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Modulation of NF-κB Activity and Apoptosis in Chronic Lymphocytic Leukemia B Cells

Richard R. Furman,* Zahra Asgary,* John O. Mascarenhas,* Hsiou-Chi Liou,2†‡ and Elaine J. Schattner2,3* ‡

Chronic lymphocytic leukemia (CLL) is an indolent malignancy of CD5+ B lymphocytes. CLL cells express CD40, a key regulator of B cell proliferation, differentiation, and survival. In nonmalignant B cells, CD40 ligation results in nuclear translocation and activation of NF-κB proteins. Based on observations that in some CLL cases, the tumor cells express both CD40 and its ligand, CD154 (CD40 ligand), we proposed a model for CLL pathogenesis due to CD40 ligation within the tumor. To evaluate this issue, we used freshly isolated CLL B cells to examine constitutive and inducible NF-κB activity by electrophoretic mobility shift assay. We consistently observed high levels of nuclear NF-κB activation in CLL B cells. The NF-κB proteins are key regulators of differentiation and survival in B cells (10, 20). In mammals this protein family includes p50, p52, p65 (RelA), c-Rel, and RelB (21–23). In the inactive state, NF-κB proteins occur as homodimeric or heterodimeric complexes in the cytoplasm bound to IκB proteins. After appropriate stimulation, IκB is phosphorylated, ubiquitinated, and degraded, which allows translocation of NF-κB to the nucleus and transcription of NF-κB target genes (24–26). Induction of NF-κB activity protects cells from apoptosis induced by a variety of stimuli including exposure to TNF-α, chemotherapy, and ionizing radiation (27–29). Recent studies have elucidated some mechanisms by which NF-κB inhibits apoptosis, such as induction of c-IAP1 and c-IAP2 (30), bcI-xL, and bfl-1 (31).

Our model predicts that CD40 ligation, delivered either by CD154+ T cells in the host, or by tumor-expressing B cells in a subset of cases, would result in augmentation of NF-κB activity and CLL cell survival (17). To test this hypothesis, we examined constitutive and inducible NF-κB activity in freshly purified CLL B cells. We evaluated the impact of CD40 ligation on nuclear NF-κB and survival in CLL cells using electrophoretic mobility shift assays (EMSA) and fluorescence flow cytometry. Through these investigations, we determined that unstimulated CLL cells have high constitutive levels of NF-κB activity, and that CD40 engagement enhances this activity and promotes survival in vitro. These results indicate that CD40-CD154 interactions may be central in CLL pathogenesis and offer new targets for therapy in this disease.

Materials and Methods

Sample isolation

Peripheral blood CLL samples were obtained from untreated CLL patients whose diagnosis was confirmed at The New York Hospital—Cornell Medical Center based on standard criteria (3). For studies in nonmalignant human B cells, we used buffy coat preparations of human peripheral blood leukocytes from The New York Blood Center (four samples) or tonsillar B lymphocytes. CLL cells express CD40, a key regulator of B cell proliferation, differentiation, and survival. In nonmalignant B cells, CD40 ligation results in nuclear translocation and activation of NF-κB proteins. Based on observations that in some CLL cases, the tumor cells express both CD40 and its ligand, CD154 (CD40 ligand), we proposed a model for CLL pathogenesis due to CD40 ligation within the tumor. To evaluate this issue, we used freshly isolated CLL B cells to examine constitutive and inducible NF-κB activity by electrophoretic mobility shift assay.
inhibitors at 0°C. The nuclear material was sonicated briefly before rotation.

PREPARATION OF CYTOSOLIC AND NUCLEAR LYSATES

Nuclear and cytosolic fractions were prepared as described previously (10). For each circumstance, a minimum of 2 × 10^7 cells were washed in cold PBS and resuspended in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.5 mM DTT) with 0.1% Nonidet P-40 and protease inhibitors (leupeptin, aprotinin, trypsin-chymotrypsin inhibitor, pepstatin A, and PMSF) at 0°C. For each sample, lysis of the plasma membrane was confirmed by trypan blue uptake before centrifugation, removal of the supernatant (cytosolic extract), and resuspension of the nuclear extract were analyzed for NF-κB activity by EMSA using a probe containing the NF-κB binding site from the Ig κ light chain promoter region. Arrows indicate specific NF-κB bands. A total of 5 µg of nuclear protein from uncultured peripheral blood B cells from healthy donors (n = 4) or CLL patients (n = 4) was evaluated by EMSA within a single autoradiograph, and the signals measured by densitometry. Each bar represents the mean densitometry result (arbitrary units). The error bars indicate the SEM.

