic DNA.
The hprt gene is expressed in all cells at low copy numbers. Therefore use of this gene gives a much better estimate of the amount of synthesized cDNA than the use of other widely used control genes such as the high or intermediate copy genes β-actin and GAPDH. By including control PCRs on known amounts of genomic DNA in each experiment we have determined that the $sst_{1\text{-}5}$ genes reproducibly could be detected at approximately 3000 copies of template and were usually undetectable at 300 copies of template under the conditions chosen. Higher sensitivities of the amplification reactions we deemed not desirable, since under the conditions used we should be able to detect the expression of sst mRNA in approximately 10% of the cell-population under study. In order to detect receptor expression in less abundant subpopulations of PBMC we followed a strategy of first isolating these subpopulations and then determining whether or not these subpopulations expressed sst mRNA, rather than increasing the sensitivity of the RT-PCR.

Quantitative PCR
Quantitative PCR was performed employing a nuclease assay (28) for real-time detection of amplification (29,30) with an ABI Prism 7700 Sequence Detection System (i.e. thermal cycler and real-time fluorescence detector, Perkin Elmer, Nieuwerkerk aan de IJssel, The Netherlands). New primer sets were designed for use in quantitative PCR of sst_{2A}, sst_3, hprt and CST with accompanying probes for the detection of the PCR products (Table 1b). These were obtained from BioSource (Nivelles, Belgium). The probes are labeled at the 3' end with a fluorophore (FAM, 6-carboxy-fluoresceïn) and at the 5' end with a quencher (TAMRA, 6-carboxy-tetramethylrhodamine) and hybridize to sequences in between the forward and reverse primers. The intact probe, whether free in solution or hybridized to the amplification product, will not emit fluorescence as a result of the vicinity of the quencher TAMRA to the FAM label. However, during extension of the primer the probe hybridized to the template will be hydrolyzed by the 5'nuclease activity of the Taq-polymerase. This removes the quencher TAMRA from the probe and consequently enables the detection of the fluorescence emitted by the FAM label. The increase in fluorescence as a result of hydrolysis of the probe will be directly proportional to the amount of amplification. The ABI Prism 7700 will record fluorescence of the FAM label (as well as of an internal standard ROX to correct for inter-well variation) during each amplification cycle. The software then determines from the increase in fluorescence for each well the threshold-cycle (Ct), which is the cycle at which the fluorescence intensity surpasses a preset threshold (related to the baseline fluorescence).
The assay was optimized with respect to concentrations of primers, probe, $MgCl_2$ and ROX as well as cycling profile, Taq-polymerase, buffer and reaction volume. Finally we performed the assays in a reaction volume of 25 µl, containing 0.25 units AmpliTaq Gold, AmpliTaq Gold Buffer (Perkin Elmer, Nieuwerkerk aan de IJssel, The Netherlands), 500 nM forward primer, 500 nM reverse primer, 100 nM probe, 200 nM ROX (5-carboxy-X-rhodamine, Molecular Probes, Leiden, The Netherlands), 2 mM $MgCl_2$, and 200 nM of each.
In each experiment standard curves for each primer set were included. Known amounts of

Table 1: Primers used for the RT-PCR and quantitative RT-PCR

A. RT-PCR

	Sequence (5'- 3') [a)]	PCR product
Sst_1 (forward)	ATGGTGGCCCTCAAGGCCGG	318 bp
Sst_1 (reverse)	CGCGGTGGCGTAATAGTCAA	
Sst_{2A} (forward)[b]	GCCAAGATGAAGACCATCAC	414 bp
Sst_{2A} (reverse)	GATGAACCCTGTGTACCAAGC	
Sst_3 (forward)	CCAACGTCTACATCCTCAACC	314 bp
Sst_3 (reverse)	TCCCGAGAAGACCACCAC	
Sst_4 (forward)	ATCTTCGCAGACACCAGACC	321 bp
Sst_4 (reverse)	ATCAAGGCTGGTCACGACGA	
Sst_5 (forward)	CGTCTTCATCATCTACACGG	226 bp
Sst_5 (reverse)	CCGTCTTCATCATCTACACGG	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	349 bp
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	
Cortistatin (forward)	GCAAATTCGCTCTAAACACAGGA	173 bp
Cortistatin (reverse)	TTGGGAAGGAGGAGAGGAAAGAT	
Hprt (forward)	CAGGACTGAACGTCTTGCTC	413 bp
Hprt (reverse)	CAAATCCAACAAAGTCTGGC	

B. Quantitative RT-PCR

Sst2A forward	ATGCCAAGATGAAGACCATCAC	171 bp
Sst2A reverse	TGAACTGATTGATGCCATCCA	
Sst2A probe	TGGCTCTGGTCCACTGGCCCTTTG	
Sst3 forward	CTGGGTAACTCGCTGGTCATCTA	86 bp
Sst3 reverse	AGCGCCAGGTTGAGGATGTA	
Sst3 probe	CGGCCAGCCCTTCAGTCACCAAC	
CST forward	GGAGAGAAGCTCCAGTCAGC	198 bp
CST reverse	GGTCCACTCAAACCACCAA	
CST probe	TATGCTCGCTGTCTCGGCCG	
Hprt forward	TGCTTTCCTTGGTCAGGCAGTAT	109 bp
Hprt reverse	TCAAATCCAACAAAGTCTGGCTTATATC	
Hprt probe	CAAGCTTGCGACCTTGACCATCTTTGGA	

[a]). The sequences of the primers for sst_1 and sst_4 are derived and adapted from (31), for sst_5 from (32), for CST from (33) and all other primers and probes were designed by use of the Primer3! software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and the appropriate GenBank entries.

[b]). The sst_2 primers are specific for sst_{2A}. In initial experiments we also included primers that amplify both sst_{2A} and sst_{2B}, but the sst_{2B} specific product was never found (unpublished results).

genomic DNA containing sst_{2A} and sst_3 or dilutions of a pool of hprt or CST containing cDNAs were amplified (in duplicate or triplicate) together with the unknown cDNA samples. A standard curve was constructed by plotting the Ct versus the logarithm of the starting quantity. With these standard curves and the measured Ct of the unknown cDNA samples the starting amounts in the cDNA samples were determined in duplicate or triplicate. To correct for differences in the efficiency of RNA isolation and cDNA synthesis the amounts of sst_{2A}, sst_3 and CST were divided by the amount of hprt in a given cDNA sample. Since the absolute copy number of hprt templates in the control cDNAs used for the hprt standard curve is not known, the starting amounts are given in arbitrary units. Reaction conditions were optimized until the standard deviation of duplicate determinations of the threshold cycle (Ct, see Materials and Methods) of standard curve samples were less than 3%. A linear correlation existed between the logarithm of the starting amount of the template and the Ct in the range of more than 300.000 copies down to approximately 100 copies. Below 100 copies duplicate measurements displayed less accuracy due to more delays and/or failures of amplification. Above 100 copies starting amount the standard deviation of the copy number calculated by means of the standard curve ranged from 5 to 20%.

Fluorescent somatostatin binding

To detect SS binding sites on PBMC the fluoresceïn-labeled SS analog Fluosomatostatin (Fluo-SS, Advanced Bioconcept, Montreal, Canada) was used. Cells were washed with PBS containing 0.5% BSA and incubated at room temperature for with 50 nM Fluo-SS or with 50 nM Fluo-SS and 5000 nM D-trp^8-SS14. After 30 minutes incubation an appropriate dilution of phycoerythrin (PE)-labeled CD3, CD19 (Becton Dickinson, Erembodegem, Belgium) or CD14 antibodies (Beckman Coulter, Mijdrecht, Netherlands) was added. Following a further 15 minutes incubation the cells were washed, resuspended in PBS containing 0.5% BSA and 20 mM sodium azide and analyzed on a FACScan cytometer (Beckton Dickinson, Erembodegem, Belgium). As a positive control for ligand binding a cell line stably transfected with sst_{2A} was used.

Results

Sst, SS and CST mRNA in PBMC

To determine whether or not sst mRNA is expressed in the PBMC cell-fraction of circulating blood we started isolation of the cells immediately after blood-withdrawal and were able to lyse the cells within two hours. This guarantees that the RNA expression pattern of the studied cells resembles that of the live cells in circulation as closely as possible. In doing so, we consistently found expression of sst_3 mRNA in PBMC by RT-PCR (Fig. 1, upper left panel).

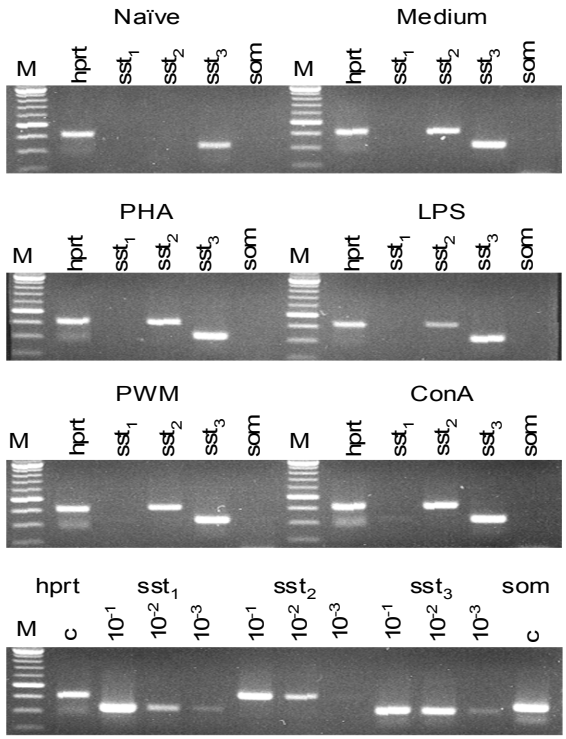

Figure 1: RT-PCR analysis of somatostatin (som) and somatostatin receptor expression in naïve and cultured PBMC. PBMC were assayed for expression of mRNA immediately after isolation (naïve) or after 24 hours culturing in culture-medium alone (medium) or with the addition of PHA, LPS, PWM or ConA. Control DNA samples contained either cDNA (lanes c) from a control cell line known to express hprt and somatostatin mRNA, or different amounts of genomic DNA (lanes 10^{-1} µg, 10^{-2} µg and 10^{-3} µg) comprising 30.000, 3.000 and 300 copies of sst genes respectively. All mock-reverse-transcribed samples and all controls with no added template showed no PCR products and none of the samples expressed sst_4 or sst_5 (not shown). Marker lanes (M) contain a 100 bp DNA ladder.

In some experiments a very weak sst_{2A} PCR product could be detected as well, but only after 40 cycles when the detection limit for sst_{2A} was better than 1% of the population. None of the other sst subtypes could be detected, nor mRNA for SS itself (Table 2). However, we detected the mRNA for CST in all cell samples. In some experiments also a PCR specific for sst_{2B} was performed, but this subtype was never detected (results not shown).

Table 2: Expression of somatostatin receptor subtypes, somatostatin and cortistatin mRNA in PBMC, purified peripheral blood T-lymphocytes, B-lymphocytes and monocytes.

	sst_1	sst_{2A}	sst_3	sst_4	sst_5	*CST*	*SS*
PBMC							
0 hr	-	-	+	-	-	+	-
24 hr medium	-	+	+	-	-	+	-
24 hr PHA	-	+	+	-	-	+	-
24 hr ConA	-	+	+	-	-	ni	-
24 hr PWM	-	+	+	-	-	ni	-
24 hr LPS	-	+	+	-	-	ni	-
	sst_1	sst_{2A}	sst_3	sst_4	sst_5	*CST*	*SS*
T-lymphocytes ($CD3^+$)							
0 hr	-	-	+	-	-	+	-
24 hr medium	-	-	+	-	-	+	-
24 hr ConA	-	-	+	-	-	+	-
B-lymphocytes ($CD19^+$)							
0 hr	-	-	+	-	-	+	-
24 hr medium	-	-	+	-	-	+	-
24 hr PWM	-	-	+	-	-	+	-
Monocytes ($CD14^+$)							
0 hr	-	-	-	-	-	+	-
24 hr medium	-	+	-	-	-	+	-
24 hr LPS	-	+	-	-	-	+	-

sst= somatostatin receptor subtype, CST = cortistatin and SS = somatostatin, ni= not investigated

After culturing the isolated PBMC for 24 hours in medium alone or in medium containing the mitogenic agents PHA, ConA, PWM or LPS the expression of sst_{2A} mRNA was clearly induced (Fig 1; Table 2, top panel). The expression pattern of the other sst subtypes and of SS and CST remained unchanged (sst_3 and CST detectable, others not, no SS).

Expression in T-lymphocytes, B-lymphocytes and monocytes

To determine in which cell-type sst_{2A} and sst_3 receptors are expressed we separated the PBMC cell-population into its main subpopulations of T-lymphocytes, B-lymphocytes and monocytes by use of paramagnetic beads coated with appropriate antibody. By keeping the cells on ice we ensured that changes in mRNA expression during handling of the cells were minimal. After separating the cells we determined the purity of the isolated cell-fractions and found that 97-99% of the cells was indeed rosetted by antigen-coated beads.

The isolated cell-populations were subsequently assayed by RT-PCR (Figure 2; Table 2, bottom panel).

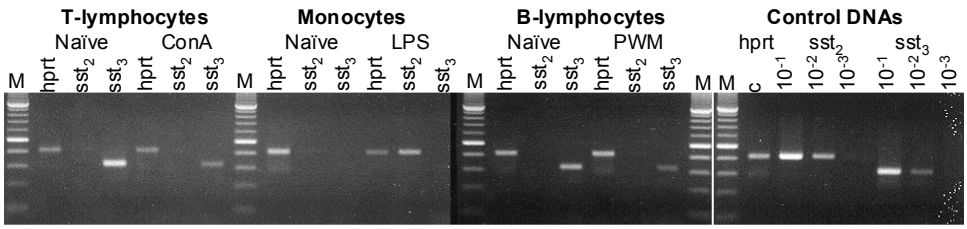

Figure 2: RT-PCR analysis of somatostatin receptor 2 and 3 expression in naïve and cultured purified T-lymphocytes, monocytes and B-lymphocytes. Cells were assayed for expression of mRNA immediately after isolation (naïve) or after 24 hours culturing in culture medium with the addition of ConA, LPS or PWM. Control DNA samples contained either cDNA from a control cell line (lane c) or different amounts of genomic DNA (lanes 10^{-1} µg, 10^{-2} µg and 10^{-3} µg) comprising 30.000, 3.000 and 300 copies of sst genes respectively. All mock-reverse-transcribed samples and all controls with no added template showed no PCR products and none of the samples expressed sst_1, sst_4 or sst_5 (not shown). Marker lanes (M) contain a 100 bp DNA ladder.

The results demonstrated that sst_3 is expressed by $CD3^+$-T-lymphocytes and by $CD19^+$-B-lymphocytes, but not by $CD14^+$-monocytes. None of the other sst subtypes nor SS is expressed in these three subpopulations, whereas the expression of CST mRNA was found in all subpopulations evaluated. Isolation of T-lymphocytes by CD2- instead of CD3-antibody-coated beads yielded similar results. Culturing the isolated cell-populations during 24 hours in medium alone or in medium containing ConA, PWM or LPS induced sst_{2A} in the monocyte-fraction only (figure 2). Sst_3 expression remained confined to T- and B-lymphocytes, whereas the other sst subtypes and SS were still undetectable in all cell-populations studied (Figure 2, Table 2, bottom panel). In the same way we determined the expression pattern of unstimulated and ConA-stimulated $CD4^+$- and $CD8^+$-T-lymphocytes and found in all populations a selective expression of sst_3 mRNA (data not shown).

Quantification of sst_{2A}, sst_3 and CST mRNA expression

To quantify the induction of sst_{2A} mRNA after activation we performed a quantitative PCR on cDNA of PBMC from 4 donors and on purified monocytes from 2 donors before and after activation (24 hr culturing in medium). The sst_{2A} and sst_3 mRNA expression (in arbitrary units) corrected for the amount of hprt in the different samples is shown in figure 3. In all PBMC samples there is a significant induction of sst_{2A} mRNA levels after activation. In the monocyte populations purified from two of the donors the induction is even higher. In three of the four donors also the sst_3 expression in PBMC is slightly increased after activation. Among the donors the expression levels of both sst_{2A} (after activation) and sst_3 showed a broad range.

Since the very low amount of sst_{2A} mRNA in naïve cells cannot be determined with the same accuracy as the higher copy numbers, it is difficult to express the induction as manifold of the level in naïve cells. However, the sst_{2A} copy number obtained after RNA

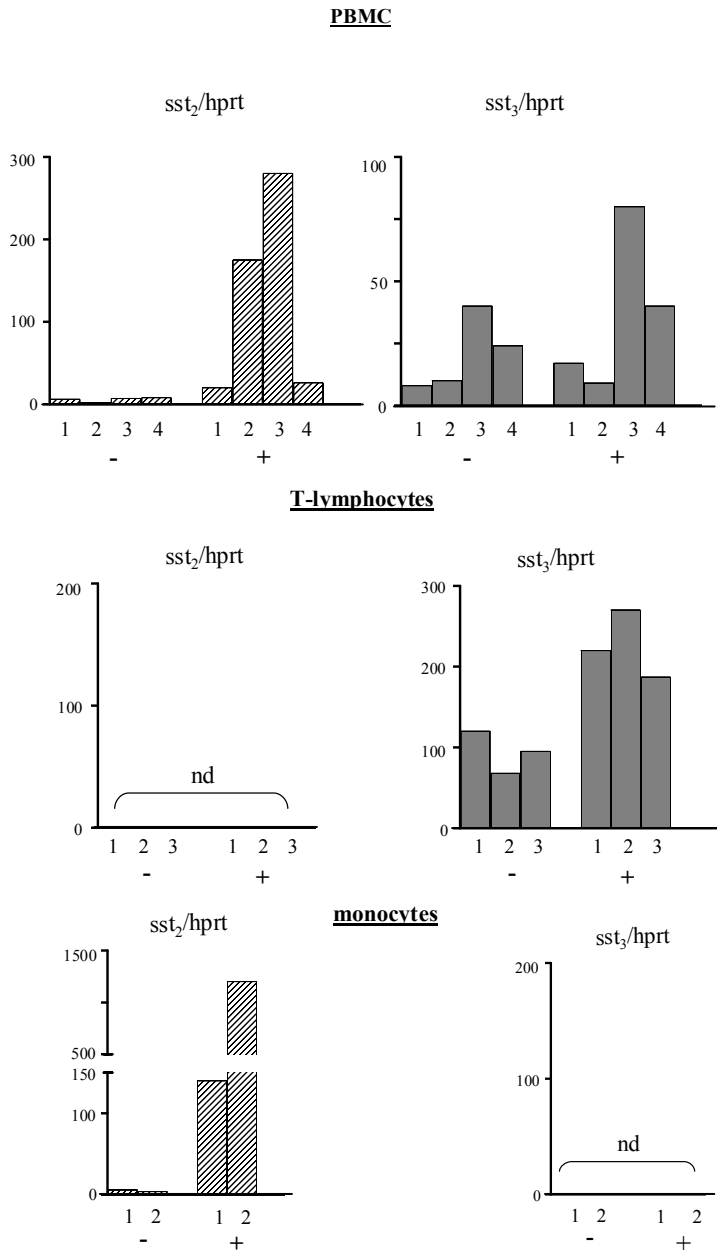

Figure 3: Quantitative PCR analysis of sst_{2A} and sst_3 mRNA expression in human immune cells. Sst_{2A} and sst_3 levels were corrected for the amount of hprt detected in each sample and are given in arbitrary units. PBMC from 4 donors, monocytes from 2 donors and T- lymphocytes from 3 donors were analysed in naïve (-) and activated (+) state. Hatched bars represent sst_{2A} mRNA, closed bars represent sst_3 mRNA. nd =not detectable.

isolation and cDNA synthesis per million PBMC rises from approximately 1000 (or less) to 25.000-150.000 copies. In contrast, the number of sst_3 copies obtained per million naïve PBMC is approximately 7.000-15.000. From 3 donors of PBMC, T and B lymphocytes were isolated and quantitative PCR was performed to quantify the expression of sst_{2a}, sst_3 and CST mRNA in these cell subsets as well.
Like already demonstrated by RT-PCR, T- and B-lymphocytes were found to express sst_3 selectively. After 24 hours of culture, expression of sst_3 mRNA was upregulated approximately 1.5-fold in T-lymphocytes. After a 24 hour incubation with of PHA, sst_3 mRNA levels were upregulated approximately 2.5 fold when compared to control cells (figure 4).

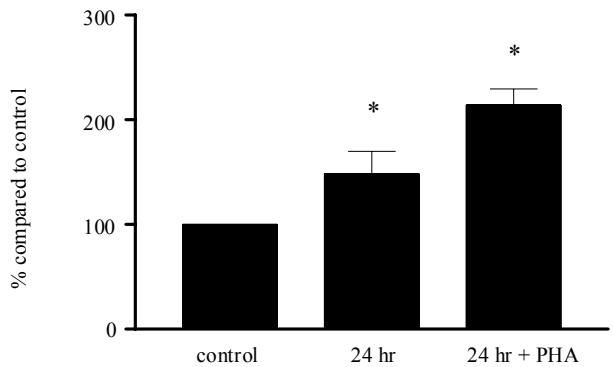

Figure 4: Expression of sst_3 mRNA in T lymphocytes. This figure represents the expression of sst_3 mRNA in T lymphocytes. Sst_3 mRNA levels were corrected for HPRT levels and bars represent the percentage of upregulation compared to control cells. Expression levels of sst_3 mRNA were evaluated in freshly isolated T lymphocytes (control), in T lymphocytes after 24 hours of culture (24 h) and after 24 hours of culture in addition of PHA (24h + PHA). Results are the mean of 3 independent experiments. $^*P<0.01$ when compared to control.

In B-lymphocytes, on the other hand, we were not able to detect expression of sst_3 mRNA by Q-PCR, even after culture and stimulation with PHA (data not shown). This is probably due to very low expression levels of sst_3 in B-lymphocytes and probably the conditions or primers for RT-PCR are more sensitive to detect these low levels.
For comparison we have determined the expression levels of sst_{2A} and sst_3 in several other normal human immune cells, in leukemic cells and in lymphoïd cell lines (Figure 5). In freshly dispersed normal human thymocytes the sst_3 expression levels are comparable to the levels found in PBMC, whereas there is slightly more sst_{2A} mRNA than in naïve PBMC. In normal human bone marrow cells the expression of sst_{2A} is much higher.

Although the expression level of sst_{2A} in human hematological tumor cells is highly variable, sst_{2A} is present in both tumor cell-populations studied. Sst_3 is just detectable in normal bone marrow cells and tumor cells studied. In immortalized T- and B-cell lines sst_{2A} and sst_3 are also both present. However, these cell lines contain approximately 10-20 times more hprt mRNA per cell than naïve PBMC. Therefore the absolute numbers of sst_{2A} and sst_3 mRNA copies obtained per million cells are much higher than in PBMC. In established T-cell lines the amount of sst_{2A} is at least 10 times higher than in naïve PBMC (10.000-60.000 copies obtained per million cells), while sst_3 expression is comparable with PBMC. In transformed B-cell lines both sst_{2A} and sst_3 levels are much higher than in naïve PBMC (500.000-800.000 copies obtained per million cells, i.e. more than 500 and 35 times higher for sst_{2A} and sst_3 respectively).

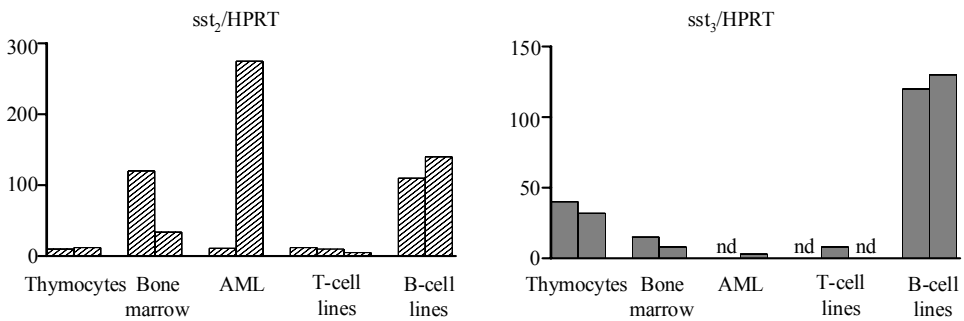

Figure 5: Expression of sst_{2A} and sst_3 mRNA in human immune cells and cell lines. Sst_{2A} and sst_3 levels were corrected for the amount of hprt detected in each sample and are given in arbitrary units. A. Expression of sst_{2A} in cell suspensions from normal human thymus, normal human bone marrow, acute myeloid leukemia (AML) cells, established T-lymphoid cell lines Jurkat, HPB-ALL and Peer and two EBV-transformed B-cell lines. B. Expression of sst_3 in the same cells. Hatched bars represent sst_{2A} mRNA, closed bars represent sst_3 mRNA. nd = not detectable.

Finally, we evaluated the expression of CST mRNA in T- and B-lymphocytes by Q-PCR. T-lymphocytes expressed CST, and CST mRNA levels were downregulated when the cells were incubated with PHA (figure 6A). CST expression in B-lymphocytes could only be detected in 2 out of 3 donors and in these 2 donors we found an upregulation after 24 hours of culture. However, when the cells were cultured for 24 hours in addition of PHA, CST levels returned to basal levels (figure 6B).

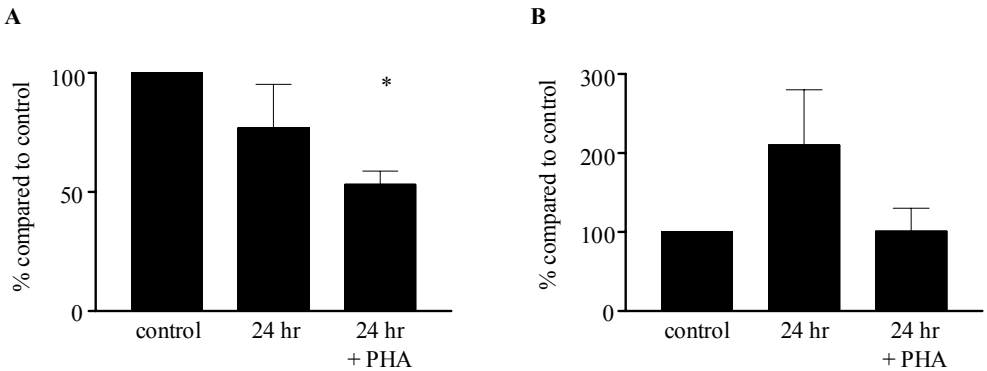

Figure 6: Expression of CST mRNA in human T-and B-lymphocytes. Expression of CST mRNA was determined in unstimulated cells (control), after 24 hours of culture (24 h) and after 24 hours of culture in addition of PHA (24 h + PHA). Figure 5A represents expression of CST mRNA in T lymphocytes (mean of 3 donors). *P<0.001 when compared to control cells. Figure 5B represents the expression of CST mRNA in B lymphocytes (mean of 2 donors). Bars represent the percentage of upregulation when compared to control.

Fluo-SS ligand binding to monocytes

To confirm that besides sst mRNA also the protein could be detected as ligand-binding sites, we performed flow cytometry analysis on cell-populations of naïve PBMC, PHA-activated non-adherent PBMC (i.e. enriched for T- and B-lymphocytes) and on LPS-activated Percoll gradient-isolated monocytes (purity 70-90%). Using a fluorescent SS14 analogue we were able to detect sst only in the activated monocyte-enriched cell population, but not in the naïve PBMC fraction, nor in the activated non-adherent PBMC fraction. The signal was specific for SS because it was displaced by addition of excess unlabeled SS14 analogue (Figure 7).

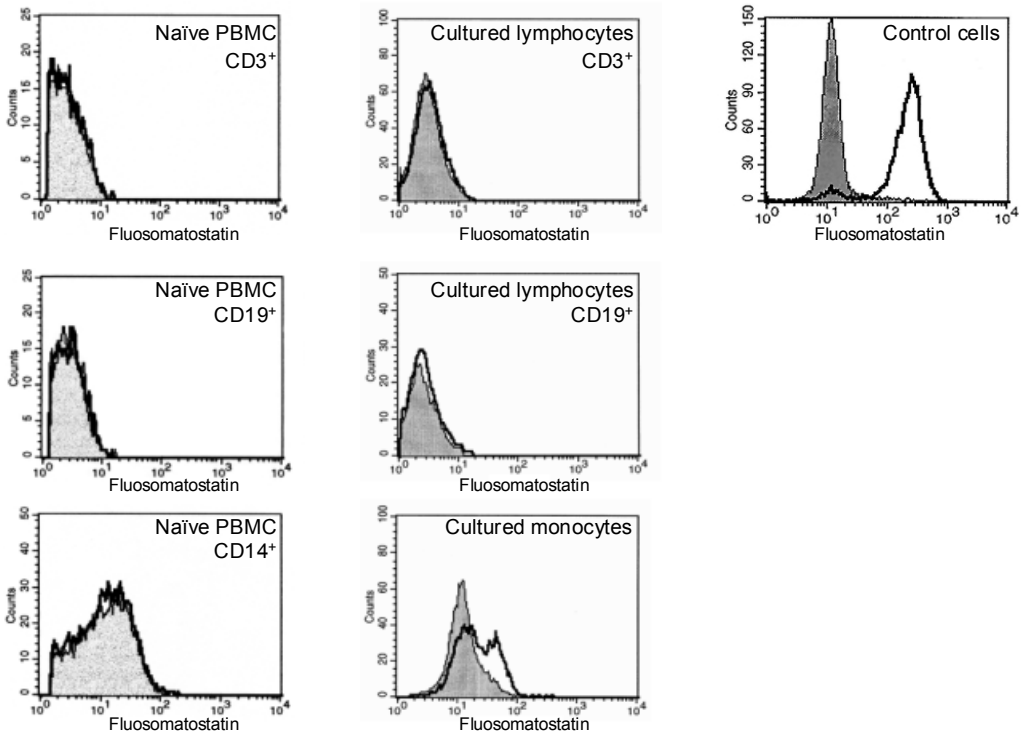

Figure 7: FACS analysis of fluorescent somatostatin binding to T- or B-lymphocytes or to monocytes. Naïve PBMC or an enriched lymphocyte population cultured for 24 hr in the presence of PHA was labeled with Fluosomatostatin and PE-conjugated CD3 or CD19 antibodies to analyze binding of somatostatin to T- and B-lymphocytes respectively. Monocytes were analyzed by labeling the naïve PBMC with Fluosomatostatin and PE-conjugated CD14 antibody and by labeling an enriched monocyte population cultured for 24 hr in the presence of LPS with Fluosomatostatin. The grey areas represent the fluorescent signal remaining after displacement of Fluosomatostatin by unlabeled somatostatin, the solid line shows the amount of fluorescence after labeling with Fluosomatostatin

Discussion

In the present study we have determined for the first time unequivocally that only sst_3 is expressed at significant levels in human PBMC resembling the in vivo situation as closely as possible. This expression is found in B-lymphocytes, as well as in T-lymphocytes, but not in monocytes. Expression of sst_3 mRNA in B-lymphocytes was significantly lower compared with the expression of this sst in T-lymphocytes. Freshly isolated monocytes derived from peripheral blood do not express any of the 5 sst, but upon activation these cells were shown to express sst_{2a} mRNA. Previously, we demonstrated that monocytes, after a Ficoll and Percoll-density centrifugation, express a low amount of sst_{2a} mRNA (34). This suggests that already minimal activation, caused by the Percoll-density gradient centrifugation step, of the cells results in upregulation of sst_{2a} mRNA. Stimulation of T- and B-lymphocytes with mitogens did not alter the expression pattern of receptor subtypes. SS mRNA was undetectable in all cell-populations studied, whereas we detected CST mRNA in lymphocytes. In a previous study we demonstrated expression of CST in monocytes and its functionally derived cells, i.e. macrophages and dendritic cells (34). CST, due to its structural similarities to SS (25), binds to the 5 sst with high affinity (26) and may therefore be an alternative ligand for sst.

Since the sensitivity of our assay was set at detection of expression in approximately 10% of the cell population, it is not known whether *all* T- and B-lymphocytes express sst_3, or that sst_3 expression is restricted to a subset of the T- and B-lymphocytes. Separation of T-lymphocytes into the major subpopulations of $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells showed sst_3 mRNA to be present in both. Using a Fluo-SS derivative in FACS analysis we were not able to detect sst on the T- or B- lymphocyte populations. This could suggest that the expression of sst_3 in these cells is very low or restricted to a very minor subset of the lymphocytes or, alternatively, that the Fluo-SS derivative shows altered specificity/affinity for binding to the different sst subtypes. It is known that Fluo-SS binds to $sst_{1,2,3}$ but not at all to sst_4 and sst_5 (according to the manufacturer). A number of studies have shown functional effects of SS on human lymphocytes (5,17,35,36), providing evidence for the presence of functional sst on these cells. Corroborating our data, sst_3 was recently shown to be present in human enriched lymphocyte fractions which also contained functional SS binding sites (37). Our data obtained with Q-PCR on PBMC samples reveal that the number of sst_3 cDNA copies obtained per million cells is approximately 15.000 or less. Assuming that the combined efficiency of RNA isolation and cDNA synthesis is in the order of 10% this suggests that only a subpopulation of the T-lymphocytes (which comprise more than 50% of the PBMC population) express sst_3.

Freshly isolated monocytes do not express sst_{2a} or sst_3 at detectable levels, but can be induced to express sst_{2A} receptor mRNA. The presence of sst on these activated monocytes was confirmed using Fluo-SS and FACS analysis. Any of the nonspecific stimuli we applied was sufficient to trigger this induction. Expression of sst_{2A} was in some samples detectable as early as one hour after stimulation. When monocytes were isolated using a Percoll gradient centrifugation, sst_2 mRNA expression could already be detected. These observations suggest that sst_{2A} upregulation is a very early event in activation of

monocytes. By Q-PCR we showed that the level of sst_{2A} induction varies among different donors, but that it is very substantial in all cases.

Translating our results of naïve and in vitro activated immune cells from the peripheral circulation to the situation for immune cells present in lymphatic tissues, it seems very likely that the receptor binding sites found with radiolabeled SS (analogs) are present on immune cells from the monocytic lineage. Whether the lymphocytes after migration into the tissues also express sufficient amounts of sst to be visualized in autoradiography is uncertain, since T-lymphocyte-rich regions in lymphoid tissues were reported to be sst negative and germinal centers of secondary lymphatic follicles rich in B-lymphocytes were shown to be receptor positive by sst scintigraphy (11,38). Our recent studies using a sst_{2A}-specific antibody for immunohistochemistry confirmed the presence of the sst_{2A} receptor on cells of the monocytic lineage and the absence of this receptor from T-lymphocytes in human rheumatoid synovium (10) and sarcoid granuloma tissue biopsies (9). However, we cannot rule out the possibility that one or more of the receptor subtypes is upregulated by other, more specific stimuli than the ones we applied. Furthermore, cells other than lymphocytes or monocytes/macrophages in lymphoid tissues are likely to express SS and sst as well, such as has been shown in endothelial cells in inflammatory lesions (10) and in thymic epithelial cells from normal human thymus (39).

Knowing these results we may now understand much better the data described so far in the literature. Whether or not sst were detectable in PBMC presumably depended on the extent to which monocytes were activated during isolation of PBMC (14-16). Moreover, our results show that sst mRNA expression in established cell-lines may not necessarily be representative for human immune cells in vivo. Most of the lymphoid cell-lines studied by Tsutsumi et al (16) expressed mRNA for subtypes 2, 3, 4 and 5, with sst_2 being the major subtype. Our own studies of a panel of lymphoid cell lines revealed only sst_{2A} and sst_3 or no somatostatin receptor expression at all (23,40). The incongruity possibly can be explained by differences in assay sensitivity. Nevertheless, it is evident that many lymphoid cell-lines express sst_{2A} as the major subtype, which is certainly at variance with lymphoid cells in circulation. Our comparison of human PBMC and human lymphoid cell lines also reveal that in immortalized cell lines the expression level of sst_{2A} is similar to or much higher than in activated PBMC. Since also the amount of hprt RNA per cell is much higher in the cell lines than in PBMC, presumably due to the enhanced proliferative capacity of the immortalized cells, the absolute numbers of sst_{2A} and sst_3 mRNA copies per cell are 10- to 800-fold higher in the immortalized cell lines.

Results reported in studies concerning the function of SS and its receptors in immune cells are not conclusive as to which receptor subtypes mediate the different effects. In vitro culturing of human peripheral blood mononuclear cells has demonstrated effects of SS or its analogues on numerous (immune) functions of these cells. However, several conflicting results regarding effects of SS (or its analogue octreotide) on proliferation, differentiation or cytokine production have been reported (discussed below), which possibly can be attributed to different experimental designs but do not clarify the function of SS in the immune system.

Sst_{2A} receptors are usually associated with inhibition of hormone-secretion (41,42). Also for LPS-activated human monocytes it has been shown that SS inhibits the secretion of pro-inflammatory cytokines (43), though another study reports stimulation of secretion

(44). Notwithstanding these conflicting accounts, the biological significance of the early induction of sst_{2A} during activation of monocytes might be that through this receptor the inflammatory response immediately could be modulated (decreased) when it is invoked. The presence of SS binding sites in rheumatoid arthritis or in granulomatous diseases (9,10,45,46) and the effects of SS in arthritis (47) also suggests that somatostatin may act in inflammation. Consistent with the suggestion that receptor subtype sst_{2A} could mediate these effects are our recent studies demonstrating the presence of the sst_{2A} receptor by immunohistochemistry in cells of the monocytic lineage in human rheumatoid synovium and sarcoid granulomas (9,10).

The identification of SS binding sites in hematological malignancies and the ability of SS (or octreotide) to inhibit proliferation of these malignant cells in vitro (15,48) hints at a role for SS in the regulation of proliferation and/or differentiation during hematopoiesis. The presence of sst_{2A} in normal human bone marrow cells, where differentiation and proliferation take place, as well as in tumor cells from hematological origin might suggest that sst_{2A} is involved in regulating the balance between proliferation and differentiation.

