Expression of Thymosinβ4 in Murine Bone Marrow Endothelial Cells, HL-60 Cells and Human Peripheral Blood Mononuclear Cells

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Abstract To investigate the relationship between the growth situation of the cells and the expression level of thymosinβ4, the specific primer of thymosinβ4 was selected to test the expression of thymosinβ4 gene in murine bone marrow endothelial cells in different proliferation states and in HL-60 cells and peripheral blood mononuclear cells by RT-PCR. The results showed that the expression level of thymosinβ4 in the murine bone marrow endothelial cells with active proliferation was high, otherwise it was low. The thymosinβ4 gene expression level was higher in HL-60 cells than that in human peripheral blood mononuclear cells. It was suggested that the expression level of thymosinβ4 mRNA is closely related to cell growth.

Key words thymosinβ4 gene expression bone marrow endothelial cell HL-60 cell peripheral blood mononuclear cell

Thymosinβ4 (β4) was isolated from the calf thymus fraction. The polypeptide sequence of β4 consists of 43 amino acids with a molecular mass of 4982 dalton. A number of investigations have suggested that β4 is ubiquitously expressed in a diverse array of cells and tissues, such as brain, embryonic lung, kidney, spleen, heart, liver, adrenal, fibroblast and so on. There were a lot of reports about the regulation of β4. For example, Gordon et al. reported that β4 expression was related to the state of differentiation of human neoplastic B lymphocytes. Moreover, β4 mRNA was stimulated by nerve growth factor in rat pheochromocytoma 12 (PC12) cells. The mitogen concanavalin A rapidly induced expression of this gene in rat thymocytes. However, Shimamura et al. found that there was no variation in β4 mRNA levels in serum deprived HL-60 cells stimulated to reenter in the cell cycle or along it. We have reported that murine bone marrow endothelial cells expressed β4. To investigate the relationship between the expression level of β4 gene and the growth situation of cells, RT-PCR was used to test the expression of β4 in murine bone marrow endothelial cells in different proliferative status and HL-60 cells and peripheral blood mononuclear cells in this study. Our findings demonstrated that the expression level of β4 was closely associated to the growth state of cells.

Materials and Methods

Cells

Endothelial cell line derived from murine bone marrow (mBMEC) has been used as the source of pure endothelial cells in this experiment (Figure 1). The mBMECs were routinely grown at 37°C and 5% CO2 in humidified atmosphere in complete culture medium consisted of Iscove’s modified Dulbecco’s medium (IMDM) (Sigma), supplemented with 20% heat-inactivated newborn bovine serum (NBS) and 100 U/ml recombinant murine granulocyte macrophage colony-stimulating factor (rmGM-CSF, Genzyme). Human peripheral blood cells were from normal donor, and mononuclear cells (PBMNCs) were separated on Ficoll-Hypaque gradient (density 1.077 g/ml). Myeloid leukemic cell line HL-60 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated NBS.

BMECs in different proliferation state

1 × 10^5/ml BMECs were cultured in different culture medium, one of which was routine culture medium (IMDM + NBS + rmGM-CSF), the second one was serum-free culture medium, complete culture medium deprived of NBS and rmGM-CSF (IMDM only), and the third one was complete culture medium supplemented with 2.5 ng/ml basic fibroblast growth factor (Genzyme) (IMDM + NBS + rmGM-CSF + bFGF). After incubation for 36 hours, the cells labeled with 3H thymidine (2μCi/well) for 8 hours were washed with cold PBS, released with 0.25% trypsin. Then the cells were vacuum-filtered through a glass-fiber filter. After washing with 5 ml PBS, 5 ml 10% trichloroacetic acid
followed by 5 ml 95 % ethanol, the filter was dried. The radioactivity of incorporated $^3$H-thymidine was measured in a liquid scintillation counter (Beckman)\textsuperscript{101}. The number of the cells in the well without $^3$H-thymidine was counted.

**Figure 1** Endothelial cells derived from murine bone marrow (× 256)

**RNA isolation**

1 × 10\(^7\) above-mentioned pure endothelial cells in good conditions were released by 0. 25 % trypsin, and then lysed by RNA extraction buffer containing 0. 14 mol/ L NaCl, 1. 5 mmol/L MgCl\(_2\), 10 mmol/L Tris- HCl (pH 8. 6). 0. 5 % NP-40, 1 mmol/L DTT, 1 000 U/ml RNasin. Total cellular RNA was extracted by the method described previously\textsuperscript{111}.

**RF PCR**

Reverse transcription-PCR (RT-PCR) was used to detect mRNA encoding $\beta$4 in mBMECs. In 50μl RT system, there were 5μg total cellular RNA isolated from mBMECs, 1μg oligo (dT)\(_{12-18}\) primer, 50 mmol/L Tris- HCl (pH 8. 3), 60 mmol/L KCl, 10 mmol/L MgCl\(_2\), 1 mmol/L DTT, 0. 1 % BSA, AMV reverse transcriptase (Promega) 40 U, 1 mmol/L dNTP, 40 U RNasin (Promega). Then $\beta$4 was amplified by polymerase chain reaction (PCR) using $\beta$4 specific primers derived from the published sequence\textsuperscript{11}. The $^5$′ $\beta$4 primer, AGAAGCTCTGCAACCAGCTTCGA, encompassed nucleotides - 19 to - 44. The $3$′ primer, CCATTTTGGATCCTGCAAGACATTT, was derived from nucleotides 476 - 458. And the cDNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also tested as a semi-quantitative control and criteria of mRNA quality. The sequence of GAPDH primer was as following: $^5$′ primer, CCAATATGATTCCACCCATG; $^3$′ primer, AGGGTCCACAGCTACGTT. In 50μl PCR system, there are RT reaction buffer 20μl, 10 ×PCR buffer 5μl, primer 50 pmol/L and Taq DNA polymerase 2. 5 U.

