IMMUNOREACTIVE NEUROPEPTIDES IN THE CELLS OF HUMAN THYMUS

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Abstract - The study was designed to explore the expression of different neuropeptides, viz. vasoactive intestinal peptide (VIP), calcitonin gene related peptide (CGRP), substance P (SP), bombesin and motilin in the cells of fetal and adult human thymus. Immunohistochemical staining revealed that cortical and medullary thymocytes were labeled by all antibodies, except those specific for motilin. Immunoreactive VIP and SP were observed in the solitary epithelial cells located in the subcapsular/subtrabecular cortex, at the corticomedullary junction and in the medulla. The cells within the subcapsular/subtrabecular monolayer, rare solitary cells in the deep cortex and epithelial cell network in the medulla, were labeled with antibodies to CGRP and bombesin. Hassall's corpuscles were labeled with all antibodies except that specific for SP. The obtained data obtained testify to the expression of different neuropeptides in human thymic lymphoid and non-lymphoid cells and suggest a role for neuroendocrine hormone-mediated mechanisms in the regulation of thymic homeostasis in humans.

Key words: VIP, Substance P, CGRP, bombesin, motilin, human thymus

INTRODUCTION

The well-documented presence of peptide neurotransmitter and hormone receptors on immune cells (Homo-Delarche and Dardenne, 1983; Bost, 1988; Jonhson and Torres, 1988; Heijnen, 2007) suggests that the peptides that regulate brain and endocrine system functions could be endogenous immunomodulatory substances, as well as bidirectional transmitters of information between the neuroendocrine and immune systems (Blalock, 1995). On the other hand, the reciprocity of receptors in the brain and endocrine system for immune system regulatory molecules such as the cytokines, has been shown (Homo-Delarche and Dardenne, 1983, Besedovsky and del Rey, 1996). Today there is a substantial body of evidence that the sharing ligands (peptide hormones, peptide neurotransmitters and cytokines) and their receptors constitute a complete biochemical information circuit between and within the immune and neuroendocrine systems (Heijnen, 2007).

The thymus is considered to be the most important lymphoid organ in the interactions between the neuroendocrine system and the immune system (Homo-Delarche and Dardenne, 1983; Blalock, 1995, Fabris et al., 1989). The thymus, apart from adrenergic (Al-Shawaf et al., 1991; Felten and Felten, 1989; Madden and Felten, 1995; Ackerman et al., 1991; Leposavić et al., 1992) and cholinergic (Al-Shawaf et al., 1991; Mićić et al., 1992 Bul-

loch and Pomerantz, 1984) nerve inputs, receives substantial peptidergic innervation (Kendall and Al-Shawaf, 1991; Bellinger et al., 1990; Weihe et al, 1989). In addition, some of neuropeptides have been found in the thymic epithelial cells (TECs) (Gomariz et al., 1990; Ericsson et al., 1990; Silva et al., 2006) and/or thymocytes (Soder and Hellstrom, 1989; Gomariz et al., 1993). Most of these findings were obtained from investigations into rodent and chicken thymi (Gomariz et al., 1990; 1993; Ericsson et al., 1990; Silva et al., 2006; Soder and Hellstrom, 1989; Weihe et al., 1989). There are only limited data available on the presence of neuropeptides in the human thymus. Therefore we examined whether the the neuropeptides VIP, SP, CGRP, motilin and bombesin are present in cells of the human thymus.

MATERIALS AND METHODS

Tissue preparation

This study included 15 human thymi from fetal to young adult subjects of both sexes, in an age range of between the 16th week of gestation and 21 years. The thymi were obtained either during autopsy (age range from 16th week of gestation to first postnatal day) or during cardiac surgery (age range from 4 to 21 years). Conventional histology did not manifest abnormalities in the architecture and cytological details of these thymi. These specimens were fixed either in 4% phosphate buffered formalin or in Bouin's fixative and embedded in paraffin. The fixative did not influence the outcome of immunohistochemistry. Serial 5 μm thick sections were cut on a slide microtome and mounted on poly-L-lysine-coated glass slides.

