Apoptosis and interleukin 7 gene expression in chronic B-lymphocytic leukemia cells

(mRNA/programmed cell death/B-cell differentiation/integrin)

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ABSTRACT mRNA for interleukin 7 (IL-7) was readily detected in leukemic cells immediately upon their removal from patients with chronic B-lymphocytic leukemia (B-CLL). IL-7 mRNA expression and IL-7 gene transcription were down regulated, however, when B-CLL cells were placed in culture at 37°C for 4 hr. Down regulation of the IL-7 gene was prevented in cells maintained at 4°C. Continued culture of B-CLL cells at 37°C resulted in programed cell death, or apoptosis, as evidenced by DNA fragmentation. The coincident kinetics of IL-7 gene down regulation and apoptosis suggested that IL-7 gene expression may be required for maintenance of CLL viability in vivo. Signals for IL-7 gene regulation and apoptosis induction were thus examined. Activation of normal B cells through their immunoglobulin receptors did not result in upregulation of IL-7 gene expression. Reagents required for CLL cell purification and culture also did not contribute to IL-7 gene regulation and apoptosis induction. IL-7 gene expression was retained and apoptosis was prevented, however, in CLL cells cultured on a monolayer of EA.hy926 human umbilical cord endothelial hybrid cells. Signals specifically presented by EA.hy926 cells supported both CLL cell viability and IL-7 gene expression, whereas culture of CLL cells on A549/8 carcinoma cells, the fusion partner used to generate the EA.hy926 cells, did not. Cell-cell contact was required, as culture supernatants did not prevent apoptosis. Specifically, IL-7 mRNA expression was retained and apoptosis was prevented only by contact with the endothelial cell hybrids. Preliminary data indicated that integrins expressed on CLL cells effected modulation of apoptosis and IL-7 gene regulation, suggesting that integrins may play significant roles in regulating viability of CLL cells.

Neoplastic cells from chronic lymphocytic leukemia (CLL) patients present an enigma in that the majority of these cells are nonproliferating yet large numbers of leukemic cells accumulate within patients. However, when removed and placed in culture, these cells fail to proliferate and tend to undergo "programmed cell death," referred to as apoptosis (1-3). Various theories have been put forth to explain the lack of leukemic cell cycling in CLL. One possibility is that a leukemic "stem cell" from an early stage of B-cell differentiation serves as the source of leukemic cells. The majority of daughter cells, however, continue to differentiate and then become blocked in an activated state and are prevented from passage into final stages of differentiation, including apoptosis. The mature leukemic cells accumulate, then, rather than continue to proliferate in a logarithmic expansion of the "early-stage" stem cells. Another theory is that autoantigens serve as cyclic triggers for the neoplastic clones (4-6). Alternatively, a burst of cytokines produced during episodes of inflammatory responses may serve as growth stimuli for the leukemic cells in a sporadic manner. Finally, endogenously produced cytokines may regulate CLL cell growth (7-12). To investigate the latter hypothesis, we have asked whether interleukin 7 (IL-7) may be involved in the regulation of CLL cell viability in vivo and death in vitro (13-15).

Our studies have demonstrated that CLL cells contain mRNA for the lymphoid stem-cell growth factor IL-7. Yet, in our hands, the majority of patients' CLL cells do not respond to this growth signal in vitro. Other features attributed to IL-7 activation, however, are evident in CLL patients, including upregulation of IL-2 (Tac) receptors which are detected in their serum (16, 17). IL-7 also upregulates IL-2 receptor (IL-2R) expression in normal cells (18, 19). We demonstrated IL-2R expression in the leukemic subset of cells isolated from most CLL patients (13, 14). Binding studies with flouochrome-labeled IL-7 showed that a subset of CLL cells expressed receptors for IL-7 (13). Here we demonstrate that CLL cells express IL-7 protein in their cytoplasm. We also examined the kinetics of IL-7 gene regulation in CLL cells, as well as apoptosis in vitro and signals that result in salvage from apoptosis. Our findings support the hypothesis that the neoplastic cells in CLL are blocked between a stage of activation and apoptosis. We demonstrate that regulation of both apoptosis and IL-7 gene expression can be mediated by cell-cell contact with endothelial cells. The regulation of the apoptotic pathway via integrins results in perpetuating CLL cell viability and IL-7 gene expression.

