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Review Article

Thymosin beta in macrophage

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Summary

Thymosin, originally discovered as a thymic hormone regulating maturation of T cells, is found in many hematopoetic and non-hematopoetic tissues including bone marrow, spleen, and lungs. While studying chicken monocyte- and granulocyte- associated peptides by Matrix Assisted Laser Desorption Ionization/ Time-of-Flight (MALDI-TOF) mass spectrometry, we found a peptide corresponding to mass/charge ratio (*m/z*) of 4963. This peptide was prominently associated with monocytes but not with the granulocyte population. Experiments revealed presence of this peptide in chicken macrophage cell lines. We purified the 4963Da peptide from the macrophages by reverse phase HPLC and identified it as thymosin β4 (Tβ4) by peptide mass fingerprinting. Tβ4 binds to G- actin, and regulates its polymerization, which is essential for cell motility. Besides, it is also involved in a multitude of other functions regulating immunity and wound healing. This review surveys the physiological significance of Tβ4 in relation to macrophage function.

Key Words: Thymosin beta, macrophages, mass spectrometry, inflammation

Abbreviations: MALDI-TOF = Matrix Assisted Laser Desorption Ionization/ Time-of-Flight; Tβ4= thymosin beta 4

Introduction

The identities of differentiated cells and tissues are often related to repertoires of proteins and peptides which not only act as structural components but also perform specialized functions. Low molecular weight proteins and peptides are parts of these repertoires which play numerous physiological roles as hormones, neurotransmitters and antimicrobial, growth and cellular signaling factors. Therefore, it is likely that all differentiated cells exhibit their own profiles of low molecular weight peptides which may act as molecular signatures. Identifying these cell - associated peptides may help to understand their function. MALDI-TOF mass spectrometry has emerged as a valuable tool for biomolecular characterization and has been used to establish bacterial identities using their molecular profiles based on "whole cell" scanning (Lay and Holland, 2000). Applying this technique of "whole cell" MALDI-TOF mass spectrometry we compared the spectral profiles of chicken peripheral blood mononuclear cells and heterophilic granulocytes.The results showed certain high intensity spectral peaks uniquely associated with each population of cells suggestive of their abundance (Fig. 1). A molecule with *m/z* 4963 was predominantly associated with mononuclear cells, but not with heterophilic granulocytes (Table 1). Subsequent experiments with different chicken macrophage cell lines showed profiles similar to mononuclear cells with a major peak corresponding to 4963Da molecule.

Table 1: Mass and intensity profiles of 10 most abundant low molecular weight peptides associated with mononuclear and granulocyte populations.

Mononuclear cells		Granulocytes	
Peaks (m/z)	Relative intensities	Peaks (m/z)	Relative intensities
4962.90	1.00	3916.59	1.00
4984.13	0.19	3933.79	0.32
4946.59	0.17	3844.68	0.27
5099.46	0.12	4503.40	0.26
5000.78	0.11	3731.63	0.18
2478.62	0.06	7892.76	0.17
9932.15	0.05	3980.14	0.16
9326.21	0.05	7841.54	0.15
4848.06	0.05	8678.48	0.09
4696.90	0.04	10240.77	0.07

To isolate and characterize this peptide, methanol/ acetic acid extracts of HTC macrophages (Rath et al., 2003) were purified on a C8 column by reverse phase HPLC and the fractions were monitored by post-column UV detection and electrospray ionization (ESI) mass spectrometry. Further experiments suggested an N-terminal blockage of the peptide precluding Edman sequencing. It was then digested with trypsin and the MALDI-TOF mass fingerprints were subjected to Mascot data base search

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(http://www.matrixscience.com) matching the peptide to thymosin β4 (Tβ4) (Kannan et al., 2007). The chicken Tβ4 appeared to have a molecular weight and an internal sequence similar to that of the mammalian peptide. However, it differed from the published amino acid sequence of chicken Tβ4 with respect to N-terminal acetylation and the absence of an extra lysine at the C-terminus (Dathe and Brand-Saberi, 2004). The similarity of chicken Tβ4 sequence with that of human peptide was further indicated by its immunolocalization in HTC cells using an anti-human thymosin β4 antibody (Fig. 2). Additionally, the Tβ4 was found to be abundant in tissues such as lung, spleen and bone marrow, involved in immunity. These are similar to the results reported using mammalian myeloid and lymphoid cells (Xu et al., 1982; Gondo et al., 1987; Gomez-Marquez et al., 1989). Therefore, we have been interested in understanding the significance of Tβ4 in macrophages, its relation to inflammation, and its role in physiological homeostasis during avian immune activation. In the following sections we discuss some aspects of the current knowledge primarily obtained from mammalian literature.

