Expression and Functional Role of Tumor Necrosis Factor Receptors on Leukemic Cells From Patients With Type B Chronic Lymphoproliferative Disorders

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Two receptors for tumor necrosis factor (TNF) with different molecular weight (75-Kd and 55-Kd) and binding affinity have been recently discovered. To investigate the distribution and the functional role of these receptors on leukemic B cells from hairy cell leukemia (HCL) and B-cell chronic lymphocytic leukemia (B-CLL), patients, we evaluated: (1) the cytofluorimetric pattern of uncultured and cultured leukemic B cells incubated with utr-1 and htr-9 monoclonal antibodies (MoAbs), which specifically recognize the 75-Kd and 55-Kd TNF receptors (TNFR), respectively; (2) the effect of TNF-α and TNF-β on leukemic B cells in an in vitro proliferation assay; (3) the role of anti-TNF MoAbs on TNF-α and TNF-β-driven B-cell growth; and (4) the proliferative effect of utr-1 and htr-9 MoAbs on in vitro cultured leukemic cells. Our study shows that the high affinity (75-Kd) but not the low affinity (55-Kd) TNFR molecules are expressed on freshly isolated leukemic B cells recovered from HCL and B-CLL patients. The expression of these receptors was neither upregulated nor downregulated by different stimuli, including TNF-α, TNF-β, B-cell growth factor, and interleukin-2. TNF-α efficiently triggers the proliferation of HC and, to a lesser extent, the growth of B-CLL cells. TNF-β was also able to transduce the proliferative signal in HCL, but not in B-CLL patients. TNF-α and TNF-β-driven B-cell proliferation was inhibited by the preincubation of leukemic B cells with utr-1 but not htr-9 MoAb. Moreover, anti-75-Kd, but not anti-55-Kd TNFR MoAbs, was able to trigger the proliferation of leukemic B cells, and in particular of HC. These results show that leukemic B cells from patients with HCL and B-CLL are equipped with a fully functional high affinity TNFR.

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SEVERAL CYTOKINES have been shown to regulate the proliferation of normal and leukemic B cells. In particular, tumor necrosis factor-α (TNF-α) is capable of inducing in vitro the growth of leukemic B cells obtained from patients with hairy cell leukemia (HCL) and B-cell chronic lymphocytic leukemia (B-CLL).1,2,13,14 Two chronic lymphoproliferative disorders of B-cell lineage whose cells are “frozen” at different stages of differentiation.2,15 The observations that leukemic B cells from both HCL and B-CLL patients proliferate in response to TNF-α and that they constitutionally produce this cytokine suggested an autocrine model of cell growth in vivo.2,13 The evidence of increased levels of TNF-α in the serum of these patients4,16 further supports the hypothesis that this cytokine plays a role in the control of neoplastic growth in these disorders. Another cytokine, called TNF-β or lymphotoxin, which shows structural and functional homologies with TNF-α,10 and which is also produced by activated B cells and B-cell lines,11,12 is able to induce the proliferation of preactivated normal B cells.13,14 Nevertheless, little information is available on the effect of this cytokine on leukemic B cells from patients with B-chronic lymphoproliferative disorders.15 The mechanisms by which TNF-α promotes the growth of leukemic cells are not still clarified. Two TNF receptors have been recently cloned and functionally characterized on different cell systems: a 75-Kd (type A) receptor binding TNF-α with high affinity and a 55-Kd (type B) receptor binding TNF-α with low affinity.16-24 It is also likely that TNF-β transduces the activation signal through TNF receptors.25 Recently, monoclonal antibodies (MoAbs)14 that recognize the 75-Kd and 55-Kd TNF receptors have been produced, thus permitting the direct analysis of the distribution and the biologic role of these receptors among different cell types. Resting normal B cells and freshly isolated leukemic B cells from patients with B-CLL have been reported to be devoid of TNF receptor (TNFR).26-28 The aim of this study was to provide new insights into the mechanisms involved in TNF-α- and TNF-β-driven proliferation of leukemic B cells through the evaluation of the expression of the above quoted TNF receptors on freshly isolated and cultured leukemic B cells from HCL and B-CLL patients. Furthermore, neoplastic cells were cultured in the presence of TNF-α and TNF-β after preincubation with anti-TNF MoAbs. The proliferative effect of anti-TNF MoAbs on neoplastic B cells was also investigated.