MATERIALS AND METHODS

Cell Sources. Blood from leukemia patients was obtained through the Hematology Service at Rush-Presbyterian St. Luke’s Medical Center. Normal donor cells were purchased asuffy coats through Lifesource (Northbrook, IL). Mononuclear cells were isolated as described (13, 14). The human umbilical vein endothelial cell hybrid line EA.hy926 and its hypoxanthine phosphoribosyltransferase-deficient fusion partner A549/8 were obtained from C.-J. S. Edgell (University of North Carolina, Chapel Hill) (20). The Epstein–Barr virus (EBV)-transformed B-cell lines were established by G.N.A.

Isolation of RNA, cDNA Synthesis, Reverse Transcription (RT), and Polymerase Chain Reaction (PCR). cDNA was synthesized from total RNA isolated by dissolving CLL cell pellets in RNAzol B (Test Tec, Friendsworth, TX). RT–PCR was performed as described (13, 14). Primers were purchased from Clontech or custom synthesized (Molecular Biotechnol-

Abbreviations: CLL, chronic lymphocytic leukemia; EBV, Epstein–Barr virus; IL, interleukin; IL-2R, IL-2 receptor; PLL, prolymphocytic leukemia; RT, reverse transcription.

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IL-7 mRNA detected in CLL and PLL patients' mononuclear cells but not in healthy donor cells by RT-PCR. PCR products are evident at 681 and 550 bp for the CLL and PLL cells (lanes 1 and 3) but not for the normal (N) cells (lane 5). Restriction digestion of the PCR products with Pvu II resulted in fragments of the expected size for IL-7 at 379 and 302 bp (lanes 2 and 4).

**Immunohistochemistry.** Cells (10^6) were centrifuged onto slides, fixed, and stained for IL-7 protein by using a monoclonal anti-IL-7 antibody (21) (Biosource International, Camarillo, CA). A minimum of 200 cells were counted per slide. A monoclonal antibody of the same isotype was used as control for specificity.

**Apoptosis.** Apoptotic cell death was distinguished by ladder patterns of DNA fragments upon electrophoresis (1). Cells were lysed in 0.2% Triton X-100/50 mM Tris/10 mM EDTA, pH 8.0. Soluble fragmented DNA was precipitated with 2-propanol, dissolved overnight at 37°C in 10 mM Tris/1 mM EDTA at pH 8.0, electrophoresed in a 2% agarose gel, stained with ethidium bromide, and then visualized and photographed on a UV-light box.

**RESULTS**

**IL-7 mRNA Detected by RT-PCR in Peripheral Blood Mononuclear Cells from Prolymphocytic Leukemia (PLL) and CLL Patients.** PCR products for IL-7 and an alternative IL-7 splice product were detected in mononuclear cells from 51 CLL patients and one PLL donor but not in cells from a normal donor (Fig. 1). Pvu II digestion confirmed the identity of the PCR products. Subsequently, the use of methods that gave higher yields of "unstable" mRNAs and more sensitive PCRs led to detection of IL-7 mRNA in a portion of normal donors as well as in some patients with infectious diseases (K. A. Herbst, A. L. Landay, and J.M.D.P., unpublished work).

**IL-7 Protein Detected by Immunohistochemical Staining.** Normal, CLL, and PLL patients' cells were examined for expression of IL-7 protein. Cytoplasmic staining with a monoclonal antibody to human IL-7 was evident in cells from CLL patients (Fig. 2 Left) and PLL patients (data not shown). Only 12.3% and 7% of cells from two normal donors were IL-7 protein-positive, whereas 17.5%, 20%, 22.5%, 35%, and 44% of cells from five CLL patients showed staining with the anti-IL-7 antibody.

