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CD39 Expression on T Lymphocytes Correlates With Severity of Disease in Patients With Chronic Lymphocytic Leukemia

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Abstract

Introduction—Chronic lymphocytic leukemia (CLL) is a B-cell disorder, but it is also associated with abnormalities in T-lymphocyte function. In this study we examine changes in T-lymphocyte CD39 and CD73 expression in patients with CLL.

Methods—Blood samples were drawn from 34 patients with CLL and 31 controls. The cells were stained for CD3, CD4, CD8, CD19, CD39, and CD73 and analyzed by flow cytometry.

Results—Overall, patients with CLL had a higher percentage of CD39⁺ T lymphocytes than did controls. The percentage of cells expressing CD39 was higher in both CD4⁺ cells and CD8⁺ cells. Higher CD3/CD39 expression was associated with a later disease stage. No correlations between T-lymphocyte CD39 levels and CD38 or Zap-70 expression were observed. In contrast, the percentage of T lymphocytes and B lymphocytes that expressed CD73 was decreased in patients with CLL. Average B-lymphocyte CD73 expression was decreased in CLL because the majority of CLL clones were CD73. However a minority of CLL clones were CD73⁺, and patients with CD73⁺ clones tended to have earlier stage disease.

Conclusion—T-lymphocyte CD39 and CD73 expression may be useful prognostic markers in patients with CLL. Expression of CD73 on the malignant cell population in CLL may be a marker of better prognosis.

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Author Contributions

Dianne Pulte designed the study, collected data, and wrote the manuscript. Richard R. Furman contributed to the design of the experiments, recruited patients, and provided input into the revision of the manuscript. M. Johan Broekman, Joan H.F. Drosopoulos, and Kim E. Olson contributed to the design and execution of experiments and provided input into the revision of the manuscript. Harold S. Ballard and Jorge R. Kizer recruited patients and provided input into the revision of the manuscript. Aaron J. Marcus provided financial support, contributed to the design of experiments, and provided input into revision of the manuscript.

Keywords

CD39; Chronic lymphocytic leukemia; Prognosis; T lymphocyte

Introduction

Chronic lymphocytic leukemia (CLL) is one of the most common leukemias in North America, representing 25%–30% of all leukemias.¹ An estimated 14,570 new cases and 4380 deaths from CLL are expected in 2011.² The leukemia is characterized by a clonal expansion of long-lived, mature-appearing B lymphocytes that coexpress the CD5, CD19, and CD23 surface antigens.³ In contrast to many other malignancies, CLL is not known to be associated with an increased risk of thrombosis in the absence of secondary reasons for thrombosis.⁴

Abnormalities in T-cell expression and function have been reported previously in CLL.^{5,6} The absolute number of T lymphocytes is often increased in CLL, largely caused by increases in the CD8⁺ population,⁷ although the relative number is usually reduced because of the large number of malignant B lymphocytes that accumulate. However the T lymphocytes induce lower proliferation and activation responses to mitogen and may not be able to stimulate normal B lymphocytes to produce immunoglobulin (Ig).⁵ Increased, decreased, or normal expression of T-lymphocyte activation markers has been reported by various investigators.⁵ An increase in T-regulatory (T-reg) cells has been reported as well.⁶

CD39 (ectonucleotidase, NTDPase1) is an ADPase and ATPase found on the surface of endothelial cells, normal lymphocytes, and other leukocytes.⁸ It is strongly expressed on peripheral B lymphocytes, weakly expressed on marginal zone B lymphocytes, and is not expressed on B lymphocytes in germinal centers.^{9,10} Its principal function on the endothelial cell surface is to decrease platelet activation and recruitment by metabolizing platelet-released adenosine diphosphate (ADP). In leukocytes the enzyme has a variety of other direct or indirect effects as well, including modulation of cytokine expression and the inflammatory response.^{11–13} CD39 will quickly metabolize the ADP produced by its metabolism of adenosine triphosphate (ATP) to adenosine monophosphate (AMP). In normal controls, CD39 is expressed on about 6%–8% of T lymphocytes and most B lymphocytes.¹⁴ Expression on T lymphocytes is higher on activated and memory/activated T lymphocytes than on naive T lymphocytes, with 30%–35% of CD25⁺ T lymphocytes (vs. < 5% CD25[−] lymphocytes) and about 10%–12% of CD4⁺/CD45RO⁺ T lymphocytes (vs. about 4% CD4⁺/CD45RO[−] lymphocytes) expressing CD39.¹⁴ Another study of CD39 expression in T lymphocytes has suggested that CD39 is expressed primarily in T-reg cells.¹⁴ However in this study, not all T-reg cells were CD39⁺, suggesting that CD39⁺ T-reg cells may be a specific subset that could be of particular interest. In CLL, CD39 is almost always expressed on the malignant clone, although activity is slightly higher than normal in early disease and may decrease to subnormal activity in later disease.¹⁵

