THE DIFFUSE NEUROENDOCRINE SYSTEM AND

ITS IMMUNO-MODULATORY ROLES IN CHICKEN T-CELL IMMUNITY

A Dissertation

by

XIAODONG ZHANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Poultry Science

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Approved by:

Chair of Committee,	Luc Berghman
Committee Members,	Ellen Collisson
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ABSTRACT

The Diffuse Neuroendocrine System and Its

Immuno-modulatory Roles in Chicken T-cell Immunity. (December 2005)

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Chair of Advisory Committee: Dr. Luc Berghman

Neuroendocrine cell populations were systematically studied and characterized in the thymus, an avian primary immune organ. The expression of the specific mRNAs for both Chromogranin A (CgA) and Carboxypeptidase E (CpE) in the thymus was first verified by RT-PCR. Additional evidence using immunofluorescent dual labeling, has demonstrated for the first time the co-existence of CgA and CpE in identical neuroendocrine cells at the protein level in a vetebrate primary lymphoid organ. These CpE- and CgA-positive cells were primarily found in the transition zone between the cortex and the medulla of the thymic lobules, an area known to contain numerous arterioles and to be heavily innervated by the autonomic nervous system, suggesting that these cell population can potentially receive input from each other, from the autonomous nervous system, from the circulation, or all of the above. (Neuro)endocrine messenger molecules produced by the thymic microenvironment, such as somatostatin (SST), seem to play a potentially important immunomodulatory role with regard to cell proliferation, differentiation, and migration, as well as cytokine production. The results showed that both SST and its receptor, SSTR2, are expressed locally within chicken thymus. The *in vitro* study showed that SST significantly inhibits IL-2 and concanavalin A (ConA)

induced proliferation of thymocytes. In comparison with controls (medium containing IL-2 and ConA but without SST), addition of SST at 10^{-9} M and 10^{-6} M resulted in a nearly 20% decrease in proliferation ($P < 0.01$). The effects of somatostatin (SST) on the immune system, the role of SST on the gene expression of cytokines (IL-1, TGF, INF), chemokine receptors (CXCR4) as well as MHC-I components was assessed by real-time PCR. The question as to exactly which stimuli trigger the release of mediators such as somatostatin remains for future study. In addition, a complete inventory of all substances stored in the thymic LDCV and their effects on the developing T-cells when released in the microenvironment of the thymus are also questions that warrant further investigation.

DEDICATION

To my mother, Guizhen Yin, and my father, Zhitian Zhang

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I would like to thank Dr. Luc Berghman for his tremendous support and help during the last four and half years. I could not have made it this far without his patience and encouragement. Thank you, Luc, for being such an excellent mentor in my doctoral studies.

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I owe so much to my beloved mother and father for their continued and unlimited support and love. I really do not know how to express my gratitude for all that you have done for me. What I know is to keep going and I hope one day you will be proud of your son.

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Finally, I want to extend my gratitude to everyone who supported and helped me in research or in daily life; to everyone who brought me joy and laughter as well as a deeper understanding of the meaning of life. Thank you all for your help and love.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The development of T-cells for protection against pathogens and other non-self antigens is an essential feature of the vertebrate cellular immune system. In birds, as in mammals, the process that creates such a diverse immunological repertoire of T-cell receptors has been shown to be critically dependent on the microenvironment of the thymus. The thymic stroma is complex and consists of epithelial cells, mesenchym, macrophages and dendritic cells, in addition to fibroblasts and matrix molecules (1). These components provide not only essential cell-cell contacts but also communicate with the developing T-lymphocytes in a humoral manner. In addition to classical cytokines, the thymus produces a number of unique humoral factors and an ever-growing list of neuroendocrine and peripheral hormones. While some of these, such as prolactin and growth hormone, have well characterized effects on thymocyte differentiation and proliferation, many have been identified but have not been assigned a defined function within the thymus(2). The neuroendocrine circuits within the avian thymus and lymphocyte-microenvironment interactions are essential for the education and balance between self-tolerance and immunity, which will be discussed in this literature review.

T-cell ontogeny

The thymus is the primary immune organ that generates naïve T-cells in birds. It consists

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This dissertation follows the style and format of Journal of Immunology.

of two rows of seven separate lobes on each side of the neck, along the jugular veins extending from the lower jaw to the thorax. These lobes are further divided in lobules, separated by septa composed of connective tissue. The lobules are the elementary units of the thymus and contain a central area, the medulla, and a surrounding area, the cortex. Unlike in the bursa of Fabricius, there is lesser clear boundary between the cortex and the medulla (3).

During embryonic development, T-cell precursors are derived from stem cells near the thoracic aorta (4). The embryonic thymus is populated with three separate waves of stem cells: at days 6-8, days 12-14, and days 18-21 of embryonic development (5). Each wave lasts for 1.5 to 2 days and is followed by intense thymocyte proliferation, maturation and seeding to the periphery, which lasts for a period of approximately three weeks. Thymic seeding continues after hatching but not in such discrete waves (6).

T-cells mature within the thymus while undergoing a series of changes of both their localization and their phenotype (7). During thymocyte maturation, they migrate from the outer thymic cortex to the medulla where they finally exit. The pattern of cluster of differentiation antigen CD 8 and CD4 expression serves as the main characteristic for distinguishing different maturational stages of TcR (T-cell receptor) $\alpha\beta$ cells. The chicken also have a TcR $\gamma\delta$ population of thymocytes that mature more rapidly and that also have a distinct, yet unknown, physiological role, except for the fact that they may be involved in the control of isotype switching(8).

Early $\alpha\beta$ thymocytes are CD8 and CD4 negative and are called triple negative as they

also lack expression of the CD3 complex. The formation of αβ T-cells in the thymic cortex requires a period of three to four days before they enter the thymic medulla. While these cells migrate from the site of T-cell precursor entry, the outer thymic cortex, to the medulla, their expression of CD4 and CD8 gradually increases and they become double positive thymocytes. This thymic population proliferates vigorously to produce the largest thymocyte population. Finally, in the medulla, thymocytes downregulate one of the two accessory moledules before leaving the thymus as single positive CD4 or CD8 αβ T-cells(6).

Additional markers of thymocyte maturation have been characterized to further examine the thymic subpopulations. The expression of another differentiation marker, CD5, starts very early in thymocyte development, possibly even before thymic colonization by precursor cells. CD5 is found at high levels on all T-cell subsets during thymocyte maturation, and is thus an ideal handle for isolation of a total T-cell population from the thymus. In contrast to the abundant CD5 expression, the CD6 antigen is only found on double positive and single positive thymocytes, suggesting it is a maturation-dependent antigen, acquired during or after positive and negative selection (6).

The thymic microenvironment and neuroendocrine factors

While a majority of cells within the primary immune organs after hatch are clearly lymphoid, the thymus also contains a diverse array of non-lymphoid cells. Taken together, these non-lymphoid elements are loosely defined as the thymic stroma. The stroma consists to a large extent of thymic epithelial cells derived from the third

pharyngeal pouch, but it is not entirely clear if all thymic (*i.e.* cortical and medullary) epithelial cells share the same developmental origin (9). In addition to cortical and medullary epithelial cells, another important stromal cell type is mesenchyme, which is derived from the neural crest (10). The importance of mesenchyme was emphasized in that extirpation of the neural crest in birds resulting in a lack of mesenchymal contribution to the developing thymus, disrupted thymic formation and function (11). The precise mechanism by which mesenchymal cells influence development of the thymus is, however, still somewhat unclear. Finally, the thymic stroma also contains bone marrow derived dendritic cells and macrophages, and fibroblasts.

Positive selection takes place in the cortex of the thymus and is largely driven by the cortical epithelial cells that provide pepide-MHC ligands for the αβ TCR and also other unknown co-stimulatory or accessory signals (9). Induction of tolerance through negative selection is taking place in the thymic medulla and is mediated by dendritic cells that reside at the cortico-medullary junction (12, 13), although medullary epithelial cells have recently been shown to express many proteins that were previously thought to be tissue- or organ-specific, indicating a potential role for these cells in tolerance to a variety of tissues (14-17). The interactions between lymphoid and stromal cells, whether through cell-cell contact or humoral interaction are thus absolutely vital for normal lymphopoiesis, as was also shown in the chicken (18-20). The humoral aspects of this microenvironment include the secretion of a plethora of cytokines (21), chemokines (22), hormones and especially neuropeptides. Arguably, the thymus has developed into the most fertile paradigm for the study of immuno-neuro-endocrine cross talk during recent

years (23, 24).

T-cells are targets for neuropeptides in several different ways. First of all, primary and secondary lymphoid organs are innervated. This is especially clear around the vasculature at the thymic cortico-medullary junction (25). The presence of parasympathetic (cholinergic) and sympathetic (noradrenergic) innervation in lymphoid organs and the immunomodulatory influences of the classical cholinergic and noradrenergic transmitters have been known for some time (26), but the importance of the complex autonomic peptidergic innervation is a topic of more recent investigations. For instance, double immunofluorescence reveals the coexistence of noradrenergic and NPY-like and/or opioid immunoreactivity, but no evidence, so far, is available of co-release of opioids and noradrenaline from the sympathetic nerve fibers of lymphoid organs (27).

In addition, considerable evidence has now been presented that the thymic stroma is an essential source of endocrine, paracrine and autocrine humoral factors (23). As mentioned above, it is a complex network consisting of epithelial cells (derived from the pharyngeal region), neural crest-derived ectomesenchym, dendritic cells, macrophages (both of bone marrow origin) and fibroblasts and each of these components has been shown to be a potential source of thymic hormones, cytokines and an ever-growing list of hormones and neuropeptides, many of which with yet unknown effects on T-cell physiology (28, 29). One of the most recent surprises includes the finding that the thymus is an important source of parathyroid hormone (30, 31), to the extent that mice without parathyroid glands have only a mildly abnormal bone phenotype, due to the parathyroid

hormone supply from the thymus. Finally, T-cells themselves produce their own set of neuropeptides such as substance P (32), calcitonin gene-related peptide (33), GnRH-I and –II (34) and many more.

The immunological significance of these neuroendocrine elements is currently an area of intensive research. A role in negative selection is one of the possibilities that have been proposed and that has recently gained significant support (16, 17, 35). For instance, expression of insulin in the thymus seems to be linked with clonal deletion of insulin-reactive T-cells, and this might also be the case for the other pancreatic hormones and potentially other peripheral tissue-specific self-antigens (36). In addition, the role of thymic pituitary hormones and neuropeptides may at least partly lay in the regulation of cytokine secretion (29).

Although much less evidence is available in birds, the avian thymus seems also to be an example of immuno-neuroendocrine interaction. Various thymic neuropeptide-positive cells, containing peptides including neurotensin, met-enkephalin, neuropeptide Y, substance P and VIP were demonstrated immunohistochemically by Atoji *et al.* (37-39). Recent studies have also demonstrated the presence of immunoreactive pro-opiomelanocortin (POMC)-related molecules in the thymus of 4-day old chickens, with cell numbers increasing with ageing (40, 41). Nitrergic, peptidergic and substance P innervation of the chick thymus was described by Gulati et al. (42, 43). The functional significance of this neuroendocrine presence within the avian cellular immune system remains elusive.

Neuroendocrine cell markers

Neuroendocrine cells secrete proteins via one of two distinctively different pathways, *i.e.* the constitutive secretory pathway and the regulated secretory pathway. In the regulated secretory pathway, proteins such as pro-hormones and pro-neuropeptides mature in the large dense-core secretory granules of the neuroendocrine cell through a number of post-translational modifications and a specific, extracellular (depolarizing) stimulus is required for delivery (exocytosis) of the secretory products to the outside of the cell. The biogenesis of secretory granules proceeds via two distinctive steps. First, the immature secretory granules are initially formed from the trans-Golgi network (TGN), separate from constitutive secretory vesicles (44). This is followed by the maturation step where the immature secretory granules become mature. Vesicles in the constitutive pathway are pruned away from the immature secretory granules to remove missorted proteins such as mannose-6-phosphate receptors and furin (45, 46).

Chromogranin A (CgA) is considered as an on/off switch controlling the biogenesis of large dense-cored vesicles (LDCV) and essential for the regulated secretory pathway in neuroendocrine cells. For instance, after inhibition of CgA expression by antisense RNAs in PC12 cells, secretory granule formation was essentially lost. On the other hand, knock-in of bovine CgA into the above CgA-deficient PC12 cells restored the regulated secretory pathway (46, 47).

Chromogranin A (CgA) is one of the most abundant acidic secretory glycoproteins ubiquitously present in neuroendocrine/endocrine cells and the major member of the

chromogranin family. About 40% of the total soluble bovine chromaffin granule proteins are CgA(48-50). It thus serves as a useful tissue and serum marker of neuroendocrine cell and neuroendocrine tumor (51). The diversity of tissues containing CgA-positive cells is enormous (including the chromaffin cells of the adrenal medulla, the parathyroid chief cells, thyroid parafollicular C cells, the pancreatic islet cells and gut neuroendocrine cells (52). The ubiquitous distribution of CgA in neuroendocrine and endocrine tissues suggests their general role in the regulated secretion.

Intracellularly, CgA is involved in sorting and packaging of peptides into secretory large dense-cored granules. These are organelles for storage of prohormones, nroneuropeptides, processing enzymes (for peptide hormone processing), and other proteins required for regulated secretion from endocrine and neuroendocrine cells (49) (53) (46). CgA controls the biogenesis of secretory granules, and hence regulated secretion in neuroendocrine/endocrine cells. For instance, 6T3 (a cell line derived from a mouse anterior pituitary tumor cell line AtT-20) lacking the regulated secretory pathway and CgA expression showed recovery of regulated secretory phenotype when CgA was introduced exogenously (47, 54, 55). CgA is also a precursor protein for several bioactive peptides, including catestatin, vasostatin, pancreastatin, and other peptide hormones with autocrine, paracrine, and endocrine activities(56).

In clinical cancer studies, CgA is a useful marker for neuroendocrine tumors (57). Recent work by Fangwen Rao and colleagues suggests that markedly elevated CgA may point to malignant pheochromocytoma. Plasma CgA concentrations may be useful to

gauge tumor response and relapse during chemotherapy of malignant pheochromocytoma (58).

Carboxypeptidase E (CpE, previously also referred to as CpH(59)) appears to be another important element in the process of sorting and processing of prohormones (see Fig.2). Carboxypeptidase E *per se* is an exoproteolytic processing enzyme exclusively present in the Golgi apparatus and secretory granules of neural/neuroendocrine cells. Its function is to remove basic amino acids residues exposed upon endoproteolyic cleavage of the hormone precursor by a specific prohormone convertase. The membrane-bound form of the enzyme is anchored in the wall of the secretory granules through its COOH-terminal end, which also serves as a sorting receptor for several prohormones and proneuropeptides in neuroendocrine cells(60, 61).

Somatostatin and somatostatin receptors in the immune system

As mentioned above, lymphoid organs can be considered preferential sites of immuno-neuroendocrine interactions. They produce several different categories of messengers, hormones or neuropeptides, which exert their local paracrine activities through their corresponding receptors on immune cells. Among the hormones and neuropeptides involved in regulating immune cell activities, we have decided to focus on somatostatin which has been shown in mammals to exert multiple effects on immune functions (62).

Somatostatin (SST, also referred as somatotropin release-inhibiting factor, or SRIF) is a cyclic neuropeptide originally found in hypothalamus, where it was identified as a potent

inhibitor of the secretion of growth hormone (GH) and thyroid-stimulating hormone (TSH) from the anterior pituitary(63, 64). In fact, SST and its receptor SSTR then have been found throughout the body, including pancreas, salivary gland, kidney, lymphoid cells, blood vessel walls, etc., and exert multiple physiological effects. For review, see (65-67).

SST, like many other neuropeptides, is initially synthesized from one single gene as a larger preprosomatostatin with, in mammals, 92 amino acids. It is then cleaved at its C-terminus to form two biologically active forms, SST-14 (See Fig. 1.1) and SST-28, composed of either 14 or 28 amino acids, respectively (68, 69). Chicken SST-14 has the exact same amino-acid sequence as in mammals, while in SST-28 there is one amino acid difference Hasegawa (70). SST has a very short half-life (1.5~3 minutes) in the systemic

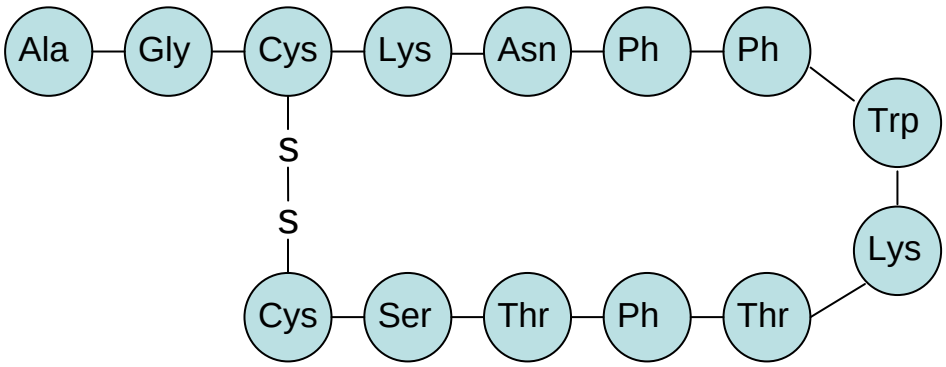


Fig.1.1. The structure of somatostatin-14. Chicken SST-14 has the exact same amino-acid sequence as in mammals.

circulation (71), indicating that SST-producing cells, or stores of SST are probably close to the target cells. Synthetic analogs were developed, such as octreotide, with a longer half-life and resistance to proteolytic degradation for clinical applications.

Five somatostatin receptor (SSTR) subtypes have been identified and characterized in mammals; they are SSTR-1, -2, -3, -4 and –5, encoded by five different, intronless genes (72, 73). In addition, rodent SSTR-2 has two isoforms, SSTR-2A and SSTR-2B, due to alternative splicing (74). SSTRs have seven α-helical trans-membrane domains, with a structure of three intra and extra cellular loops, and they are all coupled with G-protein (75). SSTRs are highly conserved between species; for example, SSTR1 of mouse and human share 99% sequence identity(76). Within the same species, there is a 45–61% sequence identity between different receptor subtypes. Both SST-14 and SST-28 bind to SSTR1–4 with equal affinity. However, SST-28 has a higher affinity for SSTR5 (76).

In mammals, the SSTRs are widely expressed in different tissues throughout the body, with different expression level and subtype combinations (77). SSTR is distributed in a tissue-specific fashion, while the majority of SST-target tissues express multiple SSTR subtypes at the same time (78).

The structure of SSTR subtypes 1-5 is highly homologous (40–60%), but evidence has supported the concept that each subtype mediates different biological actions of SST. For example, in humans, SSTR2 and SSTR5 are involved in controlling growth hormone release, and SSTR5 appears to be important in modulating insulin and glucagon release

(79). Apoptosis can be induced by activation of SSTR3, whereas SSTR1, SSTR4 and SSTR5 have an inhibitory effect on the cell cycle (80). Depending on the cell type, the five SSTR subtypes are coupled to a variety of signal transduction pathways in exerting various physiological functions, including adenylate and guanylate cyclase, phospholipase A2 and C, K^+ and Ca^{2+} channels, Na^+–H^+ exchanger, Src, Erk1/2, p38 mitogen-activated protein kinases, and tyrosine phosphatases (80).

SST has been shown to have multiple effects on various immune cells. Studies regarding the effects on immune cell proliferation, secretion, migration and apoptosis reveal that the immunomodulatory actions by somatostatin are complex and depend on various physiological and experimental conditions, as summarized in the Table 1.1.

Table 1.1. Heterogeneous effects of somatostatin on immune cells (81) (↑ stands for upreulation; ↓ downregulation).

	Thymocytes	T-cells	B-cells	Monocytes
Cell proliferation	↓	↑ ↓	↓	↔
Colony growth	–	↓	–	–
Cell secretion	–	↑ ↓	↓	↑ ↓
Apoptosis	↑	↑	↑	–
Cell adhesion	–	↑	–	–
Cell migration	↑	↑	–	↑ ↓

SST can influence the functions of immune cells in both health and disease (reviewed in (82)). An *in vivo* study on rats showed that as a consequence of repeated treatments with low doses of somatostatin-14, the volumes of thymus cortex and medulla, the total number of thymocytes, the number of thymocytes in the cortex and medulla and the numerical density of thymocytes in the deeper cortex were all decreased. Thymus size was also diminished (83). In a study of human thymuses, a negative correlation between the receptor density and the chronological age of the thymus has been demonstrated (84). The effect of SST on T-cell proliferation seems complex, as SST has been observed to both suppress and stimulate T-lymphocyte proliferation and inhibit colony formation (82). With regard to cytokine regulation, SST was reported to have inhibitory effects on IL-2 secretion from different human T-cell lines (85) and on the secretion of TNF-α, IL-1β and IL-6 in monocytes. It was also shown to inhibit the chemotaxis of human neutrophils (86). It is noteworthy that SST may regulate human T-lymphocyte migration and homing (87). Also, SST was shown to inhibit IgE and IgG4 production by plasma B-cells (88).

A potential autocrine/paracrine regulatory role has been suggested for SST in mouse (36, 89). SST and the SST analog octreotide have been shown to inhibit the proliferation of primary cultured thymic epithelial cells (90). Moreover, the hypothesis that a disturbance in the endogenous production of SST might be involved in the pathogenesis of autoimmune and neoplastic diseases was supported by *in vitro* data (91). In a more recent study, SST has been found to increase thymocyte numbers in fetal thymic organ culture, to enhance maturation and to induce the cell migration (92). In the same study, SST has

been shown to increase the cellular proliferation of total splenocytes, but to inhibit proliferation of thymocytes in suspension and purified splenic T-cells (92).

