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Natural Phosphorylation of CD5 in Chronic Lymphocytic Leukemia B Cells and Analysis of CD5-Regulated Genes in a B Cell Line Suggest a Role for CD5 in Malignant Phenotype

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Chronic lymphocytic leukemia (CLL),4 the most common adult leukemia in Western countries, is characterized by the accumulation of mature, Ag-experienced, monoclonal CD5+ B cells with an activated/memory phenotype (1–3). The accumulation of malignant cells, at least in the early stages, is due to a defect in apoptosis (4) rather than to an increase in proliferation and, indeed, most malignant cells are in the G0/G1 phase of the cell cycle (5, 6). Protein alterations at the origin of the malignant transformation are currently unknown.

The origin and nature of CLL cells are undetermined; however, there are strong arguments to suspect that they have been selected by a common Ag as suggested by surface markers and the biased, restricted repertoire of their BCR genes (7, 8). Many BCRs are characterized by the accumulation of mature, Ag-experienced, monoclonal CD5+ B cells with an activated/memory phenotype (1–3). The accumulation of malignant cells, at least in the early stages, is due to a defect in apoptosis (4) rather than to an increase in proliferation and, indeed, most malignant cells are in the G0/G1 phase of the cell cycle (5, 6). Protein alterations at the origin of the malignant transformation are currently unknown.

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was naturally phosphorylated on tyrosines in all CLL samples studied, indicating that this may reflect chronic activation in vivo. In parallel, we observed that CD5 was phosphorylated upon introduction into Daudi B cells (13), suggesting that these cell lines could be of value to study the effects of CD5 signaling on gene regulation. We compared the expression profiles of empty vector (CD5−) and CD5+ Daudi B cells by means of DNA microarrays and found that several gene families already known to be activated in CLL were induced by CD5 in Daudi B cells, suggesting that some prominent features of CLL are downstream effects of CD5 signaling. Unexpectedly, several gene families such as those involved in cholesterol synthesis were induced by CD5, whereas genes involved in RNA splicing and export from the nucleus were inhibited. Comparison of these data to microarrays generated from CLL or normal B cells revealed a CD5 signature in CLL, a finding with potential biological and clinical implications.

Materials and Methods

Patients

The patients enrolled in the study were diagnosed according to cytologic and immunologic analyses and followed up at the Pitié-Salpêtrière Hospital (Paris, France) and Antoine Béclère Hospital (Clamart, France) between 1998 and 2006. The treatment (if any)-free period was at least 3 mo. Approval for these studies was obtained from the institutional review boards of the Commissariat à l’Energie Atomique and the University Hospital Pitié-Salpêtrière in Paris, France. Among the six patients studied (Fig. 3a), four had mutated, one had unmutated, and one had undetermined IgV genes. All patients gave informed consent. B lymphocytes from leukemic and normal benevolent donors were purified and maintained in culture as previously described (21). For B cell purification a negative B cell selection was applied using a “B cell enrichment” RosetteSep kit (Stem Cell Technologies). The mean purity of B cells was 96% in donors and 92.5% (89.5% to 98%) in patients.

Constructs and selection of cell lines

TL1 and TL4 mutants were amplified from full-length CD5 cDNA using a 5′ primer, GCGCGAAGATGCCCATGCGGTCTCTGCACA, and a 3′ primer, GCACCCTCAGAATGGCTACAAGGCTGACGA, and inserted into a pNT plasmid at the XhoI and NotI sites. ΔCD5 deleted the 16-aa region encompassing Y429 and Y441 was cloned into pNT as described (15). Transfected Daudi cell lines were thawed and propagated in RPMI 1640 and 10% FCS with 0.8 mg/ml geneticin for several days and stained with anti-CD5-PE mAb (Becton Dickinson) to assess cell homogeneity. Cells were washed and cultured thereafter without geneticin for 3 days and RNA was extracted.

