Interaction with Vascular Endothelium Enhances Survival in Primary Chronic Lymphocytic Leukemia Cells via NF-κB Activation and De novo Gene Transcription

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Abstract

Chronic lymphocytic leukemia (CLL) cells rapidly undergo apoptosis in vitro, suggesting that the in vivo microenvironment provides crucial antiapoptotic signals. Overexpression of the antiapoptotic proteins Bcl-2 and Mcl-1 is a hallmark of CLL, and their expression is further enhanced in the lymphoid tissues. However, the high levels of Mcl-1 found in peripheral blood samples, coupled with its short half-life, led us to hypothesize that it must be actively maintained in the peripheral circulation. Coculture of CLL cells with human vascular endothelial cells significantly enhanced tumor cell survival, an effect that was not observed with normal B cells. This was associated with elevated levels of the antiapoptotic proteins Bcl-2, Mcl-1, and Bcl-X\textsubscript{L}, and marked increased expression of CD38 and CD49d, both of which are associated with clinically aggressive disease. Because CD38, CD49d, and some Bcl-2 family genes are transcriptional targets for NF-κB, we assessed NF-κB activation following coculture with endothelial cells. DNA binding of the NF-κB subunit Rel A was significantly increased and strongly correlated with changes in transcription of CD38, CD49d, BCL2, MCL1, and BCLXL, effects that were reversed by a peptide inhibitor of Rel A. These effects were not observed following coculture with nonendothelial cell lines. Therefore, CLL cells receive specific survival signals following interaction with endothelial cells mediated through the activation of NF-κB and the induction of downstream target genes. This type of interaction in the peripheral vasculature may explain the constitutive NF-κB activation and the overexpression of Bcl-2 family proteins commonly seen in this disease.

Introduction

Chronic lymphocytic leukemia (CLL) is a common B-cell malignancy that follows a remarkably diverse clinical course. Although numerous markers that predict outcome have been described, the reasons why disease progression occurs in one individual but not in another remain largely unclear. The fact that peripheral blood leukemic cells are generally nonproliferating led in the past to the assumption that CLL is primarily a disease of failed apoptosis. However, recent studies have shown that CLL is a highly dynamic disease with significant tumor cell turnover every day (1). Importantly, patients with progressive disease show a marked imbalance between cell production and loss of tumor cells.

Tumor proliferation is believed to mainly occur in pseudo-follicles or proliferation centers in the lymph nodes, bone marrow, and spleen, where dividing CLL cells colocalize with T lymphocytes, the microvasculature, and a variety of other stromal elements and soluble factors (2–4). We have previously shown that proliferating CLL cells colocalize with activated CD4\textsuperscript{+} T cells in the lymph nodes (5) and, in the same study, that high levels of CD38 expression on lymph node CLL cells were associated with an increased number of CD31\textsuperscript{+} microvascular cells (5). CD31 is the only known ligand for CD38, and signaling through this axis has previously been shown to support the survival and proliferation of CLL cells in vitro (6). In addition, CD38/CD31 interactions have recently been linked to a molecular pathway involving the integrin CD49d (7). Increased expression of both CD38 and CD49d is an adverse prognostic marker in this disease (8, 9), and interactions involving CD49d prevent spontaneous and drug-induced apoptosis of normal and neoplastic B cells (10, 11). A variety of other ligands and receptors expressed by various...
components of the leukemic microenvironment also influence the survival of CLL cells, including chemokines and their receptors, CD40/CD40L, BAFF, APRIL, and CD100/plexinB1 (2, 7, 12, 13). This diverse range of signals seems to be integrated at the cellular level into a restricted set of responses, resulting in maintenance and/or expansion of the leukemic clone.