Thymosin β4

Thymosin beta 4 (Tβ4) is a 5kDa peptide that was originally identified in calf thymus gland extract as a thymic hormone regulating the maturation of T lymphocytes (Goldstein, 2007; Huff et al., 2001). Subsequent research over the years showed this peptide to be present almost ubiquitously in many different cells. The mature forms of both mammalian and avianTβ4 is a 43 amino acid long peptide with an N-terminal acetylation. Both mammalian and avian Tβ4 have a stretch of amino acids LKKTETQ spanning from position 17 to 24 that bind to actin, a major cytoskeletal protein in eukaryotes, and regulate its polymerization (Safer et al., 1991; Ballweber et al., 2002; Dedova et al., 2006). Actin is responsible for a variety of cellular functions principally regulating motility, cytokinesis, adhesion, cell differentiation, and the maintenance of cell polarity (Ballweber et al., 2002; Wu and Crabtree, 2007).It exists in two forms, as the monomeric G- and the filamentous F-actin, the concentrations of the latter increasing in the event of cellular motility (Wu and Crabtree, 2007). Cytoplasmic G-actin, in the presence of ATP, polymerizes to F-actin whereas the nuclear G-actin is associated with chromatin remodeling (Blessing et al., 2004). Tβ4 sequesters G-actin and prevents its polymerization to F-actin, thereby regulating the cell dynamics (Safer et al., 1991; Cassimeris et al., 1992).

Thymosin β is a highly conserved peptide consisting of more than fifteen isoforms that are present at differential abundance in different species (Huff et al., 2001; Hannappel and Huff, 2003). The differences among

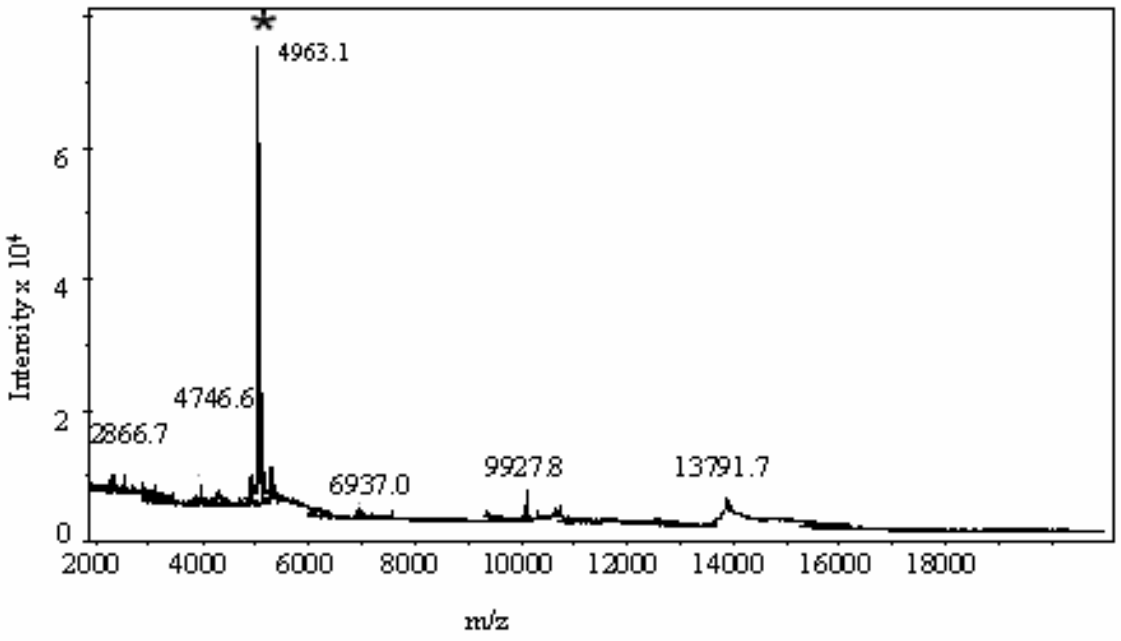

Fig. 1. MALDI-TOF mass spectrum of methanol acetic acid extract of HTC macrophage showing 4963Da peptide as a prominent peak (*).

