Decreased Actin Content of Lymphocytes From Patients With Chronic Lymphocytic Leukemia

By Richard Stark, Leonard F. Liebes, Denise Nevrla, Maryrose Conklyn, and Robert Silber

Actin, a major cytoskeletal protein, was quantitated in normal and chronic lymphocytic leukemia lymphocytes. The actin content of normal human blood lymphocytes was 2.2 ± 0.4 mg/10^9 cells and represented 6.8% ± 1.8% of the total cellular protein. A significant decrease (p < 0.001) was noted in chronic lymphocytic leukemia lymphocytes that contained 1.4 ± 0.3 mg actin/10^8 cells, constituting 4.3% ± 1.1% of the total protein. Normal T and B cells did not differ in actin content. Reduced actin levels were found in the T as well as in the B lymphocytes of "B-cell" chronic lymphocytic leukemia. The possible importance of the decreased actin level in the anomalous capping response and motility of chronic lymphocytic leukemia lymphocytes is discussed.

MATERIALS AND METHODS

Heparinized blood obtained from normal subjects and untreated patients with CLL was centrifuged on Ficoll-Hypaque gradients. The diagnosis of CLL was established by standard staging criteria. Informed consent was obtained according to the Helsinki Conference. The mononuclear cells were depleted of monocytes by adherence to Falcon plastic culture dishes or by centrifugation through percoll gradients. The degree of cellular purity was assessed by Coulter Counter sizing. The lymphocyte preparations generally contained less than 2% monocytes and less than 10% platelets. In some experiments, complete removal of platelets was achieved by defibrination rather than heparin anticoagulation. Since the actin content of these preparations did not differ from those obtained with heparinized blood, the results obtained with both types of preparations were pooled. Purified monocytes were harvested from the culture dishes using lidocaine or from the interface after percoll gradient centrifugation. The cases are summarized in Table 1.

T- and B-Lymphocyte Subpopulations

T and B cells were assayed by standard rosetting techniques using neuraminidase-treated (Eo) and complement-coated (EAC) sheep erythrocytes, respectively. Preparations were enriched in T or B lymphocytes by the combination of two methods. T cells were purified after forming Eo rosettes by centrifugation through Ficoll-Hypaque and lysis of sheep erythrocytes as previously described. In addition, B cells were further separated using a modification of a procedure based on the attachment of B cells to antibody-coated plastic dishes. For this method, anti-(Fab)_2 was purified as described by Chess and Schlossman. Five milliliters of the antibody, at a concentration of 1 μg/ml, was incubated at 4°C in 10-cm Falcon tissue culture dishes overnight. After decanting, the plates were washed 5 times with phosphate-buffered saline (PBS) prior to use for cell purification. Five milliliters of a cell suspension from which most of the T lymphocytes had been removed by formation of Eo rosettes were incubated at a concentration of 5 × 10^9/ml in RPMI containing 15% fetal calf serum (FCS) in the anti-(Fab)_2-coated dishes for 30 min at room temperature. The nonadherent T and null cells were decanted gently and washed 3 times with PBS. The B cells were eluted by incubation with 10 ml of human gamma globulin in RPMI-FCS (5 mg/ml) for 1 hr at 37°C with gentle swirling. After decanting, the plates were washed twice with RPMI-FCS. The eluted cells were washed 3 times with PBS. This procedure yielded preparations with 58%–89% B cells and 87%–99% T cells in normal subjects and from 90% to 97% B cells and 52% to 83% T cells in CLL preparations.

Actin Assays

Actin was quantitated by scanning of polyacrylamide gels following electrophoresis in SDS or after isoelectric focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Neville using a linear gradient of 10%-20% acrylamide on a slab gel. Frozen lymphocyte pellets were suspended in 5 mM Tris buffer at 4°C in a concentration of 2 × 10^9 cells/ml and sonicated for 10 sec at 60 W power using a Heat Systems Sonifier equipped with a microprobe. An aliquot containing 100–300 μg protein was mixed with 10 μl 20% SDS, 10 μl 100 mM dithiothreitol (DTT) and boiled for 5 min before application to the acrylamide gel. The electrophoresis gels were stained with Fast Green, destained using a 5:35:60 mixture of acetic acid:ethanol:H_2O, and scanned at 625 nm with a Beckman Acta II spectrophotometer. The area under the peak comigrating with rabbit skeletal muscle actin was measured and expressed as a percentage of the area under the entire scan.

