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Thymosin β4 in multiple myeloma: friend or foe

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Multiple myeloma (MM) is a malignancy characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). Because of the known involvement of thymosin β4 (Tβ4) in metastasis of tumor cells, we examined the expression and role of Tβ4 in MM disease. In a large patient population, we demonstrated that Tβ4 expression was significantly lower in myeloma cells compared to normal plasma cells and that patients with a high Tβ4 expression had a longer event free and overall survival. The decreased Tβ4 expression was also found in the murine 5TMM model. To study its function, we overexpressed the Tβ4 gene in 5T33MMvt cells by lentiviral transduction. These cells demonstrated a decreased proliferative capability and an increased sensitivity to apoptosis. Mice injected with Tβ4-overexpressing cells showed a prolonged survival compared to mice injected with controls. In contrast to its role in solid tumors, we found a decreased expression in myeloma cells compared to their normal counterpart and studies with overexpression of the Tβ4 gene indicated a tumor suppressive function of Tβ4 in myeloma development.

Keywords: multiple myeloma; thymosin beta 4; plasma cell

Introduction

Thymosin β4 (Tβ4) is a 43 amino-acid small peptide originally isolated from the thymus.[1] It was shown that Tβ4 interacts with G-actin and functions as a major actin-sequestering protein.[2] Tβ4 is considered to be a major actin-sequestering molecule, which specifically binds monomeric actin (G-actin), forming a 1:1 complex, or in a ternery complex including profilin.[3] The mechanism by which Tβ4 influences cell proliferation, migration, and differentiation is generally believed to be linked with maintaining a dynamic equilibrium between G-actin and F-actin, critical for the rapid reorganization of the cytoskeleton. Tβ4 induced cell proliferation, migration, and differentiation contribute to different physiological and pathological processes, such as angiogenesis,[4] wound healing,[5] and cardiac repair.[6]

Thymosin β4 in cancer

Multiple studies have indicated that Tβ4 was overexpressed in various tumor tissues and may play an important role by affecting tumor cell proliferation, migration, metastasis, and induction of angiogenesis. The different studies on solid tumors can be summarized as a potential role of Tβ4 in the malignant conversion of a normal cell or in a potential role in metastasis of primary tumor cells. Evidence for this role can be found from the studies in melanoma, sarcoma, and pancreas cancer. In both murine and human melanoma cells, Clark *et al.* found that Tβ4 levels were increased in tumor cells from metastatic lesions compared with the parental cells isolated from the primary site.[7] A similar observation was made in a murine fibrosarcoma model, where Tβ4 was one of genes that increased when mRNA from highly tumorigenic and metastatic cells was compared with its weakly tumorigenic precursor cell line or normal counterpart.[8,9] Furthermore, these increased levels of Tβ4 were demonstrated to regulate the migratory and invasive capacities of these cells. A adenoviral-based overexpression of Tβ4 was applied in a human colon cancer cell line and melanoma cell line. These experiments showed an increased growth, motility, and invasive

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capacities *in vitro*[10,11] and a larger tumor load (size of primary site and number of metastasis *in vivo*). In addition, Tβ4 overexpression was associated with the stimulation of blood vessel formation in these latter experiments.[12] Recently Zhang *et al.* showed that Tβ4 expression was elevated in pancreatic cancer cell lines and clinical tissue specimens, compared with control pancreatic duct cells and surrounding normal pancreatic tissues. Tβ4 was believed to be involved in stimulating human pancreatic cancer progression by promoting a proinflammatory cytokine environment and by activating JNK signaling pathway.[13] In nonsmall cell lung cancer, the Tβ4 gene was found to be upregulated in metastasizing primary tumors compared with non-metastasizing tumors and Tβ4 expression had also prognostic value: high expression levels of Tβ4 was associated with significantly worse survival of patients with stage I disease.[14]