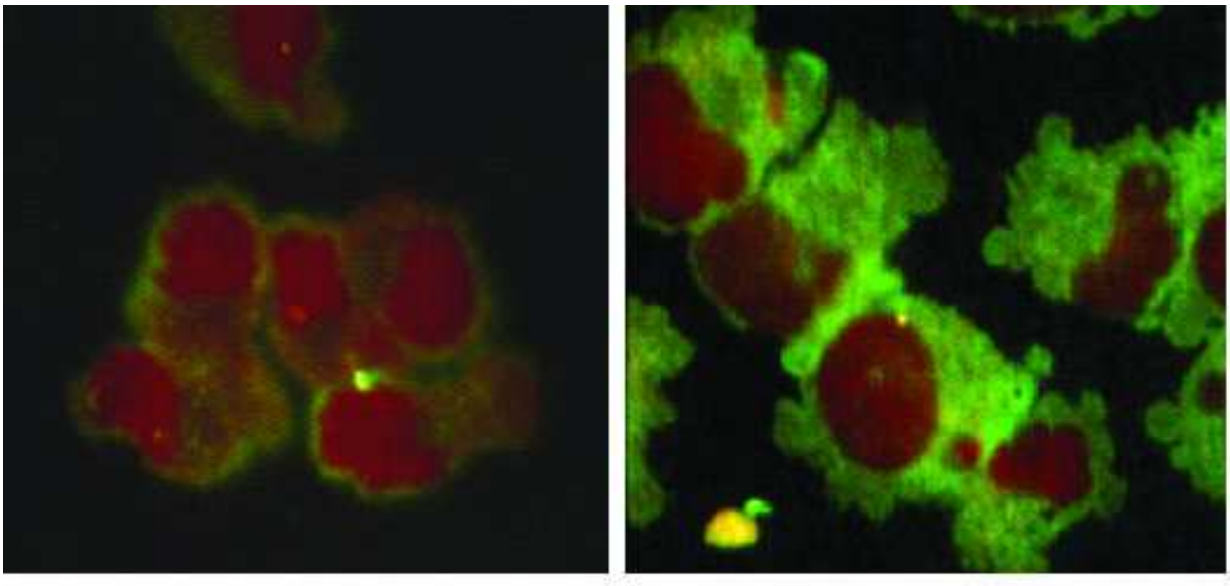

Fig. 2. Immunohistochemical localization of Tβ4 in HTC macrophages. Cytospun HTC cells were first treated with either non- immune serum (control, left panel) or anti-human Tβ4 antiserum (right panel) followed by staining with an affinity purified secondary antibody (goat anti-rabbit IgG) labeled with fluorescein isothiocynate. The nucleus was counter-stained with propidium iodide (red). The intensity of green color in the cytoplasm corresponds to the specific binding by anti-thymosin antibody. (Magnification, 400 X).

isoforms can range from a few to a stretch of amino acids in their primary sequence (Hannappel and Huff, 2003). Tβ4 appears to be the most abundant isoform and better studied than the others. Although the major function of Tβ4 has been described as sequestering G-actin, several other functions of this peptide have revealed its 'moonlighting' properties (Goldstein et al., 2005). Some of the best known functions where Tβ4 has been implicated are: wound healing, angiogenesis and inflammation (Malinda et al., 1999; Philp et al., 2004; Goldstein et al., 2005; Smart et al., 2007). In addition, Tβ4 prevents apoptosis, stimulates neurite growth, and promotes metastasis (Kobayashi et al., 2002; Choi et al., 2007). Thymosin β is one of the primary genes up-regulated following immune activation (Smith et al., 2004). The involvement of Tβ4 in wound healing has been shown and studied using cardiac, corneal, neural and dermal tissues (Vartiainen et al., 1996; Sosne et al., 2001; Bock-Marquette et al., 2004; Smart et al., 2007; Choi et al., 2007). Many of these studies involving wound healing and angiogenesis have been carried out using purified Tβ4.

