Brief Definitive Report

Transforming Growth Factor β as Endogenous Growth Inhibitor of Chronic Lymphocytic Leukemia B Cells

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Summary

Chronic lymphocytic leukemia (CLL) B cells are hyporesponsive or refractory to mitogens and growth factors in vitro. This study examined whether transforming growth factor β (TGF-β), a potent inhibitor of lymphocyte proliferation may play a role in the growth regulation of CLL B cells. CLL B cells from all donors treated expressed detectable TGF-β1 mRNA. In vitro release of TGF-β by unstimulated cultures, or cultures stimulated by antibody to cell surface immunoglobulin (anti-μ) plus phorbol 12-myristate 13-acetate (PMA) was higher in CLL than in normal B cells. High levels of TGF-β activity were also detected in plasma samples of CLL patients. The role of TGF-β in growth regulation of CLL B cells was tested in assays using different B cell activators. Purified neoplastic B cells from most CLL patients proliferated in response to anti-μ, or the combination of anti-μ plus PMA. Levels of CLL B cell proliferation were lower than observed in normal B cells. Some CLL were refractory to these stimuli. Antibody to CD40 induced proliferation of CLL B cells from all donors tested when presented on FcγRII (CDw32)-expressing L cells. Neutralizing antibodies to TGF-β increased CLL B cell proliferation in the absence or presence of additional stimuli. These effects were dose dependent and specific. Exogenous TGF-β completely inhibited CLL B cell proliferation induced by anti-μ, PMA, and anti-TGF-β. CLL B cell proliferation induced by anti-CD40 was reduced by exogenous TGF-β. However, even at high doses, TGF-β did not completely inhibit the anti-CD40 effect. In summary, TGF-β is overexpressed in CLL. CLL B cells are sensitive to TGF-β and this cytokine functions as an autocrine growth inhibitor accounting at least in part for reduced proliferative responses of these leukemic cells and for the slow progression of the malignant process in vivo.

Tumor cells are often resistant to signals that inhibit growth of their normal cell counterparts. Escape from such control mechanisms may contribute to malignant transformation. Proliferation of normal B cells is controlled in part by the endogenous production of growth inhibitors. TGF-β is a potent immunosuppressive factor and inhibitor of B cell proliferation (1). Normal B cells express TGF-β and neutralization of endogenous TGF-β results in enhanced B cell responses. Chronic lymphocytic leukemia (CLL) B cells are characterized by a very small percentage of peripheral blood and bone marrow cells in S phase (2). In vitro proliferation of CLL B cells is heterogeneous but generally characterized by hyporesponsiveness to stimulation with mitogens, Epstein-Barr virus (EBV) or cytokines (3). Low levels of CLL B cell proliferation can be induced by combinations of some mitogens or cytokines, such as IL-2, TNF, and IL-7 (4–7). Antibody to CD40 recently has been shown to stimulate proliferation in CLL B cells from most patients (8). These studies suggest that the hyporesponsiveness of CLL B cells may be the consequence of regulatory events rather than irreversible changes that are characteristic of other transformed cell types. However, regulatory pathways that may be responsible for the anergy of CLL B cells have not been identified. Furthermore, it is not clear whether this leukemic cell type is sensitive to the growth inhibitory effects of TGF-β (9). The objective of the present study was to analyze the potential contribution of TGF-β to the hyporesponsiveness of CLL B cells.

Materials and Methods

Cells. Peripheral blood samples were obtained from 18 patients fulfilling diagnostic and immunophenotypic criteria for common B cell CLL at the University of California at San Diego, Medical Center, the Veteran's Administration Hospital or the Scripps Clinic and Research Foundation (all in La Jolla, CA) (10–14). The median age of the patients was 66, ranging from 59 to 77 yr. The patients were heterogeneous with respect to clinical stage (ranging from Rai stage 0 to stage IV) and prior therapy for CLL. 14 (78%) of the patients were male, reflecting the known 2:1 male/female prevalence of common CLL (15).