**Anti-IgM-Activated Normal B Cells Do Not Synthesize IL-7 mRNA.** We considered the possibility that expression of IL-7 mRNA was a natural consequence of B-cell activation through surface immunoglobulin receptors and that IL-7 expression in CLL cells was a reflection of their state of activation. Normal donor mononuclear cells were incubated for 24 hr with goat anti-human IgM at 7 µg/ml, with or without recombinant IL-7, or with recombinant IL-7 alone (Sterling Drug, Malvern, PA) at 10,000 units/ml. Cultured cells were washed, incubated with...
a mouse monoclonal anti-CD19 antibody, and then fractionated into CD19+ positively selected samples and CD19-depleted samples by magnetic separation with goat anti-mouse immunoglobulin antibodies conjugated to iron filings. RNA was isolated from the respective fractions and RT-PCR was performed with primers specific for IL-7 cDNA, IL-2R cDNA as a positive control, or IL-2 cDNA as a negative control for B-cell purity. IL-7 mRNA was not detected in CD19+ B cells cultured either alone, with anti-IgM, with anti-IgM plus IL-7, or with IL-7 (Fig. 3 A and B). PCR products for IL-2R were detected in each sample. IL-7 upregulated IL-2R expression in B cells, similar to reports of its effect on T cells and CD56+ natural killer cells (18, 19). The purity of the CD19+ fraction was confirmed by the absence of an IL-2 PCR product. IL-2 message was detected in cells remaining after CD19 depletion.

Upregulation of both IL-2 and IL-2R mRNAs by IL-7 was confirmed in B-cell-depleted preparations (Fig. 3C). IL-7 also upregulated its own mRNA, as a weak IL-7 mRNA band was observed in CD19-depleted cells following culture with IL-7 (Fig. 3C). No IL-7 mRNA expression was observed in activated B cells, indicating that IL-7 gene expression was not a natural consequence of immunoglobulin receptor triggering.

**EBV-Transformed B-Cell Lines Express IL-7 mRNA.** Seven independently derived EBV-transformed B-cell lines from four individuals were examined for expression of IL-7 mRNA by PCR. IL-7 PCR products were detected in six of seven samples (Fig. 4). In some cases, the alternatively spliced IL-7 mRNA was observed as the primary PCR product at ~500 bp. EBV, an infectious and potential transforming agent, therefore, was capable of triggering B-cell production of IL-7 mRNA. These data are consistent with a report of IL-7 expression in lymphoblastoid lines derived from Burkitt lymphoma and patients with infectious mononucleosis and in EBV-transformed umbilical cord blood cells (22).

**Role of IL-7 in Growth of CLL Cells in Vitro.** While our studies indicated that oncogenic or viral transformation of human B cells resulted in IL-7 expression and that antigen activation of B cells did not, the functional effects of IL-7 on CLL cell growth in vitro were not revealed. Other investigators have provided conflicting evidence concerning the ability of IL-7 to support the growth of CLL cells. Our data suggest that CLL cells do not proliferate in response to IL-7 in vitro, but apoptosis becomes evident after 18–24 hr in culture. Unfractionated CLL cells as well as fractionated CLL cells were cultured with recombinant human IL-7 from 0.1 to 10,000 units/ml, either alone or in combinations with either IL-1, IL-2, IL-4, tumor necrosis factor α, stem-cell factor, interferon γ, interferon α, CD40L, or bacterial lipopolysaccharide. No dose-dependent proliferative responses were observed in any of the six CLL patients' cells with IL-7 either alone or in combinations with other cytokines or B-cell activators (data not shown).

**IL-7 mRNA Instability and Apoptosis Induction.** It had been observed previously that IL-7 mRNA was quite unstable in comparison to other cytokine mRNAs even when maintained in quinuclidin isothiocyanate solution (13). Kinetic studies were performed with CLL cells placed in culture to determine whether IL-7 mRNA was also "unstable" in whole cells (12). Incubation of CLL cells at 37°C for 3–4 hr led to the loss of IL-7 mRNA (Fig. 5). Nonspecific Rnase degradation of total mRNA was not responsible for IL-7 mRNA destruction, as other cytokine and cytokine receptor mRNAs remained intact for at least 18 hr (Fig. 5; see also Fig. 6). Maintaining CLL cells at 4°C preserved IL-7 mRNA. These results suggested that IL-7 gene transcription was downregulated in an energy-dependent manner and that IL-7 mRNA was specifically destroyed during CLL cell culture at 37°C.

