Receptors for Tumor Necrosis Factor on Neoplastic B Cells From Chronic Lymphocytic Leukemia Are Expressed In Vitro but Not In Vivo

By Werner Digel, Wolfgang Schöninger, Martin Stefanic, Hans Janssen, Christian Buck, Mathias Schmid, Anand Raghavachar, and Franz Porzsolt

Recombinant tumor necrosis factor-α (TNF-α) is a cytokine that induces proliferation of neoplastic B cells from patients with chronic lymphocytic leukemia (CLL). To gain insight into the mechanisms involved in regulating TNF responsiveness, we have examined TNF receptor expression on neoplastic B-CLL cells. We have demonstrated that freshly isolated neoplastic B cells from patients with CLL did not express TNF receptors. After 1 day of incubation in culture medium, TNF receptors were detectable in the range of 540 to 1,500/cell. Kinetic experiments revealed that receptor expression was half-maximal after 3 hours of culturing and required de novo protein synthesis. The Scatchard plots of TNF-α binding indicated a single set of high-affinity TNF receptors with a dissociation constant of 70 pmol/L. TNF receptor expression in vitro was found in all examined cases. All cytokines tested, with the exception of IL-2, did not influence the expression of TNF receptors. The TNF receptor expression is enhanced in B-CLL cells cultured in the presence of interleukin-2 when compared with the receptor expression of cells cultured in medium alone. Our data suggest that neoplastic B-CLL cells in patients with stable disease do not express TNF receptors in vivo and that an unknown mechanism suppressing TNF receptor expression in vivo may play a role in growth regulation of neoplastic B cells.

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TUMOR necrosis factor-α (TNF-α) was first discovered in the serum of mice injected with bacille Calmette-Guérin and lipopolysaccharide and was found to be a product of activated macrophages and activated T cells. In addition to its antitumor effect in vivo, TNF exerts cytotoxicity on certain transformed cell lines in vitro. It has been shown that TNF-α initiates pleiotropic effects both on neoplastic and normal cells. TNF acts as a growth factor on fibroblasts, induces differentiation in myeloid cell lines, upregulates major histocompatibility complex class I and II gene expression, mediates the cytotoxicity of natural cytotoxic cells and macrophages, and has an antiviral activity. TNF-α confers its signal to target cells via binding to specific cell surface membrane receptors.

To determine the responsiveness of a given target cell, it is necessary to demonstrate that TNF-α membrane receptors are expressed, but cell responsiveness also depends on other factors. While it was long assumed that there is constitutive expression of TNF receptors in most tumor cells, it has recently been shown that regulatory mechanisms control the membrane expression of TNF receptors in normal peripheral lymphocytes. Despite a large number of studies dealing with the expression and regulation of specific high-affinity TNF binding sites on cell lines, there is little known about TNF membrane receptors on isolated tumor cells. Therefore, the present study was initiated to analyze TNF receptor expression on neoplastic B cells that proliferated in response to rTNF-α.

We report that the expression of TNF receptors in neoplastic B cells from patients with chronic lymphocytic leukemia (CLL) was poor when observed shortly after isolation. However, high-affinity TNF receptors were expressed in the course of B-cell incubation without specific activation.

MATERIALS AND METHODS

Patients. Peripheral blood was obtained by venipuncture from eight previously untreated patients with B-CLL. Diagnosis was based on typical clinical, morphologic, and immunologic features. Most patients were in a high leukemic phase of the disease (Table 1). The male to female ratio was 7:1.

Reagents. The cell culture media used was RPMI 1640 (Gibco, Karlsruhe, FRG) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, FRG). The FCS and the medium used were tested to determine whether they contained endotoxin and were both found to be negative. This test was performed by an interleukin-1 (IL-1) production assay with six human monocyte preparations (sensitivity <10 pg lipopolysaccharide Salmonella typhi 0911/mL. Cycloheximide (CHX) was obtained from Sigma (Munich, FRG). F(ab')2 fragments of goat antibodies specific for heavy chain of human IgM (anti-μ) were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). These antibodies were insolubilized with rabbit anti-goat immunoglobulin G (IgG) antibodies (Jackson Immunoresearch Laboratories). Highly purified rTNF-α was obtained from BASF/Knoll (Ludwigshafen, FRG). The specific activity of this rTNF-α was 2 x 10^7 U/mg protein (L 929 assay actinomycin D, 20 hours). The specific radioactivity of labeled rTNF-α was 24 μCi/μg. Recombinant interferon alpha (rIFN-α, 3.3 x 10^7 IU/mg) and interferon gamma (rIFN-γ, 2 x 10^7 IU/mg) were gifts from Boehringer (Ingelheim, FRG). Further interleukins were obtained from the following sources: rIL-1β (Genzyme, Boston, MA), rIL-2 (Dr H. Wagner, Department of Immunology, University of Ulm, FRG), rIL-3 (Behring, Marburg, FRG), rIL-4 (Genzyme), rIL-5 (Amersham, Braunschweig, FRG), rIL-6 (Dr H. Wagner), and rIL-7 (Immunex, Seattle, WA).