However, an unresolved enigma is how this peptide is secreted by the cells, because it lacks a signal sequence that is essential for the release of most peptides and proteins (Horecker et al., 1985; Hannappel and Huff, 2003). Nonetheless, recent evidences suggest that Tβ4 can freely move through cell and nuclear membranes (Bock-Marquette et al., 2004). Also, Tβ4 has been shown to be present in extracellular fluids, particularly wound fluid, and its uptake by the tissues has been documented (Frohm et al., 1996; Bock-Marquette et al., 2004; Huang et al., 2006). However, it is not clear whether Tβ4 in the wound fluid is derived from dying tissues or secreted by the cells. The N-terminal domain, tetrapeptide AcSDKP (N-acetyl-seryl-aspartyl-lysyl-proline) that is derived by endoproteinase hydrolysis, appears to be biologically active and occurs at high concentrations in the wound fluid (Smart et al., 2007). Both Tβ4 and AcSDKP induce mast cell degranulation leading to the release of factors that promote wound healing and tissue remodeling (Leeanansaksiri et al., 2004; Wyczolkowska et al., 2007). AcSDKP appears to be both pro-angiogenic and anti-inflammatory (Smart et al., 2007). Tβ4 has also been shown to possess anti-fibrogenic activity, which apparently is an indirect effect mediated through its ability to release platelet derived growth factor (PDGF) and hepatic growth factor (HGF) (Barnaeva et al., 2007). The anti-fibrogenic activity likely prevents scar tissue formation that could interfere with nerve regeneration. It is also highly expressed in both ischemic tissues and postinjury-associated tissue regeneration, highlighting its role in tissue repair processes (Roth et al., 1999; Kim et al., 2006; Ferre et al., 2007).

Macrophage

Macrophages are multifaceted cells which play a critical role in both innate and adaptive immunity. The involvement of these cells in numerous physiological and pathological processes is well documented (Ross and Auger, 2002). Macrophages differentiate from hematopoetic stem cells in the bone marrow under the influence of several growth factors, particularly macrophage colony stimulating factor (M-CSF) (Hume, 2006). Released to the circulation as monocytes, these cells ultimately migrate and reside in tissues where they go by different names such as intestinal dendritic cells, alveolar macrophages in lung, Kupffer cells in liver, and microglia in brain. As antigen presenting cells, the macrophages play a very important role in the development of adaptive immunity. Tβ4 has been shown to potentiate the antigen presenting ability of macrophages (Tzehoval et al., 1989). Regardless of their tissue of residence, these cells perform a similar function that provides defense against microbial pathogens. In doing so, the macrophages produce many cytokines, chemokines, growth factors, and secretory molecules which can have both autocrine and paracrine effects.

As one of the most important sentinel cells in health physiology, the macrophages eliminate foreign pathogens, virus-infected cells, tumor cells, and apoptotic cells. Thus, they contribute to the innate defense mechanism by resolving inflammation and maintaining tissue homeostasis (Savill and Hasltt, 2001; Ross and Auger, 2002; Tosi, 2005). Although circulating macrophages are considered one of the major role players in the process of inflammation, they arrive at the site of inflammation much later than neutrophilic granulocytes (Serhan et al., 2006). Therefore, these cells do not contribute to acute inflammation but play critical roles in the management and the resolution of inflammation, most importantly by removing dying granulocytes the content of which can cause tissue damage. Macrophage-derived factors can also direct apoptosis of leukocytes and myofibroblasts, which are presumably involved in fibrosis (Wilson, 1997; Savill and Hasltt, 2001; Porcheray et al., 2005).