Immunohistochemistry

Thymic sections were dewaxed in a series of ethanol and xylene washes before rinsing three times for 5 min in 0.1 M PBS, pH 7.4. Endogenous peroxidase was blocked by incubation in 0.03% hydrogen peroxide in absolute methanol (30 min.). After a thorough rinsing (3 x 5 min) in PBS, the sections

were incubated with 100 μl of 10% non-immune goat serum to eliminate non-specific background for 30 min. The sections were incubated with 100 μl of primary antiserum supplemented with 10% non-immune goat serum for 60 min. The primary antisera applied are listed in Table 1. After washing in PBS (3 x 5 min), the sections were incubated with 100 μl of biotinylated goat anti-rabbit IgG (Zymed Labs. Inc., CA, USA) supplemented with 5% normal human serum for 10 min. The sections were then rinsed in PBS (3 x 5 min) and incubated with 100 μl of enzyme conjugated (Zymed Labs. Inc., CA, USA) supplemented with 5% normal human serum for 10 min. All incubations were performed at room temperature in a moist chamber. After washing in PBS (3 x 5 min.), the staining was visualized by incubating the sections in 0.7 mM 2,2 diaminobenzidine (DAB) – HCl/0.002% hydrogen peroxide in TRIS-HCl, pH 7.6, for approximately 5 min. The incubation time was always checked under the microscope. After washing in PBS (3 x 5 min), the sections were counterstained in Mayer's hematoxylin, rinsed in tap water, and mounted in aqueous mounting solution (Mounting media, Zymed labs., Inc., CA, USA). The sections were analyzed under an Olympus BH2 research microscope.

For determination of an optimal dilution of the antisera, fetal and adult human colon tissues served as control tissues.

