Chromatin Phospholipids in Normal and Chronic Lymphocytic Leukemia Lymphocytes

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SUMMARY

Certain phospholipids are associated with the nonhistone chromosomal proteins extracted from normal B- and chronic lymphocytic leukemia lymphocytes. The ratio of phospholipids to nonhistone chromosomal proteins was constant with the different methods used for isolating nuclei and extracting the chromatin, although the various methods allowed a different recovery of total lipids from chromatin. Three phospholipids were extractable from the nonhistone protein fraction, but their respective ratios varied in chronic lymphocytic leukemia compared to normal B-lymphocytes. The most significant variation concerns the reduction of sphingomyelin content in leukemic lymphocytes, since this phospholipid in vitro affects both DNA stability and transcription.

INTRODUCTION

It has been suggested that polyanions, such as NHCP, RNA, and phosphoproteins, play a direct role in the control of DNA replication and transcription (10, 20). Moreover, a possible involvement of phospholipids in DNA-regulating mechanisms has been postulated because of the larger phospholipid content of active chromatin compared to repressed chromatin (32), the variations in phospholipid content during different phases of the cell cycle (1, 11, 12, 14, 15, 18), and the stimulating effect of these lipids in vitro on DNA and RNA polymerase activity (13, 22, 30).

Morris hepatoma cells contain more phospholipids than normal hepatocyte nuclei (36). In particular, sphingomyelin appears to be increased in the purified chromatin fractions; it may make DNA more accessible to polymerases (7), since it exhibits in vitro a destabilizing effect on DNA (27).

The actual presence of phospholipids within chromatin has been confirmed by the demonstration of their association with NHCP in human lymphocytes (24). Therefore, to further characterize the molecules interacting with DNA to regulate its function, we examined the phospholipid content and the composition of the different chromatin fractions obtained from CLL lymphocytes, which present particular modifications of NHCP and histone fractions (25, 40).

MATERIALS AND METHODS

Sources of Materials. Heparin (Liquemin) was purchased from Roche, Milano, Italy; Dulbecco's phosphate-buffered solution containing NaCl (8 g/liter), KCl (0.2 g/liter), Na2 HPO4 (1.15 g/liter), and KH2PO4 (0.2 g/liter), Ca2+ and Mg2+ free, and Roswell Park Memorial Institute Tissue Culture Medium 1640 buffered with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid were obtained from Eurobio, Paris, France. Polyvalent antiserum to human immunoglobulin coupled with fluorescein isothiocyanate was obtained from Cappell, Downingtown, Pa. Thin-layer plates for chromatography were layered with Silica Gel G or H (Merck, Darmstadt, West Germany). Bio-Rex 70 was purchased from Bio-Rad, Richmond, Calif. Solvents were glass distilled, and all chemicals were reagent grade.

Isolation of Normal and CLL Lymphocytes. Fresh, heparinized peripheral blood was obtained from 7 patients with CLL and from normal donors. Lymphocytes were separated on Ficoll-Hypaque gradient (5) and were washed twice with Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 20% fetal calf serum. The B-cell-enriched population from normal donors was obtained by means of the nylon wool column filtration technique (19, 38). The percentage of B lymphocytes was determined both by detection of surface membrane immunoglobulins with anti-immunoglobulin serum (heavy and light chains) coupled with fluorescein isothiocyanate (31) and by the erythrocyte antibody rosette technique (4). The erythrocyte rosette test was used as membrane marker for T-lymphocytes (2).

Extraction of Chromatin Fractions. Different procedures were used for isolating nuclei and obtaining the chromatin fractions.

Nuclei were isolated from 5 × 10⁷ lymphocytes by centrifugation in 0.32 M ice-cold sucrose containing 3 mM CaCl₂, 0.5% Triton X-100, and 10 mM Tris-HCl, pH 8.0 (17). Histones were extracted from purified chromatin with 0.2 M H2SO4 (6); the NHCP-DNA complex was precipitated by centrifugation and was resuspended in 10 mM Tris-HCl, pH 8.0, and 1% sodium dodecyl sulfate. DNA was sedimented by centrifugation at 200,000 × g for 24 hr (34).