The intracellular signalling mechanisms coupled to SSR activation in immune cells have been less investigated so far. SST was previously shown to inhibit adenylyl cyclase activity, but only at very high, non-physiological concentrations (93). However, Cardoso et al. (85) have shown that SST inhibited adenylate cyclase activity in mitogen-activated human peripheral blood mononuclear cells (PBMC) and in Jurkat T cells in a dose-dependent fashion at nanomolar concentrations. Many studies have found that the effects of SST on immune secretion, proliferation or other functions, are 'biphasic'(94). That is, inhibitory effects were maximal at nanomolar concentrations of SST, and reduced or absent at higher (micromolar) concentrations. The latter effect may be the consequence of the receptor internalization process with a subsequent down-regulation, an uncoupling from second messenger activation, or even the activation of different intracellular second messenger pathways via distinct SSR subtypes.

SST is also thought to play a role in the regulation of apoptosis. SST and octreotide have been shown in human to induce apoptosis via SSTR2A and SSTR3 (95, 96), and apoptosis and chromosome breakage has been observed in activated human lymphocytes exposed to synthetic SST analogs (97). More recently, SST-14 and octreotide were demonstrated to inhibit [^{3}H] thymidine incorporation in selected human thymocyte subsets, indicating the presence of functional receptors on these cells, while SST-14, but not octreotide, induced a significant increase in the percentage of apoptotic thymocytes (98).

In summary, SSTR subtypes appear to be differentially expressed on specific cell subsets within the organs of the immune system. The expression of neuropeptide receptors on immune cells is dynamically regulated and may depend on the traffic of these cells through and within lymphoid structures and homing in tissues. SST may be involved in the regulation of a number of different immune cells but it may also regulate diverse functional aspects in the same type of immune cells (*e.g.* proliferation, secretion, migration). SST effects on immune cells, as is the case of other neuropeptides, seem to be based mainly on autocrine and paracrine modes of action contrasting with its endocrine activities along the hypothalamo-pituitary axis (81). In fact, the following chapters will further address this question on a chicken model.

CHAPTER II

IMMUNOHISTOCHEMICAL ASSESSMENT OF CGA-, CD57- AND NSE-POSITIVE CELLS IN THE CHICKEN THYMUS: DISTINCT NEUROSECRETORY CELL POPULATIONS IN A PRIMARY LYMPHOID ORGAN

Introduction

The concept that immune cells and immune response are significantly influenced by the nervous/neuroendocrine system is well established in mammals. For example, hypothalamic and pituitary hormones such as PRL, GH, ACTH, LHRH play a role in thymic cell proliferation (99). In addition, central and peripheral lymphoid organs like thymus and spleen are innervated by both sympathethic and parasympathethic components (100). Classic neurotransmitters, such as catecholamines and acetycholine, and peptide transmitters (*e.g.* NPY, VIP) from neural origin are released in the lymphoid microenvironment and participate in the immune modulation. However, the function of locally existing neuroendocrine cells within lymphoid organs still largely remain elusive. The diffuse neuroendocrine/endocrine cells are relatively poorly defined in immune system, especially in birds. Although the presence of neuropeptides (such as neurotensin and somatostatin) in the chicken thymus was reported as early as 1978 (101), a methodical analysis of neurosecretory cells in a chicken lymphoid organ, regardless of the secretory product involved, has to our knowledge, not been described. The diffuse neuroendocrine/endocrine cell populations are believed to produce major chemical

categories of messengers, hormones or neuropeptides, which exert locally their paracrine activities through their receptors on immune cells(98).

In an attempt to uncover this complex neuroendocrine-immune network, a first and essential step is to identify and characterize these neuroendocrine populations within immune organs/tissues. This study aimed to use an immunocytochemical approach to assess the molecular heterogeneity of the neuronal/neuroendocrine derived component within the chicken thymus, by employing a novel monoclonal antibody (Mab) against turkey chromogranin A (102), a commercial Mab against CD-57, i.e. HNK-1, a classical marker for neural crest-derived cells (103) and a polyclonal antiserum against neuron-specific enolase (NSE) (43).

Chromogranin A (CgA) is one of the most abundant acidic secretory glycoproteins ubiquitously present in neuroendocrine/endocrine cells and the major member of the chromogranin family (40% of the total soluble bovine chromaffin granule proteins) (48-50). It thus serves as a useful tissue and serum marker of neuroendocrine cell and neuroendocrine tumors(51). The diversity of tissues containing CgA-positive cells is enormous (including the chromaffin cells of the adrenal medulla, the parathyroid chief cells, thyroid parafollicular C cells, the pancreatic islet cells and gut neuroendocrine cells (52). The ubiquitous distribution of CgA in neuroendocrine and endocrine tissues suggests its general role in the regulated secretion.

Intracellularly, CgA is involved in sorting and packaging of peptides into secretory large

dense-cored granules, organelles that designed for storage of prohormones, proneuropeptides, processing enzymes (for peptide hormone processing), and other proteins required for regulated secretion from endocrine and neuroendocrine cells (49) (53) (46). CgA has been shown to control the biogenesis of secretory granules, and hence indirectly also regulated secretion in neuroendocrine/endocrine cells. For instance, 6T3 (a cell line derived from a mouse anterior pituitary tumor cell line AtT-20) lacking both the regulated secretory pathway and expression of CgA, showed recovery of regulated secretory phenotype when CgA was introduced exogenously (47, 54, 55). CgA is also a precursor protein for several bioactive peptides, including catestatin, vasostatin, pancreastatin, and other peptide hormones with autocrine, paracrine, and endocrine activities.

Neuron specific enolase (NSE) is a highly acidic homodimeric 78kDa protein (104) that has been found in central and peripheral nervous elements and also in many neuroendocrine cells and neuroendocrine tumors (105). NSE is thus regarded as one of the best described indicators for neurons. CD57 (the antigen recognized by the monoclonal antibodiey HNK-1) is generally considered to be a classical marker for neural crest-derived cells (106). There was evidence that the CgA-producing cells are derived from neural crest progenitor cells, a transient, migratory, multipotent precursor cell population known to generate much of the peripheral nervous system, epidermal pigment cells and a variety of mesectodermal derivatives(107). Normal development of the thymus is also dependent on correct development and patterning of neural crest cells (11, 108, 109) (110). By use of these traditional markers for (neuro)endocrine, nervous

and neural crest-derived cells, the neuroendocrine components of the chicken thymus were systematically surveyed in this report.

Materials and methods

Primary antibodies

Monoclonal anti-turkey hypophysial CgA antibodies were obtained as a side product during the production of Mabs against a preparation of highly purified turkey hypophysial prolactin that contained trace amounts of CgA. These anti-CgA Mabs have been extensively used for immunohistochemical analyses of turkey and chicken pituitary sections (references, please). The identity of the antigen recognized by these Mabs was established by tandem mass spectrometry *de novo* sequencing of seven tryptic peptides from a turkey pituitary protein purified by immunoaffinity chromatography (102). Rabbit anti-NSE antiserum was obtained from ImmunoStar (Hudson, WI) and monoclonal mouse anti-CD57 (HNK-1) was obtained from Ancell Corporation (Bayport, MN). The former is a marker for neuronal cells (43), while the latter is a marker for neural crest-derived cells (103, 111).

Tissue sampling and processing

White Leghorn chicks obtained from a local hatchery (Hy-line) at 1 day of age were raised in wire cages at the Texas A & M Poultry Science Center. The birds were euthanized at 1, 3, 4, 7, 8, 10, and 13 weeks of age. Thymic tissues were excised and fixed for 24 h at room temperature in Bouin Hollande sublimate. Bouin Hollande sublimate was prepared by adding 10 ml of saturated $HgCl_2$ solution to 90 ml of Bouin

Hollande solution. The tissue blocks were then processed for paraffin embedding using routine laboratory protocols. Seven-µm thick tissue sections were made with a rotary microtome MT 980 (Research and Manufacturing Co., Inc).

Immunohistochemical reagents

TBST is a Tris-buffered saline containing 0.1% Triton X-100, pH 7.4. Trizma base and Triton X-100 were obtained from Sigma (St Louis, MO). Biotinylated goat anti-mouse immunoglobulin (Ig) and goat anti-rabbit Ig, Rhodamine Red-conjugated goat anti-mouse Ig, peroxidase-conjugated streptavidin, and FITC- and Rhodamine Red-conjugated streptavidin were obtained from Jackson Laboratories (West Grove, PA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM, biotinylated goat anti-mouse IgG and DAB (3,3′-diaminobenzidine tetrahydrochloride) were obtained from Sigma (St Louis, MO). Cytoseal (a xylene-based mounting medium) was obtained from Stephens Scientific (Kalamazoo, MI). Vectashield (a water soluble mounting medium) was purchased from Vector Laboratories (Burlingame, CA).

Immunohistochemical detection of CgA

Immunohistochemical staining was performed on paraffin sections of thymic tissues from 1, 3, 4, 7, 8, 10, and 13 week-old chickens. The tissue sections were dewaxed and hydrated using standard laboratory procedures. The sections were first incubated in a moist chamber for 1 h with 10% (v/v) normal goat serum, and then overnight with monoclonal mouse anti-turkey CgA Mab designated PL1G7F11 at a 1:3000 dilution (starting from undiluted ascitic fluid) in TBST (rinsing and dilution buffer). The next day,

the sections were rinsed with TBST and the detection protocol employed a 30 min incubation with biotinylated goat anti-mouse IgG (1:900) followed by a 30 min incubation with peroxidase-conjugated streptavidin (1.5 μg/ml). The enzyme reaction was developed for approximately 10 min using 25 mg of DAB and 75 μl of 30% (v/v) H_2O_2 in 200 ml of 50 mM Tris–HCl, pH 7.4. A number of sections were counterstained with hematoxylin–eosin. The sections were dehydrated, coverslipped with Cytoseal, and immunoreactive (ir) cells were observed with an Olympus BX50 light microscope (Leeds Instruments, Inc., Irving, TX) and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St Sterling Heights, MI).

Immunofluorescent double staining: CgA vs. NSE and CgA vs. HNK-1

A direct comparison between the CgA- and NSE-ir cell populations was achieved by immunofluorescent double staining on the same tissue section. Upon dewaxing and rehydration, the sections were incubated overnight simultaneously with both primary antibodies, *i.e.* monoclonal mouse anti-CgA (PL1G7F11, ascitic fluid diluted 1:3000) and rabbit anti-NSE (purchased ready for use). The next day, the sections were rinsed with TBST and incubated simultaneously with Rhodamine Red-conjugated goat anti-mouse Ig (1:200) and biotinylated goat anti-rabbit Ig (1:500) for 90 min. Upon rinsing, the sections were finally incubated with FITC-conjugated streptavidin (3 μg/ml TBST) for 30 min, rinsed and coverslipped with Vectashield.

The dual staining strategy for the comparison of CgA- and HNK-1-positive cells took advantage of the fact that the former is a mouse IgG, whereas the latter is a mouse IgM.

Therefore, both primary antibodies (HNK-1 at 2 μg/ml and mouse anti-CgA ascites at 1:3000) were combined for simultaneous incubation on the same section. The next morning, the sections were rinsed and incubated with the combined secondary antibodies (FITC-conjugated anti-mouse IgM at 1:400 and biotinylated goat anti-mouse IgG at 1:300). The slides were rinsed again and incubated with Rhodamine Red-conjugated avidin (1:400) for 40 min. Upon final rinsing, the slides were coverslipped with Vectashield. Negative controls accounting for potential cross-reactivity of the secondary reagents with mouse IgG or IgM were negative.

Thymic microsomal fraction

Chicken thymic granules (microsome) were prepared by a previously described procedure (112-114). Briefly, chicken thymic lobes from a 6-week old broiler were homogenized in 0.32 M sucrose buffered with 10 mM Hepes (pH 7.4) with a glass Dounce homogenizer. Ten percent of Protease inhibitor cocktail (Sigma P2714) was added to the homogenate. All the reagents and samples were cooled on ice. The homogenate was centrifuged at 2,200g for 10 min to remove the nuclei and the rough microsomal fraction was spun down at 100,000g for 2 hours. The pellets were resuspended and stored at –80C until analysis by SDS-PAGE and western blot.

CgA SDS-PAGE and Western blotting

Chicken thymic granules, protein extracts from fresh chicken thymus, adrenal and pituitary were mixed with identical volumes of SDS-PAGE reducing sample buffer. Equal volumes of all samples were loaded onto a 12% Tris-HCl Bio-Rad precast

polyacrylamide gels, and CgA was determined by Western blot. After the proteins were electrotransferred to a nitrocellulose membrane (BioRad), the membrane was blocked with blocking buffer (Li-COR, Nebraska USA) and incubated with anti-CgA primary antibody (1:5000) for 5 hrs. Alexa Fluor 680-conjugated goat anti-mouse IgG (1:2500, Molecular Probes) was applied for signal detection. Odyssey protein weight markers were obtained from Li-Cor. The membrane was scanned with an Odyssey System (Li-COR, Nebraska USA).

Results

Immunocytochemical demonstration of chromogranin A (CgA)-positive cells

Intensely stained CgA^+ cells were readily detectable in the chicken thymus at all ages tested (Fig.2.1A–D). The overall staining characteristics of the cells varied little with age, with the exception that cells seemed to increase in number with age (results not shown). Groups of immunopositive cells were distributed in a typical ring-like fashion, one ring in every thymic lobule. Counterstaining with hematoxylin–eosin (Fig.2.1B) showed that the ring of CgA^+ cells was located at the medullary side of the cortico-medullary border. As a consequence, CgA^+ cells were located in close proximity to the arterioles and the venules that provide the blood supply to the thymus. Upon high power magnification (Fig. 2.1C and D), it became clear that the CgA^+ cells constitute a population of cells with diverse morphologies. While the majority of cells displayed a simple round to oval shape, some cells possessed very conspicuous extensions, reminiscent of a neuron-like morphology.

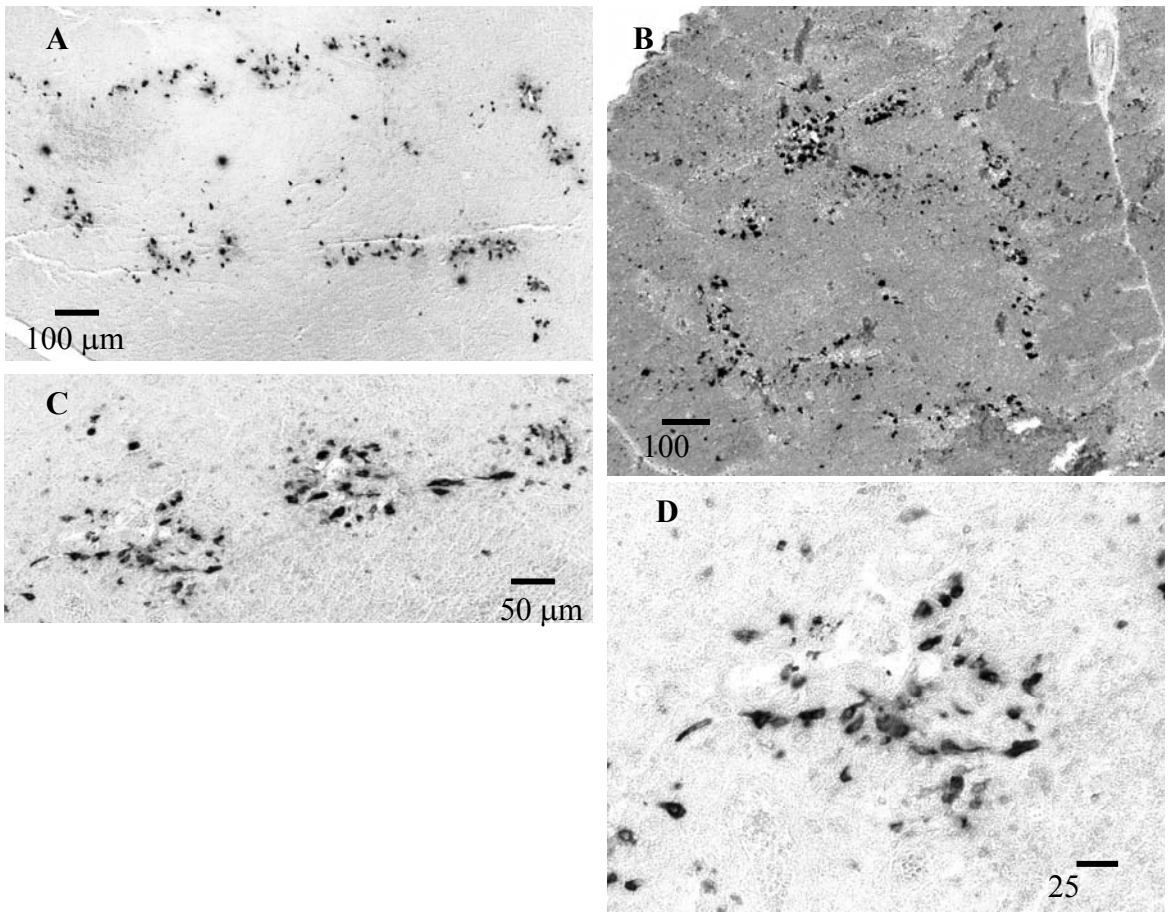


Fig. 2.1. Immunocytochemical demonstration of chromogranin A in the thymus of a 2-week old chicken. (A) Low power magnification shows intensely stained groups of immunopositive cells that are arranged in a typical ring-like distribution. There is little evolution of this picture with age, except for an apparent increase in the number and stainability of the cells. (B) Same as (A), but with hematoxylin–eosin counterstaining. The outer region (cortex, indicated by C) of each thymic lobule is stained somewhat darker by the counterstain than the inner area (medulla, indicated by M). The ring of CgA-positive cells is located at the medullary side of the corticomedullary border, in the neighborhood of arterioles and veins that are responsible for the blood supply to each of the lobules. Both the cortex and the central area of the medulla are essentially devoid of stained cells. (C) Same as (A) but at higher power magnification. This picture clearly shows the morphological heterogeneity of the CgA-positive cell population. Some cells have a simple, round or oval appearance, while other cells clearly show the neuron-like morphology with several extensions (See E). (D) Higher power amplification of the left hand cluster of cells from (C). This photomicrograph shows another illustration of the stellate appearance of some of the cells. Also note the proximity of the blood vessels near the CgA-positive cluster of cells.

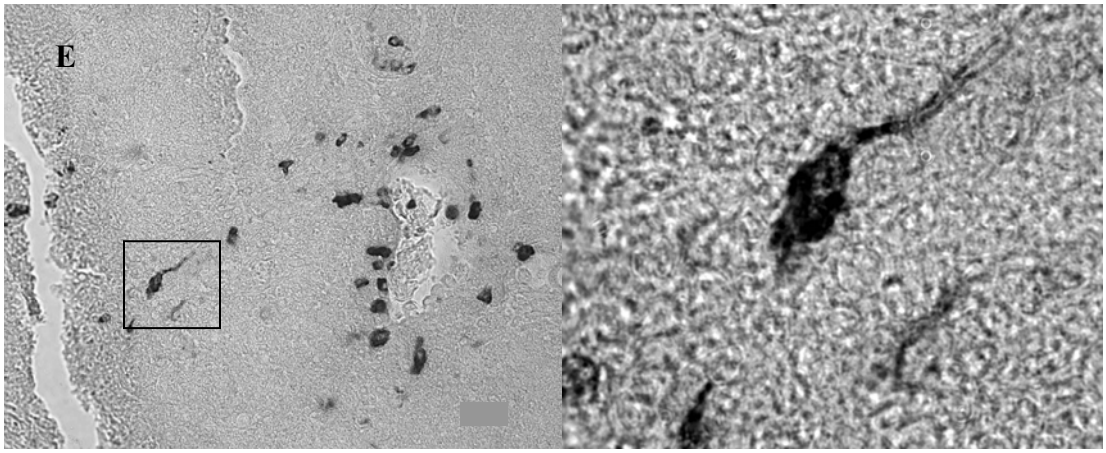


Fig. 2.1. Continued.

Western blot of CgA in thymic cell and thymic microsomal fraction

To further confirm the expression of CgA in the chicken thymus, SDS-PAGE and Western blot were carried out by using the same antibody as the one used for immunocytochemistry. A clear band with a molecular weight of approximately 70k Da was observed in the sample of total protein extracts from chicken thymus in Western blot. The CgA blotting pattern was similar to a previous study in chicken pituitary (102). Chicken adrenal and pituitary protein extracts were used as positive controls (See Fig. 2.2).

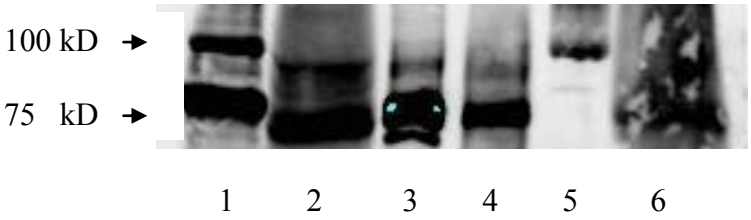


Fig. 2.2. CgA western blot of SDS-PAGE separation of chicken protein extracts from thymus, adrenal, pituitary and thymic microsomal fraction. A major band about at 70 kD was observed from samples of protein preparations and thymic microsomal granule fraction. Lane 1. Standard; 2. Thymus; 3.Adrenal; 4. Pituitary; 5. BSA; 6.Granule.

Immunofluorescent dual staining experiments

The reputation of CgA as a neuroendocrine marker protein and especially the occurrence of long cytoplasmic, axon-like extensions on some of the CgA^+ cells prompted us to explore the possibility that these cells were related with neuronal or neural crest-derived cells. For this purpose, single and dual staining experiments were performed with commercial antibodies recognizing NSE and CD57, respectively. As shown in Figs. 2.3 and Fig. 2.4 A and B, both antisera reacted with discrete thymic cell populations. NSE^+ cells were relatively scarce and fairly weakly stained, whereas $CD57^+$ cells, stained with HNK-1, were intensely stained, fairly abundant, and, more importantly, located in the exact same area as the CgA^+ cells. This is obvious when comparing the respective counterstained sections (Fig. 2.1 and Fig. 2.4).

