Immunoreactivity for thymosin beta 4 and thymosin beta 10 in the adult rat oro-gastro-intestinal tract

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Abstract

Thymosin beta 4 (Tβ4) and thymosin beta 10 (Tβ10) are two members of the β-thymosin family, involved in multiple cellular activities in different organs in multiple animal species. Here we report the expression pattern of Tβ4 and Tβ10 in rat tissues, in the gut and in annexed glands. The two peptide were differently expressed: Tβ4 was absent in salivary glands whereas Tβ10 was expressed in parotid and in submandibular glands. Tβ4 was mildly expressed in the tongue and in the esophagus, where Tβ10 was absent. A similar expression was found in the stomach, ileum and colon mucosa. In pancreas Tβ4 reactivity was restricted to the Langerhans islet cells; Tβ4 was also detected in the exocrine cells. Both peptide were not expressed in liver cells. When the rat expression pattern in rat organs was compared to reactivity for Tβ4 and Tβ10 in humans, marked differences were found. Our data clearly indicate a species-specific expression of Tβ4 and Tβ10, characterized by the actual unpredictability of the expression of these peptides in different cells and tissues. The common high expression of Tβ4 in mast cells, both in humans and in rats, represents one of the few similarities between these two species.

Introduction

Beta thymosins are a versatile family of small peptides expressed in multiple tissues in mammals, that show many intracellular and extracellular activities.[1,2] These peptides are named *thymosins* after their first isolation in the calf thymus.[3] Fifteen highly homologous beta thymosin variants, containing 40 to 44 amino acid residues, have been described.[4] Among beta thymosins, thymosin beta 4 (Tβ4)[5,6] and thymosin beta 10 (Tβ10)[7] are the most abundant in human cells and rat tissues.[8] During the years, beta thymosins have been detected inside of cells of different organs,[9-14] as well as in human blood,[15] in human saliva,[16] in tears[17] and in wound fluid after abdominal surgery.[18] Many physiological properties and cellular functions are connected to Tβ4: G-actin-sequestering,[1] promotion of cell migration,[8] angiogenesis,[9,19] stem cell differentiation,[11] modulation of cytokines and chemokines.[20] This peptide is also involved in lesion-induced neuroplasticity through microglia upregulation and it participates in the growth of neuronal processes.[21] Therefore, the mRNA encoding for Tβ4 is expressed in mouse embryonic stem cells and in mesodermal-like cells (cardiac and skeletal muscle).[22] Moreover, recent studies demonstrated a role of Tβ4 in inducing the expression of the vascular endothelial growth factor (VEGF) in colon cancer cells in experimental models.[19] Tβ4 also partecipates to the modulation of human colonic immune system[23] probably through degranulation of mucosal mast cells.[24] Contrasting results have been published on the role of Tβ10 in tumour progression. On one hand, Tβ10 diminishes tumor growth, angiogenesis and proliferation[25] and Tβ10 over-expression has been related to the increase of apoptosis in human ovarian cancer cells.[25] On the other hand, Tβ10 has been associated to the progression of papillary thyroid carcinoma[26] and over-expression of the peptide has been observed in non-small cell lung cancer[27] and in pancreatic cancer.[28] The recent report by our group of a strong expression for Tβ10 in the human salivary glands during development,[29] induced us to better analyse the protein expression pattern for Tβ10 in the oro-gastro-intestinal tract in adult rats, in order to show if Tβ10 is expressed in the gastrointestinal tract in adulthood, and to compare the expression of this peptide with that reported in the human digestive tract and in annexed glands.[24,30,31] Moreover, rat tissues were immunostained for Tβ4, with the aim of verifying the reciprocal interactions of Tβ4 and Tβ10 in the oro-gastrointestinal tract.

