Expression of the Thymosin β₄ Gene During Differentiation of Hematopoietic Cells

By Ryuji Shimamura, Jiro Kudo, Hiroko Kondo, Kazufumi Dohmen, Hisashi Gondo, Seiichi Okamura, Hiromi Ishibashi, and Yoshiyuki Nihno

Thymosin β₄ (Tβ₄) was originally isolated as a thymic hormone. Its functional properties remain obscure; however, the N-terminal peptidic sequence could have a regulatory function on hematopoietic stem cell proliferation. To investigate the mechanism of Tβ₄ expression, we studied Tβ₄ gene expression in various leukemic cells and in established cell lines. Among leukemic cell samples obtained from leukemia patients, the Tβ₄ gene was highly expressed in a lymphoid lineage, especially in adult T-cell leukemia (ATL) cells, rather than in a granulocyte lineage. The Tβ₄ gene was more transcriptionally active in chronic B-cell leukemia than in acute B-cell leukemia, while it was inactive in plasma cell leukemia. We also found that cells from one of the ATL patients transcribed a heterogeneous message, Tβ₄ messenger RNA increased in MOLT-3 during differentiation by 12-O-tetradecanoylphorbol-13-acetate (TPA), in HL60 cells induced by TPA or dimethylsulfoxide and K562 cells stimulated by cytosine arabinoside or hemin. The genomic sequence of Tβ₄ is considered to be highly conserved. Only 1 of 20 genomes from normal or hematopoietic malignant cells showed restriction fragment length polymorphism. These findings, along with previous data, suggest that Tβ₄ may be a new marker of differentiation of hematopoietic cells.

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MATERIALS AND METHODS

Leukemic cells. Peripheral blood leukocytes were obtained from leukemic patients or normal donors by venipuncture or leukapheresis. Leukemic leukocytes were collected from the patients before initiation of chemotherapy. Cytogenetic analysis was routinely performed in each case. For one acute lymphocytic leukemia (ALL) sample, leukapheresed white blood cells were used, and for other samples the mononuclear cell fraction obtained from the whole blood by Ficol-Conray density-gradient centrifugation was used. All samples contained more than 90% leukemic cells. A normal T-cell sample was obtained from the mononuclear cell fraction of a normal donor by E-rosetting. Clinical characteristics of leukemic patients are shown in Table 1.

Cell culture. Human T-cell lines CEM, HSB-2, MOLT-3, MOLT-4, TALL-1, MT-1, HUT-102, R01 (TPA-resistant MOLT-3 subclone), HL60 (human promyelocytic cell line), and K562 (human CML blast crisis cell line) cells were all maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO₂. HL60 cells were made quiescent by deprivation of fetal calf serum (FCS) in the culture medium and were maintained for 48 hours in RPMI 1640 medium. Quiescent populations were then stimulated with fresh RPMI 1640 containing 15% FCS and cells were harvested at the indicated time. For induction of differentiation to the granulocyte

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Table 1. Clinical Data of Patients

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<th>Case No.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>Karyotype</th>
<th>Leukocyte (per µL)</th>
<th>Malignant Cell (%)</th>
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Abbreviations: CML, chronic myelogenous leukemia; CMMoL, chronic myelomonocytic leukemia; AMoL, acute monocytic leukemia; AML, acute myelogenous leukemia; ATL, adult T-cell leukemia; ALL, acute lymphocytic leukemia; CLL, chronic lymphocytic leukemia; PCL, plasma cell leukemia; NE, not examined.

*French-American-British classification.
†Determined by surface marker.