Direct immunofluorescence analyses of the mononuclear cells

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from each subject substantiated the diagnosis of B cell CLL, indicating that >90% (median, 99%) of the lymphocytes in each sample coexpressed pan-B cell surface antigens, CD5, and κ or λ light chains (not shown). Of the 18 leukemia cell populations studied, 8 (44%) expressed Ig κ light chains and 10 (56%) expressed λ light chains. Normal B cells were isolated from peripheral blood of healthy volunteers.

Proliferation Assays. Proliferation studies were performed in 96-well flat bottom plates with 50,000 cells/well in RPMI 1640 supplemented with 5% FBS, L-glutamine, and antibiotics. Cells were pulsed with [^{3}H]thymidine (1 μCi/well) during the final 18 h of a 5-d culture and collected on an automated cell harvester. Radioactivity incorporated by the cells was quantified by liquid scintillation counting. Experiments on B cell responses to anti-CD40 were performed in 96-well, flat-bottom plates in RPMI 1640 supplemented with 10% FBS, L-glutamine, and antibiotics. 10^4 CDw32-L cells (kindly provided by Dr. Kevin Moore, DNAX, Palo Alto, CA) and 10^5 CLL-B cells per well were added with anti-CD40 mAb G28-5 at various concentrations with and without recombinant human IL-4 as described (16). The cells were pulsed with 1 μCi/well [^{3}H]thymidine for the final 18 h of a 5-d culture.

The results obtained with [^{3}H]thymidine incorporation were confirmed in studies where TCR-precipitated DNA from cell cultures was quantified.

Monocyte and T cell depletion were performed by incubation of PBMC with antibodies to CD3 (OKT3) and CD11c (LeuM5). Monocytes and T cells were then removed with goat anti-mouse antibody coupled to magnetic beads.

TGF-β Assays. TGF-β content in CLL plasma and conditioned media was quantified in the CLL64 and the lymphocyte activating factor assays as described (17).

Antibodies and Cytokines. Anti-TGF-β, rabbit or mouse and species-specific control Ab, and recombinant human TGF-β1 (R&D Systems Inc., Minneapolis, MN); goat anti-human IgM F(ab)$_2$ Ab and control F(ab)$_2$ (Cappel Research Products, Durham, NC); anti-CD40 mAb G28-5 (18) was a gift from Dr. Edward Clark, University of Washington, Seattle, WA; MOPC 21 (murine IgG$_1$; Caltag, Inc., South San Francisco, CA); recombinant human IL-4 was purchased from Biosource International (Camarillo, CA).

Results

TGF-β Production In Vitro and Levels in Plasma. TGF-β expression by CLL B cells was analyzed in the presence and absence of in vitro stimulation. Most of the TGF-β activity secreted by normal or CLL B cells was in latent form. The mean levels of TGF-β released from unstimulated CLL B cell preparations were approximately twofold higher as compared with normal B cells. The combination of anti-μ plus PMA increased TGF-β activity 2.8-fold in normal and 4.4-fold in CLL B cells (Fig. 1). TGF-β1 mRNA was present in high levels in all CLL B cell preparations and there were no detectable differences between the 10 patients that were tested (not shown). These results suggest that CLL B cells release more TGF-β than normal B cells and confirm a recent study (9).

Analysis of plasma showed that all of the 12 CLL samples contained > 1 ng/ml of TGF-β. Most of the TGF-β activity was in latent form but active TGF-β was detectable in 10/12 patients. Plasma samples from 20 normal volunteers contained a mean level of 1.3 ng/ml of total TGF-β activity.

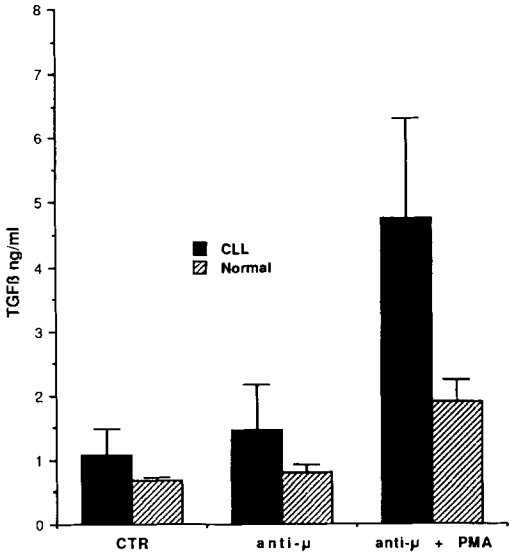

Figure 1. TGF-β production by CLL B cells. TGF was tested in 24-h conditioned media from CLL (n = 8) and normal (n = 5) B cells that were either unstimulated (control) or stimulated with anti-μ or anti-μ plus PMA. Samples were tested after transient acidification in the CCL64 assay.