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Dedicated to Professor Dr H. Heimpel, Head of the Department of Internal Medicine III (Hematology and Oncology), University of Ulm, on the occasion of his 60th birthday.

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Dibutylphthalate and bis(2-ethylhexyl)phthalate were obtained from Fluka (Neu-Ulm, FRG).

Separation of cells. Blood was anticoagulated with heparin and, in some cases, with ethylenediaminetetraacetic acid. Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Pharmacia, Freiburg, FRG) density gradient centrifugation. Plastic adherent cells were removed by incubation of the cell suspension on culture petri dishes (Nunc, Wiesbaden, FRG) for 90 minutes at 37°C. The resulting cell population was T-cell depleted by rosetting for 60 minutes at 4°C with neuraminidase-treated sheep red blood cells. Nonrosetting cells were harvested from the interface of a second Ficoll gradient. Residual monocytes and T cells were determined by indirect immunofluorescence with MO2 (CD14) and OKT3 (CD3). All of the leukemic B-cell populations used in our experiments had less than 1% MO2- and OKT3-positive cells. Indirect immunofluorescence, culture conditions, proliferation assays, and radioiodination of rTNF-α were performed as described previously.20

Binding assay. Binding assays were performed in a total volume of 0.5 mL in duplicate, with 3 to 5 x 10⁶ cells/sample. Washed cells were incubated for 2 hours at 4°C with various concentrations of [125I]rTNF-α (8 to 750 pmol/mL) in RPMI 1640 containing 10% FCS. For determination of nonspecific binding, a 100-fold excess of unlabeled rTNF-α had been mixed with the labeled ligand before adding to the cells. After incubation totals were counted, the cells were subsequently centrifuged and washed three times with ice-cold RPMI 1640 containing 10% FCS. In some selected cases, incubation mixtures were transferred to precoated polyethylene centrifuge tubes containing 500 μL phthalate oil mixture (1.5 parts dibutylphthalate, 1 part bis(2-ethylhexyl)phthalate), and centrifuged for 1 minute in an Eppendorf microfuge. The supernatants containing unbound [125I]rTNF-α were removed, and the membrane-bound radioactivity in the pellet was counted. The data from saturation binding experiments were calculated with the Equilibrium Binding Data Analysis program (Elsevier-Biosoft, Cambridge, UK) on a Commodore PC AT computer (Commodore, Frankfurt, FRG).

Removing of membrane receptor-bound TNF. To reveal possible in vivo occupation of TNF receptors, B-CLL cells (14 mL; 4 x 10⁶ cells/mL) were layered on top of a discontinuous three-step gradient consisting of the following solutions: (1) 3.5 mL of culture medium supplemented with 20% Ficoll; (2) 21 mL of 100 mmol/L NaCl, 50 mmol/L glycine/HCL, pH 3.0, supplemented with 10% Ficoll; and (3) 3.5 mL of culture medium supplemented with 5% Ficoll.

The cells were centrifuged for 20 minutes at 500g, the solution was carefully aspirated, and the cells were washed and used for binding experiments. All steps were performed at 0°C to 4°C. In control experiments, the efficiency of this pH 3.0 wash was more than 90%; i.e., more than 90% of membrane receptor-bound [125I]rTNF-α could be dissociated from the cells.

Internalization and degradation of cell-bound rTNF-α. Neoplastic B cells (5 x 10⁶ cells/0.5 mL) were incubated with [125I]rTNF-α (370 pmol) for 2 hours at 4°C. The cells were washed three times with ice-cold RPMI 1640 medium containing 10% FCS. They were then shifted to 37°C by adding prewarmed medium and further incubated at 37°C. At different incubation times, culture fluids containing degraded [125I]rTNF-α were harvested and 200 μL 0.05 mol/L glycine buffer (pH 3.0) supplemented with 0.15 mol/L NaCl was added to the cells. Subsequently, the cells were centrifuged at 500g for 5 minutes at 4°C and the radioactivity in the supernatants containing receptor-bound [125I]rTNF-α was measured as well as the cell pellets containing internalized [125I]rTNF-α. Measured counts were plotted as the percentage of total binding.