Fig 1. Northern blot analysis of the Tjδ3 message for various leukemic cells. Ten micrograms of total RNA from each sample was electrophoresed. The numbers at the top correspond to the case numbers shown in Table 1, eg., 1, CML; 2, CMMoL; 3, AMoL; 4 through 6, AML; 7 through 12, ATL; 13 through 15, ALL; 16, CLL; 17, PCL. N is a normal peripheral T cell. Upper panels in A and B show the band of 28S ribosomal RNA rehybridized with the cDNA fragment to ensure the equality of the amount of total RNA. The bands of the Tjδ3 mRNA usually appear at 0.8 kb. (A) Comparison between granulocyte lineage and lymphoid lineage. (B) Comparison among each type of lymphoid lineage. (C) Heterogenous transcript seen in patient 12. HSB-2 is a T-cell line.
TB4 GENE EXPRESSION IN HEMATOPOIETIC CELLS

mmol/L of TPA for the induction of differentiation. The extent of differentiation has been reported.26,31,32 K562 cells were treated in 5 × 10⁻⁷ mol/L of cytosine arabinoside (Ara-C)33 and in 0.03 mmol/L of hemin34,35 for differentiation to the erythroid series. The extent of differentiation was evaluated by benzidine staining.36

Flow cytometry. Cells (1 × 10⁷) were pelleted at 1,000g for 5 minutes and the supernatant was removed. Five hundred microliters of 0.1% Triton X-100 and 500 μL of 1 mg/mL RNase were added to pelleted cells and suspended to obtain the nuclei. Two milliliters of 50 μg/mL of propidium iodide was added to the solution to stain the nuclear DNA. DNA content was determined by flow cytometer, FACScan (Becton-Dickinson, Mountain View, CA), and the percentage of cells in G0/G1, S, and G2/M phase of the cell cycle was analyzed by a polynomial model (SFIT).

RNA preparation and Northern blot analysis. The collected cells were lysed in the presence of 6 mol/L guanidium isothiocyanate, and the total RNA was purified using the 5.7 mol/L CsCl density-gradient centrifugation technique described by Chirgwin et al.37 Ten micrograms of total RNA was denatured in 50% DMSO, 1 mol/L glyoxal, 10 mmol/L phosphate buffer (pH 7.0) for 1 hour at 50°C. After size-fractionation of denatured RNA samples by 1% agarose gel electrophoresis, the RNA was transferred to a nylon membrane (KYNTRAN SY-13N, Schleicher and Schuell, Dassel, West Germany). The membranes were baked at 80°C for 2 hours and hybridized with a human TB4 cDNA, 368 base pairs (bp) PsiI fragment (clone pGKS1404), labeled with [32P] deoxycytidine triphosphate by the oligoprimed method (specific activity: 1 to 2 × 10⁶ dpm/μg). Hybridizations were performed in 50% formamide, 5X SSPE, 5X Denhardt’s solution, 0.1% sodium dodecyl sulfate (SDS), and 200 μg/mL sonicated salmon sperm DNA at 42°C for 48 hours. After hybridization, the membranes were washed with 2X SSC and 0.1% SDS at 37°C for 30 minutes, then twice with 0.1X SSC and 0.1% SDS at 50°C for 30 minutes. The membranes were

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were exposed to x-ray films (Fuji RX, Tokyo, Japan) with an intensifying screen at -80°C for 16 to 24 hours.

To ensure that equal amounts of total cellular RNA were present in each lane, the membranes were rehybridized with the labeled human rDNA fragment, inserted into the plasmid pHr14E3 (developed by Dr M. Muramatsu et al), and in some cases the filters were stained with methylene blue after autoradiography.

DNA preparation and Southern blot analysis. High molecular weight DNA was isolated by an SDS/proteinase K method, digested with restriction enzymes, separated by electrophoresis through 0.7% agarose gel and transferred to a nylon membrane by the Southern blot technique. The probe used was a human Tβ3 cDNA, 556-bp PstI fragment (pGKS 1331), approximately 200 bp larger than pGKS1404 in the 3' direction. The conditions of hybridization solution, washing, and autoradiography were much the same as used for the Northern blotting described previously.

RESULTS

Tβ3, messenger RNA (mRNA) levels in various leukemic cells and T-cell lines. Northern blot analyses were performed to examine the Tβ3 mRNA levels in various leukemic cells. Leukemic cells of the lymphoid lineage showed a stronger expression of the Tβ3 gene than that of the granulocyte lineage (Fig 1A). In the lymphoid lineage, ATL cells showed a relatively strong expression (Fig 1B). In addition, T cells in peripheral blood from a normal volunteer exhibited the same level of expression as ATL cells. In one ATL cell sample, a heterogeneous transcript of 1.5 kilobases (kb) was observed (Fig 1C).