Neutralization of TGF-β Activity Causes Increased CLL B Cell Proliferation. CLL B cell proliferation induced by antibody to cell surface immunoglobulin (anti-μ) alone or in combination with PMA was reduced as compared with normal B cells (Fig. 2 *A*). B cells from some CLL patients were refractory to stimulation with combinations of these agents (Fig. 2 *E*). To test whether TGF-β is involved with the reduced proliferative responses of CLL B cells, we used antibodies that neutralize the biological activity of TGF-β. These antibodies increased CLL B cell proliferation. In some cases, proliferation was increased in the absence of other stimuli (Fig. 2 *B*), while in most CLL B cell populations the presence of anti-μ and or PMA was required (Fig. 2, *C* and *D*). CLL B cells that did not proliferate in response to anti-μ and PMA also failed to respond when anti-TGF-β was included (Fig. 2 *E*). The TGF-β antibody effects were dose dependent and specific. Stimulation was observed with antibodies from three different species (rabbit, chicken, and mouse) and the increases in CLL proliferation were directly related to their neutralizing titers. CLL B cells prepared from the different patients showed one of these four patterns of in vitro proliferative responses as shown in Fig. 2, *B–E*. These patterns of CLL B cell responses to stimulation with mitogens of antibodies to TGF-β did not correlate with the clinical stage of the patients (data not shown). To determine whether the anti-TGF-β effects were indeed on the CLL B cells, we stained the cell populations for the expression of idiotypes that characterize the CLL B cell clones. These experiments showed that after a 5-d culture in the presence of antibody to TGF-β the cell populations were >97% idiotype positive.

CLL B Cell Proliferation Induced by Anti-CD40. All CLL B cells tested in the present study proliferated to anti-CD40