Materials and Methods

In order to test Tβ4 and Tβ10 immunoreactivity in animals, 20 male wistar rats were the object of our study, divided into 10 male and 10 female. Tissues were obtained from each animal including samples from tongue, esophagus, stomach, ileum, colon, parotid gland, submandibular gland, sublingual gland, liver and pancreas. All samples were fixed in 10% formalin, paraffin-embedded and routinely processed. Paraffin sections were immunostained with anti-Tβ4 and anti-Tβ10 antibodies, using the labeled streptavidin-biotin complex system (LSAB2, Dako, Cytomation, Carpinteria, CA, USA) in a Dako Autostainer (Dako). Briefly, samples were deparaffinized, rehydrated, and endogenous peroxidase activity was quenced (30 min) by 0.3% hydrogen peroxide in

methanol. Slides were then subjected to heat-induced antigen retrieval by steaming unstained sections in a Target Retrieval Solution (Dako TRS pH 6.1) for 30 min. Slides were then incubated with 10% normal goat serum in phosphate-buffered saline (PBS) for 60 min to block non-specific binding, followed by incubation (60 min at room temperature) with a monoclonal anti-Thymosin Beta 4 antibody (Bachem-Peninsula Lab, San Carlos, CA, USA) and with a monoclonal anti-Thymosin Beta 10, respectively diluted 1:600 and 1:500 in the blocking solution. Slides were extensively washed with PBS containing 0.01% Triton X-100 and incubated with a secondary reagent

Table 1. Immunoreactivity for thymosin beta 4 (Tβ4) and thymosin beta 10 (Tβ10) in rat tissues and humans.

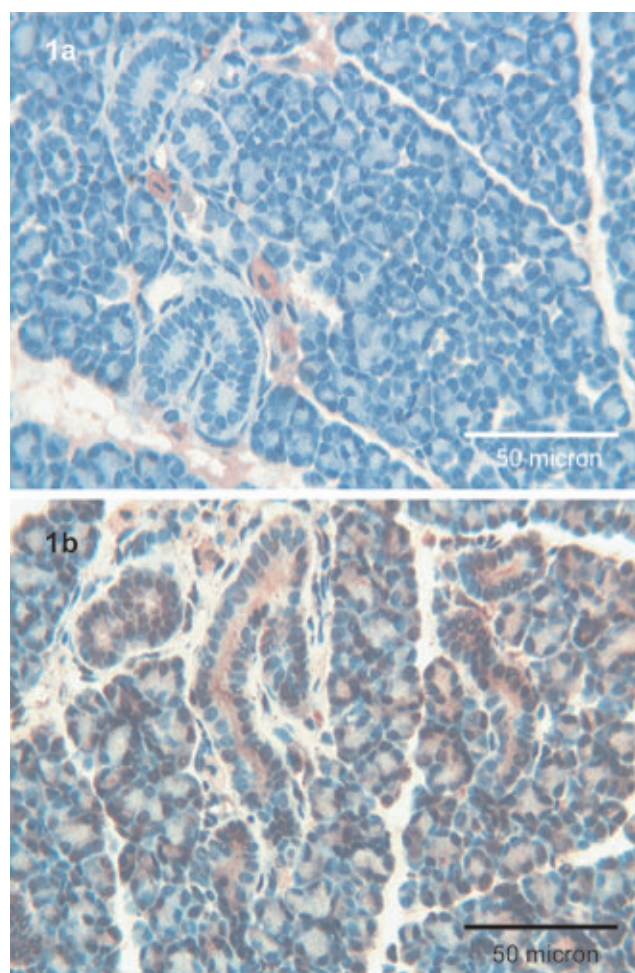| Organ	Tβ4 Rat	Tβ10 Rat	Tβ4 Human	Tβ10 Human
Parothyd	-	+++	+	+
Submandibular gland	-	+++	+	+
Sublingual gland	-	-	+	+
Tongue	+	-	+	++
Oesofagus	-	-	+	-
Stomach	+	+	+	-
Ileum	+	+	+	-
Colon	+	+	++	-
Pancreas	+	++	++	+
Liver	-	-	+++	+++