CD73 (5' nucleotidase—an ectonucleotidase not structurally related to CD39) is an extracellular enzyme that catalyzes the dephosphorylation of purine and pyrimidine ribophosphate and deoxyribonucleoside monophosphates to their corresponding nucleosides, with a preference for AMP.¹⁶ It is inhibited by ADP and ATP. Adenosine and other metabolites produced by CD73 stimulate T-cell proliferation and may mediate cell adhesion.¹⁶ CD73 is expressed on subsets of both T lymphocytes and B lymphocytes. CD73 expression and function are generally reported to be low in CLL.^{16,17}

Together CD39 and CD73 are responsible for metabolizing nucleoside triphosphatases, diphosphatases, and monophosphatases to their base equivalents. The preferred substrate of CD39 and CD73 is adenosine phosphate. However other nucleoside phosphates can be metabolized by these molecules. Fludarabine, a chemotherapeutic agent commonly used in CLL, is supplied clinically as fludarabine monophosphate and must be dephosphorylated in order to enter the cell. Thus CD73 expression on the malignant cells in CLL or on bystander cells may influence sensitivity to fludarabine. Additionally both ATP and adenosine have been implicated in alterations in the immune response, and thus changes in extracellular concentration of these molecules mediated by CD39 and CD73 may influence the immune response to CLL. In general, high levels of ATP are thought to stimulate the immune system, whereas high levels of adenosine are immunosuppressive. Thus higher levels of CD39 and lower levels of CD73 might be expected to correlate with less immune resistance to malignancy and worse outcomes in CLL as well as possibly greater vulnerability to opportunistic infections or secondary malignancies.

We previously examined abnormalities in CD39 expression and activity on the neoplastic B lymphocytes in patients with CLL.¹⁵ CD39 abnormalities have not been reported previously on the T lymphocytes of these patients. In this study we examined CD39 and CD73 expression on the nonmalignant T lymphocytes in CLL. CD73 levels on the malignant B-cell population in CLL were examined as well to confirm the observations of other investigators that showed a general decrease in CLL and further describe its role in this disease.

Methods

Patient Population

Patients were recruited from the Hematology Clinic and inpatient Hematology/Oncology Service at the New York Veterans Affairs (VA) Harbor Healthcare System and Weill Cornell Medical College. The protocol was approved by the institutional review boards at both institutions. The diagnosis of CLL was made by the presence of the characteristic immunophenotype (CD5⁺, CD19⁺, CD23⁺, sIg dim), and all patients had absolute lymphocytosis with a clonal B-lymphocyte population of $> 5 \times 10^9$. Patients with CLL were asked to participate in the study if they were able to give informed consent, had a hemoglobin value of > 8 g/dL, and had no contraindications to blood donation. After informed consent was obtained, blood was drawn into a heparinized tube. Blood was also drawn from a subset of healthy volunteers participating in the Thrombophilia In Cryptogenic stroke (THICK) study, which served as the control group. Patient data collected included Rai stage, complete blood count results, chemotherapy history, and Zap-70, CD38, and IgVH mutational status when available.