Hybridization on DNA microarrays

RNA was extracted using the RNeasy kit (Qiagen) and RNA integrity was assessed by using the Agilent 2100 Bioanalyzer. cRNA was prepared according to the manufacturer’s “one-cycle target labeling” protocol starting from 5 μg of total RNA and hybridized to HG-U133 Plus 2.0 GeneChip oligonucleotide arrays (Affymetrix). HG-U133 Plus 2.0 arrays contain 54,675 sets of oligonucleotide probes that correspond to roughly 39,000 unique human genes. Primary image analysis of the arrays was performed using GeneChip operating software 1.2 (Affymetrix), resulting in a single raw value for each probe (“signal”). Data from each different array experiment were scaled to a target value of 100 by GeneChip operating software 1.2 using the “global scaling” method. A comparison between empty vector and CD5-transfected cell samples was performed with the GeneChip operating software 1.2. Only genes found to be present in both pNT and pNT-CD5 cell lines with a “change” labeled as significantly decreased or increased and with a ratio under 0.67 or above 1.5, respectively, were retained. p < 0.05 was considered significant.

Data analysis

Comparative analysis was performed for each gene or expressed sequence tag and for each microarray separately. The first comparison value was “pairs in average,” meaning the number of probe pairs used in the computation of “average difference” and “log average.” Average difference means an intensity difference (perfect match (PM) signal/mismatch (MM) signal) for each probe pair used, which was calculated by subtracting the intensity of the MM from the intensity of the PM. “Absolute call” can be expressed as present, absent, or marginal. “Increase” means the number of probe pairs for each gene for which PM − MM is significantly greater in the experimental data (exp) than in the baseline (base) data; to be considered significant, two conditions must be met, the first being (PM − MM)exp > (PM − MM)base ≥ change threshold (CT) and the second being ((PM − MM)exp − (PM − MM)base) ≥ percentage change threshold (PCT). Similarly, “decrease” means the number of probe pairs for each gene, for which PM − MM is significantly greater in the baseline data than in the experimental data. “Difference call” can result in five possible outcomes indicating that the level of expression of a mRNA is decreased, increased, marginally increased, marginally decreased, or without significant changes.

Both the Affymetrix average difference expression data and the present/absent calls were used in the analyses. Data analysis was conducted using Affymetrix GeneChip operating software 1.2 with a MAS 5.0 statistical algorithm (Affymetrix; more detail about the Affymetrix image analysis algorithms is available at www.affymetrix.com/products/software-specific/geosaffx). Unsupervised clustering by sample and gene dimensions by hierarchical clustering was based on the Pearson correlation and the complete linkage group aggregation technique using TIGR MeV 4.0 software (22). Data on two wild-type Daudi cell lines (Fig. 3a) were generated by Affymetrix technology and obtained from Gene Expression Omnibus (GEO) DataSet (reference identifiers GSM18895 and GSM18896).
Gene Ontology analysis and analysis of biological pathways

The FatiGO (23) web interface carries out data mining using Gene Ontology database (www.geneontology.org) for DNA microarray data. The grouping of genes by functional classes and pathways provides insight that is simply not possible by looking at particular gene lists. The data mining consists of the assignation of the most characteristic Gene Ontology term to each cluster. FatiGO implements Fisher’s exact test for 2x2 contingency tables by comparing two groups of genes and extracting a list of Gene Ontology terms distributed among the groups that are significantly different. The results of the test are corrected for multiple testing to obtain an adjusted p value. The use of FatiGO help us to characterize each cluster by the significant pathways and Gene Ontology terms identified by FatiGO and also to investigate the most strongly regulated genes within each cluster.

Gene Microarray Pathway Profiler (GenMAPP) is a program for viewing and analyzing microarray data on microarray pathway profiles (MAPPs) representing biological pathways or any other functional grouping of genes (24). When a MAPP is linked to a gene-expression data set, GenMAPP automatically and dynamically color codes the genes on the MAPP according to criteria supplied by the user. GenMAPP was used to construct and modify pathways as well as to provide access to annotation for genes and a connection with a pathway using information available from the Gene Ontology Consortium (www.geneontology.org).