Thirty-five cycles of amplification were performed under the following protocol: first cycle reactions began with a 5 min 95 °C denaturation, followed by 55 °C 2 min and 72 °C 3 min. The subsequent 34 cycles consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C and 1 min of extension at 72 °C, and the last cycle was extended for another 9 min.

**Detection of RF PCR amplified product**

RT-PCR amplified product was detected by 1. 5 % agarose gel electrophoresis, and visualized by autoradiography. Quantitation of $\beta$4 mRNA level for each group was performed by normalizing relative gray-scale units for $\beta$4 to that of the respective GAPDH using Scan Analysis Software (Biosoft, Cambridge, U K).

**Statistical analysis**

Data were present as $\bar{x}$ ± s for all experiments that were performed at least three times. Significant difference was determined by ANOVA. P value of 0. 05 or less was considered significant.

**Results**

**BMECs in different proliferation state**

As shown in Figure 2, the number of BMECs in serum-free IMDM was the least, and that in bFGF supplemented complete culture medium was the most. And $^3$H-thymidine incorporation (cpm/well) in the cells cultured in serum-free medium, routine culture medium and bFGF supplemented medium was 34 466 ± 776, 57 542 ± 126 and 120 160 ± 16391, respectively.

**Differential expression of $\beta$4 in BMECs in different proliferation state and in HL-60 cells and PBMCs**

As shown in Figure 3 and Figure 4, the relative level of $\beta$4 was calculated after it was scanned by lamella scanner. The expression of GAPDH was regarded as 100 %. The relative level of $\beta$4 mRNA of BMECs cultured in serum-free medium, routine culture medium and
and bFGF supplemented medium was 8.16%, 13.26% and 17.49%, respectively. And the relative level of \( \beta 4 \) mRNA in HL-60 cells and PBMCNs was 19.51% and 15.93%, respectively.

in a T cell line\(^{13,14}\). The effect of \( \beta 4 \) on hematopoiesis has caught more and more attention. It was reported that \( \beta 4 \) resulting in a decrease of CFU-GM inhibits hematopoietic stem/progenitor cells to enter S phase. Moreover it could decrease the number of progenitors from both unfractionated bone marrow cells and purified CD34\(^+\) cells, which indicated that accessory cells are not necessary for the inhibition\(^{15}\). So it implied that \( \beta 4 \) had close relationship with hematopoietic system.

We have reported that endothelial cells from murine bone marrow could express \( \beta 4 \) mRNA\(^{16}\). In order to investigate the expression of \( \beta 4 \) mRNA in hematopoietic system, a series of experiments were designed. The mBMECs cultured in different culture medium were in different proliferation state, then the abundance of \( \beta 4 \) mRNA was tested. Furthermore, the relative level of \( \beta 4 \) in HL-60 cells and PBMCNs were tested.

In RT-PCR, GAPDH gene was selected as internal standard for its high and stable expression in all kinds of cells. Since the change of GAPDH gene was slight in cells in different growth conditions, if there was any change in \( \beta 4 \) mRNA, the relative gray-scale units for \( \beta 4 \) to that of the respective GAPDH gene could reflect such change. Furthermore, this relative unit could minimize the disturbance of the experimental elements. The results indicated that the expression level of \( \beta 4 \) was related to the proliferation state of BMECs. When BMECs were in active proliferation, their \( \beta 4 \) mRNA level was high. Otherwise, its level was low. The higher expression of \( \beta 4 \) in HL-60 cells than in PBMCNs may be result from their different differentiative status or the malignancy of HL-60 cells. Our results about the expression of \( \beta 4 \) mRNA level was consistent with some previous studies, which indicated that \( \beta 4 \) gene was regulated during cell growth. Our findings implied that \( \beta 4 \) gene may be a new molecular marker for leukemia. Since \( \beta 4 \) as an inhibitory factor protects hematopoietic stem cells from extensive differentiation, the high expression of \( \beta 4 \) in mBMECs which were in active proliferation may explain the excellent effect of endothelial cells on the expansion of primary hematopoietic progenitors\(^{16}\). However, further studies are required to investigate the mechanism and the role of \( \beta 4 \) gene control.

### Discussion

Thymosin \( \beta 4 \) (\( \beta 4 \)) was originally isolated as a thymic peptide that modified immune response: suppression of mixed lymphocyte proliferative response, induction of the expression of terminal deoxynucleotidyl transferase in T cells, inhibition of the migration of macrophages in vitro and induction of phenotypic changes in a T cell line\(^{13,14}\). The effect of \( \beta 4 \) on hematopoiesis has caught more and more attention. It was reported that \( \beta 4 \) resulting in a decrease of CFU-GM inhibits hematopoietic stem/progenitor cells to enter S phase. Moreover it could decrease the number of progenitors from both unfractionated bone marrow cells and purified CD34\(^+\) cells, which indicated that accessory cells are not necessary for the inhibition\(^{15}\). So it implied that \( \beta 4 \) had close relationship with hematopoietic system.

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