Tβ3 mRNA expressions were examined in seven T-cell lines in an exponential growth state by Northern blot analysis (Fig 2). HUT-102 and MT-1 cells were derived from ATL cells, and considered to represent the most mature differentiation stage among the seven cell lines. According to the classification of T-cell lines by T-cell receptor (TcR) gene rearrangement, there was no definite relationship between the Tβ3 mRNA expression and the maturation stage of T-cell lines defined by TcR gene rearrangement.

Tβ3 mRNA level in MOLT-3, HL60, and K562 cells during differentiation. Because we found that Tβ3 mRNA was highly expressed in ATL cells and normal T cells, we examined the change of Tβ3 mRNA level during the differen-
tiation of MOLT-3 induced by TPA (Fig 3A). Whereas E-rosette positivity of MOLT-3 cells increased up to 4 days after addition of TPA, as also noted by Nagasawa and Mak, Tβ4 mRNA expression decreased during the first 1 to 3 hours, then increased to the peak in 24 to 48 hours.

Yamauchi et al. and Mayumi et al. established the MOLT-3 subclone R01, which is resistant to TPA-induced differentiation. R01 exhibits exponential growth in the presence of TPA, whereas parental MOLT-3 cells cease growing. When R01 was treated with TPA, the Tβ4 mRNA level decreased slightly during an early phase, increased to the peak at 24 to 48 hours, and then decreased (Fig 3B). However, the peak level was approximately half of the level of parental MOLT-3 by densitometric scanning.

It was reported that Tβ4 mRNA levels increased after differentiation of HL60 cells to macrophage and granulocyte with TPA or DMSO, respectively. In this work, we observed serial changes in the Tβ4 mRNA level in HL60 cells treated with either agent (Fig 4). Approximately 90% of HL60 cells adhered to the culture flask in the presence of TPA, and 40% of the cells differentiated to mature granulocytes in the presence of DMSO at 96 hours and 120 hours, respectively. The Tβ4 mRNA level in HL60 cells decreased during an early phase, increased to the peak at 24 to 48 hours, and then decreased slightly after treatment with TPA or DMSO. Moreover, the cell population in S phase of HL60 cells did not increase at 24 hours after TPA addition when the level of Tβ4 mRNA reached maximum (data not shown).

It is known that K562 derived from CML blast crisis cells has the potential to differentiate to the erythroid series after treatment with Ara-C or hemin. However, the growth curve of the hemin-treated cells did not differ significantly from that of control cells, while the Ara-C–treated cells ceased to grow without evidence of lethality. We also studied the change of the Tβ4 mRNA level in this system (Fig 5). Benzidine-positive K562 cells accounted for approximately 70% in the presence of either agent at 96 hours. The Tβ4 mRNA level in K562 cells also decreased during an early phase, increased to the peak at 24 to 48 hours, and then decreased slightly within 96 hours.

**Growth-related expression of the Tβ4 gene.** Tβ4 expression was examined to determine whether the expression depended on the cell growth rate. To confirm the growth arrest and serum restimulation of HL60 cells, a flow cytometric assay was performed. After 48 hours of serum deprivation, cells were reincubated in medium supplemented with 15% FCS at 37°C for an additional 24 hours. As shown in Fig 6, HL60 cells resumed their cycle. Because the expression of Tβ4 in HL60 cells did not change on arrest in the G0 stage and subsequent stimulation to growth (Fig 7), the level of Tβ4 expression is not simply a reflection of the actual rate of growth.
Southern blot analysis of T-cell lines. Southern blot analyses performed using cellular DNA of seven T-cell lines showed multiple bands (Fig 8). There was only one rearranged band in HUT-102, noted with EcoRI digestion. Therefore, we speculate that variation in the Tβ4 gene was minor and the difference of the Tβ4 mRNA levels was not associated with a structural change in the genome.

