Anomalous Function of Vimentin in Chronic Lymphocytic Leukemia Lymphocytes

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Chronic lymphocytic leukemia (CLL) lymphocytes manifest anomalous motility and cap formation. Since these processes involve cytoskeletal proteins, vimentin from intermediate filaments of normal and CLL lymphocytes was investigated using hetero- and monoclonal antisera. The antisera reacted predominantly with a 60-kD polypeptide, following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of total lymphocyte proteins. When lymphocytes were stained by indirect immunofluorescence, normal lymphocytes demonstrated well defined cytoplasmic fibrils that capped spontaneously after contact with a glass surface and incubation at 37°C. This capping was dependent on energy and intact microfilaments. Lymphocytes from patients with CLL showed several patterns.

LYMPHOCYTES from patients with chronic lymphocytic leukemia (CLL) have abnormalities that suggest a defect involving cytoskeletal proteins or membranes. These include the smudging of cells on blood smears, decreased cell motility, diminished shedding of membrane proteins, and enhanced susceptibility to microtubule-disrupting drugs. Microfilaments and microtubules have been widely studied, and their major proteins, actin and tubulin, characterized in normal and CLL lymphocytes. Intermediate filaments (IF) have not been as fully investigated in lymphocytes. As in other cells of mesenchymal origin, the IF of lymphocytes are composed of vimentin, a polypeptide with a subunit molecular weight of 55-58,000. Earlier studies have shown that IF and vimentin are involved in the capping phenomenon. This article compares the organization of vimentin in lymphocytes from normal subjects and patients with CLL.

MATERIALS AND METHODS

Cell Preparation

Lymphocytes were obtained from blood of normal donors and patients with CLL by centrifugation on Ficoll-Hypaque gradients. Monocytes were depleted by adherence or by centrifugation on Pervan gradients, as previously described. Lymphocyte subpopulations were selectively obtained by rosetting lymphocytes with neuraminidase-treated sheep red blood cells and separating the rosetted cells by centrifugation on a second Ficoll-Hypaque gradient. Cells were maintained at 0-4°C during all the purification steps. The diagnosis of CLL was established by standard criteria. Fifteen of the 20 patients were untreated, and the remainder had been treated with chlorambucil (CLB). The results with cells from patients who had received therapy did not differ from those obtained with cells from the untreated patients.

Immunofluorescence Technique

Lymphocytes were suspended in RPMI 1640 medium, pH 7.4, at a concentration of 10^6 cells/ml and maintained on ice until used. One hundred microliters of the cell suspension in the center of a glass coverslip was incubated in a Petri dish at 37°C with humidification and 5% CO₂. After incubation, the supernatant was gently poured off and the coverslips sequentially immersed in methanol followed by acetone for 5 min at -20°C. The coverslips containing fixed cells were incubated with 50 μl of the primary antiserum at the appropriate dilution for 1 hr at 37°C. They were then washed 3 times in PBS, incubated with the fluorescein-labeled secondary antiserum for 1 hr at 37°C, and then washed 5 times in PBS. The coverslips were air dried at room temperature and stored under PBS at 4°C until use. Stained coverslips were mounted in PBS-glycerol and viewed on a Zeiss or Leitz microscope equipped with epifluorescence optics. Photographs were taken using Kodak-Tri-X film exposed at 400 ASA, a 63x objective, and automatic exposure control.

Antiserum

A polyclonal anti-IF antiserum obtained as a gift from Dr. E. Wang (Rockefeller University) was used at a 1:100 dilution for immunofluorescence staining and at a 1:200 dilution for immunoblotting. This antiserum reacts against vimentin-containing cells from several species. This was further confirmed by demonstrating its reactivity with filamentous structures in gerbil fibroma cells known to contain vimentin and its failure to react with PtK2 cells, an epithelial-derived cell line. A monoclonal antiserum to vimentin and tropomyosin was the gift of Dr. J.-C. Lin (Cold Spring Harbor Laboratory) and was used at a 1:500 dilution. A polyclonal anticytokeratin, obtained from Dr. T. T. Sun (New York University), and an anti-P70 neurofilament antiserum, prepared as previously described, were used at a 1:100 dilution. Fluorescein- or...
rhodamine-labeled goat anti-mouse or anti-rabbit antisera were used at a 1:20 dilution.

**Treatment of Cells**

Colchicine (Sigma, St. Louis, MO) was prepared as a 0.1 mg/ml stock solution in PBS. Cytochalasins B and D (Sigma) were prepared as a 1 mg/ml stock solution in 95% ethanol. An equal concentration of ethanol without drug was added to control cells. In some experiments, cells were maintained in suspension by constant agitation for 1 hr at 37°C and smeared on slides prior to fixation.