In human PBMC inhibition of proliferation of mitogen-activated lymphocytes has been reported (17,35,36), although also contrasting results have been obtained with enriched lymphocyte fractions (37) and with a lymphoid cell line (19). In the latter studies proliferation could be stimulated by SS. Because lymphocytes selectively express sst_3 mRNA we suggest that this receptor subtype mediates the responses observed in this cell type. Interestingly, the sst_3 subtype has been shown to be involved in SS-induced apoptosis, resulting in growth inhibition of the cell population (49). Our finding that sst_3 is the main subtype expressed in mature lymphocytes and, moreover, the upregulation of sst_3 mRNA in T-lymphocytes during culture and by activation with PHA, suggests that this receptor subtype may have an important function in these cells. Mature B- and T-lymphocytes in circulation are resting cells that start proliferation after activation. It is therefore conceivable that in mature B- and T-lymphocytes sst_3 may have an important function in regulating the balance between proliferation and apoptosis after activation. However, with respect to the possible role of sst_3 in mature B-lymphocytes we could only detect sst_3 mRNA by RT-PCR, while not by Q-PCR. This suggests that sst_3 mRNA expression in B-lymphocytes is very low and therefore it may be hypothesized that sst_3 in B lymphocytes plays a less important regulatory role, when compared to T lymphocytes, in which we found clear regulation of sst_3 mRNA expression.

Equally important for the understanding of the function of SS and its receptors in lymphoid tissues is knowledge of which cells produce the agonistic peptide SS. We demonstrated that PBMC do not synthesize SS mRNA, not even after activation. We therefore assume that SS is synthesized in other cells in the lymphoid tissues, e.g. in thymic epithelial cells (39) or in nerve endings, or perhaps in endothelial cells. Another possibility is that lymphocytes or monocyte-derived cells do express SS mRNA after more specific stimulation in vivo, conceivably after migration from the periphery into lymphoid tissues or to sites of inflammation. On the other hand, the detection of CST mRNA in various tissues and cells of the human immune system (24) and the detection of CST mRNA in the present study in T- and B- lymphocytes may suggest that CST, rather than SS, may play a regulatory role acting via the sst in these immune cells. Moreover, in monocytes, macrophages and dendritic cells, CST mRNA was upregulated upon

activation with LPS (24), whereas in B- and T-lymphocytes in general a downregulation of the CST mRNA expression upon activation was observed. The significance of this downregulation in T- and B-lymphocytes as well as the functional significance of CST in these cells remains to be determined. We hypothesized that the downregulation of endogenous CST expression may be a direct feedback mechanism following the upregulation of sst_3, in order to regulate responsiveness to the peptide. This suggests that CST and sst_3 may interact in regulation of the number of T-cells.
Summarizing, we have shown for the first time that human B- and T-lymphocytes in the peripheral blood selectively express sst_3, whereas circulating monocytes do not express sst. Expression of sst_3 mRNA can be upregulated by activation in T-lymphocytes, while sst_3 expression levels in B-lymphocytes are probably low, suggesting a less important role of these receptors in B lymphocytes, compared to T lymphocytes. Upon stimulation with nonspecific mitogens the monocytes, but not the lymphocytes, are induced to express sst_{2A} receptors. No expression of SS mRNA was detected, not even after stimulation. In line with the expression of CST mRNA in monocytes, macrophages and dendritic cells, CST mRNA was expressed in T- and B-lymphocytes as well. The differential and cell type specific regulation of the expression of sst_{2A} and sst_3 receptors in human PBMC, in combination with data on functional studies of SS on immune cells suggests that the function of SS, or more presumably CST, and its receptors in the human immune system may be found in regulating cytokine secretion in inflammatory responses and in regulating the balance of proliferation and apoptosis of (activated) lymphocytes in immune responses.

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II.4

REGULATION OF THE EXPRESSION OF SOMATOSTATIN, CORTISTATIN AND SOMATOSTATIN RECEPTORS IN HUMAN MONOCYTES, MACROPHAGES AND DENDRITIC CELLS

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Abstract

Increasing evidence suggests that neuropeptides play a role in the regulatory mechanisms between the neuroendocrine and immune systems. A differential expression of the 5 known somatostatin (SS) receptors (sst_{1-5}) has been demonstrated in human immune cells and tissues. However, little is known concerning regulation and expression of sst_{1-5} and the peptide SS. Therefore, we investigated the expression and the time-dependent regulation of sst_{1-5}, SS and cortistatin (CST), a novel SS-like peptide, in human monocytes (MO), monocyte-derived macrophages (MP) and -dendritic cells (DC), in basal and lipopolysaccharide (LPS)- or interferon-γ (IFN- γ)-activated state. MO, MP and DC selectively expressed sst_2 mRNA. SS mRNA was not detectable, while all samples expressed CST mRNA. Expression levels of sst_2 and CST mRNA showed marked differences and were in the rank order of MP>>DC>>>MO. LPS- or IFN- γ stimulation did not induce expression of SS or $sst_{1,3,4,5.}$ However, sst_2 mRNA expression was significantly upregulated by stimulation. CST mRNA was upregulated as well. During differentiation of MO into MP or DC, time-dependent, significantly increasing sst_2 and CST mRNA levels were found. By confocal microscopy, the presence of sst_2 receptors was demonstrated on MP, but not on DC. This study demonstrates for the first time a selective and inducible expression of the recently discovered CST, as well as sst_2 in human monocyte-derived cells, suggesting a role for a CST-sst_2 system, rather than a SS-sst_2 system in these immune cell types.

Introduction

Somatostatin-14 and somatostatin-28 are 14- and 28-amino acid neuropeptides, which are mainly produced in the central nervous system, gastro-intestinal tract and endocrine glands (1,2). In these systems somatostatin (SS) has a predominant inhibitory action, especially with regard to the release of mediators, such as hormones (3-5). SS acts via G-protein coupled seven transmembrane receptors of which 5 different subtypes have been cloned, named sst_{1-5} (6). In addition to their role in endocrine tissues, it is hypothesized that neuropeptides might play a regulatory role in the human immune system as well (7-9). As a neuropeptide, a role in the immune system might be ascribed to SS and its receptors (9). In contrast to the human immune system, the role of SS and its receptors has been extensively studied in granulomas induced by Schistosoma Mansoni infection in the murine immune system (10). Murine T-lymphocytes selectively expressed sst_2 (11) and macrophages expressed SS mRNA (12). Treatment of Schistosoma Mansoni infected mice with SS resulted in granuloma growth inhibition (13). Following these studies in the murine immune system, the question was addressed whether SS and sst might play a regulatory role in cells of the human immune system as well. Monocytes and its functionally derived cells, i.e. macrophages and dendritic cells are important components in different pathways in the human immune system. They play important roles in inflammatory responses by production of both pro-inflammatory and immunosuppressive cytokines, antigen-presentation and phagocytosis (14-17). Previous studies have demonstrated binding sites for SS on lymphocytes and monocytes (18,19) suggesting a role for sst in these cells of the human immune system. Immunohistochemistry studies showed that CD68-positive macrophages in human sarcoid granulomas expressed sst_2 (20). Equivocal studies have reported on functions of SS on cells of the monocyte lineage. Both inhibitory (21,22) and stimulatory (23) effects have been described on cytokine production by macrophages. SS increased cytotoxicity of macrophages against tumour cells as well (24), and SS can act as an antiangiogenic factor by inhibiting endothelial cell growth and monocyte migration (25). However, little is known about the expression and regulation of the different sst subtypes in cells of the monocyte-lineage, i.e. monocytes, macrophages and dendritic cells. Moreover, it is not known whether the natural ligand for the sst, e.g. SS, is expressed itself in cells of the monocyte lineage.
Therefore, we investigated in the present study mRNA expression of sst_{1-5} and SS in monocytes and monocyte-derived macrophages and -dendritic cells. Moreover, we investigated the regulation of the expression of the mRNA encoding a SS-like peptide, cortistatin (CST), which was previously detected in cells and tissues from the human immune system (26) and binds with high affinity to all 5 sst (27), and which, therefore, might serve as an alternative ligand to sst. Sst and CST mRNA levels were studied in monocytes, macrophages and dendritic cells in both basal and lipopolysaccharide (LPS) or interferon-γ (IFN- γ)-stimulated conditions using a TaqMan® assay. In order to investigate possible regulation of mRNA levels, expression was measured from day 1 to 6 of differentiation of monocytes into both macrophages and dendritic cells. Expression of the sst protein on cell membranes of macrophages and dendritic cells was studied using FITC-

labeled octreotate, a fluorescence-labeled sst_2 selective agonist, and visualized by confocal microscopy.

Material and Methods

Isolation of blood mononuclear cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin Blood bank, Rotterdam, The Netherlands) by Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation. Subsequently, monocytes were isolated from PBMC using a Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation as described in detail previously (28). Isolated monocytes were frozen in 10% DMSO-medium and stored in -80° C until use.

Cell-culture
A frozen suspension of monocytes was rapidly thawed in a water-bath at 37° C and viability was evaluated by Trypan-blue exclusion (Life Technologies, Grand Island, U.S.A.). Cell viability always exceeded 95%. Cells were seeded at a density of 0.5 x 10^6 cells/cm^2 in a volume of 1 ml/well in 24 well Nuncleon plates (Nalge Nunc International, Denmark) in RPMI1640+ supplemented with 10% heat-inactivated (30 min 56˚ C) fetal calf serum (FCS, Life Technologies, Grand Island, U.S.A.), L-glutamine (2 mM, Life Technologies) and penicillin (1000 U/ml, Yamanouchi Pharma BV Leiderdorp, The Netherlands). Plates were then incubated for 90 minutes at 37˚ C to allow adherence of the monocytes to the plate. Thereafter, plates were washed to remove potential contaminating T- and B-cells. To generate macrophages, fresh medium was added, containing granulocyte macrophage-colony stimulating factor (GM-CSF, 500 U/ml, Novartis Pharma BV, Arnhem, The Netherlands). The cells were cultured for 6 days, with medium refreshment at day 3. To generate dendritic cells, the isolated monocytes were incubated for 6 days with GM-CSF (500 U/ml) and IL-4 (1000 U/ml), with a medium refreshment at day 3. This method of the generation of macrophages and dendritic cells has been described previously in detail (29).
In order to study the regulation of sst_2, SS and CST mRNA expression, monocytes, 6 day macrophages or 6 day dendritic cells were incubated during 24 hours without or with LPS (Sigma Aldrich, Zwijndrecht, The Netherlands (2µg/ml)) or IFN- γ (10 ng/ml) In order to study the time course of mRNA expression levels during differentiation of monocytes into macrophages and dendritic cells, cells were collected at day 1,2,3,4,5 or 6 of culture without or with a prior 24-hour incubation with LPS (2 µg/ml). A schematic overview of the experimental set-up is shown in figure 1. At the end of each incubation period, cells were collected and used for both PCR- and FACS-analysis.

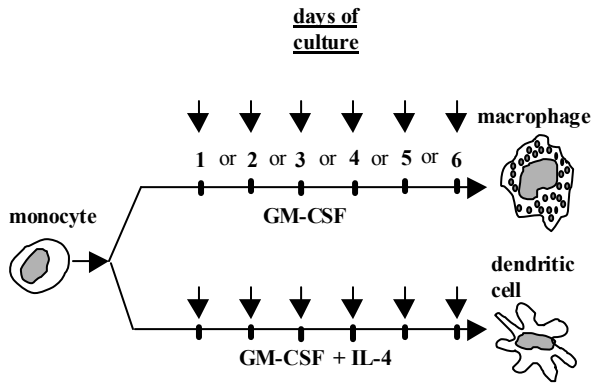

Figure 1: Experimental set-up of monocyte culture and –activation. Isolated monocytes were allowed to differentiate into macrophages and dendritic cells in 6-day cultures. Arrows indicate the different time points at which cells were collected for RT-PCR analysis. In the experiments in which cells were incubated with LPS, arrows indicate the time points at which cells were collected after a prior incubation for 24 hours with LPS.

FACS-analysis

By FACS analysis, evaluating the CD-marker pattern of the cells, the phenotypes of the cultured cells was confirmed. Cells were collected using cell-scrapers and centrifuged at 300 g for 5 min. Supernatant was removed and 25 µl of the diluted monoclonal antibody was added to each cell-pellet. The monoclonal antibodies we used included fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE) labeled antibodies purchased from Becton Dickinson (San Jose, CA, USA), CD1a-PE (diluted 1:250), CD14-PE (1:400), CD26-PE (1:120), CD68-FITC (1:10), CD71-FITC (1:10), CD80-PE (1:50), CD86-FITC (1:100) AND HLA-DR-PE (1:20). Cells were incubated for 15 min at room temperature. Cells were then washed twice with PBS/0.5% BSA, resuspended in 200 µl PBS/0.5% BSA followed by FACS-analysis on a FACSCalibur-FACS (Becton Dickinson).

RT-PCR studies

RT-PCR was performed as described previously (30). Briefly, poly A^+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from tissue samples and cells. cDNA was synthesized using the poly A^+ mRNA, which was eluted from the beads in 40 µl H_2O for 10 minutes at 65 °C, using Oligo $(dT)_{12-18}$ Primer (Life Technologies). One-twentieth of the cDNA library was used for each amplification by PCR using primer sets specific for human SS, sst_{1-5}, CST and hypoxantine-phosphoribosyl-transferase (HPRT) as a control (see Table 1).

Table 1: Sequence of primers used for RT-PCR

	Sequence (5'-3')	Size of PCR product
Sst1 (forward)	ATGGTGGCCCTCAAGGCCGG	318 bp
Sst1 (reverse)	CGCGGTGGCGTAATAGTCAA	
Sst2 (forward)	GCCAAGATGAAGACCATCAC	414 bp
Sst2 (reverse)	GATGAACCCTGTGTACCAAGC	
Sst3 (forward)	TCATCTGCCTCTGCTACCTG	221 bp
Sst3 (reverse)	GAGCCCAAAGAAGGCAGGCT	
Sst4 (forward)	ATCTTCGCAGACACCAGACC	323 bp
Sst4 (reverse)	ATCAAGGCTGGTCACGACGA	
Sst5 (forward)	CCGTCTTCATCATCTACACGG	223 bp
Sst5 (reverse)	GGCCAGGTTGACGATGTTGA	
Hprt (forward)	CAGGACTGAACGTCTTGCTC	413 bp
Hprt (reverse)	CAAATCCAACAAAGTCTGGC	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	349 bp
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	
Cortistatin (forward)	GCAAATTCGCTCTAAACACAGGA	173 bp
Cortistatin (reverse)	TTGGGAAGGAGGAGAGGAAAGAT	

The primer set used to detect CST mRNA was adapted from Ejeskar et al. (31). As positive controls for SS, CST and HPRT, cDNA of human brain RNA (Invitrogen, Groningen, The Netherlands) was used. As positive control for $sst_{1\text{-}5}$, DNA of a BLCL-BSM cell-line (an EBV-transformed B-cell line) was used. The PCR-reaction was carried out in a DNA thermal cycler with heated lid (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). After an initial denaturation at 94°C for 5 minutes, the samples were subjected to 40 cycles of denaturation at 94° C for 1 min, annealing for 2 min at 60° C and extension for 1 min at 72° C. After a final extension for 10 min at 72° C, 10 µl aliquots of the resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidiumbromide. The identity of the products was confirmed by direct sequencing using an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems) according to manufacturers protocol.

Quantitative PCR

In order to quantify sst_2 and CST mRNAs, a quantitative PCR was performed by TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence Detection System (Perkin Elmer) for real-time amplifications, according to manufacturers protocol. Q-PCR was performed for sst_2 only, because no expression of the other sst subtypes were detected in the cells we investigated.
The primer sequences that were used included:
Sst_2 forward 5'-ATGCCAAGATGAAGACCATCAC-3'
Sst_2 reverse 5'-TGAACTGATTGATGCCATCCA-3'
CST forward 5'-GGAGAGAAGCTCCAGTCAGC-3'
CST reverse 5'-GGTCCACTCAAACCACCAA-3'
HPRT forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3'
HPRT reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'
The probe sequences that were used included:
Sst2 5'-FAM-TGGCTCTGGTCCACTGGCCCTTTG-TAMRA-3'

CST 5'-FAM-TATGCTCGCTGTCTCGGCCG-TAMRA-3'
HPRT 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'
The relative amount of sst_2 mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For the RT-reaction 50 ng/µl of total RNA was used in every reaction. For determination of HPRT mRNA, a standard curve was generated of cDNA obtained from a DU45-(prostate-cancer) cell line. The amount of sst_2 mRNA was calculated relative to the amount of HPRT and is given in arbitrary units.

Statistical analysis
Data were statistically analyzed using SPSS for Windows, release 9.0 (SPSS, Chicago, IL). The differences in expression levels of sst_2 and CST mRNA in the stimulation experiments were determined using one-way analysis of variance (ANOVA). When significant overall effects were obtained by ANOVA, multiple comparisons were made using the Newman-Keuls test.

Synthesis of octreotate-FITC conjugate
The peptide was prepared on solid support by standard automated Fmoc procedure (32). The synthesis was performed on a 25 µmol scale starting with Fmoc threonine pre-loaded Wang resin. In order to avoid inadvertent labeling of the ε-K^5 amino group that is necessary for receptor binding, this amino acid was protected as Fmoc Lys(ivDDe)-OH (33). Activation of the carboxyl group and coupling of subsequent amino acids (75 µmol) were performed *in situ* by using N-hydroxylbenzotriazole (HOBt, 2 M) and 2-(1-H benzotriazole-1-yl)-1,1,1,3-tetramethyluronium hexafluorophosphate (HBTU, 2 M). Intramolecular cyclization of the acetamidomethyl-protected dithiol groups of cysteine was accomplished by adding thallium trifluoroacetate (23 mg, 42 µmol) in anhydrous DMF (1 mL). Cleavage of the peptide from the resin and concomitant removal of the side-chain protecting groups were performed with 85% TFA, 5% H_2O, 5% PhOH, and 5% thioanisole for 4 h at room temperature. The crude peptide was precipitated with cold *t*-butyl methyl ether and lyophilized in H_2O/acetonitrile (3:2). Without further purification, FITC (12 mg, 28 µmol) and $NaHCO_3$ (10 mg, 120 µmol) were added to a solution of the crude peptide in 2 mL DMF and stirred for 12 h. The mixture was filtered and the filtrate was treated with a 1 mL solution of 2% hydrazine in DMF for 20 min in order to remove the ivDde. The resulting mixture was poured into cold MBTE to precipitate the conjugate, which was lyophilized and purified by HPLC and characterized by LC-MS. HPLC purity of the final compound was >99.5%.
By autoradiography, using rat brain tissue, binding affinity of the octreotate-FITC compound was evaluated. Octreotate-FITC displaced binding of [^{125}I-Tyr^3]octreotide with relative high affinity (IC_{50} = 5.5 x 10^{-8} M).

Visualization of sst_2 receptors on human macrophages
Monocytes were cultured for 6 days on round coverslips (Ø 24 mm, Omnilabo BV, The Netherlands) in the presence of GM-CSF to obtain macrophages, or in the presence of both GM-CSF and IL-4 to obtain dendritic cells. After 6 days, cells were incubated with 20 nM octreotate-FITC, which binds to sst_2 selectively (34), in order to determine binding to sst_2 receptors and internalization of the receptor-ligand complex using confocal

microscopy (LSM 410, Carl Zeiss Instruments, Jena, Germany). A 40X/1.4 numerical aperture objective lense was used. FITC was excited with a 488 nm Ar-laser and fluorescence emission was detected using a 515-540 nm bandpass filter. Images were typically taken at a 4x zoom. As a positive control, a stably sst_2-transfected cell-line (CC2B) was used. In order to determine specificity of the binding of octreotate-FITC, binding was displaced by excess unlabelled octreotide.

Results

By FACS analysis the phenotypes of the isolated monocytes, cultured macrophages and dendritic cells were confirmed. In table 2 the mean percentages of positively stained cells are shown.

Table 2: Expression pattern of CD markers on monocytes, macrophages and dendritic cells

	Percentage of positive cells							
	CD14	**CD68**	**CD71**	**CD26**	**HLA-DR**	**CD1a**	**CD80**	**CD86**
Monocytes	82	60	80	85	83	11	**1**	77
Macrophages	90	65	93	82	95	21	77	82
Dendritic cells	78	**13**	**14**	67	95	**83**	95	**18**

Values represent the mean percentage of positively stained cells for the subsequent CD-markers and are the results of three independent experiments

Monocytes showed low expression of CD80, compared to macrophages (at day 6) and dendritic cells (at day 6). CD80 is upregulated in macrophages, with a peak at day 3 of culture and declining thereafter, whereas expression of CD80 in dendritic cells was upregulated and remained stable (29). Dendritic cells express lower CD68, CD71 and CD86 compared to
macrophages. This has been described previously (35), although also higher expression levels of CD71 expression have been reported (36). Dendritic cells show a high expression of CD1a, which is known as a dendritic cell marker (37), together with the finding that addition of IL-4 to culture results in cells becoming non-adhesive (29).
By RT-PCR, as presented in figure 2 (upper panel), a selective expression of sst_2 mRNA was found in monocytes and in *in vitro* cultured macrophages and dendritic cells. Activation of these cells with 2 μg/ml LPS did not result in a different expression pattern of the sst subtypes (figure 2, lower panel).

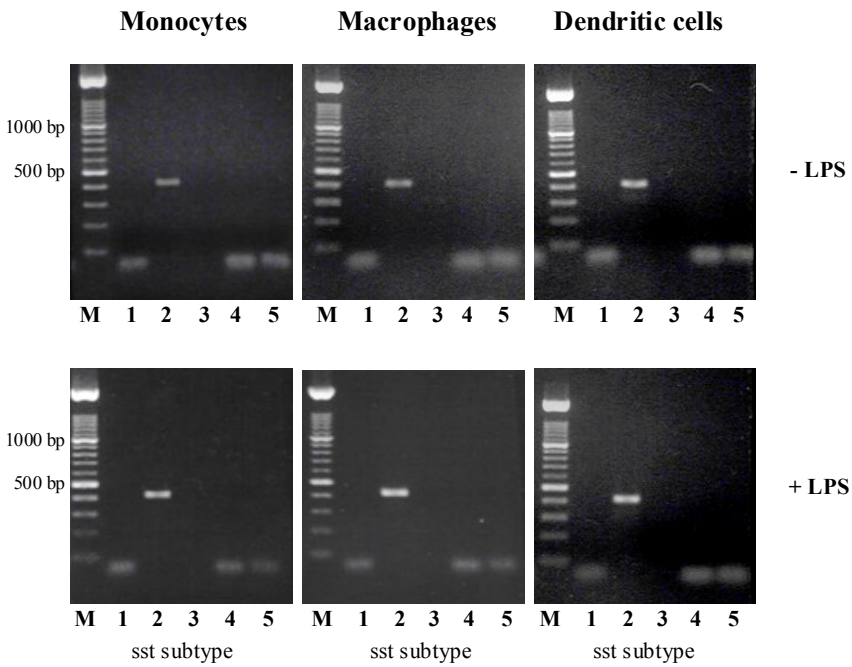

Figure 2: Sst subtype mRNA expression in monocytes, 6-day macrophages and 6-day dendritic cells. Poly A^+ mRNA was prepared from human monocytes, macrophages and dendritic cells, cultured in vitro. cDNA was synthesized and amplified using primers specific for the five different sst subtypes. Upper panel represents sst mRNA expression in unstimulated monocytes, 6-day macrophages and 6-day dendritic cells, whereas the lower panel represents sst mRNA expression in monocytes, 6-day macrophages and 6-day dendritic cells after a 24 hour stimulation with 2 µg/ml LPS. M = 100 bp DNA ladder.

At lower concentrations of LPS (range 10 ng/ml - 2 µg/ml) sst_2 mRNA was selectively expressed as well (data not shown). Cells stimulated with 10 ng/ml IFN-γ also selectively expressed sst_2 mRNA (data not shown). The expression of SS mRNA itself could not be detected in any of these cell types, neither in basal nor in LPS- or IFN-γ -activated state. Figure 3 shows the effect of LPS on SS mRNA expression. On the other hand, we detected the expression of the mRNA encoding a recently discovered SS-like peptide, i.e. CST, in monocytes, macrophages and dendritic cells. As a control, human thymic tissue was used, which expresses both SS and CST mRNA (figure 3).

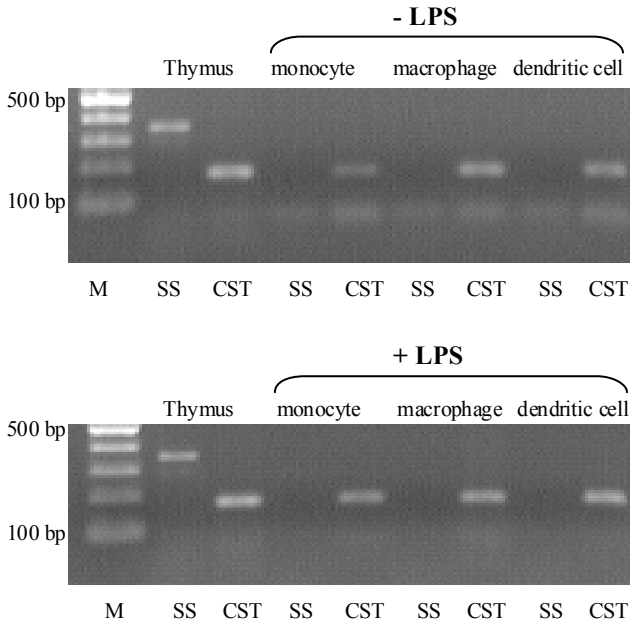

Figure 3: Expression of SS and CST mRNA in monocytes, 6-day macrophages and 6-day dendritic cells. Experiment was repeated 4 times. Upper panel represents expression in unstimulated cells, whereas the lower panel represents expression in cells after a 24 hour incubation with 2 µg/ml LPS. Human thymic tissue was used as a positive control for both SS and CST mRNA expression. M = 100 bp DNA ladder.

In order to evaluate the quantitative expression of the different mRNAs found by RT-PCR, Q- PCR was used for detection of sst_2 and CST mRNA levels. We detected relative low expression levels of sst_2 mRNA in cells under basal conditions (macrophages and dendritic cells showed an approx. 10-fold higher sst_2 mRNA expression compared to monocytes, $p < 0.001$), while sst_2 mRNA expression was significantly upregulated when cells were stimulated with 2 µg/ml LPS (figure 4).

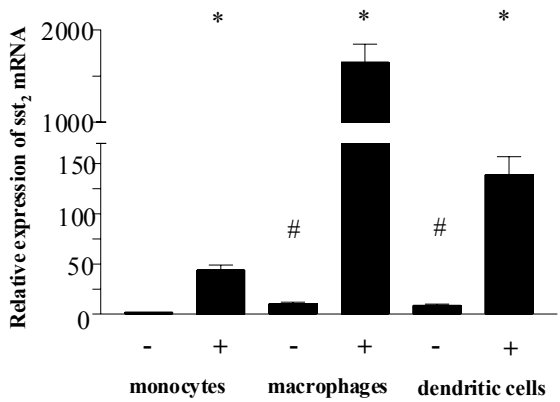

Figure 4: Regulation of sst_2 mRNA expression in monocytes, macrophages and dendritic cells Monocytes were allowed to differentiate into macrophages or dendritic cells and incubated for 24 hours with LPS at day 6 of culture. Bars represent sst_2 mRNA expression in unstimulated (-) and LPS-stimulated (+) cells, given in arbitrary units, relative to a generated standard curve from a Jurkat (T-cell) cell line and adjusted for HPRT expression. * $P<0.0001$ when compared to cells in unactivated state and $^{\#}$ $P<0.0001$ when compared to monocytes. Results are the mean of three independant experiments using cells from three different healthy donors.

In experiments using increasing concentrations of LPS (range 10 ng/ml - 2 µg/ml), 2 µg/ml LPS was shown to give maximal induction of sst_2 mRNA expression (figure 5).

As shown in figure 4, LPS induced an approx. 40- ($p<0.0001$), 150- ($p<0.0001$) and 20-fold ($p<0.0001$) increase in sst_2 expression in activated monocytes, macrophages and dendritic cells, respectively. On the other hand, stimulation of macrophages with IFN-γ resulted in an approx. 25-fold increase in sst_2 mRNA expression (figure 6).

Whereas RT-PCR demonstrated that SS mRNA was absent after stimulation of the cells with LPS or IFN-γ, expression of the CST mRNA as possible alternative ligand for sst_2 in these cells, was upregulated upon activation (Table 3).

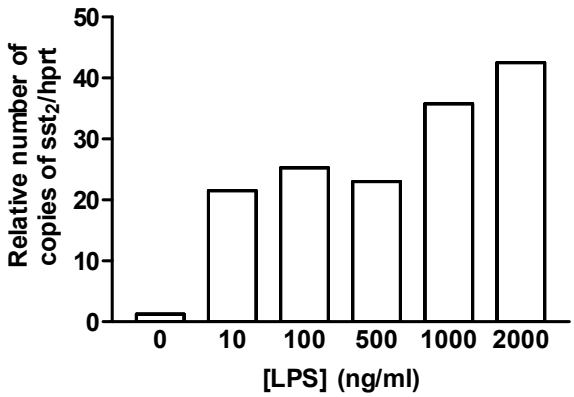

Figure 5: Upregulation of sst_2 mRNA in monocytes using different concentrations of LPS. Monocytes were incubated with different concentrations of LPS (ranging from 10 ng to 2 μg/ml). After 24 hours cells were collected and RNA was isolated. Sst_2 and HPRT mRNA levels were quantified by a TaqMan assay and results are presented as the ratio of sst_2 over HPRT.

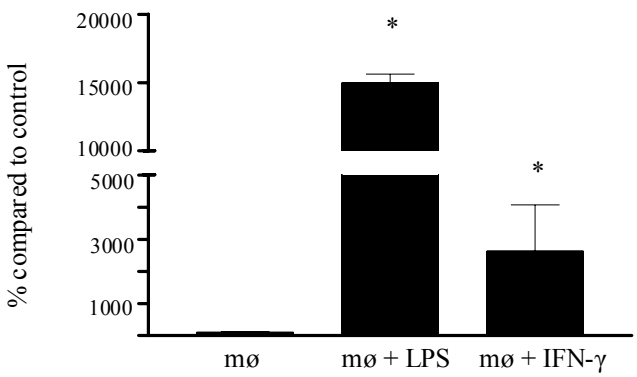

Figure 6: Upregulation of sst_2 mRNA in macrophages incubated with LPS and IFN-γ. This figure represents the expression of sst_2 mRNA in unstimulated macrophages (mø) and macrophages stimulated with either LPS (mø + LPS) or IFN- γ (mø + IFN-γ). * $P<0.001$ when compared to unstimulated cells. Results represent the ratio sst_2/HPRT, are the mean of 3 independent experiments and are shown as percentages compared to control (unstimulated) cells.

Table 3: Quantitative CST mRNA expression

	relative CST mRNA expression (fold-induction)		
	monocytes	**macrophages**	**dendritic cells**
control	0.15 ± 0.05	9.6 ± 1.4^{+}	1.7 ± 0.3^{+}
+ LPS	0.3 ± 0.04$^{\#}$ (2.0)	28.9 ± 3.2$^{*,+}$ (3.0)	16.3 ± 2.1$^{*,+}$ (9.6)
+ IFN-γ		13.8 ± 3.4$^{+,-}$ (1.5)	

Table represents CST mRNA expression in cells of the monocyte lineage. Both unstimulated as well as LPS- and IFN-γ stimulated cells were studied and differences in expression levels were measured. The results are presented as the mean ± SD of three experiments.$^{+}$ P<0.0001 vs monocytes, $^{\#}$P=0.002, *P<0.0001 and $^{-}$P<0.05 vs control cells

CST mRNA was significantly upregulated by approximately 2- (p=0.002), 3- (p<0.0001) and 10-fold (p<0.0001) in LPS-activated monocytes, macrophages and dendritic cells, respectively. Stimulation of macrophages with IFN- γ resulted in a significantly lower (± 1.5-fold) upregulation of the CST mRNA expression. To further investigate the upregulation of both sst_2 and CST mRNA by LPS stimulation in monocytes and mature macrophages and dendritic cells, expression and possible regulation of both mRNA levels were studied during differentiation of monocytes into its derived cells. Cells were collected from day 1 to 6 of culture to measure sst_2 and CST mRNA levels. During differentiation of monocytes into macrophages and dendritic cells, cells were incubated without or with LPS for 24 hours at day 1, 2, 3, 4, 5 or 6 of culture. The expression levels of both sst_2 and CST mRNA were measured during this time-dependent differentiation of monocytes into macrophages and dendritic cells. In unstimulated differentiating cells, using Q-PCR, no expression of sst_2 mRNA could be detected in macrophages and dendritic cells until day 6 (data not shown). In the LPS-stimulation experiments, sst_2 mRNA expression was detected in dendritic cells only at day 5 (p<0.0001) and 6, whereas sst_2 mRNA expression in macrophages was low and relatively constant at day 1 to 4, with a significant increase at day 5 and 6 (P<0.0001 vs. day 4). This is shown in figure 7.

In contrast to the time-dependent basal sst_2 mRNA expression, we could detect sst_2 mRNA expression in LPS-stimulated macrophages at day 1 to 4 of culture, although it was very low. However, in LPS-stimulated dendritic cells, we could not detect sst_2 mRNA expression at day 1 to 4. This finding is in accordance with the much lower expression of sst_2 mRNA in dendritic cells in both basal (data not shown) and LPS-stimulated conditions (figure 7) when compared to macrophages and suggests that sst_2 mRNA

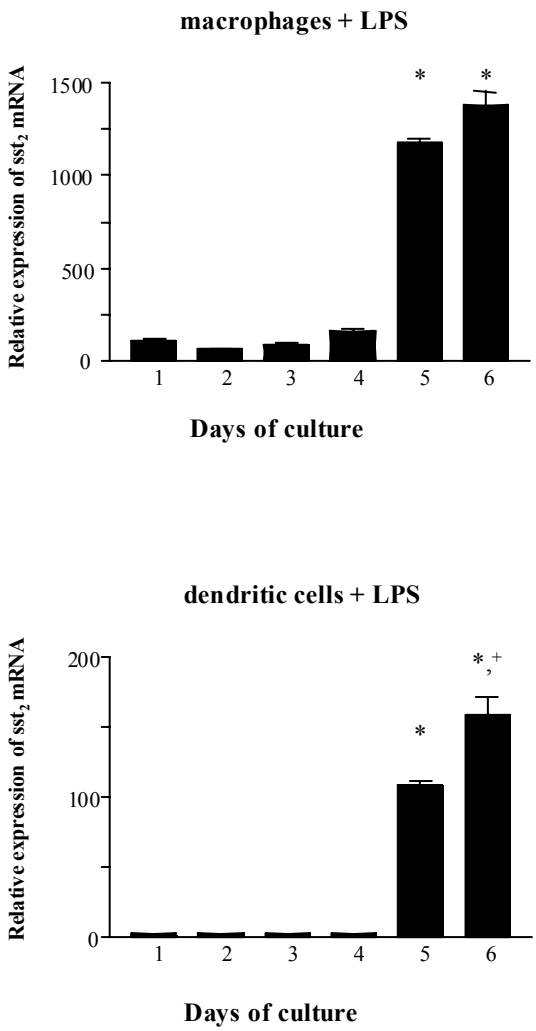

Figure 7: Time dependent increased sst_2 mRNA expression in monocytes during differentiation into macrophages and dendritic cells after stimulation with LPS (2 µg/ml) at the different time points. Bars represent sst_2 mRNA expression in macrophages (upper panel) and dendritic cells (lower panel) at day 1,2,3,4,5 or 6 of culture after a prior incubation for 24 hours with LPS, given in arbitrary units, relative to a generated standard curve from a Jurkat (T-cell) cell line and adjusted for HPRT expression. * $P<0.0001$ when compared to expression of sst_2 mRNA at day 4. $^{+}$ $P<0.01$ when compared to expression of sst_2 at day 5. Results are the mean of three independent experiments using cells from three different healthy donors.

expression levels in LPS-stimulated dendritic cells are below assay detection limit, rather than dendritic cells do not respond to the LPS-stimulation. In unstimulated cells,

expression of CST mRNA in macrophages increased after day 1 and reached its maximum already at day 2 (p=0.01) (figure 8). Dendritic cells showed maximal expression of CST mRNA already at day 1. LPS-induced CST mRNA expression during differentiation of macrophages was already maximal at day 1 of culture. In LPS-activated dendritic cells CST mRNA expression was low at day 1, $^{2}/_{3}$ of maximum at day 2 ($p<0.01$) and reached its maximum at day 3 (figure 6, $p<0.01$ compared to CST mRNA expression at day 1).

In order to demonstrate that sst_2 receptors were actually expressed on the cultured cells, macrophages and dendritic cells were incubated with the FITC-labeled octreotate, and visualized using a LSM410 confocal laser microscope (Carl Zeiss, Jena, Germany). Unstimulated macrophages and dendritic cells did not show a positive signal when incubated with octreotate-FITC (not shown). On the other hand, LPS-stimulated macrophages showed binding of the fluorescent SS-analogue, as shown by the spots in figure 9b. During the first minutes of incubation, a positive signal was detected at the cell membrane (not shown). Following the experiment in time, positive signals were also found inside the cells due to internalization of the receptor-ligand complex. When macrophages were incubated with both octreotate-FITC and a high amount of unlabeled octreotide (10^{-6} M), no fluorescent signal could be detected (figure 9d), demonstrating specificity of binding of octreotate-FITC to the sst_2 receptors. LPS-stimulated dendritic cells did not stain positive when incubated with octreotate-FITC. This is in concordance with the data found with Q-PCR in which we described an approx. 10-fold lower expression level of sst_2 mRNA in LPS-stimulated dendritic cells.

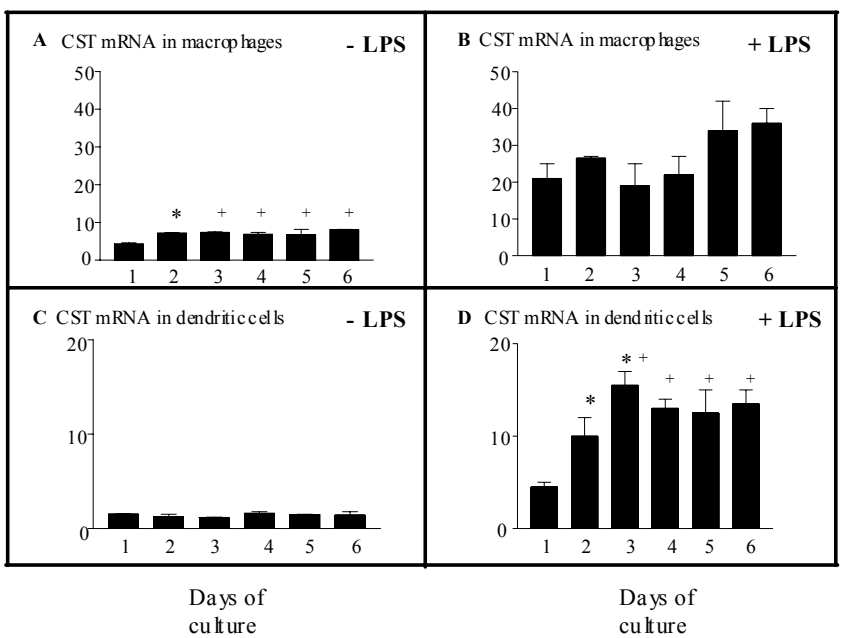

Figure 8: CST mRNA expression in monocytes during differentiation into macrophages and dendritic cells. All bars represent CST mRNA expression, given in arbitrary units generated to a standard curve from a RAJI (B-) cell line as results of quantitative RT-PCR using 50 ng/µl total RNA in the RT-reaction per sample. **A** represents CST mRNA expression in unstimulated macrophages. * P=0.01 when compared to expression at previous day, $^{+}$P=0.01 compared to expression at day 1 **B**: CST mRNA expression in macrophages at day 1,2,3,4,5 or 6 of culture after a prior incubation of 24 hours with LPS, **C**: CST mRNA expression in unstimulated dendritic cells and **D**: CST mRNA expression dendritic cells at day 1,2,3,4,5 or 6 of culture after a prior incubation of 24 hours with LPS. * P<0.01 when compared to expression at previous day and $^{+}$ P<0.01 compared to expression at day 1. The results shown are the mean from two experiments.

