BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 273–276 (1997)
ARTICLE NO. RC977277

Identification of Two Lymphotoxin Beta Isoforms Expressed in Human Lymphoid Cell Lines and Non-Hodgkin's Lymphomas[1]

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Received August 12, 1997

Two isoforms of lymphotoxin beta (LTβ) were isolated from mRNAs of a panel of human lymphoid cell lines and tumor tissues obtained from patients with non-Hodgkin's lymphoma (NHL). The truncated LTβ mRNA variant lacked 46 base pairs complementary to the complete sequence of exon 2, suggesting that both isoforms are produced by an alternative splicing mechanism. Skipping out of exon 2 causes a reading frame shift and a premature stop codon in the LTβ mRNA variant. The predictive translated polypeptide would correspond to a severely shortened LTβ protein that would lack the majority of the extracellular domain of the native molecule, thus impairing its normal complex assembly with LTα. These observations provide new insights into the molecular heterogeneity and biological function of LTβ within the tumor necrosis factor and LT ligand-receptor system.

Historically, TNF and LTα were grouped together as an apparently redundant pair of cytokines sharing identical receptors, TNF receptor type I (p55) and type II (p75). Both molecules are cytotoxic to a variety of tumor cell lines *in vitro* and can induce hemorrhagic necrosis of tumors *in vivo.* They also have a variety of immunomodulatory properties, including action on the proliferative state of a variety of cell types as well as antibacterial and antiviral responses (1). Recently, a family of ligands having structural homology to TNF and LTα has been elucidated at the molecular level. This family of proteins, implicated in the development and function of the immune system, includes also LTβ, TNF-related apoptosis-inducing ligand (TRAIL), and ligands for Fas, CD27, CD30, CD40, and murine/rat T-cell activation antigens (4-1BB and OX-40) (2).

The TNF-related ligands usually act in a membrane-bound manner, but after being shed by the cell membrane, also in their soluble forms (1,2). In contrast, LTα polypeptide lacking a transmembrane region is processed and released from cells in a manner characteristic for most secreted proteins, while its membrane form is associated with LTβ molecule (3). Membrane-associated heterotrimer formed by LTα and LTβ can exist in the 1:2 (LTα_1/LTβ_2) or 2:1 (LTα_2/LTβ_1) ratios (4). The primary surface LT form, which is expected to be a LTα_1/LTβ_2 heterotrimer, binds to a new receptor termed the LTβ receptor (LTβ-R). The LTβ-R is structurally related to both the p55 and p75 receptors yet does not recognize either TNF or LTα (5). However, both p55 and p75 receptors have been shown to bind the LTα_2/LTβ_1 heteromeric complex, but they are unable to interact with the predominant LTα_1/LTβ_2 complex (4,6).

The present study demonstrates that the human LTβ gene can be transcribed into two mRNA species, raising the possibility that the new isoform variant would encode a truncated LTβ protein which might function without being complexed with LTα.

MATERIALS AND METHODS

Samples. Five human cell lines were used as controls for LTβ gene expression, including promyelocytic leukemia (HL-60) cells, T-cell lymphoma (J-77), pre-B cell acute lymphoblastic leukemia (Nalm-6), Epstein-Barr virus-transformed B-cells (LAZ-388), and B-cell lymphoma (RL). In addition, 31 newly diagnosed NHL patients were evaluable for the study, including 29 patients with B-cell NHL and 2 with peripheral T-cell NHL.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA from cell line suspensions and lymph

[1] The nucleotide sequence data reported in this paper have been to the GenBank database under accession No. U89922.