The mechanisms that regulate the survival of CLL cells are complex, but it has long been established that CLL cells overexpress antiapoptotic Bcl-2 family proteins (14). Bcl-2 expression predicts poor outcome in patients with CLL (15, 16) and is an independent prognostic marker (17). Overexpression of Bcl-2 was originally thought to be caused by hypomethylation of the promoter region of the BCL2 gene (18). However, it is now generally accepted that this is not the only cause of constitutive Bcl-2 expression because microRNA (miR) species miR-15 and miR-16, which negatively regulate Bcl-2 at the posttranscriptional level, are deleted or downregulated in many CLL patients (19). Mcl-1, another Bcl-2 family protein, has also been shown to be an independent prognostic marker for CLL (20), with increased expression strongly related to resistance to chlorambucil, fludarabine, and rituximab (21). A growing body of evidence shows that Mcl-1 and other Bcl-2 family proteins are dynamically regulated in the lymphoid tissues through B-cell receptor engagement and CLL cell interactions with a variety of stromal cells, including mesenchymal marrow stromal cells, nurse-like cells, and follicular dendritic cells (22–25). The high level of Mcl-1 expression by peripheral blood CLL cells taken with its short half-life (26) strongly suggests that these signals occur on an ongoing basis. Although the trafficking kinetics of CLL cells between the peripheral circulation and tissues are as yet unknown, the high frequency of events required to maintain expression of such a short-lived protein indicates that at least some of these signals arise from the circulating compartment.

Over the last few years, the NF-κB family of transcription factors has increasingly been implicated in tumorigenesis (27) because of their role in the regulation of genes involved in proliferation, tissue migration, inflammation, and apoptosis. CLL cells have been shown to have high constitutive activation of NF-κB (4, 28), and the Rel A subunit has independent prognostic value (29). Furthermore, Bcl-2 family proteins are downstream transcriptional targets of NF-κB (30, 31), so it seems possible that NF-κB activation might provide a common mechanism for the maintenance and growth of the leukemic clone in CLL. In the current study, we investigated the role of NF-κB as a mediator of prosurvival signals arising from CLL cell interaction with the vascular endothelium. We hypothesized that these interactions in the peripheral circulation could maintain tumor cell viability through the induction of Bcl-2 family proteins. Here, we report a series of findings that strongly support such a relationship and showing a pivotal role of NF-κB activation in the coordination of the transcription of these molecules.

Materials and Methods

Patient samples

Peripheral blood was collected from CLL patients and normal controls with their informed consent in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation, and CLL B cells were enriched (>95%) using a human B-cell–negative selection kit without CD43 (StemCell Technologies) as previously described (5). Patient characteristics are summarized in Supplementary Table S1. In addition, a separate cohort of 88 freshly isolated CLL patient samples was analyzed for Rel A DNA binding and Mcl-1 protein expression using the methods previously described (20, 29, 32). Normal lymphocytes were derived from age-matched healthy volunteers, and B cells were enriched using the negative selection method described above.

Liquid culture conditions

Freshly isolated B cells (normal and leukemic) were cultured in HMEC-1 recommended medium supplemented with 1% bovine serum albumin (BSA). This medium had previously been tested in our laboratory and was shown to produce the same CLL cell viability as our standard CLL culture medium (RPMI 1640 supplemented with 10% FCS). Lymphocytes were incubated at 37°C in a humidified 5% carbon dioxide atmosphere for up to 7 days.

Coculture conditions

The human microvascular endothelial cell line (HMEC-1, Centers for Disease Control and Prevention, Atlanta, GA) was propagated in 24-well plates at low density (<10⁵/mL) in the recommended medium supplemented with 1% BSA (Sigma) and incubated overnight to enable adherence. Primary CLL cells were cultured alone or on HMEC-1 cells at 2 × 10⁶ per well and then harvested at the desired time points. Supernatants from control wells with just HMEC-1 cells were harvested at the same time points and processed identically to the CLL test samples to ensure that no HMEC-1 cell contamination occurred. Similar experiments were performed using a human umbilical vein endothelial cell line as well as two nonendothelial cell lines (human embryonic kidney 293T cells and the medulloblastoma cell line TFS671). For the NF-κB inhibitor assay, CLL cells were preincubated with either the inhibitory peptide NF-κB p65 (Ser²⁷⁶) or the control peptide (Imgenex) both at a concentration of 50 μmol/L for 1 hour before coculture as previously described (32). During NF-κB activation, Ser²⁷⁶ is phosphorylated, allowing Rel A nuclear translocation. This peptide functions as a competitive p65 (Rel A) decoy, preventing phosphorylation of Ser²⁷⁶ on Rel A.