Real-time PCR

Cells (2 × 10^6/ml) were incubated in complete medium for 24 h under various conditions, and RNA was purified using the RNeasy Mini Kit (Qiagen). Reverse transcription was done with random hexamers using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). Transcript expression was analyzed by PCR using the TaqMan technology as described (25). Results were analyzed using the ABI Prism 7700 sequence detection system software (PE Applied Bio System). Each reaction was normalized by the cycle threshold (Ct) of GAPDH cDNA expression. Relative expression was calculated by measuring the difference of normalized Ct between condition A vs condition B and applying the following formula: difference of expression between A and B = 2e^(−(CtA−CtB)). The following TaqMan specific primers and probes (Proligo) were selected using Primer Express Software and specificity verified using NCBI Blast software. Probes are 5'FAM and 3'TAMRA dually labeled.

The primer sequences are as follows: SC4MOL (amplicon size 146), 5'-AAGATGCTTGTGTCAGCAGCTTCA-3' (R1), and 5'-TTCCAATGGAATTGCAGACAACTCATGA-3' (P1); MVK (amplicon size 75), 5'-ACAAAGTCCCTCGCAATAC-3' (L1), 5'-CACGATCTCTGGGAACTTGA-3' (R1), and 5'-TCTGACGCCTGAGCCACAAGGG-3' (P1); SMD3 (amplicon size 107), 5'-TCAGCCTAGATGAGCGAATTT-3' (L1), 5'-TTCCACATCTCCCAAGATCA-3' (R1), and 5'-TCTGCCTCGAAGCTCTCGGTCA-3' (P1); LSM3 (amplicon size 133), 5'-TGTCTATTGGTGTGCCGATT-3' (L1), 5'-TTGGACATCTGGCAGTTTAT-3' (R1), and 5'-ACAATGTCCCTGCCATGCAA-3' (P1); CD86 (amplicon size 121), 5'-CTCTTGTGTAGATGCCCTTCT-3' (L1), 5'-AGCTACTCTAGGCTTTGTT-3' (R1), and 5'-CTGCAGACCTGCCATGCAA-3' (P1);
CD5 phosphorylation on serine residues was also investigated because it was shown to be induced in T cells following stimulation with anti CD3 or anti CD5 mAb (27). We performed CD5 immunoprecipitation and Western blotting with an anti-phosphoserine Ab and failed to detect phosphorylation on serines in fresh CLL samples even on overexposed membranes (Fig. 1a), whereas a faint phosphorylation was detected in Daudi cells. In contrast, phosphorylation on serines was clearly detected in total lysates from CLL or Daudi samples run in parallel (Fig. 1a). Thus natural phosphorylation of CD5 on tyrosine seemed to be a feature of CLL cells.

Because CD5 phosphorylation on tyrosine is essential to mediate signal in B cells (13–15), our results suggested that the CD5-transfected cell line was a bona fide model for studying the role of CD5 on gene regulation and may provide insights into the role of CD5 in CLL. This led us to analyze vector- and CD5-transfected Daudi cells by means of gene expression profiling and to compare these data with those generated from fresh CLL samples.

### Differential expression of genes involved in CD5 signaling

Comparison of (pNT) vector- and CD5-transfected B cells showed that among 38,500 potential human coding gene sequences, (>54,000 probe sets), 581 were up-regulated by CD5 (>1.5-fold and 215 were up-regulated >2-fold (supplemental information S1), whereas 525 were down-regulated >1.5-fold and 84 were down-regulated >2-fold (supplemental information S2). A Gene Ontology analysis program was used to organize the expression data of differentially expressed genes. Thirty-one gene groups were categorized according to their functions, and the number of genes up-regulated (Fig. 2 top, filled histograms) or down-regulated (open histograms) within each group is shown. It appeared that CD5 stimulated most (28/31) of metabolic pathways described here in which the number of up-regulated genes exceeds the number of down-regulated ones. In seven of these pathways all of the genes were up-regulated and none were down-regulated. These pathways included that of Jak-STAT (10/10 genes up-regulated), biosynthesis of steroids, adipocytokine signaling, and aminoacyl-tRNA syntheses (6/6), Gly-Ser-Thr metabolism (5/5), and glycolysis and tight junction (4/4). Thus, although CD5 is a negative