Control sections were routinely processed by omitting and/or replacing one of the following components: a) primary antiserum (replaced with an equivalent concentration of pre-immune rabbit serum); b) biotinylated second antibody (replaced with an equivalent concentration of pre-immune goat serum); c) enzyme conjugate; d) DAB. The specificity of the primary antisera was confirmed by preabsorbtion with homologous and heterologous peptides. The anti-SP was not absolutely specific for SP, but also recognized other tachykinins, namely neurokinin (NK) A and NKB, and therefore has to be regarded as a pan- or polytachykinin antiserum.
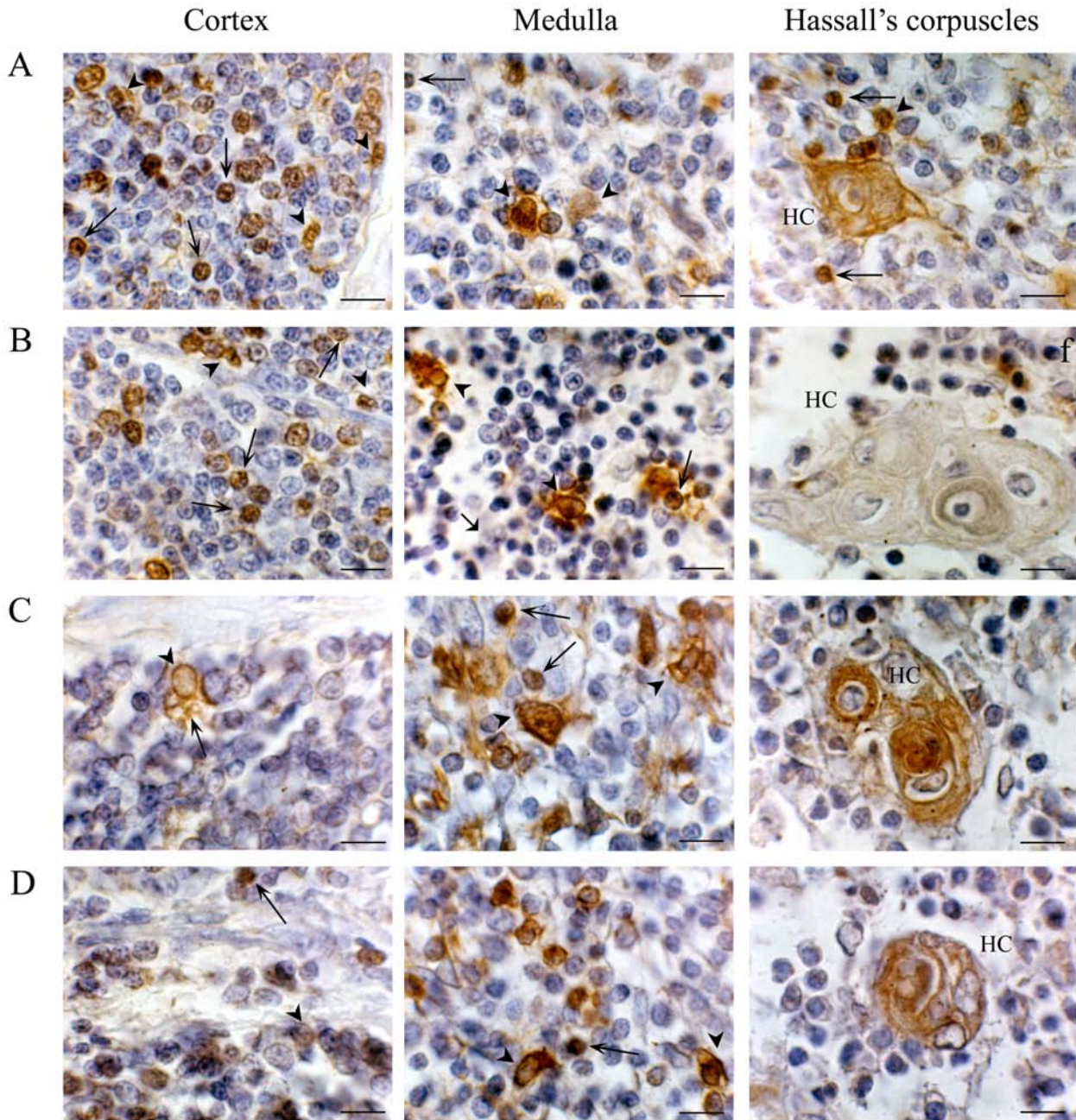
Fig. 1. Immunoperoxidase staining for neuropeptides in fetal human thymus. Thymic cortex (left photomicrographs), medulla (middle photomicrographs) and Hassall' corpuscle (right photomicrographs) stained with anti-VIP (Panel A), anti-SP (Panel B), anti-CGRP (Panel C), anti-bombesin (Panel D) antibodies. Thymocytes and epithelial-like cells exhibiting immunoreactivity are marked by arrows and arrowheads, respectively. Note immunoperoxidase staining of Hassall' s corpuscle (HC). Expression was observed for all of the neuropeptides tested when compared with the negative controls. Original magnification × 400; Bar, 20 μm

RESULTS

Thymocytes

We observed VIP immunoreactivity within subsets of both the cortical and medullar thymocytes. The highest density of thymocytes exhibiting VIP immunoreactivity was observed in the thymic deep cortex (Table 2 and Fig. 1). The same pattern of staining was demonstrated irrespective of the anti-VIP serum source (Table 1). The subsets of both cortical and medullar thymocytes also exhibited SP immunoreactivity. These cells showed a similar pattern of distribution to the VIP-immunoreactive (ir) thymocytes. The density of both VIP- and SP-ir thymocytes was very high in the fetal thymus.

Bombesin and CGRP immunoreactivity was observed in thymocytes in the thymic deep cortex and medulla. However, CGRP-ir thymocytes were more frequent in the thymic medulla than in the bombesin-ir ones (Table 2 and Fig. 1).