It had been demonstrated by others (2, 3, 23) and we confirmed that CLL cells undergo apoptosis by 18–24 hr in culture (Fig. 6). Exogenous IL-7 does not prevent apoptosis, whereas interferon γ and IL-4 do (refs. 3 and 23; data not shown). In experiments to identify signals that triggered apoptosis, we found that cells from whole anticoagulated unperturbed blood from CLL patients maintained at 37°C in vitro underwent apoptosis. We concluded that the major signals for maintenance of CLL cell viability and IL-7 gene transcription remained within the body. Since CLL cells mainly circulate in the bloodstream, it was reasoned that signals for CLL maintenance in vivo may be derived from endothelial cells lining blood vessels. CLL cells were cultured on EA.hy926 endothelial hybrid cells for 24 and 48 hr. The cells were then removed, evaluated for viability, and processed to isolate soluble DNA or mRNA. RT-PCR analyses revealed that expression of the IL-7 gene had been stabilized (Fig. 6 Upper). No or substantially reduced DNA fragmentation was observed in CLL cells cultured on the endothelial hybrid cell monolayers for 24 and 48 hr, but classic apoptosis was seen in CLL cells cultured in medium alone (Fig. 6 Lower). Viability counts confirmed that EA.hy926 cells prevented CLL cell death as only 5.6% of the CLL cells cultured with EA.hy926 cells were dead at 24 hr, whereas 31.9% were dead without EA.hy926 cells.

CLL cells cultured with A549/8 cells, the fusion partner used to generate the EA.hy926 hybrid cells, exhibited apoptosis (Fig. 7, lanes 1 and 3). Further, the addition of spent medium from confluent monolayers of either EA.hy926 or A549/8 cells did not prevent apoptosis (Fig. 7, lanes 5 and 6). These studies demonstrated that maintenance of CLL cell viability and IL-7 gene expression in vivo were active processes that could be executed via cell–cell signaling. Preliminary data from studies to delineate molecules from EA.hy926 that improved CLL cell viability in vitro and prevented downregulation of IL-7 gene expression, as well as the receptors on CLL cells.
cells cultured in EBV-transformed B cells. Although the IL-7 mRNA was transcribed into protein in a portion of these cells, exogenously added IL-7 had little effect on CLL growth. Other laboratories have had variable results with respect to CLL responses to IL-7 in vitro. Nevertheless, our data suggested that regulation of IL-7 gene expression could reveal important mechanisms with respect to maintenance of CLL cell viability in vivo. In fact, IL-7 expression in studies with IL-7 transgenic mice interfered with cellular growth control and provoked the development of lymphoproliferative disorders and induced lymphomas within 4 months of birth (24, 25). IL-7 has also been found to be expressed in human mycosis fungoides cells and to serve as a growth factor for Sezary cells (26–28). The affiliation of IL-7 expression with proliferative human diseases, therefore, may signify its importance in regulation of lymphoid cell growth and tumorigenesis.

IL-7 and other stromal cell-derived growth factors support the growth and differentiation of hematopoietic stem cells that become committed to the lymphoid lineage (29). Pre-T and pre-B cells apparently utilize IL-7 until “productive” rearrangements of genes encoding T-cell antigen receptors or immunoglobulin have been achieved (30, 31). B and T cells whose receptor genes have rearranged successfully are then positively selected as immunocompetent and are considered to be mature lymphocytes. At this point in their differentiation pathway, T and B cells are no longer responsive to the growth stimulus of IL-7. Upon activation, both become responsive again to IL-7. Lymphoid cells whose antigen receptors are negatively selected against receive a signal that regulates the expression of genes leading to the programmed cell death known as apoptosis (32). We suggest that the ability to process an IL-7 signal is germane to positive selection of competent immune cells and that loss of the IL-7 signal during differentiation follows negative selection and signals apoptosis. CLL cells appear to be frozen at this step of differentiation, between negative selection and apoptosis induction. CLL clones may have undergone carcinogenic mutations before they were negatively selected against for being autoreactive B cells. In the absence of negative selection, no signals were initiated to terminate the growth stimulus from IL-7 and to activate genes required for apoptosis. The environmental milieu of the CLL patients is critical to the maintenance of these cells. The neoplastic B cells have an autochthonous source of IL-7, as these cells express IL-7 mRNA as well as the IL-7 protein. Removal of CLL cells from the patient’s body, however, results both in the loss of the endogenous signal from IL-7 and in the expression of apoptosis. We hypothesized from these findings that there may be a direct correlation between the loss of the ability to respond to IL-7 and the subsequent expression of the apoptotic pathway not only in CLL cells but during normal T- and B-cell development and selection.