DISCUSSION

In this study we noted that the highest expression pattern of the Tβ4 gene was observed preceding the full maturation of the T cells, granulocytes, and erythrocytes, respectively. As determined in Northern blot experiments, the level of the Tβ4 mRNA is higher in the lymphoid than in the granulocyte lineage. In the lymphoid cells, ATL cells and normal peripheral T cells had a relatively higher expression. In one ATL cell sample, a heterogeneous transcript of 1.5 kb was observed. Considering previous investigations on the Tβ4 gene expression that showed a high expression of the gene in pre-B cells and a low expression in myeloma cells, the Tβ4 gene may be regulated in a maturation-related manner in each hematologic cell line. Such being the case, we performed differentiation induction experiments in vitro, using three independent hematologic cell lines.

The Tβ4 gene expression showed a sigmoid curve during MOLT-3 differentiation, a finding consistent with the result using clinical samples in which the Tβ4 expression in null cell ALL is lower than that in mature T cells or ATL cells. The Tβ4 mRNA level decreased once during an early phase (1 to 3 hours), then increased (9 to 48 hours) to the high peak. Similar results were observed in HL60 cells treated with TPA or DMSO, and in K562 cells treated with Ara-C or hemin. The observation that exactly the same expression patterns of the Tβ4 gene were noted in various cell systems

Fig 8. Southern blot analysis of genomic DNA from seven T-cell lines probed with Tβ4 cDNA. (A) EcoRI digestion; (B) HindIII digestion. The numbers at the left indicate the positions of size marker.
using different differentiation-inducing agents was interesting, as $\text{T}_4$ is a ubiquitous polypeptide existing in various species and tissues. Therefore, a relationship probably exists between $\text{T}_4$ expression and differentiation in various cell lines. The pattern of expression in the $\text{T}_4$ mRNA in TPA-resistant MOLT-3 subclone, R01, was the same as that of parental MOLT-3. However, the peak level of R01 was about a half that of the parental MOLT-3, and the $\text{T}_4$ mRNA level decreased to the same level as the pretreatment level of R01. This finding may relate to evidence that the number of phorbol ester receptors that affects the differentiation induction is low on the surface of R01 cells.32

Attempts have been made to classify T-cell lines by the rearrangement of the $\alpha$, $\beta$, and $\gamma$ TcR genes.40 However, there was no relationship between expression of the $\text{T}_4$ mRNA and such a classification of differentiation. As the increase of the $\text{T}_4$ mRNA level was transient during the process of differentiation, the amount of $\text{T}_4$ mRNA in steadily growing cells may reflect the cellular population in a specific early phase of differentiation. The $\text{T}_4$ gene was not highly expressed in HUT-102 cells derived from ATL cells, while it was highly expressed in clinical ATL cell samples. This finding suggests that the abundance of the cellular population in the specific phase of differentiation decreased during cultivation and cloning in vitro.

We examined approximately 20 samples from various leukemic cells and cell lines by Southern blot analysis. As shown in Fig 6, only one genome had a restriction fragment length polymorphism, suggesting a low frequency of sequence alteration in the $\text{T}_4$ gene locus. Multiple bands shown in Southern blot analysis could be due to either multiple exon or multi-gene structure. Additionally, MOLT-3 cells treated by TPA showed no rearrangement in the $\text{T}_4$ gene locus.

The present work showed that $\text{T}_4$ had the same gene expression pattern in several cellular differentiation systems and that it is translated abundantly in the specific phase of cellular differentiation. The $\text{T}_4$ molecule is likely to be cleaved to the tetrapeptide of Ac-Ser-Asp-Lys-Pro, as found in calf bone marrow.49 As it regulates the growth of hematopoietic stem cells and possibly of other cellular systems, the fully mature cells slowly turn off $\text{T}_4$ gene expression, the result being homeostasis of cell growth. In this context, our preliminary investigation showed that a synthetic N-terminal oligopeptide sequence of $\text{T}_4$ directly decreases approximately 5% of the cell population of $\text{S}$ phase MOLT-3 and HL60 during transition of growth in a serum deprivation experiment (manuscript in preparation).

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