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0006-291X/97 $25.00

node homogenates was isolated by a single-step method of acid guanidium thiocyanate-phenol-chloroform extraction. Extracted RNA, dissolved in diethylpyrocarbonate-saturated water, was subjected to a genomic DNA decontamination procedure in a final reaction volume of 25μl, consisting of 10 U RNase-free DNase I (Stratagene, La Jolla, CA 92037, USA), 25 U of RNasin (Promega, Madison, Wi, USA), 100mM $MgCl_2$, and 10mM dithiothreitol, performed for 15 minutes at 37°C. The reaction was stopped by adding an equal volume of DNase stop solution containing 50mM EDTA, 1.5M sodium acetate (pH 4), and 1% sodium dodecyl sulfate. Then, final phenol/chloroform/isoamyl alcohol (25/24/1) RNA extraction was performed. First strand cDNA synthesis was performed in a total volume of 20μl, consisting of 3μg of total RNA, 10pM oligo-dT (Gibco BRL), 200 U of recombinant Moloney murine leukemia virus reverse transcriptase and the recommended buffer (Gibco BRL), 500μM each dNTP (Gibco BRL), 7.5mM DTT, and 25 U of RNasin (Promega). The reaction mixture was incubated in a water bath at 37°C for 90 minutes, and then heated at 95°C for 3 minutes to inactivate the reverse transcriptase. Two microliters of cDNA were added to a final PCR reaction mixture of 50μl, containing 2.5 U of *Taq* polymerase and the recommended buffer (Gibco BRL), 5% of dimethyl sulfoxide, 1.5mM $MgCl_2$, 0.2mM deoxynucleotide triphosphates, and 25pM each of the specific forward and reverse primers. Two sets of LTβ-specific primers, designed to amplify partially overlapping fragments and spanning nucleotides (nt) 1 to 781 of the published LTβ mRNA sequence, were used: F_1 (5′CAGTCTCAATGGGGGCACTGG) with R_1 (5′GTAGCCGACGAGACAGTAGAG), and F_2 (5′CCGCAGGACGGCCTCTATTAC) with R_2 (5′CCAATATTCACGCACTCGCAC). The predicted sizes of the PCR-amplified products were 431 base pairs (bp) for the F_1-R_1 primers (nt 1 to 431), and 392 bp for the F_2-R_2 primers (nt 390 to 781). Each PCR amplification cycle (Perkin Elmer Cetus, Norwalk, CT, USA) consisted of a heat-denaturation (95°C for 1 minute), annealing (64°C for 1 minute), and extension step (72°C for 1 minute). After the last cycle, extension phase was prolonged for 9 minutes at 72°C.

Nucleotide sequence and protein structure analyses. For nucleotide sequencing, PCR products were isolated through a preparative agarose gel electrophoresis and subcloned into pGEM-T vector (Promega), and sequenced with the automated laser fluorescent sequencer (A.L.F., Pharmacia Biotech). Six clones for each fragment were sequenced in both directions to avoid errors resulting from the possible mutations caused by *Taq* polymerase. Nucleotide sequence alignements and the computation of protein characteristics were performed using BlastN and ProtParam tools of the National Center for Biotechnology Information and ExPASy servers, respectively.

RESULTS

Expression of LTβ gene in human cell lines and NHL tumor tissue. In order to define the widespread of LTβ gene's expression in NHL, we addressed the question of the presence of its transcript in tumor tissues obtained from newly diagnosed 31 NHL patients by means of RT-PCR assay. As a control, five different human cell lines (HL-60, J-77, Nalm-6, LAZ-388, and RL) were also tested. RT-PCR performed with the F_1-R_1 LTβ-specific primers showed two different-sized cDNAs, one of the predicted size (431 bp) and the other approximately 50 bp smaller. Both cDNAs were abundantly expressed in all lymphoid cell lines, but were negative in myeloid HL-60 cells (Figure 1a), and they were presented in all NHL samples examined (Figure 1b). The PCR-amplified product obtained by the use of F_2-R_2 LTβ-specific primers was of the expected size and shared identical expression pattern in the tested samples as cDNAs amplified by the use of F_1-R_1 primers (not shown). These results indicated that LTβ gene is broadly expressed in human lymphoid cell lines and tumor tissues obtained from NHL patients, and suggested the presence of two LTβ isoforms differing in their 5′ terminal nucleotides.

