Lymphotoxin β expression is high in chronic lymphocytic leukemia but low in small lymphocytic lymphoma: a quantitative real-time reverse transcriptase polymerase chain reaction analysis

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Background and Objectives. The lymphotoxin β (*LTB*) gene, localized to the major histocompatibility complex region on chromosome 6p21.3, has an important role in the formation of germinal center reactions and regulation of immune response and apoptosis. Our aim was to determine *LTB* gene expression in different hematologic neoplasias.

Design and Methods. We determined the expression of *LTB* using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) on a series of RNA samples from $CD3^+$T cells and $CD19^+$ B cells isolated from peripheral blood (n=7); $CD19^+$ B cells isolated from lymph nodes (n=11) and from patients with acute lymphoblastic leukemia (ALL; n=16), acute myeloid leukemia (AML; n=43), chronic myeloid leukemia (CML; n=12), mantle cell lymphoma (MCL; n=19), chronic lymphocytic leukemia (CLL; n=32) and small lymphocytic lymphoma (SLL; n=22).

Results. The expression level of *LTB* in $CD3^+$ T cells and $CD19^+$ B cells isolated from blood was ten to forty times lower than that in normal $CD19^+$ B cells isolated from lymph nodes. In malignant myeloid cells the expression levels were very low, whereas in malignant lymphoid cells the expression was higher than in myeloid cells, being highest in MCL and CLL (20.2±14.0 ng/μL and 81.0±116.3 ng/μL) and low in SLL (4.5±3.6 ng/μL; p=0.001). We did not find correlations between *LTB* expression and hematoclinical parameters (risk groups, immunophenotypes and overall survival).

Interpretation and Conclusions. A distinct difference in expression of *LTB* in CLL and SLL indicates that these morphologically similar B-cell malignancies are molecularly different. Further studies are needed to investigate the prognostic value of *LTB* and any role that *LTB* may have in determining whether the malignant B cells manifest a leukemia or lymphoma.

Key words: gene expression, hematologic neoplasia, lymphotoxin β.

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Lymphotoxin β (*LTB*), localized to chromosome 6p21.3 in the TNF gene cluster in the human central major histocompatibility complex region, has two isoforms and belongs to the tumor necrosis factor (TNF) ligand family.[1,2] It is a mediator of inflammatory and immune defense mechanisms, and a regulator of lymphoid organ development.[3,4] *LTB* is a type II transmembrane protein and signals through the *LTB* receptor.[5] Ware *et al.* showed that *LTB* and lymphotoxin α (*LTA*) are mainly produced by T and B lymphocytes, and by natural killer cells following cellular activation.[6] LTα monomers can form homotrimers and heterotrimers with *LTB*. The main *LTA-LTB* complex is the LTA_1-LTB_2 heterotrimer. Through the *LTB* receptor it signals to TRAF2, TRAF3, TRAF5 and other members of the TNFR-associated factors such as nuclear factor-κ and AP-1/JNK[1,2] and regulates interleukin (IL)-8 levels.[3,4,7,8]

TNF and lymphotoxins are involved in many regulatory activities.[5] At a molecular level *LTB* differs from two similar genes, *TNF* and *LTA*. The untranslated 3' region of the gene does not contain the TATT repeats known to play an important role in RNA stability.[4] Furthermore, the *SP1* site in this region is replaced by a related *EGR-1* site.[5,9-11] In the present work we analyzed the expression of *LTB* using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) on a series of 144 RNA samples from patients with different hematologic malignancies.

Design and Methods

Patients

The level of expression of LTB was measured by quantitative real-time RT-PCR on malignant cells of patients with chronic myeloid leukemia (n=12), chronic lymphocytic leukemia (CLL; n=32), acute myeloid leukemia (AML; n=43), acute lymphoblastic leukemia (ALL; n=16), mantle cell lymphoma (MCL; n=19), and small lymphocytic leukemia (SLL; n=22). The proportion of malignant cells was over 95%. Basic clinical data of the patients with leukemia or lymphoma are presented in Table 1. The WHO criteria were applied for neoplasia classification and only the diagnostically and prognostically relevant parameters are shown. All childhood ALL cases were of pre-B immunophenotype. The MCL and SLL cases were reviewed again by the same pathologist (KF), and differential diagnosis was based on cyclin D, CD5 and CD23 immunostainings. Only cyclin D positive and $CD23^-$ MCL cases, and $CD5^+$ and $CD23^+$ SLL cases were included in the study.

