Overexpression of Cyclin D2 in Chronic B-Cell Malignancies

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Tumor progression in B-cell chronic lymphocytic leukemia (B-CLL) is thought to result from the gradual accumulation of small resting G0/G1 phase lymphoid cells rather than the proliferation of actively dividing cells. The recent identification of G1 cyclins that are likely to control both the progression through G0 and G1 phase and the G1/S transition prompted us to study the mRNA expression of D-type cyclins in the peripheral blood lymphocytes from 34 patients with B-CLL, 7 patients with lymphoplasmacytic lymphoma (LPL), and 2 patients with mantle cell lymphoma (MCL). Cyclin D2 mRNA was, on average, 5- to 10-fold overexpressed in most of the samples studied (B-CLL, 25/34; LPL, 7/7; MCL, 0/2) as compared with normal resting B lymphocytes, in which cyclin D2 mRNA was barely detectable. In situ hybridization with cyclin D2 digoxigenin-labeled mRNA probe showed that all the cells from a given sample were stained with approximately the same intensity. Cyclin D3 was never detected in any of the samples tested, whereas cyclin D1 was expressed in only 3 cases (1 LPL and 2 MCL) bearing a t(11;14) translocation. A trisomy 12 was found in 4 of 19

MAJOR INSIGHTS have been accomplished during the last few years in the understanding of cell cycle regulation in eukaryotic cells. Progression through the cell cycle results from the sequential and well-ordered activation of several components that play a key role in the G1/S and the G2/M transitions.1,2 Cyclins have appeared as major regulators in this network, because their association to the cytokindependent kinases (cdks) allows the subsequent activation of the cyclin/cdk complexes and their catalytic activity. Five distinct classes of mammalian cyclins (A, B, C, D, and E) have been identified. Cyclin A is produced in late G1 and is required for the onset of DNA synthesis, whereas B-type cyclins control the passage through M phase. The regulation of the G1/S transition appears to be more complex and is not fully understood yet. The putative role of G1 cyclins, mainly D-type cyclins and cyclin E, in such issue has emerged from transfection experiments in diverse cell lines.3-13 Cyclin E is expressed during late G1 and seems to be required for entry into S phase.5,14 D-type cyclins are synthesized earlier in G1 phase and seem to be induced in response to external agents that promote entry into the cell cycle. D-type cyclins are also likely to be regulators of the G1/S transition.15-17 The cell cycle regulation has appeared even more complex since the recent identification of cdk inhibitors,18,19 one of which being deleted in numerous cancer cell lines and tumor samples, including leukemias.20,21

The clinical behavior of patients with B-cell chronic lymphocytic leukemia (B-CLL) and some related chronic lymphoproliferative disorders is quite heterogeneous.22 Most patients experience a prolonged, indolent clinical course for many months or years, whereas others present with—or rapidly develop—a more aggressive disease. The prognostic value of the lymphocyte doubling time has been established in B-CLL.23 Nonetheless, the parameters that have been used so far to study the kinetics of B-CLL are crude and related to late phases of the cell cycle. For example, even in advanced disease, cell cycle analysis by [3H]thymidine uptake or flow cytometry and immunocytochemistry with Ki67 antibody consistently showed that more than 96% of the cells were in GO/G1 phase, whatever the importance of the cells than the proliferation of actively dividing cells. This abnormally prolonged survival of malignant cells in vivo is a major biologic characteristic of B-CLL and is presumably related to deregulated programmed cell death.26 Nevertheless, no consistent anomaly of the genes involved in the control of apoptosis has been described so far in this disease. Although rearrangements of bcl-2 gene have been only occasionally reported in B-CLL,27 the malignant cells usually contain higher levels (up to 25-fold) of bcl-2 protein than do normal lymphocytes.28 However, the relevance of such a finding to the pathogeny of the disease is still unclear. Therefore, studies investigating the role of genes that potentially control G1 progression and the G1/S transition are warranted.

Some data suggest that the deregulation of G1 cyclins may be involved in oncogenesis. Cyclin D1 was cloned (initially under the name of PRAD1) from its overexpression in...
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benign parathyroid tumors. Its encoding gene (CCND1) is mapped on chromosome 11q13 in humans. This region is amplified in several solid tumors or rearranged in some hematologic malignancies, such as mantle cell lymphoma (MCL) or splenic lymphoma with villous lymphocytes. The \textit{vin-1} gene first identified as the common site of provirus integration in retrovirus-induced rodent T-cell leukemias was recently shown to be identical to the cyclin D2 gene. The possible role of \textit{vin-1}/cyclin D2 gene in oncogenesis is suggested by the overexpression that results from provirus integration.