**Immunoblotting**

Lymphocytes depleted of monocytes were sonicated in 9 M urea and 1% β-mercaptoethanol at a concentration of 10^7 cells/ml. Sodium dodecyl sulfate was added to a final concentration of 1% and the suspension heated for 5 min in a 100°C water bath. Extracts from the equivalent of 1.5 x 10^7 cells were electrophoresed through a 12.5% polyacrylamide slab gel and then transferred to nitrocellulose paper using the “Western” blotting technique.23 The nitrocellulose paper was incubated sequentially with 5% bovine serum albumin, normal goat serum, and the polyclonal antivimentin antiserum (1:200 dilution). Frequent washes using PBS were carried out between the above steps. The binding of the rabbit immunoglobulins was visualized with an anti-rabbit avidin-biotin peroxidase technique24 using a Vecta stain kit (Vector Laboratories, Inc., Burlingame, CA).

**RESULTS**

**Characterization of the Antisera**

The polyclonal anti-1F antiserum was used in most of the experiments. Peripheral blood mononuclear cells were stained with the polyclonal anti-IF antiserum after incubation on glass coverslips for 1 hr at 37°C to allow cell spreading to occur. When the cells were maintained at 4°C, the majority of the lymphocytes demonstrated numerous long fibrils running throughout the cytoplasm (Fig. 1A). Lymphocytes remained rounded, with a variable number of cells taking on a motile configuration. After the 1-hr incubation at 37°C, approximately one-half of the cells demonstrated intense staining of a cap at one pole of the cell. The remainder of the cells were unstained (Fig. 1B). When present, monocytes were easily distinguishable as large cells with well spread cytoplasm and a negatively stained bean-shaped nucleus. These cells demonstrated fine fibrils distributed throughout the cytoplasm.

The monoclonal antivimentin antiserum gave a similar staining pattern at 0°C and after incubation at 37°C. Staining by the monoclonal and polyclonal antisera was abolished by prior incubation with purified vimentin. The anti-P70 neurofilament antiserum produced faint cytoplasmic staining. The anticytokeratin antiserum did not stain any structure within the cytoplasm.

An immunoblotting technique was utilized to establish the reactivity of the antiserum with a lymphocyte polypeptide of the appropriate molecular weight (Fig. 2). The polyclonal antivimentin antiserum reacted predominantly with a 60K dalton polypeptide. There was some reactivity with two other polypeptides with approximate molecular weights of 53,000 and 90,000 daltons.

**Intermediate Filament Capping in Normal Lymphocytes**

The organization and distribution of IF were studied as a function of the duration of incubation. As noted above, cells maintained at 0–4°C demonstrated a fibrillar pattern. When incubated at 37°C, the fibrillar pattern would disappear, being replaced by intense staining at one pole of the cell, indicating cap formation. The percentage of cells with the juxtanuclear cap reached a peak after a 1-hr incubation and then began to decrease (Fig. 3). With more prolonged incubations, cells with caps or a fibrillar pattern began to reappear.

A number of experiments were performed to evaluate the mechanism of this spontaneous capping of IF (Table 1). Low temperatures and azide inhibited the process. When cells were maintained in suspension rather than being allowed to be in contact with the coverslip for the duration of incubation, few capped cells could be visualized. The majority of the cells showed persistence of a fibrillar pattern. Colchicine, over a range of 10^-6–10^-5 M, had little effect on enhanced IF cap formation. Higher concentrations of colchicine were toxic to lymphocytes. Cytochalasin B and D both prevented IF cap formation, with the cells showing a persistent fibrillar pattern. Colchicine did not reverse the inhibition induced by the cytochalasins, but caused instead a thickening and decrease in the number of cytoplasmic fibrils.

**The Organization and Distribution of IF in CLL Lymphocytes**

When lymphocytes from patients with CLL were examined, several patterns were seen. Eight patients had cells that stained weakly or not at all for the fibrillar pattern seen when normal lymphocytes are maintained at 0–4°C (Fig. 1C). When these cells were incubated at 37°C for 30 or 60 min, a much lower percentage of capped cells was seen (Fig. 1D). In 10 cases, a distinct fibrillar pattern could be discerned. In four of these cases, capping occurred, whereas in six cases, only a small percentage of cells capped. In one of these patients, capping could be induced by colchicine. Finally, in two of the patients studied, the cells demonstrated short, thick, irregular fibrils prior to incubation at 37°C (Fig. 1E). In one of these patients, capping occurred in 74% of the cells (Fig. 1F), whereas only
Fig. 1. Immunofluorescent labeling of normal and CLL lymphocytes. Rabbit antivimentin antiserum and fluorescein-labeled goat anti-rabbit IgG were incubated with lymphocytes as described in Materials and Methods. Normal lymphocytes (A, B) and CLL lymphocytes (C, D, E, and F) are shown when maintained at 4°C (A, C, E) or after incubation at 37°C for 60 min (B, D, F). The arrows indicate monocytes.
Table 1. Effect of Drugs and Incubation Conditions on Intermediate Filament Capping