EMSAs were performed as described previously (10) using a T4 kinase end-labeled probe with the NF-κB-binding sequence from the 5′-GGGACTTTCC-3′. For each EMSA, the nuclear lysates were incubated in DNA-binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol), poly(dI-dC) (100 ng/µl), Nonidet P-40 (0.25%) and 20,000 cpm of 32P-labeled probe for 15 min at room temperature before electrophoresis in a 6% native polyacrylamide gel. The gels were dried and exposed to film at −70°C, and the autoradiographs were analyzed by densitometry (SigmaGel; Jandel, San Rafael, CA). For supershift EMSAs, 10 µg of nuclear protein were incubated with 6 µg of polyclonal rabbit Ab to p50, p52, p65, c-Rel, or RelB (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 0°C before addition of labeled probe.

CELL CULTURE REAGENTS

Purified B cells were cultured at 37°C with 5% CO₂ in RPMI 1640 media supplemented with 10% FCS (Gemini Biological Products, Calabasas, CA), penicillin, streptomycin, and 1-glutamine, and the following reagents, as indicated: IL-4 (10 ng/ml; R&D Systems, Minneapolis, MN), anti-CD40 mAb (2 µg/ml, clone M3, murine IgG1, R&D Systems), anti-CD154 mAb (3 µg/ml, TRAP1 clone, murine IgG1; PharMingen, San Diego, CA), anti-CD23 (3 µg/ml EBVCS; American Type Culture Collection, Manassas, VA), and dexamethasone (10⁻⁷ M; Sigma, St. Louis, MO). In the time course experiment, anti-CD30 mAb (2 µg/ml, murine IgG1; PharMingen) was used as an isotype-matched control Ab. For some experiments, we used two Jurkat mutant cell lines in which the cells constitutively express (clone D1.1) or cannot express (clone B2.7) CD154, in coculture with target B cells after irradiation (2000 rad) of the T cells, using a T:B cell ratio of 1:4. The Jurkat lines were the kind gift of Dr. Seth Lederman (Columbia University College of Physicians and Surgeons, New York, NY) (32).

RESULTS

CLL cells have high constitutive levels of NF-κB activity that is augmented by CD40 ligation

We purified B cells from peripheral blood samples of healthy donors and CLL patients, and exposed the cells in vitro to IL-4, CD40 ligation, or dexamethasone before evaluation of nuclear NF-κB-binding activity by EMSA (Fig. 1). In this set of experiments, CD40 ligation was achieved using an agonistic, soluble anti-CD40 mAb. We chose also to examine the effects of IL-4, because this cytokine is an established growth factor for CLL, and in some circumstances acts in concert with CD40 ligation to favor B cell survival (13, 33). Dexamethasone exposure was used for purposes of comparison, as it is known to inhibit NF-κB activity and promote CLL cell apoptosis in vitro (34).

As shown in the representative analyses of Fig. 1A, CD40 ligation increased NF-κB activity in both normal and CLL B cells, and high levels of NF-κB activity were readily detected in nuclear extracts prepared from CLL cells as compared with those of non-malignant human B cells isolated and analyzed in the same manner. To demonstrate that the high levels of NF-κB activity we observed in unstimulated CLL cells were not a function of culture in media, we examined NF-κB activity in four normal peripheral blood and four CLL B cell samples immediately after purification.
but this was not the case for the majority of the samples. In four of the cases examined at the 6-h time point, NF-κB activity was augmented in CLL cells, we analyzed nuclear NF-κB activity (data not shown). To characterize which are the predominant NF-κB components in CLL B cells, we performed supershift EMSAs using Abs to specific NF-κB proteins with nuclear extracts prepared from untreated or CD40-stimulated CLL B cells. The data in Fig. 4 demonstrate that the dominant NF-κB components in stimulated CLL cells include p50, p65, and c-Rel. These results are quite similar to those observed using nuclear extracts prepared from human tonsillar B cells subjected to CD40 ligation by anti-CD40 mAb in vitro, and suggest that the regulation of NF-κB activity in CLL cells is similar to that of nonmalignant human B cells.