The goal of the present study is to investigate the expression of D-type cyclins in chronic B-cell malignancies.

MATERIALS AND METHODS

Materials. Circulating blood lymphocytes from patients with leukemic chronic B-cell malignancies and from healthy donors were collected after informed consent was obtained. Mononuclear cells were isolated by Ficoll Hypaque gradient density and were depleted of monocytes by adherence in plastic flasks (10^-6 cells/mL in RPMI 1640) for 1 hour at 37°C; samples of 10 to 20 x 10^6 cells were resuspended in GITC solution (4 mol/l guanidinium isothiocyanate, 0.5% sarcosyl, 25 mmol/L Na citrate) with 1% \textbeta;-mercaptoethanol and stored at -80°C until RNA extraction.

B lymphocytes from normal donors were separated from T lymphocytes by sheep erythrocyte rosetting before storage under the same conditions.

Cytoospin slides of freshly isolated lymphoid cells were prepared for in situ hybridization (ISH) using pretreated slides, as further described.

Additional peripheral blood or bone marrow samples from patients with acute leukemia were also analyzed.

RNA extraction and Northern blotting. RNA was extracted by the guanidinium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi. Ten micrograms of total RNA was subjected to 1% MOPS/formaldehyde agarose gel electrophoresis and blotted onto nitrocellulose membranes. The blots were hybridized overnight with 32P-labeled cDNA probes. Filters were washed twice in 2x SSC, 0.1% sodium dodecyl sulfate (SDS) at 50°C for 15 minutes and then twice in 0.2x SSC at 50°C for 20 minutes.

Autoradiography was performed for 7 to 8 days at 4°C using intensifying screens.

Murine cyclins D1, D2, and D3 and CDK4 cDNAs were kindly gifts from Dr C.J. Sherr (St Jude Children’s Research Hospital, Memphis, TN). Inserts were labeled with \gamma\textsuperscript{32}P-labeled ATP by the hexanucleotide primer technique. The oligonucleotide sequences of the coding regions of human and murine cyclins D1, D2, and D3 have approximately 90% homology.

Control of RNA loading was achieved by the subsequent rehybridization of membranes with a 26 mer oligonucleotide complementary to 28S ribosomal RNA (rRNA) labeled with \gamma\textsuperscript{32}P-ATP.

The murine erythroleukemic cell line MEL, the lymphoid cell line BaF3, and the myeloblastic cell line BTU3, which constitutively express cyclins D2 and D3, served as positive controls. BTU3 cells that derive from a myeloblastic tumor obtained after in vivo engraftment of a Friend murine leukemia virus (F-MuLV)-induced myeloblastic cell line were also used as positive control for cyclin D1 expression.

Cyclin D2 RNA slot-blot and computerized densitometric analysis. One microgram and 5 \mu g of total RNA of each sample were heat denatured and loaded onto nitrocellulose membrane using a slot-blot apparatus ( Hoefer Scientific Instruments, San Francisco, CA). Membranes were hybridized with a 32P-labeled cyclin D2 probe and subsequently with a 28S rRNA probe to normalize the amount of RNA loaded. Autoradiographs were analyzed with a Starwise software on a Compaq Deskpro 286 microcomputer (Imstar, Paris, France). Each value was expressed as the ratio between the cyclin D2 and the corresponding 28S rRNA signals. Samples from normal B lymphocytes were run in parallel for each experiment.

In situ RNA hybridization. Slides for cytoospin preparations were pretreated with 3-triethoxysilyl-propylamine and acetone, post-fixed in phosphate-buffered saline (PBS) with 1% glutaraldehyde for 1 minute, and then stored at +4°C in 70% ethanol.

Murine cyclins D1, D2, and D3 cDNAs subcloned in Bluescript plasmids were used to generate RNA probes. Sense and antisense single-strand RNA probes were synthesized by in vitro transcription using T3 and T7 RNA polymerases and digoxigenin-labeled UTP (DIG RNA labeling kit; Boehringer Mannheim Biochemica, Mannheim, Germany). For ISH purpose, RNA probes were fragmented by partial alkaline hydrolysis. After hybridization, slides were treated with proteinase K for 15 minutes at 37°C, were then treated with triethanolamine, and were thereafter dehydrated in graded ethanol. Hybridization (150 ng of RNA probe per slide) was performed overnight at 42°C. After hybridization, the slides were washed twice in 2x SSC at 50°C for 5 minutes and then in 0.2x SSC for 15 minutes. Hybridized probes were detected by enzyme-linked immunoassay using anti-DIG-alkaline phosphatase conjugate antibody and color reaction with 5-bromo-4-chloro-3-indoly phosphate (BCIP) and nitroblue tetrazolium salt (NBT) (DIG nucleic acid detection kit; Boehringer Mannheim Biochemica). Hybridization with sense RNA probe was performed in each experiment and served as negative control.