Thymosin β4 in angiogenesis

A first indication for an implication of Tβ4 in angiogenesis came from its identification in a screen for rapidly induced genes following culture of human umbilical vein endothelial cells (HUVEC) on a basement membrane matrix.[15] A fivefold induction of Tβ4 was observed during endothelial cell differentiation *in vitro* and transfection of HUVECs with Tβ4 caused an increase in the rate of attachment, spreading, and tube formation.[15] Hynda Kleinmans' group also demonstrated that Tβ4 acts as a chemo-attractant for endothelial cells, by stimulating directional migration of HUVECs *in vitro* and endothelial cell migration *in vivo* in a subcutaneous Matrigel plug assay.[4] In addition to stimulating proliferation, attachment, and differentiation of endothelial cells, Tβ4 was also able to induce tube formation on Matrigel and vascular sprouting and neo-vascularization.[16] All these studies validated the involvement of Tβ4 in angiogenesis by promoting migration of endothelial cells, but the precise mechanism by which Tβ4 directs cell migration is poorly defined and the role of actin binding versus other receptor-mediated events is still a matter of debate.[17]

Thymosin β4 in multiple myeloma

Multiple myeloma (MM) is a malignant plasma disorder characterized by the accumulation of monoclonal plasma cells in the bone marrow (BM). Despite the introduction of novel treatment strategies, MM remains an incurable disease.[18] Migration and invasion are important processes in the initial homing of the cells to the bone marrow and subsequent spreading to distant sites.[19] Moreover, angiogenesis is one of the hallmarks in MM disease progression.[20,21] Because of its involvement in these processes, we were interested in analyzing Tβ4 expression and function in multiple myeloma cells. Thymosin beta 4 was examined in a historical study that analyzed Tβ4 gene expression in lymphoid malignant cells. The authors found high levels of Tβ4 in lymphocytes and in early stages of B-cell differentiation, but Tβ4 was absent in MM cells. Since this pattern of Tβ4 gene expression was similar to that of immunologically important molecules (such as HLA class II antigen, Fc receptor, and complement receptor), a relationship between Tβ4 and B-cell differentiation state was suggested.[22] This implication in cellular differentiation was also proposed by a second study that examined Tβ4 expression in lymphoid and myeloid cells and that showed that Tβ4 gene was regulated in a maturation-related manner.[23] We studied the expression of Tβ4 in human disease and in the 5TMM mouse model of MM.[24] We investigated the *T*β-expression pattern in a large ($n = 298$) sample of primary MM cells and in normal plasma cell samples from healthy donors as previously reported.[25] We found that Tβ4 was lower expressed in MM cells compared to normal plasma cells (cf. Fig. 1). A similar finding could be seen when plasma cells from monoclonal gammopathy of undetermined significance (MGUS) patients were compared with normal plasma cells. MGUS is a premalignant form that may develop into overt MM disease. We subsequently analyzed the survival of MM patients that showed a high Tβ4 expression (termed $T\beta4^{high}$) compared to patients with a low Tβ4 expression (termed $T\beta4^{low}$). All patients were treated by high dose induction chemotherapy that was followed by an autologous stem cell transplantation. $T\beta4^{high}$ patients showed a significantly longer median EFS (38 months) than $T\beta4^{low}$ patients (26 months). The overall survival tended to differ in favor of $T\beta4^{high}$ patients.

A similar *T*β*4* expression pattern was seen in the 5TMM murine MM model where *T*β*4* was decreased compared to normal hematopoietic cells (Figs. 2 and 3A).[24] Addition of exogenous Tβ4 minorly inhibited 5T33MM cell proliferation, whereas