Fluorescence Activated Cell Sorter Analysis

Cells were single-stained with the following antibodies: CD3-APC/Cy7, CD19-FITC, CD39-PE, CD73-PE, CD4-PERCP, CD5-APC, CD69-PE/Cy7, and CD8-FITC (CD19-FITC). Antibodies were obtained as follows: all antibodies were obtained from BD Biosciences (San Jose, CA) except for the following: CD39-PE (Ancell Corporation, Bayport, MN), CD3-APC/Cy7 and CD5-APC (BioLegend, San Diego, CA). Cells were also double- or triple-stained for CD3/CD39/CD69, CD4/CD39, CD8/CD39, CD5/CD19/CD39, CD3/CD73, CD4/CD73, CD8/CD73, and CD5/CD19/CD73. Cells were incubated with antibody for 45–90 minutes at room temperature in the dark with gentle shaking. Acquisition and analysis of fluorescence activated cell sorter (FACS) data were performed on a FACSCanto using FACSDiva software (BD Biosciences). Percentage positivity and geometric mean fluorescence for normal and malignant lymphocytes were examined.

Geometric mean fluorescence was measured only for cells that were considered to be positive by FACS.

Statistics

A Student 2-sided *t* test with unequal variance was used to generate *P* values when comparing groups, except for analysis of CD69 activity in CD39⁺ vs. CD39⁻ cells, for which a Student paired 2-sided *t* test was used.

Results

Clinical Data of Participants

Data were available for 34 patients and 31 controls. Patients ranged from 35 to 86 years of age, with a median of 67 years. Eleven patients were women, and 23 were men. Among controls, the age range was 22–70 years with a median of 39 years. Sixteen controls were men and 15 were women. Seven patients were Rai stage 0, 12 were Rai stage 1, 5 were Rai stage 2, and 10 were Rai stage 3–4. Thirteen required no therapy as of the time of this writing, 8 were enrolled in a vaccine protocol for patients with early disease, and chemotherapy was planned or has been given to the remainder. Of the patients who received chemotherapy, 3 were examined either before chemotherapy or both before and after chemotherapy. Twelve patients received fludarabine as part of their treatment regimen. All but 1 patient who received fludarabine had at least a partial response. Zap-70 status was available for 16 patients and CD38 status was available for 24 patients. CD39 status was measured in all patients and CD73 status was measured in 20 patients. Four patients had experienced infectious complications of CLL, 4 had autoimmune disease related to CLL, and 9 had had secondary malignancies, including skin cancers.

CD39 Expression on T Lymphocytes

CD39 was expressed on a greater percentage of T lymphocytes from patients with CLL than from normal controls (21.9% vs. 5.7%) (Table 1). CD39 expression was higher on both CD4⁺ cells and CD8⁺ cells from patients with CLL. T-lymphocyte CD39 expression was lower in patients with stage 0 disease compared with patients with either stage 1–2 or stage 3–4 disease and in patients who did not require chemotherapy vs. those who did, with a mean percentage expression of 12% for patients who did not require chemotherapy and 28% for those who did ($2P = .01$). Only 2 patients were assessed both before and after chemotherapy, and in these patients T-lymphocyte CD39 expression decreased after chemotherapy. T-lymphocyte CD39 expression did not correlate with Zap-70 or CD38 expression (Table 2).

Mean per cell expression of CD39 on CD39-expressing cells was higher on T lymphocytes from patients with CLL than on T lymphocytes from controls (Table 3) so that not only were there more T cells expressing CD39 in CLL but also each positive T cell was more highly positive for patients than for controls (Table 3). The difference was greater for CD4⁺ lymphocytes than for CD8⁺ lymphocytes but was statistically significant in both cases. Thus in patients with CLL, a larger percentage of T lymphocytes express CD39 and those that do, express it at higher levels than do normal controls.

No correlation was observed between incidence of autoimmune disease, secondary cancer, or infectious complications and CD39 expression (data not shown).

Studies of CD39 in healthy controls demonstrated that the majority of CD39⁺ T lymphocytes were CD25⁺,¹⁴ suggesting an association between activation and CD39 expression. Therefore activation was measured by CD69 expression, an early activation

marker, because CD25 is less reliable in CLL. CD3⁺/CD69⁺ cells were slightly more likely to express CD39 than were CD3⁺/CD69⁻ cells (Table 4). As with CD39 expression overall, the standard deviation of CD39 expression in CD69⁺ cells is quite large (Table 3).