Phenotyping

CLL PBMCs were cocultured with HMEC-1 cells or control medium for 24 to 48 hours before labeling with CD19, CD5, CD38, and CD49d. For measurement of apoptosis by flow cytometry, PBMCs were incubated as above for 7 days before labeling with CD19, CD5, Annexin V (Becton Dickinson), and 7-amino-actinomycin D (7AAD; Becton Dickinson) as per the manufacturer’s instructions. Details of the Mcl-1 labeling protocol have been described previously (20). Briefly, peripheral blood CLL cells were labeled with CD19 and CD5 before treatment with Fix and Perm reagent.
performed in duplicate. Total RNA was amplified using the housekeeping control, and the results of the real-time RPS14 mRNA was quantified in all samples as an internal control for contamination by nonadhered HMEC-1 cells. Western blotting with Bcl-2, Bcl-X, Mcl-1, poly(ADP-ribose) polymerase (PARP), and BAX antibodies and a horseradish peroxidase–conjugated secondary antibody at recommended dilutions was performed using the Novex PowerEase System (Novex, Invitrogen) and NuPAGE 4% to 12% Bis-Tris gels. Proteins were visualized using ECL Plus and Hyperfilm ECL (GE Healthcare). All antibodies used are listed in Supplementary Table S2.

Western blotting
For Western blot experiments, 2 × 10^6 CLL cells were removed from the cultures at various time points, lysed on ice with 4× SDS sample buffer, and then heated to 100°C for 10 minutes. Supernatants were inspected, and cultures containing no CLL cells were harvested in the same way to control for contamination by nonadhered HMEC-1 cells. Western blotting with Bcl-2, Bcl-X, Mcl-1, poly(ADP-ribose) polymerase (PARP), and BAX antibodies and a horseradish peroxidase–conjugated secondary antibody at recommended dilutions was performed using the Novex PowerEase System (Novex, Invitrogen) and NuPAGE 4% to 12% Bis-Tris gels. Proteins were visualized using ECL Plus and Hyperfilm ECL (GE Healthcare). All antibodies used are listed in Supplementary Table S2.

Real-time reverse transcription-PCR
RNA was extracted using Trizol (Invitrogen). RNA (1 μg) was used in a 20 μL reverse transcription (RT) reaction containing 10× Buffer II, 5 mmol/L MgCl2, 0.5 μmol/L deoxynucleotide triphosphates, 2.5 units reverse transcriptase, 1 unit RNase inhibitor, and 2.5 μmol/L random hexamers. cDNA (2 μL) was placed into the RT-PCR reaction. SYBR Green was used in a 20 μL RT-PCR reaction containing 10× Buffer II, 5 mmol/L MgCl2, 0.5 μmol/L deoxynucleotide triphosphates, 2.5 units reverse transcriptase, 1 unit RNase inhibitor, and 2.5 μmol/L random hexamers. cDNA (2 μL) was placed into the RT-PCR reaction. SYBR Green technology (Roche Diagnostics) was used to quantify the amount of RNA present in each sample using primer pairs for BCL2, BAX, MCL1, BCLXL, CD38, CD49d, and RPS14. All primers were purchased from Eurogentec Ltd. The amount of mRNA was assessed using real-time RT-PCR using the LightCycler System (Roche Diagnostics). The amount of RPS14 mRNA was quantified in all samples as an internal housekeeping control, and the results of the real-time RT-PCR were expressed as normalized target gene values (e.g., the ratio between BCL2 and RPS14 transcripts calculated from the crossing points of each sample). All experiments were performed in duplicate. Total RNA was amplified using the following primers: BCL2, 5′-AGAGTGATGGGATGTTGCG-TTC-3′ (forward) and 5′-TGTGACCGAGATGCTTGGAC-3′ (reverse); BAX, 5′-TTTGGCTCTGCTTTGAGGTTG-3′ (forward) and 5′-TTGAGTGGTCGCTAGAAAA-3′ (reverse); MCL1, 5′-AAAACGAGATGTCAGGAGAGA-3′ (forward) and 5′-TGAATTCCGGGCTGAAA-3′ (reverse); BCLXL, 5′-ATC-GCAGCACCACATCTCTG-3′ (forward) and 5′-TGTCATGCTCAGTTTCCAGC-3′ (reverse); CD38, 5′-ATGGTTTCAAGGTTGATTG-3′ (forward) and 5′-TTTATGCGAGATGTTGTG-3′ (reverse); CD49d, 5′-AGAGTGCGAGATGCGGGAAAGAA-3′ (forward) and 5′-CCCCCTACAAATTTAATC-3′ (reverse); RPS14, 5′-GGCGAGACGAGATGAACTC-3′ (forward) and 5′-CCAGTTCCGAGGGTTGTTG-3′ (reverse).