Protein synthesis. For measurement of protein synthesis rates, neoplastic B cells (3 x 10⁶ cells/mL) were seeded in flat-bottom microtiter wells in a total volume of 200 μL. Cultures were incubated up to 96 hours and were pulsed with 1 μCi [3H]leucine (54 Ci/mmol, Amersham) 4 hours before cell harvesting. The further procedure has been described in detail by Okamoto and Mayer.22

Northern blot analysis. Total RNA was prepared using the LiCl/urea method.21 Poly A+ containing RNA was purified on oligo(dT)-cellulose.24 Poly A+ RNA before and after 4 hours' incubation of the cells were loaded and separated according to size on 1.0% formaldehyde gels. The fractionated RNA was transferred to nylon filters (Biodyne, Pall, Glen Cove, NY) that were exposed to [32P]dCTP (Amersham) 4 hours before cell harvesting. The further procedure has been described in detail by Okamoto and Mayer.22

Table 1. Clinical Data and Surface Marker Phenotypes of Patients Studied

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Rai Stage</th>
<th>White Blood Cell Count (10³/μL)</th>
<th>CD Immunophenotypes (%)</th>
<th>Surface Immunoglobulin (% of Positive Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>61</td>
<td>I</td>
<td>95</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>I</td>
<td>200</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
<td>I</td>
<td>334</td>
<td>3</td>
<td>1</td>
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<td>74</td>
<td>II</td>
<td>105</td>
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<td>III</td>
<td>321</td>
<td>9</td>
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<td>III</td>
<td>378</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
<td>III</td>
<td>399</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Patients no. 1, 2, 5, and 7 are patients no. 6, 3, 9, and 10 reported in our previous study.19

Abbreviation: NT, not tested.

Table 2. Number of TNF-Receptors on B-CLL Cells at Different Incubation Times

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of Receptors/Cell</th>
<th>Incubation Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;30</td>
<td>540</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>1,080</td>
</tr>
<tr>
<td>3</td>
<td>&lt;30</td>
<td>690</td>
</tr>
<tr>
<td>4</td>
<td>&lt;30</td>
<td>560</td>
</tr>
<tr>
<td>5</td>
<td>170</td>
<td>1,560</td>
</tr>
<tr>
<td>6</td>
<td>&lt;30</td>
<td>780</td>
</tr>
<tr>
<td>7</td>
<td>80</td>
<td>780</td>
</tr>
<tr>
<td>8</td>
<td>&lt;30</td>
<td>720</td>
</tr>
</tbody>
</table>

B-CLL cells were purified and binding studies were performed as described in Materials and Methods. The equilibrium dissociation constant (Kd) was approximately 70 pmol/L.

Abbreviation: NT, not tested.
human TNF-β (950 base-pairs EcoRI cDNA fragment cloned in pBR322),
human c-myc gene (ClaI-EcoRI fragment cloned in pBR322 containing the third exon),
and human c-fos gene (1.6 Kb EcoRI cDNA fragment cloned in pBR322) for 15 hours at 65°C.

Expression of TNF receptors, CLL

After washing, blots were exposed to x-ray films (Kodak X-omat S, Stuttgart, FRG) with an intensifying screen at -70°C for 1 to 4 days. Sizes of mRNAs were estimated from the positions of 28S (5.0 kb) and 18S (2.1 kb) bands in ethidium bromide-stained blot gels. A hamster β actin cDNA probe (pAct-I, a 1.25 Kb PstI fragment cloned in pBR322) was used as a control for the amount of RNA loaded on the gels.