Cytogenetic analysis. Peripheral blood was incubated at 37°C for 72 hours in the presence of phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS). Chromosome preparations were made by standard techniques and were analyzed with R- or G-banding. Karyotypes were described according to ISCN (1994).

Cell cycle analysis by flow cytometry. Lymphoid cells (1 x 10^6) were suspended in 0.5 mL propidium iodide solution (50 \mu g/mL propidium iodide, 0.1% sodium citrate, 0.1% Nonidet P-40) and analyzed with a Facstar Plus (Becton Dickinson, Le Pont-de-Clair, France). Cells were measured at a flow rate of 200 cells/s; signals were recorded in list mode and analyzed with Consort 30 software (Hewlett Packard, Evry, France).

Culture of normal B cells. Freshly isolated monocyte- and T-cell-depleted B cells from normal donors were cultured at a final concentration of 2 x 10^6/mL in RPMI supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine, and antibiotics in the presence of PMA (0.3 ng/mL) and ionomycin (0.12 mg/mL) and incubated at 37°C with 5% CO\textsubscript{2} for 24 hours. Cells were collected at 6, 12, and 24 hours and stored in GITC solution at -80°C.

RESULTS

Patient population. Peripheral blood samples from 43 patients (pts) with chronic lymphoproliferative disorder (MCL, 34 pts; lymphoplasmacytic lymphoma (LPL), 7 pts; MCL, 2 pts) were studied, as well as B lymphocytes from 7 normal individuals. In each patient, the lymphocyte count was more than 10 x 10^9/L at the time of collection. Apart from appropriate clinical and cytolologic features, the diagnosis of B-CLL relied on a characteristic phenotypic pattern with CD5 positivity, weak expression of surface Igs, and, for the most recent cases, CD23 positivity and lack of reactivity with FMC7 antibody, as shown by flow cytometry analysis. Half of the patients (B-CLL, 16 pts; LPL, 4 pts; MCL, 2 pts) had previously required therapy and received...
one or more regimens based on alkylating agents and/or anthracyclines.

The expression of cyclin D2 mRNA was also studied in bone marrow or peripheral blood samples from 2 patients with acute lymphoblastic leukemia (ALL) and 15 patients with acute myeloid leukemia (AML) (AMLI, 2 pts; AML2, 6 pts; AML4, 3 pts; and AML5, 4 pts, according to the French-American-British [FAB] classification). The circulating blast cell count was more than 50 × 10⁹/L in 11 of 17 of these cases. Nine patients were studied at diagnosis and 8 at relapse.

Expression of cyclin D2 mRNA. By Northern blot, the normal resting B lymphocytes from peripheral blood were found to express a very low amount of cyclin D2 mRNA. They were consistently negative by ISH, probably because this method is slightly less sensitive. The exposure of normal B lymphocytes to PMA and ionomycin resulted in a rapid induction of cyclin D2 mRNA, reaching a plateau after 12 hours (Fig 1).

In chronic B-cell malignancies, an overexpression of cyclin D2 mRNA was observed by Northern blot in all samples but 5 from B-CLL cases and the 2 from MCL patients, in which a low level of cyclin D2 expression comparable to that of normal resting B cells was found (Table 1). In each case, there was only one 7-kb transcript (Figs 1 and 2) and no aberrant mRNA signals were detected. By computerized densitometry on slot blot autoradiographs and after normalization with 28S rRNA probe, cyclin D2 mRNA was rapidly induced in normal B lymphocytes after exposure to PMA and ionomycin. Most patients' samples overexpress cyclin D2 as compared with normal resting B lymphocytes after normalization to 28S probe. CDK4 signal (2 kb) has roughly the same intensity as cyclin D2 signal in both stimulated normal B lymphocytes and B-CLL samples.

Expression of other D-type cyclins and CDK4 mRNA. All the samples from B-CLL or LPL cases (n = 15) and normal resting B lymphocytes (n = 3) that have been assessed for cyclin D3 mRNA expression by Northern blot and ISH were negative (Fig 2).