Nuclei, chromatin, and histones were extracted as in Method 1. The NHCP-DNA complex was resuspended in buffered aqueous-phenol, and the NHCP were extracted by the method of Teng et al. (37).
Nuclei were suspended in 0.25 M sucrose, 2 mM CaCl₂, and 10 mM Tris-HCl, pH 8.0; layered on 2.1 M sucrose in the same buffer; and purified at 50,000 x g for 50 min. Nuclear sap was extracted by centrifugation in 0.14 M NaCl. Chromatin was then homogenized in 1.0 M NaCl and 20 mM Tris-HCl, pH 7.5. Total nucleohistone was precipitated from the homogenate adjusted to a final concentration of 0.4 M NaCl by centrifugation at 200,000 x g for 2 hr.

In order to remove the residual histones, Bio-Rex 70, which was previously equilibrated with 0.4 M NaCl and 0.02 M Tris-HCl, pH 7.5, was added to the supernatant at a final amount of 20 mg Bio-Rex 70 per mg protein and was sedimented by centrifugation at 6000 x g for 15 min (21).

**Extraction and Characterization of Lipids.** Lyophilized DNA, histones, and NHCP were treated with chloroform-methanol (9); lipid separation was performed by TLC, and iodine vapors were used for detecting spots (35). Phospholipids were further separated by 2-dimensional TLC with plates of Silica Gel H (250-μm layers) containing 10% magnesium silicate (39).

Qualitative evaluation of phospholipids was performed by comparison with known standards and by specific reactions, such as ninhydrin for aminolipids and a modified Dragendorff method for choline (28). For quantitative analysis the spots were scraped off the plates directly into tubes for determination of lipid phosphorus. For background evaluation, large blank areas were scraped.

**DNA, RNA, Protein and Lipid Quantitative Determination.** For comparative purity analysis, the chromatin content in DNA and RNA was determined by the diphenylamine and orceine reactions, respectively (29). Proteins were quantitatively evaluated by the Lowry colorimetric method (23), and lipid phosphorus was evaluated by the Bartlett assay (3).

**Electron Microscopy.** The lymphocyte nuclei isolated by the different procedures were fixed with 1.7% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 15 min, postfixed with 1% OsO₄ in Veronal acetate buffer (Winthrop Laboratories, New York, N. Y.), dehydrated, and embedded in Araldite (Fluka AG, Buchs, Switzerland). Thin sections were double stained with uranyl acetate and lead citrate and examined with a Siemens Elmiskop 102 electron microscope (Siemens AG, Berlin, W. Germany) operating at 80 kV.

**RESULTS**

In all cases of CLL examined, the number of lymphocytes in peripheral blood was about 3 x 10⁸/cu mm. B-cells, identified by immunofluorescence demonstration of surface membrane immunoglobulins and by the erythrocyte antibody rosette test, were at least 92% of total lymphocytes, while T-cells, detected by the erythrocyte rosette technique, were less than 8%.

The B-cell enrichment of normal blood samples resulting from nylon wool filtration could be immunologically demonstrated to attain 88%. The 2 cell populations under study (CLL and normal), therefore, contained B-lymphocytes in almost the same percentage.

Both normal and CLL B-cell nuclei presented nuclear membrane contaminants after sucrose gradient isolation (Fig. 1, a and b), while after Triton X-100 treatment an almost complete removal of nuclear membrane was observed (Fig. 1, c and d).

The purity of normal and CLL chromatin, as revealed by UV absorbance, was nearly identical, although some differences were evident in the absorbance spectra obtained with the different extraction methods, showing a higher purity for the Triton X-100-extracted chromatin (Chart 1).

Chromatin extracted by Triton X-100 methods contained a reduced average amount of phospholipids (0.5%) compared to that obtained with sucrose gradient extraction (1.6%).

The larger phospholipid quantity recovered with the last method (Table 1) is probably due to the presence of nuclear membrane contaminants in isolated nuclei, as revealed by ultrastructural analysis (Fig. 1, a and b).