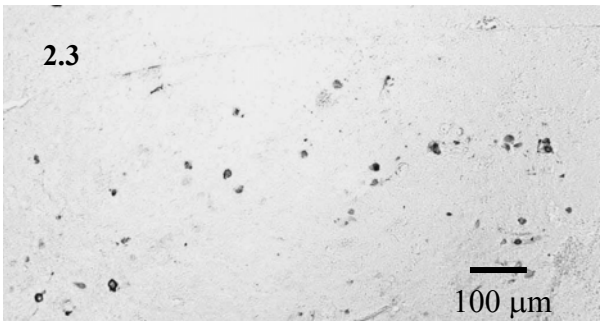


Fig. 2.3. Immunocytochemical demonstration of neuron-specific enolase (NSE) in the thymus of a 2-week old chicken. NSE-positive cells are much less abundant than CgA cells and they also stain much less intensely.

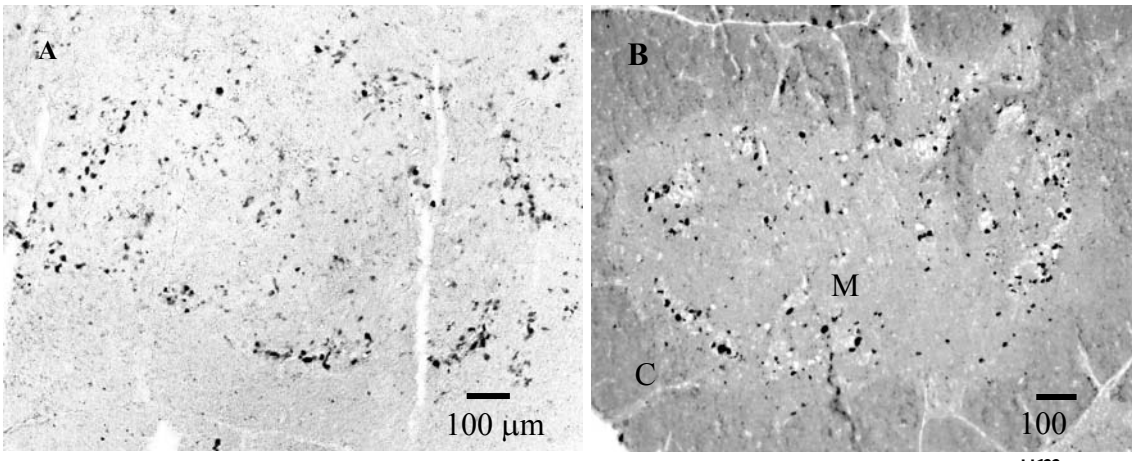


Fig. 2.4. Immunocytochemical demonstration of CD57 in the thymus of a 2-week old chicken by use of monoclonal antibody HNK-l. (B) Is the same as (A) but counterstained with hematoxylin and eosin. When compared with Fig. l(A) and (B), CD57-positive cells clearly have a very similar distribution pattern as the CgA-positive cells: more or less clustered cells in a ring-like distribution at the medullary side of the cortico-medullary border. CD57-positive cells seem slightly less numerous than CgA positive cells. C, cortex; M, medulla.

However, when compared on the same tissue section, it became clear that neither the NSE^+ cell population (Fig. 2.5), nor the $CD57^+$ population (Fig. 2.6) overlapped to a noticeable extent with the CgA^+ cells, since not a single clearly yellow stained cell could be found. This leads to the conclusion that the neural/neuroendocrine cellular component in the avian thymus is a complex entity that appears heterogeneous both in morphology and lineage.

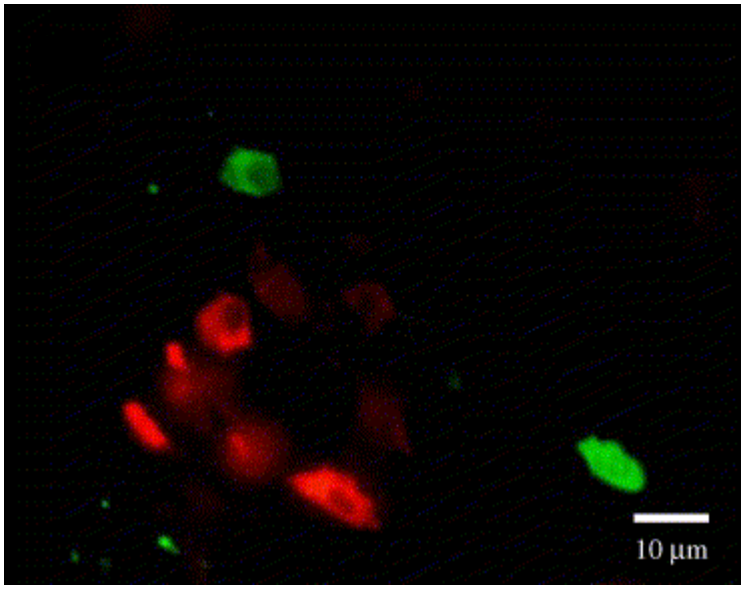


Fig. 2.5. Immunofluorescent dual staining of CgA and NSE on the same thymus section of a 2-week old bird. The picture shows red CgA-positive and green NSE-positive cells. No double-stained (yellow) cells could be observed.

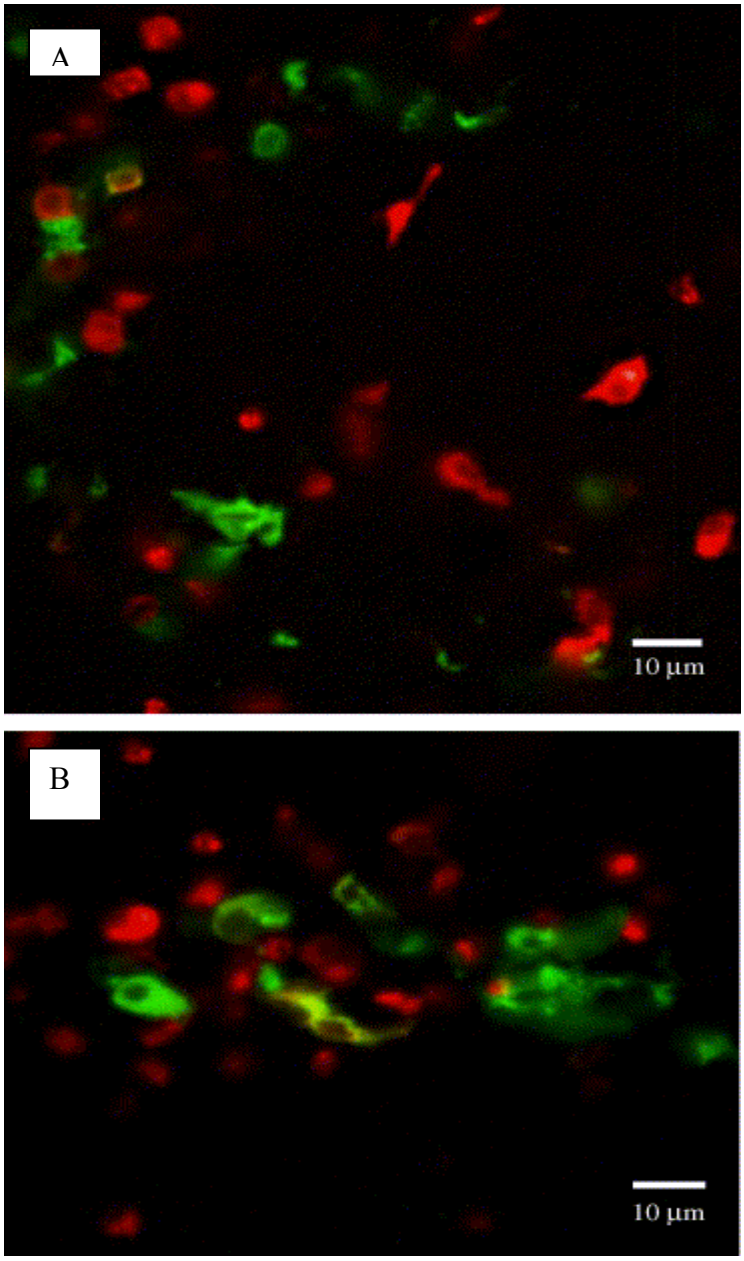


Fig. 2.6. Immunofluorescent dual staining of CgA and CD57 on the same thymus section of a 2-week old bird. CgA-positive cells are stained red while CD57-positive cells are stained green. The overlap between both populations is virtually non-existent. An occasional yellowish cell is likely a spatial superposition of a red and a green cell located in different planes.

Discussion

By the availability of a new monoclonal antibody against turkey chromogranin A (CgA), this investigation described for the first time the neuroendocrine component of the avian thymus by use of traditional markers for (neuro)endocrine, nervous and neural crest-derived tissue. In practical terms, molecular markers are invaluable in defining neuroendocrine cells and in particular chromogranin A (115), (116). The obtained immunohistochemical evidence, in combination with the identification of thymic CgA expression by Western Blot, has allowed us to demonstrate the storage and production of chromogranin A by a discrete cell population in the chicken thymus. CgA controls the biogenesis of secretory granules, and hence regulated secretion in neuroendocrine (endocrine) cells. Secretory large dense-cored granule is responsible for storage of prohormones, proneuropeptides, processing enzymes and other proteins required for regulated secretion in endocrine and neuroendocrine cells. Indeed, a number of reports have described (neuro)endocrine molecules in the avian thymus, such as neurotensin (101) (117) (37) (43) (38), neuropeptide Y, substance P, vasoactive intestinal polypeptide (43)and (37), proopiomelanocortin derivatives (41), calcitonin gene-related peptide (43) and somatostatin (101) (118).

Both the location of the CgA^+ cells (in the medulla of the lobules near the corticomedullary border) and their morphological heterogeneity (round to oval, but also stellate cells) are in remarkable agreement with the nitrergic cells that have been described earlier in the thymic microenvironment of the chicken by Gulati et al. (42, 43, 119). Therefore, immunofluorescent dual staining experiments were performed with a

neuron-specific marker, rabbit anti-NSE. While some NSE^+ cells were detected in the same area as the CgA^+ cells, it was quite clear that these were two totally separated cell populations without detectable overlap (Fig. 2.5).

Single stained sections showed a $CD57^+$ cell (i.e. Neural crest-derived cells) population with the same typical ring-like distribution as the CgA^+ cells. However, dual-staining experiments could not demonstrate the slightest overlap between the two cell populations (Fig. 2.6). LaBonne *et al.* reported that CgA-producing cells are derived from neural crest progenitor cells, a transient, migratory, multipotent precursor cell population known to generate much of the peripheral nervous system, epidermal pigment cells and a variety of mesectodermal derivatives (107). While these data confirm the presence of neural crest-derived cells in the avian thymus (120), they fail to convincingly categorize the CgA^+ cells as neural crest derivatives. A possible explanation is that these neuroendocrine phenotypes could be changed or switched during the course of cell development.

Angeletti and Hickey (121) first described CgA-positive cells as a potential link between the nervous and immunological systems. Indeed, the substantial number of CgA^+ cells and the ease with which they can be stained using a moderately sensitive, non-amplified enzymatic method is noteworthy. The profile of every lobule was characterized by a ring of clustered CgA^+ cells at the medullary side of the corticomedullary border, suggesting that in a 3-dimensional view, the medullae of the thymic lobules are surrounded by a spherical lattice of neuroendocrine cells. The cortico-medullary boundaries of the chicken are important cross-roads of communication because they have been shown to receive

vagal cholinergic (acetyl cholinesterase-positive) nerves, but they are also innervated by sympathetic perivascular catecholaminergic plexi (122). As such, the CgA-positive neuro-endocrine cells (as well as the $CD57^{+}$-positive neural crest-derived and NSE-positive neural cells) are potential targets of the autonomous nervous system and of systemically circulating signals.

In response to the stimulus, those molecules co-stored with CgA as well as CgA derivatives in the secretary granules are expected to be released intoto the microenvironment of the thymus. The biological functions of these amines or peptides are now being extensively studied. For instance, the CgA fragment corresponding to amino acids 1–76 (vasostatin-1) inhibits vascular tension, exerts antifungal and antibacterial effects, promotes cell adhesion, and affect muscle contraction(56). Another CgA-derived bioactive peptide, catestatin, is a potent catecholamine antagonist that counteracts the biological action of catecholamine, which is co-stored with CgA (123, 124). Some of the latest studies also link CgA and catestatin with hypertension, providing insight in new therapeutic agents and genetic predisposition to high blood pressure (125).

In conclusion, the neuroendocrine component of the avian thymus was characterized as acomplex system consisting of at least three apparently distinctive cell populations thatpotentially receive input from each other, from the autonomous nervous system or fromthe circulation. The nature of the messenger molecules co-stored with CgA, the mechanism by which they are released and their effects on the proliferation and differentiation of the developing T-cell populations will be topics of further investigation.

CHAPTER III

CARBOXYPEPTIDASE E, AN ESSENTIAL ELEMENT OF THE REGULATED SECRETORY PATHWAY, IS EXPRESSED AND PARTIALLY CO-LOCALIZED WITH CHROMOGRANIN A IN CHICKEN THYMUS

Overview

The study in this chapter aimed at assessing the expression of carboxypeptidase E (CpE) and its potential co-existence with chromogranin A (CgA) in the diffuse neuroendocrine system in the thymus of the chicken. Both CgA and CpE are characteristic functional and structural elements of the regulated secretory pathway. CpE is a processing enzyme that catalyzes the transformation of peptide precursor proteins into bioactive peptide hormones, but it also serves as a sorting receptor in the regulated secretion pathway, while chromogranin A is a classic marker protein for neuroendocrine cells. The immunohistochemical evidence presented in this investigation, combined with the identification of the chicken CpE mRNA by RT-PCR, has allowed us to demonstrate the co-existence of CgA and CpE in identical neuroendocrine cells in a primary lymphoid organ. The CpE- and CgA-positive cells are primarily found in the transition zone between the cortex and the medulla of the thymic lobules, an area known to contain numerous arterioles and to be heavily innervated by the autonomic nervous system. It is tempting to speculate that the diffuse neuroendocrine system may serve as a relay for nervous stimuli delivered by sympathetic and/or parasympathetic nervous system and/or for humoral factors delivered by the circulation, which would provide a mechanism for fine tuning thymopoiesis by a variety of physical and environmental factors.

Introduction

The thymus is the primary lymphoid organ for T-cell development and thus plays a pivotal role in the vertebrate cellular immune system. Considerable evidence has been presented that the thymus is also the source of an ever growing list of hormones and neuropeptides(23), for review, see(28). The immunological significance of these neuro-endocrine elements has been intensively investigated. Hormones play an essential role in establishing central T-cell tolerance (126). For example, thymic epithelial cells express insulin causing clonal deletion of insulin-reactive T-cells (127). However, mounting evidence shows that thymic neuropeptides also function as humoral signaling factors affecting T-cell physiology. In this context, neuropeptides act as immunomodulators (23) that participate in the homeostatic regulation and provide essential growth regulatory signals - ranging from stimulatory to pro-apoptotic - to thymocytes, as well as in the regulation of cytokine secretion (18, 19, 28, 29). For instance, somatostatin, a neuropeptide mainly produced in the brain, is highly expressed in murine thymus and was shown to be able to regulate thymocyte development and migration (92).

Also the avian thymus appears to be an example of immuno-neuroendocrine interaction. Various thymic neuropeptide-positive cells, containing peptides including neurotensin, met-enkephalin, neuropeptide Y, substance P and VIP were demonstrated immunohistochemically by Atoji *et al.*(37). Other studies have demonstrated the presence of immunoreactive pro-opiomelanocortin (POMC)-derived molecules in the thymus of 4-day old chickens, with cell numbers increasing with ageing (40, 41). Finally, nitrergic and peptidergic innervation of the chick thymus was described by Gulati et al.(42, 43).

While the effects of these humoral elements on T-cell physiology are now being elucidated, their releasing mechanism is largely unknown. Neuroendocrine cells secrete proteins via one of two distinctively different pathways, *i.e.* the constitutive secretory pathway and the regulated secretory pathway. In the regulated secretory pathway, proteins such as pro-hormones and pro-neuropeptides mature in the secretory granules of the neuroendocrine cell through a number of post-translational modifications and a specific, extracellular (depolarizing) stimulus is required for delivery (exocytosis) of the secretory products to the outside of the cell. Chromogranin A (CgA) is considered as an on/off switch in neuroendocrine cells, controlling the biogenesis of large dense-cored vesicles (LDCV) and essential for the regulated secretory pathway. For instance, after inhibition of CgA expression by antisense RNAs in PC12 cells, secretory granule formation was essentially lost, whereas, knock-in of bovine CgA into these CgA-deficient PC12 cells restored the regulated secretory pathway (46, 47).

In the process of sorting and processing of prohormones, carboxypeptidase E (CpE, previously also referred to as CpH(59) appears to be another crucial element. Carboxypeptidase E *per se* is an exoproteolytic processing enzyme exclusively present in the Golgi apparatus and secretory granules of neural/neuroendocrine cells. Its function is to remove basic amino acids residues exposed upon endoproteolyic cleavage of the hormone precursor by a specific prohormone convertase at the site of a dibasic pair (e.g. Lys-Lys). The membrane-bound form of the enzyme is anchored in the wall of the secretory granules through its COOH-terminal, which also serves as a sorting receptor for several prohormones and proneuropeptides in neuroendocrine cells (60, 61).

In the previous chapter, we have shown the existence of a complex neuroendocrine cell population within the chicken thymus, including a CgA-immunoreactive cell population (128). Here we further investigate whether carboxypeptidase E is also expressed in the chicken thymus, and more specifically in the previously described CgA-positive cells. The co-existence of CpE and CgA in the same cells in the chicken thymus would provide strong circumstantial evidence suggesting that the release of the neuropeptides in the thymus occurs, at least partly, through the regulated secretory pathway. This, in turn would imply that a specific stimulus is responsible for the release of neuropeptides under the proper physiological conditions. In this study, the cDNA sequence of chicken carboxypeptidase E was first assembled *in silico* and a reverse transcription polymerase chain reaction was performed to examine the production of carboxypeptidase E mRNA within chicken thymus. In addition, an immunocytochemical approach was used to confirm the presence of CpE, and to assess its cellular co-localization with CgA.

Materials and methods

Chicken carboxypeptidase E cDNA sequence

A chicken CpE cDNA contig was assembled in silico based on the public chicken Expressed Sequence Tag (EST) and chicken genome databases. The human carboxypeptidase E cDNA sequence (GI: 31565486) was compared to the available sequences in the chicken EST database (http://www.chickest.udel.edu/ Cogburn_CAP3_DB/blast.html). The resulting EST sequences with high homology were assembled into one consensus sequence using CAP3 program at

http://deepc2.zool.iastate.edu/aat/cap/cap.html. The assembled chicken CpE cDNA sequence was then verified by comparison with the chicken whole genome database (http://genome.ucsc.edu/cgi-bin/hgBlat). The predicted amino acid sequence of chicken Cpe was deduced by use of the NCBI ORF (Open Reading Frame) Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

CpE RT-PCR and sequencing

Total RNA was extracted from normal broiler chicken thymus using Trizol (Invitrogen, US) according to standard protocols. Total RNA was reverse transcribed to cDNA with Omniscript reverse transcriptase (Qiagen, US) using random hexamer-oligos as the primer (1μM). The cDNA (5μl) was then amplified by PCR for the detection of Carboxypeptidase E. The PCR reaction mixture contained 2 mM $MgCl_2$, 50 mM of KCl, dNTP mix(200 μM of each dNTP), and 1 μl BD Advantage™ 2 Polymerase Mix, [including BD TITANIUM Taq DNA Polymerase, a small amount of proofreading polymerase, and BD TaqStart Antibody (1.1 μg/μl) (Clontech, USA)] and 0.4 μM of the following gene-specific primers. The sequence of the forward primer was: *TTGGTCGAAGTAATGCCCAGG* (Tm 61^0 C) and that of the reverse primer was: *TCCTCCCAATAGCCCTTCAGA* (Tm 60^0C). The expected size of the PCR product was 550 bp. PCR reactions were carried out using the following conditions: 95 ^{0}C for 1.5 min, and 30 cycles of 95 ^{0}C for 25 sec and 68 ^{0}C for 3 min. The resulting PCR product was then sequenced in the Gene Technologies Laboratory, Texas A&M University, using an ABI Prism 377XL DNA Sequencer. Similarily, a RT-PCR was also conducted using total RNA from human thymus (Ambion, TX). Specific primers for the human CpE

sequence (gi:4503008)were designed as follows:

Forward: AGATGGAACCACCAACGGTG

Reverse: AGATGGTGGCATTCGCAATT

The primers were expected to anneal on exon 5 and 7, respectively. The RT-PCR product spans two introns (129), with an expected size of amplicon of 251 bp.

Immunocytochemistry

White Leghorn chicks were obtained from a local hatchery (Hy-line). Thymic tissues were harvested and fixed for 24 hours at room temperature in Bouin Hollande sublimate. Bouin Hollande sublimate was prepared by adding 10 mL of saturated $HgCl_2$ solution to 90 mL of Bouin Hollande solution. The tissue blocks were then processed for paraffin embedding using routine laboratory protocols. Tissue sections of 9-10 μm thick were made with a rotary microtome MT 980 (Research & Manufacturing Co., Inc).