CD40 ligation is a potent inducer of NF-κB activity in CLL cells

To investigate the kinetics of CD40-mediated NF-κB induction in CLL B cells, we analyzed nuclear NF-κB activity immediately after purification or after exposure to media alone, anti-CD40 mAb, or an isotype-matched control Ab for various time intervals (Fig. 3). As shown, NF-κB was activated within 3 h of CD40 ligation, peaked within 9 h, and was sustained at 21 h. Based on this and similar experiments, we conclude that NF-κB activity peaked between 6 and 9 h after CD40 ligation and was maintained for as long as 72 h after stimulation (data not shown). This observation is consistent with reports of sustained NF-κB activity induced by CD40 ligation in nonmalignant human B cells (8). It is noteworthy that we observed clear and reproducible NF-κB induction in these experiments in which the CLL cells were stimulated only by soluble Ab to CD40, a relatively weak effector of CD40 ligation. These observations indicate that CLL B cells are extremely sensitive to CD40-mediated transcriptional stimulation.

Characterization of specific NF-κB components in normal human and CLL B cells

Cross-linking of CD40 by anti-CD40 mAb in CLL B cells resulted in an approximate doubling of NF-κB activity in the cells (Fig. 2). In similar assays performed using four preparations of nonmalignant human peripheral blood B cells, we observed ~3-fold induction of NF-κB activity (data not shown). To characterize which are the predominant NF-κB components in CLL B cells, we performed supershift EMSAs using Abs to specific NF-κB proteins with nuclear extracts prepared from untreated or CD40-stimulated CLL B cells. The data in Fig. 4 demonstrate that the dominant NF-κB components in stimulated CLL cells include p50, p65, and c-Rel. These results are quite similar to those observed using nuclear extracts prepared from human tonsillar B cells subjected to CD40 ligation by anti-CD40 mAb in vitro, and suggest that the regulation of NF-κB activity in CLL cells is similar to that of nonmalignant human B cells.

FIGURE 1. Characterization of specific NF-κB components in normal human and CLL B cells

(p values) 0.10 0.020 0.050 0.0069

FIGURE 2. Composite results for NF-κB modulation in a series of cases. Freshly purified CLL B cells were exposed in vitro for 6 h to media alone (n = 13), IL-4 (n = 10), anti-CD40 mAb (n = 12), IL-4 and anti-CD40 mAb (n = 8), or dexamethasone (n = 11), after which nuclear extracts were prepared and NF-κB activity was measured by EMSA. For each case, the densitometry result for the NF-κB signal in each experimental circumstance was normalized to the result for the same cells exposed to media alone, evaluated in the same autoradiograph, and is represented as a percent relative to the result for media alone. Each symbol represents the results for a single case, and the horizontal bars in each column indicate the mean relative signal intensity among all of the cases. Note that the p values were determined according to Student’s t test (signal intensity for media vs each experimental circumstance).

FIGURE 3. Kinetics of CD40-mediated NF-κB induction in CLL B cells. Nuclear extracts were prepared from CLL B cells immediately after purification (no culture) or after exposure to media alone, anti-CD40 mAb, or an isotype-matched control Ab for time periods as indicated and analyzed by EMSA, as in Fig. 1.
Impedance to CD154 interaction in a CD154+ case results in inhibition of NF-κB activity and reduced survival in vitro

CLL B cells express CD154 in only a subset of cases (17, 19). To investigate the consequences of blocking CD154-CD40 interaction in a CD154+ case, we purified the B cells from the peripheral blood of a patient whose cells we had previously characterized in our laboratory and determined to be CD154+. After isolation and purification of the cells, CD154 expression was evaluated by FACS analysis. In this case, >99% of the purified cells coexpressed CD5 and CD19, and 16% of the B cells expressed CD154 (data not shown). As was evident by EMSA (Fig. 5), NF-κB activity in the CLL cells of this case was inhibited by anti-CD154 mAb to a similar extent as it was upon exposure of the cells to dexamethasone. In this particular case, mAb to CD154 also favored CLL cell death in vitro (Table I).