CD73 Expression on T Lymphocytes

In contrast to CD39, T-lymphocyte CD73 expression was lower in patients with CLL compared with controls. The differences are highly significant for both CD4⁺ and CD8⁺ lymphocytes (Table 5). Additionally total T-lymphocyte CD73 expression was lower in later (stage 3–4) vs. earlier (stage 0–2) disease (Table 5). A trend toward higher T-lymphocyte expression of CD73 was observed in patients who were Zap-70⁻ compared with those who were positive for this marker, but the difference was not statistically significant (Table 2).

Mean per cell expression of CD73 was similar between controls and patients with CLL for T lymphocytes overall and CD8⁺ lymphocytes but was higher in patients with CLL for CD4⁺ lymphocytes, despite a decreased percentage of CD4/CD73 double-positive cells (Table 5). Thus although fewer T lymphocytes were CD73⁺ in patients with CLL, those T lymphocytes that were positive had a higher per cell expression of CD73.

CD73 Expression on Malignant B Lymphocytes

CD73 expression on malignant B lymphocytes was generally lower compared with normal B lymphocytes (19.3% overall for CLL vs. 77% in normal B cells; $2P < .0001$.) However some patients had very high expression of CD73 (> 80%) on the malignant clone, whereas the majority had < 10% CD73 expression on the malignant clone, suggesting that most CLL clones are CD73⁻ but a minority of patients may have CD73⁺ CLL clones. In other words, 2 subtypes of CLL can be identified based on their CD73 expression. Most of the patients with CD73⁺ clones were stage 1, with 4/8 patients with stage 1 disease in whom CD73 expression was measured showing high levels of CD73 positivity and high expression of CD73 on the malignant clones (CD73 expression data not available for the 4 remaining patients with stage 1 disease). Although in general, CD73 expression on the malignant clone was either highly positive or very low, 2 exceptions to this pattern were observed. In 1 case, a single population of cells with low CD73 expression was observed, within which some cells could be considered CD73⁺ and others CD73⁻. In the second exceptional case, a subpopulation consisting of about 20% of the total CD5⁺CD19⁺ cells was CD73⁺, whereas the rest were clearly negative. Both of these patients had stage 2 disease. CD73 expression on the malignant clone was low in all other stage 2 CLL cases examined. Interestingly we were able to follow the second patient through several cycles of treatment with fludarabine and rituximab, and the CD73⁺ population was neither clearly more nor clearly less sensitive to chemotherapy than the CD73⁻ population, suggesting that if CD73 is involved in CLL prognosis, it is not through increased or decreased sensitivity to chemotherapy.

Mean per cell expression of CD73 on B lymphocytes was generally higher in controls than in patients with CLL (average geometric mean expression 1180 for patients vs. 5665 for controls; $2P < .0001$).

Discussion

T-lymphocyte abnormalities in CLL have been noted for many years. An increase in T-reg cells has been reported in CLL⁶ as well as in other hematologic and solid malignancies.¹⁸ However the molecular bases of the abnormalities are not completely understood. In this study we examined CD39 and CD73 expression in the T lymphocytes of patients with CLL. Our studies revealed that T-lymphocyte CD39 expression is higher in patients with CLL compared with controls. Furthermore, in contrast to the situation with the malignant B

cells,¹⁵ higher T-lymphocyte CD39 levels are associated with later stage disease. This was a cross-sectional study and therefore we could not determine whether T-lymphocyte CLL increases as the disease worsens or if higher levels are an early prognostic marker for worse disease. However data were available from 2 time points 6–12 months apart for 3 patients, and in each case little change in T-lymphocyte CD39 level was observed over time. In each of these patients the disease was stable (stage with little change in clinical state) and no chemotherapy was given (data not shown). This suggests that T-lymphocyte CD39 levels are stable, at least in patients with stable disease, although it does not address the issue of whether CD39 expression changes as the disease worsens.

CD39 expression was higher on cells that were positive for CD69, an early marker of activation. However the difference in CD39 expression in CD69⁺ vs. CD69⁻ cells was less than the overall difference in CD39 expression, suggesting that changes in activation status alone cannot explain the differences in the T-lymphocyte CD39 expression observed. Additionally the variability of CD39 expression on CD69⁺ cells was high, suggesting that activation may be of variable importance in CLL.