Table 1. Demographic and clinical data of the patients involved in the study.

Disease	*Patients (n)*	*Average age at diagnosis (years)*	*Karyotype*		*Risk group/FAB*		*Immunophenotype*	
Acute lymphoblastic leukemia (ALL)	16	5.0	Normal	1	HR	6	Pre-B	16
	(8M;8F)		Hyperdiploid	5	IR	6	CD34+	16
			t(12;21)	3			CD13+	10
			Other	7	SR	4	CD33+	10
Acute myeloid leukemia (AML)	43	47.6	Normal	18	M0	1	CD56+	15
	(20M/23F)		t(15;17)	1	M1	5	CD56-	23
			t(8;21)	7	M2	16	Not studied	5
			inv(16)	1	M3	3		
			Complex	2	M4	8		
			Other	14	M5	10		
Chronic myeloid leukemia (CML)	12	38.3	t(9;22)	10	Chr. Phase	12		
	(9M/3F)		t(9;22) variant	2	Blast. Phase	0		
Mantle cell lymphoma (MCL)	19	65.1	Not studied		IPI 1:	2	Cyclin D+	19
	(12M/7F)				IPI 2:	6	CD5+	18
					IPI 3:	5	CD23-	19
					IPI 4:	3		
					Unclassified	3		
Chronic lymphocytic leukemia (CLL)	32	64.8	Normal	12	Binet A:	13	CD5+	32
	(23M/9F)		+12	4	Binet B:	10	CD23+	32
			del 13q	4	Binet C:	9		
			del 11q	5				
			complex	7				
Small lymphocytic lymphoma (SLL)	22	61.8	Not studied				CD5+	20
	(16M/6F)						CD23+	22

According to the WHO classification, CLL and SLL are morphologically and immunophenotypically one and the same B-cell malignancy. Our patients were selected so that the disease in CLL cases appeared predominantly in circulating clonal B cells and in SLL cases in nodal cells. Unfortunately we were not able to study circulating cells in SLL, nor lymph node cells in CLL.

Source of malignant cells

Bone marrow aspirates (2 mL) from AML, ALL and CML patients were transferred into a stabilization reagent (Roche, Penzberg, Germany) and stored at -70°C for 6-12 months before RNA extraction. Peripheral mononuclear cells were separated from blood of CLL patients by Ficoll-Paque one-step density gradient centrifugation (Amersham Pharmacia Biotec, Uppsala, Sweden Country) and stored at -70°C for 2-8 months before the analysis. Lymph node biopsies from MCL and SLL patients were deep frozen (from 6 months to 5 years) prior to RNA extraction.

Controls

Normal $CD3^+$ and $CD19^+$ cells were isolated from peripheral blood of seven healthy volunteers (3M/4F) using Ficoll-Paque gradient centrifugation and microbeads conjugated to monoclonal CD3 and CD19 antibodies according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Futhermore, $CD19^+$ cells were isolated with the same kit from lymph node cells obtained from tonsillectomies of 11 children without cancer, and the cells were divided into two pools. The tonsillectomies were performed because of recurring otitis or other inflammation.

RNA isolation

RNA from the bone marrows of AML, ALL and CML patients was extracted using the mRNA isolation kit for blood/bone marrow (Boehringer Mannheim, Penzberg, Germany). Trizol Reagent (Gibco BRL, Grand Island, NY, USA) was used for RNA isolation from Ficoll-separated CLL lymphocytes, and the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) was employed for deep-frozen MCL and SLL tissue specimens and for the control samples.

Table 2. Expression of lymphotoxin β measured by quantitative real-time RT-PCR in different hematologic malignancies.

