Different Thymosin Beta 4 Immunoreactivity in Foetal and Adult Gastrointestinal Tract

Sonia Nemolato1, Tiziana Cabras2, Flaviana Cau1, Mattia Umberto Fanari1, Daniela Fanni1, Barbara Manconi2, Irene Messana2, Massimo Castagnola3, Gavino Faa1*

1 Divisione di Anatomia Patologica, Dipartimento di Citomorfologia, University of Cagliari, Cagliari, Italy, 2 Dipartimento di Scienze Applicate ai Biosistemi, Università di Cagliari, Cagliari, Italy, 3 Istituto di Biochimica e di Biochimica Clinica, Università Cattolica and/or Istituto per la Chimica del Riconoscimento Molecolare, CNR, Istituto Scientifico, Internazionale (ISI) Paolo VI, Roma, Italy

Abstract

Background: Thymosin beta 4 (Tβ4) is a member of beta-thymosins, a family of peptides that play essential roles in many cellular functions. A recent study from our group suggested a role for Tβ4 in the development of human salivary glands. The aim of this study was to analyze the expression of Tβ4 in the human gut during development, and in the adult.

Methodology/Principal Findings: Immunolocalization of Tβ4 was studied in autopic samples of tongue, oesophagus, stomach, ileum, colon, liver and pancreas obtained from two human foetuses and two adults. Tβ4 appeared unevenly distributed, with marked differences between foetuses and adults. In the stomach, superficial epithelium was positive in foetuses and negative in adults. Ileal enterocytes were strongly positive in the adult and weakly positive in the foetuses. An increase in reactivity for Tβ4 was observed in foetal colon epithelium of adults as compared with the foetuses. Striking differences were found between foetal and adult liver: the former showed a very low reactivity for Tβ4 while in the adult we observed a strong reactivity in the vast majority of the hepatocytes. A peculiar pattern was found in the pancreas, with the strongest reactivity observed in foetal and adult islet cells. Different Thymosin Beta 4 Immunoreactivity in Foetal and Adult Gastrointestinal Tract. PLoS ONE 5(2): e9111. doi:10.1371/journal.pone.0009111

Significance: Our data show a strong expression of Tβ4 in the human gut and in endocrine pancreas during development. The observed differential expression of Tβ4 suggests specific roles of the peptide in the gut of foetuses and adults. The observed heterogeneity of Tβ4 expression in the foetal life, ranging from a very rare detection in liver cells up to a diffuse reactivity in endocrine pancreas, should be taken into account when the role of Tβ4 in the development of human embryo is assessed. Future studies are needed to shed light on the link between Tβ4 and organogenesis.

Introduction

Beta-thymosins (Tβs) constitute a highly conserved family of actin-binding polypeptides [1], presenting a well conserved four-aminoacid motif, corresponding to the sequence LKKT, which interacts with actin, promoting or inhibiting actin assembly [2]. Thymosin beta-4 (Tβ4) is the archetypal member of the beta-thymosins family: it is a 43-aminoacid peptide, isolated from human blood platelets [3] which forms a 1:1 complex with actin, inhibits its polymerization [4], and acts as an extremely effective actin-monomer sequestering peptide [5]. Tβ4 has multiple functions: it moonlights to repair injured tissues [6], has anti-inflammatory efficacy in monocyte/macrophages [7], promotes wound healing [8] and mediates angiogenesis [9]. Tβ4 has been also shown to play a relevant role during the development of different neural cell types in the rat brain [10]. In particular, Tβ4 plays a neurotrophic and antiapoptotic role during the development of the nervous system [11]. In the embryo, reduced myocardial Tβ4 levels have been reported to cause a disrupted coronary vasculogenesis and a number of cardiac defects [12]. During coronary vessels development, Tβ4 should act on cardiac stem cells, also known as epicardially derived cells (EPDCs) [13] inducing their migration into the myocardium, where they differentiate into either endothelial or smooth muscle cells [9]. The theory on the putative role of Tβ4 in the physiological development of embryos, as well as in vascularization and tissue recovery in acute and chronic ischemia, has been reinforced by the discovery that Tβ4 is one of the most abundant factors secreted by embryonic endothelial progenitor cells [14]. Recently a study from our group evidenced a strong reactivity for Tβ4 in developing foetal salivary glands, with a switch from the acinar component to ductal cells in the adult [15]. Tβ4 has also been identified as a predominant transcript in intraepithelial lymphocytes (IEL) in the murine gut [16], in which it could exert a relevant anti-inflammatory effect by inhibiting neutrophilic infiltration [17].