Thymocytes labeled with anti-motilin serum were not found (Table 2 and Fig 1).

The pattern of thymocyte immunostaining with none of used antibodies changed during ontogenesis markedly.

Thymic non-lymphoid cells

In addition to thymocytes, rare non-lymphoid, as regards size and shape, thymic epithelial cells (TECs) in the subcapsular/subtrabecular cortex, and solitary TECs at the corticomedullary junction and in medulla, showed VIP-specific cytoplasmic immunolabeling. VIP immunoreactivity was also found within the Hassall's corpuscles. This pattern of labeling was observed with anti-VIP serum from both sources (Table 1). A similar pattern of TEC labeling was observed when anti-SP serum was used. However, the density of SP-containing TECs at the corticomedullary junction, where thymocyte progenitors enter the thymus through the vasculature endothelium and migrate into subcapsular cortex (Takahama,

2006), was greater when compared with that of VIP-ir TECs. Unlike VIP immunoreactivity, SP immunoreactivity was not found in the Hassall's corpuscles. (Table 2 and Fig.1).

The staining using anti-CGRP serum was observed in the flattened cells of the thymic epithelial monolayer lining the inner surface of thymic capsule/interlobular septa, where the most immature thymocytes reside (Takahama, 2006). In addition, some scattered epithelial-like cells in the deep cortex and a dense reticular network of stellate epithelial cells in medulla...?! The pattern of labeling was mainly cytoplasmic. CGRP immunoreactivity was also found within the cells in the outer layer of the Hassall's corpuscles. A similar pattern of labeling was observed with anti-bombesin serum (Table 2. and Fig. 1).

Immunoreactive motilin was only found within the Hassall's corpuscles (data not shown).

DISCUSSION

The presented results show that: i) imunoreactive VIP, CGRP and SP found in chicken and rodent thymic cells (Gomariz et al., 1990; 1993; Ericsson et al., 1990; Silva et al., 2006; Soder and Hellstrom, 1989; Weihe et al., 1989) are also detectable in the cells of human thymi; ii) immunoreactive bombesin and motilin are present in the cells of human thymi; iii) immunoreactive neuropeptides are present in both non-lymphoid and lymphoid thymic cells; iv) the pattern of their thymic distribution is not uniform;, v) these neuropeptides are detectable even in fetal 16-week-old human thymus.

Generally, the occurrence of immunoreactive neuropeptides in thymic cells can reflect either the presence of neuropeptide produced within these cells themselves or the presence of neuropeptides (from nerve terminals or circulation) that are bound to receptors on the surface of these cells. So far it has been shown that rat thymocytes, mainly those at the corticomedullary junction and medulla, express the VIP gene (Gomariz et al., 1993), and that TECs express the preprotachykinin-A gene (Geenen et

Table 1. Primary antisera used in this study.

Antiserum	Raised in	Source	Code	Working dilution
Anti-VIP	rabbit	Serotec	PEPA 41	1:800
Anti-VIP	rabbit	Zymed Labs.	08-0080	1:800
Anti-SP	rabbit	Serotec	PEPA 40	1:1000
Anti-GCRP	rabbit	Serotec	PEPA 27	1:400
Anti-bombesin	rabbit	Serotec	PEPA 23	1:500
Anti-motilin	rabbit	Serotec	PEPA 34	1:1000

Table 2. Immunoreactive (ir) – VIP; ir-CGRP, ir-bombesin, ir-motilin, and ir-SP in thymic epithelial-like cells (TECs), thymocytes (Tlys) and Hassall's corpuscles (HCs) of the human thymus.

