Expression of genes coding for the tumor necrosis factor and lymphotoxin ligand-receptor system in non-Hodgkin’s lymphomas

Abstract Excessive production of the tumor necrosis factor (TNF) ligand-receptor system has been found to contribute to the severity of non-Hodgkin’s lymphoma (NHL). We therefore investigated the expression of TNF, lymphotoxin α (LTα), lymphotoxin β (LTβ), and their receptor (p55, p75, LTβ-R) transcripts within the tumor tissue in different NHL histological subtypes. The constitutive expression of genes coding for TNF-related ligands and receptors was found in almost all 31 NHL samples studied. Semi-quantitative reverse transcription/polymerase chain reaction and computed densitometry assays revealed that the amounts of TNF, LTz, p55, and LTβ-R mRNA were higher in follicular NHL than in other histological entities. Therefore tumor cell immunopurification was performed in representative follicular NHL samples and consistent results were obtained. The pattern of LTβ gene expression was different from that of the other molecules, indicating the existence of distinct mechanisms of gene regulation. These results indicate that the transcription of genes coding for the TNF ligand-receptor system in NHL tumor tissue is more widespread than originally thought and that the heterogeneity of their expressions might be related to histological features. The expression of TNF-related ligands and receptors in tumor tissues is likely to contribute to the clinicopathological features of lymphoid-derived malignancies.

Key words TNF · Ligand · Receptor · Lymphocytes · Lymphoma

Introduction

Tumor necrosis factor α (TNF) and its functionally and structurally related partner, lymphotoxin α (LTα), are immunoregulatory cytokines produced primarily by monocytes, macrophages and activated T lymphocytes in response to various stimuli. Excessive production of these cytokines can be deleterious, mediating severe inflammatory reactions, tissue injury, and shock. Slowly released TNF and LTα are thought to contribute to fever, anemia, wasting and bone resorption in chronic diseases [26]. Elevated levels of TNF and LTα have been found in the plasma of the majority of patients with non-Hodgkin’s lymphoma (NHL) at the time of diagnosis, and are thereby associated with numerous adverse prognostic factors; they have also predicted shorter progression-free survival and overall survival [9, 20, 30].

LTz exists as a secreted molecule in a homotrimeric form but it may also accumulate on the cellular membrane when aggregated with LTβ, a type II transmembrane protein that belongs to the TNF ligand family [3]. LTβ signals exclusively through the LTβ receptor while TNF and LTz share the same cell-surface receptors, the TNF receptor type I or p55 and the TNF receptor type II or p75. These receptors are preferentially expressed on most cell types; p55 is constitutively expressed at low levels on all nucleated cells whereas p75 seems to be restricted to the hematopoietic lineage [21]. Both were found to be present at high levels in the sera of NHL patients. Interestingly, TNF and p75 values seemed to be associated with larger tumor burden while elevated p55 levels appeared to reflect the host response to tumor [30].

Earlier studies have suggested that tumor cells themselves are responsible for the excessive production of the ligands and receptors in the sera of patients [10] and that these cells have a greater tendency than non-malignant cells to produce and shed soluble forms...
of their cell-surface proteins making them difficult to detect [1].

Given the importance of the TNF/LT ligand receptor in the immune response and its putative contribution to the clinicopathological features of lymphoid-derived malignancies, we addressed the question of their gene expression in different NHL histological subtypes. We developed a semi-quantitative reverse transcription/polymerase chain reaction (RT-PCR) assay based on the coamplification of an internal standard not homologous to the cDNA target, in order to quantify the cytokine and the receptor gene transcripts in the tumor tissue and to be able to compare the expressions of the different molecules.

**Materials and methods**

**Patients**

A group of 31 newly diagnosed NHL patients were included in the study. The patients’ median age was 62 years (range, 22–86 years); 15 were female and 16 male. The patients inclusion criteria and initial medical examination were as previously described [30]. Detailed characteristics of the patients enrolled in this study are presented in Table 1. Patients’ lymph nodes, obtained before treatment initiation, were collected immediately after surgical removal and kept at the temperature of liquid nitrogen. The diagnosis was assessed by morphological and immunophenotyping analysis according to the Revised European American Lymphoma (REAL) classification.

**Cells**

The mononuclear cell fraction of a suspension of lymph node cells from 6 follicular NHL and 2 lymphocytic/lymphoplasmocytoid NHL patients was isolated by Ficoll-Hypaque (Gibco BRL, Grand Island, N.Y.) and E rosetting (BioMérieux, Marcy l’Etoile, France). The nonadherent cells were incubated with anti-CD3 (OKT3; American Type Culture Collection, Rockville, Md.) anti-CD4 and anti-CD16 monoclonal antibodies (mAb) (Immunotech, Marseille, France), then with magnetic beads (Dynabeads, Dynal, Oslo, Norway) coated with anti-mouse IgG. The isolated cells were analyzed by flow-cytometry (FACScan, Becton Dickinson, Mountain View, Calif.) after staining with fluorescein-isothiocyanate-conjugated CD3, CD14 (Immunotech) and CD19 (Dako, Glostrup Denmark) and phycoerythrin-conjugated goat anti-mouse immunoglobulin (Dako) slg and mAb light chains. All samples showed 98% cell purity and were monoclonal.

**RNA extraction and cDNA synthesis**

As a control for the expression of a given ligand/receptor gene, we used a panel of human cell lines representing B, T, and myeloid lineages, including B cell lymphoma (RL), Epstein-Barr-virus-transformed B cells (LAZ-388), pre-B-cell acute lymphoblastic leukemia (Nalm-6), T cell lymphoma (J-77), and promyelocytic leukemia (HL-60).

The total RNA from cell line suspensions, lymph node homogenates and purified cell suspensions, was isolated by a standard 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 25 μL, consisting of 10 μL RNase-free DNase I (Stratagene, La Jolla, Calif.), 25 μL RNasin (Promega, Madison, Wis.), 100 mM MgCl₂, and 10 mM dithiothreitol, performed for 15 min at 37 °C. The reaction was stopped by adding an equal volume of DNase stop solution containing 50 mM EDTA, 1.5 M sodium acetate (pH 4), and 1% sodium dodecyl sulfate. Then the final phenol/chloroform/isoamyl alcohol (25/24/1) RNA extraction was performed. First-strand cDNA synthesis was performed in a total volume of 20 μL, consisting of 3 μg total RNA, 200 U recombinant Moloney murine leukemia virus reverse transcriptase and the recommended buffer (Gibco BRL Life Technologies, Gaithersburg, Md.), 10 μM oligo (dT), 500 μM each dNTP (Gibco BRL), 7.5 mM dithiothreitol, and 25 U RNasin (Promega). The reaction mixture was incubated in a water bath at 37 °C for 90 min and then heated at 95 °C for 3 min to inactivate reverse transcriptase.

**Semiquantitative PCR**

Samples comprising 2 μL cDNA were added to a final PCR reaction mixture of 50 μL containing 2.5 U Taq polymerase and the recommended buffer (Gibco BRL), 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, and 25 pM each of the specific sense and antisense primers (Table 2). PCR for p55, p75, and LTβ were performed in the presence of 5% dimethylsulfoxide (Sigma Chemical Co, St Louis, Mo.). Specific PCR primers for each ligand and receptor were designed to span at least two exons to detect the amplification of any contaminating genomic DNA. To assess the specificity of PCR-amplified products, each of them was purified and digested.