Figure 1. Parotid. a) No immunoreactivity for Tβ4 is detected in the parotid gland; scattered mast cells are strongly immunoreactive for the peptide; OM 250x. **b)** A granular and diffuse positivity for Tβ10 is observed in all the acinar structures; ductal cells show an apical and homogeneous reactivity for the peptide; OM 400x.
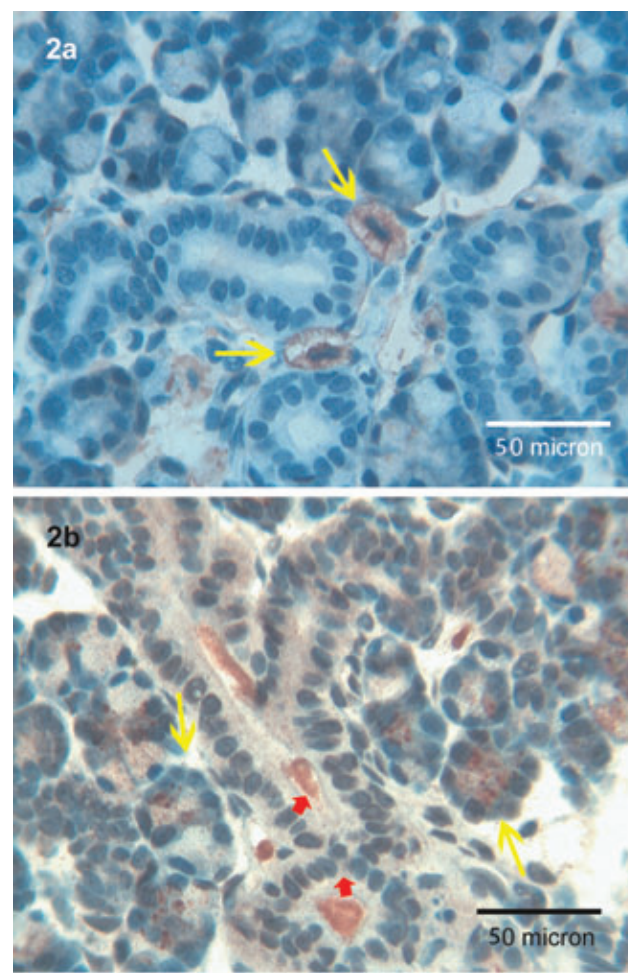
Figure 2. Submandibular glands. a) Tβ4 is not expressed in the structures of the submandibular glands; only a fine positivity could be detectable in the surrounding stroma; mast cells show a strong granular cytoplasmic positivity (yellow arrows); OM 400x. **b)** Coarse granules of Tβ10 are observed in the acini of the submandibular gland (yellow arrows); ductal cells present a fine cytoplasmic immunoreactivity for the peptide mainly localized in the lumen (red arrows); OM 400x.

(En Vision kit) according with the manufacturer instructions (Dako, Glostrup, Denmark). Diaminobenzidine (DAB) was used as chromogen. After additional washes, colour was developed using the AEC reagent (Dako), sections were counterstained with Mayer's hematoxylin and mounted. Sections of reactive lymph nodes with Tβ4-immunoreactive histiocytes were utilized as a positive control. As a negative control, the same procedure was applied omitting the primary antibody.

Results

Parotid

Tβ4 immunoreactivity was completely absent both in acini and in ducts (Figure 1a). Tβ10 was expressed both in acinar serous

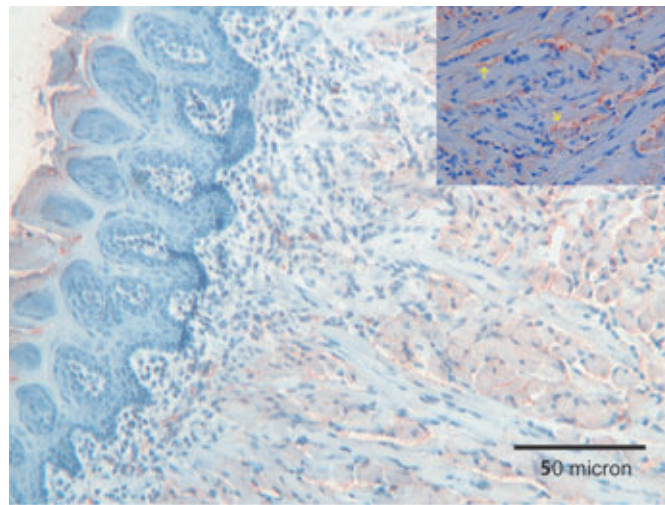Figure 3. Tongue. A weak immunoreactivity for Tβ4 is observed only in the superficial layers of the tongue's epithelium; a weak immunostaining for the peptide was observed in cell membranes of muscle cells (see inset, yellow arrows); OM 250x.