MATERIALS AND METHODS

Patients. Seventeen patients with chronic lymphoproliferative disorders were studied. Seven cases (5 men and 2 women, aged from 45 to 62 years) suffered from HCL and 10 patients (6 men and 4 women, aged from 46 to 67 years) had B-CLL. The diagnosis was established on the basis of typical clinical, morphologic, histologic, and immunologic features. All HCL patients had the leukemic form of the disease with white blood cell (WBC) counts ranging from 2,300 to 13,250/mm³. B-CLL patients were graded according to Rai’s staging system29 as follows: stage I (5 cases), stage II (3 cases), stage III (2 cases). The total lymphocyte count ranged from 14,400 to 32,840/mm³. All patients were studied at diagnosis, before any therapy.
Cell separation and B-cell enrichment. Peripheral blood lymphocytes (PBL) from patients were obtained from freshly heparinized blood by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. Control B cells were derived from the spleen of normal subjects who experienced a posttraumatic splenectomy. After mechanical disruption, spleen cells were centrifuged through a Ficoll-Hypaque gradient. The cells were then washed three times with phosphate-buffered saline (PBS) and were resuspended in RPMI 1640 medium (GIBCO, Paisie, Scotland).

The samples from patients under study and normal splenocytes were enriched for B lymphocytes using a modification of the method previously described. After removal of E-rosetting cells by the neuraminidase (Sigma Chemical Co, St Louis, MO) treated sheep red blood cell (RBC) rosetting technique, the cell suspension was depleted of adherent cells by incubation for 45 minutes in plastic Petri dishes at 37°C in an atmosphere of 95% air and 5% CO2. According to the method of Lee et al.,31 cells were further purified by removing CD3+ and CD66+ lymphocytes using magnetic microspheres coated with antiserum IgG (Dyneahains, Dynal, Norway), as previously described in detail.32 Briefly, after incubation (45 minutes at 4°C) of the cell suspension obtained as above with CD3 (OKT3; Ortho, Raritan, NJ) and CD66 (Leu 19; Becton Dickinson, Sunnyvale, CA) MoAbs, 40 x 10^6 beads were incubated with 10 x 10^6 cells/mL for 30 minutes at 4°C under continuous slow rotation. The CD3+ and CD66+ cells rosetting with antibody-coated beads were then isolated and removed applying a magnetic system on the outer wall of the test tubes for 2 minutes. Following this multistep negative selection procedure, more than 96% of the cells were viable as judged with trypan blue exclusion test and were found to be positive for trtarate-resistant acid phosphatase and for staining with CD19, CD1 IC (SHCL3/LeuM5), and CD5 MoAbs in HCL patients, for CD19 and CD5 MoAbs in B-CLL subjects, and for CD19 MoAb in normal subjects.

Reagents. TNF-α and TNF-β antibodies were purchased from Genzyme (Cambridge, MA). Recombinant interleukin-2 (IL-2) was obtained from Biogen (Cambridge, MA). Polyclonal rabbit anti-human TNF-α and TNF-β antibodies were purchased from Genzyme Corp (Cambridge, MA). These reagents have been proven in vitro to block the activity of TNF-α and TNF-β, respectively. Low molecular weight B-cell growth factor (LMW-BCGF) was purchased from Cellular Products Inc (Buffalo, NY). Phycoerythrin (PE)-conjugated TNF-a protein was kindly provided from the Knoll AG/BASF (Ludwigshafen, Germany), the TNF-α with specific activity of 10 X 10^10 cpms/mg from Biogen (Cambridge, MA). Polyclonal rabbit anti-human TNF-α and TNF-β antibodies (purchased from Genzyme Corp) were used. These latter reagents have been proven to block in vitro the activity of TNF-α and TNF-β, respectively. Proliferation was determined by pulsing plates with 1 µCi/well of [3H]-Thymidine (CEA Ire Sorin, Saluggia, Italy) for the last 12 hours of culture; cells were then harvested and [3H]-Thymidine incorporation measured in a β-scintillation counter. Results are expressed as stimulation index (SI) according to this formula: mean cpm stimulated cells/mean cpm cultured cells in medium alone.