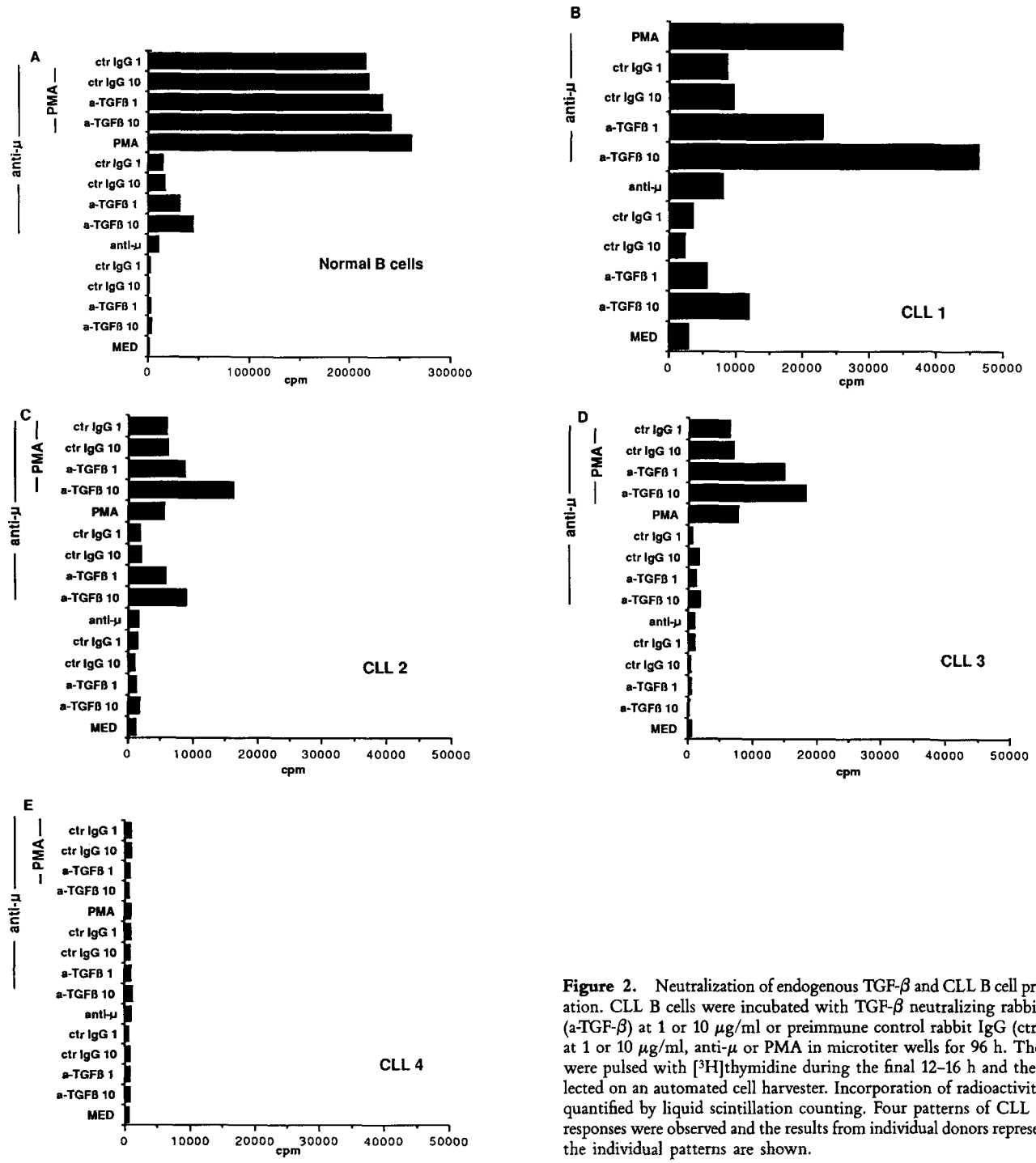

Figure 2. Neutralization of endogenous TGF-β and CLL B cell proliferation. CLL B cells were incubated with TGF-β neutralizing rabbit IgG (a-TGF-β) at 1 or 10 μg/ml or preimmune control rabbit IgG (ctr IgG) at 1 or 10 μg/ml, anti-μ or PMA in microtiter wells for 96 h. The cells were pulsed with [^{3}H]thymidine during the final 12–16 h and then collected on an automated cell harvester. Incorporation of radioactivity was quantified by liquid scintillation counting. Four patterns of CLL B cell responses were observed and the results from individual donors representing the individual patterns are shown.

presented on L cells expressing the human FcγRII (CD32) in the absence or presence of IL-4. The magnitude of proliferation induced by anti-CD40 was greater than that induced by the other stimuli. Even those CLL B cells that did not respond to the other stimuli (CLL4) were induced to proliferate (Fig. 3). This group of CLL B cells also responded to TGF-β antibodies in combination with suboptimal concentrations of anti-CD40 (Fig. 3). These findings suggest that all CLL B cell populations respond with increased proliferation to neutralizing antibody to TGF-β despite their heterogeneity in responsiveness to anti-μ and PMA.

Inhibition of CLL B Cell Proliferation by Exogenous TGF-β. Results obtained from studies with TGF-β neutralizing antibody indirectly suggested that CLL B cells can be growth inhibited by TGF-β. To test TGF-β responsiveness of CLL B cells directly, cells were stimulated with anti-TGF-β or anti-μ plus anti-TGF-β or PMA and cultured in the presence of exogenous TGF-β. Recombinant human TGF-