It is interesting to note that T-lymphocyte CD39 is not associated with Zap-70 or CD38 status, suggesting that it may be an independent marker of prognosis. Longitudinal studies of T-cell CD39 status as the disease progresses and examination of outcomes, particularly in patients with stage 0–2 disease, and varying levels of T-lymphocyte CD39 may resolve this issue. IgVH mutational status was not examined in this study because it was not routinely available for patients at the VA at the time the study was open and so would be missing on the majority of patients.

Coexpression of CD39 and CD73 on T-helper cells has been suggested as a marker of T-reg cells in mice.¹⁹ Another study examining CD39 expression on human T-helper cells suggested that CD39 is expressed on a subset of T-reg cells, the T-reg or activated effector/memory-like suppressor cells.¹⁴ However the same study did not demonstrate a strict correlation between T-reg/rem cells and CD73.¹⁴ CLL is known to be associated with an increase in T-reg cell numbers,⁶ although the increase in T-reg cells in the published study was only about 2-fold compared with controls, whereas the percentage of CD4⁺ cells that are CD39⁺ is increased by > than 3-fold, suggesting that CD39⁺ T-reg cells may be associated with CLL to a greater extent than CD39⁻ T-reg cells. T-reg cells have been reported to be immunosuppressive in vitro.¹⁴ This suggests that 1 mechanism by which high T-lymphocyte CD39 may affect outcome in CLL is through increased immunosuppression.

Of note, several lines of evidence suggest that the increase in T-reg cells is not the only factor leading to higher T-lymphocyte CD39 levels in patients with CLL. First, expression of CD39 is increased on CD8⁺ cells as well as on CD4⁺ cells. The significance of CD39 in CD8⁺ cells is less well characterized than in CD4⁺ cells. However 1 publication suggests that CD8⁺CD39⁺ cells exhibit more specific cytotoxic activity than do CD8⁺CD39⁻ T cells, which exhibit more natural killer (NK) cell-like activity.²⁰ Additionally, CD4⁺CD39⁺ cells seem to represent only a subset of T-reg cells. Further research is needed to determine whether this subset of T-reg cells is of particular significance in CLL and cancer in general and how it differs from the CD39⁻ T-reg population.

CD73 levels are generally lower in patients with CLL compared with controls. Most CLL clones are CD73⁻, leading to a low expression of CD73 on the total B-lymphocyte population in patients with CLL. However a subset of patients had a CD73⁺ clone and we identified at least 1 patient with 2 subclones, 1 CD73⁺ and 1 CD73⁻. The patients we identified who expressed CD73 highly on their malignant clones (4/35) had stage 1 disease, suggesting that CD73⁺ CLL may be associated with less aggressive disease. The patient

with 1 CD73⁺ and 1 CD73⁻ clone had stage 2 disease and required chemotherapy. Interestingly the CD73⁺ clone was neither more nor less chemosensitive than the CD73⁻ clone (data not shown). This suggests that if CD73 is associated with less aggressive disease, the mechanism is not through increased sensitivity to chemotherapy-induced apoptosis.

CD73 levels are decreased in the T-lymphocyte population of patients compared with controls, with a decrease in both the CD4/CD73 and the CD8/CD73 populations. In contrast to results with CD39, there is not a strong correlation between T-lymphocyte CD73 status and stage, although overall there is lower expression of CD73 on T lymphocytes in patients with CLL compared with controls, and a trend toward lower expression in both CD4⁺ and CD8⁺ cells is observed.

The physiologic mechanisms by which T-lymphocyte CD73 and CD73 on malignant cells mediate severity of disease in CLL are unclear at this time. One mechanism might be through an increase in biologically available fludarabine in the tumor milieu when CD73 expression and activity are higher. A previous publication examining CD73 mRNA levels on malignant cells in CLL found that higher CD73 mRNA conferred a higher probability of response to fludarabine in a univariate analysis but not in a multivariate analysis.²¹ However mRNA levels may not correlate with protein expression or activity and therefore it is not possible to entirely rule out a role for CD73 in induction of fludarabine sensitivity. Because the majority of the patients we evaluated responded to fludarabine, we could not evaluate this hypothesis. The 1 patient who did not respond clinically had high CD39 and low CD73 levels (data not shown). However in 1 patient who had both CD73⁺ and CD73⁻ clones, both clones responded about equally to fludarabine, suggesting that the CD73 status of the malignant clone is not in itself enough to cause differential sensitivity to this agent. Possibly other factors, including amount of CD39-produced AMP in the environment and CD73 expression in T lymphocytes, may interact to confer higher or lower sensitivity to fludarabine. Further research is needed to evaluate this possibility.