Nucleotide sequencing of two LTβ-related mRNAs. To define which cDNA represented a predicted 5′ termi-

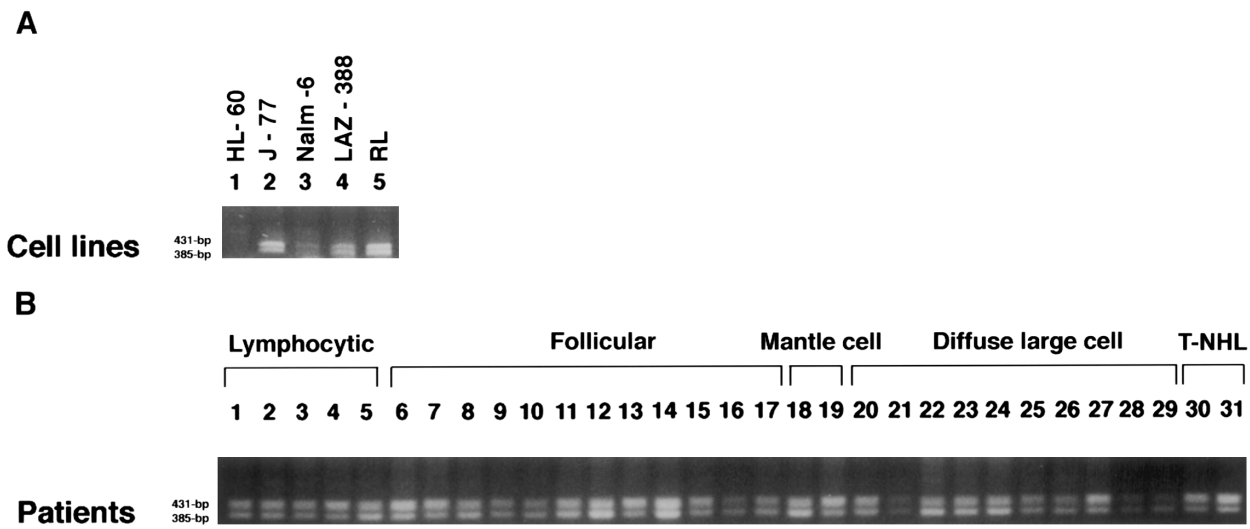

FIG. 1. RT-PCR analysis of the LTβ gene's expression in a panel of human cell lines representing myeloid-, T-, and B-cell lineages (A), including promyelocytic leukemia (HL-60) (lane 1), T-cell lymphoma (J-77) (lane 2), pre-B cell acute lymphoblastic leukemia (Nalm-6) (lane 3), Epstein-Barr virus-transformed B-cells (LAZ-388) (lane 4), and B-cell lymphoma (RL) (lane 5), and in tumor tissues obtained from 31 patients with NHL (B), including lymphocytic (lanes 1-5), follicular (lanes 6-17), mantle cell (lanes 18-19), diffuse large cell (lanes 20-29), and peripheral T-cell (lanes 30-31) NHL. The positions and sizes of PCR-amplified products are indicated on the left side of the figure.