<table>
<thead>
<tr>
<th>Condition</th>
<th>Percent Capped Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44</td>
</tr>
<tr>
<td>Low Temperature (4°C)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Azide (10^{-2} M)</td>
<td>12</td>
</tr>
<tr>
<td>Cells in suspension (1 hr at 37°C)</td>
<td>2</td>
</tr>
<tr>
<td>Colchicine (5 x 10^{-6} M)</td>
<td>50</td>
</tr>
<tr>
<td>Cytochalasin-B (4 μg/ml)</td>
<td>5</td>
</tr>
<tr>
<td>Cytochalasin-D (4 μg/ml)</td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2. Reaction of antivimentin antiserum with lymphocyte proteins shown by immunoblotting. The proteins from a normal lymphocyte extract (shown in lane 3) were transferred to nitrocellulose paper and reacted with normal rabbit control serum (lane 1) and the antivimentin antiserum (lane 2). The paper was stained as described in Materials and Methods. Molecular weight markers are shown for comparison in lanes 4 and 5.

13% capped in the other. There was no apparent correlation between the staining pattern and clinical stage, according to the Rai classification.

Overall, of 20 CLL patients studied, 21% ± 5.1% (mean ± SE) of the cells had IF caps compared to 47% ± 3.9% in 12 normal donors (Fig. 4). The difference was significant with a p < 0.002.

Since CLL lymphocytes are predominantly B cells and normal peripheral blood lymphocytes are predominantly T cells, it seemed possible that this difference in percentage of capped cells reflected inherent differences between T and B cells. To exclude this possibility, normal blood lymphocytes were partially purified to obtain populations of cells enriched in T and B cells. In two experiments, preparations of lymphocytes enriched to 72% and 80% of normal B cells demonstrated 41% and 38% capped cells, respectively. This did not differ from the percentage seen in the enriched T cell subpopulations, which were 35% and 43%.

DISCUSSION

The studies reported here demonstrate that peripheral blood lymphocytes contain a network of IF composed of vimentin. At 37°C and after contact with a
glass surface, these IF are reorganized to form a cap at one pole of the cell. This cap formation is an active process, requiring energy and intact microfilaments. The enhancement of IF cap formation by colchicine suggests that intact microtubules may retard the capping process.

The spontaneous capping of IF that occurs in lymphocytes after surface contact has been described in other cell types after replating of cells in culture. In many other cell types, IF can be induced to form caps or perinuclear whirls after treatment with an agent that disrupts microtubules. This, along with a more direct demonstration of an association between microtubules and IF, has suggested that IF are organized actively around microtubules. The basis for the reorganization of IF after treatment that disrupts microtubules is poorly understood. The present studies suggest that, at least for lymphocytes, this reorganization is an active contractile process involving microfilaments. Prior studies have demonstrated that actin in lymphocytes similarly accumulates at one pole of the cell to form a “cap” after surface contact. In addition, Felix and Strauli have described the rearrangement of 100 Å filaments from large bundles to a loose distribution pattern in the cytoplasm of L5222 leukemic cells associated with locomotion.

The reorganization of IF may be required for lymphocyte motility and adherence to a surface. Contact with a surface may induce the dissociation of IF and microtubules or disrupt microtubules. Following this dissociation, the IF may be able to bind to actin containing microfilaments. Such binding may then lead to activation of actinomyosin-mediated contraction of the entire complex. The recognition of a common antigenic site on vimentin and tropomyosin (which binds to microfilaments) suggests that such a microfilament binding site might be present.

Studies of CLL lymphocytes demonstrated heterogeneity in both initial staining patterns and in the percentage of capped cells visualized after a 1-hr incubation. A similar variation has been observed for both the shedding of several membrane receptors and in the capping of C3 receptors by CLL lymphocytes. In the latter study, there was no correlation between C3 capping and clinical stage, but there was a correlation with response to therapy.

This heterogeneity suggests that there are a number of mechanisms that result in the anomalous motility of CLL lymphocytes. In many cases, the weak initial staining implies a decreased amount of IF or vimentin in the cells. The failure to visualize a cap in these cells may simply result from the small amount of vimentin present. However, in association with the defective motility in CLL lymphocytes, this decreased amount of protein is likely to be functionally important. A decrease in actin content in CLL lymphocytes has been previously reported.

In some cases, there appears to be normal or increased amounts of vimentin based on the intensity of initial staining. These instances may have an alternative explanation. The aberrant organization visualized in two patients suggests that, in these patients' lymphocytes, the IF subunit polypeptides might be functionally abnormal. Cases in which IF organization was normal and capping decreased might, perhaps, be explained on the basis of an abnormal interaction between the IF and other cytoskeletal components.

The present report adds another example to the growing list of cytoskeletal abnormalities in CLL lymphocytes. Further characterization of interactions between IF and other components of the cytoskeleton, as well as a detailed characterization of the proteins involved, may help clarify the mechanism of the anomalous motility in this cell.

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