	CORTEX				MEDULLA		
	Outer		Deep				
	TECs	T lys	TECs	T lys	TECs	T lys	HCs
VIP	+	+	+	++	+	+	+
CGRP	+	+	+	+	+++	++	+
bombesin	+	+	+	+	+++	+	+
motilin	-	-	-	-	-	-	+
SP	+	+	++	+++	+	+	+

Immunoreactivity was evaluated on a 4-graded scale where dense immunoreactivity is designated as **+++,** moderate immunoreactivity **as ++,** sparse immunoreactivity **as +,** while - signifies lack of immunoreactivity. The presence of immunoreactivity within the Hassall's corpuscles is marked by **+.**

al., 1991). Also, neurokinin A,– a peptide encoded by the preprotachykinin-A gene, shares a common immunodominant epitope with SP and neurokinin B and therefore possibly represents the tachykinin family during the process of T-cell differentiation and shape T-cell repertoire, so that any potential tachykinin autoreactive T-cell clone would be unenergized or deleted (Geenen et al., 1991). The physiological significance of neuropeptide synthesis in thymic cells could be regarded as a possible means for intrathymic communication. The thymus is an organ characterized by a unique specialized three-dimensional microenvironment that allows bone marrow precursors to proliferate and mature into self-tolerant T cells with both effector and regulatory activities (Anderson et al., 1996; Taka-

hama, 2006). T-cell development is a strictly compartmentalized multistep process. TECs have been regarded as the main drivers of T-cell differentiation/maturation (Anderson et al., 1996; Takahama, 2006). They produce a variety of thymic hormones, steroids and cytokines, as well as extracellular matrix components, that influence migration, differentiation, apoptosis and maturation of T-cells (Anderson and Jenkinson, 2001; Savino and Dardenne, 2000), but to date only a few of the signals that drive thymopoiesis have been fully characterized. Thus, the neuropeptides released from thymic cells most likely modulate the process of T-cell differentiation/maturation, acting directly on maturing thymocytes. This assumption is corroborated by data showing that the thymocytes and/or T cells express

receptors for various neuropeptides, such as VIP, SP, CGRP (Homo-Delarche and Dardenne, 1983; Ottaway, 1984; Payan et al., 1994; Delgrado et al., 1996). In line with these findings are data showing: i) that VIP, by preventing the apoptosis of CD4+CD8+ thymocytes, can influence maturation of rat T-cells in a receptor-dependent manner (Delgrado et al., 1996), and that ii) CGRP (Bulloch et al., 1995) and tachykinins (Soder and Hellstrom, 1989; Payan et al., 1983) can influence T-cell development by modulating thymocyte proliferative capacity. On the other hand it was shown that VIP and CGRP can directly influence the proliferative and secretory activity of cultured rat thymic epithelium (Head et al., 1998). These data fit into the concept of a bidirectional symbiotic relation between thymocytes and TECs, so that thymic stroma is necessary for T-cell development, while developing T cells provide maturation and survival signals that are necessary for the development and maintenance of their microenvironment. (Ritter and Boyd, 1993). Additionally, they suggest that neuropeptides in thymus may act in both an autocrine and paracrine manner.

Finally, given the evidence of crosstalk between the immune and neuroendocrine systems (Homo-Delarche and Dardenne, 1983, Besedovsky and del Rey, 1996; Heijnen, 2007) one can hypothesize that the neuropeptides released by thymic lymphoid and non-lymphoid cells are involved in transmitting signals from the immune to the neuroendocrine system.

The expression of neuropeptides already in the fetal thymus further emphasizes their important role in the thymus, and suggests their possible significance in the development of this organ.

In conclusion, this study indicates that many neuropeptides are endogenous to the human thymus where they most likely serve as signals in intrathymic cell-to-cell communication, as well as signals in bidirectional communications within neuroendocrine-immune circuits. Thus, understanding the functional consequences of neuropeptide expression in the human thymus might be of interest for eluci-

dating mechanisms controlling not only local T-cell development, but also immune system homeostasis. In addition, identifying changes in neuropeptide-mediated thymic communications may contribute to our understanding of thymic based immunopathology.

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