CD40-CD154 interaction promotes CLL B cell survival

To determine whether CD40-mediated NF-κB induction was associated with CLL cell survival, we measured apoptosis in cells exposed to the anti-CD40 mAb, or to anti-CD154 mAb, by annexin V binding and fluorescence flow cytometry (Fig. 5). In this case, there was some baseline expression of CD154 in the CD19+ B cells (9%) which was augmented after exposure to anti-CD40 mAb (17%). This result is similar to what we have reported previously regarding CD40-mediated induction of CD154 in CLL B cells (17). As shown, there was only a modest survival benefit in CLL cells exposed to anti-CD40 mAb, which was typical of the results for similar experiments using cells of other cases. In our laboratory, we have observed much more dramatic prosurvival effects of CD40 ligation in CLL B cells when the stimulus to CD40 is cell-bound CD154 (Fig. 7, below) as compared with anti-CD40 mAb. However, there was a dramatic reduction in survival among cells placed in culture with an Ab to CD154 (TRAP clone), which blocks intercellular CD40-CD154 interaction, such that 77% of the CLL cells bound annexin V at this time point. Strikingly, the degree of cell death induced by anti-CD154 mAb was similar to that observed in cells exposed to dexamethasone, which is concordant with the inhibition of NF-κB activity upon exposure to anti-CD154 mAb documented for another case in Fig. 5. Taken together, these data suggest that CD40-CD154 interactions are crucial in CLL B cell survival, and that soluble Ab to CD154 can accelerate CLL cell death in cases that express CD154.

Finally, to examine the effect of cell-bound CD154 on CLL cell survival, we employed two Jurkat T cell mutant lines that constitutively express (clone D1.1) or cannot express (clone B2.7) CD154. Fig. 7A shows a representative experiment in which we evaluated apoptosis in CLL cells after coculture with irradiated CD154-expressing T cells in the presence or absence of blocking Ab to CD154. In this case, there was considerable baseline apoptosis in the cells cultured with media alone, such that only 45% of the cells were viable at 48 h. Survival was improved (60%) after exposure to CD154-deficient Jurkats, possibly due to soluble factors such as IL-4 or other cytokines provided by these T cells. However, the proportion of surviving CLL cells was greatest after coculture with CD154-expressing T cells (72%). This prosurvival effect of the T cells was completely abrogated in the presence of Ab to CD154 (14%), but not in the presence of an isotype-matched control Ab to CD23 (62%). Fig. 7B indicates the compiled results for this and three similar experiments, which demonstrate that CD154-expressing T cells promote the survival of CLL B cells in coculture via a CD154-dependent mechanism. The mean survival value among CLL cells exposed to irradiated CD154+ T cells in this system was 71%, as compared to 31% among cells exposed only to media (p = 0.0028, Student’s t test). In cells exposed to CD154+ T cells in the presence of anti-CD154 mAb, there was a significant reduction in the mean value for the surviving cells (38%, p = 0.039), as compared with the results for the same cells without blocking mAb.

Table I.  CLL cell viability

<table>
<thead>
<tr>
<th>Culture Circumstance</th>
<th>Viable % (day 3)</th>
<th>Viable % (day 6)</th>
</tr>
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<tbody>
<tr>
<td>Media only</td>
<td>82</td>
<td>45</td>
</tr>
<tr>
<td>Anti-CD154 mAb</td>
<td>41</td>
<td>20</td>
</tr>
<tr>
<td>Anti-CD40 mAb</td>
<td>84</td>
<td>51</td>
</tr>
<tr>
<td>Dexamethasone (10⁻² M)</td>
<td>60</td>
<td>22</td>
</tr>
</tbody>
</table>

*a CLL B cells (case 28, as in Fig. 5) were placed in culture and examined after 3 and 6 days for viability by hemocytometry and trypan blue exclusion. The results shown indicate the percentage of viable cells at each time point relative to the initial number of viable cells.