NF-κB Rel A detection by ELISA
Nuclear proteins were assayed for Rel A DNA binding with a TransAM NF-κB kit according to the manufacturer’s instructions (Active Motif). The consensus oligodeoxynucleotide used for NF-κB binding was 5′-GGAGACTTTCC-3′, Wild-type and mutated consensus oligodeoxynucleotides were used to monitor the specificity of the assay. The absorbance reading at 450 nm (A450) was read on a microtiter plate reader (Bio-Rad). A450 values were converted into ng Rel A NF-κB/μg of nuclear protein for each sample tested from a standard curve constructed using known quantities of recombinant Rel A. We have previously shown a strong correlation between this ELISA-based assay and electrophoretic mobility shift assay using NF-κB–specific probes (33).

Statistical analysis
All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software). All of the paired data were tested for normality and considered Gaussian, so data sets were compared using the paired t test.

Results

Coculture with endothelial cells markedly enhances CLL cell in vitro survival
CLL cells cocultured with vascular endothelial cells for up to 7 days showed significantly higher viability compared with those cultured in standard liquid culture conditions (n = 15, P < 0.0001; Fig. 1A and B). Coculture with two nonendothelial cell lines (293T and TE671) also enhanced CLL cell viability but to a significantly lesser extent than that observed following coculture with the vascular endothelial cell lines (Supplementary Fig. S1A). This was consistent in all the samples tested irrespective of immunoglobulin gene mutational status or initial CD38 expression. In addition, coculture with endothelial cells inhibited PARP cleavage under the same conditions after 24 hours of coculture compared with CLL cells incubated in control medium (Fig. 1C). In contrast, coculture of purified normal B cells failed to be cytoprotective (Fig. 1B). Analysis of Bcl-2 family protein expression in purified CLL cells cocultured with endothelial cells for 24 hours revealed a marked increase in the expression of the antiapoptotic proteins Bcl-2, Bcl-X, and Mcl-1 with no significant change in the proapoptotic protein BAX (Fig. 1D). This resulted in marked increases in the ratio between antiapoptotic and proapoptotic proteins and provides a plausible mechanism for the antiapoptotic phenotype exhibited by CLL cells cocultured on endothelial cells.