Specimens	*Number of samples (n)*	*Mean lymphotoxin concentration (ng/μL±SD)*	*The level of significance (p) compared to:*		
			CD3+ blood	*CD19+ blood*	*CD19+ lymph node*
CD3+ blood	7	8.1±10.4	–	0.337	0.04
CD19+ blood	7	2.2±3.0	0.337	–	0.04
CD19+ lymph node (pooled)	2 poles 11 pts	76.9±53.6	0.04	0.04	–
Chronic myeloid leukemia	12	0.02±0.02	0.128	0.001	0.028
Acute myeloid leukemia	43	0.09±0.2	0.071	0.001	0.018
Acute lymphoblastic leukemia	16	4.1±2.5	0.6	0.095	0.025
Chronic lymphocytic leukemia	32	81.0±116.3	0.004	0.001	0.421
Small lymphocytic lymphoma	22	4.5±3.6	0.721	0.067	0.022
Mantle cell lymphoma	19	20.2±14.0	0.014	0.001	0.042

Based on the kit suppliers' instructions as well as on our experience of lymphoma specimens, RNA extraction and cell suspension were performed using different kits.

DNase I treatment

DNase I (Boehringer Mannheim, Penzberg, Germany) enzyme was used to remove the genomic DNA contamination in the RNA preparations. The quality and integrity of RNA were checked by electrophoresis using 1% agarose gel with ethidium bromide staining and UV transillumination. RNA concentrations were measured using spectrophotometry at 260 nm.

Quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR)

Quantitative RT-PCR was performed on all samples to determine the expression of the lymphotoxin β gene (*LTB*) and one housekeeping gene, β-actin (*ACTB*) (Table 2). Complementary DNA (cDNA) was synthesized using 0.5 μg DNase-I-treated total RNA and a First Strand cDNA Synthesis kit for RT-PCR (Roche Diagnostics Corp., Indianapolis, IN, USA). Gene-specific primers were designed and synthesized by TIB MOLBIOL (Berlin, Germany) (forward: AggAgCCACTTCTCTggTgA and reverse: CTCTggCAgCTTCTgAAACC). Polymerase chain reactions were performed using a LightCycler (Roche). Each PCR consisted of 1 μL of DNA Master SYBR Green I mix (LightCycler FastStart DNA Master SYBR Green I kit, Roche; containing Taq polymerase, dNTP, $MgCl_2$, and SYBR Green I dye), 1 μL of cDNA and 2.5 pmol of primers. The amplification program included initial denaturation at 95°C with 8 min hold, followed by 50 cycles with denaturation at 95°C with 10 s hold, annealing at 65°C with 5 s hold and extension at 72°C with 10 s hold. Amplifications were followed by melting curve analysis using one cycle at 98°C with 0 s hold, 65°C with 10 s hold, and 95°C with 0 s hold in the acquisition step mode. A negative control without cDNA template was run simultaneously with every assay. The PCR from each cDNA sample was run in duplicate and the average was used in the calculations. Standard curves were obtained by using serial dilutions of the β-globulin gene (DNA Control kit, Roche) according to the supplier's instructions. The concentration of each gene product was determined on the basis of a kinetic approach using the LightCycler software (Roche). The levels of *ACTB* were used for normalization of RNA quantity and quality differences in all samples.

Gel electrophoresis of QRT-PCR products

Following QRT-PCR we isolated the PCR products from the glass capillaries by centrifugation. We then used agarose gel electrophoresis (1.5% agarose, 80 V, and 2 hours run) followed by ethidium bromide staining and visualized the DNA fragments with UV transillumination in each case.

Statistical analysis of data

Statistical analysis was performed using SPSS for Windows (Version 9.0), with the Mann-Whitney test for gene expression comparisons. The overall survival related to gene expression was analyzed by the Kaplan-Meier method.

Results

The *LTB* expression levels in different leukemias, lymphomas and controls are shown in Table 2. The expression in nodal cells was significantly higher than that in circulating B cells, whereas circulating B cells showed higher (but not significantly) expression than did circulating T cells.

Clonal B cells in CLL (p=0.004) and in MCL (0.014) had significantly higher *LTB* expression than did circulating B cells, whereas expression in these cells was equal to that in normal nodal B cells. Clonal cells in SLL showed significantly lower expression than did clonal cells in MCL and especially in CLL.

LTB expression was not associated with the following clinicopathologic parameters in any of the neoplasia subtypes (Table 2): age, sex, risk group in ALL, International Prognostic Index (IPI) in CLL, cytogenetic aberrations, CD56 immunophenotype in AML, CD13/33 in ALL, and deletion 11q and survival in MCL.