Immunohistochemical reagents and primary antibodies

TBST is a Tris-buffered saline containing 0.1% Triton X-100, pH 7.4. Trizma base, Triton X-100 and DAB were obtained from Sigma (St. Louis, Mo). The peroxidase-conjugated secondary antibodies, biotinylated goat anti-rabbit Ig, rhodamine red-conjugated goat anti-mouse Ig, and FITC- and rhodamine red-conjugated streptavidin were obtained from Jackson Laboratories (West Grove, PA). Cytoseal (a xylene-based mounting medium) was obtained from Stephens Scientific (Kalamazoo, MI). Vectashield (a water soluble mounting medium) was purchased from Vector Laboratories

(Burlingame, CA). Monoclonal anti-CgA antibodies were previously characterized (102, 128). Rabbit anti-CpE antibodies were a generous gift from Dr. Peng Loh (61).

CgA and CpE immunofluorescent double staining

A direct comparison between the CgA- and CpE-ir cell populations was achieved by immunofluorescent double staining on the same tissue section. A detailed procedure was described elsewhere (128). Briefly, upon dewaxing and rehydration, the sections were incubated overnight simultaneously with both primary antibodies, ***i.e.*** monoclonal mouse anti-CgA (ascites fluid diluted 1:3000) and rabbit anti-CpE (1: 1000). The next day, the sections were rinsed with TBST and incubated simultaneously with Rhodamine Red-conjugated goat anti-mouse Ig (1:600) and biotinylated goat anti-rabbit Ig (1:500) for 90 minutes. Upon rinsing, the sections were finally incubated with FITC-conjugated streptavidin (3μg/ml TBST) for 30 minutes, rinsed and coverslipped with Vectashield. Immunoreactive cells were observed with Zeiss Axioplan Microscope and Zeiss Axiophot Camera Module. Photomicrographs were taken with a Spot camera.

SDS-PAGE and Western blot

Protein extracts from fresh chicken thymus, adrenal and pituitary were prepared mixed with identical volumes of SDS-PAGE reducing sample buffer(130). The procedure was previously described in the first chapter. Equal volumes of all samples were loaded onto a 12% Tris-HCl Bio-Rad precast polyacrylamide gel. The proteins were separated for 1 h at a constant voltage of 100 V. Immediately after the separation, the proteins were electro-transferred to a nitrocellulose membrane, using a NOVEX blotting system at 0.8 mA per

cm^2 for 1 hour. The membrane was blocked with blocking buffer (Li-COR, Nebraska USA) and incubated with primary antibody (1:4000) for 5 hrs. Alexa Fluor 680-conjugated goat anti-mouse IgG (1:5000, Molecular Probes) was applied for signal detection. Odyssey protein weight markers were obtained from Li-Cor. The membrane was scanned with an Odyssey Infrared Imaging System (Li-COR, Lincoln, Nebraska USA).

Results

Chicken CpE structure and synthesis

A chicken CpE cDNA contig of 2240 bp was assembled (data not shown). The positions of exons and introns were examined by aligning the cDNA contig with the chicken genomic sequence at http://genome.ucsc.edu/cgi-bin/hgGateway. The chicken CpE gene is located on chromosome 4 and consists of 9 exons. Synthesis of CpE mRNA in the chicken thymus was proved by RT-PCR (Fig. 3.1) and confirmed by sequencing of the resulting PCR product.

The predicted Chicken CpE protein consisted of 469 amino acids, while mouse and human have been reported to consist of 476 amino acids. The calculated theoretical pI and molecular weight of chicken CpE is 5.1 and 52.4 kDa, respectively. The cDNA and protein sequence of chicken CpE, as well as the secondary structure, are highly conserved (Fig. 3.2 and Fig. 3.3). The C-terminal of CpE is believed to anchor the molecule to the membrane of the secretory granules and its sequence is identical between species.

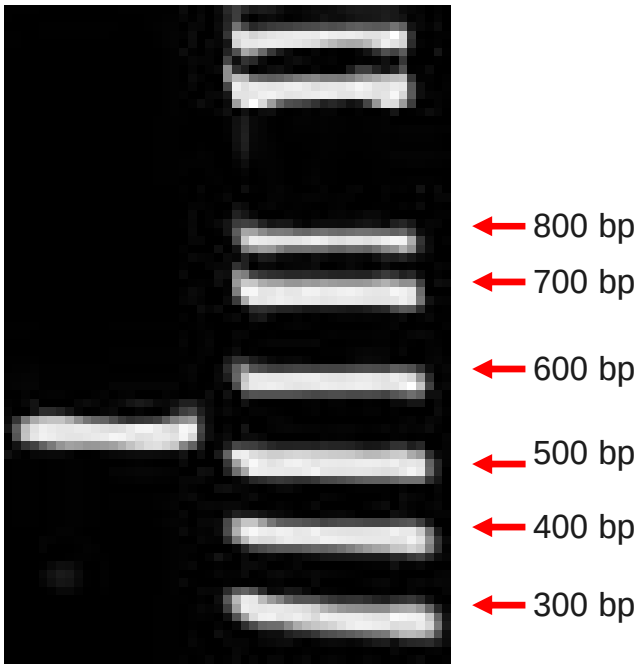


Figure 3.1. Agarose gel electrophoresis of CpE RT-PCR product. The specific amplicon with the expected size of 550 bp was sequenced and was shown 98% of identity with the predicted cDNA sequence.

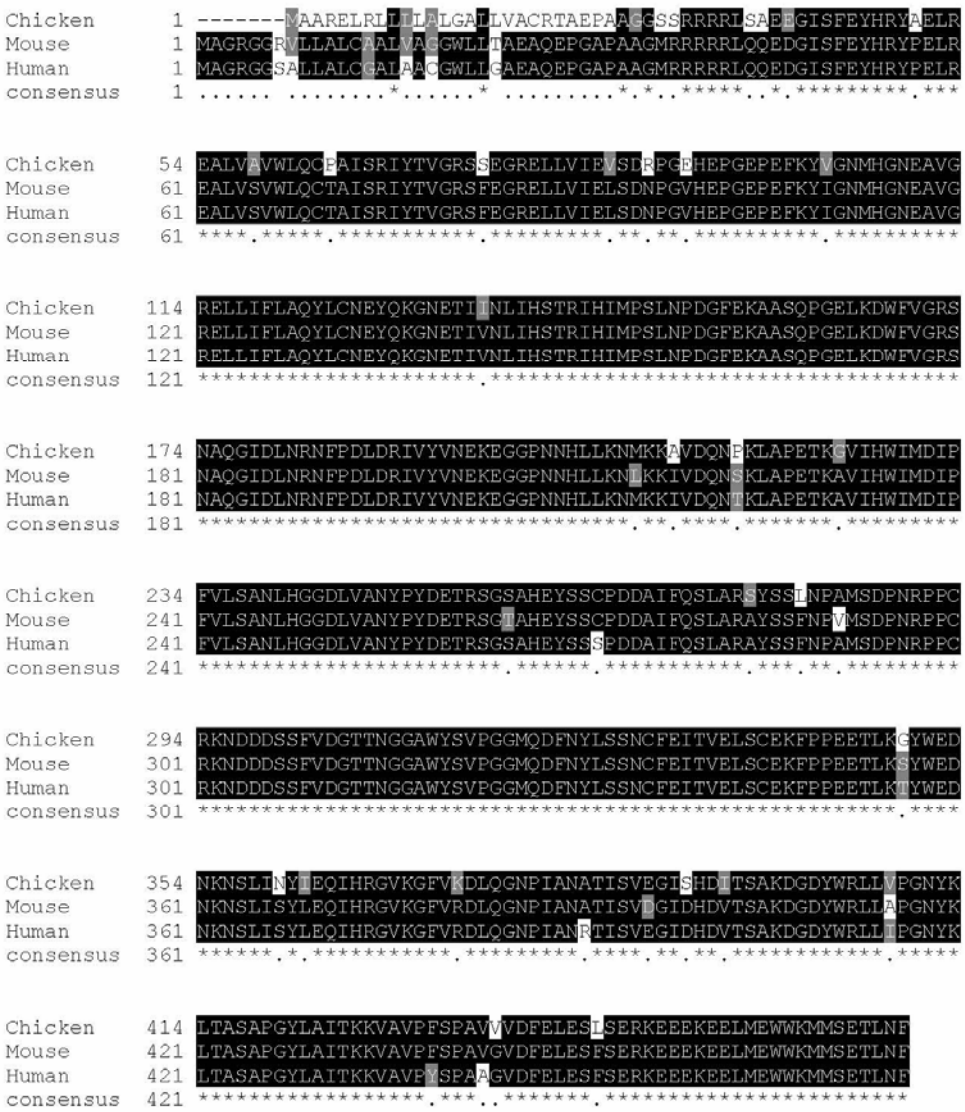


Figure 3.2. The predicted chicken CpE protein sequence, aligned with human (gi:6429043) and mouse (gi:22203763) CpE sequence. The amino acid sequence of CpE appears to be highly conserved among chicken, mouse and human. Dark-shaded sequences shown are identical, as noted * in the consensus. ~ noted for high homology.

Figure 3.3. Secondary structure of chicken CpE. The chicken CpE contains a Zn_pept domain from 168 and ends at position 458. Zn_pept domain (a Zn-binding catalytic domain of Zinc metallopeptidases) is conserved among many other members of the carboxypeptidase protein family. Structural analysis was conducted using SMART (http://smart.embl.de/).

CpE protein expression in chicken thymus

A. Immunocytochemical localization

Chicken CPE was shown to be expressed in chicken thymus at the protein level by immunocytochemistry (Fig. 3.4), using pituitary as a positive control tissue. Paraffin sections of chicken thymus and pituitary were stained with an anti-CpE C-terminal antibody (61). Cells displaying CpE-like immunoreactivity (CpE-ir) were located primarily on the cortico-medullary border, a region that is heavily innervated by the autonomous nervous system and numerous blood capillaries. While the morphology of the CpE-ir cells is heterogeneous, most of the cells are round but some of them have star-like extensions. Fig. 3.5 shows a picture of double staining of CPE and CgA. Although a few single-stained CgA- and CpE-positive cells could be found, the large majority of cells were double-stained.

As a positive control, the pituitary showed specific, very typical cytoplasmic staining in groups of adjacent cells sharing an almost identical morphology reminiscent of the

POMC cells, although the phenotype of these cells in pituitary was not determined in this experiment. However, both lobes contained CpE-positive cells (results not shown).

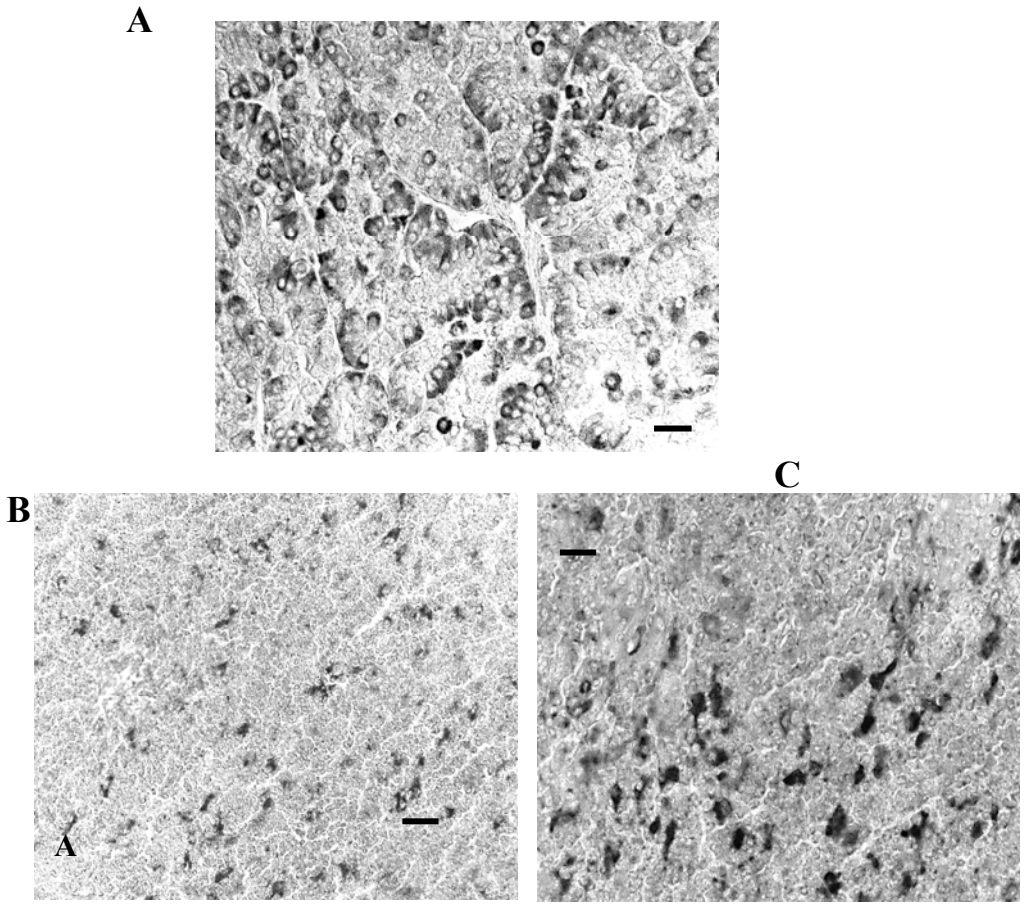


Figure 3.4. Six-week-old chicken pituitary (top) and thymus (bottom) was stained with rabbit anti-CpE antiserum. (A, pituitary; B-C, thymus. The bar in A and C equals to 25 micrometes; the bar in B equals to 50 micrometers)

In thymus, CpE immuno-reactive cells (Fig. B and C) were located primarily on the cortico-medullary border, a region that is heavily innervated by the autonomous nervous system and where numerous blood capillaries can be found. While the morphology of the CPE-immuno-reactive cells is heterogeneous, most of the cells are simple, round and some of them have star-like extensions. In pituitary, typical cytoplasmic staining was readily detectable in groups of adjacent cells sharing an almost identical morphology.reminiscent of the POMC cells (Fig. A).

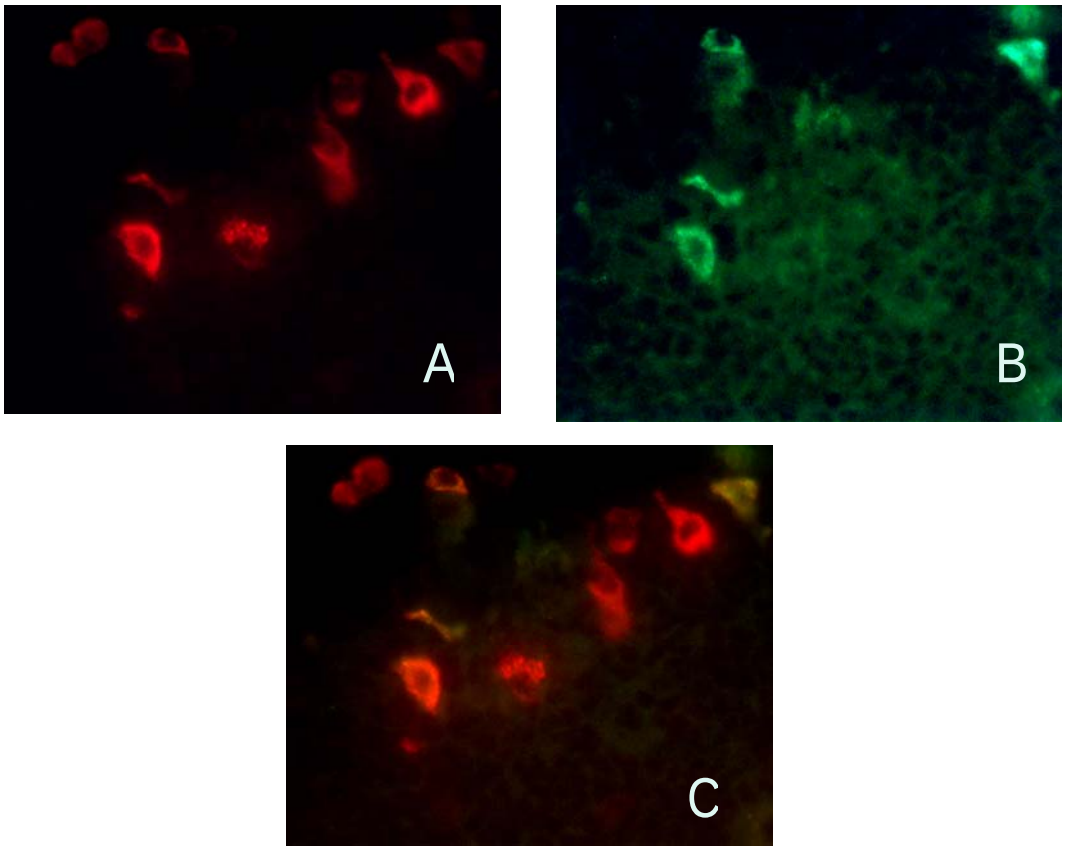


Figure 3.5. Immunofluorescent CgA and CpE double staining. Thymic cells were stained with a monoclonal antibody against CgA and a rabbit polyclonal antiserum against a synthetic C-terminal peptide of CpE. Cells labeled in red (rhodamine) were the CgA-immunoreactive cells and the green labeled cells (FITC) were CpE-positive cells. Fig. 3.5 (A) shows the single red channel and (B) the single green. (C) shows the composite image. Although a few single-stained CgA- and CpE-positive cells could be found, many cells was double-stained. Arrow in (C) points to a double-stained yellow cell. Arrow in (A) points to the single-stained red CgA positive cell.

B. Western blotting

To further confirm the expression of CpE in the chicken thymus, SDS-PAGE and Western blot were carried out by using the same antibody as the one used for immunocytochemistry. A clear band with a molecular weight of approximately 53k Da

was observed in the sample of total protein extracts from chicken thymus in Western blot. Chicken adrenal and pituitary protein extracts were used as positive controls (See Fig. 3.6).

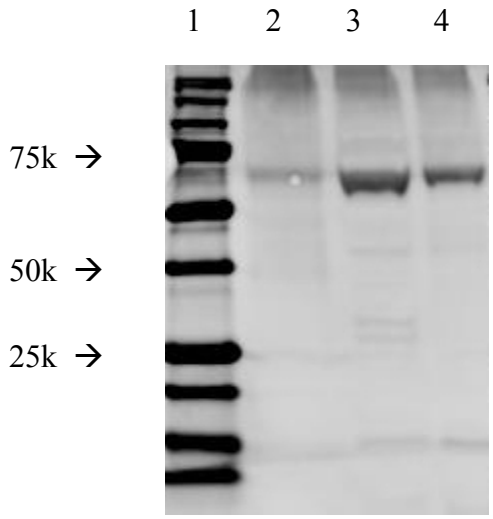
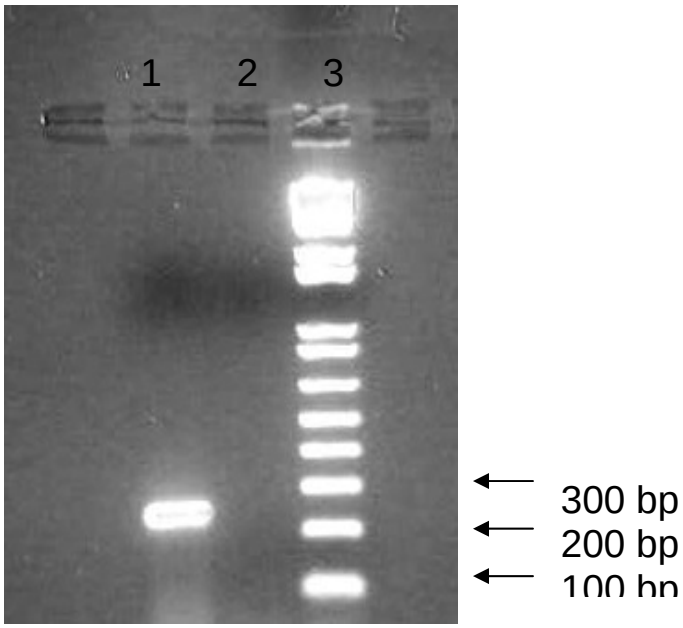


Figure 3.6. Western blot identification of CPE. Lane 1: Protein standards; Lane 2: Pituitary; Lane 3: Adrenal; Lane 4: Thymus. Protein extracts from chicken thymus, adrenal gland and pituitary were separated by SDS-PAGE on a 12% Tris-HCl precast polyacrylamide gel, blotted onto a nitrocellulose membrane and analyzed with a rabbit polyclonal antiserum (1:4000) against against a synthetic C-terminal peptide of CpE. Alexa Fluor 680-conjugated goat anti-mouse IgG antibody was used as the detection reagent. CPE was expressed in chicken thymus, as well as in adrenal gland and pituitary (positive controls).



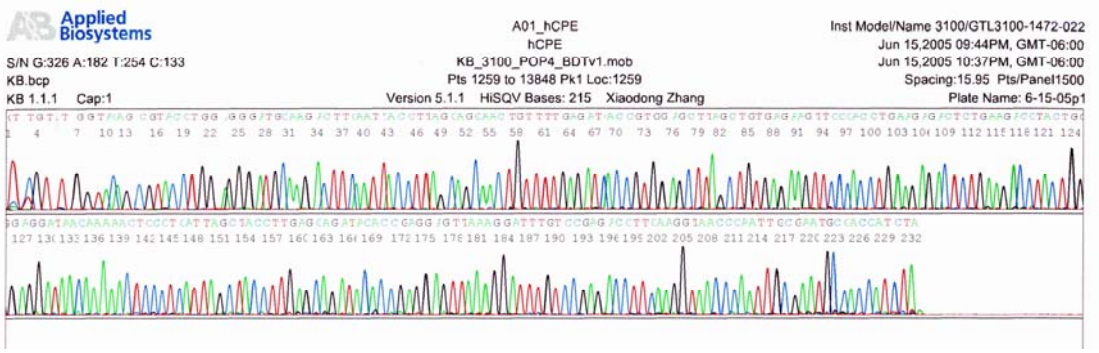


Fig. 3.7. RT-PCR of CpE on human thymus total RNA. Lane 1: specific amplicon; lane 2: negative control; lane 3: molecular markers. An expected amplicon size of 251 bp was observed. The specificity of the PCR product was further confirmed by DNA sequencing.