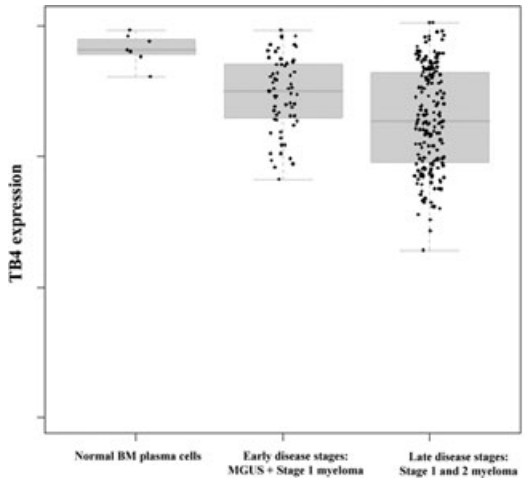

Figure 1. **Shows the micro-array data obtained for the *Tβ4* expression in CD138$^+$ sorted BM plasma cells from healthy donors and MM patients. These results were validated by quantitative RT-PCR. The premalignant form, MGUS, and Salmon & Durie Stage 1 were considered as early myeloma disease stages and Salmon & Durie Stage 2 and 3 as late disease stages. Figure illustrates the expression in plasma cells from normal healthy volunteers, patients with early disease stage and patients with late disease stage.[24]**

addition of the AcSDKP tetrapeptide had no effect on proliferation. Because of its low expression in 5T33MMvt cells, the Tβ4 gene was overexpressed using a lentiviral expression vector. In a proliferation assay, 5T33MMvt$^{Tβ4+}$ cells showed a significant decrease in DNA synthesis compared to control cells. 5T33MMvt$^{Tβ4+}$ cells showed a significantly increased sensitivity to different anti-MM agents such as the NF-κB inhibitor Bortezomib, dexamethasone, or melphalan (Fig. 3B). The quantification of G-Actin and F-Actin by Western blotting showed a lowered G-Actin–F-Actin ratio after *Tβ4* overexpression. F-Actin is of particular importance in cytoskeleton changes involved in cellular migration and in microtubuli organization controlling the mitotic spindle. In line with these results, vinca-alkaloids with micro-tubulin inhibitory activity had more effect on the proliferation capacities of 5T33MMvt$^{Tβ4+}$ cells than on control cells. After intravenous injection of 5T33MMvt$^{Tβ4+}$ or control cells, the survival of mice injected with control cells was significantly shorter: 65.9 days compared to 88.9 days for mice injected with 5T33MMvt$^{Tβ4+}$ cells.

In contrast to what is seen in solid tumors, we found in MM cells a decreased expression of Tβ4 compared to their normal counterpart (plasma cells). Tβ4 expression had some prognostic value; patients with a high Tβ4 expression had a better outcome after intensive chemotherapy compared to a low Tβ4 expression. We observed *in vitro* differences in proliferation and sensitivity to anti-MM agents, which indicated a direct effect on proliferation. Quantitative RT-PCR showed that the Tβ4 gene expression could be correlated to the malignant phenotype of 5TMM cells: 5T33MMvt cells are highly proliferative cells with limited migratory capacities (and showed a very low *Tβ4* expression),

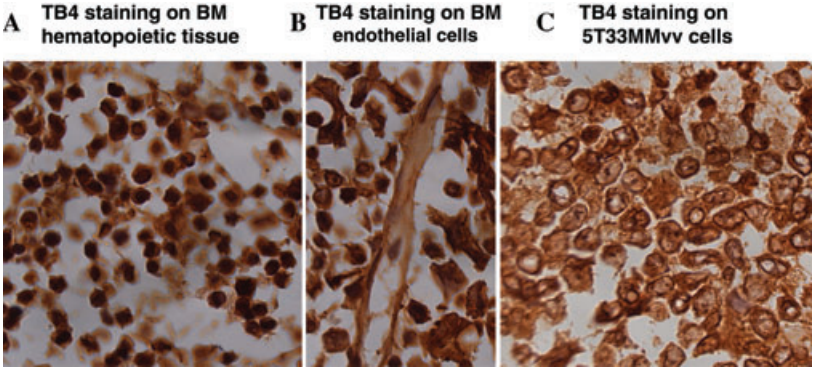

Figure 2. **Tβ4 staining on normal hematopoietic cells, bone marrow endothelial cells, and 5T33MMvv cells. In normal hematopoietic cells, a strong nuclear staining was seen (A). MM disease progression is associated with a neo-vascularization. BM endothelial cells also showed strong positivity for Tβ4 (B). MM cells stained positive intra-cytoplasmatic for Tβ4 (C), which was less intense as in normal hematopoietic cells.**