Coculture with endothelial cells increases antiapoptotic BCL2 family gene transcription
We next assessed whether the increases in Bcl-2 family protein expression were associated with a change in gene transcription following coculture with endothelial cells. RT-PCR analyses of RNA isolated from purified CLL cells from all six patients tested showed a significant increase in BCL2 gene transcription in the presence of endothelial cells after just 4 hours of coculture, with a further increase at 24 hours (P = 0.009 and 0.0004, respectively; Fig. 2A). In contrast, BCL2 transcription in CLL cells cultured alone was not significantly altered over the same period when compared with time 0 (P = 0.94 and 0.93, respectively). MCL1 gene transcription was also significantly increased at 4 and 24 hours when compared with the time 0 samples (P = 0.005 and 0.006, respectively;
Fig. 2B). Consistent with the short half-life of Mcl-1 protein, the transcription of MCL1 in CLL cells cultured without endothelial cells was reduced at both 4 and 24 hours compared with time 0 ($P = 0.02$ and $0.002$, respectively). The relative transcription of BCLXL at time 0 was the lowest of all the Bcl-2 family members analyzed. This is in keeping with the fact that Bcl-XL protein is expressed at low levels in peripheral blood CLL cells (24). However, BCLXL gene transcription was significantly increased in the presence of endothelial cells at 4 and 24 hours ($P = 0.01$ and $0.002$, respectively; Fig. 2C). Once again, the transcription of this gene was not significantly altered in the absence of endothelial cells. The transcription of the proapoptotic gene BAX did not significantly change in CLL cells cocultured with endothelial cells at both the 4- and 24-hour time points ($P = 0.23$ and $0.21$, respectively; Fig. 2D). In contrast, CLL cells cultured alone showed a significant increase in BAX transcription after 4 and 24 hours ($P = 0.01$ and $0.002$, respectively). The BCL2/BAX, MCL1/BAX, and BCLXL/BAX ratios were thus all significantly elevated in the presence of endothelial cells and reduced in CLL cells cultured alone. In the case of CLL cells cocultured on endothelial cells, the increased ratios were almost entirely due to the increased transcription of the antiapoptotic genes (Fig. 2E–G). Coculture of CLL cells with
Figure 2. Coculture with endothelial cells increases ant apoptotic BCL2 family gene transcription. Purified CLL cells were cultured in the presence or absence of endothelial cells, and RNA was prepared at 4- and 24-h time points. Samples were analyzed by RT-PCR for transcriptional levels of the BCL2 family members BCL2 (A), MCL1 (B), BCLXL (C), and BAX (D). A to D, the bar charts in the left columns are composite results for all six patients studied and show a statistically significant increase in the transcription of BCL2, MCL1, and BCLXL in CLL cells when cocultured with endothelial cells at both 4 and 24 h. BAX transcription in CLL cells remained unaltered when cultured with endothelial cells, but levels were increased in CLL cells cultured alone. The right columns show the composite percentage change in transcription from time 0. Coculture with endothelial cells resulted in an increase in transcription of BCL2, MCL1, and BCLXL at both 4 and 24 h. E to G, the BCL2/BAX, MCL1/BAX, and BCLXL/BAX transcription ratios showed statistically significant increases at both 4 and 24 h.
293T cells failed to induce the transcriptional activation of BCL2, MCL1, or BCLXL but did repress the transcription of BAX (Supplementary Fig. S2B–E). This suggests that BAX transcription is regulated by a different mechanism to that of BCL2, BCLXL, and MCL1 and that these genes are specifically modulated by coculture with endothelial cells.

**Nuclear DNA binding of the NF-κB subunit Rel A is increased in CLL cells cocultured with endothelial cells**

Given that Bcl-2 family members are transcriptional targets of NF-κB and nuclear DNA binding of the subunit Rel A is associated with *in vitro* resistance to apoptosis (31–33), we next determined whether coculture with endothelial cells resulted in nuclear activation of Rel A. Once again, primary CLL cells were cultured in the presence or absence of endothelial cells (or the nonendothelial 293T cells), and nuclear extracts were made following 4 and 24 hours of culture. After just 4 hours, CLL cells cultured alone showed a significant reduction in Rel A DNA binding compared with time 0 samples (*P* = 0.005; Fig. 3A). In contrast, CLL cells cocultured with endothelial cells maintained levels of Rel A DNA binding at 4 hours and significantly increased nuclear Rel A levels at 24 hours (*P* = 0.003). Coculture of CLL cells with 293T cells failed to maintain Rel A DNA binding, indicating that the activation of Rel A in primary CLL cells is specifically induced by interaction with endothelial cells (Supplementary Fig. S2A). Furthermore, we found a significant positive correlation between Rel A DNA binding and the transcription of BCL2, BCLXL, and MCL1 (Fig. 3B–D).