### Table I. Surface Ags and cytokines modulated by CD5 in Daudi B cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Unigen Identifier</th>
<th>Public Identifier</th>
<th>pNT Signal</th>
<th>CD5 Signal</th>
<th>CD5/pNT Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3</td>
<td>Hs.75703 NM_002984</td>
<td>48.8</td>
<td>125.8</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>Hs.514107 NM_002983</td>
<td>88.4</td>
<td>228.8</td>
<td>2.58</td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>Hs.75703 NM_002984</td>
<td>48.8</td>
<td>125.8</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td>CCR7</td>
<td>Hs.370036 NM_001838</td>
<td>253</td>
<td>397.9</td>
<td>1.57</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>Hs.87968 AB045180</td>
<td>211.7</td>
<td>479.3</td>
<td>2.26</td>
<td></td>
</tr>
<tr>
<td>CD80</td>
<td>Hs.838 BC042665</td>
<td>139.1</td>
<td>75.6</td>
<td>0.54</td>
<td></td>
</tr>
<tr>
<td>CD86</td>
<td>Hs.171182 NM-006889</td>
<td>175.6</td>
<td>110.1</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>Hs.11805 AL050262</td>
<td>128.1</td>
<td>68.2</td>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

* Ratios of absolute signals from CD5-/vector-transfected Daudi B cells as shown from DNA microarray analysis.

CD5 phosphorylation and Western blotting

CLL cells were processed from fresh blood and washed at room temperature in serum-free RPMI 1640 with 10 mM HEPES. CLL and Daudi cells were stimulated or not stimulated with 2 μg/ml rabbit polyclonal F(ab′)2 anti-IgM (BD Pharamingen) for 5 min and lysed. Cells (10 × 105 Daudi, or 15 × 106 CLL) were lysed at 4°C for 30 min in lysis buffer (20 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1 mM EDTA, 50 U/ml aprotinin, 1 mM PMSF, and 1 mM sodium orthovanadate) containing 1% Nonidet-P-40 detergent. Lysates were precipitated with 1 μg of the 0490D anti-CD5 mAb (26) plus protein G-Sepharose beads (Sigma-Aldrich). Proteins were separated by electrophoresis on SDS-polyacrylamide gels, electrotransferred onto PVDF membranes (Amersham Biosciences), and blotted with polyclonal rabbit anti-phospho-serine Ab (Zymed Laboratories), anti-phosphotyrosine 4G10 mAb (Upstate Biotechnology), or anti-CD5 Ab (Bio-Rad) and ECL detection (Amersham Biosciences). For tailless CD5 phosphorylation on serine residues was also investigated.
regulator of BCR-mediated early events, it is nevertheless able to stimulate the transcription of several hundred genes in B cells and to inhibit the expression of many others.

When gene array data were analyzed through a “biological process” program the above picture was confirmed as shown from the percentage of genes overexpressed or underexpressed within each category in CD5⁺ B cells vs vector-transfected cells (Fig. 2 bottom). A selection of immune genes for which signal was present in both empty vector and CD5⁺ cells is shown in Table I. Except for a few discrepancies, most of the surface Ags, cytokines/chemokines, and their receptors are also strongly expressed in CLL cells, whereas genes down-regulated by CD5 are underexpressed in CLL as inferred from the literature (see Discussion). If Gene Ontology or biological process programs were applied to compare CLL to normal B cells, a similar picture as that in Fig. 2, emerged (data not shown).

Other up-regulated genes (fold enrichment) include the following: Bcl2 (2.55) and the vascular endothelial growth factor (VEGF) (1.88); Wnt signaling pathway genes WNT10A (1.78), LDLR (1.82), and CSNK1E (1.91); MAPK cascade genes SIPS1 (2.28), MAP2K2 (2.14), MAP2K3 (1.98), and MAP2K7 (1.72); IκB/NF-κB-cascade genes RELB (2.28), IL-10 (1.70), Bcl3 (2.1), TLR8 (1.85), and STAT1 (1.74); TGFβ signaling genes TGBF1 (1.59), STAT1 and STAT3 (2.0), MAPK9 (1.60), and RUNX3 (1.88); oncogenes PIM1 (1.52) and PIM2 (1.57); cholesterol synthesis genes (14 of 16 genes were stimulated and none was inhibited, see MAPP data in supplemental information S3); and genes involved in adipogenesis such as RARA (5.0), CEBPB (2.6) and PCK2 (3.0). In brief, the overall CD5 activity can be viewed as inhibitory for RNA and protein processing and stimulatory for metabolic processes such as amino acid, steroid, and lipid metabolism and for several intracellular signaling pathways.