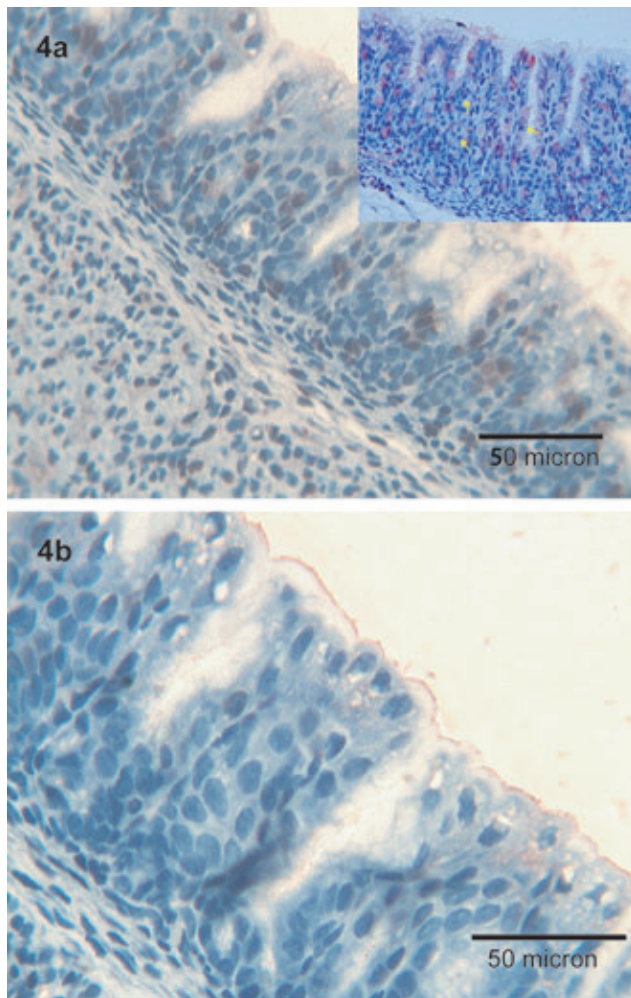Figure 4. Stomach. a) Immunoreactivity for Tβ4 is expressed in scattered foveolar cells (see inset, yellow arrows) and in the luminal surface of the stomach; OM 400x. b) Tβ10 is mainly localized in the luminal border of the foveolar cells of the stomach; OM 250x.
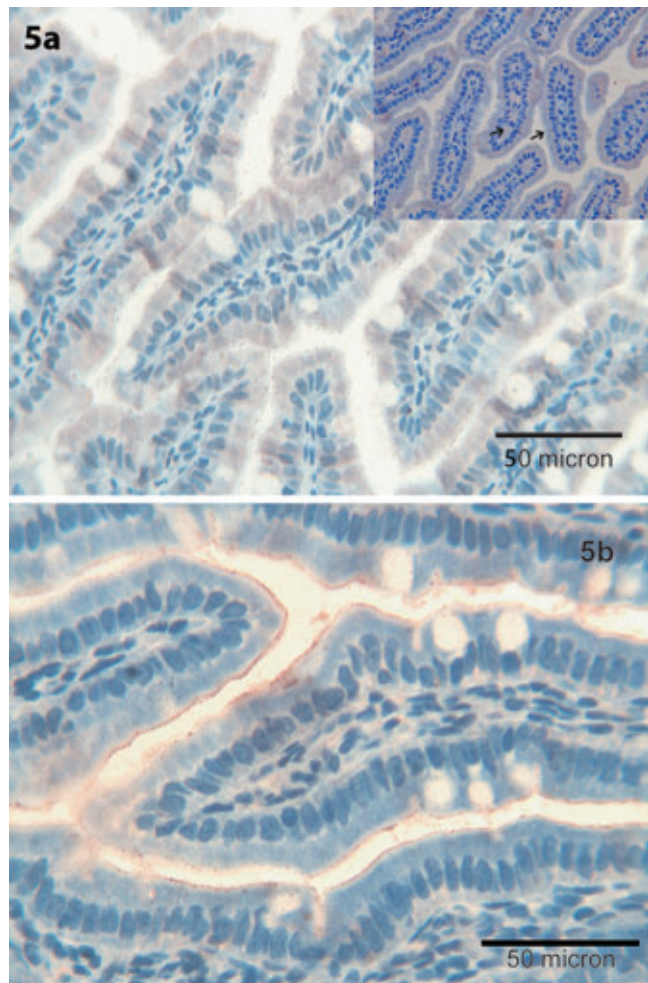