Discussion

In the regulated secretory pathway (RSP), neuropeptide precursors in the trans-Golgi network are transported and sorted to the secretory granules of neuroendocrine cells. They are then processed and mature into active peptides and stored in large dense-core vesicles (LDCVs) until secreted upon delivery of a specific stimulus to the cell (50, 131). The present study aimed at assessing the co-existence of two essential elements of the regulated secretory pathway, chromogranin A and carboxypeptidase E, in the diffuse neuroendocrine system in the thymus of the chicken. The immunohistochemical evidence presented in this investigation, combined with the identification of the chicken CpE mRNA by RT-PCR, has allowed us to demonstrate this co-existence of CgA and CpE in identical neuroendocrine cells. To our knowledge, this has not been shown previously in any lymphoid tissues of any vertebrate species, including in human thymus.

Recently, a predicted carboxypeptidase E mRNA of 2034 bp was added to the GenBank (XM_420392.1 GI:50746181) based on the chicken (jungle fowl) genome sequence. Compared to the nucleotide sequence we assembled *in silico* (based on publicly available chicken ESTs), the open reading frames of both sequences were almost identical but the EST-based sequence was about 200 bp longer in the 3' non-coding region. The chicken CpE gene consists of nine exons, as is also the case in humans. The predicted chicken CpE protein sequence consists of 469 amino acids and shows 87, 87, 88 and 90% homology with rat, mouse, human and bovine CpE, respectively. The C-terminal sequence of CpE, which serves as an anchor to the membrane of secretory granule, is perfectly conserved between birds and mammals. The major portion of the chicken CpE

protein structure consists of a Zn_pept domain (residues 168 through 458). This domain is conserved in many other members of the carboxypeptidase protein family in mammals(132). The highly conserved CpE sequence between species may suggest a conserved function and distribution of this molecule. Preliminary RT-PCR experiments demonstrated expression of CpE mRNA in human thymus (see Fig 7), further exploration on mammalian species in a comparative view is underway.

Immunofluorescent dual staining clearly showed partial co-localization of the CpE and CgA in the identical cells, although single stained cells (either CgA- or CpE-positive) were occasionally observed. The latter observation proves that dual stained cells are authentic and not an artifact caused by cross-reacting secondary reagents.
Importantly, this evidence suggests that in the chicken thymus, some of the locally produced neuroendocrine molecules may be processed and released through the regulated secretory pathway. If this hypothesis is correct, it follows that extra-cellular stimuli must be regulating the function of at least part of the thymic diffuse neuroendocrine system.

As previously described, the CpE-CgA double positive cells are primarily found in a transition zone between the cortex and the medulla of the thymic lobules. This area is known to contain numerous arterioles and is heavily innervated by the autonomous nervous system (133). Therefore, it is tempting to speculate that the diffuse neuroendocrine system may serve as a relay for nervous stimuli delivered by sympathetic and/or parasympathetic nervous system and/or humoral factors delivered by the

circulation, allowing thymopoiesis to be fine tuned by a variety of physical and environmental factors (134).

An area of future research is to isolate and purify LDCV from the crude microsomal fraction described in this study, by immuno-affinity chromatography, magnetic sorting or even immunoprecipitation, using a monoclonal antibody against C-terminal end of CpE starting. This will pave the way of identification of those neuropeptides and hormones stocked in the neurosecretory granules.

CHAPTER IV

INTRA-THYMIC EXPRESSION OF SOMATOSTATIN AND ITS EFFECTS ON PROLIFERATION AND DIFFERENTIATION OF CHICKEN THYMIC CELLS

Introduction

The immune system and neuroendocrine systems are now known to have intense bi-directional interactions in order to maintain physiological homeostasis. More and more evidence is showing that the cross-talk between these two systems is fundamentally important to their physiological functions. In fact, primary and secondary lymphoid organs are innervated by nerve fibers and neuropeptides are released from the nerves to signal the immune system (135, 136). In addition, lymphoid organs contain an ectopic population neuroendocrine cells producing hormones or neuropeptides, as shown in the chicken in the previous chapters, and in other studies in mammals as well.(82, 137). Various neuropeptides have been shown to act as immunomodulators of the immune organs in mammals (87, 138). In this study, we will focus our attention on somatostatin in chicken thymus, which has been shown in mammals to exert multiple effects on immune functions within the microenvironment (62) (65, 67)

Somatostatin (SST, also referred as somatotropin release-inhibiting factor, or SRIF) is a cyclic neuropeptide originally found in hypothalamus, identified as a potent inhibitor of the secretion of growth hormone (GH) and thyroid-stimulating hormone (TSH) from the anterior pituitary(63). In fact, later on, SST and its receptor SSTR have been found throughout the body, including in the pancreas, salivary gland, kidney, lymphoid cells,

blood vessel walls, etc., and it became clear that this neuropeptide exerts multiple physiologic effects. For review, see (65).

SST, like many other neuropeptides, is initially synthesized from one single gene as a larger preprosomatostatin containing 92 amino acid in mammals. It is then cleaved at C-terminus to form two biologically active forms, SST-14 and SST-28, composed of either 14 and 28 amino acids, respectively (68, 69). Chicken SST-14 has the exact same amino acid sequence as in mammals, while in SST-28 there is one amino acid difference(70).

In mammals, five SSTR subtypes (SSTR-1, -2, -3, -4 and –5) have been identified and characterized; they are encoded by five different, intronless genes (72, 73). In addition, rodent SSTR-2 has two isoforms, SSTR-2A and SSTR-2B, due to alternative splicing (74). SSTRs have seven α-helical trans-membrane domains, with a structure of three intra- and extra-cellular loops, and all of them are G-protein-coupled receptors (75). The SSTRs are widely expressed in different tissues throughout the body, with different expression level and subtype combinations (77) depending on tissue and stage of development.

As mentioned earlier, SST has been shown to have multiple effects on various immune cells. Studies regarding the effects on immune cell proliferation, secretion, migration and apoptosis reveal that the immunomodulatory actions by somatostatin are complex and depend on various physiological and experimental conditions. For review, see (81). SST has a very short half-life (1.5~3 minutes) in the systemic circulation (71), indicating that

SST-producing cells, or stores of SST are probably close to the target cells. It is thus that SST can exert regulatory roles on the chicken developing T-lymphocytes in the thymus, through local paracrine secretion and their receptors on immune cells.

In the previous chapters, we have provided strong evidence that chicken thymus, a primary lymphoid organs contains neuroendocrine cells. In this study, we examined if somatostatin and its receptor are locally expressed within the chicken thymus and their effects on the neighboring thymic cells. In particular, we have evaluated the physiological role of somatostatin *in vitro* with respect to thymic cell proliferation, differentiation and apoptosis.

Materials and methods

Animal and tissue processing

Four to 6-week old broiler chickens were sacrificed by cervical dislocation and tissues were dissected on site. For immunohistochemical purposes, the tissues were immediately immersed in fixative (Bouin Hollande sublimate) for 24h at room temperature. The fixative contained 10 ml of saturated $HgCl_2$ solution and 90ml of Bouin Hollande solution. The tissue blocks were then paraffin-embedded using routine protocols. Eight to 10 μm thick tissue sections were made with a rotary microtome MT 980 (Research and Manufacturing Co., Inc.). All experiments followed Animal Use Protocols approved by Animal Care and Use Committee at Texas A&M University.

Immunocytochemistry

A. SST single staining

Immunocytochemical single staining was performed on paraffin sections of thymus tissue from 43 days-old Single Comb White Leghorns. The sections were de-waxed and hydrated according to standard lab procedures and then incubated overnight with polyclonal rabbit anti-SST-14 antibody (ImmunoStar, Lot 216002) at 1:800 in Tris Buffer Saline (TBS) containing 0.1% (v/v) Triton-X100 (TBST) buffer. The next day, the sections were rinsed with TBST and the secondary antibody, GaRIgG-peroxidase (Jackson ImmunoResearch Lab) was applied with final dilution 1/600 and incubated for 30 minutes. The enzymatic reaction was developed for approximately 10 minutes using 25 mg of DAB and 75 µl of 30% (v/v) H_2O_2 (source) in 200 ml Tris buffer (50 mM Tris-HCl, pH 7.4). The sections were dehydrated and cover-slipped with Cytoseal. To verify the specificity of the staining, the diluted primary antibody was pre-incubated with somatostatin-14 peptide (Peninsula Laboratories, Belmont, CA).

B. Immunofluorescent double staining of SST and CgA

Immunofluorescent staining procedure is previously described elsewhere (128). Briefly, after dewaxing and rehydration as in 1.1, the sections were incubated overnight with both primary antibodies simultaneously, *i.e.* monoclonal mouse anti-CgA (1:3000) (102) and rabbit anti-SST-14 (1:800). The next day, the sections were rinsed with TBST and incubated simultaneously with Rhodamine Red-conjugated goat anti-mouse IgG (1:600) and FITC-goat anti-rabbit IgG (1:600) (Jackson ImmunoResearch Lab) for 45 minutes. Upon rinsing, the sections were coverslipped with Vectashield (Vector Laboratories,

CA). Immunoreactive cells were observed with an Olympus BX50 light microscope (Leeds Instruments, Inc.; Irving, TX) equipped for epifluorescence with a dual band filter set for FITC and Rhodamine (Chroma Technology Corp, Rockingham, VT) and photographed with a Spot 110 digital camera (Diagnostic Instruments, Inc., St. Sterling Heights, MI).

SSTR2 RT-PCR

Total RNA was isolated from chicken thymus and pituitary, using TriZol reagent (Invitrogen, Carlsbad, CA). RNA samples were quantified with a spectrophotometer (Eppendorf BioPhotometer), and treated with RNase-free Dnase I (Invitrogen) to digest any residual DNA, according to the manual provided by the manufacturer. The first strand cDNA was synthesized from approximately 1.2 μg of total RNA by RETROscript reverse transcriptase (Ambion, Austin, TX) using either random hexamers or dexamers according to the manufacturer's protocol. Gene-specific PCR primers were designed by using Primer Express 2.0 (Applied Biosystems, Foster City, CA), based on the cDNA sequence of chicken somatostatin receptor type 2 (*Gallus gallus*, gi:50757970). PCR primer oligonucleotides were synthesized by Intergrated DNA Technologies Inc. Primer sequences were as follows:

Forward: GCGGATGAGCTGTTCATGCT

Reverse: GATGCCTGAGGACTTGACTTTGA

The first-strand cDNA was amplified in a 50 μl PCR reaction that contained 2 mM $MgCl_2$, 200 μM dNTP mix, 1.0 μM of each of the forward and reverse primers, PCR buffer and 1.25 U AmpliTaq Gold DNA polymerase (Perkin Elmer). The polymerase was

activated by pre-heating at 94 ^{0}C for 10 min. The PCR protocol used 30 cycles consisting of 30s denaturation at 94 ^{0}C, and 70s at 65 ^{0}C, with a final extension step at 65 ^{0}C for 10 min. The products from the RT-PCR reaction were analyzed by electrophoresis on a 1.5% agarose gel using ethidium bromide for visualization. Photos were taken using a MultiDoc-It Digital Imaging System (UVP, USA).

Proliferation assay

Chicken thymic lobes were collected from 1-day old male Leghorns (Hy-Line International). A thymic cell suspension was prepared in AIM-V serum-free medium (Gibco, Carlsbad, CA). Cells were cultured in 96-well plates at a density of approximately 50,000 cells/well, 50 µl in each well; cells were incubated with somatostatin (Peninsula Laboratories, Belmont, CA) at a concentration of 10^{-12} M, 10^{-9} M or 10^{-6} M for 24h, respectively. Two µg Concanavalin A per ml and 1.5 ng chicken IL-2 (kindly donated by Dr. Ellen W. Collisson) per ml were also added to the medium to stimulate the cell proliferation. Cell proliferation was assessed using the CellTiter96 Cell Proliferation Assay Kit (Promega, WI, USA), based on conversion of a tetrazolium salt into a formazan product by the cellular metabolism. Two trials of the assay was conducted according to the manufacturer's instructions. Absorbance at 580nm was recorded with a 96-well plate reader (Wallac Victor^2, PerkinElmer Life and Analytical Sciences, Shelton CT).

Flow cytometric measurement of T-cell differentiation

Initially, thymic cell fragments about 1 mm^3 were incubated at 37 ^{0}C and 5% CO_2 in the presence of SST at concentrations of 10^{-12} M, 10^{-9} M and 10^{-6} M in AIM-V serum-free medium for 5 days. Thymic cell suspensions were then prepared and incubated with two directly labeled primary antibodies: mouse anti-chicken CD8α-PE (0.2 μg/10^6 cells) and mouse anti-chicken CD4-FITC (1 μg/10^6 cells) (Southern Biotechnology, Birmingham, AL) on ice for 40 min. Cells were washed twice with cold PBS. Flow cytometric analysis was conducted by Dr. Roger Smith, III in the core lab for flow cytometry in the Veterinary Phathobiology Department at Texas A&M University. Briefly, labeled cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, CA, USA) using CELLQuest software. Data were analyzed with the FlowJo software packet (Treestar Incl, CA).

CD5 positive T-cell panning

Two or three thymic lobes were collected from a day-old chicken. Dispersed cells were prepared as described previously. The thymic cells were incubated in 10 ml RPMI 1640 with 0.125% (w/v) collagenase D (Sigma, MO) at 37 °C for 15 min with gentle agitation every 5 min. Density gradient centrifugation was used to remove red blood cells, dead cells and cell debris. Five ml of diluted cell suspension was mixed with an equal volume of Histopaque 1077 (Sigma, MO) and centrifuged at 1,500 rpm for 20 min. Viable cells were collected from the interface and washed with PBS (pH 7.2) and further incubated with 1:500 rat anti-CD5 antibody (Southern Biotechnology, AL) for 1.5h at room temperature. The day before panning, a 75 cm^2 tissue culture flask was prepared and

coated overnight with the secondary antibody rabbit anti-rat IgG (50 μg/ml in PBS containing 2% (w/v) bovine serum albumin (BSA, Sigma, St. Louis, MO), Southern Biotechnology, Birmingham, AL). The thymic cell suspension was then transferred to the secondary antibody-coated flask and was incubated for 1 hour at room temperature. After 3 rinses with PBS (plus 2% (w/v) BSA), cells were immediately subjected to RNA extraction using Trizol, as described above.

Apoptosis detection and quantification

Cells were prepared as previously described under 4 and cultured in the presence of 10 ng/ml dexamethasone (Sigma, St. Louis, MO) in the presence or absence of somatostatin (SST) at concentrations of 10^{-12} M, 10^{-9} M and 10^{-6} M, respectively, for 4h and 8h. Detection and quantification of apoptosis was carried out using an Annexin-V-FLUOS staining kit (Roche Applied Science, Indianapolis, IN). About 10^{6} cells were washed with PBS at 200g for 5 min. The resulting cell pellet was resuspended in 100 μl of Annexin-V-FLUOS labeling solution and incubated for 10-15 min at room temperature. Samples were then analyzed by flow cytometry with the help of Dr. Roger Smith at Texas A&M University.

Statistical analysis

Statistical significance was assessed by ANOVA and Student's two-tailed t-test (For proliferation assay, n=12). Values were considered statistically significant at $p < 0.01$.

Results

Immunocytochemical demonstration of SST in chicken thymus

The presence of SST was assessed at the protein level by immunocytochemistry on paraffin-embedded thymus sections of adult chickens. Immunocytochemical data (Fig.1) confirmed the presence of SST in the chicken thymus. Numerous SST-positive cells were readily observed, localized predominantly in the cortico-medullary junction of the thymic lobules, in a typical ring-like area, similar to the distribution of CgA and CpE described in previous chapters. These cells displayed diverse morphologies. Most cells were round to oval in shape, but some cells had stellate appearance with clear extension (see also Fig. 2A). Specific staining was abolished by pre-adsorption of the primary antiserum with SST-14, confirming the specificity of staining.

Immunofluorescent double staining for both SST and chromogranin A (CgA), a marker for neuroendocrine cells, showed a partial overlap between these two cell populations. (Fig. 2). In addition, lots of single-stained SST positive cells (green) can be observed, without the co-localization of CgA, but also single stained green, non-cellular structures were labeled, some of which might be axons or nerve endings.

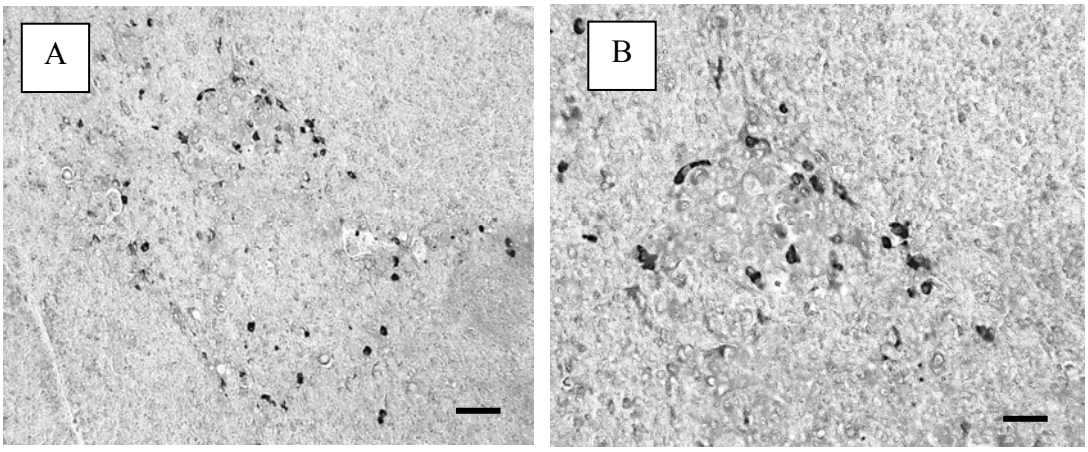


Fig. 4.1. Immunohistochemical staining for somatostatin (SST) in the thymus of a 43-day old Leghorn. A. Paraffin sections were stained with rabbit anti-SST; SST-positive cells were detected mainly on the cortico-medullary border, in a typical ring-like area (micrometer bar = 100 μm). At higher magnification (B), neuron-like SST-immunopositive cells were readily observed. Some cells had a stellate appearance with at least one extension (see also Fig. 4.2). Specific staining was eliminated by the pretreatment of the antibody with SST-14 (micrometer bar = 50 μm).

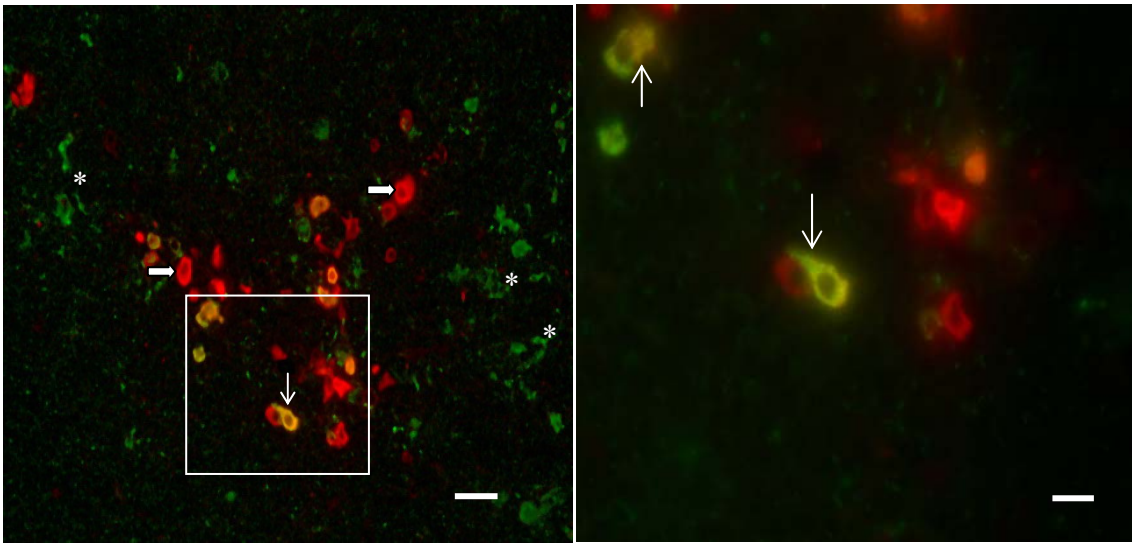


Fig 4.2. Double stained chicken thymus section. Primary antisera were against rabbit anti-SST (FITC-labeled) and monoclonal mouse anti-CgA (rhodamine labeled). This photomicrograph shows that some of the CgA-positive cells expressed somatostatin. A dual stained cell with a conspicuous extension is indicated with a vertical arrow (see inset and panel to the right). However, many cells were positively labeled for CgA without expressing SST (horizontal arrows). In addition lots of structures (indicated with asterisks) were stained single green; some of these may be axons or nerve endings. Micrometer bars: left = 50μm; right = 17 μm.

Expression of the somatostatin receptor type2 (SSTR2) in chicken thymus

For SST to have direct effects on the thymus, more particularly on the T-cells, it is essential that the target cell or target tissue expresses the corresponding receptor. Since previous studies have shown that SSTR2 is expressed in mammalian thymus and the sequence for chicken SSTR2 was described in the chicken pituitary(63) , it was a logical step to assess the expression of SSTR2 in the thymus of the chicken.