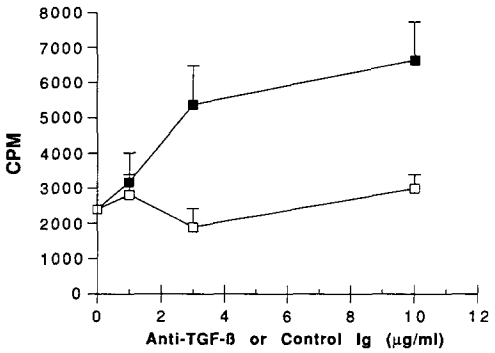

Figure 3. Neutralizing antibodies to TGF-β augment anti-CD40–induced proliferation of CLL B cells. 10^5 CLL-B cells were cultured on a monolayer of CDw32-L cells in the presence of 1 μg/ml anti-CD40 mAb and 10 ng/ml rhIL-4 and increasing concentrations of rabbit anti-human TGF-β mAb (*closed squares*) or control rabbit Ig (*open squares*) were added at the initiation of cultures as indicated on the abscissa. Proliferation was measured during the final 18 h of a 5-d culture as cpm of incorporated [^{3}H]thymidine. Data points, means of triplicate wells, error bars, SD.

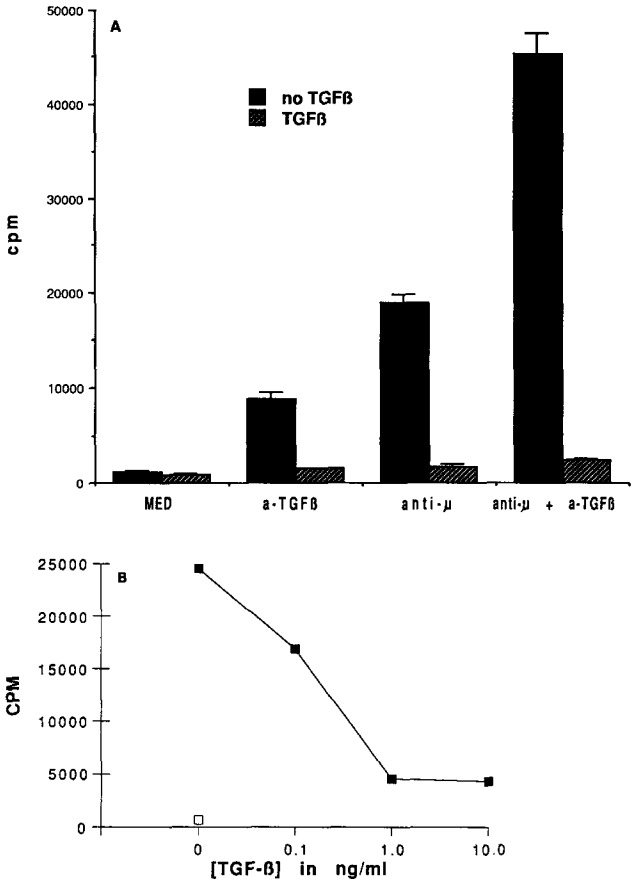

Figure 4. Exogenous TGF-β and CLL B cell proliferation. (*A*) Exogenous TGF-β completely inhibits proliferation induced by anti-μ, PMA, and anti-TGF-β. CLL B cells were stimulated with antibodies to TGF-β in the presence or absence to anti-μ. Recombinant TGF-β1 (10 ng/ml) was added at the initiation of a 5-d culture. (*B*) Exogenous TGF-β does not completely inhibit anti-CD40–induced proliferation of CLL B cells. 10^5 CLL B cells were cultured on a monolayer of CDw32-L cells in the presence of 1 μg/ml MOPC 21 control murine IgG (*open squares*) or anti-CD40 mAb (*closed squares*). Increasing concentrations of human TGF-β1 were added at the initiation of cultures as indicated on the abscissa. Proliferation was measured during the final 18 h of a 5-d culture as counts per minute of incorporated [^{3}H]thymidine. Data points are means of triplicate wells. The proliferation measured at all concentrations of exogenous TGF-β plus anti-CD40 is significantly higher than that observed in the presence of control mAb and CDw32-L cells ($p \leqslant 0.03$).