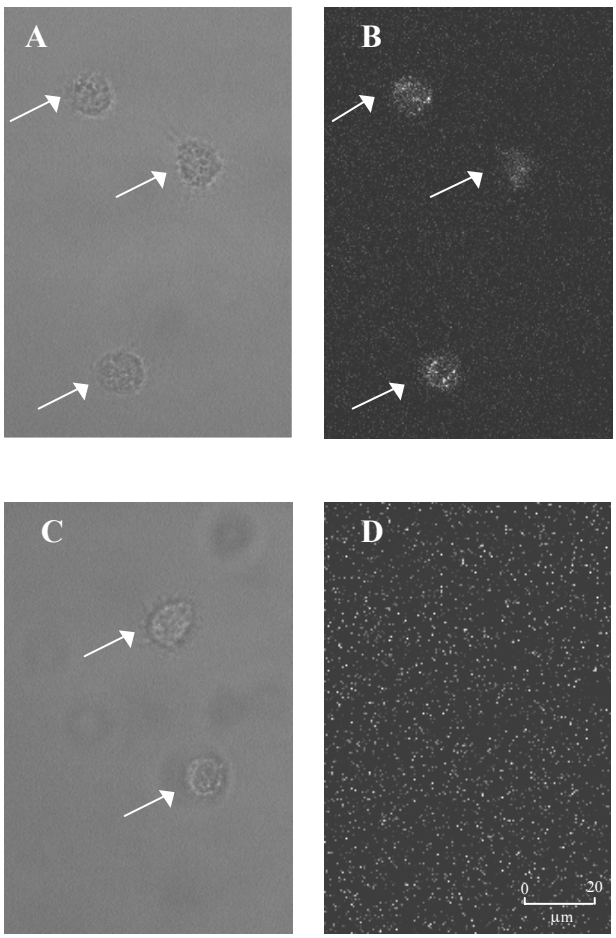

Figure 9: Visualization of sst_2 receptors on LPS-stimulated macrophages. LPS-stimulated macrophages were incubated with FITC-labeled octreotate, a sst_2 selective analogue and visualized by confocal microscopy. **A & B** Visualization of macrophages incubated with 20 nM FITC-octreotate. **A** Transmitted light image (arrows indicate macrophages), **B** Fluorescence signal of octreotate-FITC accumulated inside the macrophages, showing membrane binding and subsequent internalization of the labelled compound. **C & D** Macrophages incubated with FITC-octreotate and an excess of unlabeled octreotide. **C** Transmitted light image. **D** in the fluorescence channel no signal was detected in the cells. Scale bar is 20 µm.

Discussion

SS-14 and SS-28 are neuropeptides, playing an important role in inhibition of hormone release. SS also functions as a neurotransmitter, immunomodulator, and suppressor of angiogenesis and cell proliferation (1,2,38-41). Neuropeptides are involved in the interactions that exist between the human neuroendocrine and immune system (7,8). A role might be ascribed to SS and its receptors in this network as well. Ssts have been demonstrated in various endocrine and lymphoid tissues by classical ligand binding studies (42-44). The role of SS and its receptors in human immune system is still unclear. In contrast to the human immune system, the role of SS and sst in the murine immune system has been extensively studied by the group of Weinstock et al. (10). In their studies the expression and role of SS and sst in a model of mice infected with a Schistosoma Mansoni infection was evaluated. In granulomas formed following this Schistosoma infection, T-lymphocytes expressed $sst_{2\ (11)}$, while macrophages in these granulomas expressed SS mRNA (12). Stimulation of murine splenic macrophages with IFN-γ induced upregulation of the SS mRNA within 4 hours (45). Treatment of Schistosoma infected mice with SS resulted in granuloma growth inhibition by decreasing the IFN-γ production by T-cells. Moreover, in rat lymphoid tissues SS is expressed (46) and rat T- and B-lymphocytes from the thymus and spleen synthesize and secrete SS (47). In the human immune system, the role of SS and sst and the regulation of their expression remains still unclear. It is not known whether SS plays a regulatory role in the human immune system and whether this is comparable to the situation in the experimental rodent models. Cells of the monocyte lineage, i.e. monocytes, macrophages and dendritic cells, are known to be an important component in the human immune system (14-17).
Therefore, in the present study we investigated by RT-PCR the expression and regulation of the mRNA levels encoding the 5 known sst and SS in cells of the human monocyte lineage. We detected expression of only sst_2 mRNA in monocytes, macrophages and dendritic cells. Freshly Ficoll density-gradient isolated monocytes showed no expression of sst_2 mRNA (Unpublished results, Ligtenauer-Kaligis et al.). When monocytes were subsequently isolated from PBMC by Percoll density-gradient centrifugation, cells get already activated and express sst_2 mRNA, explaining the expression of sst_2 mRNA in our Fercoll-Picoll isolated monocytes. In addition, LPS- or IFN- γ -activated macrophages and dendritic cells were studied. LPS was chosen as activating factor, because it is known as an activator of dendritic cells, by inducing maturation and migration (48,49), and LPS effectively activates macrophages and induces tumoricidal activity (50,51). IFN- γ is known to induce macrophage stimulation, increasing the capacity of macrophages to promote T-cell proliferation (52). It is hypothesized that LPS and IFN-γ play regulatory roles in the innate and adaptive immune response, respectively (53,54). The innate immune response includes all host defense mechanisms that are encoded in the germ-line genes of the host. Adaptive responses are based on antigen-specific receptors, expressed on T- and B-lymphocytes. These receptors are assembled by somatic rearrangement of germ line gene elements (55). When cells were activated with LPS or IFN-γ, no expression of other sst subtypes could be detected, suggesting a selective role for sst_2 in these cells. As SS itself has a very short half-life, it is expected that SS to which the sst-

bearing cells respond is produced locally, probably by the sst-positive cells itself. However, despite expression of sst_2, no expression of the SS mRNA itself could be detected in any of the cell samples tested. Even following activation of the cells with LPS or IFN-γ, no SS mRNA could be detected, ruling out the possible autocrine role of SS in the human immune system, in contrast to the rodent immune systems. Interestingly, we found the expression of the mRNA encoding CST, a recently discovered SS-like peptide in all three cell types that were evaluated. CST is a 17 amino-acid peptide, which shows structural resemblance to SS (56) and has high binding affinity to the 5 known sst (27). In one preliminary report the expression of CST mRNA has been demonstrated in different human tissues, including leukocytes (31). Previously we demonstrated a selective expression of CST mRNA in cells and tissues of the human immune system, while no expression of SS mRNA was detected (26).

In the present study we mainly focussed on the expression and regulation of the 5 sst in cells of the monocyte lineage. As shown, only the sst_2 mRNA could be detected by RT-PCR, in both activated and non-activated cells. A previous study showed that sst_2 expression in leukocytes was upregulated after PHA stimulation (57). In this study, however, the regulation of sst_2 expression in cells of the monocyte lineage was not addressed. To investigate this regulation, monocytes were cultured into macrophages or dendritic cells, as schematically shown in Figure 1 and expression levels of sst_2 mRNA were measured using Q-PCR. Under basal conditions, expression of sst_2 was very low. Macrophages and dendritic cells expressed approximately 10-fold higher sst_2 mRNA levels than monocytes. In both IFN-γ and LPS-stimulated cells, expression of sst_2 mRNA was significantly upregulated, suggesting a potential role of the sst_2 in more mature and activated cells. Upregulation of sst_2 mRNA in IFN-γ treated macrophages was much lower compared with LPS-stimulated macrophages. Therefore, it may be hypothesized that sst_2 plays a more important role in the onset of innate immune responses, rather than in adaptive immune responses. On the other hand, the levels of sst_2 in IFN-γ stimulated cells may already be of functional significance in the immune response. We demonstrated that sst_2 mRNA is upregulated relatively late during culture. In this respect the dramatic rise in sst_2 mRNA between day 4 and 5 of culture is obvious. We observed this dramatic rise during maturation of monocytes into dendritic cells, as well as into macrophages. Therefore, we hypothesize that sst_2 expression only reaches significant levels after full maturation of monocytes, irrespective of the direction of differentiation. In the murine immune system it has been demonstrated that sst_2 plays an important role in granuloma formation in Schistosoma infected mice and that SS treatment inhibited granuloma growth through binding to sst_2 on T-cells and subsequent decline of IFN-γ production by these T-cells (11,13). The question is now addressed whether sst_2 also plays a role in the human immune system. In previous studies sst_2 expression was detected in granulomatous diseases as sarcoid granulomas and rheumatoid arthritis (58,59). Recently, in a preliminary study 10 patients with rheumatoid arthritis have been treated with the long-acting SS analogue, octreotide. Significant clinical improvement was found in these patients (60). In one study, 2 patients with sarcoidosis were treated with octreotide, a sst_2-selective SS-analogue (20). One patient responded very well, while the second showed no clinical response. These results, in combination with the expression of sst in cells of the monocyte-lineage indicate that SS or its analogue octreotide might have clinical

significance in diseases affecting the human immune system, acting via the sst_2 expressing macrophages or dendritic cells. As we demonstrated expression of sst_2 mRNA in cells of the monocyte lineage and no expression of SS mRNA itself, we also investigated the expression of the mRNA encoding CST, as a possible autocrine ligand for the sst_2, which has been proposed for SS in the rodent immune system (10-13). As previously reported (26), we found CST mRNA expression in all cell samples. Therefore, we evaluated the regulation of CST mRNA during differentiation of monocytes and by LPS and IFN-γ stimulation.

Monocytes showed low expression levels of CST mRNA, which were considerably upregulated in macrophages and dendritic cells. LPS-activation of these cells resulted in a significant upregulation of the CST mRNA levels as well. This upregulation during differentiation and by LPS activation suggests a possible role for CST in these human immune cells, especially in the mature cells and in cells in activated state. CST mRNA expression was also measured during differentiation of monocytes under basal and LPS-stimulated conditions. Overall, CST mRNA expression was upregulated earlier during differentiation, both in basal and LPS-stimulated conditions, than sst_2 mRNA expression. IFN-γ stimulation also resulted in significant upregulation of CST mRNA in macrophages. Whereas an autocrine SS-sst_2 regulatory pathway has been described in the murine immune system, the absence of SS and presence of CST mRNA suggests a more likely autocrine CST-sst_2 pathway in the human immune system. However, different lymphoid organs are innervated by nerves of the sympathetic nervous system and by sensory nerves containing neuropeptides such as SS (61). SS from these nerves might interact with the sst present in the human immune tissue, explaining the absence of SS itself in the immune cells. Moreover, CST is expressed in neurons as well. It may not be ruled out that CST as well can reach the sst via this route. The presence of CST mRNA in the immune cells itself, however, suggests an autocrine role of CST and the sst in addition to a possible paracrine role of CST from the neurons. The presence of functional sst_2 binding sites on membranes of the cells, both activated as well as non-activated, was investigated by confocal laser microscopy using the new compound, FITC-labelled octreotate. By autoradiography, we confirmed that FITC-octreotate was able to displace binding of [^{125}I- Tyr^3]octreotide to sst_2 in rat brain tissue with relative high affinity. In LPS-stimulated dendritic cells no binding of FITC-octreotate to the sst_2 could be visualized. These findings are in concordance with the relative low expression levels of sst_2 mRNA in these cells. On the other hand, on LPS-stimulated macrophages expression of the sst_2 protein was visualized and the binding of FITC-octreotate was successfully displaced when cells were also incubated with an excess amount of unlabeled octreotide, demonstrating specificity of binding. Thus sst_2 receptor levels on unstimulated cells and in LPS-activated dendritic cells are probably below the detection level of our assay. Unfortunately, at this moment we are unable to detect the protein for CST in the cells expressing CST mRNA. However, the expression of the mRNA and moreover, the regulation of this expression during differentiation and by activation suggests that CST indeed may play a role in the human immune system. Further functional studies should be performed to elucidate the possible effects of CST on these cells.

In conclusion, we investigated the mRNA expression of the 5 known sst in cells of the monocyte lineage, as well as its endogenous ligand SS and an alternative ligand CST. In

monocytes, macrophages and dendritic cells only expresion of sst_2 mRNA could be detected, whereas no SS mRNA was expressed. Interestingly, CST mRNA was expressed in all cells.
When cells were stimulated with LPS or IFN-γ, sst_2 expression was upregulated significantly, pointing to a possible role of at least these receptors in the human immune system. These receptors might show interaction with CST in the immune system, but it is also possible that SS reaches the place of inflammation by nerve-endings, in which SS is produced. During differentiation of monocytes into macrophages and dendritic cells, both sst_2 and CST mRNA levels were upregulated as well, pointing to a more important role of the sst_2-CST system in more mature cells. Interestingly, it seemed that CST mRNA was upregulated earlier during differentiation than sst_2 mRNA did. Finally, the expression of sst_2 has been visualized on membranes of LPS-activated-macrophages, while not on unstimulated cells and LPS-activated dendritic cells, in concordance with the lower expression levels of sst_2 mRNA found with Q-PCR. The functional significance of the expression of CST and sst_2 in these cells needs to be further investigated.

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II.5

THE EFFECTS OF GLUCOCORTICOIDS ON SOMATOSTATIN RECEPTOR EXPRESSION IN HUMAN MACROPHAGES

Abstract

Receptors for the neuropeptide somatostatin (SS) are widely expressed throughout the human body, both in physiological and pathophysiological tissues. The expression of somatostatin receptors (sst) in disease has led to the development of γ-camera scintigraphy, using radionuclide labeled SS analogues to visualize affected sites for diagnosis. Preliminary results have been shown that sst scintigraphy is promising in visualization of sarcoid lesions. It has been hypothesized that sst scintigraphy may be used in the evaluation of therapy efficiency in sarcoidosis. Uptake of radionuclide SS analogues is usually decreased after therapy. However, treatment of sarcoidosis is mainly based on the use of corticosteroids, which are known to have down-regulatory effects on transcription. Therefore, we investigated in the present study whether the decreased uptake of radionuclide labeled SS analogues may be due to a downregulation of sst_2 expression on human macrophages, which are known to be key mediators in sarcoidosis.
By quantitative PCR, we evaluated the expression levels of sst_2 mRNA on both LPS- and IFN-γ activated macrophages, which were cultured with different concentrations of dexamethasone (range: 10^{-13} to 10^{-7} M).
We found that expression levels of sst_2 mRNA were significantly downregulated by culturing with dexamethasone. This suggests that the decreased uptake of radionuclide labeled SS analogues after therapy may be the result of a down-regulatory effect of corticosteroids on the sst expression, rather than selectively being the result of reduction of inflammatory cells. Therefore caution should be taken in considering sst scintigraphy as the selective tool for evaluation of therapy efficiency in sarcoidosis patients.

Introduction

Somatostatin receptors (sst) are widely expressed in tissues throughout the human body. These receptors, of which 5 subtypes exist (sst_{1-5}) are mainly involved in the inhibitory actions of the peptide somatostatin (SS) on secretion processes, for instance in the pituitary, thyroid gland, pancreas and the gastro-intestinal tract (for a review see: (1)). Sst are not only expressed in physiological target tissues of SS, but have also been described in several malignancies (2-4). Detection of sst expression in human tumours has led to the development of sst scintigraphy for diagnostic purpose, by which sst-expressing tumours can be visualized by γ-camera scintigraphy using radionuclide labeled SS analogues (5,6). Moreover, different neuroendocrine tumours can be treated effectively with SS analogues (7), and more recently, also by peptide receptor radionuclide therapy (PRRT) (8).
Sst have also been described in cells and tissues of the human immune system in physiological state (9,10). In addition, sst were found to be expressed in various malignancies originating from immune cells, like Hodgkin's and non-Hodgkin's lymphomas (11,12), but also in inflammatory diseases, like sarcoidosis (13) and rheumatoid arthritis (14,15). More specifically, in sarcoid granulomas and rheumatoid synovium a selective expression of sst_2 has been found. The expression of sst_2 was mainly localized on macrophages (16,17), that are major effector cells in both sarcoidosis and rheumatoid arthritis. The detection of sst expression in these diseases has led to studies evaluating whether these receptors may have therapeutical implications (18), or whether the expression of these receptors may be useful as markers of successful treatment (19) or disease activity (14).
It was shown that treatment of sarcoidosis resulted in decreased uptake of the radionuclide labeled SS analogue (19). This suggests that treatment of sarcoidosis induces a decrease of sst_2-expressing effector cells. Therefore it was hypothesized that sst scintigraphy may be used in follow-up of treatment (19). However, corticosteroids are currently the standard of care for symptomatic patients, suffering from sarcoidosis (20). It is well known that corticosteroids can downregulate the transcription and expression of different genes (21), including sst (22). Therefore, the lower uptake of the radionuclide labeled SS analogue after treatment, may also be influenced by a downregulation of the sst_2 receptor on the cell membranes of the sst_2-expressing macrophages, rather than being the result of successful treatment (= eradication of effector cells).
Therefore, we investigated in the present study the effects of corticosteroids (dexamethasone (DEX)) on the expression pattern of sst in macrophages by RT-PCR. By quantitative PCR the effects of DEX on sst_2 mRNA expression levels in activated macrophages was evaluated.

Material and Methods

Isolation of blood mononuclear cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin Blood bank, Rotterdam, The Netherlands) by Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation. Subsequently, monocytes were isolated from PBMC using a Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation step as described in detail previously (23). Isolated monocytes were frozen in 10% DMSO-medium and stored in -80° C until use.

Cell-culture
A frozen suspension of monocytes was rapidly thawed in a water-bath at 37° C and viability was evaluated by Trypan-blue exclusion (Life Technologies, Grand Island, U.S.A.). Cell viability always exceeded 95%. Monocytes were seeded at a density of 0.5 x 10^6 cells/cm^2 in a volume of 1 ml/well in 24 well Nuncleon plates (Nalge Nunc International, Denmark) in RPMI1640+ supplemented with 10% heat-inactivated (30 min 56˚ C) fetal calf serum (FCS, Life Technologies, Grand Island, U.S.A.), L-glutamine (2 mM, Life Technologies) and penicillin (1000 U/ml, Yamanouchi Pharma BV Leiderdorp, The Netherlands). Plates were then incubated for 90 minutes at 37˚ C to allow adherence of the monocytes to the plate. Thereafter, plates were washed to remove potential contaminating T- and B-cells. To generate macrophages, fresh medium was added, containing granulocyte macrophage-colony stimulating factor (GM-CSF, 500 U/ml, Novartis Pharma BV, Arnhem, The Netherlands). The cells were cultured for 6 days, with a medium refreshment at day 3. This method of generation of macrophages has been described previously in detail (24).
In order to study the regulation of sst_2 mRNA expression, 6 day macrophages were incubated during 24 hours with lipopolysaccharide (LPS, Sigma Aldrich, Zwijndrecht, The Netherlands (2μg/ml)) or interferon γ (IFN-γ, 10 ng/ml), with or without increasing concentrations of dexamethasone (DEX, concentrations varying between 10^{-13} and 10^{-7} M). After 24 hours of incubation, macrophages were collected using cell scrapers and scraped cells were used for further PCR analysis.

RT-PCR analysis
Poly A^+-mRNA was isolated as described in detail elsewhere (25). In order to evaluate the effects of DEX on sst subtype expression patterns, RT-PCR was performed as previously described for sst_{1-5} (26).

Quantitative PCR analysis
In order to quantify sst_2 mRNA, a quantitative PCR (Q-PCR) was performed by TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence Detection System (Perkin Elmer) for real-time amplifications, according to manufacturers protocol. Q-PCR was performed for sst_2 only, because no mRNA expression of the other sst subtypes were detected in the cells we investigated.
The primer sequences that were used included:

Sst_2 forward 5'-ATGCCAAGATGAAGACCATCAC-3'
Sst_2 reverse 5'-TGAACTGATTGATGCCATCCA-3'
HPRT forward 5'-TGCTTTCCTTGGTCAGGCAGTAT-3'
HPRT reverse 5'-TCAAATCCAACAAAGTCTGGCTTATATC-3'
The probe sequences that were used included:
Sst2 5'-FAM-TGGCTCTGGTCCACTGGCCCTTTG-TAMRA-3'
HPRT 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3'
The relative amount of sst_2 mRNA was determined using a standard curve generated from known amounts of human genomic DNA. For the RT-reaction 50 ng/µl of total RNA was used in every reaction. For determination of hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA, which was used to normalize sst_2 mRNA expression levels, a standard curve was generated of cDNA obtained from a DU45- (prostate-cancer) cell line. The amount of sst_2 mRNA was calculated relative to the amount of HPRT and results are presented as percentage compared to control cells. Control cells were either LPS- or IFN-γ stimulated macrophages.

Results

First, we determined whether DEX treatment could influence the expression pattern of sst subtypes. We found that DEX-stimulated macrophages selectively expressed sst_2 mRNA (data not shown), which is in concordance with our findings in unstimulated macrophages (26). DEX, therefore, did not have any effects on the sst subtype expression pattern. Our second question concerned the effects of DEX treatment on sst_2 expression levels. By Q-PCR, we found a clear upregulation of sst_2 mRNA levels, when cells were incubated with LPS or IFN-γ. LPS upregulated the sst_2 mRNA approximately 150-fold, whereas the upregulation by IFN-γ was lower, amounting a 30-fold upregulation (data not shown, see in chapter II.4). When LPS-stimulated macrophages were incubated with different concentration of dex (10^{-13} to 10^{-7} M) we found a biphasic effect of DEX on sst_2 mRNA expression (figure 1).
A high concentration (10^{-7} M) or a low concentration (10^{-13} M) did not have statistically significant effects on sst_2 mRNA expression, but the other concentrations significantly decreased the LPS-stimulated sst_2 mRNA levels, with a maximum inhibitory of 65 % induced by 10^{-9} M. Different concentrations of DEX did not have statistically significant effects on the expression levels of sst_2 mRNA in unstimulated macrophages (data not shown). When macrophages were stimulated with IFN-γ, we found a comparable inhibitory effect of DEX (figure 2).

DEX biphasically decreased the sst_2 mRNA levels in IFN-γ stimulated macrophages, with a maximum effect (50 % inhibition) at a concentration of 10^{-9} M, as we observed in LPS-stimulated macrophages as well.

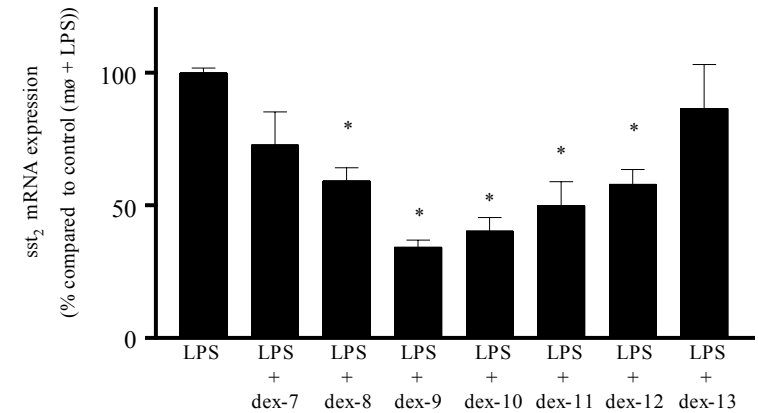

Figure 1: Effect of dexamethasone on lipopolysachharide-stimulated macrophages. This figure represents the expression of somatostatin receptor subtype 2 (sst_2) mRNA in macrophages (mø), either stimulated with lipopolysaccharide (LPS) alone or with LPS plus different concentrations of dexamethasone (DEX, 10^{-7} to 10^{-13} M). The expression levels of sst_2 mRNA were investigated by quantitative PCR and are corrected for HPRT. This figure represents the mean ± standard deviation of 4 independent experiments and data are presented as percentage compared to control (mø + LPS). * P<0.001 when compared to mø + LPS.

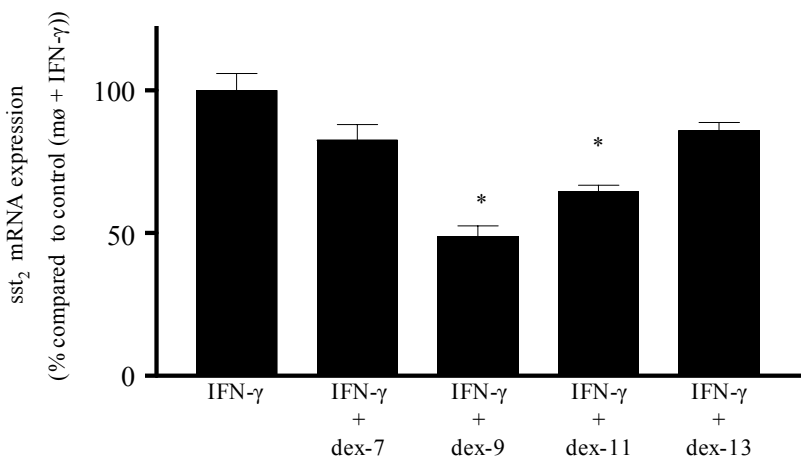

Figure 2: Effect of dexamethasone on interferon-γ stimulated macrophages.This figure represents the expression of somatostatin receptor subtype 2 (sst_2) mRNA in macrophages (mø), either stimulated with interferon-γ (IFN-γ) alone or with IFN-γ plus different concentrations of dexamethason (DEX, 10^{-7} to 10^{-13} M). The expression levels of sst_2 mRNA were investigated by quantitative PCR and are corrected for HPRT. This figure represents the mean ± standard deviation of 3 independent experiments and data are presented as percentage compared to control (mø + IFN-γ). * P<0.001 when compared to mø + IFN-γ.

Discussion

As the expression of sst has been described in affected tissues in diseases of the human immune system (11-15) it has been hypothesized that SS or its analogues can play a role in either evaluating the stage of disease or in treatment (11,13,14,19,27,28). Sst scintigraphy has been shown to visualize Hodgkin's and non-Hodgkin's lymphomas (27,28). In addition, affected sites in patients suffering from sarcoidosis and rheumatoid arthritis can be visualized using sst scintigraphy (13,14). In rheumatoid arthritis it was demonstrated that the degree of pain and swelling, which are the major symptoms in rheumatoid arthritis, correlated well with the positive findings by sst scintigraphy (14). However, no data exist on sst scintigraphy after treatment of rheumatoid arthritis, in order to see the effects of treatment on sst_2 expression. One study reported that the uptake of radiolabeled SS analogues decreased after therapy of sarcoidosis using corticosteroids (19). Clinical improvement was evaluated by X-ray as well. It was suggested that the decrease in uptake of radionuclide labeled SS analogues was due to successful therapy, thus a decrease in disease-involved sst-expressing cells. However, in the present study we found that DEX significantly decreased the sst_2 mRNA levels on either IFN-γ or LPS-activated macrophages. A previous study demonstrated that the inhibitory effects of dexamethasone on sst_2 mRNA expression were mainly due to changes in the transcription rate of the sst_2 gene (29). However, the exact mechanism is still unknown. In one study, in which stimulatory effects of dexamethasone on sst_2 mRNA expression were found in mice, it was demonstrated that in the second sst_2 promotor a functional glucocorticoid response element (GRE) exists, which could explain the stimulatory but not the inhibitory effects (30).

Our findings suggest that therapy of sarcoidosis by corticosteroid-treatment may decrease sst_2 expression on the cell membranes of the effector cells. Thus, a lower uptake found by sst scintigraphy, may not fully represent improvement. Sclerodizing sarcoid lesions can be visualized at radiological examination, suggesting active disease, while fibrotic lesions are supposed to be negative at scintigraphy. In a study by Kwekkeboom et al. (19), sst scintigraphy was performed before and after treatment of 10 sarcoidosis patients with corticosteroids. Clinical improvement, as investigated by chest X-ray, was found in 5 patients. In 4 out of these 5 patients, a decreased uptake was found by sst scintigraphy. Although no clinical improvement was found by chest X-ray in 5 patients, uptake of radionuclide labeled SS analogues decreased after therapy in a number of these patients (2 out of 5). This may thus be explained by the downregulatory effect of corticosteroids on sst_2 expression, as observed in our study.

Since sst_2 are expressed on cells of the monocyte lineage, including macrophages and dendritic cells (chapter II.4), it may be hypothesized that SS or its analogues can play a role in therapy of immune-related diseases like rheumatoid arthritis and sarcoidosis as well (13,14). Sst_2 is the sst subtype, which is known to be involved in the regulation of secretion by SS and its analogues (31,32). As sst_2 was found to be expressed on macrophages (26), SS or its analogues may play a role in regulating expression of cytokines and growth factors by these cells. One pilot study showed clinical improvement in patients suffering from rheumatoid arthritis after treatment with the SS analogue

octreotide (18). In sarcoidosis, until now, there is no experience in treatment with SS analogues. However, it may be hypothesized that SS analogues, probably in combination with corticosteroids, can play an immunoregulatory role in sarcoidosis. As mentioned previously, SS may possibly downregulate the secretion of pro-inflammatory cytokines and growth factors by macrophages (33). On the other hand, to achieve this potential beneficial effect of SS analogues, expression of abundant sst on the cells is necessary. Since glucocorticoids are frequently used in treating immune diseases like rheumatoid arthritis (34) and sarcoidosis (20), the downregulatory effect of glucocorticoids on sst_2 expression in macrophages could theoretically influence the results of SS treatment. This may be of importance in PPRT as well.

In conclusion, we found that DEX significantly decreased sst_2 mRNA levels in activated macrophages. Therefore, a decrease in uptake of radionuclide labeled SS analogues after treatment of sarcoidosis may be the result of downregulation of the sst as well, rather than a therapeutical effect on the number of inflammatory cells. Sst scintigraphy may, therefore, not be a good solitary parameter to evaluate the success of therapy. Other measurements should always be included, like for instance X chest. For the future, speculating about the possible therapeutical implication of SS and its analogues in immune-mediated disease, it should be mentioned that corticosteroids may influence the sst expression status and thus the efficiency of treatment with SS or its analogues.

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Chapter III

THE FUNCTIONAL SIGNIFICANCE OF SOMATOSTATIN RECEPTOR EXPRESSION IN HUMAN IMMUNE CELLS

III.1a

QUANTITATIVE AND FUNCTIONAL EXPRESSION OF SOMATOSTATIN RECEPTOR SUBTYPES IN HUMAN THYMOCYTES

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Abstract

We recently demonstrated the expression of somatostatin (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 isolated 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 purified human thymocytes at various stages of development. Thymocytes represent a heterogeneous population of lymphoid cells displaying different levels of maturation and characterized by specific cell surface markers. In the present study, we first demonstrated specific high affinity [^{125}I-Tyr^{11}]-SS-14 binding on thymocyte membrane homogenates. Subsequently, by RT-PCR, sst_{2A} and sst_3 mRNA expression was detected in the whole thymocyte population. After separation of thymocytes into subpopulations, we found by quantitative RT-PCR that sst_{2A} and sst_3 are differentially expressed in intermediate/mature and immature thymocytes. The expression of sst_3 mRNA was higher in the intermediate/mature $CD3^+$ fraction compared with the immature $CD2^+CD3^-$ one, while sst_{2A} mRNA was less abundant in the intermediate/mature $CD3^+$ thymocytes. In 7 days cultured thymocytes SSR subtype mRNA expression was lost. SS-14 significantly inhibited ^{3}H-Thymidine incorporation in all thymocyte cultures, indicating the presence of functional receptors. Conversely, octreotide significantly inhibited ^{3}H-Thymidine incorporation only in the cultures of immature $CD2^+CD3^-$ thymocytes. Sst_3 is mainly expressed on intermediate/mature thymocyte fraction and most of these cells generally die by apoptosis. Since SS-14, but not octreotide, induced a significant increase in the percent of apoptotic thymocytes, it might be that sst_3 is involved in this process. Moreover, sst_3 has been recently demonstrated on peripheral human T lymphocytes, which directly derive from mature thymocytes, and SS analogs may induce apoptosis in these cells. Interestingly, $CD14^+$ thymic cells, which are cells belonging to the monocytes-macrophage lineage, selectively expressed sst_{2A} mRNA.
Finally, SSR expression in human thymocytes seems to follow a developmental pathway. The heterogeneous expression of SSR within the human thymus on specific cell subsets and the endogenous production of SS as well as SS-like peptides emphasize their role in the bi-directional interactions between the main cell components of the thymus involved in intrathymic T-cell maturation.

Introduction

The immune and neuroendocrine systems cross talk by using similar 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) represents one of the most relevant neuropeptides involved in neuro-immuno-endocrine interaction (5,6). The wide spectrum of actions of SS and the presence of SS receptor (SSR) in lymphoid organs implies a broad functional role of this peptide in the immune system (2,6-8).

We have recently demonstrated the expression of SS itself and of three different SSR subtypes (sst) within the human thymus (9). 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 (9), while recently, our preliminary data have demonstrated a low number of SS-binding sites on freshly isolated human thymocytes (10). On the other hand, SSR are expressed on thymocytes of different animal species (11-13), and in humans SS is known to modulate different functions of T lymphocytes, which directly derive from thymocytes (6). Moreover, sst_3 mRNA has been recently demonstrated to be constitutively expressed in human resting peripheral T lymphocytes (14). 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. 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 (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 (16). Thus, thymocytes undergo maturation through a series of stages that can be distinguished 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 (5). Finally the $CD3^+CD4^+CD8^+$ subset further differentiates into mature $CD4^+$ or $CD8^+$ single positive thymocytes (16,17).

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 13 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 parents of patients. All samples were histopathologically normal and were taken fresh at the operation.

Protocol of the study. Thymocytes were freshly isolated from the thymic samples and used for binding studies on membrane homogenates with iodinated SS-14 (Table 1). Thymocytes derived from 4 samples were separated in subpopulations for RT-PCR studies (nos. 5-8, Table 1; see below). Thymocytes from 5 samples of the same series (nos. 5-7, 10, 11, Table 1) were used for the *in vitro* primary cell cultures. Thymocytes from 3 samples were used for study the induction of apoptosis (nos. 11-13, Table 1; see below).

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 (18). To confirm this we performed FACS® analysis 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 intermediate/mature $CD3^+$ thymocyte subset and the $CD14^+$ monocyte-macrophage fraction. Thymic cells (10^6) were sorted by setting appropriate electronic gates with the dual-laser FACS® system (Beckton Dickinson & Co.) For RT-PCR analysis and for functional studies, 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

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

Samples	[^{125}I-Tyr^{11}]-somatostatin-14 binding	
	K_d (nM)	B_{max} (pmol/mg protein)
1.	3.1±1.0	59±5.1
2.	0.7±0.02	11±1.1
3.	1.2±0.2	31±3.2
4.	0.4±0.2	12±1.1
5.	0.3±0.1	52±5.2
6.	2.1±0.3	47±3.1
7.	0.6±0.2	36±2.1
8.	0.8±0.2	29±3.0
9.	0.4±0.1	18±1.1
10.	0.3±0.1	32±2.5
11.	0.8±0.2	29±3.1
12.	0.9±0.1	35±2.2
13.	0.8±0.3	42±4.0
rat brain	1.2±0.1	470±67.1

K_d, dissociation constant; B_{max}, maximum binding capacity. Specific binding ranged between 12.7 and 45.8% of total binding (n=3).

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 ($CD2^+CD3^-$). Additional freshly isolated thymocytes (from 5 cases), which did not undergo beads separation, as well as negatively selected $CD3^+$ and $CD2^+CD3^-$ cell fractions (from 2 cases) were seeded (5 x 10^6 cells per well) 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 (9). [^{125}I-Tyr^{11}]-SS-14 (Amersham, Houten, The Netherlands) 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 with and without excess (1 μM) of unlabeled SS-14 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, and ranged between 12.7 and 45.8% of the total binding (32.4±3.3).

Functional studies

In all experiments SS-14 (Bachem Inc., Hannover, Germany) and octreotide (Novartis Pharma, Basel, Switzerland), dissolved in the culture medium (RPMI-1640 supplemented with 10% heat-inactivated FCS, penicillin and fungizone), were used at a concentration of 10^{-13}, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M. The culture medium was added to the control wells to evaluate the possible effects of the vehicle. 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 then 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 (9). 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 hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Table 2).

Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation the cDNA reactions were also performed without reverse transcriptase and amplified with each primer-pair (no introns have yet been found in the coding region of these genes, which include the oligonucleotide amplimers used in this study). 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 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 were amplified, because these primer-pairs did enclose introns in the genomic DNA. In thymocyte preparation 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 and probe sequences that were used for the quantitative RT-PCR are reported in Table 3.

Table 2: Primers used for RT-PCR studies

	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)	GCCAAGATGAAGACCATCAC	214	414 bp
sst_{2A} (reverse)	GATGAACCCTGTGTACCAAGC	627	
sst_3 (forward)	CCAACGTCTACATCCTCAACC	236	314 bp
sst_3 (reverse)	TCCCGAGAAGACCACCAC	549	
sst_4 (forward)	ATCTTCGCAGACACCAGACC	547	321 bp
sst_4 (reverse)	ATCAAGGCTGGTCACGACGA	867	
sst_5 (forward)	CCGTCTTCATCATCTACACGG	596	226 bp
sst_5 (reverse)	GGCCAGGTTGACGATGTTGA	819	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	-1	349 bp
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	348	
HPRT (forward)	CAGGACTGAACGTCTTGCTC	132	413 bp
HPRT (reverse)	CAAATCCAACAAAGTCTGGC	544	

a) The sequences of the primers for sst_1, sst_4 and sst_5 are derived and/or adapted from Kubota *et al*. (19) and Wulfsen *et al*. (20). All other primers and probes were designed by use of the Primer3! software (http://www-genome.wi.mit.edu/genome_software/ other/primer3.html) and the appropriate GenBank entries. ***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

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.