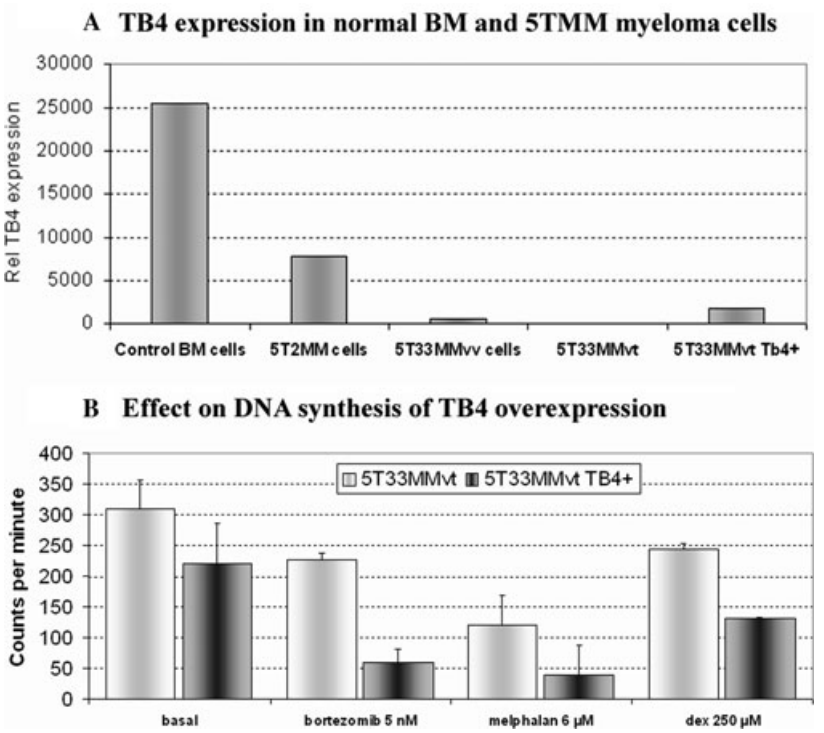

Figure 3. (**A**) **In the 5TMM model, a similar gene expression pattern was observed as in humans.** *Tβ4* **mRNA expression in 5T33MM and 5T2MM invaded BM was lowered compared to normal BM cells. 5T33MMvt cells showed the lowest mRNA expression. Transfection of Tβ4 using a lentiviral vector resulted in overexpression of the Tβ4 gene. (B) 3-H thymidine uptake revealed a decreased DNA synthesis rate in 5T33MMvt$^{T\beta 4+}$ cells compared to wild-type cells. Incubation with the anti-MM agents bortezomib (5 nM), melphalan (6 μM), and dexamethasone (250 μM) had stronger effects on 5T33MMvt$^{T\beta 4+}$ than on control cells.[24]**

whereas 5T2MM and 5T33MMvv cells have a lower proliferative, but higher migratory index (with *Tβ4* expression that was higher in a low proliferative 5T2MM cells). These results may suggest that Tβ4 may be involved in the differentiation status of MM cells, as indicated by the earlier studies on lymphoid and myeloid cells. However, its mechanism of action is unclear and currently under investigation.

In conclusion, our results propose a tumor suppressive function of *Tβ4* expression in MM with impact on survival. *Tβ4* was downregulated in MM cells of patients compared to the normal BM plasma cells and studies with the murine 5T33MM model show a decreased *in vitro* and *in vivo* tumor growth for cells overexpressing the *Tβ4* gene.

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Conflicts of interest

The authors declare no conflicts of interest.

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