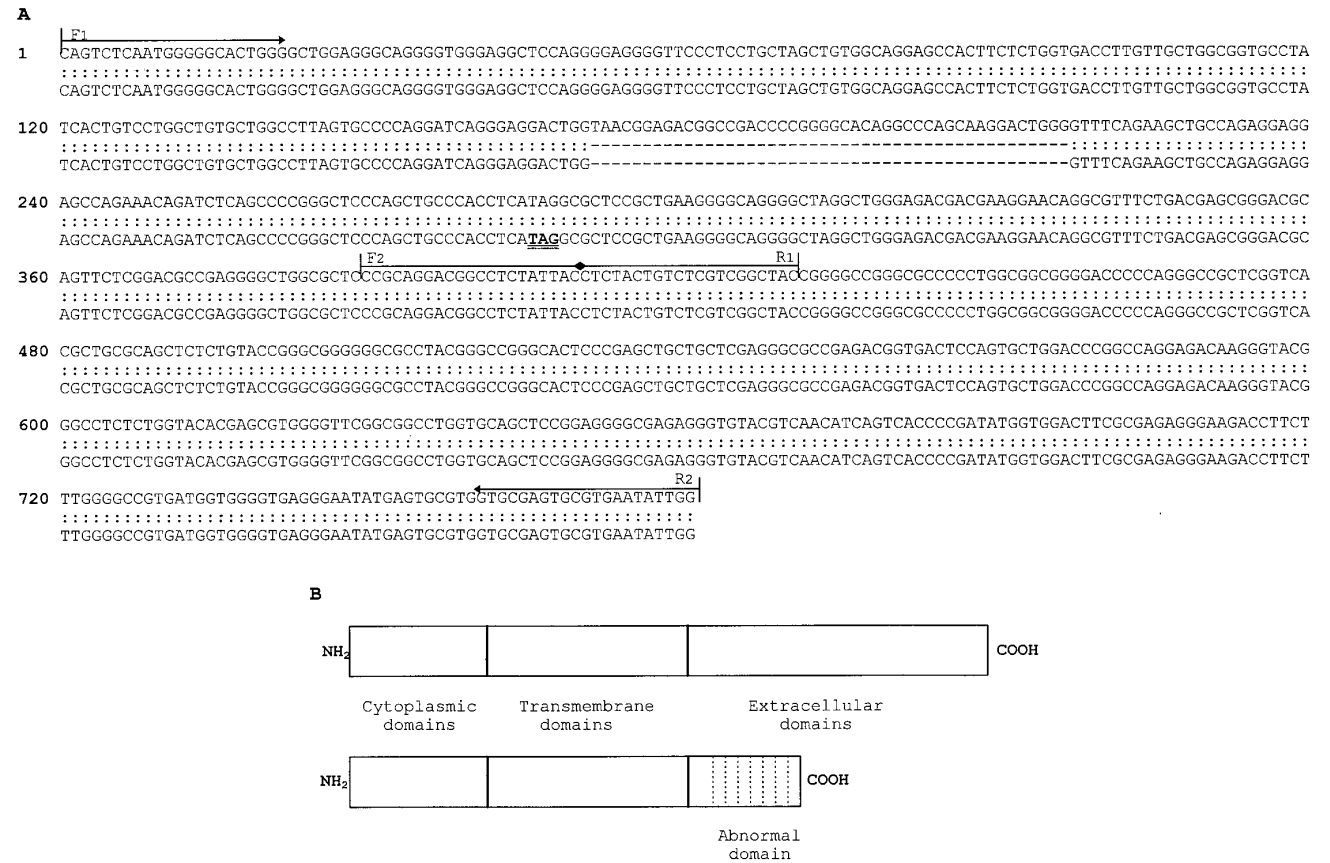

FIG. 2. Nucleotide sequence (A) and predicted protein structure (B) of the truncated form of human LTβ (GenBank accession number U89922). The nucleotide sequence of the truncated LTβ mRNA (Figure A, lower panel) is shown with the corresponding sequence of the native molecule (Figure A, upper panel) (Genbank, accession number L11015). Nucleotides' sequences marked by strikeout and bold double underline indicate the deleted sequence and premature stop codon in the truncated LTβ isoform, respectively. The arrows indicate the positions and directions of primers which were used for the PCR and cloning. Nucleotide numbers on the left side of the figure corresponds to the ordinary LTβ mRNA sequence.