Figure 5. Ileum. a,b) Tβ4 and Tβ10 are detected in the surface epithelium of enterocytes covering the villi of the ileum (see inset, black arrows); OM 400x.

cells, showing a granular pattern, as well as in ductal cells, in which a homogeneous cytoplasmic staining was detected (Figure 1b).

Submandibular gland

Fine Tβ4-immunoreactive granules were observed in the periglandular stroma and in scattered mast cells, in the absence of any reactivity inside the salivary gland cells (Figure 2a). A strong reactivity for Tβ10 was observed in acinar serous cells, appearing as coarse granules and in mucous cells appearing as fine granules. The ducts show a homogeneous cytoplasmic staining and intraluminal granular deposits (Figure 2b).

Sublingual glands

No reactivity was detected for Tβ4 and for Tβ10.

Tongue

A mild immunoreactivity for Tβ4 was detected in the superficial layers of the stratified epithelium. Moreover, a weak immunostaining for the peptide was observed in muscle cells, mainly localized at the cell membrane (Figure 3). No reactivity for Tβ10 was found.

Esophagus

Scattered Tβ4-immunoreactive granules were detected inside the esophageal lumen. No reactivity for Tβ10 was observed.

Stomach

Immunoreactivity for Tβ4 was restricted to scattered foveolar cells and to intraluminal granular deposits (Figure 4a). A similar expression pattern was observed for Tβ10 (Figure 4b).

Ileum

Tβ4 was maily expressed in the cytoplasm of enterocytes covering ileal villi (Figure 5a). A similar pattern characterized immunoreactivity for Tβ10 (Figure 5b).

Colon

Tβ4 was maily expressed at the apical pole of enterocytes, and in fine granular deposits inside the intestinal lumen (Figure 6a), paralleling the expression pattern observed for Tβ10 (Figure 6b).

