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Original contribution

Occurrence of thymosin $\beta 4$ in human breast cancer cells and in other cell types of the tumor microenvironment☆

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Summary Previous studies have shown that the G-actin sequestering polypeptide thymosin $\beta 4$ frequently is overexpressed in cancers and that such overexpression correlates to malignant progression. However, the localization of thymosin $\beta 4$ in human cancers has not been determined. We now demonstrate that there is a considerable heterogeneity in the cellular distribution of thymosin $\beta 4$ in breast cancer. In most tumors examined, cancer cells showed low or intermediate reactivity for thymosin $\beta 4$, whereas leukocytes and macrophages showed intense reactivity. In addition, endothelial cells showed variable reactivity to thymosin $\beta 4$, whereas myofibroblasts were negative. There was no correlation between the intensity of tumor cell staining and histological grade, whereas there was a tendency toward a correlation between endothelial cell staining and grade. These results demonstrate that multiple cell types within the tumor microenvironment produce thymosin $\beta 4$ and that such expression varies from tumor to tumor. Such heterogeneity of expression should be taken into account when the role of thymosin $\beta 4$ in tumor biology is assessed.

1. Introduction

β-thymosins were originally isolated from the thymus and constitute a highly conserved family of polypeptides [1-3]. In humans, thymosins $\beta 4$, $\beta 10$, and $\beta 15$ have been identified. Thymosin $\beta 4$ is the most abundant of these molecules and has been linked to a number of important biological actions, including actin polymerization [4,5], angiogenesis [6,7], wound healing [8,9], inflammation [10-13], and signaling through the Akt pathway [14]. The role of thymosin $\beta 4$ in actin polymerization is complicated and appears to involve both sequestration of monomeric G-actin as well as binding to polymerized F-actin [4,5]. Recent studies have shown that thymosin $\beta 4$ frequently is overexpressed in malignant tumors [6,15-18]. In addition, forced overexpression of thymosin $\beta 4$ in melanoma cells was found to be associated with increased metastastic capability, increased angiogenesis, and increased production of vascular endothelial growth factor [6]. Wang et al [19,20] also presented data showing that cultured colon carcinoma cells progressed toward a more malignant phenotype after overexpression of thymosin $\beta 4$. In contrast, however, Yamamoto et al [21] described down-regulation of thymosin $\beta 4$ in metastatic cells of colorectal carcinomas. The role of thymosin $\beta 4$ in angiogenesis is supported by experimental

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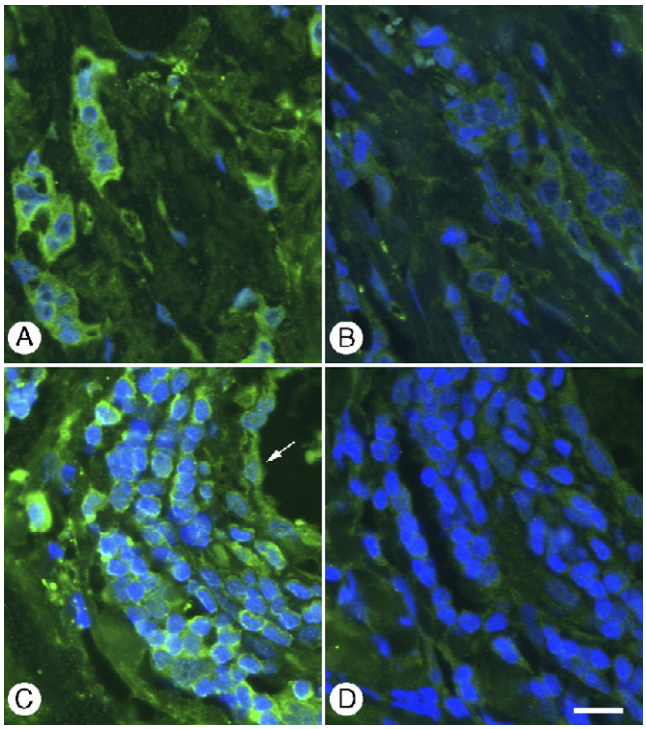