Discussion

Our analyses of *LTB* expression by real-time quantitative PCR showed first that the expression level of normal CD19+ B lymphocytes from lymph nodes was high being more than ten times higher than in normal CD3+ peripheral lymphocytes. Second, the expression was low in acute leukemias and in CML. Third, the average *LTB* expression in B cells of CLL was the same as in normal CD19+ lymph node cells and forty times higher than in circulating CD19+ cells isolated from blood. In MCL the expression was observed to be 50% lower than in normal nodal B cells. Fourth and intriguingly, the expression level of *LTB* in SLL was 20 times lower than that in CLL.

Our results fit well with the findings of previous studies that peripheral and lymph node B lymphocytes, leukemic B cells in CLL as well as some normal T-cell subsets express *LTB*.[12,13] However, a comparison of normal peripheral and nodal CD19+ B cells and CD3+ T cells has not been reported earlier. Furthermore, there are no previous reports indicating the lack of *LTB* expression in leukemic cells in AML and CML, or low expression in ALL and in MCL. *LTB* expression has been reported in one half of patients with non-Hodgkin's lymphoma[14] and high expression has been suggested to be associated with the severity of the lymphoma.[5]

CLL and SLL are considered to have the same morphologic and immunophenotypic features and to present the same disease (*WHO Classification: Tumours of Haematopoietic and Lymphoid Tissues*).[15] Thus our finding that the expression of *LTB* is very different is intriguing, giving evidence that CLL differs molecularly from SLL. Both CLL and SLL belong to the series of *mature* B-cell neoplasias.[16] Lymphocytes from both diseases express CD19, CD20, CD5 and CD 23 immunophenotype. However, differences have been reported in CD11a/CD18, *BCL-2*, *cyclin D2* and *MDM2* expression.[17-19]

Most cytogenetic changes, except deletions of 6q, 13q and 11q, seem to be the same in both CLL and SLL.[20] del(6q21-23) has been reported to be the most frequent abnormality in SLL and del(13q) less frequent in SLL than in CLL.[21-24] Our earlier studies demonstrated that del(11q) is common in CLL but rare in SLL.[25]

In the present study *LTB* expression was lower (although not statistically significantly so) in CLL patients with del(11q) than in those with normal chromosome 11. Our previous cDNA studies from the same patients gave similar results.[25] Other reported findings from del(11q) in CLL are that it is a poor prognostic sign and associates with the absence of hypermutation in the IGV region.[26] To test our hypotheses: 1) that hypermutation status in CLL differs from that in SLL; 2) that there is an association between del(11q), the absence of the hypermutation in the IGV region and expression of *LTB*; 3) that expression of LTB is a poor prognostic sign not only in CLL/SLL but also in MCL; and 4) that *LTB* expression plays an essential role in dispersing leukemic cells from germinal center to blood stream or homing them there, we are at present conducting gene expression, gene mutation and chromosome deletion analyses on CLL, SLL and MCL specimens.

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Pre-publication Report & Outcomes of Peer Review

Contributions

BN and SK designed the study, analyzed the data, interpreted and assessed the results, and drafted the manuscript. BN and AF performed the QRT-PCR. SG participated in the statistical analysis of data, creating the Kaplan Meier curves. MLL, YA, SC, JV, TR, KV, and KF were responsible for the clinical studies. All authors contributed to the intellectual content of the manuscript and approved its final version. SK as the senior author is cited last: he supervised the project and was responsible for its funding. Otherwise the order of names is based on time, work and scientific contribution given by the authors.

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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Terry Hamblin, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Hamblin and the Editors. Manuscript received November 11, 2002; accepted April 16, 2003.

In the following paragraphs, Professor Hamblin summarizes the peer-review process and its outcomes.

What is already known on this topic

Lymphocytotoxin β (*LTB*) is known to be expressed in peripheral blood and lymph node B cells, CLL cells and some normal T-cell subsets. About half of non-Hodgin's lymphomas express *LTB*, the level of expression being correlated with severity.

What this study adds

Using real-time PCR the authors have shown that *LTB* expresion is low in acute leukemias and CML, that the expression in normal lymph node B lymphocytes is ten times higher than in normal circulating T cells, and forty times higher than circulating B cells. In CLL cells the expression was the same as in normal lymph node B cells, whereas in mantle cell lymphoma the expression was only half this. The surprising finding was that the cells of small lymphocytic lymphoma, ostensibly the same disease as CLL, expressed only one twentieth the amount expressed by CLL cells.