The absolute quantities of the single chromatin components were similar in normal B and in CLL lymphocytes, except for the larger histone amount in CLL cells (Table 1). Almost the same quantity of phospholipids could be extracted with the NHCP fraction in both normal B and CLL lymphocytes with all the methods used. Phospholipids were not present in detectable amounts in DNA, RNA, or histone fractions in either normal B or CLL lymphocytes, as previously reported for total human lymphocytes (24).

The reduced recovery of phospholipids with Method 3 (Table 1) could indicate a loss of membrane lipids during purification and isolation of the single chromatin components.

However, although some differences were obtained in phospholipid recovery by applying the different chromatin extractions (Table 1), phospholipids always represented about 2% of NHCP (Table 2).

Qualitative analysis of phospholipids extractable from NHCP of normal B- and CLL lymphocytes indicated that 3
Table 1

Comparison of nuclei isolated by Triton X-100 (Methods 1 and 2) and sucrose (Method 3), showing the DNA, RNA, chromosomal protein, and phospholipid content

<table>
<thead>
<tr>
<th></th>
<th>% in chromatin</th>
<th>Isolated single fractions (mg)</th>
<th>Recovery in %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>CLL</td>
<td>B</td>
</tr>
<tr>
<td>DNA</td>
<td>35.5±4.6</td>
<td>31.5±4.2</td>
<td>4.5±0.6</td>
</tr>
<tr>
<td>RNA</td>
<td>5.6±0.6</td>
<td>5.0±0.6</td>
<td>0.7±0.09</td>
</tr>
<tr>
<td>Chromosomal proteins</td>
<td>58.3±6.9</td>
<td>63.0±7.6</td>
<td>7.2±0.9</td>
</tr>
<tr>
<td>Histones</td>
<td>4.0±0.6</td>
<td>6.4±0.8</td>
<td>34.8±10.6</td>
</tr>
<tr>
<td>NHCP</td>
<td>3.2±0.4</td>
<td>4.0±0.5</td>
<td>34.8±10.6</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.5±0.06</td>
<td>0.4±0.05</td>
<td>0.07±0.001</td>
</tr>
</tbody>
</table>

Method 1 and 2 (Triton X-100)

Method 3 (sucrose gradient)

<table>
<thead>
<tr>
<th></th>
<th>% in chromatin</th>
<th>Isolated single fractions (mg)</th>
<th>Recovery in %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>CLL</td>
<td>B</td>
</tr>
<tr>
<td>DNA</td>
<td>37.6±4.5</td>
<td>31.6±4.1</td>
<td>4.1±0.5</td>
</tr>
<tr>
<td>RNA</td>
<td>5.3±0.5</td>
<td>5.4±0.7</td>
<td>0.6±0.07</td>
</tr>
<tr>
<td>Chromosomal proteins</td>
<td>55.5±6.6</td>
<td>61.0±7.9</td>
<td>6.6±0.8</td>
</tr>
<tr>
<td>Histones</td>
<td>3.6±0.4</td>
<td>6.0±0.9</td>
<td>34.3±10.6</td>
</tr>
<tr>
<td>NHCP</td>
<td>3.0±0.3</td>
<td>3.5±0.4</td>
<td>28.6±10.6</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>1.6±0.17</td>
<td>1.8±0.23</td>
<td>0.07±0.001</td>
</tr>
</tbody>
</table>

* Recovery referred to total chromosomal proteins.

Table 2

Phospholipids extracted from the NHCP of normal B- and CLL lymphocytes

<table>
<thead>
<tr>
<th></th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids:NHCP (weight ratio)</td>
<td>0.021</td>
<td>0.019</td>
<td>0.023</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>50.0±2.5</td>
<td>20.1±1.7</td>
<td>19.5±1.2</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>12.3±1.5</td>
<td>38.0±2.4</td>
<td>37.8±2.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>39.8±2.0</td>
<td>40.0±2.9</td>
<td>41.7±3.6</td>
</tr>
</tbody>
</table>

Phospholipids were present: sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine. Their average ratio for all methods was 50:10:40, respectively, in normal B-lymphocytes and 20:40:40, respectively in CLL lymphocytes (Table 2).

Therefore, in CLL lymphocytes, sphingomyelin was reduced to about 40%, whereas phosphatidylcholine was increased 4-fold with respect to normal B-cells; the amount of phosphatidylethanolamine was similar in the 2 cell populations.