Additional evidence that Mcl-1 expression is associated with Rel A was provided by a retrospective analysis of cohort of 88 CLL patients in whom we had measured both Mcl-1 protein and nuclear extracts were made following 4 and 24 hours of culture. After just 4 hours, CLL cells cultured alone showed a significant reduction in Rel A DNA binding compared with time 0 samples (*P* = 0.005; Fig. 3A). In contrast, CLL cells cocultured with endothelial cells maintained levels of Rel A DNA binding at 4 hours and significantly increased nuclear Rel A levels at 24 hours (*P* = 0.003). Coculture of CLL cells with 293T cells failed to maintain Rel A DNA binding, indicating that the activation of Rel A in primary CLL cells is specifically induced by interaction with endothelial cells (Supplementary Fig. S2A). Furthermore, we found a significant positive correlation between Rel A DNA binding and the transcription of BCL2, BCLXL, and MCL1 (Fig. 3B–D).

**Figure 3.** The presence of endothelial cells maintains or increases nuclear NF-κB binding in CLL cells, and it correlates positively with transcription of BCL2, MCL1, and BCLXL and negatively with BAX. A, purified CLL cells were cultured in the presence or absence of endothelial cells and nuclear lysates were made at 4- and 24-h time points. The level of NF-κB subunit p65 Rel A DNA binding was quantified. The figure shows a composite chart of all six patients showing a consistent decrease in Rel A binding at 4 h (*P* = 0.005, paired *t* test) and 24 h (*P* = 0.002, paired *t* test) when CLL cells were cultured alone compared with time 0. In contrast, coculture with endothelial cells maintained Rel A binding levels at 4 h and increased them at 24 h (*P* = 0.003, paired *t* test) when compared with time 0. B, C, D, and F, correlation analysis within samples from all six patients showed strong positive correlations between nuclear Rel A and BCL2 (B), BCLXL (C), and MCL1 (D) and a statistically significant negative correlation with BAX (F). E, a retrospective analysis of a separate cohort of 88 CLL patients in whom we had previously measured both Mcl-1 protein expression and Rel A DNA binding confirmed the strong positive correlation between Mcl-1 protein levels and nuclear Rel A levels.
Coculture with endothelial cells induces specific changes in CLL cell phenotype mediated by NF-κB activation

The striking increase in CLL cell survival following coculture with vascular endothelial cells led us to examine whether CLL cell phenotypic changes were associated with this phenomenon. We noted consistent increases in both CD38 and CD49d protein expression on CLL cells compared with those cultured alone irrespective of initial levels (n = 10, P = 0.001 and n = 6, P = 0.01, respectively; Fig. 4A). Both of these proteins are associated with poor prognosis in this disease, and high expression is linked to the activation of CLL cells (8, 9). These changes were seen as both increased percentage expression and elevated antigen density, as shown by enhanced mean fluorescence intensity (MFI) values. Importantly, neither of these proteins was significantly altered following coculture with 293T cells (Supplementary Fig. S2F and G). These data provide further evidence that the phenotypic and transcriptional changes in CLL cells that we observed following coculture with endothelial cells are selectively induced by this cell type. We went on to show that the changes in protein expression were mirrored by increased transcription of both CD38 and CD49d (Fig. 4B). Given that previous studies in other cell systems have shown that CD38 and CD49d are regulated by NF-κB (34, 35), we assessed whether changes in transcription were associated with altered nuclear Rel A DNA binding. We found a very strong correlation between both genes and the nuclear DNA binding of this transcription factor (Fig. 4C). These results indicate that Rel A plays an important role in regulating the expression of these genes and that CLL cell interaction with vascular endothelium can facilitate their transcription.