Evidence for a CD5 signature in CLL and validation of individual genes

Unsupervised hierarchical clustering was performed using 22,277 gene probes across B-CLL, healthy B cells, wild-type Daudi, and transformed Daudi samples. Our transformed Daudi cells clustered with the wild-type ones previously described by others (as evidenced from data available on line; see Material and Methods), thereby validating our own cell line. Thus, transfection of vector or CD5 did not significantly change the whole pattern of gene expression in Daudi cells (Fig. 3a).

Supervised hierarchical clustering was then performed on significantly CD5-modulated genes by using a very stringent test (false discovery rate = 0). One hundred twenty-two genes were selected that constitute a true CD5 signature. As shown in Fig. 3b, CLL samples are closely grouped with the CD5 signal, whereas healthy samples cluster with empty vector cells. Expression level is shown by color scale ranging from 0.5 (dark green) to 15 (red).

Table II. Seven Differentially expressed genes that were validated by real-time PCR

<table>
<thead>
<tr>
<th>Gene Designator</th>
<th>Full Gene Name</th>
<th>Relative Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC4MOL</td>
<td>Sterol C4 methyl oxidase-like</td>
<td>2.72</td>
</tr>
<tr>
<td>MVK</td>
<td>Mevalonate kinase</td>
<td>2.23</td>
</tr>
<tr>
<td>UBE2D1</td>
<td>Ubiquitin-conjugating enzyme E2D1</td>
<td>0.22</td>
</tr>
<tr>
<td>SMD3</td>
<td>26S proteasome subunit non-ATPase 3</td>
<td>0.42</td>
</tr>
<tr>
<td>LSM3</td>
<td>U6 small nuclear RNA-associated</td>
<td>0.57</td>
</tr>
<tr>
<td>CCL3</td>
<td></td>
<td>8.26</td>
</tr>
<tr>
<td>CD86</td>
<td></td>
<td>0.25</td>
</tr>
</tbody>
</table>

* Ratios of absolute signals from six CLL/four normal adult B cells and CD5-transfected/vector-transfected Daudi B cells as shown from DNA microarrays. Confirmation was obtained by quantitative RT-PCR (Fig. 4).
Thus B-CLL cells shared a common gene expression pattern with CD5-transfected cells, but not with empty vector-transfected B cells.

To further validate these results, seven genes, the expressions of which were regulated in the same manner in CD5+ Daudi cells and in CLL cells according to DNA microarray data (Table II), were studied by real-time PCR. By looking at Table I, it is intuitive that genes up-regulated by CD5 are strongly expressed in CLL and, conversely, that genes down-regulated by CD5 are underegulated in comparison to normal B cells. We selected genes with less obvious functions in B cells such as those involved in cholesterol synthesis (MVK and SC4MOL) or those involved in proteasome pathway (UBE2D1 and SMD3) or mRNA processing (LSM3). CCL3 and CD86 were selected as controls. We show (Fig. 4) that the expression of CCL3, SC4MOL, and MVK was increased whereas that of CD86, UBE2D1, SMD3, and LSM3 was decreased in both CLL and CD5+ cells in comparison to normal B cells and empty vector cells, respectively. For all seven genes, the ratios of CLL/healthy controls and CD5/vector (Fig. 4) were close to those obtained from microarrays (Table II), thereby validating the DNA microchip data described here.

CD5 phosphorylation is necessary to regulate gene expression

Our previous results demonstrated that consecutive to BCR engagement, CD5 must be phosphorylated on tyrosines within its cytoplasmic domain to regulate the BCR signal (14, 15). To further dissect the role of CD5, real-time PCR experiments were performed in Daudi cells transfected with various CD5 mutants and expressions of the above seven genes were quantified. Tailless TL1-CD5 and TL4-CD5 were deleted from the domains starting at residues 380 and 468, respectively, and ΔCD5 was deleted from the 16 residues encompassing Y429 and Y441 (14, 15). As shown in Fig. 5, TL4-CD5 deleted of the 3 serines at the C-terminal end (28) had similar regulatory activity than full length CD5. In contrast, TL1-CD5 and ΔCD5 had no significant regulatory activity. Because BCR engagement phosphorylated CD5 on tyrosines, the role of which was further highlighted in the above experiments, we analyzed the effect of BCR stimulation on gene expression.