The activation of macrophage is mediated through specific pattern recognition receptors called 'Toll-Like Receptors (TLR)' whereby the macrophages recognize specific ligand molecules elicited by the pathogens (Kaisho and Akira, 2006). The recognition leads to the activation of complex signaling pathways culminating in the production of appropriate response factors such as cytokines, acute phase proteins and metabolites, which modulate inflammatory events and, eventually, immune responses. Over 10 TLRs have been described in different vertebrate species (Werling and Coffey, 2007). Recent evidences from our laboratory show that avian macrophages can possibly secrete Tβ4 in response to certain pro-inflammatory factors such as lipopolysaccharide and peptidoglycans (Kannan et al., unpublished). In addition to the classical TLR activators, there are other stimulators of macrophages which may work through different pathways.

Inflammation

A variety of injurious stimuli such as infection, toxins and physical trauma induce inflammation, which involves interplay of vascular, neural, endocrine and immune systems. Inflammation is a defense strategy to neutralize the causative agents in order to protect the affected area from excessive damage and prevent deleterious systemic effects.It involves recognition of causative stimulus, signaling and the execution of events such as production of cytokines, growth factors and metabolites, eventually mitigating inflammation.The heterophilic granulocytes, though have a short half life, are the first populations of cells which migrate to the inflammatory site and are the major determinants of acute inflammation (Tosi, 2005). Substances released from these cells and by the damaged tissues provoke neuroendocrine responses such as pain and tenderness that are hallmark characteristics of inflammation. Some of the major mediators of inflammation are cytokines such as interleukins 1, 6, and 8, tumor necrosis factor-α, complements, kinins, proteases and leukotrienes, most of which have short half lives. Inflammatory mediators activate nuclear factor kappa B NFκB, a cytoplasmic transcription factor, which on translocation to nucleus, interacts with regulatory genes to produce inflammatory proteins (Perkins, 2007).

Recently, Tβ4 has been shown to suppress NF Kappa B activity in the corneal epithelium (Sosne et al., 2007). Edema of inflammation results from vascular leakage leading to the migration of leukocytes to the damaged area.Thymosin β4 has been shown to induce exocytosis of mast cells which are the likely source of histamine and other vasodilatory agents that facilitate vasodilation and recruit other cells and blood-derived substances to promote wound healing (Leeanansaksiri et al., 2004; Wyczolkowska et al., 2007; Leslie, 2007). Along with other wound fluid factors such as fibrin, immunoglobulins, lipoxin, complements and growth

factors, Tβ4 possibly plays an important role in regulating inflammation. As a modulator of inflammation, Tβ4 down-regulates cytokine production, protects regenerating tissues, and attenuates oxidative stress-induced tissue damage (Girardi et al., 2003). Tβ4 also stimulates keratinocyte and epithelial cell migration, and promotes corneal epithelial healing (Huang et al., 2007).

Significance of Tβ4 in macrophages

From the above discussion it is clear that Tβ4 is an important macrophage component involved in a plethora of physiological and immunological processes. As migratory cells, the macrophages require constant cytoskeletal organization such as polymerization and depolymerization of actin necessary for their motility, adhesion, and polarity maintenance which is facilitated by Tβ4. Actin polymerization is also required for their phagocytosis function. At the site of inflammation, the macrophages encounter inflammatory mediators leading to their activation and the release of Tβ4. Besides its ability to stimulate tissue remodeling and angiogenesis (Philp et al., 2006), Tβ4 is also capable of acting as an antioxidant and antimicrobial factor that prevent exacerbation of inflammation. Monocytes and macrophages also produce Tβ4 and Tβ4 sulfoxide in response to several non-steroidal and steroidal anti-inflammatory drugs (Chettibi et al., 1994; Young et al., 1999; Jain et al., 2004). Tβ4 sulfoxide is an oxidized form of Tβ4 produced in response to glucocorticoid and it is shown to exhibit superior anti-inflammatory efficacy (Young et al., 1999). Also, as discussed in the preceding section, Tβ4 down-regulates NF Kappa B activation (Sosne et al., 2007) suppressing inflammation. Therefore, it is appealing to consider that Tβ4 is a significant remedial factor produced by the macrophages to counter inflammation and restore physiological homeostasis.

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