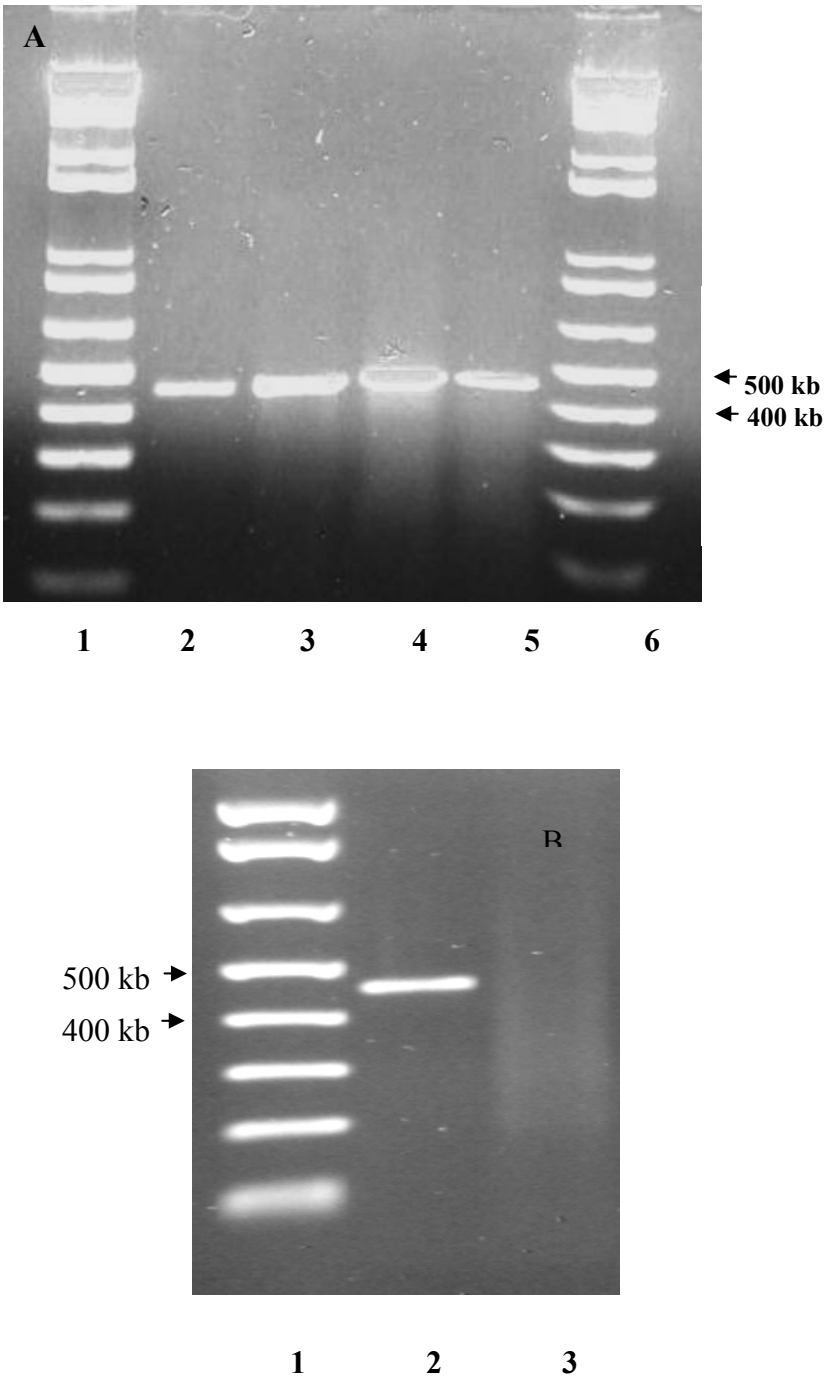


Fig. 4.3. RT-PCR of SSTR2 in total RNA in chicken thymus (A) and in $CD5^+$ cells (B). An amplicon with expected size of 456 bp was synthesized. Crude total RNA was treated with DNase I, which was also used as a template in PCR as a negative control.
Fig. A. Lane 1 and 6: DNA ladder; 2-5: PCR products with 5 µl or 10 µl loading volume.
Fig. B. Lane 1: DNA ladder; 2: PCR product; 3. Negative control (DNase I treated RNA as template).

Expression of SSTR2 mRNA was demonstrated within chicken thymus. In addition, CD5-positive cells produce SSTR2 mRNA, suggesting that developing T-cells are a potential target for SST. Hence, somatostatin and its receptor may be important for thymocyte development. Consequently, the biological role(s) of SST were examined by incubating primary thymic cell suspension *in vitro* with different concentrations of SST.

Biological effects of somatostatin (SST) on primary thymocytes in vitro

For this purpose, primary thymocyte suspensions, stimulated with Con A and IL-2, and treated with SST in picomolar, nanomolar and micromolar concentrations. The results of these experiments (Fig. 4.4) suggest that SST significantly inhibits IL-2 and ConA induced proliferation of thymocytes. In comparison with controls (medium containing IL-2 and ConA but without SST), addition of SST at 10^{-9} M and 10^{-6} M resulted in a nearly 20% decrease in proliferation. At 10^{-12} M of SST, however, this anti-proliferative effect was no longer statistically significant.

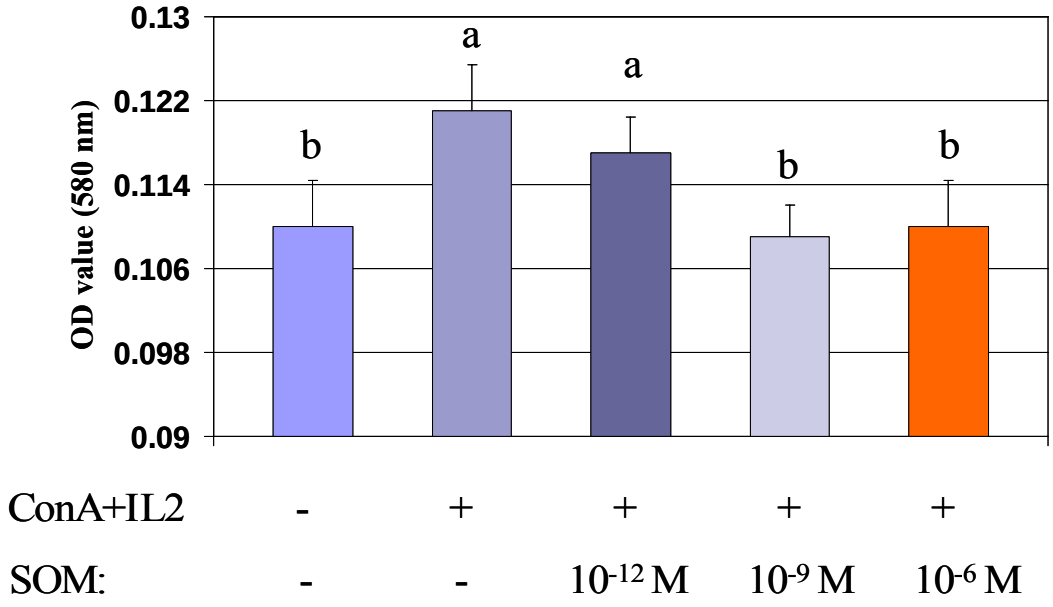


Fig. 4.4 Anti-proliferative effects of SST on chicken primary thymocytes *in vitro.* The proliferative effect of IL-2 (1.5 ng/ml) and Con A (2 μg/ml) was blocked by a 24h incubation with SST at micro- and nanomolar concentrations. Picomolar levels of SST were not effective in this respect.

In addition to the regulation of thymocyte proliferation, the possible involvement of SST in the T-cell maturation was also examined. Our data (Fig. 4.5) showed that with a higher SST concentration, the percentage of CD8 single positive cells tended to increase in a dosage-dependent manner, although this numerical trend was not statistically significant. This finding suggests that SST could play a role in the differentiation of double positive T-cells into CD8 single positive cells in chicken thymus.

Finally, apoptosis induced by dexamethasone (as assessed by binding of Annexin-V-FLUOS), was not significantly decreased by SST at any of the concentrations used in this study (results not shown).

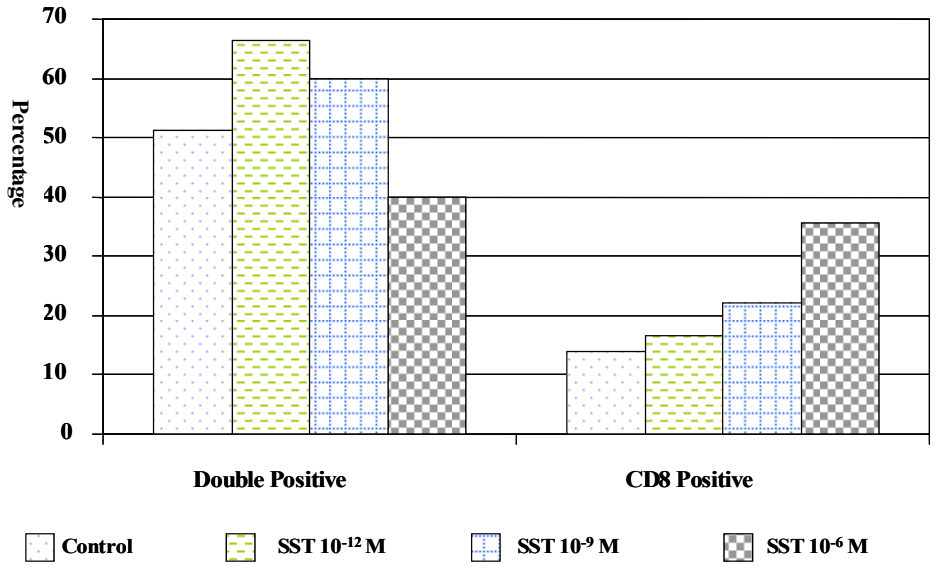


Fig. 4.5. SST facilitates thymic double positive T-cells differentiate into CD8 single positive cells.

Discussion

In this study, we showed both at the protein level (by immunocytochemistry) and at the mRNA level (by RT-PCR) that SST and its receptor SSTR2 are expressed in the chicken thymus, and provided evidence that SST plays a regulatory role in thymocyte development.

The existence of five different SSTRs and the fact that these subtypes might serve different functions raises the question as to which SSTR subtype(s) is/are expressed in chicken thymic cells. RT-PCR, being very specific and sensitive, is an ideal tool to investigate this problem. In the past, different RT-PCR studies of SSTR subtype expression in immune cells have provided inconsistent results between species: human peripheral blood T-lymphocytes selectively express SSTR3 (139), while expression of SSTR2 and SSTR4 was found in mouse peripheral blood mononuclear cells (PBMC) (140). Moreover, in primary lymphoid tissues, SSTR2 expression was found in mouse but not in rat thymus (88, 92, 140). However, in general terms, SSTR2 was shown to be the most prominently expressed SST receptor subtype in lymphoid organs and in several lymphoid and myeloma cell lines (141). The RT-PCR results in our study clearly showed that chicken total thymic cells and CD5-positive thymic cells express SSTR2. As to the question of which cell type(s) express(es) SSTR2 in the chicken thymus, the final answer is yet to be conclusively established. In a lymphoid organ such as thymus, in addition to developing T-cells at various stages of development, there are many accessory cell types such as epithelial cells, dendritic cells, fibroblasts, nerve cells, making the question of which cell type expresses SSTR2 relatively complex. CD5 is a transmembrane

glycoprotein expressed by all T-cells and on a subpopulation of B-cells (142). Although we can not rule out the presence of B-cells in the purified cell population, it is notable that the overwhelming majority of the cells in a CD5-enriched cell preparation from chicken thymus were T-lymphocytes; in addition, the SSTR2-specific mRNA was readily detectable with a routine RT-PCR protocol. It is thus tempting to conclude that chicken developing T-cell do express SSTR2. This is in agreement with the study of Solomou *et al*. (92), reporting that SSTR2 is also expressed in adult mouse thymocytes.

Interestingly, in human, different developmental stages of T-cells express different SSTR subtypes, adding potentially an additional level of regulation and complexity to the SST-SSTR interaction. In combination with the observed endogenous production of SST, this suggests and corroborates the putative role of SST and SSTR in a bi-directional interaction between the cell components of the thymus, including in intrathymic T-cell maturation (98).

In the proliferation assay described in the present study, the interaction of SST and its receptor SSTR2 was further demonstrated to be implicated in the regulation of chicken thymocyte development. . The proliferation of thymocytes induced by ConA and IL-2 was significantly inhibited by somatostatin. In comparison with controls (medium without peptide but with Con A and IL-2), addition of SST at 10^{-9} M and 10^{-6} M resulted in a nearly 20% decrease in proliferation, but proliferation was not statistically inhibited at 10^{-12} M. This is in agreement with other studies reporting the inhibitory effects of SST on cell proliferation (81, 140). It has been demonstrated that the SST-SSTR2 interaction

controls T-cell proliferation via either arresting cells at S-phase of the cell cycle or by terminating the G1 phase progression (143). These intracellular signalling transduction pathways could also be shared among other species, including the birds. In addition, the number of double positive T-cells was found to be decreased after the treatment with SST. Similarily, an *in vivo* study showed that SST analogue octreotide accelerates the elimination of thymic cells with $CD4^{+}CD8^{+}$ double positive T-cells(144).

An annexin-V assay was used to detect potential effects of SST on protection against apoptosis in thymic cells. Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). The detection of cell-surface PS with annexin-V serves as a marker for apoptotic cells because in a viable cell, PS is restricted to the inner leaflet of the plasma membrane by an energy-dependent transport from the outer to the inner leaflet of the bilayer(145). In the current investigation, no significant decrease was recorded in the percentage of apoptotic cells treated with SST after induction of apoptosis by dexamethasone. These results are in contrast with a study using mouse fetal thymic lobes that found that SST protects thymocytes from dexamethasone-induced apoptosis (92), although this apparent contradiction may simply be the result of the species (and class) difference. In addition, this discrepancy could be mostly due differences in the experimental protocol: in the mouse study, thymocytes in the thymus tissue fragments were still capable of interacting with other cell types in the thymus, could thus be affected by a different set of signals compared to cells in a cell suspension, as used in our study. Remarkably, under certain conditions, SST can also have pro-apoptotic effects: earlier studies have shown that an over-production of SST in the thymic

cells can lead to an over-production of SSTR2 and in turn, resulting in an increase of apoptosis of thymocytes (146).

In general, this study confirms the presence of somatostatin at the cortico-medullary border in the chicken thymus and shows, for the first time, that somatostatin plays a role in chicken thymic cell proliferation and maturation, an effect that can be explained by the expression of its specific receptor SSTR2 (but potentially also other SST receptor subtypes) on developing T-cells. Furthermore, these data suggest that in order to fully understand avian immune function, neuroendocrine factors cannot be ignored.

CHAPTER V

SOMATOSTATIN MODIFIES GENE EXPRESSION OF IL-1B, TGF-B1 AND CXCR4 IN A CHICKEN MITOGEN-ACTIVATED T CELL LINE

Introduction

In chapters II and III, we have demonstrated the existence of complex neuroendocrine cell populations within the thymus, a chicken primary lymphoid organ. Furthermore, as shown in the chapter four, somatostatin (SST), a classical neuropeptide, is an effective immuno-modulatory substance locally produced within chicken thymus. These data clearly indicate that in addition to a role as transmitters/mediators in the nervous system, SST and other neuropeptides have the potential to directly interact with their corresponding receptors on chicken immune cells, such as T-cells. To further unravel the effects of SST on the immune system, the role of SST on the induction of cytokines, a chemokine receptor as well as on MHC components gene expression was assessed.

Cytokines are central regulators of the immune response. Cytokines are produced and released by various cells in the body, responding to external stimuli and inducing reactions through their specific receptors. They can act in an autocrine manner or paracrine manner. Some cytokines can affect distant cells acting in an endocrine manner, although this depends on their ability to enter the circulation and on their half-life (147).

Cytokines can be generally categorized into Th1 and Th2 cytokines in mammals, according to the type of response they generate. Th1 cytokines include, to name just a

few, IL-1, IFN-γ, tumor necrosis factor (TNF), IL-12 and transforming growth factor (TGF). They are mainly involved in the cell-mediated immunity. For instance, IFN-γ and TNF are macrophage activating cytokines (148). The recombinant chicken IL-1β has been shown to exert biological activities similar to those of its mammalian homologue. For instance, it induces fibroblasts to secrete chemokines and upregulates corticosterone production (149). On the other hand, Th2 cytokines like IL-4, IL-5, IL-10 and IL-13 mainly regulate humoral immune responses (148) (27).

Knowledge of avian cytokines is lagging far behind as relatively few cytokines were cloned and functionally characterized. So far, all of the cytokines characterized in the chicken are categorized as Th1-like. Whether Th1-type or Th2-type immune responses can be defined in the chicken (150) is still a matter of debate, as classical Th2 cytokines have not been determined until very recently. A study reported last year (151) characterized the first non-mammalian Th2 cytokine gene cluster based on a genomics approach. The cluster contains functional single-copy genes for IL-3, IL-4, and IL-13. Another study showed an increased expression of IL-13 and IL-4 mRNA in the ileum 14 days after infection with a helminth, which is expected to induce a humoral immune response (152). This seems to suggest that the Th1-Th2 polarization was evolutionarily conserved for over 300 million years.

The chemokines are a family of small, secreted proteins. They were initially characterized through their chemotactic effects on leucocytes. Based on structural and genetic considerations, chemokines are grouped into CXC, CC, C and CX_3C families.

Chemokines play a fundamental role in the recruitment and function of T-lymphocytes. T-lymphocytes themselves are also a source of a number of chemokines. At the same time, they also express most of the known CXC and CC chemokine receptors to an extent that depends on their state of activation/differentiation and/or the type of stimuli they are receiving. The expression of CXCR4, together with the production of its ligand, stromal cell-derived factor 1 (SDF-1), appears to be extremely important HIV-1 infection of T-cells (153, 154). In human, (SDF-1) signaling (SDF-1/CXCR4) is critical for early T-cell development (155). Treated with neutralizing antibodies against SDF-1 or CXCR4, human thymocytes showed a significant reduction of the number of the cells as well as an arrested thymocyte differentiation.

To evaluate the function of somatostatin (SST) on chicken T-cells, we have studied the effects of SST *in vitro* on the gene expression profile of genes encoding the cytokines interleukin-1β (IL-1β), IFN-γ and transforming growth factor-β_4 (TGF-β_4), and also the components of the major histocompatibility complex class I (MHC-I), β_2 microglobulin (β_2M) and the MHC class I α-chain (MHC-IA), as well as the newly characterized chicken chemokine receptor, CXCR4 (156).

Materials and methods

Phenotypic characterization of T-cell line

A chicken T-cell line (ATCC CRL-12357, US Patent 5,691,200) for the study of mature splenic T-cells was kindly donated by Dr. Mike Kogut (USDA-ARS, College Station, TX). This chicken spleen T-cell line was generated by incubating splenocytes with

Concanavalin A and subsequently transforming the cells with avian reticulo-endotheliosis virus. Cell suspensions were prepared and incubated with two directly labeled primary antibodies: mouse anti-chicken CD8α-PE (0.2 μg/10^6 cells) and mouse anti-chicken CD4-FITC (1 μg/10^6 cells) (Southern Biotechnology, Birmingham, AL) on ice for 40 min. Cells were washed twice with cold PBS. Flow cytometric analysis was conducted by Dr. Roger Smith, III in the core lab for flow cytometry in the Veterinary Pathobiology Department at Texas A&M University. Briefly, labeled cells were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, CA, USA) using CELLQuest software. Data were analyzed with the FlowJo software packet (Treestar Inc., CA).

SSTR2 RT-PCR from chicken T-cell line

Total RNA was isolated from chicken thymus and pituitary, using TriZol reagent (Invitrogen, Carlsbad, CA). RNA content of the samples were quantified with a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany), and treated with RNase-free Dnase I (Invitrogen, CA) to digest any residual DNA. The first strand cDNA was synthesized from approximately 1.2 μg of total RNA using RETROscript reverse transcriptase (Ambion, Austin, TX) and random hexamers or dexamers according to the manufacturer's protocol. PCR primers were designed by using Primer Express 2.0 (Applied Biosystems), based on the cDNA sequence of chicken somatostatin receptor type 2 (Gallus gallus, gi:50757970 XM_425384). Based on the predicted sequence, the expected amplicon size is 456 bp. PCR primer oligonucleotides were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). Primer sequences were as follows:

Forward:GCGGATGAGCTGTTCATGCT

Reverse: GATGCCTGAGGACTTGACTTTGA

The first-strand cDNA was amplified in a 50 µl PCR reaction that contained 2 mM $MgCl_2$, 200 µM dNTP mix, 1.0 µM of each of the forward and reverse primers, PCR buffer and 1.25 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Wellesley, MA). The polymerase was activated by a pre-heating step at 94 ^{0}C for 10 min. The PCR amplification protocol comprised 30 cycles consisting of 30 s at 94 ^{0}C and 70 s at 65 ^{0}C, with a final extension step at 65 ^{0}C for 10 min. The products from the RT-PCR protocol were analyzed on a 1.5% agarose gel using ethidium bromide for visualization. The results were photographed with a MultiDoc-It Digital Imaging System (UVP, Upland, CA).

Cell culture

Cell cultures were propagated in Dulbecco's Modified Eagle's medium (DMEM) containing 2 mM glutamine, 10% (v/v) fetal bovine serum, and 100 U of penicillin and 10 mg of streptomycin per ml. Cells were counted and plated in 6-well plates at a density of approximately 5 x 10^6 cells /ml, at 37 ºC in a 5% CO2-balanced air environment Plates were incubated for 2h, 4h, 8h, 12h, 24 h and 48h, with three different concentrations (pM, nM, µM) of SST (Sigma, St. Louis, MO). Treatments were tested in duplicate. At the end of the respective treatments, the cells were harvested and processed individually for total RNA extraction.

Real-time polymerase chain reaction (real-time PCR)

Total RNA was extracted from each group of cells using Trizol (Invitrogen, Carlsbad, CA). Total RNA concentrations were determined by OD 260. cDNA synthesis was carried out using the 2-step reverse transcriptase polymerase chain reaction (RT-PCR) method (RETROscript Protocol; Ambion, Austin, TX). Briefly, RNA was combined with 1 µl of oligo dT (1 µM final concentration) and denatured at 70 ºC for 10 min. Then, 1 µl of RNase inhibitor (10 U/ml), 2 µl 10X RT buffer, 4 µl of dNTP mix (2.5 µM each dNTP final concentration), and 1 µl of MMLV reverse transcriptase (100 U/ml) were added to a final volume of 20 µl. The mix was incubated at 42 ºC for 60 min. The resulting cDNA was denatured at 92 ºC for 10 min and stored at –80 ºC.