On the basis of these data, suggesting a relevant role for Tβ4 during the development of the foetus and the embryo, it seemed of some interest to investigate the expression of the peptide in the...
gastrointestinal tract of human fetuses and in adults, with the aim to gain insights into the expression of Tβ4 in the human gastrointestinal tract during development.

**Materials and Methods**

Two human fetuses of 20 weeks (male) and 21 weeks (female) of gestational age, and two adults 65 and 74 year old respectively were the subject of the present study. In each subject, we obtained, at autopsy, multiple samples from the following segments of the gut: tongue, oesophagus, stomach, ileum, colon. Samples were also obtained from liver and pancreas. Tissue samples were fixed in 10% formalin, routinely processed and paraffin-embedded. Immunohistochemistry was performed on 5 μm-thick sections, using the labeled streptavidin-biotin complex system (LSAB2, Dako) in a Dako Autostainer (DakoCytomation, Carpintera, CA, USA). Heat-induced antigen retrieval was carried out by steaming unstained sections in Target Retrieval Solution (Dako TRS pH 6.1) for 30 min. Tissue sections were incubated (30 min at room temperature) with the monoclonal anti-thymosin beta 4 antibody (Bachem, Bubendorf, Switzerland). Sections of a reactive human adult lymph node with activated macrophages were used as positive controls. As a negative control, we utilized sections of foetal oesophagus: immunohistochemistry was performed using isotype antibody (Fig. 1). The immunostaining was interpreted as positive when at least 10% of cells expressed the antigen. The positive expression was further categorized into focal (10–33%) and diffuse (>33%).

All cases were independently reanalyzed by two pathologists specialized in gastrointestinal pathology (GF, SN).

**Ethics Statements**

The study protocol and written consent forms were approved by the Ethics Human Studies Committee of University Medical Centre of Cagliari (according to the instructions of the declaration of Helsinki). Full written consent forms were obtained from the parents of the newborns and all rules were respected. For the specimens from adults, we obtained written consent to research use by their next of kin.

**Results**

The immunostaining for Tβ4 appeared granular or homogeneously diffuse, always restricted to the cytoplasm of positive cells; no nuclear reactivity was observed in this study. Reactivity for the peptide was also observed, in some cases, in the interstitial spaces, as fine granules, mainly located around the epithelial structures. No significant differences were found in the immunohistochemical pattern for Tβ4 between the two foetuses and the two adults analyzed.

**Tongue**

**Foetus (20 weeks of gestation).** The epithelium covering the tongue appeared constantly negative in its deeper layers. The superficial epithelial cells showed a diffuse immunoreactivity for Tβ4, localized in the cytoplasm (Fig. 2a). The underlying corion was negative. A weak positivity was also observed in the muscular cells.

**Adult.** The immunohistochemical pattern paralleled that detected in the foetuses. The superficial layers of the stratified epithe

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**Figure 1. Negative control.** Foetal oesophagus immunostained by using isotype antibody. L = oesophageal lumen. Arrows indicate the surface epithelium. doi:10.1371/journal.pone.0009111.g001

**Figure 2. Immunohistochemical detection of thymosin β4 in foetal and adult tongue.** a) Foetal tongue: the superficial epithelial cells (arrow) shows a diffuse immunoreactivity for Tβ4 localized in the cytoplasm. The underlying corion is negative. (Original Magnification ×250) b) Adult tongue: the superficial layers of the stratified epithelium show a strong diffuse cytoplasmic reactivity for Tβ4 (arrow). (Original Magnification ×250) doi:10.1371/journal.pone.0009111.g002
epithelium of the tongue showed a diffuse cytoplasmic reactivity for Tβ4 (Fig. 2b).

Oesophagus

**Foetus (21 weeks of gestation).** The epithelium covering the oesophageal lumen appeared negative. The oesophageal lumen appeared coated with a thin Tβ4-immunoreactive layer, which resulted diffuse to the entire oesophageal lumen (Fig. 3a). A weak reactivity for the peptide was observed in the muscular cells.

**Adult.** Immunoreactivity for Tβ4 was restricted to epithelial cells of the superficial layers covering the oesophageal lumen. No reactivity for Tβ4 was found in the intermediate and deep layers (Fig. 3b).

Stomach

**Foetus (21 weeks of gestation).** The gastric epithelium showed the presence of Tβ4-immunoreactive granules in the cytoplasm of the majority of columnar cells. Immunoreactive deposits were mainly found aggregated in the perinuclear regions. Abundant deposits were also found dispersed throughout the mucous covering the gastric surface (Fig. 4a).