Table 3: Primers and probes used for quantitative RT-PCR studies.

	Sequence (5'- 3')[a]	Position[b]	Size of PCR product
sst_{2A} (forward)	ATGCCAAGATGAAGACCATCAC	212	171 bp
sst_{2A} (reverse)	TGAACTGATTGATGCCATCCA	382	
sst_{2A} (probe)	TGGCTCTGGTCCACTGGCCCTTTG	308	
sst_3 (forward)	CTGGGTAACTCGCTTGGTCATCTA	178	86 bp
sst_3 (reverse)	AGCGCCAGGTTGAGGATGTA	263	
sst_3 (probe)	CGGCCAGCCCTTCAGTCACCAAC	218	
HPRT (forward)	TGCTTTCCTTGGTCAGGCAGTAT	437	109 bp
HPRT (reverse)	TCAAATCCAACAAAGTCTGGCTTATATC	545	
HPRT (probe)	CAAGCTTGCGACCTTGACCATCTTTGGA	489	

Legend: see Table 2.

Effects of SS and octreotide on apoptosis of thymocytes

For studying induction of apoptosis, thymocytes were incubated during 24 h without or with different concentrations of SS-14 or octreotide. After 24 h, the cells were collected and pelleted by centrifugation. Thereafter, the medium was carefully removed and the cells were lysed in lysis buffer (Roche Diagnostics GmbH) during 30 min at room temperature. Subsequently, the lysate was centrifuged at 200 x g for 10 min and 20 µl of each lysate was used for photometric measurement of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) using a Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics GmbH, Mannheim, Germany). Values are expressed as absorbance units [A_{405nm}-A_{490nm}].

Statistical analysis

Data are expressed as Mean ± SEM. In functional studies $n = 4$ 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. 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 0.3±0.1 and 3.1±1.0 nM and a low maximum binding capacity (B_{max}) ranging between

11±1.1 and 59±5.1 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.

SS and SSR subtype expression

The method of beads separation showed that thymocytes after cell counting were more than 95% (≥ 98% of rosetted cells), while $CD14^+$ cells were less than 5% among the filtered thymic cells, in all the cases examined. This finding was confirmed by FACS® analysis (data not shown). Moreover, the data are in agreement with other authors, which performed this evaluation on a larger series of age- and sex-matched paediatric thymuses (21). The percent of thymocytes in each subgroup after beads separation ranged from 95.5 to 99.2%. By RT-PCR, sst_{2A} and sst_3 mRNA expression was detected in freshly isolated thymocytes from 4 of 4 cases tested (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 (9). 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 4 summarizes the results of RT-PCR analysis and an example is shown in Figure 2.

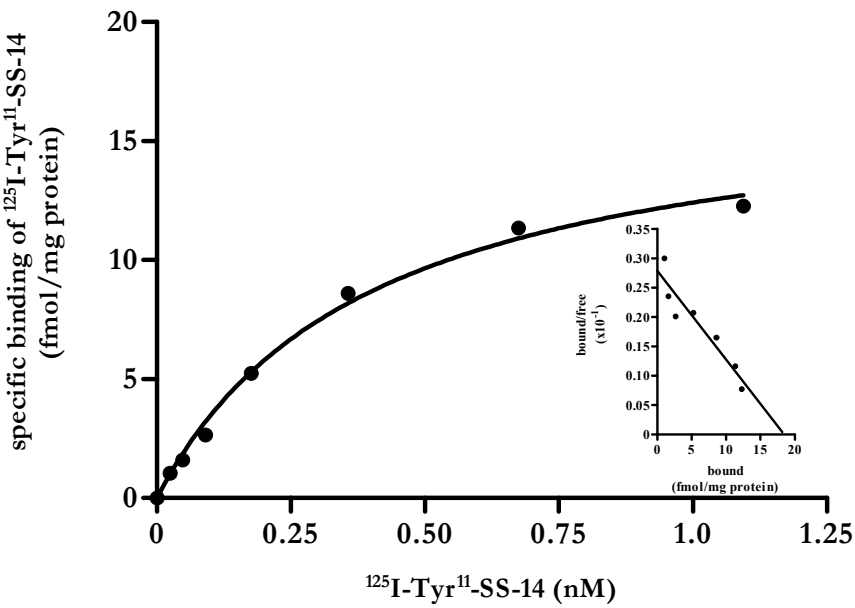


Figure 1: Binding of [^{125}I-Tyr^{11}]-SS-14 to a membrane homogenate preparation of human thymocytes. ●, Specific binding (total minus non-specific binding in presence of 1 μM of SS-14). Inset: Scatchard analysis of the binding data (K_d, 0.4 nM and B_{max}, 18.4 fmol/mg membrane protein; no. 9, Table 1).

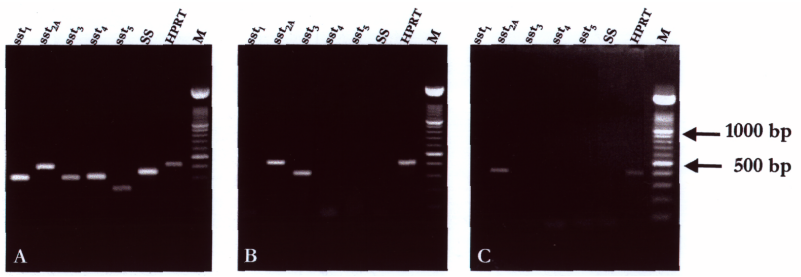


Figure 2. Heterogeneous expression of SS and sst_{1-5} mRNAs in human thymocytes and CD14 cells. Poly A^+ mRNA was reverse transcribed and cDNA was amplified by PCR. PCR products of the sst_{1-5} were separated on 1% agarose gel and stained with ethidium bromide. M, 100-bp ladder; ***A)*** control; ***B)*** 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 4: Heterogeneity of somatostatin and somatostatin receptor subtype mRNA expression in different fractions of human thymocytes and $CD14^+$ thymic cells as determined by RT-PCR.

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.

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).

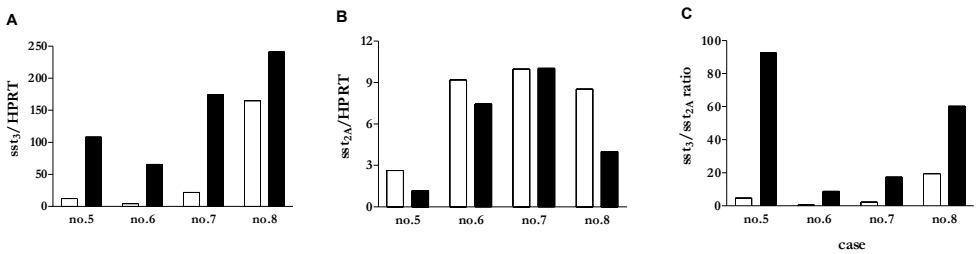
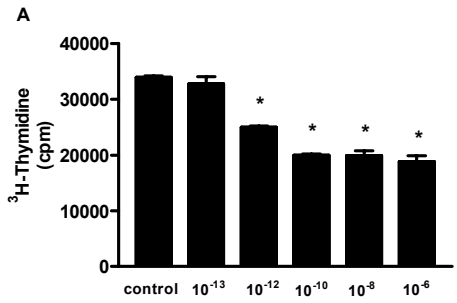


Figure 3: Quantitative SSR RT-PCR in human thymocytes. Quantitative analysis of RT-PCR data showed a different amount of sst_{2A} and sst_3 mRNA in immature $CD2^+CD3^-$ and intermediate/mature $CD3^+$ thymocytes calculated relative to the amount of HPRT and given in arbitrary units. ***A)*** sst_3/HPRT mRNA ratio; ***B)*** sst_{2A}/HPRT mRNA ratio; ***C)*** sst_3/sst_{2A} mRNA ratio. Data derived from 4 different thymuses. sst_{2A} assay: correlation coefficient standard curve (R^2) 0.980±0.005, slope of the curve -3.47±0.11, (n=3); sst_3 assay: correlation coefficient standard curve (R^2) 0.971±0.0045, slope of the curve: -3.27±0.15, (n=3).
Immature $CD2^+CD3^-$ ■ , and intermediate/mature $CD3^+$ □ thymocytes.

Effect of SS and octreotide on ^{3}H-Thymidine incorporation in thymocytes
SS-14 significantly inhibited ^{3}H-Thymidine incorporation in all the 5 cultures (nos. 5-7, 10, 11 Table 1) of freshly isolated thymocytes (whole population) in a dose dependent manner. The inhibition was statistically significant at a concentration of 10^{-6} (ranging between 45 and 77%) and 10^{-8} M (ranging between 27 and 41%) in all 5 cases, at a concentration of 10^{-10} M in 4 of 5 (ranging between 21 and 64%), and at a concentration of 10^{-12} M in 3 of 5 (ranging between 26 and 42%) (Fig. 4A-E).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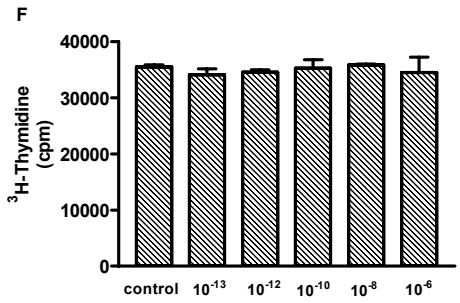
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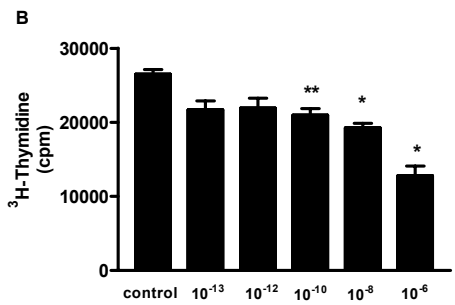
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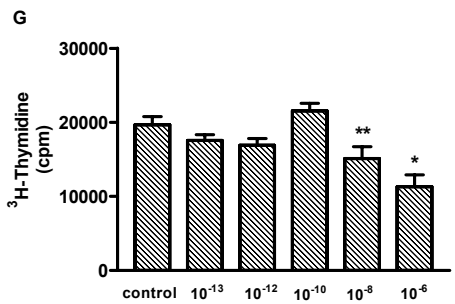
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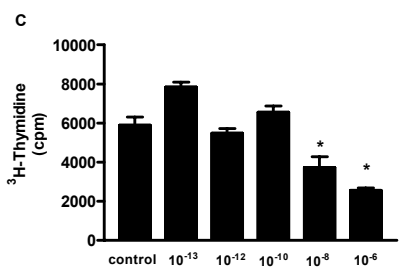
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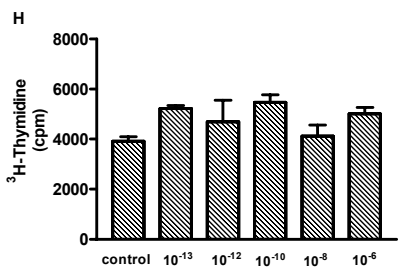
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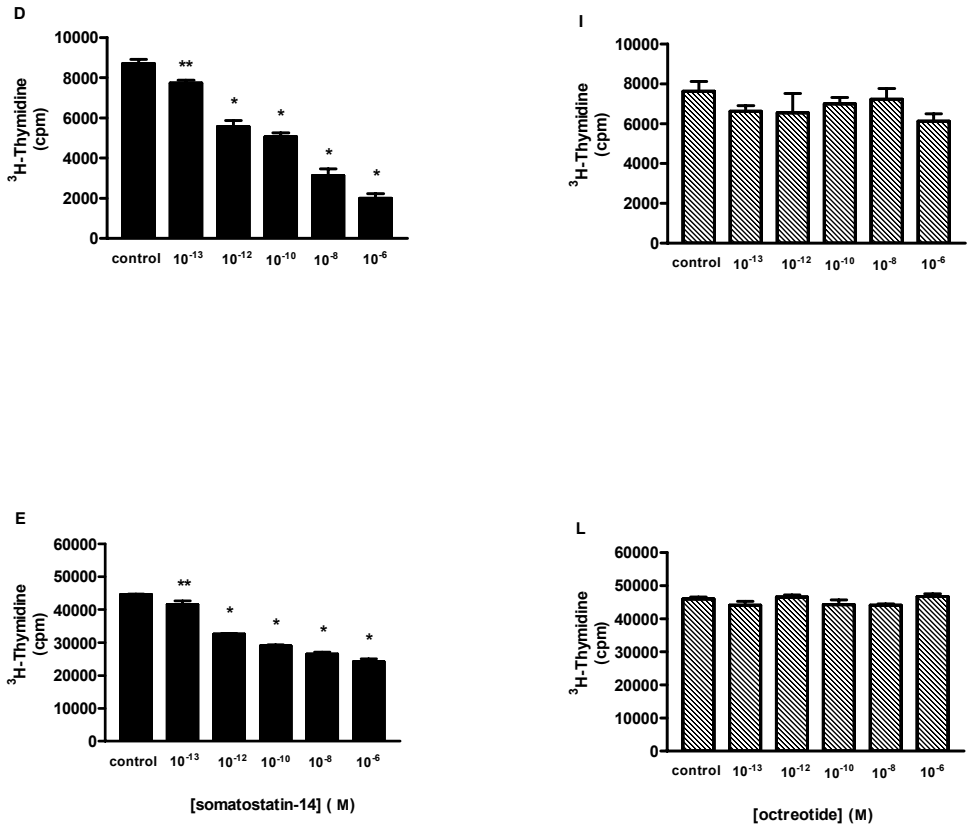

Figure 4: Effects of somatostatin (*A-E*) and octreotide (*F-L*) on ^{3}H-Thymidine incorporation in thymocyte cultures from 5 different thymuses (whole population). Thymocytes were incubated in RPMI-1640 supplemented with 10% heat-inactivated FCS, penicillin and fungizone during 24 h in quadruplicate 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 ▨

The SS analog octreotide significantly inhibited ^{3}H-Thymidine incorporation only in 1 culture of thymocytes (whole population) at concentrations of 10^{-6} (43%) and 10^{-8} M (23%) (Fig. 4F-L). In the $CD2^+CD3^-$ thymocyte cultures, both SS-14 and octreotide significantly inhibited ^{3}H-Thymidine incorporation in the 2 cases evaluated (Fig. 5A-D). The inhibition was dose dependent and statistically significant at all concentrations (except at the concentration of 10^{-13} M in one case). Conversely, in the $CD3^+$ thymocyte cultures only SS-14 significantly inhibited ^{3}H-Thymidine incorporation in a dose

dependent manner in both cases evaluated, whereas octreotide was ineffective (Fig. 6A-D).

Effects of SS and octreotide on the apoptosis of thymocytes

SS-14, but not octreotide, significantly increased the amount of histone-associated DNA fragments, which are measurable following induced cell death (apoptosis), in all the early cultures of freshly isolated and purified thymocytes (whole population). The number of apoptotic cells was significantly higher at all concentrations in 1 case (Fig. 7C) and at the concentration of 10^{-6}, 10^{-7} and 10^{-8} M, and at the concentration of 10^{-6} and 10^{-7} M in the remaining 2, respectively (Fig. 7A, B).

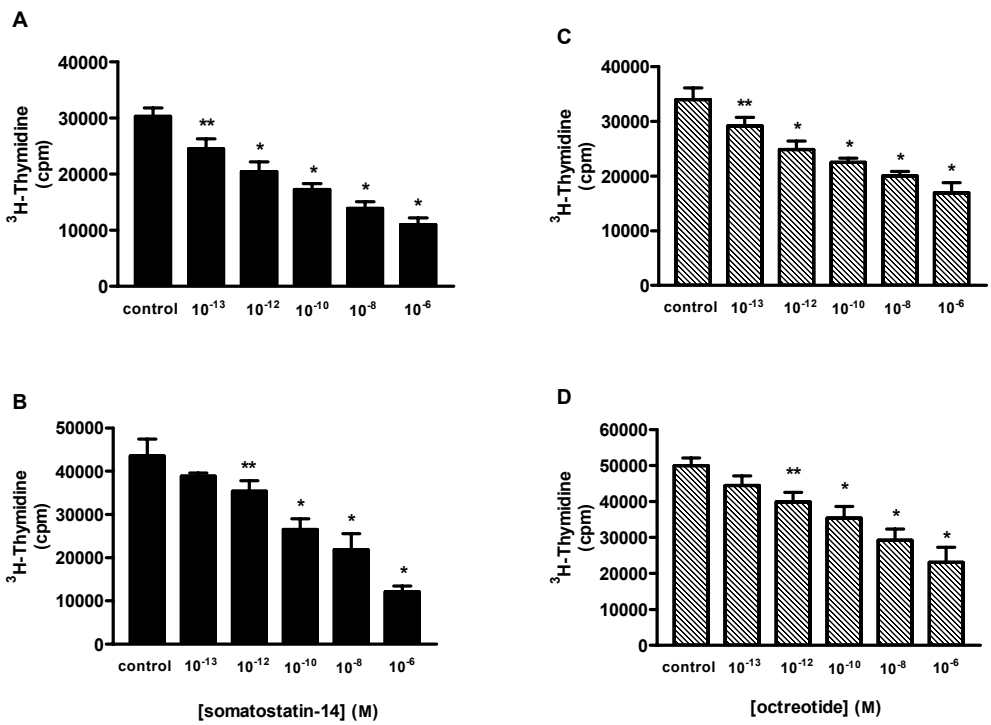

Figure 5: Effects of somatostatin (***A, B***) and octreotide (***C, D***) on ^{3}H-Thymidine incorporation in isolated $CD2^+CD3^-$ thymocyte cultures from 2 different thymuses Culture conditions and legends see Figure 4. Mean±SEM (n=4 well per treatment group; nos. 10, 11, Table 1).
*, $p<0.01$ *vs*. control**;** **, $p<0.05$ *vs*. control. SS-14 ■ and octreotide ▨

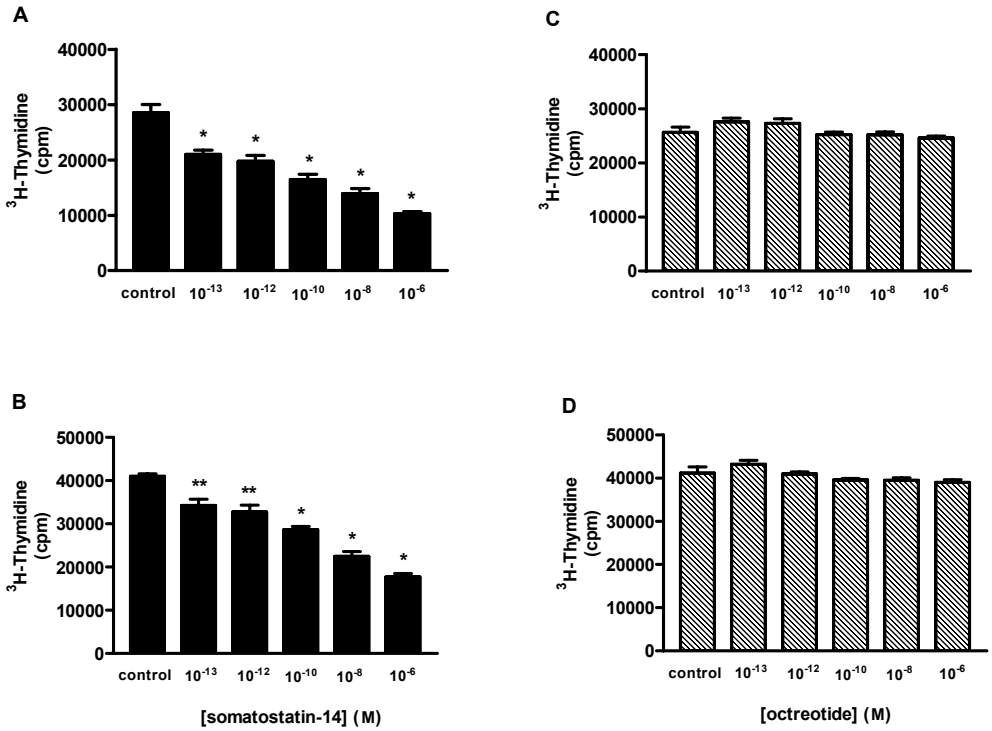

Figure 6: Effects of somatostatin (***A, B***) and octreotide (***C, D***) on ^{3}H-Thymidine incorporation in isolated CD3$^+$ thymocyte cultures from 2 different thymuses. Culture conditions and legends see Figure 4. Mean±SEM (n=4 well per treatment group; nos. 10, 11, Table 1).
*, p<0.01 *vs*. control**;** **, p<0.05 *vs*. control. SS-14 ■ and octreotide ▨

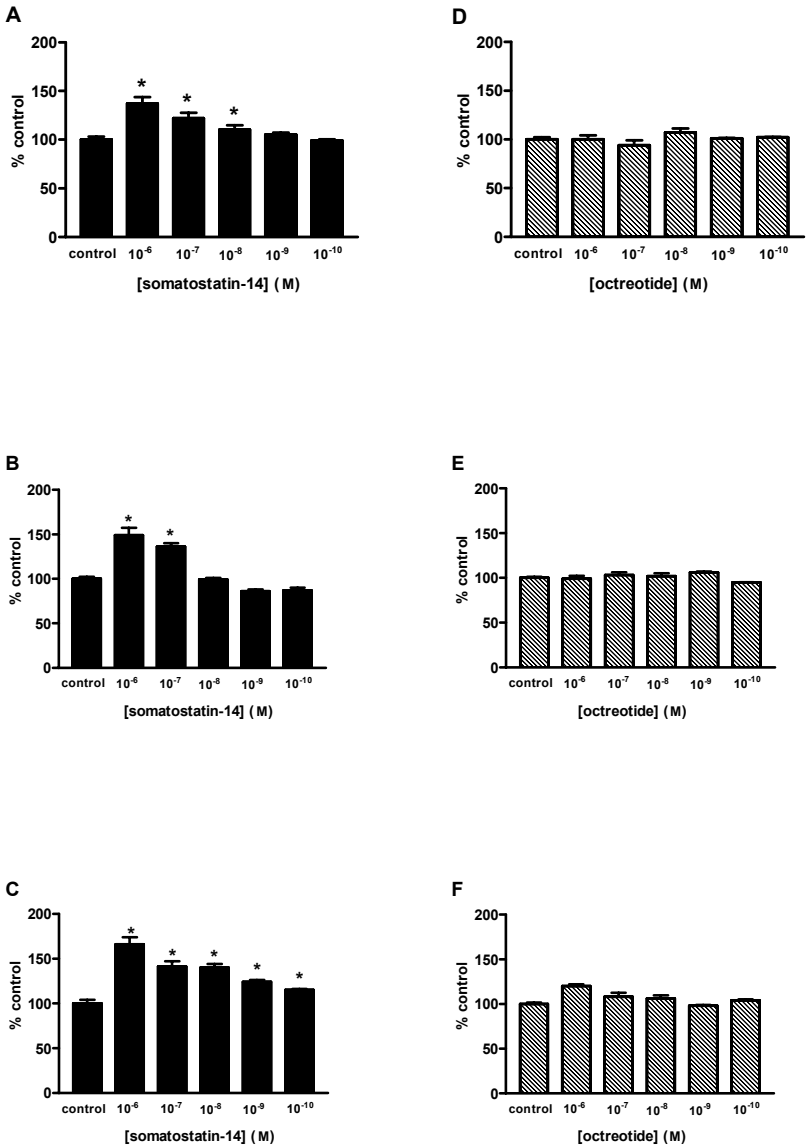

Figure 7: Effects of somatostatin (***A-C***) and octreotide (***D-F***) on apoptosis in early thymocyte cultures (whole population). Thymocytes were incubated during 24 h without or with the drugs indicated at the concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M. Values are absorbance units and are expressed as percent of the control (n=3, triplicate determination on cells obtained from nos. 11-13, Table 1).
*, $p<0.05$ *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 thymic macrophages (22,23). 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 (24,25). Among the soluble factors, neuropeptides have been demonstrated to be 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 neuropeptide with a wide spectrum of actions (26). The biological effects of SS are mediated *via* five specific, high-affinity, G-protein coupled receptors (5). The presence of the neuropeptide and its receptors has been demonstrated in the human thymus (2,9,27). 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 (9). Sst_2 mRNA has been detected in murine resting thymocytes (11), in contrast with the expression in the rat, where these cells selectively express sst_3 and sst_4 mRNAs (13). 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 (12). It should be mentioned that many extrinsic factors and changes in the microenviromental conditions might regulate the expression of SSR (28,29). SS itself could be involved in the regulation of receptor expression (30). This might explain why, although we found SS-binding sites on freshly isolated thymocytes (10), in long-term cultured thymocytes SSR mRNA was lost (9).

In the present study, using freshly isolated thymocytes, we firstly demonstrated specific [^{125}I-Tyr^{11}]-SS-14 binding on thymocyte membrane homogenates. The number of SS-binding sites was very low on these thymic cells, which are a heterogeneous population mainly formed by intermediate/mature thymocytes (16,21). Subsequently, we characterized the SSR subtype expression by RT-PCR. In the whole population of 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, confirming our previous findings (9). 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 expressed in the diverse stages of maturation. We separated the whole thymocyte population 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 (17). Interestingly, sst_3 mRNA has been found constitutively

expressed in peripheral T lymphocytes, which directly derive from mature thymocytes (14). Conversely, a predominant expression of sst_{2A} mRNA was found in the $CD2^+CD3^-$ thymocytes, which are the immature fraction. The $CD2^+CD3^-$ thymocytes form in the developed thymus a very small but highly heterogeneous pool of cells, while the $CD3^+$ intermediate/mature cells represent the major proportion of thymocytes (31). Most of these cells are destined to die as a consequence of failing selection (32). 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 (32). The sst_3 expressed on these cells might be involved in SS-mediated apoptosis (33). In fact, we have found that SS-14, but not octreotide which has a lower affinity for sst_3, may increase the amount of apoptotic cells when incubated in human thymocytes cultures, as whole population. Moreover, preliminary data indicated that the number of apoptotic cells is significantly higher in the intermediate/mature $CD3^+$ fraction compared to the $CD2^+CD3^-$ one, when tested separately (Ferone D, et al., unpublished observations). It is intriguing that another synthetic SS analogue has been found to induce apoptosis in cultured human lymphocytes as well (15). Moreover, it has been recently shown that octreotide has a modulatory effect on anti-CD3 and dexamethasone-induced apoptosis of murine thymocytes (34). However, 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. Furthermore, the data presented in this study demonstrate that both SSR subtypes on human thymocyte subsets may be activated upon binding with their own ligands. In fact, after the administration of SS-14 we found an inhibition of ^{3}H-Thymidine incorporation in all the early cultures of either whole population or isolated $CD2^+CD3^-$ and $CD3^+$ thymocytes fractions. Conversely, the inhibition of ^{3}H-Thymidine incorporation by the SS analog octreotide occurred in only one out of the five cultures of whole thymocyte population, and only at high concentrations. On the contrary, octreotide administered in the $CD2^+CD3^-$ isolated thymocytes induced a significant inhibition of ^{3}H-Thymidine incorporation in a dose dependent manner, whereas this SS analog was ineffective in cultures of $CD3^+$ isolated thymocyte fractions. This evidence is in line with the higher affinity of this SS analog for sst_{2A} compared with that for sst_3 (5). The possibility that at high concentration, octreotide may act *via* the sst_3 can not be fully ruled out. On the other hand, emerging data pointed to the role of either sst_{2A} or sst_3 in promoting apoptosis by p53-dependent and –independent mechanisms, respectively (35). However, the intracellular pathways mediating the SS-dependent activities regulating cell growth, proliferation and death might be still partially inactive in the immature thymocyte fraction.

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 (36-38). SSR are widely distributed within the human thymus on the different cell subsets forming this organ. The stromal compartment preferentially express sst_1 and sst_{2A} mRNA, whereas lymphoid cells express mainly sst_3 and, with lesser extend, sst_{2A} mRNA. Preliminary observations seem to confirm this evidence at protein level. In fact, using polyclonal antibodies we have

recently studied SSR subtype expression by immunohistochemistry in the normal as well as neoplastic thymic tissues, where this pattern of receptor distribution is basically maintained (39). However, in thymic tumor, sst_{2A} immunoreactivity has been found on the endothelium of intratumoral vessel as well, whereas sst_3 immunoreactivity has been clearly observed on normal reactive thymocytes, but also on some tumor cells (40,41). These data support the evidence of strong compartmentalization of neuroendocrine peptide receptors in lymphoid tissue (2), as it has also been shown for the expression of vasoactive intestinal peptide (VIP) receptors on murine and rat thymocytes (42). 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* (42).

We have previously demonstrated that SS mRNA is present in the human thymus in TEC (9), while, as it is shown in the present study, SS mRNA was undetectable in thymocytes. However, preliminary data from our group have shown that the SS-like peptide cortistatin-17 is highly expressed in human lymphoid cells, including thymocytes (43). These evidences suggest that SS produced by a subset of TEC, but perhaps endogenous cortistatin produced by thymocytes as well, may affect thymic cells populations in a paracrine and/or autocrine manner. Therefore, SS and SS-like peptides may participate in regulating T-cell differentiation and selection in the thymus.

In conclusion, the heterogeneous expression of SSR in different cell subsets within the human thymus together with the endogenous production of SS, SS-like peptides, as well as of other neurohormones, emphasizes once more the pivotal role played by neuropeptide hormones in this organ. 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 (9), 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 (44). 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, which are well recognized factors participating at the sophisticated and elegant process leading to the maturation of cellular immunity (18,45). In this light SS represents an important molecule involved in the chain of events, resulting in the generation of T cell repertoire.

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III.1b

EFFECTS OF CORTISTATIN ON FRESHLY ISOLATED HUMAN THYMOCYTES

Introduction

In the human thymus, immature thymocytes undergo maturation into specific T-lymphocyte subsets (1,2). Selection of thymocytes by regulation of cell death, called programmed cell death or apoptosis, plays an important role in thymocyte development (3). It is hypothesized that neuropeptides play a regulatory role in these complex processes as well (4). Previously, it was demonstrated that thymocytes express somatostatin receptor subtype 2 (sst_2) and sst_3 mRNA (5), and the sst subtype expression in thymocytes seemed to follow a developmental pathway (6). It has been shown that sst_3 is involved in apoptosis (7) and, thus, sst_3 may play a regulatory role in thymocyte selection and development. In the previous paragraph, we showed that SS significantly decreased [methyl-^{3}H]-thymidine incorporation in whole-population thymocytes. The SS analogue octreotide, which preferentially binds to sst_2, on the other hand, did not decrease [methyl-^{3}H]-thymidine incorporation in these whole-cell populations, suggesting that the effect of decreased thymocyte proliferation was mediated via sst_3 in these cells. Indeed, SS induced an increase in the number of apoptotic thymocytes (6).
Previously, we demonstrated that another SS-like peptide, named cortistatin (CST), was expressed in cells and tissues of the human immune system (chapter II.1). CST was found to be expressed in human thymic tissues, both in thymic epithelial cells (TEC) and isolated thymocytes. Moreover, SS expression was found only in TEC, but not in thymocytes. CST binds with high affinity to all sst (8) and, therefore, it was hypothesized that CST as well may play a regulatory role in thymocyte development, probably via interaction with sst_3. Until now, no specific receptor for CST has been detected. In the central nervous system differential effects of SS and CST have been demonstrated (9), suggesting that both compounds act through their own specific receptors. In the present study, we compared the effects of SS and CST on cell proliferation of whole-population thymocytes, in order to evaluate whether there is a comparable effect of SS on human thymocytes as compared with CST. In addition, SS binding sites on human thymocytes were characterized by displacement studies using SS and CST.

Material and Methods

Isolation of human thymocytes
Human thymic tissues (n=5) were removed from 5 patients (ranging in age from 1 to 7 months) to allow adequate exposure of the heart during cardiovascular surgery. The protocols were in accordance with the Helsinki Doctrine on Human Experimentation. Informed consent was obtained. Thymocytes were isolated using a filter chamber (NPBI, Emmer-Compascuum, The Netherlands) and collected 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), as described in detail previously (6). Cell viability was determined before each study and was more than 95%.

Functional experiments
Cells were seeded at a density of 2 to 5 x 10^6 cells/ ml/ well in 24 wells plates (Costar Corning bv, Schiphol, The Netherlands). Cells were incubated for 24 hours without or with different concentrations of SS or CST.
In all experiments SS-14 (Bachem Inc., Hannover, Germany) and CST-17 (Phoenix Pharmaceuticals, USA), dissolved in the culture medium (RPMI-1640 supplemented with 10% heat-inactivated FCS, penicillin and fungizone), 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 then 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).

Sst membrane binding studies
Binding studies were performed as described previously (10). The radioligand used was the [^{125}I-Tyr^{11}]somatostatin, kindly provided by Dr. Wout Breeman (Department of Nuclear Medicine, Erasmus MC, The Netherlands). Briefly, membrane preparations (corresponding to 15-30 μg protein) of freshly dispersed human thymocytes were incubated in a total volume of 100 μl at room temperature for 90 min with 30,000 to 50,000 cpm radioligand and increasing concentrations of unlabeled SS or CST in HEPES-buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.2 g/l bacitracin, pH=7.6) containing 0.2% BSA (Sigma Aldrich bv, Zwijndrecht, The Netherlands). After the incubation, 1 ml ice-cold HEPES buffer was added to the assay 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 with ice-old HEPES-buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be the total binding minus binding in the presence of 10 μm unlabeled SS and CST.

Statistical analysis
Data are expressed as mean ± SD. 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.
Calculations of IC_{50} values for displacement of [^{125}I-Tyr^{11}]somatostatin were made uding GraphPad Prism (San Diego, CA, USA).

Results

Figure 1 shows that both SS and CST induced a significant and dose-dependent inhibition of cell proliferation as measured by [methyl-^{3}H]-thymidine incorporation.

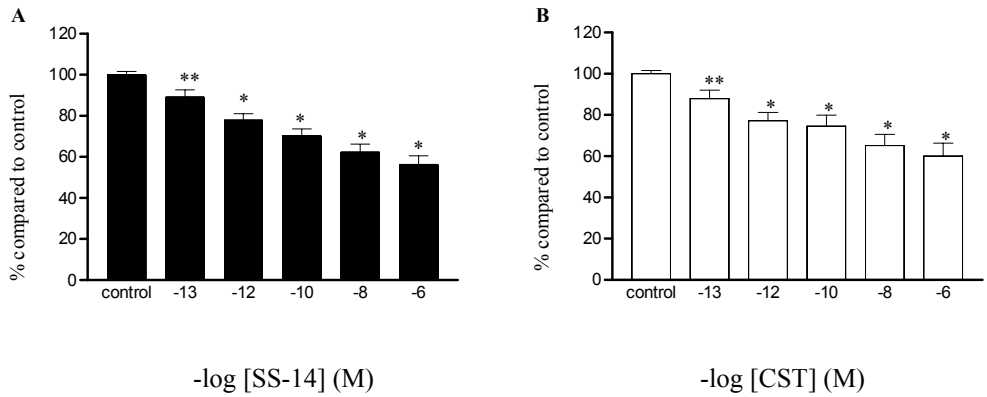

Figure 1: Effects of somatostatin (A) and cortistatin (B) on [^{3}H-methyl] thymidine incorporation in thymocyte cultures from 5 different human thymuses (whole population). Thymocytes were incubated in RPMI-1640 supplemented with 10% heat-inactivated FCS, penicillin and fungizone during 24 h in quadruplicate without or with the drugs indicated at the concentrations of 10^{-13}, 10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M. Values were measured in counts per minute and control values were set at 100% (mean±sd). The results represent the mean of 5 independent experiments and are expressed as percentage compared to control.
*, $p<0.01$ *vs*. control**;** **, $p<0.05$ *vs*. control. SS-14 ■ and cortistatin □ .

The IC_{50} values of inhibition of [methyl-^{3}H]-thymidine incorporation were 1.4 x 10^{-10} M and 0.9 x 10^{-10} M by CST and SS, respectively. In addition, also the maximal inhibitory effects induced by both compounds were comparable and amounted 41 and 44 % respectively, for CST and SS.

Figure 2 shows displacement of [^{125}I-Tyr^{11}]somatostatin-14 binding to membrane preparations of whole thymic tissues and freshly dispersed thymocytes. IC_{50} values for displacement of [^{125}I-Tyr^{11}]somatostatin-14 binding amounted 2 x 10^{-11} and 8 x 10^{-10} M respectively, for CST and SS in whole thymic tissue membranes and 2 x 10^{-10} and 7 x 10^{-12} M, respectively, on isolated thymocytes, showing that both SS and CST bound to sst in human thymic tissues and isolated thymocytes with affinities in the low-nanomolar range.

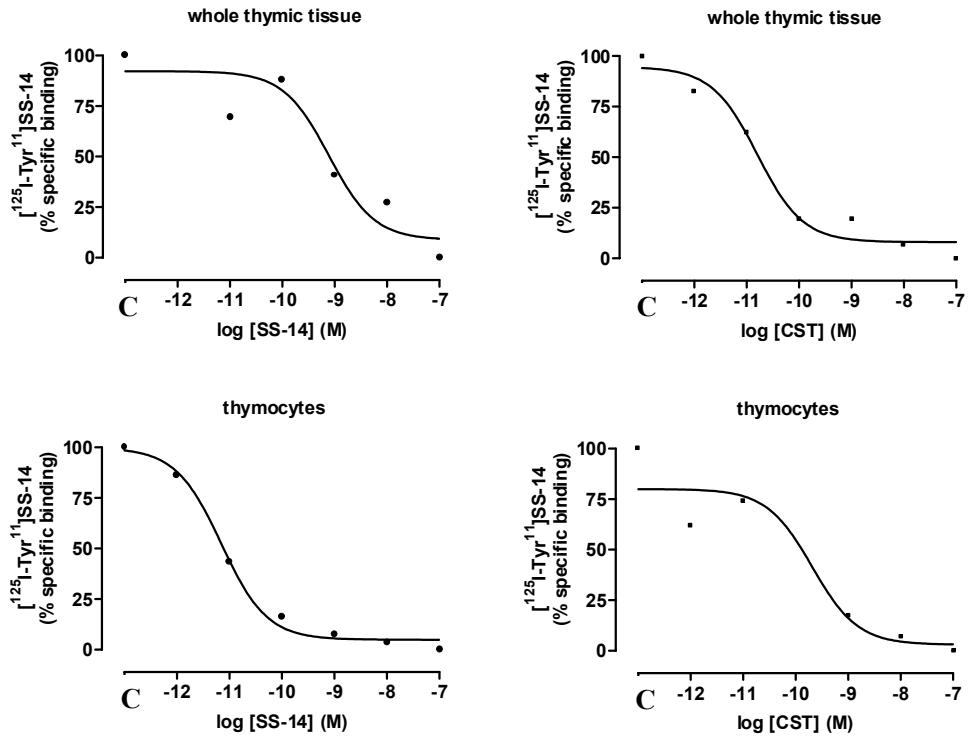

Figure 2: Displacement of binding of [^{125}I-Tyr^{11}]somatostatin-14 to membrane preparations of 2 whole thymic tissues (upper panel) and of their isolated whole populations thymocytes (lower panel).