Isoelectric focusing and two-dimensional gel electrophoresis were performed according to the method of O’Farrell using the modification of Planagan and Lin. The isoelectric focusing gels were 3 × 13 mm. Cell proteins were solubilized by sonicating cell pellets in a lysis solution containing 9.5 M urea, 5% β-mercaptoethanol, and 0.2% NP-40 at a concentration of 10^6 cells/ml. Some experiments were carried out in which 1 mM diisopropylfluorophosphate (DFP) (Sigma Chemical Co., St. Louis, Mo.) or 3 mM EGTA were present in the lysis buffer. Rabbit skeletal muscle actin, prepared according to the method of Spudich and Watt, was used as a standard.
ACTIN IN NORMAL AND CLL LYMPHOCYTES

Table 1. Clinical Characteristics of Patient Population

<table>
<thead>
<tr>
<th>Patient</th>
<th>Stage</th>
<th>WBC (x 10^3/μl)</th>
<th>Lymphocytes</th>
<th>Actin (mg/10^6)</th>
<th>Mean Cell Volume (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.N.</td>
<td>III</td>
<td>56</td>
<td>70</td>
<td>3.7</td>
<td>1.4</td>
</tr>
<tr>
<td>P.K.</td>
<td>I</td>
<td>16</td>
<td>87</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>H.S.</td>
<td>IV</td>
<td>106</td>
<td>87</td>
<td>3</td>
<td>1.0</td>
</tr>
<tr>
<td>E.S.</td>
<td>0</td>
<td>24</td>
<td>65</td>
<td>28</td>
<td>1.0</td>
</tr>
<tr>
<td>D.P.</td>
<td>0</td>
<td>18</td>
<td>—</td>
<td>—</td>
<td>1.8</td>
</tr>
<tr>
<td>S.M.</td>
<td>0</td>
<td>24</td>
<td>—</td>
<td>—</td>
<td>1.4</td>
</tr>
<tr>
<td>M.G.</td>
<td>I</td>
<td>24</td>
<td>90</td>
<td>6</td>
<td>1.5</td>
</tr>
<tr>
<td>D.F.</td>
<td>IV</td>
<td>650</td>
<td>82</td>
<td>4</td>
<td>0.8</td>
</tr>
<tr>
<td>D.M.</td>
<td>0</td>
<td>22</td>
<td>67</td>
<td>28</td>
<td>1.9</td>
</tr>
<tr>
<td>B.B.</td>
<td>0</td>
<td>24</td>
<td>54</td>
<td>10</td>
<td>1.2</td>
</tr>
<tr>
<td>S.B.</td>
<td>III</td>
<td>33</td>
<td>90</td>
<td>8</td>
<td>1.4</td>
</tr>
<tr>
<td>L.B.</td>
<td>I</td>
<td>22</td>
<td>59</td>
<td>36</td>
<td>1.7</td>
</tr>
<tr>
<td>M.W.</td>
<td>IV</td>
<td>55</td>
<td>89</td>
<td>8</td>
<td>1.3</td>
</tr>
<tr>
<td>H.L.</td>
<td>0</td>
<td>21</td>
<td>57</td>
<td>12</td>
<td>1.5</td>
</tr>
<tr>
<td>L.R.</td>
<td>II</td>
<td>45</td>
<td>90</td>
<td>9</td>
<td>1.7</td>
</tr>
<tr>
<td>R.B.</td>
<td>I</td>
<td>83</td>
<td>85</td>
<td>8</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Patients 159 ± 20.9

Normal subjects 177 ± 6.5

Cell volumes were obtained as previously described and corrected for the hyperosmolarity of the isoton suspending fluid.

Isolelectric focusing gels, stained with Coomassie blue and destained as above, were allowed to expand in 10% acetic acid for 2 hr prior to scanning at 576 nm. The area under the peaks corresponding to the β and γ isoelectric point of nonmuscle actins was measured with a Houston Omniscribe 2 channel recorder equipped with an electronic integrator. Purified actin from normal and CLL lymphocytes consisted entirely of β and γ components (unpublished observations). A direct measurement of the actin present on the gels was obtained by relating the area with values obtained from a standard curve generated from 0.5-5.0 µg purified rabbit skeletal muscle actin. The assay was linear from 0.5-5.0 µg actin. For all experiments, only β and γ peak values falling within this range of linearity were used for quantitation. Total cell protein was measured by the method of Lowry et al. using bovine serum albumin as a standard.