Real-time PCR amplification was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems), in 384-well plates. The target genes included in this study were: IFN-γ and transforming growth factor-β_4 (TGF-β_4), MHC-β_2 microglobulin (β_2M) and the MHC class I α-chain (MHC-IA), as well as a newly characterized chicken chemokine receptor, CXCR4, chicken IL-1, and β-actin as the endogenous control. Primers used for the PCR (Table 5.1) were designed using Gene Express 2.0 (Applied Biosystems, Foster City, CA, USA) or based on literature data (157). The target specificity of primer sequences was verified by comparison with genomic sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/ Blast.cgi). Each PCR reaction included 1 µl of cDNA sample, 10 µl of SYBR Green master mix (PE-Applied Biosystems) and 500 nM of the appropriate gene-specific forward and reverse primers, in a final reaction volume of 20 µl. Samples were loaded using the

Eppendorf epMothion 5070 robotic workstation. The thermal cycling conditions included an initial denaturing step at 95 ^{0}C for 10 min, 40 cycles at 95 ^{0}C for 15 s, and 65 ^{0}C for 1 min. All mRNA samples were analyzed in triplicate. The effect on the gene expression profile of SST in the chicken T cell line was calculated using the ΔΔCT method (User Bulletin #2 for PRISM Sequence Detection System, ABI). The results represent the averages of three samples, each run in triplicate, meaning each data point is the result of 6 measurements.

Statistical analysis

The effect on the relative gene expression that resulted from SST treatment of chicken T-cells was calculated using the ΔΔCT method(158) (159), using the following formula:

(treatment mRNA levels) / (control mRNA levels) = $2^{-\Delta\Delta CT}$, where:

$\Delta\Delta CT = (CT \text{ target gene} - CT\ \beta\text{-actin})_{\text{treated}} - (CT \text{ target gene} - CT\ \beta\text{-actin})_{\text{control}}$

Table 5.1. Real-time PCR primers

The primers for real-time PCR were designed based on chicken gene sequences using Gene Express or based on literature data (157). *Gene sequences were obtained from http://www.ncbi.nlm.nih.gov/

Gene name	**Accession Number***	**Forward 5'— 3'**	**Reverse 5'—3'**	**Exon boundaries**
IL-1	AJ245728	GCTCTACATGTCGTGTGTGATGAG	TGTCGATGTCCCGCATGA	5/6
TGF β	M31160	AGGATCTGCAGTGGAAGTGGAT	CCCCGGGTTGTGTTGGT	6/7
β_2 M	Z48921	CTACAAGTGGGATCCCGAGTTC	TCATTTCAACTTGGAATGCAGAA	2/3/4
MHC-IA	M84766	CAGCGGCGCTACAACCA	GATGTCACAGCCGTACATCCA	2/3
CXCR4	AF294794	CCTTGCGTTCTTCCATTGCT	GCATTTTGTGCTGATGTTTTGAA	1/2
Fas Ligand	AJ890143	AAGGCATGACCAGAGACAGGTT	AAGCCAGTGAAAAAGGAAGCAA	3/4
INF-γ	Y07922	GTGAAGAAGGTGAAAGATATCATGGA	GCTTTGCGCTGGATTCTCA	3/4
β-actin	L08165	CTGATGGTCAGGTCATCACCATT	TACCCAAGAAAGATGGCTGGAA	1/2

Result

Phenotypic characterization of T-cell line ATCC CRL-12357

Flow cytometric analysis of the phenotype of the chicken splenic T-cell line ATCC CRL-12357 using fluorescently (directly) labeled anti-CD4 (fluorescein-labeled) and anti-CD8 (phycoerythrin-labeled) antibodies (Fig. 5.1.). The results of the analysis reveal a heterogeneous cell mixture, in that 66.9 % of cells are strongly CD4-positive, while 32.3 % of cells do not express CD4 noticeably above background level, and can technically be considered as double negative cells, although it is more likely that these immortalized cells have de-differentiated and lost expression of CD4 after multiple cell divisions.

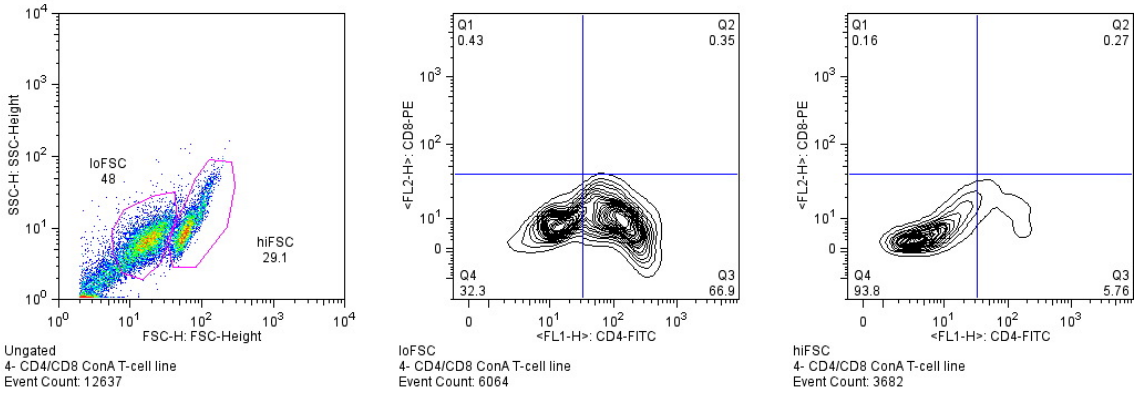


Fig. 5.1. Flow cytometric analysis of the phenotype of the chicken splenic T-cell line ATCC CRL-12357 using fluorescently (directly) labeled anti-CD4 and anti-CD8 antibodies. The results of the analysis show that 66.9 % of cells were strongly CD4-positive, while 32.3 % of cells did not express CD4 above background level.

Expression of SSTR2 in virally immortalized splenic T-cells

Analysis of the total RNA isolated from the splenic T-cell line by RT-PCR showed that SST receptor subtype 2 (SSTR2) is expressed in the chicken ConA-activated T-cell line used in this study. An expected amplicon of 456 bp was observed (Fig. 5.2). This provides the basis for the further in vitro study of the potential effects SST on the gene expression profile cell line.

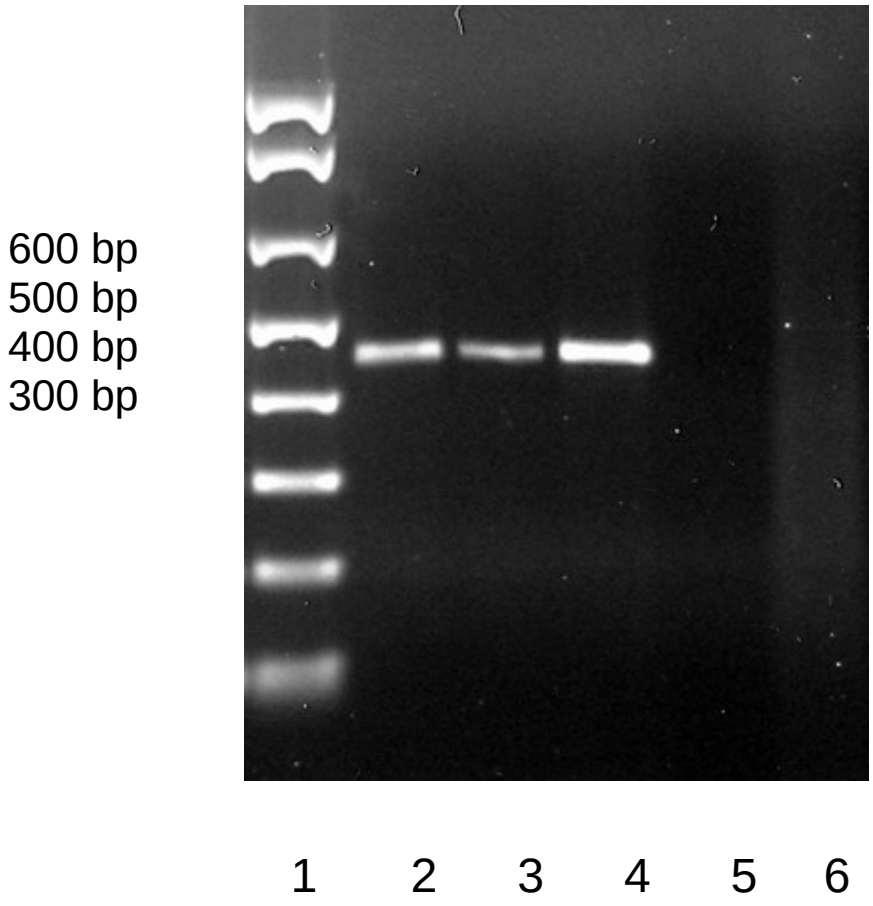


Fig. 5.2. Analysis of splenic T-cell line RNA for the presence of SSTR2 mRNA by use of RT-PCR. An expected amplicon of 456 bp was observed. Lane 1. 100 bp DNA ladder; 2. Sample A (using hexamers as primers in RT); 3. Sample B (using oligo-dT as primers); 4. Positive control; 5. Negative control (H_2O); 6: DNase treated RNA (negative control).

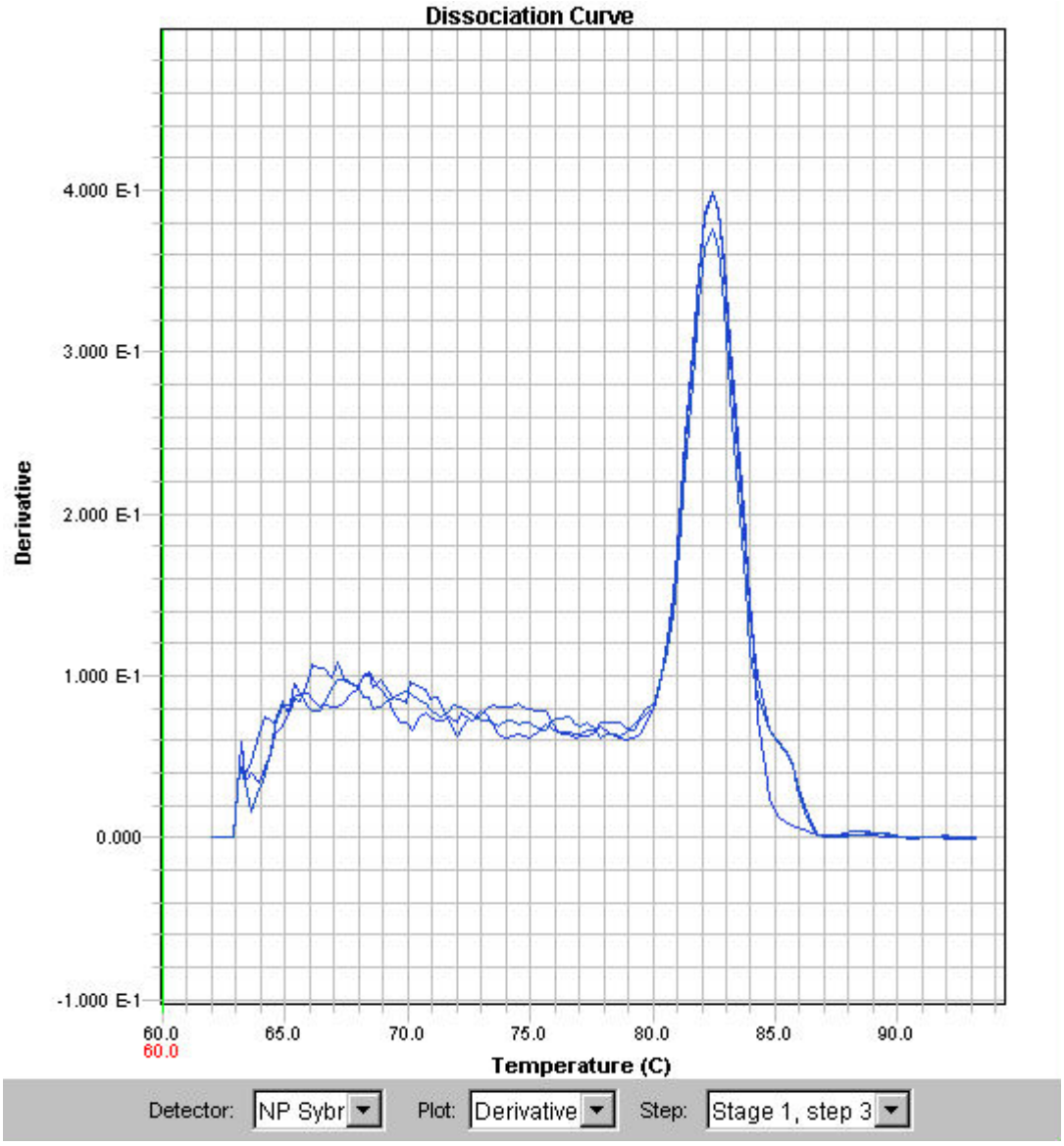


Fig. 5.3. Examples of dissociation curve of different genes in real-time PCR reactions. The figure shows that a specific amplicon (one peak) was synthesized in each reaction. Each figure includes three repeats of the same gene amplification, showing the high repeatability of the real-time PCR analysis.

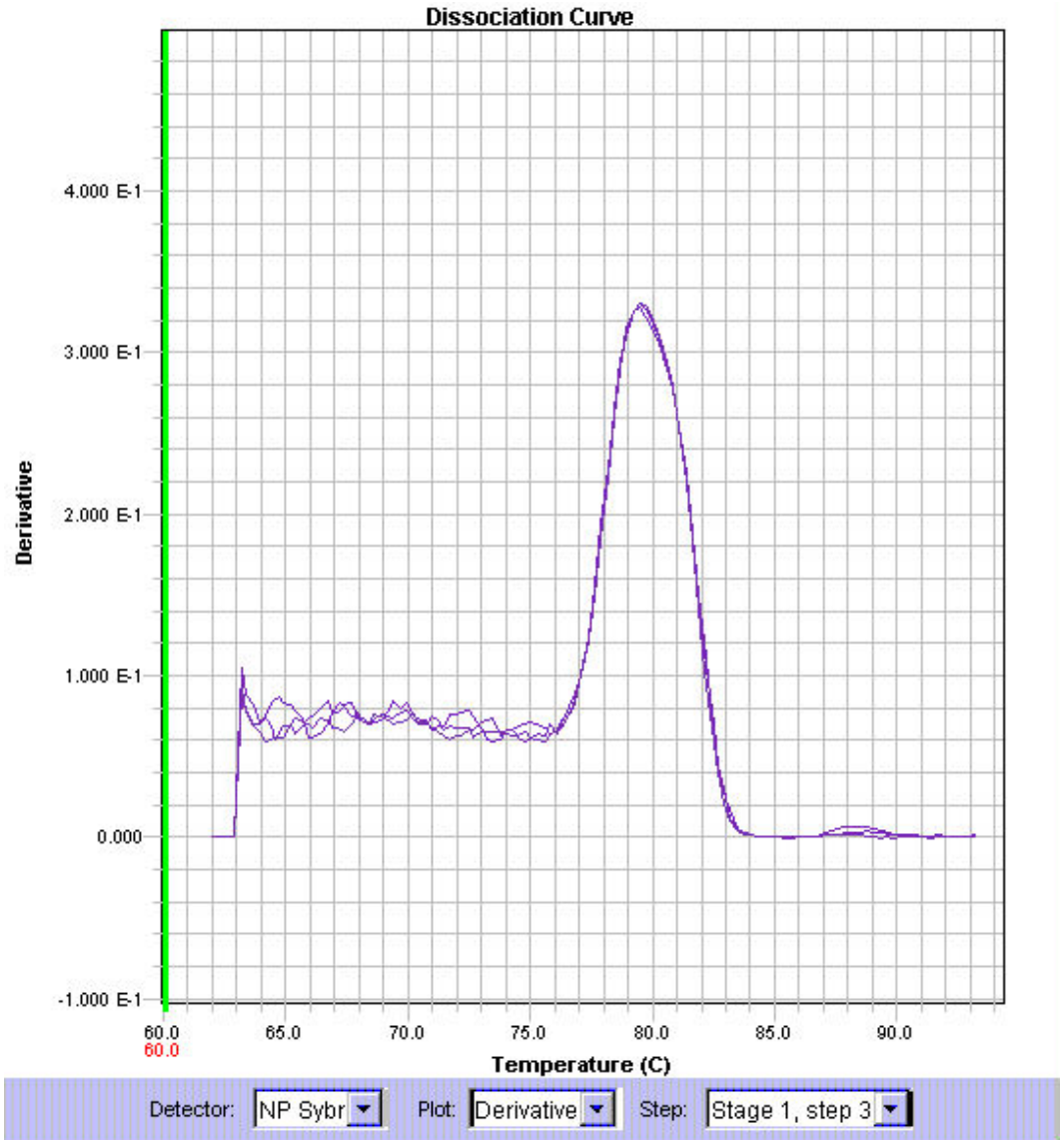


Fig. 5.3 Continued

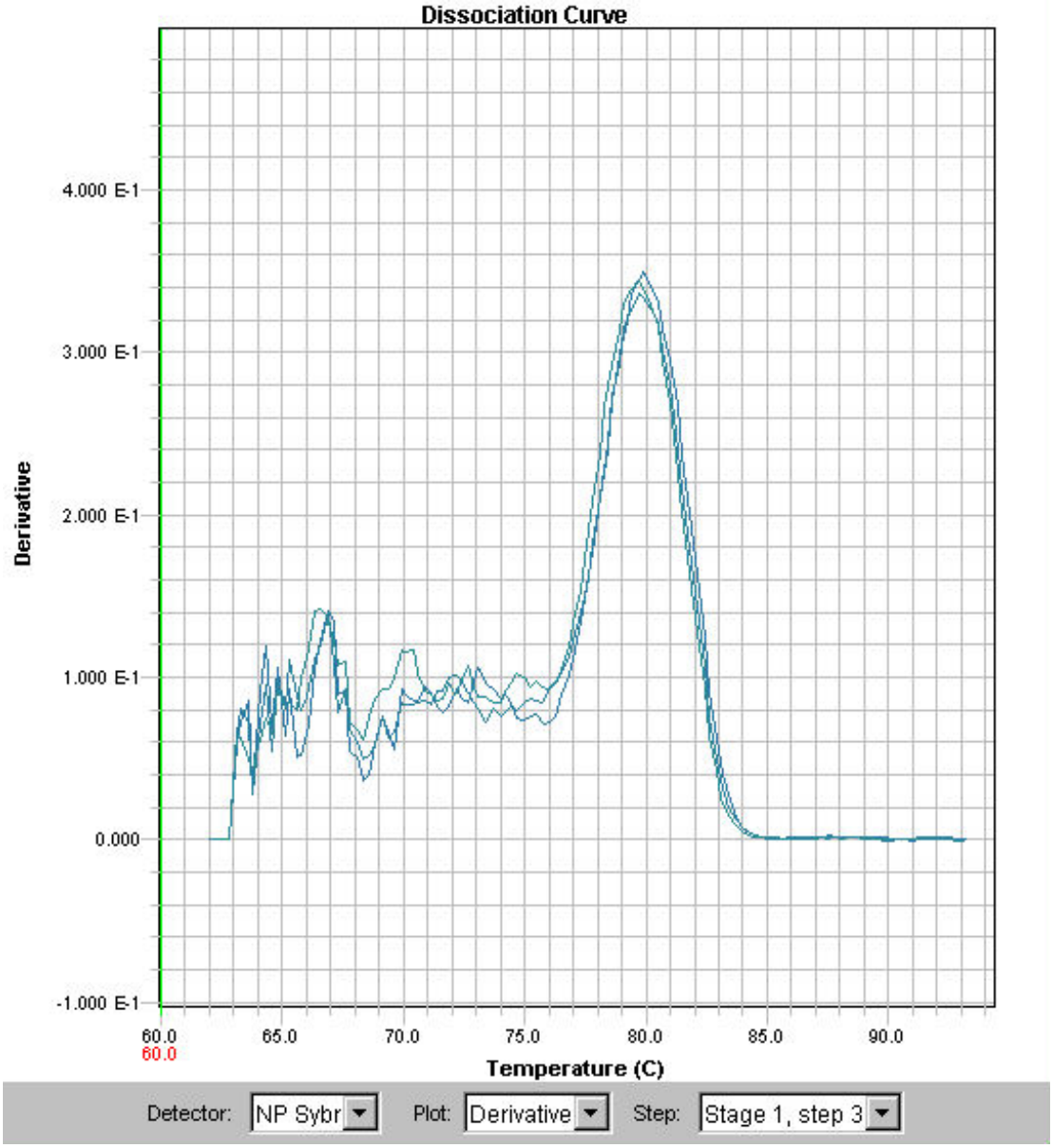


Fig. 5.3. Continued

Real-time PCR analysis of SST-induced gene expression changes in T-cells in vitro

From the dissociation curves of the real-time PCR analyses (see examples in Fig. 5.3), it was clear that a specific amplicon was synthesized in each reaction, as only one peak was observed in every plot. Each panel of Fig. 5.3 includes three repeats of the same gene amplification, indicating that the overall PCR reaction was highly reproducible. Loading quality is high as an automatic robotic loading workstation was used instead of manual pipeting.

A. Short term effects (observed within 4 hours post-treatment)

Since there was only time to run one independent trial (although the *in vitro* stimulation was run in duplicate in separate plates, we decided to disregard any shifts that were not at least 2-fold different from control levels (up- or down-regulated) as potentially not biologically relevant fluctuation. As shown in Figs. 5.4-10, the observed effects of SST on chicken T-cells were highly complex.