Pancreas

Tβ4 immunoreactivity was restricted to Langherans islets, in the absence of any significant immunostaining in the esocrine pancreas (Figure 7a). On the contrary, Tβ10 was
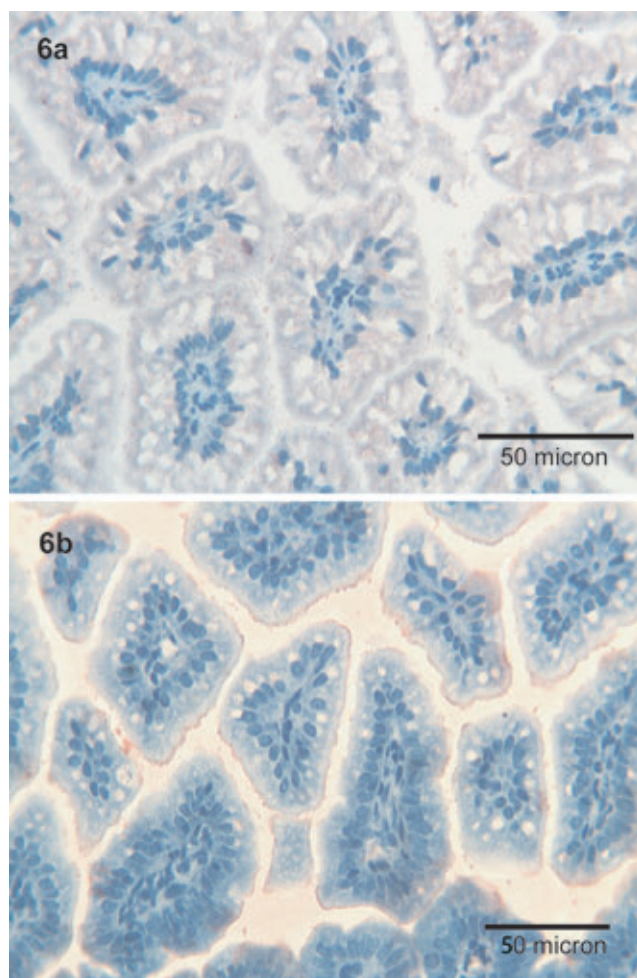
Figure 6. Colon. a,b) A fine granular positivity for Tβ4 is observed in the brush border of the enterocytes in the surface epithelium of the colon and in the intestinal lumen; Tβ10 parallels the immunoreactivity of Tβ4; OM 400x.
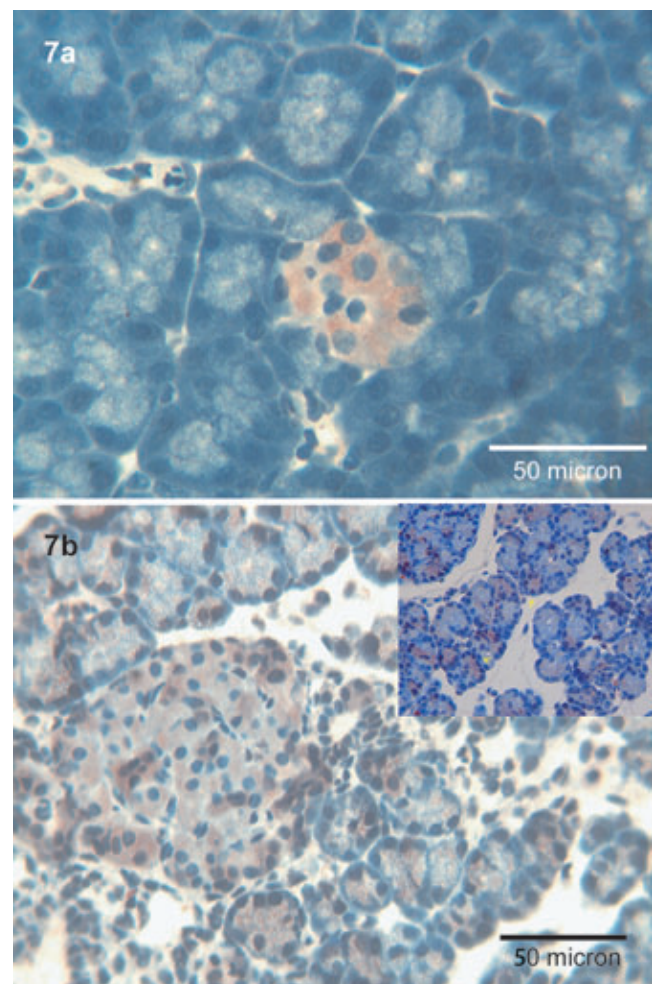
Figure 7. Pancreas. a) Tβ4 is diffusely immunoexpressed in the islets of Langherans; no immunoreactivity is observed in the rest of pancreatic parenchima; OM 400x. b) Immunoreactivity for Tβ10 is localized both in islets of Langherans and in the acini (see inset); the latest show a fine granular positivity in the absence of ductal reactivity; OM 400x.

detected both in the exocrine and in the endocrine pancreas. Endocrine cells of the Langherans islets showed a strong cytoplasmic reactivity, whereas in acinar cells Tβ10 was mainly detected in granular deposits. No immunostaining was found in ductal cells nor inside the tubular lumen (Figure 7b).