CD5 phosphorylation was dramatically augmented in anti-IgM-stimulated CD5+ Daudi cells (Fig. 6a) but not in cells transfected with TL1-CD5 (13). In contrast, CD5 phosphorylation was moderately augmented in anti-IgM-stimulated CLL samples (Fig. 6a), as expected from their well-documented poor BCR responsiveness (16, 17, 29).

We analyzed the CCL3 and MVK mRNA that were shown above to be up-regulated by CD5. Similar levels of transcripts were found in vector vs CD5-TL1 cells stimulated or not stimulated with anti-IgM. This is at variance with CD5+ cells, which displayed much higher amounts of MVK and CCL3 transcripts (Fig. 6b). In parallel, transcript levels were higher in unstimulated CLL than in control B cells, and the ratios of CLL/controls were significantly enhanced in anti-IgM-stimulated cells.

Similar results were observed with SMD3 and LSM3, two genes down-regulated by CD5. We found no difference between vector and CD5-TL1 cells for the expression of SMD3 and LSM3, whereas CD5 cells displayed lower levels of both transcripts. Inhibition of SMD3 and LSM3 was even more pronounced in anti-IgM-stimulated CD5 cells (Fig. 5b). Note also that CLL cells displayed significantly lower amounts of transcripts than normal B cells, this difference being amplified by anti-IgM stimulation.

In conclusion, these experiments discriminated between BCR- and CD5-mediated signals. We found that CD5 needed to be phosphorylated in Daudi-cells to amplify BCR-mediated signal, whether its effect was stimulatory or inhibitory on gene expression. Although not formally demonstrated in CLL, it is likely that CD5 played a role in these cells as well. Indeed, respective amplification

![FIGURE 4](http://www.jimmunol.org/)

![FIGURE 5](http://www.jimmunol.org/)
or inhibition of the four genes studied here is very similar between CLL cells and CD5-transfected B cells.

Discussion
Based on our comparison of two cell lines that differed only for CD5 expression, we have described several gene groups either up-regulated or down-regulated by CD5. We found that many of them have already been described in CLL and were augmented or diminished in comparison to normal B cells, suggesting that the difference can be at least partly attributed to CD5 expression and phosphorylation in B-CLL cells. Having previously demonstrated that CD5 phosphorylation is needed to regulate a BCR-mediated signal (13–15), we have now shown that CD5 needs to be phosphorylated to regulate gene expression in B cells. In this respect, the finding that CD5 is phosphorylated on tyrosines but not on serines in CLL may have important implications. CD5 associates naturally to casein kinase (CK)-2 (28, 30), a serine/threonine kinase shown to play a role in cell survival and dysregulated in many cancers (31) but whose activity, if any, is unknown in CLL. In turn, CK2 phosphorylates CD5 at a cluster of three serine residues at its C-terminal end (28). Sustained CD5 phosphorylation on serine is observed if lymphocytes are treated with anti-CD5 mAbs but not after BCR or TCR stimulation (27, 30), consistent with the activation of CK2 by the cross-linking of CD5 but not of the BCR or the TCR complexes (30). Our results therefore imply that CLL cells are activated in vivo through the BCR rather than CD5.

An interesting question not addressed here is whether CD5 is differentially phosphorylated in mutated vs unmutated cases. Indeed, anergy is more prominent in mutated CLL and may therefore correlate with CD5 activation. In contrast, because CD5 is activated following BCR engagement it may be strongly phosphorylated in unmutated CLL. Unfortunately, we do not yet understand the molecular mechanism of CD5 phosphorylation, the discovery of which would help us understand the BCR signaling defects in CLL.