nal LTβ cDNA sequence and what was the origin of the second band, we cloned and sequenced both fragments. LTβ 3′ terminal cDNA, amplified by the use of F_2-R_2 primers, was also cloned and sequenced. To avoid errors resulting from the possible mutations caused by *Taq* polymerase, six independent clones for each fragment were sequenced in both directions. Nucleotide sequencing showed that the larger-sized 5′ terminal cDNA corresponded to the predicted LTβ cDNA sequence (nt 1 to 431), whereas the smaller one lacked 46-bp. The sequence comparison showed that the deletion was localized at positions 171 to 216 of the published LTβ cDNA sequence, corresponding to a complete loss of exon 2 and alternative mRNA formation with exon 1 precisely abutted to exon 3 (3). LTβ 3′ terminal cDNA sequence was identical with the 3′ moiety of the ordinary sequence (nt 390 to 781) (Figure 2a). Several PCR, cloning, and sequencing experiments from distinct cell lines and patients' samples were performed, and each of them showed the presence of the same nucleotide sequences. These results indicate the presence of two LTβ isoforms in the human lymphoid cell lines and tumor tissues obtained from NHL patients. LTβ mRNA variant was determined to be truncated by a complete loss of nucleotides complementary to the exon 2 of genomic DNA. Skipping out of the exon 2 in the the truncated form of LTβ mRNA suggests that both isoforms are produced by an alternatively splicing mechanism.

Analysis of the truncated LTβ polypeptide. The sequences of the 5′ terminals of both LTβ isoforms had unique nucleotide sequences ranging from nt 1 to 170, but the smaller-sized LTβ lacks downstream 46-bp. Following these nt, the mRNA sequence of the latter matched perfectly with that of the native molecule. However, skipping out of the 46-bp in the smaller-sized LTβ mRNA causes a reading frame shift and a premature stop codon 70 nt downstream from the junction point (nt 286 to 288) (Figure 2a). The predictive translated polypeptide would correspond to a severely shortened LTβ protein consisting of 77 amino acids, with

unchanged cytoplasmic and membrane-anchoring domains. This polypeptide, ending with 24 different amino acids, would lack however the majority of extracellular domain which is present in an ordinary transmembrane form of LTβ (Figure 2b). These results suggest that although the potential transmembrane segment exists in the truncated LTβ variant, the 46-bp deletion of its coding mRNA is probably too drastic to allow normal complex assembly of the truncated LTβ variant with LTα or its recognition by antibodies specific for the ordinary LTβ epitopes.

DISCUSSION

In this study we isolated two LTβ isoforms from mRNAs of the human lymphoid cell lines and tumor tissues obtained from NHL patients. The truncated LTβ mRNA variant lacked 46-bp complementary to the complete sequence of exon 2, which suggests that both isoforms are produced by an alternatively splicing mechanism. Recent findings of genomic organization of the murine and human LTβ genes demonstrated that although sequence upstream of the transcription initiation sites are highly conserved between mouse and human LTβ genes, the donor splice site at the distal end of the presumptive exon 2 is absent in the murine gene. This suggested that human and murine LTβ genes may be differently spliced. The sequence analyses have confirmed that the murine LTβ gene contains three exons and encodes a 244-amino acid polypeptide with a 66-amino acid insert that is absent in the human homologue (3,7-9). The results of the present study indicate that mechanisms involved in the processing of LTβ mRNA are even more complex, confirming the general concept of more compound evolutionary events of LTβ as compared with TNF/LTα genomic loci.

The dramatic conversion of the LTβ aggregates into LTα/LTβ trimers when LTβ is co-expressed with LTα is interpreted as strong biochemical evidence favoring the heteromeric complex as the physiological LT ligand (10). Previous studies on cell surface LT were also consistent with a complex that was at least as large as the trimeric LTα_1/LTβ_2 structure (3,4,11). The results of the present study suggest that an open reading frame of the LTβ mRNA variant can code for a severely shortened LTβ protein that would lack the majority of the extracellular domain of the native molecule, thus impairing its complex assembly with LTα. Consequently, the possibility that this isoform would encode a protein which might function without being complexed with LTα is very intriguing. These data provide new insights into the molecular heterogeneity and biological function of LTβ within the TNF/LT ligand-receptor system.

ACKNOWLEDGMENTS

This work was supported by the Programme Hospitalier de Recherche Clinique, Paris (HCL PNRC Lyon-004). K.W. was supported by a Yamagiwa-Yoshida Memorial International Cancer Study Grant supported by the Olympus Optical Company and the Japan National Committee of the International Union Against Cancer (Geneva, Switzerland).

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