RESULTS

The results of initial experiments using SDS-PAGE, summarized in Table 2, indicated that CLL lymphocytes contained significantly less actin than normal lymphocytes. Three other protein peaks (designated 1, 2, and 3 in Fig. 1A) analyzed as a control, revealed no differences between CLL and normal lymphocytes.

Despite the resolution obtainable by a 10%-20% acrylamide gradient, the possibility remained that other proteins ranging in molecular weight from 40,000 to 45,000 were not distinguished from actin. We therefore adapted the use of isoelectric focusing to improve the resolution of actin from other cellular proteins. The use of a narrow pH gradient of 4.5-6.5 served to exclude a large percentage of the cellular proteins from the region where the β and γ nonmuscle actins are focused. This is demonstrated in Fig. 1B, which shows their separation in a lymphocyte extract. Over 95% of the protein in the segment of the isoelectric focusing gel containing the β and γ actins migrated with rabbit skeletal muscle actin when subjected to SDS-PAGE in a second dimension. It was therefore concluded that isoelectric focusing of lymphocyte extracts was suitable as a quantitative method for determining actin content.

The results obtained with this method, also summarized in Table 2, confirmed a lower actin content in CLL lymphocytes (p < 0.001). These differences were found whether the levels were expressed per 10^6 cells or as the percentage of the total cellular proteins. While the average volume of lymphocytes from patients with CLL was slightly lower than that of normal subjects (159 versus 177 fl), this difference is within 10%. Furthermore, when the actin content was plotted against cell volume, an r value of 0.31 indicated no significant correlation. The data therefore indicate that cell volume is not a major parameter controlling the actin content per cell. The extent of the decrease was not related to the absolute lymphocyte count or percentage of B cells (Table 1). Diminished levels were observed in patients with stage 0 disease as well as in the patients in other stages. The data suggest a correla-

Table 2. Actin Levels in Normal and CLL Lymphocytes

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Percentage Total Protein (mg/10^6 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>7</td>
<td>8.4 ± 2.1</td>
</tr>
<tr>
<td>IEF</td>
<td>20</td>
<td>6.6 ± 1.8</td>
</tr>
<tr>
<td>C.LL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>8</td>
<td>4.5 ± 1.8†</td>
</tr>
<tr>
<td>IEF</td>
<td>16</td>
<td>4.3 ± 1.0‡</td>
</tr>
</tbody>
</table>

*Method used to quantitate actin: SDS, densitometric scanning of SDS-PAGE gels; IEF, densitometric scanning of isoelectric focusing gels.

†p < 0.002 (Student's t test), p < 0.01 (Mann-Whitney U test) when compared with normal values obtained by SDS method.

‡p < 0.001 (Student's t test), p < 0.011 (Mann-Whitney U test) when compared with normal values obtained by IEF method.
tion between disease stage and decrease in actin level, \( r = -0.46, p < 0.05 \). By and large, lower levels were found in patients with advanced disease. However, the study of a large series is needed to establish such a relationship. Similar ratios of \( \beta \) to \( \gamma \) actin were found in normal (2.2 ± 0.3, \( n = 10 \)) and in CLL (2.0 ± 0.5, \( n = 10 \)) lymphocytes.

The results of experiments determining the actin content of lymphocyte preparations enriched with T or B cells obtained from normal subjects and patients with CLL are shown in Fig. 2. Normal B and T lymphocytes had a similar concentration of actin. CLL B lymphocytes had a significantly lower actin content than normal B lymphocytes. The actin level in CLL T lymphocytes was the same as in CLL B lymphocytes and significantly lower than in normal T lymphocytes. Similar results were obtained when actin was expressed as percent of total cell protein or on a per-cell basis.

Although all cell populations were depleted of monocytes by adherence to plastic, the possibility was considered that the higher levels observed in normal
lymphocytes reflected monocyte contamination. The actin content of monocytes was determined to be 2.8 ± 0.8 mg/10⁷ cells or 4.9% ± 0.9% of the total protein (n = 4). As such, even a level of 10% monocyte contamination of normal lymphocyte populations, which is 5 times higher than normally observed in our preparations, could not account for the increased actin content.