Discussion

In the human thymus, thymocytes undergo maturation into T-lymphocytes (1,2,11). This maturation is under control of many different factors and neuropeptides may be involved in thymocyte maturation as well (4). Previously, the expression of sst on human thymocytes has been demonstrated (5), which suggests that their ligand, SS, may play a regulatory role on thymocyte development. In chapter III.1a we demonstrated that SS significantly decreased the [methyl-^{3}H]-thymidine incorporation and induced apoptosis in human thymocytes, suggesting a regulatory role for SS and its receptors on selection processes of thymocytes. However, in the human thymus, SS is not the only sst ligand that is expressed. We detected the expression of a novel SS-like peptide, CST, in cells and tissues of the human immune system (chapter II.1) and CST was found to be expressed in the human thymus in isolated TEC as well as in thymocytes. Therefore, it may be hypothesized that in the human thymus, CST may play a role in thymocyte development as well, probably integrated with the effects of SS, as they can both bind with high affinity to sst_2 and sst_3, which are the sst expressed on human thymocytes (6). In the present study, we investigated whether CST exerts effects on thymocytes. In line with our previous study using SS, we investigated whether CST negatively controlled the cell proliferation in whole-cell populations of isolated thymocytes as well. We found that CST, comparable to SS, decreased the [methyl-^{3}H]-thymidine incorporation in a dose-dependent manner. These findings suggest that CST as well may play a role in the selection of thymocytes during their development, as described in the previous paragraph for SS. Looking at the effects of both SS and CST we conclude that both peptides exert comparable effects. In addition, both SS and CST displaced binding of radiolabeled SS in the low nano-molar range. This suggests that SS and CST both exert their effects through the sst, rather than CST exerting its effects through its specific receptor in the inhibition of proliferation. However, the existence and expression of a distinct CST receptor may not be ruled out on basis of our findings, as we have only investigated one aspect of the potential actions of CST on human thymocytes. A CST receptor may play a more important role in other functions as well, i.e. secretion and/or migration processes. Already back in 1989, the expression of SS was demonstrated in the human thymus, and it was hypothesized that SS plays a role in T-lymphocyte development (12). In the murine immune system it has indeed recently been demonstrated that SS, via sst_2, is involved in T-lymphocyte development (13). SS inhibited thymocyte proliferation, elicited chemotactic responses on thymocytes and could thus play an important role in thymocyte development (13). Migration of T-lymphocytes in the thymus during development is essential for maturation. By this mechanism, T-lymphocytes interact with different stromal cells, from which they can receive signals for survival, differentiation or apoptosis (14). As we described the expression of both SS and CST, their actions on T-lymphocyte development may well be integrated in these processes. In conclusion, we described for the first time a functional effect of CST on proliferation of normal human immune cells. The effects of CST on thymocyte proliferation are comparable with the effects of SS, which suggests that SS and CST, both exerting their effects via the sst, may be involved in the generation of the T-cell repertoire during development.

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III.2

cDNA MICRO-ARRAY ANALYSIS OF GENE EXPRESSION CHANGES INDUCED BY OCTREOTIDE IN HUMAN MACROPHAGES

Introduction

Macrophages play an important regulatory role in the human immune system in host defense. They can directly phagocytose and destroy foreign material, like microorganisms (1,2), but they can also act as antigen-presenting cells to other immune cells, like T-lymphocytes, to activate the adaptive immune system (3). In addition to their antigen-presenting capacity, macrophages are major regulators of immune system activity by secreting over a hundred biologically active molecules, including cytokines and growth factors (4). Moreover, macrophages can respond to cytokines and growth factors themselves, via specific receptors for these molecules (4). These features make macrophages important regulatory cells in the human immune system. In physiological state the secretion of both pro-inflammatory and anti-inflammatory molecules by macrophages is balanced. When macrophages become activated, they can play a protective role by phagocytosing microorganisms or activating other immune cells to eradicate the invading agents. On the other hand, macrophages are known to play a central role in the pathogenesis of different immune diseases, like sarcoidosis and rheumatoid arthritis, in which the responses of macrophages to as yet poorly understood factors stimulate different cascades which will result in tissue destruction (5,6). Therefore, it is of major importance that the activity of macrophages is strictly regulated, as their disturbed activity may result in severe disease. Many different factors are involved in controlling macrophage activity (7). In recent years a large number of studies have focussed on neuroendocrine-immune interactions and it has been demonstrated that neuropeptides play regulatory roles in the human immune system (8-10). Different neuropeptides have been described to influence macrophage function (9). Recent studies showed that macrophages express the receptors for somatostatin (SS) (11), a 14 amino acid neuropeptide. Of the 5 known somatostatin receptor (sst) subtypes, macrophages selectively express sst_2 (11). The expression of these receptors suggests that SS may have a regulatory role on macrophage function, both in physiological, as well as in pathophysiological state. A preliminary study demonstrated that treatment of patients suffering from rheumatoid arthritis with the SS analogue octreotide showed clinical improvement (12). SS and its analogues may, therefore, play a role in the treatment of immune mediated diseases. However, the exact functional role of SS and sst on human macrophages is largely unknown. In order to get more insight into the potential role of SS and its receptors in human macrophages, we performed micro-array analysis of changes in gene expression induced by octreotide treatment of cultured peripheral blood derived human macrophages. Octreotide is a sst_2 preferring SS analogue (13). Macrophages were incubated with or without octreotide for 6 hours, after which the differences in expression levels of over 44,000 genes were evaluated. In this way the effects of octreotide on sst_2 expression in 6-day macrophages were investigated.

Material and Methods

Isolation of blood mononuclear cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin Blood bank, Rotterdam, The Netherlands) by Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation. Subsequently, monocytes were isolated from PBMC using a Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation step as described in detail previously (14). Isolated monocytes were frozen in 10% DMSO-medium and stored in -80° C until use.

Cell-culture
A frozen suspension of monocytes was rapidly thawed in a water-bath at 37° C and viability was evaluated by Trypan-blue exclusion (Life Technologies, Grand Island, U.S.A.). Cell viability always exceeded 95%. Cells were seeded at a density of 0.5 x 10^6 cells/cm^2 in a volume of 1 ml/well in 24 well Nuncleon plates (Nalge Nunc International, Denmark) in RPMI1640+ supplemented with 10% heat-inactivated (30 min 56˚ C) fetal calf serum (FCS, Life Technologies, Grand Island, U.S.A.), L-glutamine (2 mM, Life Technologies) and penicillin (1000 U/ml, Yamanouchi Pharma BV Leiderdorp, The Netherlands). Plates were then incubated for 90 minutes at 37˚ C to allow adherence of the monocytes to the plate. Thereafter, plates were washed to remove potential contaminating T- and B-cells. To generate macrophages, fresh medium was added, containing granulocyte macrophage-colony stimulating factor (GM-CSF, 500 U/ml, Novartis Pharma BV, Arnhem, The Netherlands). The cells were cultured for 6 days, with medium refreshment at day 3. This method of the generation of macrophages has been described previously in detail (15).
6 day macrophages were incubated with or without octreotide (10^{-8} M) for 24 hours. 4 experiments, using 4 independent donors, were performed. After the 24 hour incubation, cells were collected by scraping and obtained cell pellets were stored at –80° C until further use.

RNA-isolation and micro-array
RNA was isolated of the different cell pellets using a Total RNA Isolation Kit (Qiagen) according to manufacturers protocol. The RNA concentration was determined by spectroscopy, and the quality was confirmed by gel electrophoresis.

cRNA preparation and array hybridization
Total RNA was converted into double-stranded cDNA using a custom kit (SuperScript II Double-Stranded cDNA Synthesis Kit, Life Technologies, Gaithersburg, MD, USA). After ethanol extraction, in vitro transcription reactions were performed (BioArray HighYield RNA Transcript Labeling Kit, Enzo Biochemicals, Inc., Farmingdale, NY, USA) according to manufacturer's protocol. Purified, labeled cRNA was quantified by spectophotometric analysis and fragmented to 30 to 60 base fragments with Tris-acetate (pH 8.1, 40 mM), KOAc (100 mM), and MgOAc (30 mM) in a 20 µl volume heated for 35 min at 94˚ C. Protocols and instrumentation set-ups, including total RNA samples,

hybridization to human U133a/b microarrays, washing, staining and scanning were followed as recommended in the manufacturers technical manual (GeneChip Microarrays, Affymetrix, Santa Clara, CA, USA).

Data analysis
The resultant arrays were scanned in the system's confocal scanner (HP GeneArray Scanner; Hewlett Packard, Palo Alto, CA, USA). Data analysis was first performed with the software accompanying the microarrays (GeneChip Expression Analysis Software, version 3.3, Affymetrix) to obtain average difference intensities. A change P-value between 0.003 and 0.997 was regarded as significant different expression of a gene.

Results

By the micro-array experiment, the expression levels of over 44,000 genes were determined. In particular, differences in expression levels between the untreated and octreotide-treated macrophages were evaluated. First, expression of cytokines, cytokine receptors and growth factors was evaluated in untreated in vitro cultured macrophages. The results are summarized in table 1.

Table 1: Gene expression profiles of cytokines, cytokine receptors and growth factors in human in vitro cultured 6 day macrophages.

Cytokines	Cytokine receptors		Growth factors
IL-1β	IL-1	IL-13	VEGF
IL-6	IL-2	IL-17	PDGF
IL-7	IL-4	IL-21	IGF-1
IL-8	IL-6	IL-22	
IL-15	IL-7		
IL-16	IL-8		
IL-18	IL-10		

This table represents the expression as detected by micro-array of cytokines, cytokine receptor and growth factor mRNAs in human 6 day macrophages.

As shown in table 1, macrophages only expressed a low number of growth factors. They expressed a significant number of cytokine receptors and cytokines, including the expected cytokines (IL-1β, IL-6 IL-8).

In order to investigate the possible functional significance of SS analogues on macrophages, the cells were treated for 6 hours with the SS analogue octreotide. The significant differences in gene expression levels between control and octreotide-treated macrophages were determined.
In the octreotide-treated macrophages we found 35 genes that were significantly upregulated when compared to untreated macrophages and 39 genes that were downregulated. Of these 74 genes that were regulated, 18 genes are associated to a known function in man and further analyzed. Of the other 56 regulated genes no specific function is currently known. The 18 genes that were further evaluated are presented in table 2, in which their functional significance, as known until now, is indicated as well.

As indicated in table 2 (next page), octreotide has no direct influence on the expression of any of the cytokines, cytokine receptors and growth factors expressed in cultured 6 day macrophages. Mainly gene expression of molecules involved in cell cycle regulation or apoptosis was modified by octreotide treatment. Of particular interest is the induction of the expression of chemokine receptor CCR6, which is involved in chemotaxis and anti-pathogen response.

Table 2: Changes in gene expression in macrophages induced by treatment with the sst_2-preferring SS analogue octreotide.

Gene encoding:	Fold induction	↑/↓	Functional significance
Arginase type II	3.5	↑	nitric oxide synthesis/ arginine catabolism
Ankyrin	3.9	↑	male gonad development
Cold autoinflammatory syndrome 1	3.1	↑	encoding a protein causing familial cold autoinflammatory syndrome and Muckle-Wells disease
TATA box binding protein (TBP)-associated factor	3.0	↑	RNA polymerase I and II transcription factor
Cystein-rich hydrophobic domain	2.0	↑	gene translocated in leukemia
Tp53TG3 protein	2.5	↑	growth suppressing
Serine/threonine kinase	2.0	↑	cell stress
Protection of telomeres 1	1.8	↑	protection of telomeres and chromosome ends
APR1-protein	1.7	↑	cell death/apoptosis
Chemokine (C-C motif) receptor 6 (CCR6)	1.3	↑	immune response, chemotaxis
Cullin 2	1.3	↑	induction of apoptosis, cell cycle arrest, negative control of cell proliferation
Synuclein A	1.0	↑	anti-apoptotic
Zinc finger protein 192	-3.6	↓	zinc finger protein plays a role in hetero and homo-dimerization
Solute carrier family 26	-1.7	↓	chloride/sulfate/iodide transporter
Cell division cycle 2-like 1	-1.3	↓	control of cell proliferation
Thioredoxin reductase 2	-1.2	↓	catalyzes reduction of physiologically important substrates, stimulates tumour proliferation & phenotype change of the tumour
Apolipoprotein B mRNA editing enzyme	-1.1	↓	new gene expressed in hematopoietic stem and progenitor cells from patients with Myelodysplastic Syndromes
Thyroid hormone receptor-associated protein	-1.0	↓	transcription co-activator

This table summarizes the cDNA expression of genes with a known function that are upregulated (↑) or downregulated (↓) in octreotide treated macrophages, when compared to untreated macrophages. The number in column "fold-induction" represents the percentage x 100 of up- or downregulation, when compared to expression levels under basal conditions. Upregulation of 1.0 indicates that expression levels of the gene in octreotide-stimulated macrophages are upregulated 100%, thus doubled, when compared to unstimulated macrophages.

Discussion

Macrophages are the key modulators of the human immune response. They not only phagocytose invading microorganisms by themselves (1,2), but they can also present antigens to other immune cells, regulating the eradication of invading agents (16,17). The immune-regulatory functions of macrophages are under tight control of many factors (3). In recent years, much focus has been addressed to the interplay between the neuroendocrine and immune systems (8-10). The expression and functional significance of various neuropeptides and their receptors has been evaluated in different cells of the human immune system (8-10). Sst have been detected on macrophages, the sst expression is upregulated upon activation (11), suggesting that SS may have a regulatory role on macrophage function (11). As SS is known for its anti-secretory effects in endocrine systems (18-21), it may be hypothesized that SS controls macrophage function by regulating the secretion of various molecules. In pathophysiology, SS may regulate macrophage function by inhibiting the over-secretion of pro-inflammatory molecules, in this way protecting the organism for an overly vigorous immune reaction, which may destroy the host (22). SS is also described to have apoptotic effects, either mediated via sst_3 (23) or sst_2 (24). In this way SS may regulate the number, and thus activity, of pro-inflammatory cells.

In order to further elucidate the effects of SS on human macrophages, we evaluated by a micro-array experiment gene expression levels in macrophages incubated with or without the sst_2 preferring SS analogue octreotide. After an incubation of 6 hours with octreotide, gene expression levels were determined of over 44,000 genes. First, we determined the expression of cytokines, cytokine receptors and growth factors in the untreated macrophages. The expression pattern of cytokines and growth factors was in accordance with reports in literature, i.e. macrophages expressing IL-1β, IL-6, IL-8, IL-15, IL-16 and IL-18 (4). Although IL-10 and IL-12 also have been described to be expressed in human macrophages, we could not detect their presence in our in vitro cultured macrophages. The macrophages we studied only expressed genes of a low number of growth factors, i.e. IGF-1, VEGF and PDGF. It may be hypothesized that expression of other growth factors, like EGF and FGF may only be detected in macrophages that are in activated state. Octreotide did not have any direct effects on the expression levels of these growth factors, which suggests that in basal conditions the expression of these factors is not under SS control. However, it would be of interest to investigate the effects of SS analogues on gene expression levels of these factors in macrophages in activated state, i.e. stimulated with LPS or IFN-γ. Firstly, because sst_2 are expressed at higher levels when macrophages are activated (11) and secondly, because the expression of cytokines and growth factors may be upregulated in activated state as well (25). In a preliminary study, successful treatment of rheumatoid arthritis with octreotide has been described (12). We hypothesize that a possible target for octreotide may have been the regulation of secretion of pro-inflammatory cytokines or growth factors by macrophages in the affected joints.

In our micro-array experiment, expression levels of only 74 genes were either up- or downregulated when macrophages were incubated with octreotide. Of these 74 genes, 18 genes were further investigated, because of the other genes no functional significance is

currently known. However, when the function of these genes will be elucidated in the future, these genes may play an important role in macrophage function as well.
Of the 18 different genes that were regulated, from 4 of them it is not known which role they can play in macrophage function, and, in addition, what the significance of their regulation by octreotide is.
Ankyrin, for instance, is involved in male gonad development (26). Apolipoprotein B mRNA editing enzyme is a recently detected gene of which the function is still unknown, but is found to be expressed in hematopoietic stem and progenitor cells from patients with myelodysplastic syndromes. It was also demonstrated in normal peripheral leukocytes (27). The 2 other genes with unknown functional significance in human macrophages are the cold autoinflammatory syndrome 1 gene (28) and the cysteine-rich hydrophobic domain, which is translocated in leukemia (29).
The other 14 genes that we found to be up- or downregulated upon stimulation with octreotide can be summarized as genes involved in cell-proliferation and apoptosis.
Tp53TG3 protein is protein that is involved in suppression of cell growth (30), serine/threonine kinase (31), cullin2 (32) and APR-1 (33) are genes that are involved in the induction of apoptosis. The cell division cycle 2-like 1, which is downregulated, is known for its function in the control of cell-proliferation (34). Its downregulation may be involved in the induction of apoptosis as well. The regulation (either up or down) of these 5 genes all point to a potential role of sst_2 in inducing apoptosis or in the inhibition of cell proliferation. Previous studies have demonstrated apoptotic effects of SS analogues, exerting their effects via sst_2 (24). These effects were described on transfected pancreatic carcinoma cell lines. Our findings may suggest that sst are involved in the regulation of apoptosis of unstimulated macrophages as well. These findings seem in agreement with the observation that SS induces apoptosis in peritoneal macrophages via activation of p53, Bcl-2, Fas and caspase-8, and via downregulation of inducible nitric oxide synthetase (iNOS) expression and NO production (35). In this respect, SS may be involved in monitoring the number of macrophages, thus preventing an overly vigorous reaction of the immune system.
Thioredoxin reductase 2 stimulates tumor cell proliferation (36). Octreotide downregulated the expression of this gene, which may implicate that octreotide has an anti-proliferative effect as well. Interestingly, the regulation of synuclein A (37) and arginase type II (38) gene expression point to a proliferation-stimulating and anti-apoptotic effect of octreotide, which seems contradictory to the above mentioned effects. The significance of these contrasting findings remains unknown. Moreover, these findings should be validated by quantitative PCR, in order to elucidate the true significance of these findings.
The POT1 gene was upregulated as well. This gene is known for its telomere-protecting properties (39). The function in macrophages is unknown, as macrophages normally do not proliferate. The zinc finger protein is down regulated, when macrophages are incubated with octreotide. Zinc finger proteins are involved in transcription processes. They potentially may regulate transcription of different factors during macrophage development (40).
Finally, the regulation of the chemokine receptor 6 (CCR6) is discussed. CCR6 is

involved in chemotaxis and anti-pathogen response. CCR6 is important for B-lineage maturation, antigen-driven B-cell differentation and regulation of the migration of dendritic cells and T-cells during inflammatory responses (41,42). The cloning and characterization of the dendritic cell CCR6 suggests a role for chemokines in the control of the migration of dendritic cells and the regulation of dendritic cell function in immunity and infection (42). Octreotide upregulated the expresion of the CCR6 gene in human macrophages. Therefore, it may be hypothesized that SS or its analogues play a role in chemotaxis of macrophages, for instance in immune response, towards the site of infection. In this respect, it is of interest that recent studies demonstrated that SS can elicit chemotactic responses on thymocytes as well (43) and that octreotide was found to stimulate migration of primitive hematopoietic progenitor cells (44).

The function of a high number of genes that changed their expression under octreotide is unknown at present. However, knowledge about these genes will be available in the near future and will give more insight in their regulation in relation with sst_2 activation.

Summarizing our findings, it seems that the effects of SS analogues on unstimulated macrophages are bi-directional. It may either repress the immune cell function, but on the other hand, it may also increase the efficiency of the immune reaction. However, further detailed studies using quantitative PCR methods are required to confirm the microarray data and elucidate the exact role of SS analogues on macrophage function. Moreover, it will be of major interest to evaluate the role of SS analogues on activated macrophages, especially with regard to the production of cytokines and growth factors. It should, however, be mentioned that SS analogues may influence the production and secretion of different molecules (45), rather than influencing the gene expression.

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III.3

MACROPHAGE-FIBROBLAST INTERACTIONS : AN IN VITRO MODEL FOR SARCOIDOSIS AND THERAPEUTICAL IMPLICATIONS FOR SOMATOSTATIN ANALOGUES ?

Abstract

Sarcoidosis is an immune-mediated disease of unknown etiology. Fibrosis in the lung is a severe complication, which results in increased morbidity and mortality. Macrophages, which are involved in the development of sarcoidosis, secrete many cytokines and growth factors, which may be involved in the stimulation of fibroblast proliferation, in this way stimulating fibrosis. Because of the increased morbidity and mortality, prevention of fibrosis would be of major importance. As macrophages are involved and recent studies have demonstrated the expression of somatostatin receptors (sst) on human macrophages, a role for somatostatin (SS) or its analogues in the treatment of sarcoidosis may be proposed. Previously, it has been demonstrated that SS may inhibit the secretion of pro-inflammatory growth factors and cytokines. SS and its analogues may, therefore, have a role in the inhibition of factors by macrophages that stimulate fibroblast proliferation.
In order to evaluate the effects of macrophages on fibroblast proliferation and to study whether SS analogues may influence the activity of macrophages in this respect, we performed experiments in which human peripheral blood derived macrophages (either stimulated or unstimulated) incubated without or with SS, cortistatin (CST) and octreotide, -or their conditioned media-, were co-cultured with human fetal lung fibroblasts (HLF1). Proliferation of the cultured fibroblasts was measured by determining the DNA content or by [methyl-^{3}H]-thymidine incorporation.
We found that SS analogues significantly inhibited macrophage- induced fibroblast proliferation. However, we were not able to identify the factors, secreted by human macrophages, that were responsible for the stimulatory effect on fibroblast proliferation. Nevertheless, our findings may provide a basis for therapy of sarcoidosis using SS analogues, thereby lowering or preventing fibrosis. Future studies are required to determine the factors that are involved.

Introduction

Sarcoidosis is a granulomatous disease of unknown etiology that affects multiple organs. The disease most commonly affects the lungs, lymph nodes, skin, eyes, spleen, bone, and glandular tissue (1). Sarcoidosis is histologically characterized by the presence of non-caseating granulomas. Macrophages and T-lymphocytes form a major cellular component of these granulomas (2). The T-lymphocyte population contains both CD4+ and CD8+ cells and intracellular cytokine staining profiles demonstrated a T_H1 (interferon-γ (IFN-γ)) predominant cytokine pattern (3). It has been demonstrated that activated T cells in the lung secrete interleukin (IL)-2 (4). This activation is associated with activation of macrophages and IFN-γ production (5) and may lead to granuloma formation. In more than 60 % of the patients the granulomas resolve in 2-5 years but aggregated granulomas may also result in pseudotumour lesions and they may become enclosed by fibrous tissue. Fibrosis may seriously impair lung function and is related to poor prognosis with increased morbidity and mortality (6). Therefore, it would be of great importance if fibrosis in sarcoidosis could be prevented or at least decreased. In recent years many studies have been performed in order to find satisfying therapeutical tools for the system treatment of sarcoidosis. Different approaches have been used. Effects of corticosteroids have been studied (7-9), however, showing only modest efficacy. T lymphocytes and macrophages, being the cells predominantly present in sarcoid granulomas, have also been objects of study (10-14). However, until now, no conclusion can be made with respect to successful treatment of sarcoidosis. Recent studies have demonstrated that macrophages express somatostatin receptors (sst). From the 5 known sst subtypes, sst_2 is the subtype selectively expressed on these cells (15). Immunohistochemistry studies demonstrated that macrophages in sarcoid granulomas indeed expressed sst_2 (16). It is known that somatostatin (SS) can play a regulatory role in the inhibition of hormone secretion and sst_2 is associated with this inhibitory role of SS (17,18). Moreover, macrophages can secrete growth factors, like FGF (19), EGF (20), IGF-1 (21) and cytokines like, IL-β (22), IL-6 (23) and TNF-α (24). It is known that some of these factors can stimulate fibroblast proliferation (24-26). Therefore, it would be of interest to investigate the role of SS or its analogues, like octreotide or the recently discovered SS-like peptide cortistatin (CST) (27), on the secretion of the above mentioned growth factors and cytokines by human macrophages and to evaluate the role of macrophages in the control of fibroblast proliferation. The results of such experiments may provide a potential basis for the treatment of sarcoidosis with SS analogues. Therefore, we investigated in the present study the effects of SS, octreotide and CST on cytokine and growth factor secretion by in vitro cultured macrophages. In order to investigate the effects of macrophages on fibroblast proliferation, and the possible inhibitory effects of SS or its analogues, macrophages or their conditioned media were incubated with fibroblasts.

Material and Methods

Fibroblast cultures
Human fetal lung fibroblasts (HFL1) were purchased from ATCC and cultured in F-12K Medium (Kaign's Modification, Life Technologies, Grand Island, U.S.A.) supplemented with 10% fetal calf serum (FCS, Life Technologies), L-glutamine (2 mM, Life Technologies) and streptomycin. Fibroblasts were kept in culture during approximately 12 weeks in 75 cm^2 culture flasks (Costar Corning bv, Schiphol, The Netherlands). For experiments, fibroblasts were seeded at a density of 15.000 cells/cm^2 in a volume of 1 ml/well in 24 well Costar plates (Corning Costar) for 24 hours to allow adherence. Thereafter, medium was renewed and fibroblasts were incubated with interleukin-6 (IL-6, 10 ng/ml), insulin-like growth factor (IGF-1, 10^{-8} M), tumor necrosis factor α (TNF-α, 10 n/ml), fibroblast growth factor (FGF, 10 ng/ml) or epidermal growth factor (EGF, 10 ng/ml) for 72 hours, in order to evaluate the effects of these compounds on fibroblast proliferation. After 24 hours DNA content was measured. Also the effects of LPS, IFN-γ and octreotide on fibroblast proliferation were investigated, in order to rule out that the effects of macrophages activated by these compounds were caused by a direct effect of these compounds on fibroblast proliferation, rather than exerting their effect via macrophages.

DNA content
Medium was removed from the wells and cells were incubated with 150 µl lysis buffer (7.5 ml 13.4 N NH_3 + 2 ml 10% triton X-100 + 90.5 ml H_2O) for 10 minutes at 4 ° C. Thereafter, cells were sonified using a Soniprep 150 (amplitude=14000 micron, 5 seconds). Plates were incubated at 4 ° C for 10 minutes again. 1 ml assay buffer (1 M NaCl, 0.1 M EDTA and 0.1 M Tris in H_2O) is added to each well to neutralize the lysis buffer. 20 µl of the standards (prepared from 5 mg calfs thymus DNA in 100 ml assaybuffer = 50 µg/ml), in concentrations varying between 312.5 to 5000 ng/ml, and 20 µl of the samples were transferred to a microtitre plate (Costar Special Optics 96-well plates) and 200 µl color reagent (1 µg Hoechst 33258/ 10 ml H_2O) was added to each well. Plates were analyzed using a Victor^2 1420 multilabel counter (Wallac, Turku, Finland) at extinctions of 340 and 460 nm. Measured values were calculated relative to the standard curve, corrected for dilution and are presented as ng DNA/well of culture plate.

Isolation of blood mononuclear cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Sanquin Blood bank, Rotterdam, The Netherlands) by Ficoll (Pharmacia, Uppsala, Sweden; density 1.077 g/ml) gradient centrifugation. Subsequently, monocytes were isolated from PBMC using a Percoll (Pharmacia; density 1.063 g/ml) density gradient centrifugation as described in detail previously (28).

Macrophage culture
Viability of the isolated monocytes was evaluated by Trypan-blue exclusion (Life Technologies, Grand Island, U.S.A.). Cell viability always exceeded 95%. Cells were

seeded at a density of 0.5 x 10^6 cells/cm^2 in a volume of 1 ml/well in 24 well Nuncleon plates (Nalge Nunc International, Denmark) in RPMI1640+ supplemented with 10% heat-inactivated (30 min 56˚ C) FCS (Life Technologies), L-glutamine (2 mM, Life Technologies) and penicillin (1000 U/ml, Yamanouchi Pharma BV Leiderdorp, The Netherlands). Plates were then incubated for 120 minutes at 37˚ C to allow adherence of the monocytes to the plate. Thereafter, plates were washed to remove potentially contaminating T- and B-cells. To generate macrophages, fresh medium was added, containing granulocyte macrophage-colony stimulating factor (GM-CSF, 500 U/ml, Novartis Pharma BV, Arnhem, The Netherlands). The cells were cultured for 6 days, with a medium refreshment at day 3. This method of generation of macrophages has been described previously in detail (29). After 6 days, macrophages were either collected by scraping or incubated with either lipopolysaccharide (LPS) or interferon-γ (IFN-γ), with or without octreotide for 24 hours.
After 24 hr conditioned media were collected and stored at -80° C until further use.
Macrophages were also collected for quantitative PCR analysis. In 24 hour LPS- and IFN-γ activated macrophages we evaluated the expression of sst_2 mRNA by quantitative PCR as described previously in detail (15).

Growth factor and cytokine production
In vitro cultured macrophages were incubated with 2 μg/ml LPS for 24 or 48 hours, with or without SS, octreotide and CST (10^{-8} M) in order to investigate the effects of cell activation and incubation with SS-like compounds on growth factor and cytokine production. Macrophages were also stimulated with IFN-γ (10 ng/mL) with or without SS, octreotide and CST. After 24 and 48 hours media were collected and stored at –80° C until further use. ELISA-assays were performed for IL-1β, IL-6, TNF-α, EGF and FGF using LPS-stimulated macrophages and for EGF and FGF using IFN-γ activated macrophages according to manufacturer's protocol. ELISA Kits were purchased from Roche Diagnostics.

Co-culturing
To evaluate the direct effects of macrophages on fibroblast proliferation, fibroblasts were incubated with either the macrophages, collected by scraping, or with the conditioned macrophage media.
Incubation with conditioned media: Fibroblasts were seeded at a density of 15.000 cells/ cm^2 in a volume of 1 ml/well in 24 well Costar plates (Corning Costar) for 24 hours to allow adherence. Thereafter, medium was removed and 500 μl macrophage culture medium (see above) was added. To each well, 500 μl of conditioned macrophage medium was added as well, and fibroblasts were cultured for 72 hours in these conditioned media after which the effects on cell proliferation were measured by determining the DNA content, representing the number of cells.
Incubation with macrophages: Fibroblasts were pre-incubated for 24 hours to allow adherence, as described above. Thereafter, medium was removed and fibroblasts were incubated with the scraped macrophages. 250.000 macrophages in 1 ml of macrophage medium were added to each well and cells were co-cultured for 72 hours. After 66 hours, [mathyl-^{3}H]-thymidine was added for 6 hours. Thereafter, [methyl-^{3}H]-thymidine

incorporation was measured as described below. As a control, [methyl-^{3}H]-thymidine incorporation was also measured in cultured macrophages.

[methyl-^{3}H]thymidine incorporation
In the 72 hour [methyl-^{3}H]-thymidine incorporation experiments, cells were incubated after 66 hours with 1 µCi [methyl-^{3}H]-thymidine (91 Ci/mmol, Amersham) for 6 hours. Thereafter, the cell suspension was transferred to 5 ml tubes and precipitated with 10 % trichloroacetic acid (TCA), and the pellet was washed once again in TCA. 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, USA).

Results

Fibroblasts were incubated with TNF-α, IL-6, IGF-1, EGF and FGF in order to investigate the effects of these compounds on fibroblast proliferation. DNA content was measured after 72 hours of incubation and results are shown in figure 1A. FGF, IGF-1, EGF and TNF-α significantly stimulated fibroblast proliferation. IL-6 did not have statistically significant effects on cell proliferation. Secondly, in order to investigate the direct effects of LPS, IFN-γ and octreotide on fibroblast proliferation, these cells were incubated for 72 hours with these three compounds. LPS, IFN-γ and octreotide did not have statistically significant effects on fibroblast proliferation (figure 1B).

Because macrophages were incubated with SS analogues in the absence or presence of LPS or IFN-γ, we investigated the expression levels of sst_2 mRNA after activation with both compounds. LPS induced an approximately 150-fold ($p<0.0001$) increase in sst_2 expression in activated macrophages. On the other hand, stimulation of macrophages with IFN-γ resulted in an approximately 25-fold increase in sst_2 mRNA expression (data not shown, see chapter II.4). In addition, we evaluated the expression of sst subtype mRNAs in fibroblasts and found that sst_1 mRNA was selectively expressed. No other sst subtype mRNAs were found (data not shown). In order to evaluate the potential production of factors by macrophages, which interact with fibroblast activity, fibroblasts were incubated for 72 hours with different conditioned macrophage media. Incubation of fibroblasts with media of unstimulated 6-day macrophages resulted in an approximately 2-fold induction of fibroblast proliferation. When macrophages were incubated for 24 hours with LPS, these conditioned media further stimulated proliferation of fibroblasts approximately 2.5-fold (figure 2A). Conditioned media of macrophages incubated with IFN-γ, did not significantly upregulate fibroblast proliferation when compared to unstimulated macrophage conditioned medium (figure 2B).

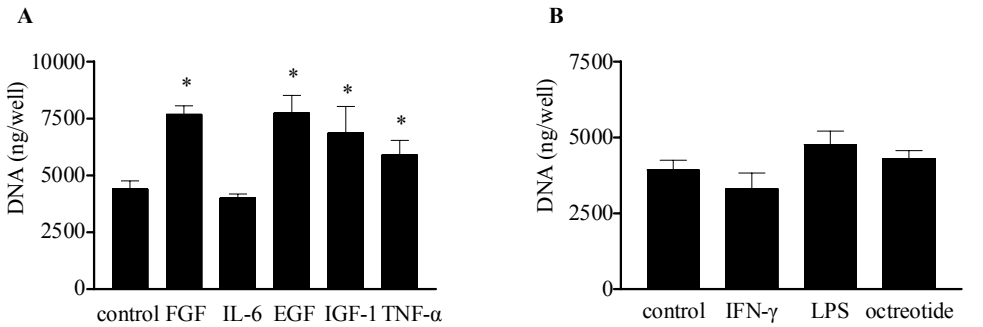

Figure 1: Effects of different compounds on fibroblast (HFL1) proliferation
A: Effect of different groeth factors and cytokines on fibroblast proliferation.15.000 fibroblasts were seeded per well and incubated for 72 hours with the different compounds. FGF= fibroblast growth factor, 10 ng/ml, IL-6 = interleukin-6 10 ng/ml, EGF= epidermal growth factor 10 ng/ml, IGF-1 = insulin-like growth factor 1 10^{-8} M and TNF-α = tumor necrosis factor α 10 ng/ml. After 72 hours, DNA content of the different wells was measured. Bars represent the mean of 3 independent experiments. * $P < 0.01$ when compared to control.
B: Effect of LPS, IFN-γ and octreotide on fibroblast proliferation. The effects of lipopolysaccharide (LPS, 2μg/ml), interferon γ (IFN-γ, 10 ng/ml) and octreotide (10^{-8} M) were evaluated in 72 hour cultures of 15.000 fibroblasts per well. Bars represent the mean of 2 independent experiments.

In order to investigate the potential effects of SS analogues on the fibroblast stimulatory effects of macrophages, fibroblasts were incubated with conditioned media of octreotide-treated macrophages. The results of these experiments are shown in table 1. Compared with macrophage medium alone, octreotide did not significantly influence macrophage induced proliferation of fibroblasts. Moreover, IFN-γ induced fibroblast proliferation was not influenced by octreotide as well. On the other hand, conditioned media from octreotide-treated, LPS-activated macrophages showed a statistically lower activation of fibroblast proliferation (table 1).

Table 1: The effects of octreotide on macrophage-induced fibroblast proliferation

	without octreotide	with octreotide (10nM)	P-value
Macrophages	100 ± 13	94 ± 22	0.3
Macrophages + IFN	100 ± 12	103 ± 23	0.8
Macrophages + LPS	100 ± 6	89 ± 13	0.046

This table represents the percentage of proliferation of fibroblasts after incubation with octreotide, when compared to control (set at 100 %). Proliferation induced by macrophages, macrophages + IFN-γ or macrophages + LPS was set at 100 % and the effect of octreotide is presented as percentage to these controls. Results represent the mean of 4 (macrophages), 3 (macrophages + IFN-γ) and 5 (macrophages + LPS) experiments respectively and are presented as mean percentage ± standard deviation.

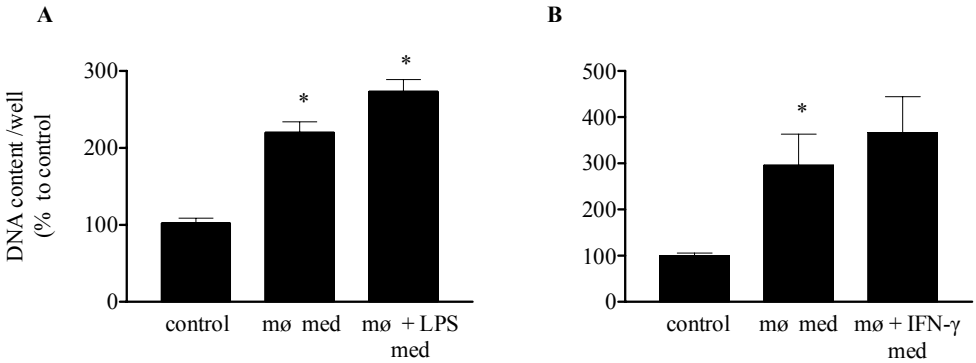

Figure 2: Effects of macrophages on fibroblast proliferation. This figure represents the effects of unstimulated, LPS-stimulated (A) and IFN-γ stimulated (B) macrophages on fibroblast proliferation. 6 day macrophages were cultured with or without LPS or IFN-γ. Culture media were collected after 24 hours, fibroblasts were incubated for 72 hours with the culture media and thereafter the DNA content of the wells was measured. Bars represent the mean of 5 experiments and are shown as the percentage compared to control cells. * $P < 0.01$ compared to control.