RESULTS

Expression of TNF-α receptors after B-cell isolation.
As we have recently reported, neoplastic B cells from CLL patients proliferated in a dose-dependent manner in response to rTNF-α. Binding studies of iodinated rTNF-α to purified neoplastic B cells were performed as described in Materials and Methods. As shown in Table 2, freshly isolated B cells from eight patients did not express significant numbers of TNF receptors. After 1 day of incubation in culture medium, TNF receptors in the range of 540 to 1,560/cell were detectable (Table 2). MNCs from the same patients isolated at ice-cold temperatures also did not express significant numbers of TNF receptors. TNF receptor expression of cultured MNCs was comparable to cultured isolated B cells. Kinetic experiments revealed that receptor expression was half-maximal after 3 hours of culture (Fig 1). The Scatchard plots of TNF-α binding (Fig 2) indicated a single set of high-affinity TNF receptors with a dissociation constant (Kd) of approximately 70 pmol/L at each time point analyzed (Fig 2). In the course of further incubation, TNF receptor expression was maintained in all examined cases (Table 2). To detect a class of lower-affinity receptors, binding assays were performed in parallel in four of eight tested cases by three washes and centrifugation through an oil gradient. Low-affinity binding sites were not detectable on freshly isolated B cells or on incubated B cells. Otherwise, there was no difference in receptor expression of the high affinity between the two methods.

To address questions concerning receptors occupied by in vivo bound TNF on freshly isolated B cells, a possible membrane-associated ligand was removed by a pH 3.0 wash over a three-step gradient. Binding capacity was not recovered in these experiments, providing evidence that B-CLL cells expressed no, or only very few, receptors in vivo. LEAF binding sites were not detectable on freshly isolated B cells or on incubated B cells. Otherwise, there was no difference in receptor expression of the high affinity between the two methods.

Table 2. To detect a class of lower-affinity receptors, binding assays were performed in parallel in four of eight tested cases by three washes and centrifugation through an oil gradient. Low-affinity binding sites were not detectable on freshly isolated B cells or on incubated B cells. Otherwise, there was no difference in receptor expression of the high affinity between the two methods.
for TNF-α and TNF-β was found to be undetectable in the B-CLL cells from patients no. 7 and 8 (ie, both in primary cells and in cells incubated for 4 hours).

Binding of the 125I rTNF-α to cultured B cells was performed at 4°C and 37°C. Maximal specific binding was rapid within the 20 minutes maintained at 4°C and briefly declined approximately 15% at 37°C (data not presented). To study internalization, isolated neoplastic B cells were preincubated with saturating concentrations of 125I rTNF-α (570 pmol) for 2 hours at 4°C. Internalized, degraded, and bound 125I rTNF-α was determined as described in Materials and Methods. The internalization of surface-bound 125I rTNF-α was extremely rapid, and the degradation was half-maximal at 30 minutes and more than 90% complete by 60 minutes (Fig 4).

Expression of TNF receptors requires de novo protein synthesis. Binding studies of iodinated rTNF-α were performed in parallel in the presence or absence of CHX over a period of 24 hours. CHX treatment completely suspended the expression of TNF receptors (Fig 1), which indicates that in vitro expression of TNF receptors requires de novo protein synthesis. In two patients, we determined the turnover rate of TNF receptor protein and found a receptor half-life of 2.5 hours (Fig 5).

Influence of cytokines on the expression of TNF-α receptors. Isolated B-CLL cells were incubated for 20 hours with rIFN-α (100 IU/mL), rIFN-γ (10 IU/mL), or 10 U/mL of interleukin-1 to interleukin-7. After washing, the binding experiments indicated no enhancement in receptor expression of cells cultured with rIFN-α, rIFN-γ, rIL-1, rIL-3, rIL-4, rIL-5, rIL-6, or rIL-7. Only rIL-2 (10 U/mL) enhanced the TNF receptor expression when compared with neoplastic B cells cultured only with medium (Table 4).

Table 3. Protein Synthesis of Neoplastic B-CLL Cells During In Vitro Incubation

<table>
<thead>
<tr>
<th></th>
<th>2H Leucine Incorporation (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After Isolation 24 h 96 h</td>
</tr>
<tr>
<td>Medium alone</td>
<td>2,184 ± 442 2,569 ± 100 2,541 ± 224</td>
</tr>
<tr>
<td>Anti-μ</td>
<td>2,047 ± 303 3,701 ± 90 4,753 ± 577</td>
</tr>
</tbody>
</table>

Table 3. Protein Synthesis of Neoplastic B-CLL Cells During In Vitro Incubation

B-CLL cells from patient no. 5 were purified and incubated with and without anti-μ (10 μg/mL). Protein synthesis was measured as described in Materials and Methods. The data shown represent the cpm ± SE from triplicate wells.