<table>
<thead>
<tr>
<th>Cyclin D2</th>
<th>Cyclin D1</th>
<th>Cyclin D3</th>
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<tr>
<td>B-CLL</td>
<td>29/34</td>
<td>0/20</td>
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<tr>
<td>LPL</td>
<td>7/7</td>
<td>1/5*</td>
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<tr>
<td>MCL</td>
<td>0/2</td>
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<td>AML</td>
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In each column are denoted the number of samples in which an overexpression of each cyclin D has been observed by Northern blot (on left) and the number of samples tested (on right). Abbreviation: ND, not done.

* Karyotype included a t(11;14)(q13;q32) in the 3 cases in which cyclin D1 was expressed.
Cyclin D1 mRNA was not detected in patients with B-CLL (n = 20) or in normal B cells. However, a high expression of cyclin D1 was observed in 1 of the 5 LPL samples tested and in the 2 MCL samples. In these 3 cases, both cyclin D1 transcripts were found by Northern blot at the expected sizes of 4.5 and 1.8 kb.

Cohybridization of Northern blot filters with both cyclin D2 and CDK4 probes showed that the intensity of CDK4 signals (1 single 2-kb transcript) roughly paralleled those of cyclin D2 in normal resting and stimulated B lymphocytes as in B-CLL samples (Fig 1).

Correlation of cyclin D2 expression with clinical, cytogenetic, and kinetics findings. There was a trend toward a higher expression of cyclin D2 in lymphoid cells from patients with advanced B-CLL or LPL than in cells from newly diagnosed and/or untreated patients, but this was not statistically significant (Fig 3). Among the patients in whom a 20-fold or greater overexpression of cyclin D2 was observed, 5 of 7 had a long-lasting, advanced, and resistant disease. Nevertheless, in some instances, the tumor cells from patients with such clinical characteristics were found to express a low level of cyclin D2 mRNA.

In AML, cyclin D2 expression did not appear to correlate with either the white blood cell (WBC) count, the WBC doubling time, or the status of the disease.

Cytogenetic analysis from peripheral blood was performed in 21 patients from this series. No metaphases were obtained in 3 cases and karyotype was normal in 3 cases. A clonal change involving chromosome 12 was found in 5 cases. Among these, trisomy 12 was the sole abnormality in 4 cases (B-CLL, 3 pts; LPL, 1 pt) and in 1 LPL case, karyotype was complex with t(5;12)(p15;q13) and t(11;14)(q13;q32) translocations, and malignant cells were found to overexpress both cyclins D1 and D2. Other cytogenetic abnormalities included del(13)(q13;q21) in 2 cases, del(11)(q22) in 2 cases, and miscellaneous changes in 4 cases. The 2 MCL cases had a complex karyotype including a t(11;14)(q13;q32). Although the number of cases bearing a chromosome 12 abnormality was limited, the finding of trisomy 12 was apparently not correlated with a particular pattern of cyclin D2 expression.

Analysis of the cell cycle repartition of peripheral blood lymphocytes was performed by flow cytometry after staining with propidium iodide in 18 B-CLL cases. It consistently showed that 96% cells or more were in GO/G1 phase, whatever the level of cyclin D2 overexpression was. These results did not differ from what we observed using the same method in normal resting B lymphocytes.

DISCUSSION

In the present study, we investigated the mRNA expression of D-type cyclins in peripheral blood lymphocytes from patients with chronic B-cell malignancies. Cyclin D2 mRNA was found to be overexpressed in 29 of 34 B-CLL cases and in all cases of LPL (n = 7). The level of cyclin D2 expression in these disorders was, on average, 5- to 10-fold higher than in normal resting B lymphocytes. Cyclin D3 was not detected in any sample from B-CLL or LPL patients, whereas cyclin D1 was expressed only in the 3 cases (LPL, 1; MCL, 2) associated with a t(11;14) translocation.42 We failed to demonstrate the expression of cyclin D2 protein in these samples using in vivo 35S methionine incorporation and immunoprecipitation. Such a failure was also reported by others in various experiments on B-CLL cells, most likely because they are resting cells and do not incorporate 35S methionine in short culture without mitogens. However, in stimulated...
human normal T lymphocytes, the level of cyclin D2 protein has been shown to be closely correlated with RNA levels.\textsuperscript{16} There was a trend toward a higher cyclin D2 mRNA amount in patients with advanced and resistant disease, but the number of patients tested was not sufficient to reach statistical significance. In other hematologic malignancies, such as AML or ALL, cyclin D2 mRNA was inconstantly detected by Northern blot, and the level of cyclin D2 expression in the positive cases was moderate, even though the diseases were highly proliferative ones.