Several limitations of the study should be mentioned. First, the control group and the patient group differed with respect to age and sex ratio. However no correlation between sex or age and CD39 expression has been observed, making it unlikely that either of these factors changed the outcome significantly. Additionally the modest sample size makes it difficult to evaluate subgroups such as stage or patient characteristics fully. Finally, a larger study, preferably with a longitudinal design, is needed to evaluate changes in T-lymphocyte CD39 that may occur during disease progression.

In summary, CD39 expression is increased in the nonmalignant T-lymphocyte population in patients with CLL. There is a greater increase in patients with later stage disease. In contrast CD73 expression is decreased in patients with CLL compared with controls, but the level of CD73 expression is not strongly associated with stage or the requirement for chemotherapy. CD73 expression on the malignant clone may be associated with less aggressive disease, but this observation needs confirmation in a larger study.

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CD39 Expression (Standard Deviation) As Percentage Expression on Total T Lymphocytes and T-Lymphocyte Subsets

Table 1

Variable	Controls	All Patients	Stage 0	Stage 1–2	Stage 3–4
T Lymphocytes	5.7 (4.1)	21.9 ^a (14.9)	11.2 (5.6)	21.3 ^b (15.0)	31.1 ^b (14.8)
CD4 ⁺	7.5 (3.5)	23.7 ^a (19.3)	10.4 (8.9)	18.3 (14.8)	40.1 ^b (21.5)
CD8 ⁺	5.0 (5.2)	21.0 ^a (19.6)	13.1 (8.5)	21.1 (20.7)	24.6 (22.6)

^a₂*P* 0.05 vs. control.

^b₂*P* 0.05 vs. stage 0.

Table 2

CD39 and CD73 Expression on T Lymphocytes Compared With Zap-70 and CD38 Expression

Variable	Zap-70 ⁻	Zap-70 ⁺	CD38 ⁻	CD38 ⁺
CD39	20.2	28.4	22.0	21.1
CD73	27.7	11.6	18.7	10.6

2P> .05 for all comparisons.

Table 3

Geometric Mean Per Cell Expression (Standard Deviation) of CD39 and CD73 in CLL Patients and Controls As Measured in Arbitrary Units

Variable	CD39 Controls	CD39 Patients With CLL	CD73 ⁻ Controls	CD73 ⁻ Patients With CLL
T Lymphocytes	1759 (608)	2129 (782)	1577 (325)	1785 (506)
CD4⁺	1987 (535)	2792 ^a (1019)	1118 (383)	1613 ^a (703)
CD8⁺	1129 (392)	1490 ^a (585)	2236 (594)	2232 (1392)

^a₂*P* < .05 vs. control.

Table 4Percentage Activated (CD69⁺) Vs. Nonactivated (CD69⁻) T Lymphocytes That Express CD39

Variable	CD3 ⁺ /CD69 ⁺ (SD)	CD3 ⁺ /CD69 ⁻
% CD39 ⁺	28.9 (20.4)	15.0 ^a (7.2)

* $P < .05$ for comparison, with N = 12 samples examined.

Table 5

CD73 Expression on Total T Lymphocytes and T-Lymphocyte Subsets in Patients With Early and Later Stage Disease

Variable	Controls (SD)	All Patients (SD)	Stage 0–2 (SD)	Stage 3–4 (SD)
T Lymphocytes	28.8 (6.6)	12.9 ^a (11.1)	15.4 ^a (12)	6.6 ^{a,b} (3.7)
CD4⁺	19.0 (6.5)	10.1 ^a (6.2)	11.4 ^a (8.1)	7.6 ^a (2.1)
CD8⁺	48.6 (18.6)	17.1 ^a (13.9)	20.9 ^a (7.6)	7.6 ^a (10.8)

Abbreviation: SD = standard deviation.

^a₂*P* < .0001 compared with control.

^b₂*P* < .05 vs. stage 0–2.