DISCUSSION

Cell surface receptors for TNF have been previously characterized. These receptors are involved in the stimulation and down-regulation of cellular growth processes. We and others have demonstrated that neoplastic B cells from CLL proliferated in response to rTNF-α. The action of TNF-α requires binding to specific cell surface receptors. Thus, TNF sensitivity could be controlled at the level of receptor expression. On the other hand, control at a postreceptor level is also conceivable.

We have demonstrated that neoplastic B cells from CLL observed shortly after isolation did not express a significant number of TNF receptors. We therefore conclude that neoplastic B cells from CLL do not express a significant number of TNF binding sites in vivo. This assumption is supported by the fact that minimally processed unseparated MNCs also did not express TNF receptors in vitro. Moreover, we found no in vivo masking of possible TNF receptors by endogenous TNF.

In vitro culture of B-CLL cells in medium alone led to the...
Lymphokines produced by B-CLL cells in vitro may also regulate TNF-receptor expression. IFN-γ, a product of T cells, has been shown by other investigators to upregulate the number of TNF receptors, but incubation with IFN-γ did not change the "spontaneous" expression of TNF receptors in our study. According to our tests, IL-2 was the only lymphokine that enhanced TNF receptor expression. This is in accordance with the report of Owen-Schaub et al, who described regulation of lymphocyte TNF receptors by IL-2.

The expression of TNF receptors may possibly be regulated by a ligand up-regulation as described for IL-2.

However, Northern blot analysis indicated no expression of TNF-α mRNA in the tested cells. Hahn et al found increased spontaneous TNF production by malignant B cells from all stage 0 patients, but TNF was not detected in the supernatants of cultured B-CLL cells from higher stages, ie, the stages that were represented in this study. Unstimulated tonsillar B cells cultured in medium did not make TNF mRNA, whereas some established B-cell lines constitutively express TNF mRNA. Lindemann et al found TNF mRNA expression in hairy cell leukemia cells, but not in the three CLL cases tested.

The mechanisms involved in the in vitro induced expression of TNF receptors on B-CLL cells are unclear, but may include the loss of an in vivo inhibitor of TNF receptor expression. The failure to detect TNF receptors on minimally processed B-CLL cells does not exclude the possibility that TNF does act as an autocrine tumor growth factor for stable stages of chronic B-cell malignancies, if TNF acts in vivo internally with the cell without the full TNF receptor expression on the surface of the cells. However, the possibility of the in vitro expression of TNF receptors may suggest that TNF receptors are also inducible in vivo under special circumstances and that TNF may then interact with their receptor.

After incubation in vitro, TNF receptor expression was found in all cases examined. The concentration of rTNF-α producing maximal [3H]-Tdr incorporation (ie, 25 ng/mL [500 pmol/L]) turned out to be in the range of receptor saturation. TNF binding sites on B-CLL cells had a high binding affinity. This binding affinity is comparable to that described for TNF receptors on other human leukemia cells. Since receptor numbers are mean values of one cell population, we cannot exclude the possibility that a subpopulation has high numbers of binding sites while the majority of cells express only few receptors.

In conclusion, we show the induction of high-affinity TNF receptors on neoplastic B cells without activation. This regulatory mechanism of TNF receptor expression may play an important role in the growth regulation of tumor cells such as B-CLL cells, either directly or by TNF-linked induction of growth factors. Previous reports concerning TNF receptor expression of cultured tumor cells and their relevance in vivo may now be discussed with respect to this view.

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Table 4. Number of TNF Receptors on B-CLL Cells After Incubation With rIL-2

<table>
<thead>
<tr>
<th>Patient</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>rIL-2</td>
</tr>
<tr>
<td>1</td>
<td>524</td>
<td>662</td>
</tr>
<tr>
<td>3</td>
<td>265</td>
<td>391</td>
</tr>
<tr>
<td>5</td>
<td>1,264</td>
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<td>782</td>
</tr>
<tr>
<td>8</td>
<td>1,264</td>
<td>1,564</td>
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B-CLL cells were purified and binding studies were performed after incubation with and without rIL-2 (10 IU/mL) for 24 and 48 hours as described in Materials and Methods.
REFERENCES


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Receptors for tumor necrosis factor on neoplastic B cells from chronic lymphocytic leukemia are expressed in vitro but not in vivo

W Digel, W Schoniger, M Stefanic, H Janssen, C Buck, M Schmid, A Raghavachar and F Porzsolt