Inhibition of Rel A causes transcriptional repression of CD38, CD49d, and BCL2 family genes

Preincubation of CLL cells with a Rel A peptide inhibitor, which we have previously shown to inhibit nuclear Rel A and induce apoptosis in CLL (32), blocked nuclear activation of Rel A in the presence of endothelial cells (Fig. 5A). The inhibitor also prevented endothelial cell-mediated transcriptional changes in BCL2, BAX, MCL1, and BCLXL (Fig. 5B–E) and repressed the transcription of CD38 and CD49d (Fig. 5F and G), indicating that the expression of all of these genes is coordinated by the transcription factor Rel A. Furthermore, the inhibitor abrogated the cytoprotective effect of coculture with endothelial cells (Supplementary Fig. S1B). Taken together, these data suggest that the antiapoptotic effect of endothelial cells on primary CLL cells is, at least in part, regulated through NF-κB activation and the subsequent transcriptional activation of its antiapoptotic target genes BCL2, MCL1, and BCLXL. The role of CD38 and CD49d in this context has not yet been fully elucidated, but these proteins are clearly associated with an aggressive clinical phenotype and play a role in CLL cell survival and transendothelial migration (7, 36, 37).

Discussion

The molecular mechanisms that regulate apoptosis in CLL are complex, but the importance of the Bcl-2 family of apoptosis-regulating proteins in CLL has been established for over a decade (17, 38–40). Although CLL cells accumulate in vivo, they rapidly undergo apoptosis in vitro, suggesting that prosurvival signals emanating from the microenvironment play a major role in preventing cell death (41–43). In this regard, the bone marrow and lymph node microenvironments have received most attention, with the peripheral circulation largely considered as a passive transit compartment (11, 44–46). However, interaction of CLL cells with vascular endothelium in the peripheral circulation may also provide important tonic signals that maintain survival of the CLL clones.

In this present study, we showed that in vitro coculture of primary CLL cells with endothelial cells specifically induced the expression of the antiapoptotic proteins Bcl-2, Mcl-1, and Bel-Xₐ and profoundly inhibited CLL cell apoptosis. In addition to changes in Bcl-2 family proteins, CLL cells cocultured on endothelial cells markedly induced the expression of CD38 and CD49d on the surface of CLL cells. Importantly, we were able to show that the increase in protein expression was caused, at least in part, by transcriptional activation of these genes that was mediated via increased DNA binding of the NF-κB subunit Rel A. Although coculture with nonendothelial cell lines conferred some cytoprotection to CLL cells, this was significantly less marked than that induced by coculture with endothelial cells (Supplementary Fig. S1A). Furthermore, we were able to show that coculture with nonendothelial cells did not induce Rel A DNA binding or increased transcription of BCL2, MCL1, or BCLXL (Supplementary Fig. S2).

We have previously shown that Rel A is an independent prognostic marker and a potential therapeutic target in this disease (29, 32). Despite its constitutive activity in CLL, Rel A is also inducible through engagement of the B-cell receptor (47). Here, we show for the first time that CLL cell interaction with vascular endothelium can elicit a similar response. These new data provide a biological rationale for the poor prognosis associated with high Rel A DNA binding (i.e., the transcriptional induction of the antiapoptotic genes BCL2, MCL1, and BCLXL). BCL2 and BCLXL are known transcriptional targets for NF-κB (30, 31), and here, we show that MCL1 is also regulated by NF-κB in primary CLL cells. The notion that Rel A is a critical regulator of MCL1 in CLL was further supported by the strong correlation between Mcl-1 protein expression and Rel A DNA binding in a separate
cohort of 88 CLL patients (Fig. 3E). Furthermore, to confirm that Rel A regulates the transcription of CD38, CD49d, BCL2, MCL1, and BCLXL, we used a peptide inhibitor of Rel A. We have previously shown that this peptide can block nuclear Rel A in primary CLL cells (32), so here we evaluated its effects on CLL cells cocultured with endothelial cells. The inhibitor significantly reduced Rel A DNA binding in CLL cells even in the presence of endothelial cells. Crucially, this inhibition also resulted in significant transcriptional repression of CD38, CD49d, and all three antiapoptotic genes.