Fig. 1 Staining for thymosin β4 (green immunofluorescence) in breast cancer. Sections shown in panels (A) and (C) have been stained with unpretreated antiserum, whereas sections shown in panels (B) and (D) represent controls stained with antigen-preabsorbed antiserum. Sections have been counterstained with bisbenzimide to reveal the nuclei (blue). A-B, Breast cancer cells (patient 2) showing strong (+++) staining for thymosin β4 when stained with unpretreated antiserum (A) and no staining when stained with antigen-preabsorbed antiserum (B). C-D, Intratumoral vessel (arrow) surrounded by a cuff of lymphocytes. Note staining of both lymphocytes (+++) and endothelial cells (++) (patient 6) (C) that disappears when the antiserum is preabsorbed with antigen (D). Scale bar, 30 μm.

data [6,7] as well as by studies localizing the polypeptide to the endothelium of developing blood vessels and to the heart [14,22,23]. At later stages of development, not all endothelial cells were positive for thymosin β4, suggesting that the peptide may play a role in the developing endothelium rather than in sustaining the mature vessel [22]. The effects of thymosin β4 on actin polymerization have also been linked to its influence on transcriptional regulation through chromatin remodeling events (reviewed by Goldstein [24] and Olave et al [25]). In addition, cells are able to take up exogenously administered thymosin β4 and respond to the peptide by activation of integrin-linked kinase [14]. Thymosin β4 is present in blood plasma and, at much higher concentrations (estimated to 13 μg/mL), in wound fluid where it may function as a wound-healing factor [26,27]. The polypeptide is also present at high concentrations in mononuclear and polymorphonuclear white blood cells [27]. It occurs at lower concentrations in platelets, from which it may be released in response to wounding but is not detectable in erythrocytes [27].

Thus, thymosin β4 may influence malignant progression by effects on angiogenesis, tumor cell migration, and adhesion as well as through its antiinflammatory and wound-healing properties. The role of thymosin β4 in cancer has, so far, been analyzed by experimental overexpression or by biochemical and microarray studies of whole tissue extracts. The exact distribution of thymosin β4 within human tumors has never been examined. Using immunocytochemistry on human breast cancers, we now report that this peptide is expressed by tumor cells as well as by several other cell types of the tumor microenvironment, including endothelial cells, macrophages, stromal cells, and white blood cells. There was no correlation between the degree of tumor cell staining for thymosin β4 and histological grade.

2. Materials and methods

Nineteen specimens from invasive breast cancers, obtained from Hvidovre University Hospital, were fixed in neutral-buffered formalin and embedded in paraffin. Dewaxed 5-μm sections underwent antigen retrieval by microwaving in a Polar Patent PP780 microwave oven (Miehle Axlab, Copenhagen, Denmark) thrice for 5 minutes in 10 mmol sodium citrate buffer (pH 6.0) at 780 W and were then preblocked for unspecific protein binding in 10% normal goat serum and exposed for 2 hours at room temperature to a rabbit antiserum raised to the synthetic thymosin β4(1-14) sequence (Immundiagnostik AG, Bensheim, Germany), diluted 1:200 in 0.05 mol/L Tris buffer, containing 0.15 mol/L NaCl and 0.25% bovine serum albumin (tris-buffered saline [TBS]). The site of antigen antibody reaction was revealed with Alexa-488–labeled goat antirabbit immunoglobulin G (IgG; Molecular Probes Inc, Eugene, OR), diluted 1:400 in TBS for 30 minutes at room temperature. After washing in TBS, sections were stained for DNA using bisbenzimide (Sigma, St. Louis, MO) and mounted in fluorescence mounting medium (DakoCytomation, Glostrup, Denmark). Controls consisted of conventional staining controls [28] as well as of absorption of the antiserum with 100 μg/mL of synthetic thymosin β4(1-43) (Immundiagnostik AG) overnight at 4°C. Double immunofluorescence was performed by mixing the rabbit antithymosin β4 antiserum with mouse monoclonal antibodies specific for cytokeratin ("pancytokeratin," clone MNF116), CD31 (clone JC/70A), von Willebrand factor (clone F8/86), CD68 (clone PG-M1), or smooth muscle actin (clone 1A4) (all from DakoCytomation), followed by detection using Alexa-488–labeled antirabbit IgG and Alexa-594–labeled antimouse IgG. Specimens were examined in a DMRB epifluorescence microscope equipped with selective filter blocks for Alexa-488, Alexa-594, and bisbenzimide and with Q-FISH software for image acquisition (Leica, Mannheim, Germany). Digital images were assembled in Adobe Photoshop CS2 (Adobe, San Jose, CA). Sections