In order to get a better insight into the direct effects of unstimulated or activated macrophages on fibroblast proliferation in vitro, fibroblasts and macrophages were co-cultured. Because we found that octreotide, LPS and IFN-γ did not have significant direct effects on fibroblast proliferation, fibroblasts were cultured together with macrophages, which were then incubated with the different compounds. In these experiments we also used the natural ligand SS and the SS-like peptide cortistatin (CST). We found that octreotide, SS and CST in these experiments significantly inhibited the macrophage-induced proliferation of fibroblasts (figure 3A). Octreotide and SS also significantly inhibited fibroblast proliferation that was induced by LPS-stimulated macrophages (figure 3B). The increase in [methyl-^{3}H]-thymidine incorporation was due to increase of fibroblast proliferation, because macrophages alone showed no [methyl-^{3}H]-thymidine incorporation (data not shown). The inhibition found in these experiments was more profound then in the experiments using conditioned media, especially with respect to inhibition of proliferation induced by basal macrophages.

In order to investigate the potential factors that are responsible for the stimulation of fibroblast proliferation by (activated) macrophages, we evaluated by ELISA the effects of SS, CST and octreotide on secretion of IL-1β, IL-6, TNF-α, FGF and EGF by basal, LPS- or IFN-γ activated macrophages. LPS significantly upregulated the secretion of IL-1β, IL-6 and TNF-α. However, SS, octreotide and CST did not have any inhibitory (or stimulatory) effect on LPS-stimulated secretion (data not shown). SS, octreotide and CST also had no effects on basal secretion of these factors by macrophages. No significant effects of LPS or IFN-γ were found on EGF, IGF-1 and FGF production. SS and its

analogues did not show consistent effects on the secretion of IGF-1, EGF and FGF (not shown).

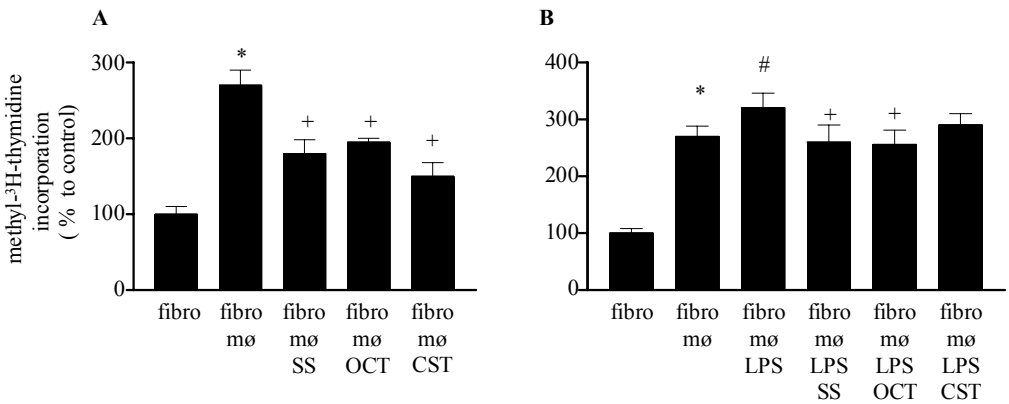

Figure 3: Co-culture experiments of fibroblasts and macrophages. Fibroblasts and 6 day macrophages were co-cultured in the same wells. The cells were either unstimulated (A) or stimulated with lipopolysaccharide (LPS, 2 µg/ml, B) and incubated with somatostatin (SS, 10^{-8} M), octreotide (OCT, 10^{-8} M) or cortistatin (CST, 10^{-8} M) for 72 hours. After 66 hours 1 µCi [methyl-^{3}H]-thymidine was added to the wells for 6 hours. Thereafter [methyl-^{3}H]-thymidine incorporation was measured, representing proliferation activity of the fibroblasts. Bars represent the mean of 2 different experiments and are presented as percentage of [methyl-^{3}H]-thymidine incorporation in the different samples compared to [methyl-^{3}H]-thymidine incorporation in control fibroblasts. * $P < 0.001$ compared to control fibroblasts, $^{\#}$ $P < 0.01$ compared to fibroblasts incubated with macrophages and $^{+}$ $P < 0.01$ compared to fibroblasts incubated with macrophages (A) or fibroblast incubated with macrophages plus LPS (B).

Discussion

Sarcoidosis is a granulomatous disease, in which immune cells play an important regulatory role (30,31). Especially T-lymphocytes and macrophages seem to be important in the onset and maintenance of granuloma formation. In bronchoalveloar lavage (BAL) fluid, high proportions of CD4-positive lymphocytes have been demonstrated (32). These activated T lymphocytes secrete IL-2 (32) and IL-18 (5), factors that are thought to play a central role in granuloma formation in sarcoidosis. This activation is associated with macrophage activation, IFN-γ production and formation of granulomas (33). Many studies have been performed in order to elucidate the etiology of sarcoidosis. Association with environmental or occupational exposure (34), infectious agents (35) and autoimmunity (36,37) have been hypothesized. However, until now, it is not clear to which extent these factors contribute to the development of sarcoidosis. Although the course of disease is self-limiting in about 60 % of sarcoidosis patients, progressive sarcoidosis may result in fibrosis and fibrosis can seriously impair lung function (6). To prevent this serious complication it is important to find an option for treatment for sarcoidosis. Many studies have already been performed using different strategies to attack fibrosis in sarcoidosis.
Two studies (7-9) showed beneficial effects of treatment with corticosteroids, although the degree of improvement was only modest. The finding that T lymphocytes are involved in the inflammatory process in sarcoidosis has led to studies investigating therapeutical possibilities by targeting these cells, however, with little success. Treatment with cyclosporin resulted in decreased activity of T-lymphocytes, but was not related to clinical improvement (13). Also macrophages are important components of the granulomas formed in sarcoidosis and therefore, these cells have become subjects of study. In particular, TNF-α has been investigated in detail. Alveolar macrophages have been shown to secrete large amounts of TNF-α (38,39) and increased secretion of TNF-α has been demonstrated to be predictive for persistent disease (33). Different groups have evaluated the effects of anti-TNF-α treatment. This treatment was successful in a number of studies. Successful treatment of sarcoidosis with methotrexate or corticosteroids resulted in decreased TNF-α secretion (11). Pentoxyfilline, that blocks TNF-α secretion, was shown to have beneficial effects on sarcoidosis as well (14). However, no control group was studied and pentoxyfylline caused gastrointestinal intolerance as important side effect. Other studies using anti-TNF-α compounds showed moderate to little effects (10,12). Because secretion of TNF-α is associated with persistent disease and TNF-α is secreted by macrophages, macrophages are still a major subject of study. Macrophages are also known to produce other growth factors and cytokines, like EGF, FGF, IGF-1, IL-1β and IL-6 (19-24) that can stimulate proliferation of fibroblasts (24-26).
Regulation of the secretion of these compounds by macrophages may therefore be an alternative pathway in order to decrease fibroblast proliferation and, thus, fibrosis.
Recently, we have demonstrated that macrophages express sst. Sst_2 was found to be the sst subtype selectively expressed on these cells (15). The natural ligand for sst, SS, is known to have an inhibitory effect on secretion processes in many tissues, like pituitary, thyroid gland, pancreas and gastro-intestinal tract (for review: (40)). The SS analogue octreotide is nowadays widely used in the treatment of neuroendocrine tumours (41). The inhibitory

effects of octreotide on secretion processes are predominantly mediated via sst_2 [17, 18]. Because macrophages have been shown to express sst_2, we addressed in the present study the question whether SS analogues may also have a therapeutical implication in sarcoidosis, possibly by inhibiting secretion of factors that stimulate fibroblast proliferation. Therefore, we used a human fetal lung fibroblast cell line. These cells responded with increased proliferation when they were incubated with FGF, EGF, IGF-1 and TNF-α. The most potent stimulatory effects were found for FGF and EGF. Both factors are produced by macrophages (19,20). We found that unstimulated macrophages were able to stimulate proliferation of fibroblasts, most probably by secreting proliferation-stimulating growth factors or cytokines. When macrophages were stimulated with IFN-γ, no significant difference was found in fibroblast stimulation when compared to the unstimulated macrophages. However, LPS-activated macrophages stimulated fibroblast proliferation even more when compared to unstimulated macrophages. This in vitro finding is in concordance with the finding that a persistent activation of macrophages results in fibrosis in sarcoidosis patients in vivo (42). Interestingly, stimulation of macrophages with IFN-γ does not result in a further upregulation of fibroblast proliferation. This implies that IFN-γ and LPS stimulate macrophages in different ways, resulting in different regulatory pathways. Fibroblasts did not respond with increased proliferation to LPS, IFN-γ and octreotide, ruling out direct effects of these compounds, which are present in the conditioned media of cultured macrophages, on fibroblasts. The absence of a direct effect of the sst_2-preferring SS-analogue octreotide on fibroblast proliferation, is in agreement with our observation that fibroblasts selectively expressed sst_1, to which octreotide does not bind (40).

In order to investigate the possible inhibitory effects of SS analogues on secretion of proliferation-stimulating factors by macrophages, macrophages, both unstimulated and stimulated, were incubated with 10^{-8} M octreotide. When fibroblasts were incubated with these conditioned media, we found that octreotide had no significant inhibitory effect on the proliferation of fibroblasts induced by basal macrophages. Octreotide did not have any inhibitory effects on the proliferation induced by IFN-γ stimulated macrophages as well. On the other hand, the proliferation of fibroblasts, which was induced by LPS-stimulated macrophages, was significantly reduced by octreotide, providing evidence for an inhibitory effect of octreotide on secretion of different (unknown) factors by macrophages. The fact, that octreotide had no inhibitory effect on fibroblast proliferation induced by basal and IFN-γ activated macrophages may be explained by the sst_2 expression levels in the different macrophages. Expression levels of sst_2 mRNA are much higher in LPS-activated macrophages, when compared to the IFN-γ activated and basal macrophages. When macrophages and fibroblast were co-cultured a direct cell-cell interaction was possible, we observed that the inhibitory effects of octreotide on macrophage-induced fibroblast proliferation were even more profound. This may be explained by a more direct interaction between the different cell types, resulting in paracrine interactions between both cell types, compared to the effects of fibroblast-conditioned media. Having observed these inhibitory effects of octreotide on macrophage-induced fibroblast proliferation, we evaluated which factors could be responsible for these effects. By ELISA we investigated the secretion of IL-1β, IL-6, TNF-α, IGF-1, EGF and FGF by LPS- and IFN-γ stimulated macrophages. IL-1β, IL-6 and TNF-α secretion could

well be measured. On the other hand, the secretion of IGF-1, EGF and FGF was hardly detectable, also in the media of activated macrophages.
Under our conditions we could not find effects of SS, OCT or CST on the secretion by macrophages of any of the factors investigated. This suggests that the factors we evaluated are not involved in the inhibition of fibroblast proliferation induced by SS analogues under the culture conditions used. Moreover, it should be taken into account that the macrophages we used, which are PBMC-derived macrophages of healthy donors, may differ from sarcoid macrophages. It is suggested that other factors may play a role in the regulatory process of SS analogues on macrophages as well. Platelet derived growth factor (PDGF) (43) or transforming growth factor-β (TGF-β) (44) are factors that can also stimulate fibroblast proliferation, and possibly, these factors may be influenced by SS analogues. The role of these factors should be further evaluated.
On the other hand, also factors, that are unknown until now, can play a role in the mechanisms of SS on macrophage-induced fibroblast proliferation. Further studies are required to determine these possible new factors. In sarcoidosis, both T-lymphocytes and macrophages play important regulatory roles (2). Previously, we demonstrated that T-lymphocytes express sst_3 (Chapter II.3), whereas macrophages selectively expressed sst_2 (Chapter II.4). Recently, a SS analogue showing high binding affinity to $sst_{1,2,3\ and\ 5}$ has been introduced, named SOM230 (45). It may be hypothesized that this compound may have therapeutical implications in the treatment of sarcoidosis as it can bind to both T-lymphocytes and macrophages and probably inhibit the secretion of factors that stimulate fibroblast proliferation and/or cell proliferation.
In conclusion, we found in an oversimplified in vitro model for sarcoidosis, that macrophages stimulate the proliferation of human lung fibroblasts, probably by secreting proliferation-stimulating growth factors. Conditioned media of macrophages significantly stimulated fibroblast proliferation, but in experiments co-culturing macrophages and fibroblasts the stimulatory effect of macrophages was even larger, probably due to a more direct interaction in these co-cultures. It may be hypothesized that different intracellular signals in macrophages or direct macrophage-fibroblast interactions play a role in these more profound effects in direct cell-cell interactions. When macrophages are stimulated with LPS, they are capable of stimulating proliferation even more, in contrast to IFN-γ activated macrophages. Treatment of activated macrophages with SS analogues decreased proliferation of fibroblasts slightly, but statistically significant. This may be explained by the inhibitory effect of SS-analogues on the secretion of currently unknown pro-inflammatory cytokines or growth factors. PDGF and TGF-beta may be candidates but also other factors may be involved. The finding, that SS-analogues can decrease macrophage-induced fibroblast proliferation may be very interesting with regard to their potential future use in treatment of sarcoidosis. Further studies should be performed in order to evaluate the potential beneficial effects of these compounds in sarcoidosis.

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CHAPTER IV

THE SIGNIFICANCE OF SOMATOSTATIN RECEPTOR EXPRESSION IN DIAGNOSIS AND THERAPY OF DISEASES ORIGINATING FROM THE HUMAN IMMUNE SYSTEM

IV.1

THE ROLE OF OCTREOTIDE SCINTIGRAPHY IN NON-HODGKIN'S AND HODGKIN'S LYMPHOMAS, RHEUMATOID ARTHRITIS AND SARCOIDOSIS

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Abstract

Somatostatin receptors are widely expressed on cells and tissues throughout the human body. Apart from their expression in the physiological target organs of the peptide, somatostatin receptors are also expressed in various tumours. The expression of somatostatin receptors on neuroendocrine tumours led to the development of somatostatin receptor scintigraphy using [^{111}In-DTPA-D-Phe^{1}]-octreotide (^{111}In-pentetreotide) in order to visualize somatostatin receptor positive tumours and their metastases in vivo. Previous studies reported the expression of somatostatin receptors in both normal and pathological cells and tissues of the human immune system as well. Somatostatin receptors have been demonstrated in Hodgkin's disease, malignant lymphomas, sarcoidosis and rheumatoid arthritis. In this paper we discuss the (possible) role of somatostatin receptor scintigraphy in diagnosis, staging or follow-up of patients suffering from these diseases.

Somatostatin and its receptors

Somatostatin (SS) is a neuropeptide, which was first discovered by Brazeau et al. (1) as a growth hormone release inhibiting factor. SS was shown to exist as two different polypeptides, consisting of 14 or 28 amino acids (SS-14 and SS-28, respectively). SS is widely distributed throughout the human body. The central nervous system, gastro-intestinal tract and endocrine glands are the major sites of production (2,3). Throughout the human body, SS has a predominantly inhibitory action, especially with regard to the release of mediators, such as hormones (4,5). SS acts via the family of G-protein-coupled seven transmembrane receptors of which, to date five different subtypes have been cloned, sst_{1-5} (6). Both natural ligands, SS-14 and SS-28 show high binding affinity to all five receptor subtypes (6). Because of the inhibitory effect of SS on hormone production, it was suggested that SS could play a role in therapy of diseases caused by overproduction of hormones by endocrine tumours (7). Because SS itself has a very short half-life, it turned out to be unsuitable for use in treatment (8). D amino acids have been introduced to decrease enzymatic degradation. The SS-analogue octreotide, which is an eight amino acid peptide, is now widely used in the treatment of endocrine active tumours, for instance growth hormone-producing pituitary adenomas and gastroenteropancreatic tumours (9). The positive effects of octreotide in these diseases –inhibition of hormone overproduction- is likely to be mediated via the somatostatin receptors, which have been demonstrated on most endocrine active tumours (9).

Somatostatin receptor scintigraphy

Following the detection of sst expression on neuroendocrine tumours, in 1987 peptide receptor scintigraphy was introduced for in vivo visualization of these sst-positive tumours in patients (10,11). After intravenous injection of radionuclide-labeled SS analogues, primary tumours and metastases could be visualized by this scintigraphy

technique. At first [^{123}I-Tyr^3]-octreotide was used for scintigraphy. However, the use of this compound was shown to have several drawbacks for its use in sst scintigraphy (12). Some of its major drawbacks were the short half-life, rapid clearance from the blood, which results in a short effective residence time for the accumulation in sst positive tumour sites, and the accumulation of radioactivity in the intestines, due to the clearance of [^{123}I-Tyr^3]-octreotide via the liver and biliary system. The accumulation in the intestines makes the interpretation of planar- and single-photon emission computed tomography (SPECT) images difficult. [^{111}In-DTPA-D-Phe^1]-octreotide (^{111}In-pentetreotide) is a radiopharmaceutical that also binds to $sst_{2,3,5}$ specifically (13). This compound has a longer half-life and a lower target/backgound ratio, because of its renal excretion. 90 % of the dosage injected can be found in urinal excretion after 24 hours. Because of the longer half-life and its renal clearance, ^{111}In-pentetreotide can be used to visualize sst-positive tumours efficiently at 24 and 48 hours after injection, when the interfering background radioactivity is minimized by renal clearance (11,13,14). These features of ^{111}In-pentetreotide make it now widely used in clinics for visualization of neuroendocrine tumours (15-20). Both planar and SPECT images are preferentially obtained 24 hours after injection of a dose of approximately 200-250 MBq of ^{111}In-pentetreotide (16,21). It has been demonstrated that sst are also expressed in normal spleen (22,23) and bone marrow (24). Therefore these organs can be a target for ^{111}In-pentetreotide when injected in the patient. Octreotide scintigraphy labeled the spleen and this labeling can be reduced in patients pre-treated with unlabeled octreotide (16,25). It should be taken into consideration that the radioactivity can cause damage in these sst-positive organs, which play an important role in hematopoiesis. From birth, hematopoiesis in humans is almost exclusively situated in the bone marrow (26). Bone marrow and kidneys are the dose-limiting organs because of their uptake of radioactivity. In previous studies depression of bone-marrow function has been described after treatment with high doses of ^{111}In-pentetreotide, leading to necessary blood transfusions (27).
Apart from the visualization of sst-positive neuroendocrine tumours, several other tumour tissues have been successfully visualized by sst scintigraphy as well, like thyroid cancers (16,28,29), breast cancer (16,30) and small cell lung cancer (16,31-33). In the present paper we will focus on the role of sst scintigraphy of malignancies of the immune system and inflammatory diseases in which the human immune system is involved.

Somatostatin receptors in normal lymphoid cells and tissues

Back in 1981, the group of Bhathena et al. first identified expression of sst on human mononuclear leukocytes (34). They found low-affinity binding sites on resting monocytes and lymphocytes. Subsequently, using fluorescent SS, sst were identified on mitogen-activated human peripheral lymphocytes (35). Resting peripheral blood lymphocytes, granulocytes and red blood cells did not express sst. Various cell lines of different origin (T cell, B cell, myeloma and leukemic origin) have been shown to express sst (36,37) as well. Interestingly, expression of sst mRNA in normal peripheral blood mononuclear cells was very low compared to expression of sst mRNA in peripheral blood mononuclear cells from leukaemia patients or in these cells after stimulation with PHA (37). The sst expression pattern in human lymphoid cells is summarized in table 1.

By in vitro receptor autoradiography using [^{125}I-Tyr^{3}]-octreotide, SS binding sites have been demonstrated in lymphoid organs in human. Human gut-associated lymphoid tissues (palatine tonsils, ileal Peyer's patches, vermiform appendix, and colonic solitary lymphatic follicles) (46), thymic tissue and spleen (23,25) clearly showed sst expression.

Table 1: Expression of somatostatin receptor subtypes in cells from the human immune system, including human leukemic cell lines as demonstrated by RT-PCR, immunohistochemistry and FACS analysis.

Cell	Sst1	Sst2a	Sst3	Sst4	Sst5
CD 34+ stem cells	-	+	-	-	-
Thymocytes	-	-	+	-	-
T lymphocytes	-	-	+	-	-
B lymphocytes	-	-	+	-	-
T lymphoid cell lines	-	+	+	-	-
B lymphoid cell lines	-	+	+	-	-
Monocytes	-	-	-	-	-
Macrophages	-	+	-	-	-
Dendritic cells	-	+	-	-	-
Myeloid leukemia	-	+	-	-	-
Malignant lymphoma	-	+	+	-	-

Based on (38-45). Expression of the different somatostatin receptor subtypes is indicated with +. Absence is indicated by -.

Octreotide scintigraphy in diseases affecting the human immune system

Apart form their expression in neuroendocrine and previously mentioned tumours, sst are also expressed on cells derived from several hematological malignancies (35,36,47,48) and inflammatory diseases affecting the human immune system, like sarcoidosis (39, 49) and rheumatoid arthritis (40,50,51). Therefore the question is addressed whether sst scintigraphy may also play a role in visualization of sst-positive sites of disease in these patients for diagnosis, staging or treatment follow-up purposes.

Octreotide scintigraphy in non-Hodgkin's Lymphomas and Hodgkin's disease

Somatostatin receptors have been demonstrated in both Hodgkin's and non-Hodgkin's lymphomas (52-54). In vitro autoradiography studies have demonstrated that sst are mainly expressed in the lymphoblastic areas of the lymphomas, which are the most active parts of these tumours. In non-Hodgkin's lymphomas, sst were detected in vitro in 92% of

low-grade malignancies, 100% of intermediate-grade malignancies and 70% of high-grade malignancies. Sst were expressed in about 100% of the Hodgkin's lymphomas (52). Moreover, it has recently been shown that Hodgkin's lymphomas and non-Hodgkin's lymphomas expressed sst_2 and sst_3 mRNA selectively (Chapter IV.2). The expression of sst_2 might be of significant importance in sst scintigraphy because the ^{111}In-pentetreotide shows preferential high binding affinity to the sst_2. Following the in vitro detection of sst in these diseases several sst scintigraphy studies have been performed to evaluate the possible role of this technique in visualization of these lymphomas for diagnosis, staging and follow-up of the disease.
In an initial study 8 non-Hodgkin's (table I) and 2 Hodgkin's (table II) lymphomas were studied by sst scintigraphy and all of the scintigraphic pictures were positive (53). ^{111}In-pentetreotide scintigraphy also detected lesions that had been missed on chest radiographs and CT. An example of sst scintigraphy in a patient with Hodgkin's disease is shown in figure 1. By the use of in vitro receptor autoradiography on tissue samples, which were surgically removed from the patients scanned, it was confirmed that positive sites by scintigraphy did consist of sst-positive lymphoma tissue.

Table 2: sst scintigraphy studies in Hodgkin's disease

First-named author	Reference:	Number of patients	Sensitivity per patient (%)	Sensitivity per site (%)
Van Hagen	53	2	100	100
Goldsmith	55	2	100	100
Bares	56	5	NA	78
Bong	57	9	NA	91
Sarda	58	3	100	67
Lipp	54	11	73	70
Ivancevic	59	6	57	57
Van den Anker-Lugtenburg	60	56	98	NA
Van den Anker-Lugtenburg	61	126	100	94

Sensitivity per patient represents the percentage of patients visualized by sst scintigraphy. Sensitivity per site represents the percentage of tumour sites visualized successfully.
NA= not applicable

Table 3: sst scintigraphy studies in Non-Hodgkin's lymphomas

First-named author	Reference:	Number of patients	Sensitivity per patient (%)	Sensitivity per site (%)
Reubi	52	4	100	NA
Van Hagen	53	8	100	100
Goldsmith	55	5	20	NA
Bares	56	13	NA	63
Bong	57	NA	NA	37
Sarda	58	23	91	57
Lipp	54	23	57	34
Ivancevic	59	26	85	38
Van den Anker-Lugtenburg	62	50	84	60
Van den Anker-Lugtenburg	63	94	90	71

Sensitivity per patient represents the percentage of patients visualized by sst scintigraphy.
Sensitivity per site represents the percentage of tumour sites visualized successfully.
NA= not applicable

The results of 3 studies are not shown in detail here, but summarized in table I and II because of the low number of patients involved (55) or the missing numbers of patient-related sensitivity (56,57). Sarda et al. (58) showed positive sst scintigraphy in 3 out of 3 Hodgkin's diseases and in 91% of 23 non-Hodgkin's lymphomas. In a study by Lipp et al. (54) in a series of 11 Hodgkin's and 23 non-Hodgkin's lymphomas, positive scintigraphy was found in 8 out of 11 (73 %) Hodgkin's and 13/23 (57%) non-Hodgkin's lymphomas and they showed striking differences in sensitivity within different parts of the human body. In general, sensitivity was higher for lesions localized above the diaphragm. The lower sensitivity below the diaphragm may be due to the uptake of ^{111}In-pentetreotide by the liver and spleen, as well as its elimination via the kidneys. These background signals might influence the identification of target sites in the respective area. Also the group of Ivancevic (59) reported a relative low sensitivity (as shown in table I and II) of sst scintigraphy in both Hodgkin's and non-Hodgkin's disease and they concluded that sst scintigraphy is not useful in the staging of malignant lymphomas, because of the low detection rates. In a prospective study by the Rotterdam group (60) a total of 56 previously untreated patients with histologically proven Hodgkin's disease were studied. The results of the sst scintigraphy were compared with physical and radiological examinations. Sst scintigraphy was positive in 55 out of 56 patients (98%). In 20 patients, previously undetected tumour sites were detected by sst scintigraphy. In a larger study by

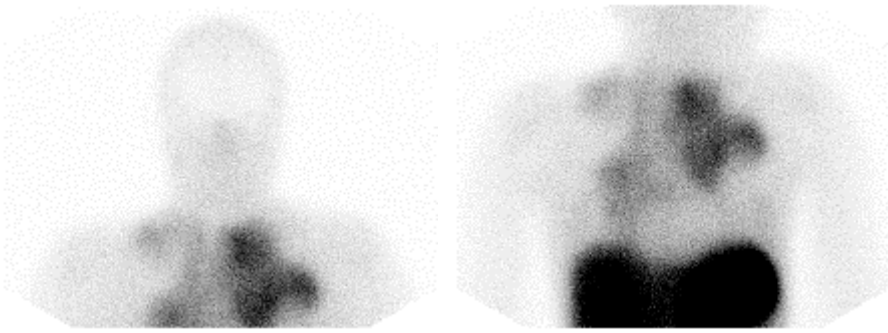

Figure 1: Visualization of multiple somatostatin receptor positive lymph nodes in a patient with Hodgkin's disease (anterior view, scanned 24 h after injection).

the same group (61), which included 126 newly diagnosed patients with Hodgkin's disease, a high patient-related sensitivity was found of 100%, also the lesion-related sensitivity was very high with 94%. In both studies it was shown that sst scintigraphy provided superior results for the detection of supradiaphragmatic Hodgkin's localizations, when compared to CT scanning and ultrasonography. Below the diaphragm sst scintigraphy appeared to be less sensitive, again due to accumulation of the ^{111}In-pentetreotide in liver, spleen, bowel and kidneys. In two other studies by the group of van den Anker-Lugtenburg et al. (62,63) a group of 50 untreated patients with low-grade and a group of 94 untreated patients with intermediate- and high-grade non-Hodgkin's lymphomas were studied. 42 out of 50 (84%) low-grade patients were positive by sst scintigraphy, whereas the lesion-related sensitivity was 60 % (62). 85 out of 94 (90%) patients in the intermediate and high-grade group showed positive scintigraphy (63). In this group lesion-related sensitivity was 71 %. In both groups, in 20% of the patients new lesions were detected by sst scintigraphy. However, lesion-related sensitivity was relatively low. Generally, sensitivity to detect Hodgkin's disease by sst scintigraphy was higher than for non-Hodgkin's lymphomas. In almost all cases of Hodgkin's lymphomas lesion localizations were found by sst scintigraphy, making Hodgkin's disease a possible target for sst scintigraphy in diagnosis and staging. Moreover, it would be of great importance if sst scintigraphy could be used for imaging and evaluation of active sites of disease after treatment of Hodgkin patients.

Octreotide scintigraphy in granulomatous disease

Granulomas can be defined as collections of modified macrophages and lymphocytes as a result of the reaction of the host organism to a variety of agents. Fibroblasts and granulocytes may also be present at sites of granuloma formation. They may occur in mycobacterial, parasitic, fungal or other infectious diseases as a reaction to non-infectious,

poorly digestible agents and in diseases of obscure origin like sarcoidosis and Wegener's disease. Aggregated granulomas often result in pseudotumour lesions and in advanced stages they may become enclosed by fibrous tissue and can eventually be replaced by hyaline scars. In the murine immune system it was shown that Schistosoma mansoni infection-induced granulomas were postive for sst (64-66). Following these findings in mice, sst have been localized by in vitro receptor autoradiography using [^{125}I-Tyr^{3}]octreotide in biopsies of lesions of patients suffering from sarcoidosis (49). Binding of [^{125}I-Tyr^{3}]octreotide was mainly found in the region of epitheloid cells and giant cells. In further detail, sst_2 expression was demonstrated in granulomas formed in sarcoidosis in man (39). The expression of sst_2 was associated with cells of the mononuclear phagocyte lineage, including epitheloid cells and multinucleated giant cells. In a prospective study the value of sst scintigraphy in granulomatous disease was investigated in a group consisting of 13 patients with sarcoidosis, 4 patients with tuberculosis and 3 patients with Wegener's disease (49). In all patients (20/20) uptake of radioactivity was found by sst scintigraphy. Interestingly, patients with Wegener's disease showed lower uptake of ^{111}In-pentetreotide, which was hypothesized to be due to their pre-treatment with corticosteroids, resulting in a reduction in the size of the granulomatous lesion. However, it cannot be ruled out that the lower uptake of ^{111}In-pentetreotide is due to a down-regulation of sst on the cell membranes, rather than a decrease in the number of affected cells as it is known that glucocorticoids are able to downregulate transcription and expression of different genes (67). In vitro receptor autoradiography studies confirmed the expression of sst in the region of epitheloid and giant cells of the granulomas. In a second study known mediastinal and hilar lymph nodes, and interstitial lung diseases were detected by sst scintigraphy in 36 out of 37 (97%) patients (68). Moreover, previously undetected involved sites of inflammation were detected by sst scintigraphy (figure 2).

Patients were treated and thereafter sst scintigraphy was repeated. In patients, that showed radiological improvement, also a decreased uptake of the radiolabeled somatostatin analogue was found, suggesting that sst scintigraphy might play a role in treatment follow-up (figure 3).
However, because of the small group of treated patients in this study, further prospective studies should be performed to address this question. A very recent study compared the use of Gallium (^{67}Ga) scintigraphy, which is widely used in the evaluation of the extent of sarcoidosis (69-77) to sst scintigraphy in 18 patients suffering from sarcoidosis (78). It was shown that in 18 out of 18 (100%) patients sst scintigraphy revealed abnormalities.

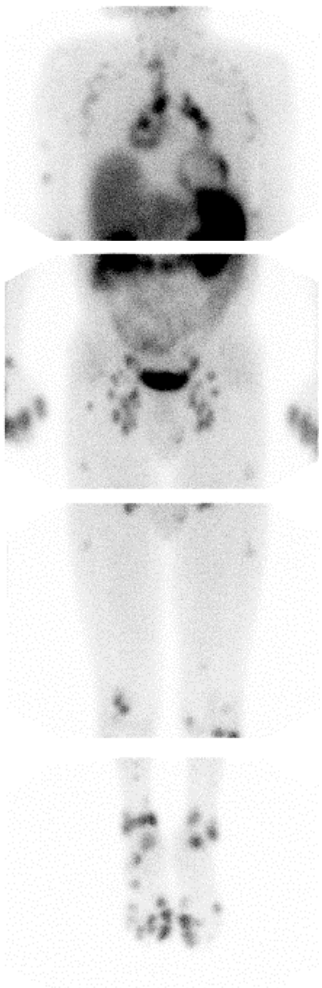

Figure 2: Granuloma localizations in a patient with sarcoidosis (scanned 24 h after injection).
These scans show widely spread localizations of sarcoidosis in lymph nodes in the axillary regions, thorax, mediastinum, iliacal region and also in the lung, skin and joints

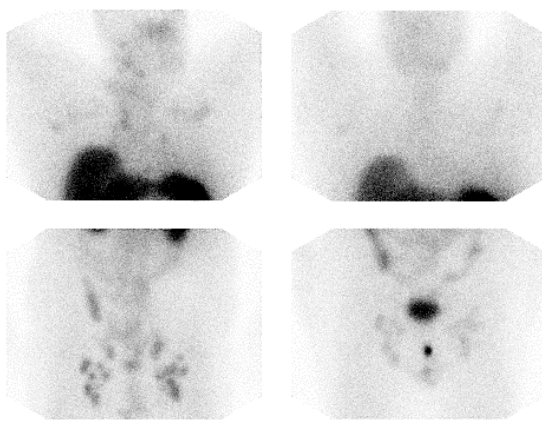

Figure 3: Localizations of sarcoidosis before and after treatment with prednison (scanned 24 h after injection).
The left panel shows localizations before treatment and the right panel shows the remaining localizations after treatment with prednison. A significant difference in uptake after treatment is seen in the head, neck, thorax and iliacal region.

Gallium scintigraphy detected abnormalities in 16/18 (89%) patients. The major drawback in the use of ^{67}Ga scintigraphy is the higher background caused by physiological uptake in the liver, spleen, bone marrow, lacrimal and salivary glands, breast and lung, which makes it more difficult to detect lesions with low uptake. Sst scintigraphy also demonstrated a higher lesion-related sensitivity (83% vs. 65%). In this study 9 treated patients were included and of these, ^{67}Ga scintigraphy found abnormalities in 7 out of 9 (78%) patients, whereas by sst scintigraphy, abnormalities were detected in 9/9 (100%) patients. This report demonstrated that, compared to the ^{67}Ga scintigraphy, sst scintigraphy detected significantly more sites of sarcoidosis involvement. However, it was shown that still 40% of extrathoracic sites were missed by sst scintigraphy. This was due to uptake of the radiolabeled compound in surrounding areas of the lesions, which led to difficulty in analyzing the area of interest. Although sst scintigraphy appears to be more accurate than ^{67}Ga scintigraphy for evaluating the extent of sarcoidosis, additional (larger) studies need to be performed to further evaluate the role of sst scintigraphy in follow-up of treated patients.

Octreotide scintigraphy in rheumatoid arthritis

The most characteristic manifestations of rheumatoid arthritis (RA)- joint pain, joint swelling and reduced mobility- are the result of synovitis, an inflammatory process in the synovial tissue. Joint structure destruction, which results in consequent deformation and loss of function, is caused by this inflammation. Rheumatoid arthritis is characterized by persistent immunological activity, in which diffuse lymphocytic infiltration (predominantly CD4-positive lymphocytes) and/or macrophages are present in most cases. It was hypothesized that these cells, which are in the activated state in synovitis, might express sst, which might allow the detection of the inflammatory joints by sst scintigraphy. In a study by van Hagen et al. (50) sst scintigraphy was studied in a series of 14 consecutive patients with RA. In all patients suffering from RA, uptake of ^{111}In-pentetreotide was observed in a number of joints (figure 4).
The lesion-related sensitivity was 74%. In this study it was supposed that the clinical inflammation activity is represented by pain and swelling of the joints. It was shown that this activity was correlated with the sst scintigraphy results. Recently it was demonstrated by immunohistochemistry that sst_2 was expressed in synovia of affected joints in RA patients. Sst_2 immunoreactivity was found both on the endothelial cells of venules and on cells of the monocyte/macrophage lineage (40). Until now, no other studies have been performed evaluating sst scintigraphy in rheumatoid arthritis. However, this initial study looked very promising and larger studies should be performed to further investigate the role of sst scintigraphy in diagnosis of rheumatoid arthritis. Moreover, Takeba et al. (79) demonstrated that proliferation of synovial cells of patients suffering from rheumatoid arthritis could be inhibited by somatostatin in vitro. In a clinical trial by Paran et al. (80), significant clinical improvement was found in patients with active, refractory rheumatoid arthritis. These results might be the basis for treatment of rheumatoid arthritis with

somatostatin or its analogues. In this respect, the development of a novel somatostatin analogue, the SOM-230, which binds with high affinity to all sst subtypes, except for sst_4 (81,82), is of high interest, as in rheumatoid arthritis expression of $sst_{1,2,3}$ has been demonstrated (83). Refractory rheumatoid arthritis might also be a target for radiotherapy, in which radionuclide-coupled SS analogues are injected in synovia of affected joints. Because of the expression of sst, the radiolabeled SS analogue is internalized in the cells and the radioactivity causes cell death of the infiltrating synoviocytes. This might be particularly important in rheumatoid arthritis patients that do not respond to conventional therapies. The role of radiotherapy is currently under investigation.

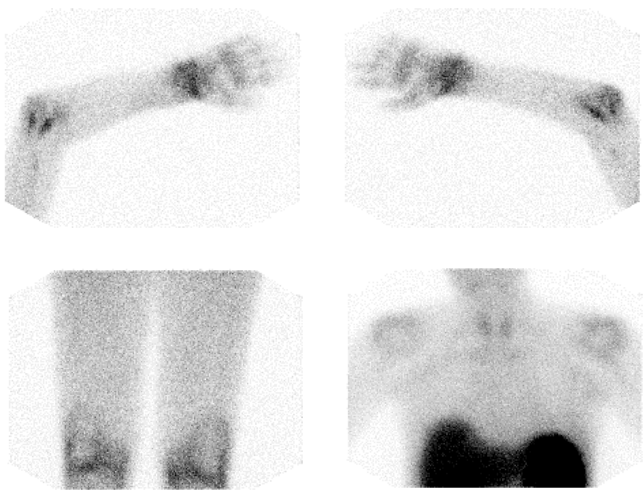

Figure 4: Visualization of the joints of the arms, shoulders and knees in a patient with rheumatoid arthritis (scanned 24 h after injection). Uptake of ^{111}in-pentetreotide is demonstrated symmetrically in the joints of the hands, wrists, elbows, shoulders and knees.

Conclusion

Following the successful visualization of neuroendocrine tumours by sst scintigraphy, different studies, mainly on malignant lymphomas, have addressed the question of whether this technique might play a role in diagnosis, staging and follow-up of patients suffering from diseases originating from cells of the immune system. Although promising results have been reported with regard to the high patient-related sensitivity of sst scintigraphy, the lesion-related sensitivity appears not to be satisfactory in all studies. As for Hodgkin's disease, the best results have been described with regard to sensitivity and detection of previously undetected lesions and therefore it appears that sst scintigraphy can play an important role in staging of this disease. Sensitivity in indolent non-Hodgkin's lymphomas is much lower and compared to CT scanning sst scintigraphy is inferior. Sst scintigraphy in these patients therefore will be clinically of little importance. On the other hand, in aggressive non-Hodgkin's lymphomas, sst scintigraphy detected more lesions when compared to conventional techniques. This can mean that the clinical stage of the disease is upstaged, which can result directly in different treatment approaches. In aggressive non-Hodgkin's lymphomas, sst scintigraphy might play a role. Due to the limited reports on granulomatous diseases and rheumtoid arthristis it is difficult to make a conclusion at this point. For rheumatoid arthritis, further studies should be performed in order to gain more insight in its possible role, as there is until now only one report on the evaluation of sst scintigraphy in this disease. For granulomatous disease, sst scintigraphy might be interesting, as it was shown that it can play a role in treatment follow-up of patients. It was demonstrated that sst scintigraphy followed the results found by conventional techniques after therapy. However, caution should be taken, because we previously showed (chapter II.5), that glucocorticoids can decrease the expression of sst on immune cells, which may influence uptake of the radionuclide labeled SS analogue as well.