The report that proteases could complicate the quantitation of contractile proteins in human PMNs²⁰ raised the possibility that the observed differences in actin content could stem from lower proteolytic activity in normal than in CLL lymphocytes. For this reason, actin assays were performed in the presence of 1 mm DFP. Under these conditions, the average actin content was 11% higher than in control normal or CLL samples. The addition of DFP, however, had no effect on the relative actin levels of CLL and normal lymphocytes. The actin content of CLL lymphocytes was still 70% that of the normal cell. The presence of EGTA in the lysing buffer had no effect on this difference.

DISCUSSION

The present studies demonstrate that actin is a major protein in human lymphocytes, which is decreased in the B and T cells of patients with CLL. While work from several laboratories has documented alterations in the organization of this protein with cell transformation or differentiation,²¹,²² to the best of our knowledge, this is the first report of a decrease in the content of this protein in a human neoplastic cell. The lower level may stem from diminished synthesis or increased degradation. No evidence for increased proteolysis in CLL was obtained. A generalized decrease in protein synthesis would be unlikely, since other proteins in CLL lymphocytes were not significantly reduced. Although differential expression of actin genes may occur, as demonstrated for T lymphocytes in culture,²³ the lack of a difference in the β-γ ratio between CLL and normal lymphocytes does not favor this as a possible explanation. A preferential loss of actin as a result of membrane shedding is an alternate mechanism. Such shedding has been described in a variety of neoplastic cells,²⁴ including lymphoid malignancies.²⁵,²⁶ Since actin is a major protein component of membranes,²⁷,²⁸ it may be lost along with membrane particles if shedding occurs in vivo.

Actin appears to play a fundamental role in nonmuscle cell motility by forming microfilaments and interacting with other proteins such as myosin.²⁵ Several lines of evidence also implicate actin as functioning in the capping of certain membrane ligands.²⁵,²⁶ Capping has been shown to be affected by agents such as cytochalasin-B and local anesthetic drugs, which are known to perturb microfilaments.²⁵,²⁶ Through the use of electron microscopy²⁵,²⁶ or immunofluorescence,²⁵,²⁷ actin or microfilaments have been shown to be concentrated in the region underlying the cap. Other studies

attachment:Fig. 2. The actin content of T and B lymphocytes from normal or CLL donors. B and T lymphocytes were purified and the actin content measured as described in Materials and Methods. Percentage "B" and "T" cells were determined using EAC and E₁ rosette techniques. The values listed represent the range of purity of the enriched preparations on which the data were obtained. Each point is the mean of 2 or 3 determinations from a given sample. Mean value is indicated by horizontal line. CLI B or T lymphocytes were different, respectively, from normal B or T lymphocytes. p < 0.002 (Student's t test); p < 0.01 (Mann-Whitney U test).
have demonstrated that cross-linking surface receptors with ligands results in increased actin associated with the cell membrane.37,38
The method employed in the present study quantitates total cell actin independent of polymerization state, interactions with other proteins, or distribution within the cell. Since polymerization of actin is concentration dependent, it is tempting to speculate that the decreased actin observed in CLL lymphocytes results in the impaired motility and capping in these cells. The polymerization of actin may be inadequate to initiate or sustain what is required for capping or cell movement to occur. This interpretation must be tempered by the realization that factors such as energy-dependent mechanisms,39 other cytoskeletal42,43 or actin regulatory proteins44,45 are also involved in capping and cellular motility.

While CLL is generally considered a B-cell dyscrasia, a number of studies have shown that CLL T cells differ from normal T cells in several respects. These include decreased responsiveness to T-cell mitogens,46,47 defective “helper” function,47 intracellular enzyme activity,48 isoenzyme distribution,49 and surface receptor anomalies.50 The decreased actin content of CLL T cells provides further evidence of the abnormality of this population. It remains to be established whether the differences observed reflect T-cell involvement by the neoplastic process or a response by normal T cells to the abnormal B-cell clone.

The pathophysiologic significance of this finding can be viewed in the light of a recent report that the intracellular actin content may affect cell deformability and influence the recirculating properties of lymphocytes.52 Since the CLL lymphocyte kinetics are known to differ markedly from the in vivo recirculation of normal lymphocytes,53,54 the decreased actin content may contribute to this anomalous behavior of the CLL lymphocyte.

REFERENCES

40. Pozza T, Corps AN, Montecucco C, Hesketh TR, Metcalfe JC: Cap formation by various ligands on lymphocytes show the same dependence on high cellular ATP levels. Biochim Biophys Acta 602:558–566, 1981
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