At variance with CLL, we failed to detect constitutive phosphorylation on CD5 from mantle zone lymphoma samples (Fig. 1) although CD5 underwent phosphorylation following anti-IgM stimulation in vitro (our unpublished results), suggesting that CD5 may not signal in this disease in vivo. Indeed, although for a few genes such as CCL4 (see below), the pattern of gene expression of CLL is very different from that published in mantle zone lymphoma.

Likewise, comparison of CD5⁺ and CD5⁻ B cells on a single CD5 expression criteria would be inappropriate for defining a CD5 signature because these cells differ by thousands of transcripts, including

![FIGURE 6. The role of CD5-phosphorylation in the regulation of gene expression.](image-url)
CD5 inducers, making it impossible to discriminate the effect of CD5 from that of other molecules. Possibly the best validation of our model is the finding that most transcripts, the expressions of which were increased or decreased by CD5, are especially relevant to CLL physiology. Previous gene array studies demonstrated that B-CLL shared a common signature related to memory B cells irrespective of their Ig mutational status (1–3). Noteworthy, it has been shown that CCR7 expression is higher in CLL than in normal memory B cells (3). In CLL, interaction of CCR7 with its ligand synergizes with integrins for the migration of malignant cells into lymph nodes (32). This is in agreement with our finding that both CCR7 (Table I) and integrin-mediated cell adhesion are up-regulated by CD5 (Fig 2). Integrins also synergize with VEGF to enhance the chemokine-induced motility of CLL cells, but not of normal B cells, through the endothelium (32). Angio genesis is indeed augmented in the bone marrow and lymph nodes of CLL patients (33), partly due to the autocrine effect of VEGF. Interestingly, VEGF enhances survival in CLL likely through interaction with the VEGF receptor, which associates with STAT1 and STAT3 (34). Our finding that CD5 up-regulated several STAT genes in addition to VEGF is therefore relevant to CLL.

Among chemokines, a slight decrease in CXCL12/SDF1 (0.59 ratio) was observed in the CD5+ cells, possibly in agreement with the finding that CLL patients had lower SDF1 plasma levels than normal controls (35). Intriguingly, two chemokines, CCL3 and CCL4, were strongly up-regulated by CD5 (Table I), whereas their respective counter receptors (CCR1 and CCR5) were unchanged. Although unexplored yet in CLL, CCL4 is increased in (CD5-) MCLs (36). Of note, B cells recruit regulatory T cells through CCL4 (37). Also relevant to CLL physiology is the enhancement of IL-10 and IL-10R by CD5 as expected from our previous results (13). IL-10, together with TGFβ signaling, may participate in the immune defect in CLL. TGFβ is released by CLL cells (38) and inhibits both hematopoietic progenitors in synergy with CCL3/MIP1α (39) and CLL B cell proliferation through interference with proliferative T cell signals (40). In this respect, normal and leukemic CD5+ B cells respond to IL-2 and IL-15 in vitro (18, 28, 41) in agreement with CD5-enhanced IL-2Rγ (Table I), IL-2Rβ, and IL-15Rα expressions (supplemental information).

The recently described Wnt pathway in CLL (42) is also induced by CD5 and may contribute to the defect in apoptosis that characterizes this disease. Several other genes pertaining to immune regulation and expressed in memory B cells and/or in CLL are up-regulated by CD5 as shown in Table I: the CD27-ligand CD70 (43), CD72, another negative regulator of BCR signaling (44), and TLR9 (45). TLR9 is of special interest as it is constitutively expressed in memory cells and able to induce B cell proliferation in these cells independently of BCR stimulation (46, 47). Because many B-CLL clones are autoreactive (17, 43, 47), they might be vigorously selected by dual BCR and TLR engagement.

CLL is characterized by the poor Ag-presenting capacity of malignant B cells, possibly in part due to low B7 Ag expression (48) which is reversed by CD40-activation (49). Given the inhibition of CD80, CD86, and several genes involved in proteinaceous processing by CD5, malignant cells may fail to efficiently load MHC Ags with peptides and deliver a second signal to effector cells. Among immune deficits in CLL, an impaired Ig production and a compromised class switching are prominent. The 2-fold increase in Bcl3 induced by CD5 should be relevant to CLL physiology inasmuch as Bcl3-transgenic mice developed altered Ig production and B cell expansion (50).