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IV.2

SOMATOSTATIN RECEPTORS IN MALIGNANT LYMPHOMAS: TARGETS FOR RADIOTHERAPY?

Submitted

Abstract

Somatostatin (SS) receptor (sst) scintigraphy is widely used in the visualization of neuroendocrine tumours expressing sst and radiotherapy using radionuclide labeled SS analogues has been introduced for treatment of patients with neuroendocrine tumours. Previous sst scintigraphy studies revealed that malignant lymphomas can also be visualized using this technique. The question has been addressed whether lymphomas might also be possible targets for radiotherapy using radionuclide labeled SS analogues. Therefore, we investigated in vitro the characteristics of lymphoma tissues and lymphoid cell lines in order to evaluate whether lymphomas can be targets for radiotherapy.

Six orbital lymphomas, two Hodgkin- and two non-Hodgkin lymphomas from the neck region were collected. RT-PCR and quantitative RT-PCR were performed to detect and quantify the expression of sst_{1-5} mRNA. Receptor autoradiography studies using [^{125}I-Tyr^3]octreotide were performed to evaluate binding to sst to cryostat sections of lymphomas. By immunohistochemistry expression of sst_2 and sst_3 was investigated. Membrane binding studies and in vitro internalization experiments using [^{125}I-Tyr^3]octreotide were performed to study binding and uptake of [^{125}I-Tyr^3]octreotide by lymphoid cell lines (JY, TMM, APD) and primary cells from a B-CLL.

A selective expression of sst_2 and sst_3 mRNA was demonstrated. By quantitative RT-PCR expression levels of sst_2 and sst_3 mRNA were relatively low. Autoradiography studies revealed low binding of [^{125}I-Tyr^3]octreotide, whereas immunoreactivity could not be detected for sst_2 and sst_3 by immunohistochemistry. On the lymphoid cell lines only low numbers of high affinity SS binding sites were found. In vitro uptake of [^{125}I-Tyr^3]octreotide by these cells was very low as well.

Lymphomas appear not to be good candidates for radiotherapy using radionuclide labeled SS analogues. However, lymphomas are highly radiosensitive tumours and further clinical studies should be performed to evaluate whether the low receptor density is sufficient target for treatment in these highly radiosensitive tumours.

Introduction

The actions of somatostatin (SS), a 14 amino-acid neuropeptide, are mediated through G-protein coupled seven-transmembrane receptors, of which, until now, five different subtypes have been cloned, named sst_{1-5} (1). SS has a predominant inhibitory effect throughout the human body, especially with regard to the release of mediators, such as hormones (2,3). Several studies have shown antiproliferative (4) and anti-angiogenetic (5) effects of SS as well. Apart from the physiological expression of sst in normal tissues, sst are upregulated in several human malignancies (6). Sst have been demonstrated in most neuroendocrine neoplasms (7), but also in tumours that originate from the central nervous system, breast and lung (8). Following in vitro detection of sst expression in human tumours, peptide receptor scintigraphy using radionuclide labeled SS analogues was introduced for tumour-imaging. Sst-positive neuroendocrine tumours and their metastases could be visualized by scintigraphy after intravenous administration of radionuclide labeled analogues of SS (9,10).
Following successful imaging of neuroendocrine tumours, it has been previously demonstrated that affected tissues in patients with malignant lymphomas could be visualized in vivo with sst scintigraphy as well (11). However, when compared to neuroendocrine tumours, uptake by lymphomas is around 10-fold lower (12) and differences in sensitivity were reported in several studies (13,14) and it was suggested that a tumour should have a minimum size to allow detection (13). In addition, sensitivity to visualize Hodgkin's lymphomas was substantially higher than that for non-Hodgkin's lymphomas (13,15). On the basis of the visualization of lymphoma tissues by sst scintigraphy, it is hypothesized that β-emitting SS-analogues might play a future role in radiotherapy (16). However, until now, little is known about receptor subtype expression and, even more important, with respect to radiotherapy, receptor-density of the sst in lymphoma tissues. In order to study the receptor density and sst subtype expression in malignant lymphomas, we investigated in the present study the expression of the mRNAs encoding the 5 different sst subtypes in a series of human lymphomas. In a previous study it has been shown that no SS mRNA itself was expressed in normal cells and tissues of the human immune system, however, expression of the mRNA encoding cortistatin (CST) (17), a SS-like peptide, was detected. CST is also capable of binding with high affinity to all 5 sst (18). In spite of a differential effect of CST in the brain, a specific receptor for CST has not been found yet (19). In the present study we investigated the mRNA expression of both SS and CST in lymphomas as well. Previous studies showed that not all lymphomas could be visualized by sst scintigraphy. This could be due to technical imperfections, as in different studies too low dosages of radioactivity were used and in other studies uptake of radioactivity was counted for a very short time period. On the other hand, it was questioned whether this was due to a relatively low receptor-density or to absent or differential sst subtype expression. Therefore, sst mRNA expression levels were measured quantitatively, using quantitative PCR (Q-PCR) and compared to sst mRNA levels in GH-secreting pituitary adenomas, which express high levels of sst mRNA. Receptor-ligand internalization and membrane binding studies were performed with a SS-like radiolabeled compound, [^{125}I-Tyr^{3}]-octreotide, in order to evaluate

internalization of SS-coupled radioactivity by different lymphoid cell-lines as a model for binding and internalization of the receptors expressed in the lymphoma tissues. By autoradiography, specific binding of [^{125}I-Tyr^{3}]-octreotide, which binds with high affinity to sst_2, was evaluated and quantified in a series of lymphoma sections. The expression of sst_2 and sst_3 was studied by immunohistochemistry as well. Finally, in order to exclude possible mutations in the sst_2 receptor, DNA of 4 lymphomas was analyzed by sequencing.

Material and Methods

Tissues Orbital lymphomas (n=6), Hodgkin's lymphomas (n=2) and non-Hodgkin's lymphomas from the neck region (n=2) were collected during diagnostic biopsies and surgical intervention, quickly frozen in liquid nitrogen and stored at -80 °C until use or selected on the presence of archived frozen material. Histopathological classification of the tissues studied is shown in table 1. The tissues were numbered, to assure anonymity of the patients. Informed consent was obtained from all patients involved.

Table 1: Histopathological classification of the different tissues studied and their somatostatin receptor subtype and ligand expression

		sst_1	sst_2	sst_3	sst_4	sst_5	SS	CST	
1	ML-MALT	-	+	+	-	-	-	+	orbital region
2	Follicular center lymphoma	-	+	+	-	-	-	+	
3	Mantle cell lymphoma	-	+	+	-	-	-	+	
4	Diffuse large B-cell lymphoma	-	+	+	-	-	-	+	
5	Mantle cell lymphoma	-	+	+	-	-	-	+	
6	Follicular center lymphoma	-	+	+	-	-	-	+	
7	Hodgkin's stage I	-	+	-	-	-	-	+	neck-region
8	Hodgkin's stage II	-	+	-	-	-	-	+	
9	Low-graded non-Hodgkin's	-	+	-	-	-	-	+	
10	Intermediate graded non-Hodgkin's	-	+	-	-	-	-	+	

Table represents expression of the different somatostatin receptor subtype mRNAs (sst_{1-5}) and their ligands (somatostatin (SS) and cortistatin (CST)) in a series of malignant lymphomas from the orbital region and neck-region, + indicates a positive signal for the mRNA using RT-PCR, ML-MALT= extranodal marginal zone B-cell lymphoma. The two Hodgkin's from the neck region were classified according to the Ann Arbor Classification and the two non-Hodgkin's from the neck region were classified according to The National Cancer Institute Working Formulation.

RT-PCR studies

RT-PCR was performed as described previously (20). Briefly, poly A^+ mRNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from malignant lymphoma samples. cDNA was synthesized using the poly A^+ mRNA, which was eluted

Table 2: Sequence of primers used for RT-PCR

	Sequence (5'-3')	Size of PCR product
Sst1 (forward)	ATGGTGGCCCTCAAGGCCGG	318 bp
Sst1 (reverse)	CGCGGTGGCGTAATAGTCAA	
Sst2 (forward)	GCCAAGATGAAGACCATCAC	414 bp
Sst2 (reverse)	GATGAACCCTGTGTACCAAGC	
Sst3 (forward)	TCATCTGCCTCTGCTACCTG	221 bp
Sst3 (reverse)	GAGCCCAAAGAAGGCAGGCT	
Sst4 (forward)	ATCTTCGCAGACACCAGACC	323 bp
Sst4 (reverse)	ATCAAGGCTGGTCACGACGA	
Sst5 (forward)	CCGTCTTCATCATCTACACGG	223 bp
Sst5 (reverse)	GGCCAGGTTGACGATGTTGA	
Hprt (forward)	CAGGACTGAACGTCTTGCTC	413 bp
Hprt (reverse)	CAAATCCAACAAAGTCTGGC	
Somatostatin (forward)	GATGCTGTCCTGCCGCCTCCAG	349 bp
Somatostatin (reverse)	ACAGGATGTGAAAGTCTTCCA	
Cortistatin (forward)	GCAAATTCGCTCTAAACACAGGA	173 bp
Cortistatin (reverse)	TTGGGAAGGAGGAGAGGAAAGAT	

The primer set we used for the detection of CST mRNA was adapted form Ejeskar et al. (21). As positive controls for SS, CST and HPRT, cDNA of human brain RNA (Invitrogen, Groningen, The Netherlands) was used. As positive control for sst_{1-5}, DNA of a BLCL-BSM cell-line (an EBV-transformed B-cell line) was used. The PCR-reaction was carried out in a DNA thermal cycler with heated lid (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). After an initial denaturation at 94°C for 5 minutes, the samples were subjected to 40 cycles of denaturation at 94° C for 1 min, annealing for 2 min at 60° C and extension for 1 min at 72° C. After a final extension for 10 min at 72° C, 10 µl aliquots of the resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidiumbromide. The identity of the products was confirmed by direct sequencing using an ABI Prism™ 3100 Genetic Analyzer (Applied Biosystems) according to manufacturers protocol.

from the beads in 40 µl H_2O for 10 minutes at 65 °C, using Oligo $(dT)_{12-18}$ Primer (Life Technologies). One-twentieth of the cDNA library was used for each amplification by PCR using primer sets specific for human SS, sst_{1-5}, CST and hypoxantine-phosphoribosyl-transferase (HPRT) as a control (see table 2).

Quantitative RT-PCR (Q-PCR)

In order to quantify expression of sst mRNAs, a quantitative PCR reaction was performed by TaqMan® Gold nuclease assay (Perkin Elmer Corporation, Foster City, CA, USA) and the ABI PRISM® 7700 sequence Detection System (Perkin Elmer) for real-time amplifications, according to manufacturers protocol. Q-PCR was performed for sst_2 and sst_3 only, because no expression of the other sst subtypes could be detected in the tissues and cells we investigated by RT-PCR. Primer sequences we used are summarized in table 3.

Table 3: Primers used for quantitative PCR

	5'-sequence-3'
Sst2 forward	-ATGCCAAGATGAAGACCATCAC-
Sst2 reverse	-TGAACTGATTGATGCCATCCA-
Sst2 probe	-FAM-TGGCTCTGGTCCACTGGCCCTTTG-TAMRA-
Sst3 forward	CTGGGTAACTCGCTGGTCATCTA-
Sst3 reverse	AGCGCCAGGTTGAGGATGTA-
Sst3 probe	-FAM-CGGCCAGCCCTTCAGTCACCAAC-TAMRA
HPRT forward	-TGCTTTCCTTGGTCAGGCAGTAT-
HPRT reverse	-TCAAATCCAACAAAGTCTGGCTTATATC-
HPRT probe	-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-

The relative amount of sst mRNAs were determined using a standard curve generated from known amounts of human genomic DNA. For determination of HPRT mRNA, a standard curve was generated of cDNA obtained from a DU45(prostate-cancer) cell line. The amount of sst mRNAs was calculated relative to the amount of HPRT and is given in arbitrary units.

Internalization

Internalization experiments were performed as described in detail previously (22) using [^{125}I-Tyr^3]-octreotide (kindly provided by Dr. W. Breeman, Dept. of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands) (23). In short, 10^6 cells were seeded/well in 24-well plates (Costar Corning, Schiphol, The Netherlands) and incubated with approximately 400,000 cpm/ml [^{125}I-Tyr^3]-octreotide, with or without an excess of unlabeled peptide. After different periods of incubation (t=30, 60, 120) cell-surface bound radioligand was removed with 1 ml acid wash for 10 min (22). Internalized radioligand was measured as acid-resistant counts in pellets of the acid-washed cells. The cell-lines

we studied included: one myeloid leukemic cell line: TMM, a lymphoid B-cell line: JY, an Epstein Barr-Virus (EBV)-transfected APD cell-line and EBV-immortalized primary B-cells from a patient suffering from a B-cell derived chronic lymphatic leukemia (B-CLL). As a positive control for internalization via sst_2, stably sst_2-transfected CC531 colon adenocarcinoma cells were used (CC2B). CC531 cells were established from an adenocarcinoma and maintained by serial passage after trypsinization in culture medium (24). The human sst_2 cDNA in pBluescript was a kind gift from Dr. G.I. Bell (Howard Hughes Medical Institute, Chicago, IL). This sst_2 cDNA was excised from pBluescript and inserted into the Nhe-I and Sal-I cloning sites of the retroviral vector pCI-neo (Promega Corp., Madison, WI). This vector was used to generate the CC2B cells.

Sst binding on membrane homogenates

Membrane isolation and binding studies were performed as described before (25), on the above indicated cells. In short, membrane preparations (corresponding to 30-50 µg of protein) 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/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 14000 rpm in an Eppendorf microcentrifuge. The remaining pellet was washed twice in ice-cold HEPES buffer and the pellet was counted in a γ-counter (1470 Wizard, Wallac, Turku, Finland). Specific binding was taken to be the total binding minus binding in presence of 1 µM unlabeled octreotide.

Sst autoradiography

Binding of [^{125}I-Tyr^3]-octreotide to sst in the malignant lymphoma tissues was investigated by autoradiography on unfixed cryosections. Malignant lymphoma biopsies were taken, immediately frozen and small parts were embedded in TissueTek (Miles Inc., Elkhart, USA) and processed for cryosectioning. 20 µm thick sections were mounted on gelatine-coated glass slides and stored at -80° C for 3 days to improve adhesion of the tissue sections to the slides. Autoradiography was performed on cryostat sections of malignant lymphoma tissue and sections of neuroendocrine tumours, serving as a positive control for binding to sst_2 specifically, as described previously (26). Binding of [^{125}I-Tyr^3]-octreotide was displaced by an excess of unlabeled octreotide to show specificity of binding. The number of pixels was quantified automatically using a Phosphor Imager. Specific binding was defined by > 50% displacement of the autoradiographic signal by excess unlabeled octreotide.

Immunohistochemistry

Immunohistochemistry was performed on 5µm thick sections cut on a cryostat (Jung CM3000, Leica, Germany) as described in detail previously (27). Sections were incubated overnight at 4 ˚C with sst_2 (Biotrend, Cologne, Germany) and sst_3 (Biotrend) antibodies, using 1:2000 and 1:3000 dilutions in PBS + 5% BSA respectively. A standard streptavidin-biotinylated-HRP complex (ABC kit, Biogenex, San Ramon, CA, USA) was

used according to manufacturers protocol. Finally sections were developed with diaminobenzidine (DAB) 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 minutes, rinsed in tap water followed by PBS. Subsequent steps were performed as in the protocol for frozen sections. The antibodies were used in dilutions of 1:2000 in PBS + 5 % BSA and sections were incubated overnight at 4 ˚C. Human pancreatic tissue served as a positive control for expression of sst.

Sequencing of the sst_2 gene encoding region
Purified PCR products were sequenced on a ABI Prism 310 Genetic Analyzer, using a BigDye™ Terminator Cycle Sequencing Ready Reaction DNA sequencing kit (Applied Biosystems, Warrington, United kingdom).

Results

Results by RT-PCR are summarized in table 1. In the two Hodgkin's and two non-Hodgkin's lymphomas from the neck region, only expression of sst_2 mRNA could be detected. In all orbital lymphomas, sst2 as well as sst_3 mRNA were demonstrated. SS mRNA could not be detected in any of the tissues tested. However, CST mRNA was expressed in all tissue samples. Results of the Q-PCR are shown in table 4.
By Q-PCR, expression levels of sst_2 and sst_3 mRNA were studied. Sst_2 mRNA levels in the lymphoma tissues were compared to the expression of sst_2 mRNA in GH-secreting pituitary adenomas, which express high levels of sst_2 mRNA. As shown, the expression levels of $sst_{2\ \text{and}\ 3}$ mRNA in the lymphoma tissues are relatively low, the highest relative expression of sst_2 mRNA in the lymphomas being 208 copies when adjusted for hypoxanthine-guanine phosphoribosyl transferase (HPRT). When compared to GH-secreting pituitary adenomas, these sst_2 mRNA levels were 6- to 200-fold lower in the malignant lymphomas. Although we found positive signals for sst_2 and sst_3 mRNA in the different tissues using RT-PCR technique, we detected by Q-PCR that these bands represented only a very low expression level of sst_2, whereas the sst_3 mRNA expression levels were very low as well, or even below the detection limit in most tissues tested.

Autoradiographic studies were performed in order to investigate and quantify binding of a sst_2 specific agonist, [^{125}I-Tyr^3]-octreotide, to sst_2 to malignant lymphomas. Rat brain and a human GH-secreting pituitary adenoma served as positive controls. Both control samples showed high specific binding of [^{125}I-Tyr^3]-octreotide. A series of 6 orbital lymphomas (no. 1-6) were incubated with [^{125}I-Tyr^3]-octreotide without or with unlabelled octreotide. Specific binding of [^{125}I-Tyr^3]-octreotide was detected in all orbital lymphomas. However, compared with rat brain and a GH-secreting pituitary adenoma, total binding of [^{125}I-Tyr^3]-octreotide was much lower in the malignant lymphomas, as shown in figure 1. The binding of [^{125}I-Tyr^3]-octreotide was quantified using a Phosphor-Imager. The intensity of black spots, which represent binding of [^{125}I-Tyr^3]octreotide to sst_2, was set at 100% in the GH-secreting pituitary adenoma and intensity in the

lymphomas was calculated relative to the intensity in the positive control, as shown in table 5.

Table 4: Quantitative expression of sst mRNA in lymphoma tissues compared to sst mRNA expression in two GH-secreting pituitary adenomas

Lymphomas	Sst_2/HPRT	Sst_3/HPRT	GH-secreting pituitary adenomas	Sst_2/HPRT	Sst_3/HPRT
8	208	-	1	1714	1260
1	115	<	2	1020	2140
10	65	-			
7	32	-			
9	30	-			
5	28	26			
2	13	<			
6	13	<			
4	8	9			
3	6	<			

Values represent arbitrary units, generated to a standard curve. Values are presented as the ratio of number of copies of the sst over the HPRT copy number, both relative to a standard curve. - = Quantitative RT-PCR not performed, because no expression of the sst_3 was found by RT-PCR, < = expression was below detection limit of quantitative RT-PCR.

Table 5: Autoradiography experiments on lymphoma tissues

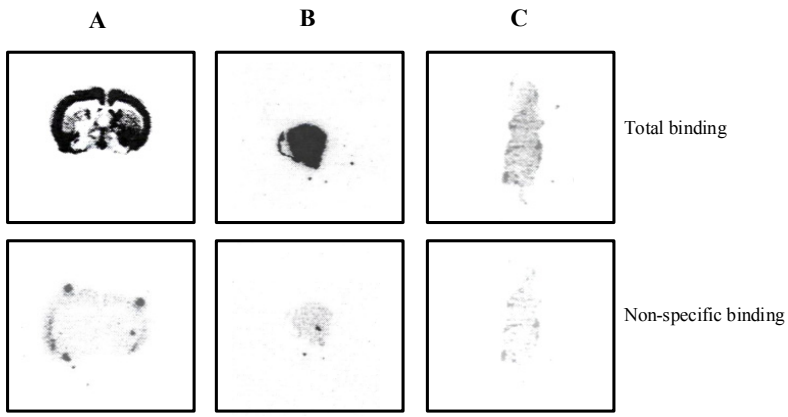
Tissue	% binding
GH-producing tumour	100
Rat brain	72
5	13.8
1	13.7
2	9.5
6	7.7
3	7.1
4	7.0

Binding percentages of [^{125}I-Tyr^3]-octreotide in lymphoma tissues are measured using a phosphor imager, in which the number of pixels was quantified. The pixels represent binding of [^{125}I-Tyr^3]-octreotide to the tissue. Specific binding of [^{125}I-Tyr^3]-octreotide to the GH-producing tumour was set at 100 % and the amount of binding of the radiolabeled compound to the different lymphoma tissues is presented relative to that of the GH-producing tumour.

All malignant lymphomas studied have very low total binding with the highest being less than 14% of the binding to the GH-secreting pituitary adenoma and less than 20% of the binding in rat brain cortex, indicating a very low expression of the sst_2 receptors in these tissues. These findings correspond to the relatively low expression levels of sst_2 mRNA found by Q-PCR.

By immunohistochemistry using sst_2 and sst_3 specific polyclonal antibodies an attempt was made to visualize sst_2 and sst_3 receptors in malignant lymphoma tissue. Human pancreas served as a positive control. Sst_2 and sst_3 immunoreactivity was clearly detected

Figure 1: Autoradiography experiments on lymphomas



This figure shows in the upper panel the binding of [^{125}I-Tyr^3]-octreotide, represented by the black spots, to rat brain (A), a GH-producing tumour (B), both used as positive controls, and an example of one of the lymphomas studied (C), lymphoma no. 6. In the lower panel binding of [^{125}I-Tyr^3]-octreotide is displaced by excess unlabelled octreotide.

in the pancreatic islets with a pattern of expression as described previously (7). This immunostaining could be completely abolished by pre-absorption of the respective antibodies with 100 nM of the peptide antigen (data not shown). However, under the conditions used, we were not able to detect immunoreactivity for sst_2 and sst_3 in the different lymphomas. To further evaluate this conclusion, membrane binding and internalization-studies were performed to evaluate the amount of radio-labelled octreotide, [^{125}I-Tyr^3]-octreotide, bound and internalized by the sst_2 on the cell membranes. For these experiments we used B-lymphoid and myeloid cell lines, as a model for cells in malignant lymphoma tissues. As a positive control the stably sst_2- transfected colon adenocarcinoma cells (CC2B) were used. Q-PCR was performed to investigate the sst_2 mRNA expression levels in the cell lines we used. Data are summarized in table 6.

All cell lines expressed sst_2 mNA, expression levels were relatively low compared with the stably sst_2-expressing CC2B cell line. To investigate the expression levels of the sst_2 protein on the different cell lines, membrane binding studies were performed. The results are shown in figure 2.

Table 6: Quantitative expression of sst_2 in different B- and myeloid cell-lines

Cells	Sst_2/750.000 cells
APD	11770
JY	11230
TMM	8155
B-CLL	490
CC-531	<
CC2B	72000

Table represents relative amount of sst_2 mRNA expression in different cell lines, calculated relative to a standard curve, generated from a Jurkat cell line and presented as the relative amount of sst_2 mRNA copy numbers per 750.000 cells isolated. CC-531 represents the untransfected cell-line. < = below detection limit

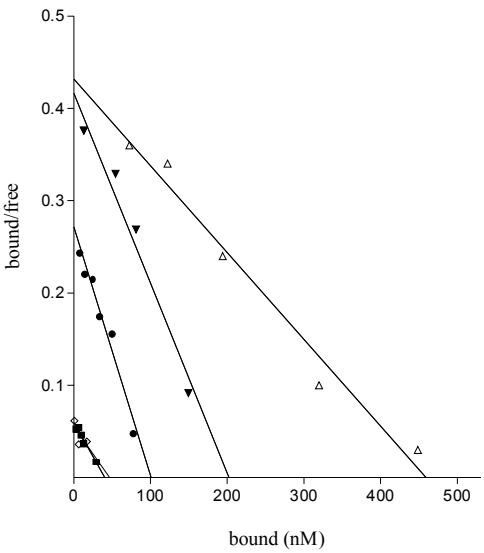


Figure 2: Membrane binding studies. Scatchard analysis of [^{125}I-Tyr^3]octreotide binding on human B and myeloid cell lines ◊ B-CLL (Kd = 0.8 nM, n= 50 fmol/mg), ■ APD cell line (Kd= 0.7 nM, n=40 fmol/mg), ● TMM cell line (Kd= 0.4 nM, n=100 fmol/mg), ▼ JY cell line (Kd= 0.4 nM, n=195 fmol/mg), Δ CC2B cell line (Kd= 0.8 nM, n=460 fmol/mg)

All cells investigated showed high affinity binding sites for SS, however, the number of receptors was found to be low. To investigate to which extent these receptors are able to internalize the radionuclide coupled SS-analogue, internalization experiments were performed using [^{125}I-Tyr^3]-octreotide.

As shown in figure 3, the CC2B cells internalized a significant higher amount of [^{125}I-Tyr^3]-octreotide than the JY, TMM and APD cell lines did, in which internalization was very low, whereas no internalization could be detected in the primary B-CLL cells.
Finally, DNA obtained from lymphoma tissues was used for sequencing in order to detect possible mutations in the DNA sequence in the sst_2-coding region in malignant lymphomas. Four orbital lymphomas were studied (no 1,3,4 and 6). Mutations could not be detected in the sst_2 coding region (data not shown).

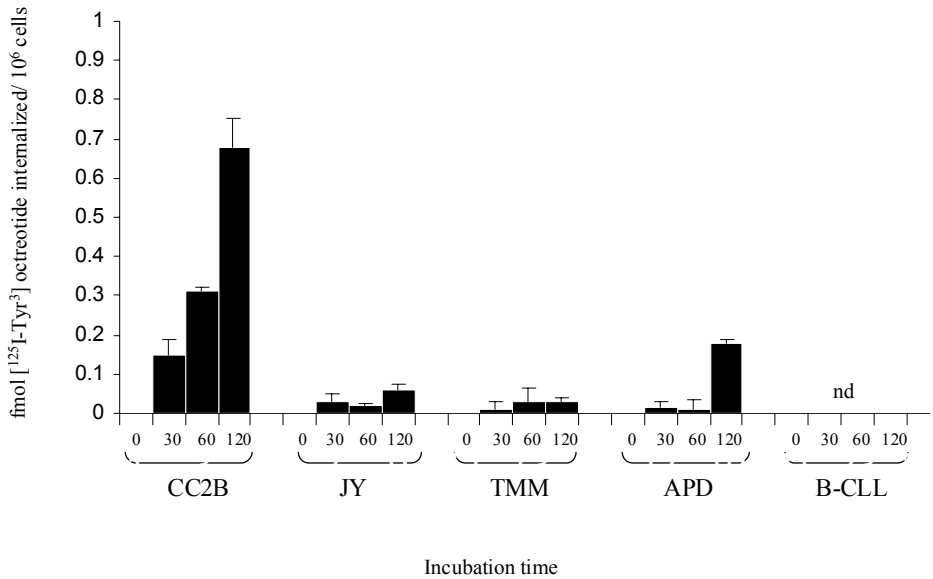


Figure 3: Internalization experiment using CC2B, JY, TMM, APD and B-CLL cells. Cells were incubated with either [^{125}I-Tyr^3]-octreotide alone or with [^{125}I-Tyr^3]-octreotide and an excess of unlabeled octreotide (1 μM) for 30, 60 and 120 minutes respectively. At each time-point internalization was measured and is presented as the amount of radiolabeled compound internalized by the cell in fmol/mg. nd = no internalization detectable.

Discussion

Previously, it has been demonstrated by sst scintigraphy, that malignant lymphomas express sst (11,28). Sensitivity of sst receptor scintigraphy for Hodgkin's disease lies around 95-100 % (29,30). Sensitivity for Non-Hodgkin's lymphomas is around 80 % (29). It was shown that uptake of [^{111}In-DTPA^{0}]octreotide in lymphomas was lower compared with the uptake in neuro-endocrine tumours (12,31). As the lymphomas of a number of patients with malignant lymphoma cannot be visualized by sst scintigraphy, we addressed the question what the cause of this non-visualization might be. In addition, our studies involved the question whether patients with malignant lymphomas might be candidates for radionuclide labeled SS-analogue therapy.

In the present study we investigated the expression of the different sst in a series of malignant lymphomas, using both RT-PCR and Q-PCR. By RT-PCR, in two Hodgkin's and two non-Hodgkin's lymphomas from the neck region, expression of sst_2 mRNA was detected, whereas the 6 lymphomas originating from the orbital region expressed sst_2 and sst_3 mRNA. Until now, sst has been demonstrated in lymphoma tissue using autoradiography in a few studies (11,28,32) reporting a differential sst subtype expression in malignant lymphoma tissue. In one study expression of sst_2 mRNA was detected in one Hodgkin and one non-Hodgkin lymphoma, whereas one non-Hodgkin lymphoma expressed both sst_1 and sst_3 mRNA (28). Another study showed that extra-gastric MALT-type lymphomas expressed sst_2 mRNA whereas intra-gastric MALT-type lymphomas expressed sst_3 and sst_4 mRNA but not sst_2 mRNA (32). In our previous studies we never detected expression of sst_4 in the human immune system and in human malignancies, while little is known concerning the expression of the different sst subtypes in malignant lymphomas.

The expression pattern of the sst subtypes was further studied by Q-PCR and results were compared to sst expression in 2 GH-secreting pituitary adenomas, which expressed high levels of sst. We found relatively low levels of sst_2 and sst_3 mRNA expression in all lymphomas studied, compared to the pituitary adenomas. Autoradiography and immunohistochemistry further supported these findings. By autoradiography, very low specific binding of [^{125}I-Tyr^{3}]-octreotide was demonstrated and by immunohistochemistry sst_2 or sst_3 receptors could not be visualized. This low or absent specific binding can be explained by low receptor levels in these tissues, in agreement with the low sst mRNA levels encoding the receptor proteins found by Q-PCR. The variable positive results obtained by sst scintigraphy in malignant lymphomas in previous studies (29-31) have been attributed to a too small size of the lesion (13,30). These findings might be explained by technical imperfections in different studies as well. The technique has been described previously (33-35), but in different studies too low dosages or too short periods of counting ahsve been used. Moreover, the negative findings may also be explained by very low expression of the receptors on the cell membranes as well, as demonstrated in our study. In order to study the internalization of radionuclide coupled SS analogues we used a number of cell lines and one sample of primary B-CLL cells. This was done, because

fresh lymphoma cells were difficult to obtain. Scatchard analysis of these cell lines and B-CLL cells revealed that all cells used showed relatively low levels of high affinity binding sites and a very low internalization, which is in agreement with the low number of SS binding sites. The sst_2 expressing CC2B cells internalized significant higher amount of the radiolabeled compound. An explanation for the relatively low sst levels and low internalization of radiolabeled SS analogues may be endogenous SS production as has been demonstrated in pheochromocytomas (36). Endogenous SS production may facilitate internalization of the sst_2 in an autocrine fashion (36), thus a lower uptake of radiolabeled SS analogues may be expected in SS expressing tissues. We evaluated expression of SS mRNA in lymphoma tissues, in order to find an alternative explanation for the low uptake by these cells of radionuclide labeled SS analogues. No SS mRNA in any of the lymphoma tissues studied was detected, however. Recently we demonstrated that a SS-like peptide, named cortistatin (CST), is expressed in normal cells of the human immune system (17). CST might act as alternative ligand to sst, rather than SS itself, because of its high binding affinity to all 5 sst (18). Therefore, we also investigated the expression of CST mRNA in the lymphomas. We detected expression in all samples tested, instead of the expression of SS mRNA. Although until now we could not detect the protein CST,it might be suggested on basis of the expression of CST mRNA, that CST can also facilitate internalization of the sst_2 on the cell membrane and cause a lower uptake of radioactively labeled SS analogues as well. Further studies should be performed to elucidate this issue.
In order to exclude that mutations in the encoding region of the sst_2 gene form the basis for possible changes in amino acid arrangement in the sst proteins in malignant lymphomas and subsequently lower efficiency in internalization, DNA of 4 malignant lymphomas was sequenced. Mutations in the sst_2 receptor encoding region could not be detected, ruling out that the low internalization rate could be caused by a defect in the receptor. Based on our findings of low sst numbers, binding and internalization, it seems that lymphomas are not ideal candidates for radiotherapy using radiolabeled SS analogues. Recently, advances have been made in gene transfer of sst in cancer models (37-39). It has been demonstrated that the induction of sst_2 in both primary and metastatic pancreatic cancer models results in a significant antitumor effect characterized by an increase of apoptosis and an inhibition of cell proliferation (38). In the sst_2-transfected CC2B cells we found indeed a very effective internalization of the radionuclide coupled SS analogue used. This finding might be promising with respect to a possible combination of sst gene therapy and SS analogue radiotherapy in for example lymphoma tissues. Further studies have to be performed to elucidate this interesting topic as in solid tumours it has been demonstrated that gene therapy using either retroviral or adenoviral vectors with the p53-gene in non-small cell lung cancers resulted in tumour regression or stabilization in most patients (40). On the other hand, recent advances have been made with respect to the development of new (better) radionuclide labeled sst_2 agonists. Recently, [^{177}Lu-DOTA^{0}Tyr^3]octreotate has been developed, which shows around 9-fold higher affinity to sst then ^{111}In-octreotide does (41). The uptake of this compound by tumours was much higher when compared to the uptake of ^{111}In-octreotide (41). Treatment of gastro-entero-pancreatic tumours with [^{177}Lu-DOTA^{0}Tyr^3]octreotate has already been shown to be

successful (42). The higher binding affinity of this new compound may be of functional significance in treatment of the low sst-expressing lymphomas as well.
In conclusion, we investigated the expression of sst, both qualitative and quantitative, in different malignant lymphomas, as well as the expression of the natural ligands SS and CST, in order to answer the question whether SS or its analogues might play a future role in radiotherapy treatment of these diseases. We demonstrated very low expression levels of sst mRNA and subsequently, low or absent specific binding to these tissues in our autoradiography and immunohistochemistry experiments. Although previous studies showed expression of sst in different lymphoma tissues (11), it was also clearly demonstrated that not in all patients lesions were detected using the sst scintigraphy (29,31). We hypothesize that these negative results might be due to a very low expression of the sst in malignant lymphomas. In the literature it was hypothesized that SS or one of its analogues might play a future role in treatment of malignant lymphomas, either as 'cold' peptides (43) or as β-emitting radionuclide coupled peptides (16). In our cell-line models we demonstrated that, despite expression of the sst_2, internalization capacity was very low, but could be enhanced by overexpression of sst_2-receptors. These data suggest that lymphomas in vivo might not be a target for radiotherapy with SS or its analogues. However, malignant lymphomas are the human tumours, which are most sensitive to radiation (44). In contrast, neuroendocrine tumours like carcinoids and gastrinomas, are considered to be rather radio-resistant. Still successful tumour shrinkage has been reported in neuroendocrine tumours after ^{177}Lu-DOTA-Tyr^3-octreotate administration (41). Future clinical trials will show, whether, unless their low number of sst and presumably low efficiency of the internalization, lymphomas may still be a target for therapy using radionuclide labeled SS analogues. Lymphomas are very radiosensitive and this may possibly compensate for their low receptor number and low internalization, when compared to neuroendocrine tumours, which express higher numbers of sst and show more effective internalization, but are much more radio-resistant.

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Chapter V

GENERAL DISCUSSION

During the last decades a large number of studies have demonstrated that hormones and neuropeptides can play a regulatory role in the human immune system. On the other hand, immune-derived products, like cytokines, may influence neuro-endocrine mechanisms (1). The interactions between the neuroendocrine and immune systems are known to be important in maintaining organ homeostasis. Bi-directional signalling pathways are involved in immune reactions against invading agents. In these reactions, the neuroendocrine system monitors and, when necessary, regulates immune cell function, while immune cells also need the neuroendocrine system in order to determine their appropriate response against the invading agent (2). Different ways exist by which the neuro-endocrine and immune system can interact. Neuroendocrine cells in the vicinity of immune cells can produce hormones and neuropeptides, but immune cells can also secrete these signalling molecules themselves, and interact with these molecules via specific receptors (3). Moreover, lymphoid organs are highly innervated tissues (4), through which the signalling molecules can reach the immune cells.
Somatostatin (SS) is a neuropeptide, of which the functional significance has been extensively investigated in the endocrine and nervous systems. In these systems SS plays a predominant inhibitory role in secretion processes, while inhibitory as well as excitatory actions have been described in the central nervous system (5). Expression of receptors for SS has been demonstrated at sites of inflammation and more specifically, on human immune cells as well (6). However, the functional significance of SS and somatostatin receptors (sst) in the human immune system is currently still largely unknown. A large number of studies have reported on the expression and functional significance of SS and sst in the rodent immune systems (7,8). Interestingly, it was shown that expression patterns of sst subtypes in rodents do not reflect the expression pattern in the human immune system (9). Therefore, caution should be taken when interpreting studies using animal models and translating the results to the situation in the human immune system. With respect to functional properties of SS on human immune cells, equivocal effects have been described. For instance, both stimulatory (10) and inhibitory (11) effects on the secretion of cytokines have been demonstrated. Such differential effects may be explained by the expression of distinct sst subtypes on immune cells (12). However, the expression pattern of sst in the different human immune cell subsets remains still unclear.

Expression of somatostatin and cortistatin in immune cells.

In order to investigate the possible functional significance of SS in the human immune system, the first step was to determine the expression of SS and its receptor subtypes in different cells of the immune system (Chapter II). In this thesis the focus of the studies has been on T- and B-lymphocytes, and especially on cells of the monocyte lineage, i.e. monocytes, macrophages and dendritic cells.
These cells were studied because of their major importance in human host defense and maintenance of organ homeostasis (13,14). Expression of different hormones, neuropeptides and their receptors has previously been demonstrated in these cells (15) and in this thesis we have focussed on the role of the neuropeptide SS.