β1 completely blocked the stimulatory effects of anti-TGF-β antibody as well as proliferation induced by anti-μ or anti-μ plus PMA (Fig. 4) with an ED_{50} between 0.1 and 1 ng/ml. Anti-CD40 induced proliferation also is reduced by exogenous TGF-β in all cases (Fig. 4 *B*). However, even at high concentrations, TGF-β cannot completely inhibit proliferation induced by CD40 cross-linking. In experiments with optimal concentrations of anti-CD40 in the absence or presence of IL-4, we observed an average 52.9% (±24.7 SD) inhibition with maximal doses of TGF-β1 (10–20 ng/ml). Complete inhibition by TGF-β was not observed in any of 12 separate experiments using cells from 6 different CLL patients.

Discussion

The present study shows that CLL B cells produce TGF-β in vitro and that plasma samples from CLL patients contain increased levels of TGF-β. B cells from all CLL patients tested in this study were sensitive to growth inhibition by TGF-β. Endogenously produced TGF-β serves as an autocrine growth inhibitor of CLL B cells. Increased proliferation was induced by antibody to TGF-β in all cases of CLL B cells.

In vitro proliferative responses of CLL B cells from the different patients in the present series were heterogeneous as observed in most previous studies (19, 20). Heterogeneity was observed in the responses to stimulation with anti-μ and PMA. CLL B cells from some donors responded to a single stimulus whereas others proliferated only when treated with several stimuli. The effect of CD40 cross-linking was qualitatively uniform. CLL B cells from all donors tested responded to anti-CD40. The magnitude of the anti-CD40 response was greater than the effect of the other stimuli, and varied among the different patients, but the levels of the responses to anti-CD40 and the other stimuli correlated.

TGF-β appears to function as an endogenous growth inhibitor in CLL, since neutralizing antibodies increased proliferation in all cases. In some cases TGF-β-neutralizing antibodies caused increased proliferation in the absence of other added stimuli, suggesting that these CLL B cells are sufficiently activated to enter S-phase and that this is prevented by endogenous TGF-β. This is in contrast to normal B cells where antibodies to TGF-β do not increase proliferation in the absence of mitogens. In other cases of CLL anti-TGF-β augmented the reduced proliferative responses to mitogens and growth factors, suggesting that, in the context of appropriate stimulation, in vivo proliferative responses are reduced by TGF-β. There were no apparent correlations between the magnitude of the proliferative responses, the response patterns to anti-TGF-β and clinical variables in a retrospective

analysis of the results. However, a prospective analysis would be more appropriate to formally address this question.

The results obtained with antibodies to TGF-β suggested that these leukemic B cells are sensitive to the antiproliferative effects of this cytokine. Direct evidence for this was obtained in studies where proliferation induced by anti-μ or anti-μ and PMA was completely inhibited by exogenous TGF-β.

The proliferative response to anti-CD40 also was reduced in a dose-dependent fashion. However, complete inhibition was never observed. Even in the presence of the highest concentrations of TGF-β there was significant residual proliferation. This partial resistance of the anti-CD40 proliferative effect is consistent with a recent study that showed that anti-CD40 can override the TGF-β inhibition of CD23 expression (21). As CLL B cell proliferation induced by stimuli other than CD40 cross-linking is inhibited by TGF-β, a factor expressed in CLL, it is conceivable that stimulation through CD40 may be the primary mechanism driving expansion of these cells in vivo. Cross-linking of CD40 induces phenotypic changes on CLL B cells in vitro which are not seen on leukemic B cells isolated from peripheral blood of CLL patients (16). Identification of anatomical sites other than blood where CD40 ligand is expressed as well as the precise location where CLL B cells proliferate in vivo will advance this hypothesis.

The present results indicate that TGF-β is present at increased circulating levels in CLL patients and that this may originate from the tumor cells. Patients with this leukemia have signs of impaired immune function as indicated by defective delayed-type hypersensitivity reactions, autoimmune phenomena, and an increased frequency of secondary malignancies. It is possible that the increased levels of TGF-β contribute to these defects and also may impair the immune responses to these tumor cells. Overexpression of TGF-β in other conditions such as glioblastoma, adult T cell leukemia (ATL) (22), and HIV infection (23) has been suggested to lead to immunosuppression.

The findings from the present study provide new insight into the pathogenesis of CLL and define a negative autocrine circuit that is responsible for some of the functional characteristics of CLL B cells in vitro and may influence the course of the disease process in vivo.

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