It is of particular interest that the cytoprotective effects of endothelial cells were specific to CLL cells, as coculture with
Figure 5. In the presence of endothelial cells, the addition of a Rel A inhibitor (Rel Ai) significantly induced BAX transcription and suppressed the transcription of BCL2, MCL1, BCLXL, CD38, and CD49d. A, purified CLL cells were preincubated with a NF-κB inhibitor and cultured in the presence or absence of endothelial cells for 24 h, and then nuclear lysates were prepared before the quantification of DNA binding of the NF-κB subunit p65 Rel A. The panels represent composite charts of all six patients showing that the inhibitor consistently represses the increase in Rel A binding induced by coculture with endothelial cells. B to G, purified CLL cells from the same patients were treated as in A, and RNA was extracted and reverse transcribed to assess changes in the transcription of BCL2 (B), BAX (C), MCL1 (D), BCLXL (E), CD38 (F), and CD49d (G). For every gene analyzed, the Rel A inhibitor was able to reverse the effects of coculture with endothelial cells.
purified normal B cells failed to enhance their survival. Recent studies have highlighted the impaired transendothelial migration of CLL cells, reinforcing the idea that there are significant differences in the way in which CLL cells interact with the vascular endothelium (48, 49). This is further supported by the work of Till and colleagues (36) who found that CLL cells, but not normal B cells, are dependent on vascular endothelial growth factor and α4β1 integrin (CD49d) for chemokine-induced motility on and through endothelium. Given these findings, efforts to discover the fundamental differences between CLL cells and normal B cells in terms of their interactions with endothelial cells may allow us to develop novel targeted strategies in these tumors.

Clearly, there are many potential ligand/receptor interactions that can contribute toward Rel A induction and prosurvival signaling, but there is an increasing realization that specific ligand/receptor pairs may not work in isolation but rather collaborate to promote survival and growth in CLL. In this regard, recent work has elucidated the cooperation between CD49d/VCAM-1 and CD38/CD31 interactions in CLL following coculture with endothelial cells (7). This group linked the CD38/CD31 interaction with CD49d and the overexpression of the chemokines CCL3 and CCL4. In keeping with these findings, the present study showed that both CD49d and CD38 expression were significantly increased on CLL cells following coculture with endothelial cells. In addition, we have previously shown that CCL3 is differentially expressed in purified CD38-positive CLL cells when compared with CD38-negative CLL cells derived from the same patient (37). It is worthy of note that CCL3 is also a putative NF-κB transcriptional target gene (50), so it is conceivable that the induction of all of these molecules is orchestrated through the induction of NF-κB. The precise mechanism(s) for the regulation of CD38 and CD49d following interaction with endothelial cells is the subject of ongoing investigations in our laboratories, in which we are systematically blocking ligand/receptor interactions to ascertain the critical molecular interactions that lead to their transcriptional activation.

In this study we have shown for the first time that CLL cell interaction with vascular endothelium markedly enhances the viability of CLL cells through the activation of the NF-κB transcription factor Rel A. This is associated with the up-regulation of NF-κB target genes including CD38, CD49d, and antiapoptotic BCL2 family members. Given the short protein half-life of Mcl-1, these results also provide a rational explanation for the maintenance of Mcl-1 protein levels in the peripheral blood of CLL patients. Two functional compartments can thus be envisaged in CLL: an intravascular “maintenance” compartment in which prosurvival signals mediated by NF-κB activation predominate, and an extravascular tissue–based environment in which additional factors provided by T cells and other stromal cells provide the signals required for proliferation and expansion of the neoplastic clone. NF-κB may also be involved in the regulation of migration into the tissues, as its activation induced the expression of CD38 and CD49d, both of which are involved in this process. To understand how CLL persists and progresses, it will be necessary to unravel the complex network of signals that mediate the survival and proliferation of CLL cells and how transit between the peripheral circulation and lymphoid tissue compartments is controlled. The data presented here strongly suggest that NF-κB is a central player in these events. Because normal B cells are not protected from apoptosis by interaction with vascular endothelial cells and do not show constitutive NF-κB activation, this pathway represents a promising drug target with a favorable therapeutic index.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Interaction Promotes CLL Cell Survival via NF-κB


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