FIGURE 4.  Comparison of NF-κB proteins in human tonsillar and CLL B cells. Nuclear extracts were prepared from purified human tonsillar or CLL B cells after exposure for 12 h to anti-CD40 mAb and analyzed for specific NF-κB proteins by supershift EMSA using polyclonal rabbit Abs to p50, p52, p65, RelB, c-Rel, or control rabbit Ig, as indicated.

FIGURE 5.  Inhibition of NF-κB activity in a CD154-positive case by anti-CD154 mAb. CLL cells were isolated from the peripheral blood of patient 28. After the purified cells were evaluated by FACS for coexpression of CD5 and CD19 (99% double positive) and CD154 (16% positive), nuclear extracts were prepared immediately (“unstimulated”) or after exposure for 12 h to media alone, anti-CD154 mAb (murine IgG1), anti-CD40 mAb (murine IgG1), or dexamethasone (10⁻² M) and examined for NF-κB-binding activity by EMSA, as in Fig. 1.
We have reported that CD154 expression in CLL B cells diminishes over the initial days in culture, such that it is usually undetectable within 72 h after isolation of the cells in a positive case (17). In the example shown in Fig. 7A, the degree of apoptosis that occurred in the stimulated cells exposed to anti-CD154 mAb exceeded that which occurred in cells exposed to media only, and was similar in degree to that observed in the experiment shown in Fig. 6. In our laboratory, we have observed that CD154 expression is readily apparent at the cell surface in ~15% of CLL cases and can be detected at some level in up to one-third of cases (17, 19). The data included in Figs. 6 and 7 suggest that anti-CD154 mAb can block endogenous CD154 expressed by tumor B cells. In cases that express CD154, impedance to CD154 results in inhibition of NF-κB activity and CLL cell death. In cases that do not express

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**FIGURE 6.** Impact of CD40-CD154 interaction on CLL cell survival in vitro. Purified B cells from the peripheral blood of a patient with CLL were exposed to media, anti-CD40 mAb, anti-CD154 mAb, or dexamethasone. A, Flow cytometric analysis for coexpression of CD5 and CD19. B, After 48 h of culture in each circumstance, the cells were evaluated with CD19-FITC (x-axis, top and middle rows) and CD154-PE (y-axis, top row), or as a control, CD3-PE (y-axis, middle row). The percentage of double-positive cells in each dot plot is indicated. Apoptosis of the same cells was measured (bottom row) using annexin V-FITC and CD19-PE, and the percentage of annexin V-positive (apoptotic) B cells in each circumstance is indicated.

**FIGURE 7.** Engagement of CLL B cells by CD154-expressing T cells promotes CLL B cell survival. A, CLL B cells were purified and exposed to media alone, irradiated (2000 rad) CD154-deficient mutant Jurkat T cells (clone B2.7, CD154<sup>-</sup> T<sub>xr</sub>), or to irradiated CD154-positive Jurkat T cells (clone D1.1, CD154<sup>+</sup> T<sub>xr</sub>) in a ratio of 4:1 (B:T cells) in the absence of Ab, with anti-CD154 mAb (3 μg/ml, TRAP clone), or with an isotype-matched control Ab (3 μg/ml anti-CD23). After 48 h, the B cells were analyzed for apoptosis by FACS after exposure to a PE-conjugated Ab to CD19 and annexin V-FITC. The percentage of annexin V-negative (viable) B cells in each circumstance is indicated. B, Similar experiments were conducted using CLL cells from three additional cases. The columns represent the mean results for the percentage of viable B cells in each circumstance, and the error bars indicate the SD.
CD154, anti-CD154 mAb might impede survival signals conferred to CLL cells via CD154-expressing T cells or possibly by other cells such as endothelial cells in the host (35).