To verify whether anti-TNFR MoAbs mimic the effect of these cytokines, leukemic B cells recovered from three HCL patients and three B-CLL subjects were also cultured in the presence of utr-1 and htr-9 MoAbs only, at the concentration of 0.05 and 5.0 µg/mL. Results are expressed as SI according to this formula: mean cpm stimulated cells/mean cpm cultured cells in medium alone.

RESULTS

Expression of TNF receptors on normal B cells at resting conditions. Flow cytometry analysis of the expression of TNF receptors on unseparated PBL samples is reported in Fig 1. Panels A through C show the overlayed histograms of the control, utr-1, and htr-9 MoAbs on CD19+ gated normal B-cell population in two representative patients (one HCL patient; Fig 1A; and one B-CLL patient; Fig 1B) and on normal B cells from a control subject (Fig 1C). Although the
percentage of positive cells was calculated to range from 14.5% to 63%, the histograms of 75-Kd TNFR, recognized by utr-1, in leukemic B cells were totally shifted as compared with the control histogram. This indicates that the whole population of leukemic B cells expresses the 75-Kd TNFR, although to different degrees of density. By contrast, freshly isolated B cells (Fig 1C) from control subjects were not able to bind the utr-1 MoAb, thus indicating that the 75-Kd TNFR is lacking on normal B cells. The histograms of leukemic and normal B cells stained with htr-9 MoAb recognizing the 55-Kd (low affinity) TNFR were not shifted with respect to the control, thus indicating that this MoAb works as a control MoAb. Because utr-1 but not htr-9 MoAbs bind leukemic B cells, blocking experiments of PE-TNF-a binding with these antibodies were performed. Unseparated PBL were treated with these antibodies either individually or in combination and successively stained with PE-TNF-a and anti-CD19-FITC MoAb. Figure 1 shows data for TNF-a staining of CD19+ gated PBL from two representative cases (Fig 1D: one HCL patient; Fig 1E: one B-CLL subject) demonstrating that utr-1 MoAb blocked the binding of TNF-a to leukemic B cells, whereas htr-9 MoAb did not affect the binding of TNF-a to these cells. The histogram obtained following the block with utr-1 and htr-9 MoAbs was superimposable to that obtained with utr-1 alone (data not shown). Normal B cells show a pattern that is consistent with the results of the binding of utr-1 and htr-9 MoAbs (Fig 1F). Superimposable results were obtained on separated and purified leukemic and normal B cells.

Biologic effects of TNF-α and TNF-β on leukemic B cells. To evaluate the functional properties of TNF-α and TNF-β in HCL and B-CLL patients, leukemic B cells were cultured in the presence of these cytokines (Fig 2). When the cells were cultured with different concentrations of TNF-α (Fig 2A), a proliferative response was observed both in HCL patients and to a lesser extent in B-CLL subjects. Because TNF-α slightly induced B-CLL leukemic cells to proliferate, a dose-response curve of B-CLL proliferation after TNF-α stimulation was performed in four patients and the data are reported in Table 1, which shows an individual variability in terms of proliferation. To test the effect of TNF-β on leukemic B cells, neoplastic B cells were cultured in the presence of different concentrations of this cytokine. As illustrated in Fig 2B, TNF-β induced a significant ³H-Thymidine incorporation by HC but not by leukemic B cells recovered from B-CLL patients. TNF-α- and TNF-β--induced proliferations of leukemic B cells were highly inhibited by anti-TNF-α and anti-TNF-β MoAbs. In particular, anti-TNF-α antibody showed an inhibitory effect ranging from 46% to 72% on TNF-α-cultured HC, while anti-TNF-β antibody exhibited an inhibitory effect ranging from 68% to 79% on HC cultured in the presence of TNF-β.