First, expression of SS was evaluated in various tissues and cells of the immune system (chapter II.1). Because the peptide SS has a very short half-life (16) it is suggested that SS should be expressed in close vicinity to its receptors, and probably acting in an autocrine or paracrine way. Human thymus, isolated thymocytes and thymic epithelial cells (TEC), bone marrow, spleen, monocytes, macrophages, dendritic cells, peripheral blood mononuclear cells and B- and T-lymphocytes were studied for expression of SS mRNA by RT-PCR.
No expression of SS could be detected in any of the samples tested, except for whole human thymic tissue. Previously, it was demonstrated that expression of SS mRNA in human thymic tissue was due to its expression in the epithelial component and not the lymphoid component of the thymus (17). These findings were confirmed as expression of SS mRNA was detected in isolated TEC and not in thymocytes. The absence of SS mRNA expression in cells and tissues of the human immune system suggests that the source of SS may be from cells outside the immune system, like innervating nerve endings that can secrete SS (4). On the other hand, recently, the cDNA encoding a novel SS-like peptide has been cloned from the brain cortex. This 17 amino acid neuropeptide was named cortistatin (CST) (18). CST shows high structural similarity to SS and binds with high affinity to the $sst_{1\text{-}5}$, suggesting that CST may be an alternative ligand for sst (19). However, in preliminary studies expression of CST was found to be more restricted to certain tissues than the expression of SS (20). Because no expression of SS mRNA in the human immune tissues and cells could be found, the expression of CST mRNA, as alternative ligand for sst, was investigated as well.
Interestingly, CST mRNA was found to be expressed in all cells and tissues tested. Moreover, by autoradiography studies, binding of synthetic CST to sst_2 in human thymic tissue was confirmed. By quantitative PCR, significant differences in expression levels were detected between the various tissues and cell types. In addition, it was shown that activated and the more mature cells expressed higher levels of CST mRNA, which suggests that CST may play a more important role in immune cells in activated and differentiated state, i.e. in host defense. By RT-PCR, 2 CST mRNA isoforms were detected. In T- and B-cell lines, expression of only one isoform was detected in most of the B-cell lines, while most T-cell lines expressed both isoforms. The significance of the existence and differential expression of the 2 isoforms is still unclear. These data suggest that in the human immune system CST, as it binds to sst in thymic tissue, may be the ligand for sst, rather than SS itself, which is not expressed in any immune cells and tissues investigated. CST could therefore play an immune-regulatory role via the sst. However, the functional significance of the CST mRNA expression remains to be further evaluated.
SS is known for its predominantly inhibitory actions with respect to secretion processes in general in man (21,22) and in more detail it has been shown that SS in the murine immune system can suppress secretion of pro-inflammatory cytokines (7). Therefore, it may be hypothesized that CST could have anti-secretory effects in the human immune system as well. As CST mRNA expression is upregulated in activated cells, CST could regulate the immune response in a way, that no overly vigorous response occurs, which may affect the host. A possible mechanism may be the inhibition of secretion of pro-inflammatory cytokines and growth factors in an autocrine or paracrine way. The observation that CST mRNA was expressed in immune cells and tissues also raised the question whether CST

expression was restricted to the central nervous (18) and immune systems, or that the expression of CST showed a more widespread pattern.
Therefore, by quantitative PCR expression of both SS and CST mRNA was investigated in a large number of other peripheral tissues as well (chapter II.2). SS mRNA was widely expressed throughout the human body, with an expression pattern in concordance with previous reports in literature (23,24). CST mRNA was also widely expressed, with a much broader expression pattern than previously described (20). Among the different human central and peripheral tissues that were evaluated, immune cells and tissues expressed an average amount of CST mRNA. Only in cells and tissues of the human immune system a true selective expression of CST mRNA was observed, pointing to a selective regulatory role for CST in human immune cells only. The broad expression of CST mRNA suggests that CST may have more functional properties in tissues throughout the human body than previously assumed. As both SS and CST mRNA were expressed in the majority of tissues evaluated, it may be hypothesized that the mechanisms of actions of SS and CST are integrated acting via the same receptors, or, probably, CST and SS may exert their (differential) effects through their distinct receptors. However, no specific CST receptor has been described yet. No correlation between CST and SS mRNA levels in the various tissues was found.

Expression of somatostatin receptor subtypes in immune cells.

Having evaluated the expression pattern of natural ligands for sst in the human immune system, the expression patterns of sst subtypes in human immune cells were studied (chapter II.3 & II.4). Moreover, the regulation of expression of CST mRNA was studied in more detail in these cells as well.
A selective expression of sst_{2A} and sst_3 mRNA was detected in human peripheral blood mononuclear cells (PBMC). When cells were further isolated and evaluated, expression of sst_3 mRNA was detected in T- and B-lymphocytes by RT-PCR. However, by Q-PCR only significant levels of sst_3 mRNA were found in T-lymphocytes, indicating that expression of sst_3 in B-lymphocytes is extremely low. It should be questioned, therefore, whether sst_3 expression in B-lymphocytes may be of functional significance. Activation of T-lymphocytes resulted in an upregulation of sst_3 mRNA levels. Sst_3 in general is associated with induction of apoptosis (25), although the functional significance of upregulation of sst_3 in T-lymphocytes remains unknown. Because of its stimulatory effects on apoptosis, sst_3 may play a regulatory role in maintaining the balance between proliferation, which starts after activation of lymphocytes, and apoptosis. A previous study demonstrated that SS inhibited proliferation of human T-lymphocytes (26), but the expression of sst_3 mRNA we detected suggests that apoptosis could be involved in the regulation of the cell number in host defense or maintaining homeostasis as well. Expression of CST, on the other hand, was downregulated after activation of T-lymphocytes. It was hypothesized that downregulation of endogenous CST expression could be a direct feedback mechanism following activation of sst3 receptors, in order to regulate responsiveness to the peptide. This suggests that CST and sst_3 could interact in regulating the number of T-cells. No clear effects on CST mRNA expression were found in activated B-lymphocytes. As

previously described, SS also has anti-secretory effects and CST derived from human immune cells and tissues may therefore have regulatory effects on secretion of cytokines as well. In monocytes, macrophages and dendritic cells, a selective expression of sst_2 mRNA was found (chapter II.4). Expression levels were upregulated during differentiation and after activation. Although studies are equivocal, it has been previously demonstrated that SS was able to inhibit secretion of pro-inflammatory cytokines by activated monocytes (11). These effects may be explained by the expression of sst_2 receptors we found on these cells. Literature data indeed suggest that the anti-secretory effect of SS is mediated predominantly via sst_2 (27,28). Using fluorescent labeled SS analogues, expression of sst_2 proteins was visualized on cell membranes of activated macrophages, showing the successful translation of mRNA into functional protein. The functional significance of sst_2 expression in cells of the monocyte lineage remains to be further evaluated, although anti-secretory effects of CST and/or SS may be involved in preventing an overly vigorous immune reaction. Sst_2 in these cells may also be a potential target for therapy using SS analogues. In for instance granulomatous or autoimmune diseases, macrophages activate the immune response, by secreting cytokines (29,30). SS analogues, binding to the sst_2, may inhibit this secretion and diminish the destructive immune response.
In conclusion, it is now clearly demonstrated that lymphocytes selectively express sst_3 mRNA, whereas cells of the monocyte lineage selectively express the sst_2 mRNA. In none of the cells or tissues of the human immune system SS mRNA could be detected, whereas CST was widely expressed. Its expression could be regulated and thus seems to be the immune ligand for sst. These findings suggest immune-regulatory actions of CST acting via the different sst (figure 1).

Functional significance of somatostatin receptors on immune cells.

Sst expression in human immune cells may have several clinical implications. Expression of sst in neuroendocrine tumours has led to the development of sst scintigraphy to visualize sst-positive tumour sites using radionuclide labeled SS analogues. Also affected sites in immune-derived malignancies (31,32) and inflammatory diseases like sarcoidosis and rheumatoid arthritis (6,33,34) can be visualized by sst scintigraphy. It has been hypothesized that expression of sst in granulomatous disease may be a marker for clinical improvement (= eradication of effector cells) after therapy with for instance corticosteroids (6). Expression levels of sst_2 mRNA were found to be upregulated by LPS and IFN-γ in macrophages (chapter II.4). Glucocorticoids have been shown to regulate the expression of sst in (neuro)endocrine tumour cells and to modulate responsiveness to SS in endocrine cells (35). Therefore, it is hypothesized that corticosteroids may have a regulatory effect on the sst expression levels in immune cells as well.
In chapter II.5 it was shown that physiological concentrations of dexamethason downregulated the expression of sst_2 mRNA in activated macrophages, which are major effector cells in immune-mediated granulomatous disease. This suggests that the lower uptake of radiolabeled SS analogues in sst scintigraphy after therapy could be influenced by a downregulation of sst number. Therefore, certain caution should be taken in

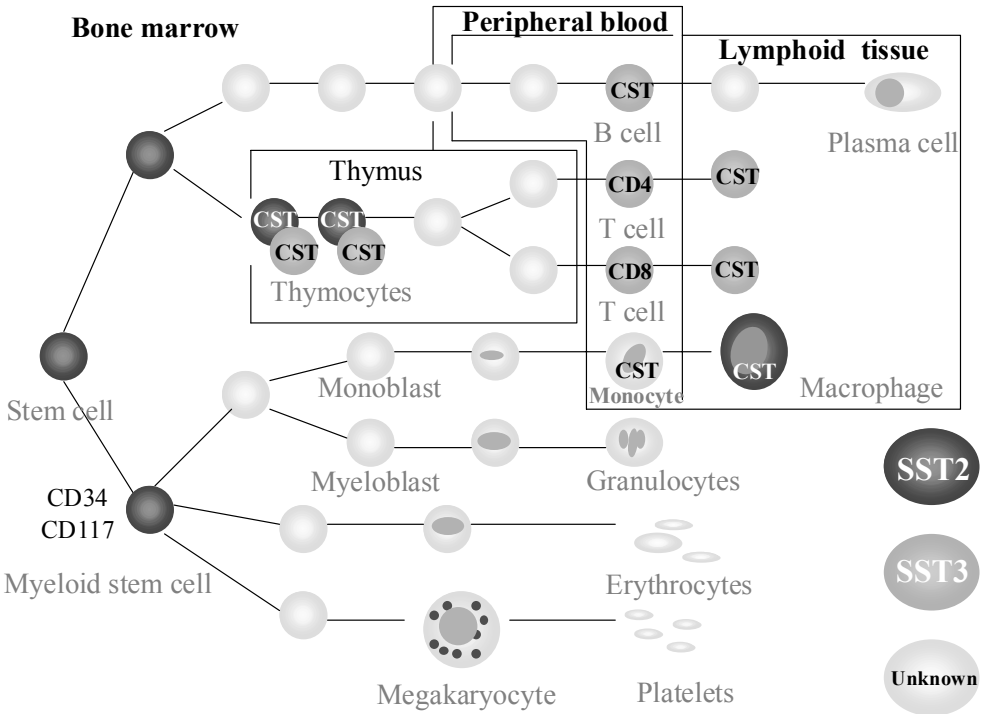

Figure 1: Expression of sst and its ligands in human immune cells. The expression of the ligands for sst in human immune cells, cortistatin, is indicated with its abbreviation (CST) in the appropriate cells.

considering sst scintigraphy a stand-alone tool in evaluating treatment efficiency. Other diagnostic tools in addition to sst scintigraphy, like X-ray, should be used to evaluate therapy efficiency.

As the expression of sst and a possible ligand for these sst, CST, were detected in different cells and tissues of the immune system, the question was addressed which functional significance these receptors may have in the human immune system.

In the murine immune system, it has been demonstrated that SS is involved in thymocyte maturation (36). Most profound effects of SS have been described on the regulation of thymocyte proliferation in the murine thymus. A regulatory role has been proposed for SS in the development of human thymocytes into T-lymphocytes as well. If so, thymocyte development in man may be under control of either SS or CST, as both peptide mRNAs were found to be expressed in the human thymus.

It was shown (chapter III.1) that SS, octreotide and CST significantly inhibited thymocyte proliferation. Interestingly, SS and CST were found to have similar effects on proliferation, which suggests that both compounds act on the same receptor (sst), rather

than that CST binds to a specific CST receptor, at least with respect to this anti-proliferative action. These findings suggest that thymocyte development in the human thymus may be under CST and/or SS control as well. Like SS (36), CST may contribute to thymocyte development by selection of cells. CST could inhibit the development of certain immature T-lymphocyte subsets by inhibiting their proliferation and possibly inducing apoptosis.

Moreover, CST, as a ligand to sst, may be involved in the regulation of migration of immature thymocytes, which is an essential process in T-lymphocyte maturation. Previously, in the murine thymus, SS was found to induce migration of thymocytes (36), which allows interaction of T-lymphocytes with different stromal cells, from which they can receive signals for survival, differentiation or death (37). As both SS and CST are expressed in the human thymus, both peptides may be involved in the regulation of migration as well.

In order to further explore the functional significance of sst on macrophages, a micro-array experiment was performed (chapter III.2), in which human unstimulated macrophages were incubated for 24 hours with or without octreotide.

Octreotide mainly influenced expression levels of genes involved in regulation of the cell cycle and apoptosis, suggesting that octreotide could play a role in this respect. These findings may implicate that SS analogues in human immune cells can regulate the activity of the immune response by regulating the number of active human immune cells. In macrophages, inhibition of proliferation seems not to play an important role as these cells do not proliferate. However, induction of apoptosis may again contribute to regulation of an appropriate immune response and moreover, may be a basis for therapy of immune-mediated diseases with SS analogues. SS analogues may eradicate in this way the cells that cause an auto-immune reaction. It should be mentioned that macrophages selectively express sst_2, which is more involved in anti-secretory effects than apoptosis. Nevertheless, we observed in our micro-array experiment effects of octreotide on genes involved in apoptosis, which are mediated via the sst_2. Recently, it has been demonstrated that indeed sst_2 as well may be involved in the induction of apoptosis in man (38), which makes the sst_2 in human macrophages a potential regulator of apoptosis as well. In addition, SS induces apoptosis in murine peritoneal macrophages, known to express sst_2, via an upregulation of p53, Bcl-2, iNOS and NO production (39). In the micro-array experiment, no clear effects of octreotide on any growth factor or cytokine expression was found. This may be due to the fact that unactivated macrophages probably secrete very low levels of growth factors, which are not influenced by octreotide. It should be mentioned, however, that the macrophages were in an unactivated state, thus expressing relatively low levels of sst_2 mRNA (chapter II). It can be hypothesized that sst_2 levels are too low to mediate effects of octreotide on growth factor and cytokine expression. On the other hand, sst_2 on macrophages may regulate secretion processes, rather than gene expression. Of particular interest was the finding of upregulation of the expression of the gene encoding chemokine receptor 6 (CCR6). CCR6 is known to be involved in chemotaxis (40). Our findings suggest that SS or the endogenously expressed sst-ligand, CST, by stimulation via sst_2, may play a role in migration of cells of the monocyte lineage, as has previously already been suggested for dendritic cells during inflammation (40). Moreover, previous studies have already demonstrated that SS, via sst_2, plays a role in migration processes during the

development of thymocytes in mice (36) and migration of human hematopoietic progenitor cells was induced by SS as well (41). These findings suggest that SS plays a very important role in migration processes, either during development or during activation of immune cells.
In granulomatous disease, uncontrolled proliferation of fibroblasts forms the cause of tissue damage. Macrophages have been proposed to play a stimulatory role due to the production of cytokines and growth factors (29).
In chapter III.3 we demonstrated that SS, octreotide and CST had a slight, but significant inhibitory effect on the stimulation of fibroblast proliferation induced by macrophages . As macrophages selectively express sst_2, this effect must be mediated via this receptor, probably by decreasing the secretion of pro-inflammatory growth factors. Until now, we have not been able to determine the specific factors that were involved in the stimulation of fibroblast proliferation and whether the production of such factors could be influenced by SS or its analogues. On the other hand, we found that IL-1β, IL-6, TNF-α, IGF-1, EGF and FGF are not mainly involved. In rheumatoid arthritis, in which macrophages also play an important role in mediating disease activity, it has been demonstrated that treatment with SS analogues resulted in significant clinical improvement (42) and currently studies are ongoing evaluating in more detail the possible role of SS analogues in treatment of rheumatoid arthritis.
The findings described in chapter III.3 may form a basis for future therapy of sarcoidosis, or other inflammatory diseases, as they often result in fibrosis, with SS analogues, because SS and its analogues may decrease fibrosis, which is a major cause of increased morbidity. However, further clinical studies are required to investigate the potential beneficial effects of SS analogues in this disease. In addition, studies investigating the factors that are involved in these mechanisms are required. Studies in rodents on the role of sst in immune disease seem not suitable, because the sst subtype expression pattern in rodent immune cells shows a pattern which is strikingly different from that in humans (9).
Finally, the potential role of radiolabeled SS analogues in treatment of malignancies affecting the human immune system was evaluated (chapter IV). Using radionuclide labelled SS analogues, tumour sites can be visualized in a high number of malignant lymphoma patients (31,32). In patients suffering from neuroendocrine tumours, radiotherapy using radionuclide labelled SS analogues has recently been introduced with promising results (43,44). The question was addressed whether lymphomas may also be treated with radionuclide labelled SS analogues in the nearby future. It was shown that lymphomas only expressed low levels of sst mRNA and protein. In addition, cell lines, as a model for cells in lymphomas, showed low uptake of the radionuclide labelled SS analogue (chapter IV.2).
These results suggest that due to the relatively low expression of sst and consequently low uptake of radionuclide labeled SS-analogue, lymphomas may not be a suitable target for therapy using SS analogues coupled with β-emitting radionuclides. However, some caution should be taken, because lymphomas are tumours that are highly radiosensitive (45) and due to this high radiosensitivity, the relatively low uptake of radioactivity may still result in clinical beneficial effects. Further clinical studies should be performed to address this issue. Moreover, the recent advances in the development of new radionuclide labeled SS analogues, like ^{177}Lu-DOTA^{0}Tyr^{3}]octreotate, which shows much higher

affinity to sst_2 then ^{111}In-octreotide (44), are of major interest with respect to their potential use in the low sst-expressing lymphomas as well.

Future perspectives:

As now a detailed insight is gained with respect to the expression pattern of sst subtypes and their ligand(s) in cells of the human immune system, a basis for further research is provided.
It has clearly been demonstrated that no SS mRNA is expressed in cells and tissues in the human immune system, but CST seems to be the endogenous ligand for sst. However, until now, it has not been possible to demonstrate expression of the CST protein. Not only in the immune system, but also in other organs the protein CST has not been isolated yet. Although regulation at the mRNA level suggests a functional significance, detection of the CST protein is of major importance. The development of antibodies to CST will be of high interest with respect to this topic. In addition, the recently developed technique of RNA interference (RNAi), allowing selective silencing of gene expression, may be used to evaluate the relevance CST expression in immune cells as well. Until now, no specific receptor for CST has been described, and it has been hypothesized in literature that CST exerts its effects via the sst. However, in the central nervous system differential effects have been described for both SS and CST, which may suggest that both compounds have their own specific receptor. Future studies are required to address the question whether a specific CST receptor exists.
The expression pattern of sst, SS and CST has been investigated in detail, but further functional studies are required to unravel the functional significance of sst and its ligand(s) in the human immune system, both in physiology and in pathophysiology. Further studies with respect to regulation of secretion of growth factors and cytokines by macrophages or lymphocytes, as well as studies to the regulatory effect of SS and its analogues on cell cycle mechanisms are needed to gain insight in the functional significance of sst in this respect. As we found by microarray analysis of macrophages a potential role for SS analogues in inducing expression of a chemokine receptor involved in migration processes and because previous studies demonstrated involvement of SS in migration of developing murine thymocytes and in migration of human hematopoietic progenitor cells, a chalenge will be to further study in more detail the functional significance of SS and CST on the migration of human immune cells, both in physiological maturation, as well as in inflammatory reactions.
Immune-mediated granulomatous disease, like sarcoidosis, may be a future target for SS analogues, as the main effector cells in sarcoidosis, i.e. macrophages, express sst and disease development may be influenced by targeting sst on these cells. Other diseases of the immune system, like sst-expressing lymphomas, may be targets for (radionuclide labelled) SS analogue therapy, although it is not clear, whether the low sst number expressed on lymphomas is sufficient to allow successful treatment. Future studies will provide the answers whether SS analogues might contribute to the treatment of immune-mediated diseases.

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Chapter VI

SUMMARY & SAMENVATTING

Summary

In recent years it has become evident that the neuroendocrine and immune systems interact in maintaining homeostasis of the organism. Many neuroeptides have been described which play a regulatory role in the human immune system as well. Somatostatin (SS) is a neuropeptide with mainly inhibitory actions in the endocrine and nervous systems. SS exerts its effects via specific somatostatin receptors (sst). Sst have also been described on cells and tissues of the human immune system, although data on the expression pattern of the different subtypes on immune cell subsets are equivocal. In addition, little is known about the functional significance of SS in the immune system. Studies in human and rodent models have shown a differential expression pattern of sst between the different species.
In this thesis the question was addressed which sst subtypes are expressed on cells of the immune system. Secondly, the expression of natural ligands for sst were studied. The primary focus was on T- and B-lymphocytes and cells of the monocyte lineage, i.e. monocytes, macrophages and dendritric cells, because these cells are known to play an important role in human host defense. In addition, studies have been performed in order to evaluate several aspects of the functional significance of SS and its analogues in the immune system. The answers to these questions may have consequences for a better understanding the potential role of SS and its receptors in the human immune system, both in physiological and in pathophysiological state.

Chapter I gives an introduction on the expression and functional properties of sst and its ligands, particularly focussing on cells and tissues of the immune system.

In **chapter II** the expression of SS and cortistatin (CST), an alternative ligand for sst, in cells and tissues of the human immune system has been described. SS was not expressed in cells of the monocyte lineage, and in T- and B-lymphocytes. Subsequently, it was demonstrated that CST was widely expressed and bound with relative high affinity to sst_2 in human thymic tissue, indicating that CST may be the ligand for sst in the human immune system, rather than SS (**chapter II.1**). Evaluating the expression of CST and SS mRNA in a large number of tissues throughout the human body, we found a broad expression of both SS and CST mRNA (**chapter II.2**), which demonstrates that CST potentially has a much broader functional significance than its previously presumed selective actions in the central nervous system. In human immune cells, a selective expression of CST was found, while all other tissues expressed both SS and CST mRNA.
As we determined the expression pattern of the potential ligands for sst in the human immune system, the regulation of the expression of sst subtypes and CST has been studied in more detail in lymphocytes, monocytes, macrophages and dendritic cells. T- and B-lymphocytes selectively expressed sst_3 mRNA, as determined by RT-PCR (**chapter II.3**). Expression of sst_3 mRNA was upregulated in T-lymphocytes by activation, while CST mRNA expression was downregulated. In B-lymphocytes we could not detect sst_3 by quantitative PCR, which suggests that sst_3 does not play an important role in B-lymphocyte function. On the other hand, in monocytes, macrophages and dendritic cells a

selective expression of sst_2 and CST mRNA was detected (**chapter II.4**). None of the other subtypes, nor SS were expressed. Expression of sst_2 was visualized on cell membranes of activated macrophages using a fluorescent labeled SS-analogue, which binds preferentially to sst_2. Both sst_2 and CST mRNA were upregulated during differentiation and activation of the cells, demonstrating that the potential CST-sst_2 immune-regulatory circuit plays a more significant role in mature macrophages as well as in activated macrophages.
In **chapter II.5** we demonstrated that glucocorticoids downregulated the expression of sst_2 mRNA in activated macrophages. In previous studies it was hypothesized that sst receptor status may be indicative for determining disease activity after therapy in patients suffering from granulomatous disease, like sarcoidosis. **Chapter II.5** demonstrates that the results of sst scintigraphy in patients with sarcoidosis may have been influenced by previous glucocorticoid treatment of the patients.

In **chapter III** we evaluated several aspects of potential functional significance of sst expression in human immune cells. In **chapter III.1** the functional significance of SS on human thymocytes is described. Previously, it was hypothesized that SS is involved in T-lymphocyte maturation. We showed that SS and CST inhibited thymocyte proliferation. These findings indicate that sst and their ligands, as both SS and CST are expressed in the human thymus, have a regulatory role on thymocytes. In order to gain more insight into the effects of SS analogues on macrophages, macrophages were cultured with or without octreotide, after which a micro-array experiment was performed, evaluating changes in the expression of over 40,000 genes (**chapter III.2**). Of the genes with a known function, octreotide mainly influenced the expression of genes involved in cell cycle mechanims and apoptosis. On the basis of these findings we hypothesized that SS, acting on the sst_2, may regulate the number of cells present at sites of inflammation, thereby probably playing a regulatory role in the immune reaction. Moreover, it was shown that octreotide upregulated expression of the chemokine 6 receptor (CCR6), which is known to be involved in chemotaxis. SS and/or CST might regulate migration of macrophages, for instance in inflammatory reactions, via this receptor. Finally, we performed experiments in which macrophages and fibroblasts were co-cultured, as a simplified in vitro model for sarcoidosis (**chapter III.3**). Macrophages stimulated the proliferation of fibroblasts. SS analogues were able to inhibit the fibroblast proliferation induced by macrophages, providing a potential basis for future therapy of sarcoidosis with SS analogues. Nevertheless, it is not known until now, which factors are involved in the stimulation of proliferation, and to which extent the production of such factors can be regulated by SS analogues.

In **chapter IV.1** the role of sst scintgraphy in diseases affecting the human immune system, like Hodgkin's and non-Hodgkin's lymphomas, sarcoidosis and rheumatoid arthritis is introduced. Current knowledge with respect to the use of sst scintigraphy in the diagnosis of these diseases is discussed.
Following the use of sst scintigraphy in diagnosis of lymphomas and treatment of neuroendocrine tumours with radionuclide labeled SS analogues, we evaluated whether lymphomas may be a potential target for treatment with radioactively labeled SS

analogues as well (**chapter IV.2**). Lymphomas were found to express only low levels of sst mRNA and protein. Moreover, lymphoid cell lines only showed a relatively low uptake of the radionuclide labeled SS analogue, which indicates that lymphomas are not targets for this therapy. On the other hand, their relatively high radiosensitivity may compensate for the low number of receptors and corresponding lower efficiency in uptake.

In **chapter V** we discussed the results described in this thesis, focussing on their significance for understanding the potential role of sst and its ligands in the human immune system. Moreover, plans for further research are postulated.

Samenvatting

In de laatste jaren is het meer en meer duidelijk geworden dat het neuroendocriene en immuunsysteem interactie met elkaar vertonen ter behoud van homeostase van het organisme. Er zijn vele neuropeptiden beschreven, die ook een regulerende rol spelen in het menselijk immuunsysteem. Somatostatine (SS) is een neuropeptide met een voornamelijk remmende werking in het endocriene systeem en zenuwstelsel. SS werkt via specifieke somatostatine receptoren (sst). Sst zijn ook beschreven op immuuncellen en –weefsels, hoewel de data over de expressiepatronen van de verschillende sst tegenstrijdig zijn. Bovendien is er weinig bekend over de functionele betekenis van SS in het immuunsysteem. Studies in humane- en knaagdiermodellen hebben verschillen in expressiepatronen van sst tussen de verschillende soorten aangetoond.
In dit proefschrift werd de vraag gesteld welke sst subtypen tot expressie komen in cellen van het humane immuunsysteem. Ten tweede werd de expressie van de natuurlijke liganden voor de sst bestudeerd. De primaire aandacht ging hierbij uit naar T- en B-lymfocyten en cellen van de zogenaamde monocyten-lijn, te weten monocyten, macrofagen en dendritische cellen, omdat deze cellen bekend zijn om de belangrijke rol die zij spelen in de humane afweerreactie. Bovendien zijn er studies uitgevoerd om verscheidene functionele aspecten van SS en haar analoga in het immuunsysteem te evalueren. De antwoorden op deze vragen hebben mogelijk consequenties voor een beter begrip van de potentiële rol van SS en haar receptoren in het humane immuunsysteem, niet alleen in fysiologische condities maar ook in ziekte.

Hoofdstuk I geeft een introductie met betrekking tot de expressie en functionele eigenschappen van sst en haar liganden, voornamelijk gericht op immuuncellen en –weefsels.

In **hoofdstuk II** wordt de expressie van SS en cortistatine (CST), een alternatief ligand voor sst, in immuuncellen en –weefsels beschreven. SS komt niet tot expressie in cellen van de monocyten-lijn en in T- en B-lymfocyten. Vervolgens werd aangetoond dat CST wijd verspreid tot expressie komt en CST bond met relatief hoge affiniteit aan sst_2 in humaan thymus weefsel. Deze bevindingen suggereren dat CST, in plaats van SS, het ligand is voor sst in het humane immuunsysteem (**hoofdstuk II.1**). De aanwezigheid van SS en CST mRNA werd geëvalueerd in een groot aantal humane weefsels, waarbij een wijd verspreide expressie van zowel SS en CST werd gevonden (**hoofdstuk II.2**). Dit suggereert dat CST een bredere functionele betekenis heeft dan voorheen aangenomen werd, namelijk een selectieve rol in het centrale zenuwstelsel. Alleen in humane immuuncellen werd een selectieve expressie van CST mRNA gezien, terwijl de andere onderzochte weefsels zowel SS als CST mRNA bevatten.
Na het expressiepatroon van de potentiële liganden voor sst in het humane immuunsysteem bestudeerd te hebben, werd de regulatie van de expressie van sst subtypen en CST in meer detail bestudeerd in lymfocyten, monocyten, macrofagen en dendritische cellen. Met behulp van RT-PCR wer een selectieve expressie van sst_3 mRNA gevonden in T- en B-lymfocyten (**hoofdstuk II.3**). Sst_3 expressie vertoonde upregulatie na activatie van T-lymfocyten, terwijl CST mRNA juist downregulatie vertoonde. In B-

lymfocyten kon geen expressie van sst_3 mRNA aangetoond met behulp van quantitatieve PCR. Dit suggereert dat sst_3 geen belangrijke rol speelt in de functie van humane B-lymfocyten. Aan de andere kant werd in monocyten, macrofagen en dendritische cellen een selectieve expressie van sst_2 mRNA gevonden (**hoofdstuk II.4**). Geen andere subtypen of SS werden aangetoond. Gebruik makend van een SS analoog, gelabeld met een fluorescent, dat selectief bind aan sst_2 werd de expressie van sst_2 op celmembranen van geactiveerde macrofagen gevisualiseerd. Zowel sst_2 als CST expressie vertoonden upregulatie tijdens differentiatie en activatie van de cellen, wat suggereert dat het mogelijke CST-sst_2 immuunregulerende circuit een belangrijkere rol speelt in uitgerijpte of geactiveerde immuuncellen.
In **hoofdstuk II.5** hebben we aangetoond dat glucocorticoiden een downregulerend effect hebben op de expressie van sst_2 mRNA in geactiveerde macrofagen. Voorgaande studies hebben gesuggereerd dat de mate van expressie van sst indicatief kan zijn met betrekking tot het vaststellen van de activiteit van ziekte na behandeling van patiënten lijdend aan granulomateuze aandoeningen, zoals sarcoidose. De resultaten, beschreven in **hoofdstuk II.5** suggereren dat de uitkomsten van sst scintigrafie in sarcoidose patiënten beïnvloed kunnen worden door voorafgaande behandeling met glucocorticoiden.

In **hoofdstuk III** worden verschillende aspecten van potentiële functionele betekenis van de sst expressie in humane immuuncellen bestudeerd. In **hoofdstuk III.1** wordt de functionele betekenis van SS op humane thymocyten beschreven. Eerder werd de hypothese opgeworpen dat SS betrokken is bij de maturatie van T-lymfocyten. Wij vonden dat SS en CST de proliferatie van thymocyten remden. Deze bevindingen geven aan dat zowel SS en CST, aangezien beide tot expressie komen in de thymus, een regulerende werking kunnen hebben op thymocyten. Aangezien alleen CST, en niet SS, tot expressie komt in thymocyten, wordt gesuggereerd dat CST betrokken kan zijn in de complexe processen bij de generatie van het T-lymfocyt repertoire tijdens de ontwikkeling.
Om meer inzicht te verkrijgen in de effecten van SS analoga op macrofagen werden macrofagen geincubeerd met of zonder octreotide, waarna een micro-array experiment werd uitgevoerd, waarbij de verschillen in expressie niveaus van meer dan 40.000 genen werden bestudeerd (**hoofdstuk III.2**). Octreotide beïnvloedde met name genen die betrokken zijn in celcyclus mechanismen en apoptose. Gebaseerd op deze bevindingen werd gesuggereerd dat SS, via sst_2, mogelijk een regulerende werking heeft op het aantal cellen, dat aanwezig is op de plek van ontsteking en dus een regulerende rol speelt in de immuunreactie. Behandeling met octreotide leidde ook tot upregulatie van de chemokine receptor subtype 6 (CCR6). Deze receptor is betrokken bij chemotaxis. Voorgaande studies hebben aangetoond dat SS migratie van muizen thymocyten en humane hematopoietische voorganger cellen stimuleert. SS of CST kunnen daarom mogelijk ook de migratie van humane macrofagen beïnvloeden, bijvoorbeeld in ontstekingsreacties.
In **hoofdstuk III.3** worden experimenten beschreven waarbij macrofagen en humane fibroblasten in co-culture werden gebracht, als een gesimplificeerd model voor sarcoidose. Macrofagen stimuleerden de proliferatie van fibroblasten. SS analoga waren in staat de door macrofagen gestimuleerde proliferatie van fibroblasten te remmen. Deze resultaten vormen mogelijk een basis voor toekomstige behandeling van sarcoidose met SS analoga.

Echter, tot op heden is het nog onbekend welke factoren verantwoordelijk zijn voor de stimulatie van de fibroblast proliferatie en in welke mate de productie van deze factoren gereguleerd kan worden door SS analoga.

In **hoofdstuk IV.1** wordt de rol van sst scintigrafie in ziekten die uitgaan van het humane immuunsysteem, zoals de ziekte van Hodgkin, non-Hodgkin lymfomen, sarcoidose en reumatoïde artritis, geïntroduceerd. Huidige kennis met betrekking tot het gebruik van deze techniek in de diagnose van bovengenoemde ziekten wordt besproken.
In navolging van het gebruik van sst scintigrafie in de diagnose van lymfomen en behandeling van neuroendocriene tumoren met radionuclide gelabelde SS analoga, werd onderzocht of lymfomen mogelijk ook behandeld kunnen worden met radioactief gelabelde SS analoga (**hoofdstuk IV.2**). In lymfomen werden slechts lage niveaus van sst mRNA en eiwit expressie gevonden. Lymfoide cellijnen namen slechts een lage hoeveelheid van het radionuclide gelabelde SS analoog op. Dit suggereert dat lymfomen geen kandidaat zijn voor behandeling met deze SS analoga. Echter, de hoge radiosensitiviteit van lymfomen kan mogelijk compenseren voor het lage aantal receptoren en de lage opname van radionuclide gelabelde SS analoga.

In **hoofdstuk V** worden de bevindingen van dit proefschrift bediscussieerd, gericht op hun betekenis voor het begrip van de potentiële rol van sst en haar liganden in het humane immuunsysteem.

ABBREVIATIONS

α-MSH	alpha-melanocyte stimulating hormone
ACh	acetylcholine
ACTH	corticotropin
ANOVA	one-way analysis of variance
BLCL-BSM	B-lymphoblastoid cell line-BSM
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosine monophosphate
CCR	chemokine receptor
CD	cluster of differentiation
Ci	Curie(s)
ConA	concavalin A
CRF	corticotrophin releasing factor
CST	cortistatin
D2DR	dopamine receptor D2
DC	dendritic cells
DEX	dexamethasone
DMF	dimethylformamide
DNA	deoxyribonucleic acid
EBV	epstein-barr virus
EEG	electroencephalogram
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethoxycarbonyl
FSH	follicle-stimulating hormone
GH	growth hormone
GM-CSF	granulocyte macrophage-colony stimulating factor
GnRH	gonadotropin-releasing hormone
GRIF	growth hormone release inhibiting factor
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HLA-DR	human leukocyte antigen class II
HPA	hypothalamic-pituitary-adrenal axis
HPO	hypothalamic-pituitary-ovary axis
HPRT	hypoxanthine guanine phosphoribosyltransferase
IFN-γ	interferon gamma
Ig	immunoglobulin
IGF-I	insulin-like growth factor 1
IL	interleukin
IvDde	1-(4,4-dimethyl-2,6-dioxocyclo-hex-1-ylidene)-3-methylbutyl

LC-MS	liquid chromatography-mass spectrometry
LH	luteinizing hormone
LPS	lipopolysaccharide
MAPK	MAP kinase
MBTE	methyl tertiary butyl ether
MHC	major histocompatibility complex
MO	monocytes
MOR1	mu-opioid receptor
MP	macrophages
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
OCT	octreotide
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PHA	phytohemagglutinin
PhOH	phenol
PRL	prolactin
PTP	protein tyrosine kinase
PWM	pokeweed mitogen
Q-PCR	quantitative polymerase chain reaction
RA	rheumatoid arthritis
REM	rapid eye movement
RT-PCR	reverse transcriptase polymerase chain reaction
SP	substance P
SPECT	single photon emission computed tomography
SS	somatostatin
Sst	somatostatin receptor
TCA	trichloroacetic acid
TEC	thymic epithelial cells
TFA	trifluoroacetate
TGF- β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
Tris	tris(hydroxymethyl)-aminomethane
TSH	thyroid stimulating hormone
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 27 juli 1978 geboren te Dordrecht. In 1996 behaalde hij het gymnasium diploma aan het Johan de Witt Gymnasium in Dordrecht. In september 1996 begon hij aan de studie geneeskunde aan de Erasmus Universiteit Rotterdam. In juli 2000 werd het doctoraal diploma behaald, na een afstudeeronderzoek op de afdeling Inwendige Geneeskunde aan de Erasmus Universiteit Rotterdam onder leiding van Dr. L.J. Hofland, getiteld: Preliminary studies on the expression and functional significance of somatostatin and its receptors in human macrophages. In navolging hiervan werd in september 2000 aangevangen met het in dit proefschrift beschreven onderzoek op de afdeling Inwendige Geneeskunde aan de Erasmus Universiteit Rotterdam, in het kader van het NWO-project: The role of somatostatin receptors in neuroendocrine immunology and –hematology, onder leiding van Prof. Dr. S.W.J. Lamberts, Dr. L.J. Hofland en Dr. P.M. van Hagen. In april 2003 startte hij met zijn co-schappen aan de Erasmus Universiteit Rotterdam teneinde zijn studie geneeskunde af te ronden.