DISCUSSION

The presence of a phospholipid fraction bound to NHCP has been observed with different chromatin extraction procedures with or without the use of Triton X-100, extensive extraction with acids and salts, or purification with ion-exchange resins. On the other hand, phospholipids were absent in DNA, RNA, and histone fractions (24, 25).

Moreover, the relative composition of the phospholipids extractable from NHCP of normal B- and CLL lymphocytes was quite different from that of nuclear membrane (41) or total chromatin (10).

The total amount of phospholipids bound to NHCP was similar in normal B- and CLL lymphocytes with all methods. However, the 3 phospholipids were present in different ratios in CLL lymphocytes compared to normal B-cells.

In other diseases, such as Morris hepatoma, a 2-fold increase in sphingomyelin content occurs in chromatin, although the amount of other phospholipids remains unmodified. The higher sphingomyelin concentration of hepatoma chromatin has been interpreted as affecting DNA synthesis and cell replication (7). This hypothesis is based on the influence that sphingomyelin exhibits on DNA stability in vitro. In fact, when present in low concentrations, sphingomyelin increases the stability of the DNA double helix, whereas in higher concentrations, the same phospholipid reduces DNA stability (27). Moreover, DNA duplication is strongly enhanced in vitro by the addition of sphingomyelin at a high concentration to DNA polymerase systems (30).
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served a noticeable decrease in the amount of spherogomelynlin extractable from NHCP, associated with a 4-fold increase of the phosphatidylcholine content.

Some other alterations affecting chromatin structure and composition have been reported in CLL, such as a reduced DNA:histone ratio, an increase of the intermediate-molecular-weight NHCP (8, 25, 40), a consistent enhancement of some histone fractions (25), and an increased heterochromatin content (8, 25). All these changes can be related to cellular abnormalities displayed by CLL lymphocytes, mainly consisting of an immunological deficiency that causes an accumulation of old cells in peripheral blood.

The reduced percentage of spherogomelyn in phospholipids extractable from CLL lymphocyte NHCP is probably related to the reduced transcriptional ability of these cells. In fact, the marked reduction affects only spherogomelyn, which reduces DNA thermal stability (27) and promotes DNA polymerase activity (30). Phosphatidylcholine, which does not cause comparable effects on DNA (26), appears to be increased.

In which way phospholipids and, in particular, sphingomyelin may interact with DNA and influence gene regulation is still a matter of theoretical speculation. Phospholipids may participate in derepression of RNA synthesis, acting as counterligands by limiting the DNA:histone interactions (10, 32) and probably inducing an increase of the histone molecule α-helical content, as observed for apolipoproteins (33).

It can therefore be concluded that, besides the previously reported changes affecting the chromatin components of CLL lymphocytes, the altered relative ratios of phospholipids bound to NHCP could influence both chromatin assembly and gene transcription.

REFERENCES


Chromatin Phospholipids in CLL Lymphocytes


Fig. 1. Electron micrographs of isolated lymphocyte nuclei. a, normal B-lymphocyte nuclei isolated with sucrose gradient centrifugation (Method 3). Note the presence of nuclear membrane contaminants. x 30,000. b, CLL nuclei isolated with sucrose gradient centrifugation (Method 3). The inner and outer membranes are clearly visible. x 30,000. c, Triton X-100-isolated normal B-lymphocyte nuclei (Methods 1 and 2). Note the absence of nuclear membranes. x 15,000. d, Triton X-100-isolated CLL nuclei (Methods 1 and 2). The complete absence of the nuclear membranes is evident at high magnification. x 40,000.

Fig. 2. Two-dimensional TLC of the phospholipids bound to the NHCP. a, normal B-lymphocytes; b, CLL lymphocytes. Phospholipids, 200 µg, were applied. Or, origin; Sph, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; I, 1st run; II, 2nd run. Solvents used for the 1st run were chloroform:methanol:aqueous ammonia (65:25:5, by volume); solvents used for the 2nd run were chloroform:acetone:acetic acid:methanol:water (3:4:1:1:0.5, by volume). For photography the plates were stained with H2SO4.
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2a

2b

PE
PC
Sph

Or

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