Binding of anti-TNFR antibodies to leukemic B cells after in vitro triggering with different cytokines. To investigate the possibility that different cytokines can modulate TNFR expression, the positivity for utr-1 and htr-9 MoAbs was assessed on leukemic and normal B cells after their culture in the presence of cytokines that have been shown in vitro to
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Fig 2. Proliferative effect of TNF-α and TNF-β on leukemic B cells in patients with HCL and B-CLL in the presence of different concentrations of TNF-α and TNF-β (A and B, respectively). Data are expressed as stimulation index ± SE. The 3H-Tdr incorporation of leukemic B cells cultured in medium alone ranged from 284 to 568 cpm in HCL patients and from 213 to 951 in B-CLL patients.

stimulate the growth of leukemic B cells, including TNF-α, TNF-β, LMW-BCGF, and IL-2. Leukemic B cells cultured in the presence of TNF-α and TNF-β for 3 days did not show any modification of utr-1 and htr-9 histograms with respect to the cells cultured in medium alone and to freshly obtained cells. A similar pattern was also observed in normal B cells. Moreover, no significant variation of the binding of anti-TNFR antibodies to leukemic B cells was observed after culture with TNF-α and TNF-β for 24 and 48 hours (data not shown). The binding of anti-TNFR MoAbs was also evaluated after culturing neoplastic cells in the presence of LMW-BCGF and IL-2. As illustrated in Fig 3, leukemic B cells cultured in the presence of LMW-BCGF and IL-2 did not show any change in the binding of utr-1 and htr-9 MoAbs with respect to the cells cultured in medium alone. Also, normal B cells did not demonstrate any shift in the binding of utr-1 and htr-9 MoAbs following incubation with IL-2 and different batches of LMW-BCGF. Only the combination of anti-IgM and LMW-BCGF was able to induce the expression of the 75-Kd TNFR; the 55-Kd TNFR was not expressed even in these conditions.

Effects of anti-TNFR antibodies on leukemic cells. To determine whether utr-1 and htr-9 antibodies block or mimic the biologic effects induced by TNF-α and TNF-β, leukemic B cells were cultured in the presence of different concentrations of the above quoted cytokines and MoAbs. The anti-TNFR utr-1 MoAb was able to significantly reduce the proliferation of leukemic B cells both to TNF-α and TNF-β in HCL patients tested (Fig 4A). Similar results were obtained in B-CLL patients when leukemic B cells were cultured in the presence of TNF-α (Fig 4A), whereas little inhibitory effect was observed on B-CLL leukemic lymphocytes cultured in the presence of TNF-β. This effect was probably caused by the low proliferative rate of leukemic B-CLL cells in response to this latter cytokine. htr-9 MoAb did not inhibit the proliferation of leukemic cells induced by both cytokines (Fig 4B).

To evaluate whether these antibodies show activity on their recognized receptor, 3H-Thymidine incorporation assays were also assessed after culturing leukemic B in the presence of different concentrations of utr-1 and htr-9 MoAbs (Fig 5). utr-1 has been observed to trigger the proliferation of leukemic HC and to a very low rate the proliferation of B-CLL neoplastic lymphocytes, while htr-9 did not trigger the proliferation of HC and B-CLL leukemic cells.

DISCUSSION

Our study shows that freshly isolated leukemic B cells from HCL and B-CLL patients react with anti-75-Kd TNFR MoAb that recognizes the high-affinity TNFR and lack the low-affinity 55-Kd TNFR. The expression of these receptors was neither upregulated nor downregulated by incubating leukemic cells with TNF-α, TNF-β, IL-2, and LMW-BCGF. Functional in vitro studies showed that leukemic B cells, and notably HC, proliferate not only in the presence of TNF-α, but also in the presence of TNF-β. The TNF-α– and TNF-β–driven proliferation of neoplastic B cells was dramatically inhibited by anti–75-Kd TNFR MoAb, but not by anti–55-Kd TNFR; the only utr-1 MoAb has been observed to trigger the proliferation of leukemic B cells, and in particular of HC. The evidence herein provided strengthens the hypothesis that this cytokine might act as an autocrine factor in these disorders.