Unexpectedly, CD5 enhanced the expression of most cholesterol synthesis genes and genes involved in adipogenesis (supplemental information). This may seem anecdotal, but the ability of statins (inhibitors of HMG CoA) to induce apoptosis of CLL cells was reported years ago (51). One possible explanation for the role of lipids in the survival of malignant cells is the enhanced ability of the CD5- and BCR-associated tyrosine kinase Lyn, which resides constitutively in lipid rafts, to associate with the BCR in lipid-enriched membranes (52). Lipid-rich membranes may also help cells migrate through endothelium and lipid synthesis may favor the metabolic modifications of proteins such as those involved in the Wnt pathway (53), which are also shown to be activated in CLL cells and contribute to malignant cell survival.

The discovery that CD5 inhibited RNA processing was also surprising. Although not trendy, this subject has been studied by a few groups. It was indeed shown that CLL cells displayed RNA processing abnormalities (54, 55). Strikingly, thymidine kinase mRNA is 100-fold higher in CLL than in normal lymphocytes without the corresponding enzymatic activity (56). These results are relevant to what we observed in CD5+ cells, which may help our understanding of how the lack of ribosomal and mRNA processing and trafficking leading to the accumulation of abnormal RNAs in the nucleus might disturb the cell’s normal feedback systems or generate abnormal proteins, thereby favoring the development of malignant conditions.

Based on our (13–15) work and that of others (7–10) we hypothesize that, in CLL, chronic activation through the BCR and/or other as yet undetermined surface molecules promote cell survival. Recently, sustained signaling through the BCR by immobilized anti-IgM promoted CLL survival (57), although the status of CD5 was not investigated in this study. A likely hypothesis is that activated CD5 regulates redundant signals that all concur in B cell survival. For instance, STAT genes are involved in both TGFβ signaling and adipsogenesis pathways, whereas suppressor of cytokine signaling (SOCS) genes are involved in both cell growth and adipogenesis. In this respect it has been demonstrated that STAT5 phosphorylates PIM (58), which in turn interacts with SOCS proteins (59) It is tempting to ascribe to these genes a role in malignant cell survival, although their precise mechanisms of action need to be determined. It is worth mentioning here that acquisition of CD5 is associated with poor prognosis in diffuse large B cell lymphomas, supporting the view that CD5 is a survival factor in B cells (60).

One could note, however, that several genes, namely IL4, IL4R, CD23, and CXCR4, although present in CLL (47), are not influenced by CD5. It is intuitive that CLL cells differ from normal B cells in many aspects other than CD5 expression and activation and that several other genes may concur with the malignant phenotype. This is inferred from our quantitative data indicating that for most genes described in this article the differences between CLL and normal B cells were more significant than those between CD5- and vector-transfected cell lines. Yet, analysis of the entire set of genes modulated by CD5 clearly showed that CD5-transfected cells were closer to CLL cells whereas vector-transfected cells were closer to normal B cells (Fig. 3). Although different from CLL B cells, Burkitt lymphoma-derived Daudi cells have an intact signaling machinery and many constitutively phosphorylated proteins including Lyn (our unpublished observation), an Src kinase that couples the BCR to downstream signaling and is constitutively active in CLL (61).

Our data suggest that CD5 can be targeted in CLL either with molecules that would inhibit its phosphorylation or with Abs that may prevent its interactions with the BCR as already described (52). Thus, inhibiting CD5 expression by RNA interference should be able to induce the apoptosis of CLL B cells, provided that CD5 recycling is not too slow in this disease. In this respect it would be
worse tolerating and interesting to elicit the combined effect of an altered ubiquitin-proteasome pathway, previously shown to be affected in CLL at protein and enzymatic levels (62), with an altered control of the gene expression of several mRNA transcripts, on protein half-life and trafficking in CLL. Although we bring attention to many relevant pathways, interesting genes might have been discarded due to our elimination of transcripts absent in one cell line but present in the other and that merit further analysis. Our data provide a new useful cell model that may help to further investigate and possibly target CD5-emanating signals contributing to B-CLL leukemogenesis.

Disclosures

The authors have no financial conflict of interest.

References