Because the human cyclin D2 gene (CCND2) has been mapped to chromosome 12p13\textsuperscript{,13} and trisomy 12 is the commonest chromosomal change in B-CLL and immunocytomas,\textsuperscript{43} we looked for a possible relationship between the presence of a chromosome 12 abnormality and the level of cyclin D2 overexpression. A trisomy 12 was found in 4 of 19 (21\%) of B-CLL and LPL cases for which cytogenetic analysis was available, and a translocation involving the long arm of chromosome 12 was observed in an additional LPL case, but these samples did not exhibit a particular pattern of cyclin D2 expression. In B-CLL, the trisomy 12 is usually detected early in the course of the disease and does not appear to be a secondary chromosomal change.\textsuperscript{44} Among the structural abnormalities of chromosome 12 also described in B-CLL, few of them specifically involve the 12p13 region.\textsuperscript{45} Despite the frequency of chromosome 12 abnormalities in B-CLL, no critical gene located on this chromosome has been identified so far that could contribute to the pathogenesis of the disease. Cyclin D2 gene might be a potential candidate for such a role and deserves further studies.

The overexpression of any G1 cyclin by transfection into fibroblasts\textsuperscript{14,17,45} or myeloid cells\textsuperscript{15} results in a shortening of the G1 phase, but the total length of cell cycle remains unchanged because of a compensatory prolongation of S phase. However, this effect is more obvious for the cyclins D1, D3, and E than for cyclin D2. Cyclin D2 overexpression
in B-CLL was not associated with such alterations in cell cycle repartition. Our cell cycle analysis data confirmed the results of previous kinetic studies in B-CLL by showing that only a very low subset of B-CLL cells were in S phase, whatever the level of cyclin D2 expression was.

Cyclin D2 is likely to exert a distinctive function among G1 cyclins. We have previously shown that normal human resting T lymphocytes contained very low levels of cyclin D2 mRNA. Both cyclin D2 mRNA and protein were upregulated very quickly in G1, as soon as 2 hours after mitogenic stimulation, suggesting that cyclin D2 might play a role in G0/G1 transition. Other G1 cyclins were not expressed in G0 lymphocytes (except cyclin C) and appeared much later in G1. Similarly, in normal human B lymphocytes (our data) and in mouse B lymphocytes (C. Sautès, personal communication, May 1994), cyclin D2 mRNA was rapidly induced after stimulation by PMA plus ionomycin and anti-IgM or LPS, respectively. Therefore, in B-CLL, the overexpression of cyclin D2 might reflect the progression of lymphocytes in an early G1 phase, without implicating further passage into S phase. One can hypothesize that being in early G1 instead of in a G0 state could allow these cells to be responsive to some external signals such as cytokines or to activate a set of genes that play a role in survival.

In human normal lymphocytes or cancer lymphoid cell lines, cyclin D1 and cyclin D2 were found to be differentially expressed, whereas cyclin D3, cyclin E, and all other cell cycle regulatory genes were almost ubiquitously detected. Cyclin D1 was silent in cell lines that do not bear 11q13 chromosomal rearrangements, whereas cyclin D2 was expressed in various cell lines without any association with peculiar chromosomal changes, the highest levels being observed in cell lines immortalized by Epstein-Barr virus. Both cyclins were growth factor dependent in different cell models and could be considered good candidates for potential oncogenes.

Some transfection experiments have suggested that cyclin D2 could act more as a survival factor than a proliferative one. In the interleukin-3 (IL-3)-dependent 32D myeloid cell line, cyclin D2 was quickly downregulated when the cells were deprived in growth factor, and cells died through apoptosis. Constitutive overexpression of cyclin D2 or D3 by stable transfection into this cell line appeared to be able to differ cell death after IL-3 deprivation. In the murine erythroleukemic cell line MEL, exposure to HMBA induced cell differentiation and was associated with loss of proliferation and ultimately cell death. In MEL cells stably transfected with cyclin D2, HMBA still induced differentiation but cell viability was sustained for weeks. These experiments have provided evidence that overexpression of cyclin D2 may alter the cell death program by preventing its normal activation after growth factor deprivation or induction of differentiation.

We observed that overexpression of cyclin D2 mRNA was an almost constant feature in B-CLL and the closely related lymphoproliferative disorder LPL, whereas its detection was far less consistent in other hematologic malignancies characterized by a more proliferative activity such as AML. As shown by ISH experiments, this overexpression was uniformly detected in all B-CLL cells and not only a subset of them. Whether it may play a prominent role in pathogenesis and/or progression of the disease or represent only a secondary change remains to be elucidated. Further studies should allow us to know if cyclin D2 prevents B-CLL cells from undergoing programmed cell death and allow us to investigate its relationship with other partners involved in the control of apoptosis.

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