Liver

No reactivity for Tβ4 and Tβ10 was observed in the liver samples. Data regarding immunoreactivity for both thymosins in the different rat organs are summarized in Table 1.

Discussion

The role of Tβ4 and Tβ10, the beta-thymosins expressed virtually in all mammalian tissues and cells, has not been completely clarified yet. Previous studies on their expression in the rat central nervous system evidenced that temporal and cellular patterns of their expression are different, suggesting that each beta-thymosin could play a specific physiological function during development and in adulthood.[32] Our study confirms the existence of marked differences in the distribution of Tβ4 and Tβ10 in the oro-gastro-intestinal tract of the adult rat. The most striking differences were found in the parotid and submandibular glands, in which Tβ4 was absent whereas immunostaining for Tβ10 was strong and diffuse. On the contrary, no reactivity for both beta-thymosins was detected in sublingual glands. These differences in beta-thymosin expression between different salivary glands confirm that each beta-thymosin probably plays a specific role in each salivary gland, irrespectively of their common embryogenesis.

No significant difference in Tβ4 and Tβ10 expression was present in the gastrointestinal tract: the absence of reactivity for both beta-thymosin in the oesofagus contrasts with the presence of both peptides in the enterocytes of the remaining gastrointestinal tract, confirming that a patchy distribution of these peptides should be expected, even in different parts of the same system.

The peculiar pattern for beta thymosins detected in pancreas deserves some considerations: Tβ10 was strongly expressed both in the exocrine and in the endocrine cells, whereas Tβ4 reactivity was restricted to the Langerhans islet cells. These findings taken together clearly indicate the presence of a complex modulation in the expression of beta-thymosins inside the same organ, each thymosin playing different functions in different cell types, confirming the β-*thymosin enigma*.[33]

When data obtained in rat tissues were compared with immunoreactivity for Tβ4 and Tβ10 in human tissues, significant differences were evidenced, supporting the hypothesis that beta-thymosin expression in cells and tissues is species-specific. The most striking differences were found in liver specimens, characterized by the complete absence of both thymosins in rat, contrasting with the strong and diffuse reactivity for Tβ4 and Tβ10 previously reported in humans.[34] However, which is the role of Tβ4 and Tβ10 in adult rat tissues? The detailed mechanisms of the action of Tβ4 and Tβ10 in different mammalian cells and tissues are not fully understood, as well as the similarities and differences between these isoforms.[35] Previous studies evidenced that activities of these two beta-thymosins are paradoxically different, Tβ4 promoting cell migration and angiogenesis, and Tβ10 inhibiting angiogenesis.[35] Opposing effects on angiogenesis are likely to be mediated via Tβ4 stimulation and Tβ10 inhibition of VEGF production.[36]

Moreover, in contrast to Tβ4, Tβ10 has been shown to be a negative regulator of tumor development and progression.[37] In short, Tβ4 might promote cell survival by blocking apoptosis,[38] whereas Tβ10 might exert a proapoptotic activity, by accelerating apoptotic cell death.[39] According with these data, we may speculate that the complex and pleiotropic expression of Tβ4 and Tβ10 here reported in different tissues and cell types might reflect different direct and indirect effects on the actin cytoskeleton, as well as modulation of signaling pathways that impact on different cellular functions. In particular, over-expression of Tβ4 could be related to prevention of apoptosis by blocking early apoptotic signals,[40] whereas overexpression of Tβ10 might be related to its pro-apoptotic activity and to a down-regulation of cell growth[35] and of angiogenesis.[36]

Finally, this study, one of the few in which the immunohistochemical expression pattern of Tβ4 and Tβ10 has been paralleled in the same tissues, evidenced that detailed mechanisms of the action of beta-thymosins in different cells and tissues are not fully understood, and show that our lack of knowledge is particularly evident regarding mature adult tissues, the vast majority of studies on the role of beta-thymosins having been carried out in fetal or tumoral tissues. Further studies exploring the molecular events that are associated with Tβ4 and Tβ10 overexpression or down-regulation are required, in order to give a solution to the thymosin enigma.

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