The most surprising result of this entire series of analyses was certainly the observation that somatostatin at micromolar levels increased the expression of IL-1β in splenic chicken T-cells *in vitro* dramatically (> 25-fold) and fast (within 2 hours or less) (Fig. 5.4.). While the response was fast and big, it was clearly transient, since expression levels were reduced to control levels by 4 hours after SST exposure.

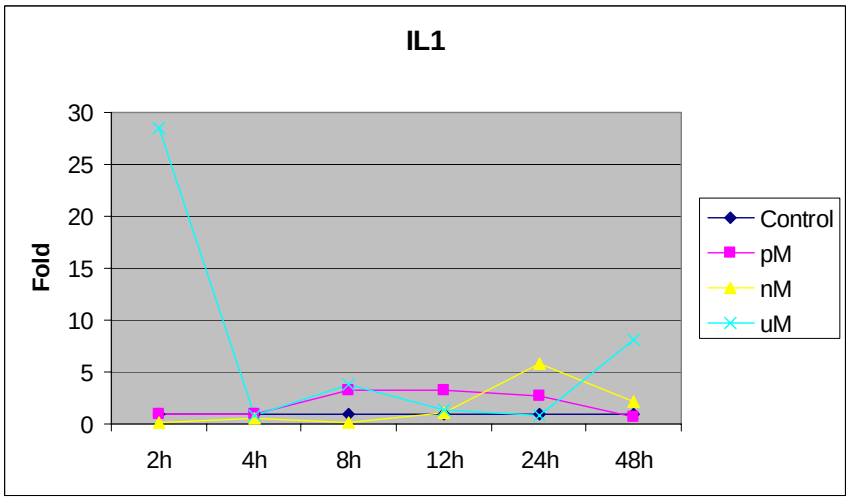


Fig. 5.4. Real-time PCR analysis of IL-1 mRNA levels in immortalized splenic T-cells after stimulation with somatostatin as a function of time and dose. Each data point represents the result of 6 measurements (duplicate *in vitro* incubation and analysis of individual mRNA samples in triplicate). The expression level of actin was used as a calibrator.

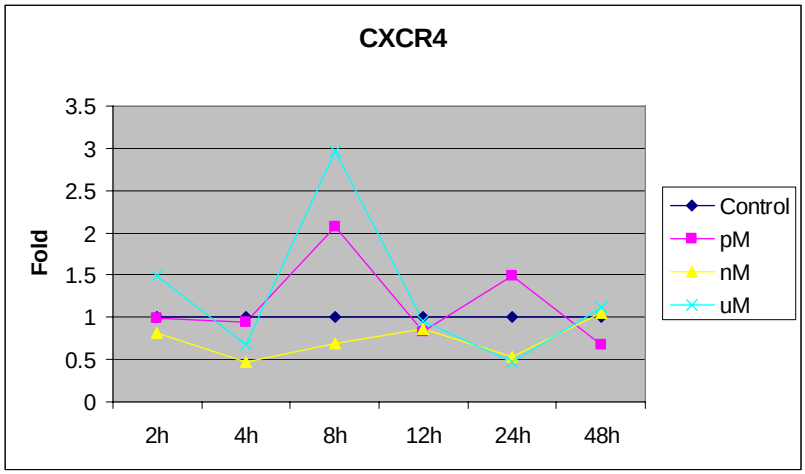


Fig. 5.5. Real-time PCR analysis of CXCR4 mRNA levels in immortalized splenic T-cells after stimulation with somatostatin as a function of time and dose. Each data point represents the result of 6 measurements (duplicate *in vitro* incubation and analysis of individual mRNA samples in triplicate). The expression level of actin was used as a calibrator.

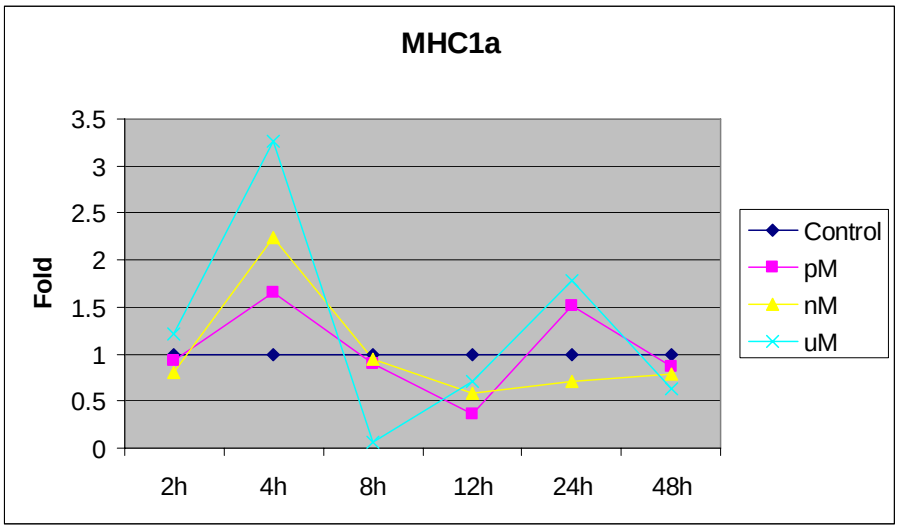


Fig. 5.6. Real-time PCR analysis of MHC-1α mRNA levels in immortalized splenic T-cells after stimulation with somatostatin as a function of time and dose. Each data point represents the result of 6 measurements (duplicate *in vitro* incubation and analysis of individual mRNA samples in triplicate). The expression level of actin was used as a calibrator.

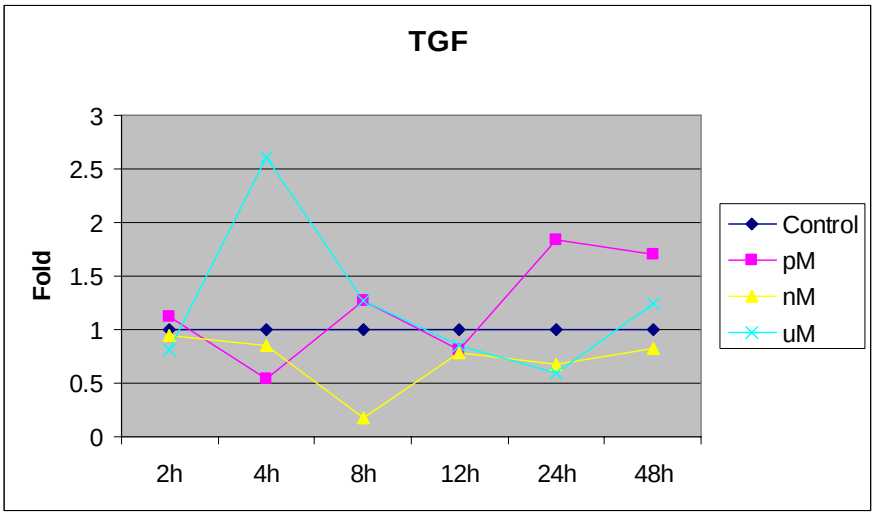
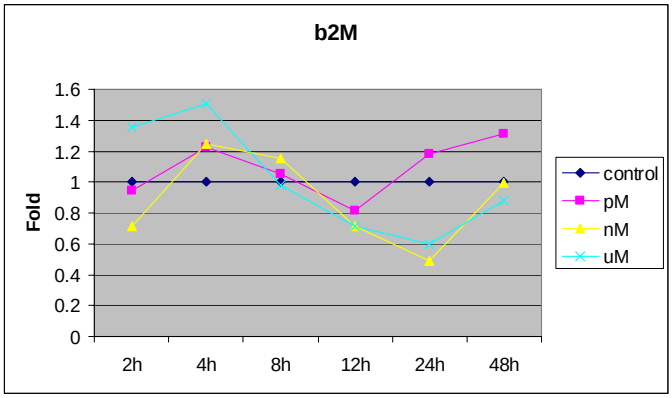


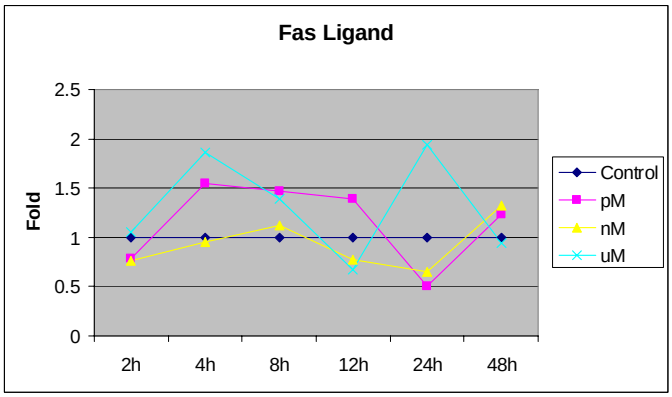
Fig. 5.7. Real-time PCR analysis of TGF-β4 mRNA levels in immortalized splenic T-cells after stimulation with somatostatin as a function of time and dose. Each data point represents the result of 6 measurements (duplicate *in vitro* incubation and analysis of individual mRNA samples in triplicate). The expression level of actin was used as a calibrator.

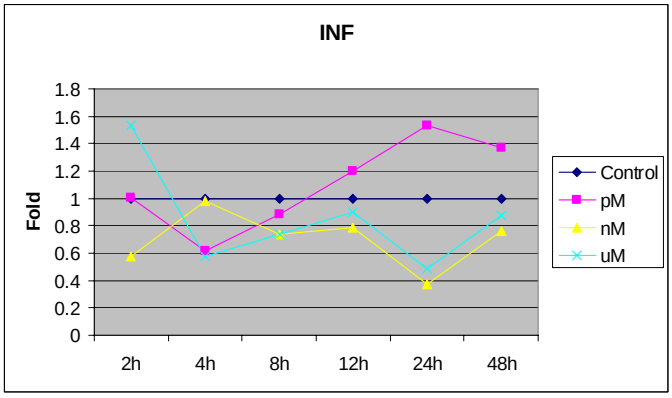
T-cell CXCR4 mRNA levels were increased nearly 3-fold after 8h treatment (Fig. 5.5). MHC-Iα mRNA levels (Fig. 5.6) were increased at 4 h in a dose-dependent manner and reached a more than 3-fold increase at micromolar concentrations of SST. At micromolar levels, SST upregulated the gene expression of TGF-β4 after 4 hours of treatment (Fig. 5.7). At picomolar levels, however, the observed effect was opposite, *i.e.* a downregulation to approximately 50% of the control level was measured. No dependable effects on Fas Ligand, β2-Microglobulin and INF-γ production were observed (Figs. 5.8-10).

B. Long-term effects (after 8 – 48 hours of treatment with SST)

Although several apparently significant gene expression shifts were observed 8 hours or more after the start of the SST treatment (Figs. 4-10), these may very well be indirect consequences of previous short term effects of SST on IL-1. However, since we did not have access to an immunoassay (or bioassay) for the quantification of IL-1 protein levels in the cell culture media, it remains to be seen whether any long-term effects are actually SST-mediated effects or IL-1 effects.







Figs. 5.8. Real-time PCR analysis of β2-microglobulin, Fas ligand and interferon γ mRNA levels, resp., in immortalized splenic T-cells after stimulation with somatostatin as a function of time and dose. Each data point represents the result of 6 measurements (duplicate *in vitro* incubation and analysis of individual mRNA samples in triplicate). The expression level of actin was used as a calibrator.

Discussion

The effect of SST on cytokine mRNA expression of lymphocytes is clearly a complex phenomenon. One of the remarkable findings in this study is the induction of IL-1β expression by SST. Although the response to SST was fast and transient, SST (in μM concentrations) dramatically (> 25-fold) increased the expression of IL-1β in splenic chicken T-cells *in vitro*. Micromolar quantities of any neuropeptide may seem excessive and thus pharmacological in nature, but given the fact that the SST that reaches the T-cells in the thymus or in the spleen can perfectly be produced by a paracrine source, exposure of T-cells to micromolar SST concentrations may be quite realistic in an *in vivo* situation. Unfortunately, such a claim is nearly impossible to verify. However, also picomolar concentrations proved effective at enhancing IL-1 mRNA levels in the present study: a sustained approx. 3-fold upregulation was observed, albeit only after 8 hours of exposure to SST. It is at this point unclear which of the two observed effects is more plausible *in vivo*, although both might occur under different physiological conditions.

The significantly elevated mRNA level of this pro-inflammatory cytokine is surprising, as it is in contrast with the known anti-inflammatory effects that have been reported for SST (160-162). Theoretically this apparent contradiction could be explained by the hypothesis that SST might promote a compensatory overproduction of IL-1β in response to its consumption and/or blockade in mitogen/antigen-activated T-cells. A similar phenomenon was observed in previous studies on a rat arthritis model, where *i.v.* administration of a synthetic SST analogue (octreotide) did attenuate the clinical inflammatory symptoms, but at the same time SST also significantly increased both the mRNA levels of IL-1 β in local

tissues (163) as well as IL-1β protein levels in serum (164). The mechanism and the physiological meaning of this effect of SST still needs to be further studied.

On the other hand, one needs to recognize that increased mRNA levels may not necessary lead to significant translation of IL-1 protein. Clinical studies have indeed shown that measurements of elevated levels of IL-1β mRNA in cells do not necessarily reflect elevated protein levels of IL-1 β. A dissociation of transcription and translation seems to be characteristic for IL-1β, as the propeptide of IL-β requires proteolytic cleavage by caspase 1 in order to become bioactive (165, 166). In other words, SST may stimulate transcription rather than translation of IL-1. Unfortunately, we did not have access to an immunoassay for the measurement of chicken IL-1 in the culture supernatants produced in this study. In contrast, other studies have shown that SST can actually *inhibit* IL-1β secretion by LPS-activated human monocytes (86) and rat hepatic stellate cells (Lang A, 2005).

CXCR4 is the specific receptor for chemokine SDF-1(Stromal-cell Derived Factor-1). In the present study, the expression of CXCR4 was shown to be up-regulated after 8h of treatment with SST. Interestingly, SST has been described to inhibit SDF-1-α-induced T-cell infiltration in humans (167). Thus, it is hard to ascribe the up-regulation of CXCR4 to the direct effect of SST. However, when looking back at 2h, a significant increase of the IL-1 mRNA was observed, and if translation did in fact take place, the effect on CXCR4 may be indirectly due to the SST-induced increase of IL-1 concentrations in the medium. This hypothesis is in line with previous studies describing the enhancement of CXC

chemokine receptor 4 (CXCR4) mRNA expression upon treatment with the cytokine IL-1β (168).

Taken together, our data demonstrate that SST can and does affect the cytokine and chemokine receptor transcription in chicken T-cells; however such effects appear to be highly context-dependent (162). They clearly depend on the cell type under study and the stage of the activation of this cell type, as well as on the cytokine in question.

CHAPTER VI

SUMMARY

For a long time, immune and nervous/neuroendocrine systems have been viewed as independently functioning entities, “hidden” from each other, so to speak. However, accumulated evidence obtained during the last few decades has profoundly changed this concept (For reviews, see(100, 169).In fact, the communication between the immune and nervous/neuroendocrine systems has proven to be extensive(2, 162) and, more importantly, the cross-talk between the two systems is crucial to maintain homeostasis, and therefore for the survival of the organism(169). In this study, we focused on the diffuse neuroendocrine cells in central immune organ (the thymus) in the chicken and aimed at investigating how the neuroendocrine factors (*e.g.* somatostatin) regulate or modulate immune function.

In birds, as in mammals, the process that creates the diverse immunological repertoire of T-cells recognizing non-self antigens has been shown to be critically dependent on the microenvironment of the thymus. The thymic stroma is complex and consists of epithelial cells, mesenchyme, macrophages and dendritic cells, in addition to fibroblasts and extracellular matrix molecules. These components provide not only essential cell-cell contacts but also communicate with the developing T-lymphocytes in a humoral manner. The thymus produces a number of unique humoral factors and an ever-growing list of neuroendocrine and peripheral hormones. While some of these, such as prolactin and growth hormone, have well characterized effects on thymocyte differentiation and proliferation, many have been identified but have not been assigned a defined function within the thymus, and arguably many messenger molecules remain to be identified.

Moreover, the function of local neuroendocrine cells within lymphoid organs remains still largely elusive. Although the presence of neuropeptides (such as neurotensin and somatostatin) in the chicken thymus was reported as early as 1978 (101), a methodical analysis of neurosecretory cells in a chicken lymphoid organ, regardless of the secretory product involved has, to our knowledge, not been described. The diffuse neuroendocrine/endocrine cell populations are believed to produce major chemical categories of messenger molecules, hormones, neurotransmitters or neuropeptides, which locally exert their paracrine activities through their specific receptors on immune cells (98).

In an attempt to uncover this complex neuroendocrine-immune network, a first and essential step is to identify and characterize these poorly defined neuroendocrine populations within immune organs/tissues. One of the most important landmarks that are extremely helpful in defining neuroendocrine cells is the presence of dense-core secretory granules(170). Numerous studies have provided evidence that the large dense-core secretory granule, also known as large dense cored vesicle (LCDV) is an organelle for storage of prohormones, pro-neuropeptides, processing enzymes, and other proteins required for regulated secretion in endocrine and neuroendocrine cells. Both Chromogranin A (CgA) and Carboxypeptidase E (CpE) are characteristic functional and structural elements of the secretory granule in the regulated secretory pathway. CpE is a processing enzyme that catalyzes the transformation of peptide precursor proteins into bioactive peptide hormones, but it also serves as a sorting receptor in the regulated secretion pathway, while chromogranin A is a classic marker protein and is considered an ‘on/off’ switch for the biogenesis of the LDCV. The expression of the specific mRNAs for both

CgA and CpE in the thymus was first verified by RT-PCR. Additional evidence at the protein level, using immunofluorescent dual labeling, has allowed us to demonstrate the co-existence of CgA and CpE in identical neuroendocrine cells in an avian primary lymphoid organ. These CpE- and CgA-positive cells were primarily found in the transition zone between the cortex and the medulla of the thymic lobules, an area known to contain numerous arterioles and to be heavily innervated by the autonomic nervous system. Interestingly, additional but clearly different neuroendocrine cells with similar distribution were identified using two other markers, one for neurons (neuron-specific enolase) and one for neural-crest derived cells (HNK-1). For the remainder of the study, we chose to focus on the CgA-positive population, because those are more likely to take part in regulated secretory activity. Based on its neuro-anatomical location and the presence of two hallmark molecules typical for cells involved in regulated secretory activity, it is tempting to speculate that the thymic diffuse neuroendocrine system may serve as a relay, for nervous stimuli delivered by the sympathetic and/or parasympathetic nervous system, for humoral factors delivered by the circulation, or both, which would provide a mechanism for fine tuning thymopoiesis by a variety of physical and environmental factors perceived by the nervous system.

The existence of complex neuroendocrine cell populations within chicken thymus has led us to further study the distribution and biological effects of releasing factors, such as neuropeptides, produced by these cells. Particularly, our attention was drawn to somatostatin (SST), which in mammals has been shown to exert multiple effects on immune functions within the thymic microenvironment (62, 65, 67). In chapter 4 we have

examined if both somatostatin and at least on of its receptors (SSTR2) are locally expressed within the chicken thymus and we have investigated their potential effects on the neighboring thymic cells. The results showed that both SST and SSTR2 are expressed locally within chicken thymus. Furthermore, we have evaluated the physiological role of somatostatin *in vitro* with respect to thymic cell proliferation, differentiation and apoptosis. The results suggest that SST significantly inhibits IL-2 and ConA induced proliferation of thymocytes. In comparison with controls (medium containing IL-2 and ConA but without SST), addition of SST at 10^{-9} M and 10^{-6} M resulted in a nearly 20% decrease in proliferation ($P < 0.01$). At 10^{-12} M of SST, however, this anti-proliferative effect was no longer statistically significant.

To further unravel the effects of somatostatin (SST) on the immune system, the role of SST on the gene expression of cytokines, chemokine receptors as well as MHC-I components was assessed. In chapter 5, RNA samples from a somatostatin treated splenic chicken T-cell line were assayed *in vitro* for the production of mRNA encoding the cytokines interleukin-1β (IL-1β), IFN-γ and transforming growth factor-β_4 (TGF-β_4), and also components of the major histocompatibility complex (MHC), β_2 microglobulin (β_2M) and the MHC class I α-chain (MHC IA), as well as a newly characterized chicken chemokine receptor, CXCR4. Somatostatin at micromolar level dramatically (> 25-fold) increased the expression of IL-1β in splenic chicken T-cells *in vitro*. The response was fast (within 2 hours or less) and transient. However, at the picomolar level, a sustained but more moderate (up to 3-fold) upregulation was observed between 8 and 24 hours after the SST treatment was started. T-cell CXCR4 mRNA production was increased nearly 3-fold after 8 hours of SST treatment.

MHC-Iα mRNA levels increased at 4h in a dose-dependent manner and reached more than 3-fold upregulation at micromolar concentrations of SST. No significant effects on Fas Ligand, β2-Microglobulin and INF-γ gene expression were observed.

In conclusion, the diffuse neuroendocrine component of the avian thymus was characterized as a complex cell group, existing of at least three different subpopulations that potentially receive input from each other, from the autonomous nervous system, from the circulation, or all of the above. Moreover, this study has provided evidence that, also in birds, neuroendocrine circuits within the thymus may be important, if not essential, for the education and balance between self-tolerance and immunity, as appears to be the case in mammals. (Neuro)endocrine messenger molecules produced by the thymic microenvironment, such as somatostatin, seem to play a potentially important immunomodulatory role with regard to the cell proliferation, differentiation, migration, as well as cytokine production. However, the question as to exactly which stimuli trigger the release of mediators such as somatostatin remains a field of future study. In addition, a complete inventory of all substances stored in the thymic LDCV and their effects on the developing T-cells when released in the microenvironment of the thymus are also questions that warrant further investigation.

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