**Adult.** Gastric mucosa was covered by a thin superficial layer intensely reactive for Tβ4 extending to gastric foveolae. Immunoreactivity for Tβ4 in gastric glands was restricted to chief and oxyntic cells (Fig. 4b).

Ileum

**Foetus (21 weeks of gestation).** A granular reactivity for Tβ4 was detected in the epithelium covering the ileal villi, more evident in the cytoplasm of mucous cells (Fig. 5a). A reactivity for the peptide was also observed in the mucous occupying the intestinal lumen.

**Adult.** A mild but diffuse reactivity for Tβ4 was present in the cytoplasm of enterocytes covering villi (Fig. 5b). Fine granular deposits were also observed in the cytoplasm of mucous cells.

Colon

**Foetus (21 weeks of gestation).** Coarse granules immunoreactive for Tβ4 were observed in the cytoplasm of superficial colon epithelial cells. No reactivity was found in the...
crypts (Fig. 6a). The muscular layer did not show any significant reactivity for the peptide. A weak positivity was detected in the nervous cells.

**Adult.** An intense and diffuse reactivity with coarse supranuclear granules immunoreactive for Tβ4 were present in the superficial and crypt epithelium. (Fig. 6b)

**Liver**

**Foetus (21 weeks of gestation).** No significant reactivity for Tβ4 was found in hepatocytes and in biliary ducts nor in biliary epithelial cells of the ductal plate in the foetal liver examined in this study. A weak reactivity was frequently observed in the red blood cells in the dilated sinusoids. Only occasionally, scattered large cells in immature portal tracts showed a strong Tβ4 immunoreactivity (Fig. 7a).

**Adult.** The expression pattern for Tβ4 changed completely in immunostained adult livers. A strong granular immunoreactivity was found in the cytoplasm of the majority of hepatocytes in all acinar zones and in activated Kupffer cells (Fig. 7b). Portal tracts, including bile ducts were constantly negative.

**Pancreas**

**Foetus (20 weeks of gestation).** Immunoreactivity for Tβ4 appeared focal, mainly localized inside the islets of Langerhans. A part of the endocrine cells showed the entire cytoplasm strongly immunoreactive for the peptide. Few Tβ4-reactive cells were also present in the exocrine pancreas, inside the tubular structures, intermingled among the tubular epithelium. A weak granular reactivity for the peptide was also found inside the cytoplasm of the tubular cells, a pattern suggestive for a secretion of Tβ4 in the pancreatic juice (Fig. 8a).

**Adult.** The distribution of Tβ4 immunoreactivity paralleled that observed in the foetus: the highest reactivity was observed in the islet cells which showed diffuse fine cytoplasmic granular deposits in the majority of endocrine cells. Only rare acinar cells contained cytoplasmic vacuoles reactive for Tβ4 (Fig. 8b).

**Discussion**

Tβ4, the most abundant member in human cells of the thymosin family, has been initially embraced as the ideal actin monomer-
sequestering peptide [18], and its function was restricted to regulate actin polymerization of non-muscle cells [19]. Further data, suggesting a role of Tβ4 in modulating stem cell migration [9], activation [20] and inhibition [21], as well a relation between Tβ4 and integrin signalling [22] induced some authors to speak about “the β-thymosin enigma” [9]. The oxidized form of Tβ4, thymosin beta 4 sulfoxide, has been demonstrated to have an important anti-inflammatory activity inhibiting neutrophil chemotaxis in vitro [23] and in vivo [7].

This study represents the first comprehensive analysis of Tβ4 immunoreactivity in the human gastrointestinal tract, pancreas and liver of human foetus in comparison to adult. Interesting differences were found between foetus and adult Tβ4 immunoreactivity: in some organs, such as liver, the immunoreactivity of Tβ4 was higher in the adult, while in others, such as endocrine pancreas, it was lower. Differences in Tβ4 expression between the foetus and the adult have been previously reported by our group in human salivary glands [15]. In that study developing major and minor salivary glands showed a marked reactivity for Tβ4, which contrasted with the low levels of immunoreactivity observed in the adult glands. In this study liver showed an opposite pattern, characterized by low reactivity for the peptide in the intrauterine life and much higher levels in the adult life. All together, these data seem to indicate the existence of an exquisite cell type- and differentiation stage-specific regulation and expression pattern of Tβ4 in the human gut and annexed glands during development. As a consequence, Tβ4 could play different roles in different organs during the organogenesis, and these roles should change during adult life.