The demonstration that leukemic cells from HCL and B-CLL patients bind utr-1 MoAb but not htr-9 MoAb coupled to the evidence that the TNF-α binding is blocked by utr-1 MoAb but not by the htr-9 MoAb indicates that leukemic B cells from these patients discretely express the high-affinity
75-Kd TNFR. Our observations that the 75-Kd TNFR is expressed not only on in vitro activated leukemic cells but also on unseparated PBL as well as on freshly isolated lymphocytes are not consistent with the findings reported by other groups who failed to demonstrate this receptor on "resting" leukemic B cells. Differences in methods used, patient disease stage, and/or previous therapies are likely to account for these discrepancies. Moreover, using immunohistologic techniques the constitutive expression of the 75-Kd high-affinity TNFR on leukemic B cells has been recently reported in high-grade B-cell non-Hodgkin's lymphoma. Undetectable or low amounts of the 75-Kd TNFR have been detected on freshly isolated normal B cells and on tissue normal B cells, while its upregulation was observed on stimulation of anti-μ or Sac. Whether the 75-Kd TNFR plays a definite role in the biology of cells under study or it represents an abnormally expressed receptor structure on these leukemic cells that might be regarded as a prognostic marker remains to be clarified. As far as the low-affinity TNFR, the inability of HCL and B-CLL to bind htr-9 MoAb indicates that this receptor is lacking, thus suggesting that the two TNFR are not co-expressed on these neoplastic cells, neither at resting conditions nor following in vitro stimulation. These data are in agreement with the findings reported by Ryffel et al who demonstrated that in normal lymphoid tissues TNFR are present on the surface membrane of different cell types, with the 75-Kd TNFR being preferentially expressed in T-cell areas while the 55-Kd TNFR being commonly located in the germinal center.

The observation that several cytokines, including TNF-α and LMW-BCGF, can be involved in the regulation of leukemic cell growth but were unable to regulate (up or down) the expression of TNF receptors indicates that they do not transduce signals closely related to the modulation of these receptors. Because leukemic B cells from HCL and B-CLL patients express the low-affinity IL-2 receptor and because IL-2 is able to transduce a proliferative signal, we analyzed the ability of leukemic B cells to bind utr-1 and htr-9 MoAbs also on IL-2 incubation. Results indicate that the CD25 molecule and its binding to IL-2 does not affect the regulation of TNFR expression on patients' leukemic cells.

To investigate the biologic role of TNF receptors in leukemic B cells, proliferative assays were performed in the presence of different concentrations of TNF-α, TNF-β, and anti-TNFR MoAbs. Our data extend the previous observations related to TNF-α demonstrating that TNF-β is successful in triggering the proliferation of leukemic B cells and in particular HCL, thus indicating that, in addition to TNF-α, TNF-β might also represent a growth factor for leukemic HC. The proliferative effect of TNF-β we observed on leukemic HC is not consistent with the data reported by Buck et al, who failed to demonstrate a TNF-β-induced HCL growth. Possible explanations for these discrepancies might lie on the heterogeneity of the two study populations. In this regard, all tests in our patients were performed before therapy, while the majority of the patients reported by Buck et al received previous treatment. Further validation of our findings comes from tests performed in the presence of TNF-β and anti-TNF-β antibodies showing that the proliferation was actually related to the effect of this cytokine (data not shown).

The evidence that the block of the 75-Kd TNFR by utr-1 MoAb inhibited both TNF-α- and TNF-β-induced leukemic B-cell growth substantiates the concept that this receptor is constitutively expressed on freshly isolated leukemic B cells from B-CLL and HCL patients and indicates that the 75-Kd TNFR is the biologically active receptorial structure that regulates both TNF-α and TNF-β growth of HC and further points to the role of this molecule. The demonstration that the TNF-β-induced proliferation is inhibited by the utr-1 MoAb indicates that lymphotoxin transduces the proliferative signal through the 75-Kd TNFR in a similar way to TNF-α.
and is consistent with the concept that TNF-α and TNF-β bind to the same receptors. The evidence herein provided that in HCL patients the utr-1 MoAb is also able to mimic the proliferative effect of TNF-α through the high-affinity TNFR indicates that this MoAb recognizes the structure(s) of a TNF receptor that is(are) involved in signal transduction in these cells.

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