Our findings suggested that a signal for apoptosis may not have been transduced in vitro but that a signal remaining within the body may have blocked an apoptotic signal already triggered in vivo. If the blocking signal could be identified, then perhaps that block could be pharmacologically reversed so that CLL cells would self-destruct in vivo. One mechanism that holds apoptosis in abeyance involves integrin signaling (33). Our data demonstrated that cell–cell interactions between CLL cells and endothelial hybrid cells prevented the expression of apoptosis. It had been demonstrated that adhesion through a β2-integrin prevented apoptosis of germinal-center B cells, and it was suggested that this mechanism may play a role in B-cell selection (34). Preliminary data from our laboratory indicate that a β2-integrin on CLL cells may be involved in regulating apoptosis expression. Monoclonal antibodies directed against the β2-integrin, CD11b/CD18, also known as Mac-1 or CR3, modulate the interaction between CLL cells and endothelial hybrid cells (J.M.D.P. and B.W.L., unpublished work). CD11b/CD18 is a myelomonocytic marker ex-

**FIG. 6.** Prevention of IL-7 gene downregulation and mRNA destruction, as well as attenuation of apoptosis, in CLL cells cultured on EA.hy926 endothelial hybrid cells. (Upper) RNA isolated from CLL cells before culture (A), after 24 hr of culture (B), or on EA.hy926 cells (C). Culture of CLL cells on EA.hy926 resulted in ablation of specific IL-7 gene downregulation and mRNA destruction (C) evident in CLL cells cultured in medium (B). (Lower) Culture of CLL cells for 24 and 48 hr on EA.hy926 cells inhibited apoptosis of CLL cells (lanes 2 and 4 as opposed to lanes 3 and 5).

**FIG. 7.** Apoptosis expression specifically held in check by contact between CLL and EA.hy926 endothelial hybrid cells. DNA laddering was not visible in preparations from CLL cells cultured for 24 hr on endothelial hybrid cells but was apparent in cultures with either the parental carcinoma line, A549/8, or spent medium from confluent cultures of either line. Lanes 1 and 2, soluble DNA isolated from CLL no. 34 cultured on A549/8 and EA.hy926, respectively; lanes 3 and 4, soluble DNA isolated from CLL no. 53 cultured on A549/8 and EA.hy926, respectively; lanes 5–7, soluble DNA isolated from CLL no. 22 cultured with 100% spent medium from A549/8 or EA.hy926 or with fresh medium, respectively.

### DISCUSSION

We found that the gene for the growth factor IL-7 was expressed in leukemic cells from PLL and CLL patients and in
pressed on CLL cells and reportedly is associated with an unfavorable prognosis (35, 36). Furthermore, the expression of CD11b/CD18 on leukemic cells has been correlated with the ability of leukemic cells, including CLL cells, to adhere to human umbilical vein endothelial cells (37). Our experiments suggested that counter-receptors (ligands) expressed on endothelial cells could activate a signal in CLL cells via a β2-integrin to prevent the expression of apoptosis and the down regulation of the IL-7 gene. The survival of chronic-phase leukemic cells such as CLL cells in vivo may result from interactions between integrins expressed on the leukemic cell surfaces and ligands in their immediate environment. These interactions prevent some genes, such as the IL-7 gene, from being downregulated and others, such as those associated with apoptosis, from being upregulated.

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