Immunoreactivity for Tβ4 was detected in all different intestinal segments and in all glands examined, but with striking differences among different sites: pancreas and liver of adults showed the highest levels of reactivity for Tβ4, while the lowest reactivity was observed in the developing liver. A remarkable heterogeneity of Tβ4 immunoreactivity within the developing gastrointestinal tract was observed, ranging from diffuse immunoreactivity in pancreas and enteroctyes, to peptide absence in the foetal hepatocytes. It should be outlined that some differences observed in Tβ4 immunoreactivity between fetuses and adult organs may be

Figure 7. Immunohistochemical detection of thymosin β4 in foetal and adult liver. a) Foetal liver: no significant reactivity for Tβ4 is found in hepatocytes and in ductal cells. Scattered large cells in immature portal tracts show a strong immunoreactivity for the peptide (arrow). (Original Magnification ×250) b) Adult liver: a strong granular immunoreactivity for Tβ4 is found in the cytoplasm of the majority of hepatocytes in all acinar zones (arrow) and in activated Kupffer cells (arrowhead). Portal tracts, including bile ducts are negative. (Original Magnification ×400).

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Figure 8. Immunohistochemical detection of thymosin β4 in foetal and adult pancreas. a) Foetal pancreas: immunoreactivity for Tβ4 is localized inside the islets of Langherans. Some of endocrine cells show the entire cytoplasm strongly immunoreactive for the peptide (arrows). Few Tβ4-reactive cells are also present in the exocrine pancreas, inside the tubular structures (arrowhead). (Original Magnification ×400). b) Adult pancreas: the highest reactivity for Tβ4 is observed in Langherans islet cells, which show diffuse fine granular deposits in their cytoplasm (arrows). (Original Magnification ×400).

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related to the different degree of cell differentiation. For example, chief and oxytic cells, the site of Tβ4 storage in the adult stomach, are not well differentiated yet in the foetal stomach glands. Noticeable individual differences during gut development, regarding the intensity of the positivity for Tβ4, and its subcellular localization, were also observed (data not reported).

On the whole, the high amounts of Tβ4 here reported in the human gut during development are in agreement with previous experimental studies on the role of Tβ4 in organogenesis. High levels of Tβ4 mRNA had been indeed reported in early mouse postimplantation embryos [24] and in the ventricular myocardium of mice at embryonic day 10 [25]. The essential role of Tβ4 in heart development had been demonstrated by the generation of mice with RNAi-mediated cardiac specific knockdown of Tβ4.

Tβ4-mutant hearts showed severe impairment in the mobilization of cardiac progenitor cells, resulting in impaired cardiac development and survival [26]. Our data clearly indicate a major expression of Tβ4 during the development of the human gastrointestinal tract. Such a strong expression should be related to relevant roles in gut development. The well known function of Tβ4 in the regulation of the equilibrium between globular and filamentous actin [27] could partly explain the strong Tβ4 immunoreactivity found in the cytoplasm of enterocytes in this study. Tβ4 has been hypothesized to also function as a sentinel of the cell oxidative stress, its oxidation reflecting an oxidizing environment that often correlates with cell damage [7]. Lymphoid Tβ4, a splice variant of Tβ4 produced by intrathepial lymphocytes normally present in the cytoplasm of enterocytes, has been shown to have a great capacity to reduce oxidative stress [17]. Following this hypothesis, a strong immunohistochemical reaction for Tβ4 could be interpreted as a cell’s response to stress, also considering that antibodies utilized in this study cannot discriminate the oxidized Tβ4 from the non-oxidized one. Recently, Tβ4 has been shown to be involved in regulating phagocytosis of apoptotic cells mediated by stabilin-2 [28]. Given the fundamental role of apoptosis in embryology, our finding of a strong reactivity for Tβ4 in the developing gut as well as in developing salivary glands [13] reinforces the hypothesis of an important role of Tβ4 in organ development during embryogenesis. Future studies will be necessary to shed light on the link between Tβ4 and organogenesis, with particular emphasis on the role played by the peptide in cell response to oxidative stress, in apoptosis, in actin metabolism in the different phases of fetal, embryo and adult life. The heterogeneity of Tβ4 immunoreactivity detected in different organs of gastrointestinal tract and the differences observed between intrauterine and adult life should be taken into account when the role of Tβ4 in human physiology is assessed.

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Author Contributions
Conceived and designed the experiments: SN TC FC MUF DF BM IM GF. Performed the experiments: SN FC MUF IM GC. Analyzed the data: SN TC FC MF BM IM GC. Contributed reagents/ materials/analysis tools: FC MUF. Wrote the paper: SN TC IM MF.

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