Discussion
We have demonstrated that unstimulated CLL B cells have high levels of NF-κB activity relative to nonmalignant human B cells and that this activity is significantly induced by CD40 engagement. The dominant NF-κB components in CLL cells appear to be p50, p65, and c-Rel, which is similar to the results reported by Romano et al. (36). Augmented NF-κB activity upon CD40 ligation was associated with improved CLL cell survival. In a CD154+ case, the addition of anti-CD154 mAb resulted in dramatic inhibition of NF-κB activity in the CLL cells to a similar degree as did dexamethasone exposure. In the majority of cases examined, enhanced NF-κB activity upon CD40 engagement was associated with CLL cell survival, and blocking CD40-CD154 interactions by anti-CD154 mAb resulted in accelerated CLL cell death in vitro. Taken together, our results indicate that the CD40-CD154 interaction can have profound effects on transcriptional regulation and tumor cell survival in CLL.

Previous investigators have identified NF-κB activity in lymphocytic tumors associated with transforming viruses. For example, the Tax protein of the human T cell leukemia virus-1, which occurs in certain human T cell leukemias and lymphomas, interacts with the IκB kinase complex, resulting in enhanced IκB phosphorylation, degradation, and induction of NF-κB (37). Recently, high levels of NF-κB activity were detected in EBV-associated posttransplant B cell lymphoproliferative disorders (38). Other investigators have observed NF-κB activity in cell lines derived from B cells such as endothelial cells in the host (35). Our observation that NF-κB activity is constitutively turned on in primary CLL B cells is significant because these cells are not infected by a virus. These findings implicate an alternative mechanism for NF-κB induction in this tumor.

We have reported that in some CLL cases the B cells coexpress CD40 and CD154 (17, 19). However, the data do not support that levels of NF-κB activity in the tumor cells correlate with B cell-derived CD154. Rather, we have observed relatively high levels of constitutive NF-κB activity in most CLL cases examined, whether CD154 positive or negative. There are three broad possible explanations for the lack of correlation: 1) that the high levels of NF-κB activity are due to CD154-independent mechanisms in some cases; 2) that most CLL B cell express CD154 in vivo, which exerts powerful effects, but that the molecule is shed rapidly upon isolation of the cells such that it is usually not detected; or 3) that the observed activity occurs as a function of CD40 engagement in vivo by CD154 derived from nonmalignant cells in the host such as CD4+ T cells.

Despite that B cell-derived CD154 appears to be a factor in only a minority of cases, our data are consistent with the hypothesis that constitutive NF-κB activity in CLL cells is due, at least in some cases, to the continuous engagement of CD40 by its ligand in vivo. These results are consistent with and build upon those recently reported by two other groups, who have identified that CD40 ligation inhibits chemotherapy and dexamethasone-induced apoptosis in CLL B cells, and that this effect may be dependent on NF-κB (36, 42). We would emphasize that the CD154-CD40 interaction is unlikely to be the primary pathogenic mechanism in CLL. Rather, our data support that this molecular interaction promotes growth of a tumor that has already arisen due to a separate, transforming event. In CLL cases that express both CD40 and CD154, autoligation of CD40 within a tumor cell, or ligation of CD40 by a nearby CLL cell, would result in NF-κB activation in vivo. In cases that do not express CD154, CD40 ligation might occur due to tumor-infiltrating CD4+ T cells. Such a mechanism for NF-κB induction and CD40 engagement via CD154 on T cells would be consistent with clinical observations regarding the efficacy of adenosine analogues in this disease. These chemotherapeutic agents effectively deplete the host’s CD4+ T cells for prolonged periods (43) and could function, at least in part, by the elimination of T cell “help” that drives the B cell tumor (19).

Finally, these results offer two possible targets for new therapies in this disease. First, we have demonstrated that in some cases blocking the CD154-CD40 interaction results in inhibition of NF-κB activity and CLL cell death. Therefore, new approaches might include the use of Ab to CD154, such as is being tried in patients with autoimmune diseases such as systemic lupus erythematosus and idiopathic thrombocytopenic purpura. This treatment might be targeted to cases in which the CLL cells express CD154, or cases in which the patients suffer from associated autoimmunity. Second, our results also support the investigation and trial of specific inhibitors to NF-κB, which might in themselves cause apoptosis of the malignant cells, or would facilitate death in CLL cells exposed to conventional chemotherapy agents.

Acknowledgments
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