AgNOR clusters as a parameter of cell kinetics in chronic lymphocytic leukaemia

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Abstract

Aims—To study correlations between the pattern of silver stained nucleolar organiser regions (AgNORs) in chronic lymphocytic leukaemia (CLL) and parameters of tumour kinetics. To investigate whether quantitation of the AgNOR pattern can be used to discriminate between patients with stable and progressive disease.

Methods—Peripheral blood smears from 48 patients with CLL, classified as having either stable or progressive disease (Rai stage III or IV; bulky lymph nodes or massive splenomegaly; or peripheral lymphocytes >100 x 10⁹/L), were studied. For each patient, total tumour mass (TTM) and for patients undergoing a period of observation without treatment, the TTM duplication time (DT) and the lymphocyte doubling time (LDT) were calculated.

Results—Four cell types could be distinguished according to their AgNOR pattern: (1) cells with a single cluster; (2) cells with a single compact nucleolus; (3) cells with two compact nucleoli; and (4) cells with several scattered dots. The percentage of cells with clusters was the AgNOR parameter which correlated best with TTM and LDT. Correlations were also seen between the proportion of cells with clusters and age and haemoglobin concentration. A significant correlation with DT could be detected only when age was kept constant. Linear discriminant analysis revealed that the percentage of cells with clusters was the most important prognostic factor. This alone classified 94% of the patients correctly (jackknife procedure) as either stable or progressive CLL.

Conclusions—The percentage of circulating lymphocytes with clusters of AgNORs can be used as a parameter of tumour kinetics in CLL and helps to discriminate between patients with stable and progressive disease. For practical purposes, a value of more than 13% of cells with clusters is suggestive of progressive disease.

Keywords: nucleolar organiser regions, discriminant analysis, prognostic factors, chronic lymphocytic leukaemia, cell kinetics, lymphocyte doubling time.

Silver staining of nucleolar organiser regions (AgNORs) in interphase cells has been used to evaluate cell proliferation in many tumour types.¹⁻¹⁰ Quantification of AgNORs has a predictive value in several neoplasias, such as non-Hodgkin's lymphoma,¹¹ breast carcinoma,¹² neuroblastoma,¹³ and multiple myeloma.¹⁴ The AgNOR pattern can be regarded as a parameter of cell kinetics in normal haemopoiesis,¹⁵ acute leukaemia¹⁶ and chronic myeloid leukaemia.¹⁷ The few studies on AgNORs in chronic lymphocytic leukaemia (CLL)¹⁸⁻¹⁹ have described the morphology of the method but did not investigate the physiological significance of AgNORs as a parameter of cell kinetics.

CLL is a lymphoproliferative disease of mainly quiescent lymphocytes in G₀ phase.¹⁴⁻¹⁵ In patients with stable disease there is a small tumour mass at diagnosis and the disease runs a relatively stable course, sometimes for years. Under these conditions, treatment is not necessary.¹⁵⁻¹⁶ Chemotherapy is started only when the patient presents with progressive disease. The classification into stable and progressive disease is based mainly on clinical parameters,¹³ such as anaemia, thrombocytopenia, bulky lymphadenopathy, and/or splenomegaly. Several studies have shown the importance of proliferation parameters in the prognosis of CLL.¹⁵⁻²⁰⁻²³

The aim of the present study was to describe the AgNOR pattern in peripheral lymphocytes of patients with CLL and to investigate possible correlations with the total tumour mass (TTM),¹⁴ and lymphocyte (LDT),¹⁵ and tumour doubling time (DT).²⁰ Furthermore, we were interested to see whether quantitation of the AgNOR pattern could be used to discriminate between patients with stable and progressive disease.

Methods

All patients with CLL followed at the Haematology Service of the State University of Campinas, Brazil, between August 1994 and January 1996 were included in the study. Patients were included at initial diagnosis or during follow up (at least three months) without treatment. Clinical data such as age, sex, extent and size of lymph node enlargement, and liver and spleen involvement were collected. All patients were examined by one of the authors (ILM) at each visit. Peripheral blood counts, bone marrow cytology and serum protein electrophoresis were done. In all patients, the Rai stage¹⁷ and the TTM were determined. For newly diagnosed patients who underwent a period of observation without treatment, the TTM duplication time (DT) and the LDT were also calculated. We assigned...
the patients to one of two categories: stable disease or progressive disease. A patient was diagnosed as having progressive disease when at least one of the following criteria was fulfilled: (1) Rai stage III or IV; (2) systemic symptoms; (3) bulky lymph nodes or massive splenomegaly on clinical examination; and (4) peripheral lymphocytes >100 x 10⁶/l. Patients with stable disease were followed but not treated. Those with progressive disease underwent chemotherapy. All patients had been assigned to one of the two categories prior to the analysis of the AgNOR pattern.

AGNOR STAINING
Peripheral blood smears taken from the same sample used for blood counts were fixed in acetone (seven minutes at room temperature) and incubated in a colloidal silver solution as described by Smith and Crocker (30 minutes in the dark at room temperature). They were then washed in deionised water (10 minutes), counterstained with Harris’s haematoxylin (30 seconds) and mounted in resin.

COUNTING PROCEDURE
One hundred consecutive lymphocytes were analysed in each case and a differential count was made considering four classes of the AgNOR pattern (fig 1) as described by Wachtler et al: (1) cells with a single (or rarely two) AgNOR cluster (several silver dots in a matrix inside the nucleolus); (2) cells with a single compact nucleolus; (3) cells with two compact nucleoli; (4) cells with several silver dots scattered throughout the nucleus.

Each smear was counted by two observers in a double blind manner. Variability in the number of each AgNOR pattern counted for each case by the two observers never exceeded 5%. In a preliminary study we found that the cumulative percent distribution curves of all AgNOR patterns in most of the cases stabilised after 70–100 cells had been counted. Therefore, we decided to count 100 cells/smear in order to simplify the calculations without losing accuracy. Rüschoff and Barth have also suggested this number.

TOTAL TUMOUR MASS, TUMOUR DOUBLING TIME AND LYMPHOCYTE DOUBLING TIME
The TTM was calculated as described by Jaksic and Vitale, using the following formula:

$$TTM = (L)^{0.3} + LN + S$$

where L is the peripheral lymphocyte count, LN is the diameter of the largest palpable lymph node in cm and S is the size of the spleen below the left costal margin in cm.

For patients followed for at least three months, the TTM doubling time was calculated according to the formula:

$$DT = M \times TTM_1 \times (TTM_2 - TTM_1)^{-1}$$

where TTM₁ is the TTM at the beginning and TTM₂ the TTM at the end of the observation period. M is the period of observation in months.

The LDT was calculated using the best fit equation for simple regression on WinSTAT (version 3.1) software (Kalma Inc., Cambridge, MA, USA) between the variables “date” and “lymphocyte count”. This parameter was calculated only when four or more peripheral cell blood counts were available over a treatment-free period of at least three months.

STATISTICAL ANALYSIS
WinSTAT software was used for statistical analysis. First, we determined which of the different AgNOR patterns correlated best (Spearman correlation coefficient) with the parameters TTM, LDT and DT. Second, this AgNOR parameter was correlated with age, peripheral platelet count and haemoglobin concentration. The results of previous studies led us to assume a negative correlation between AgNOR measurements and age, haemoglobin concentration and platelet count, but a positive correlation with TTM. Therefore, we were able to use one sided tests to minimise type II errors. To control for type I errors, the individual significance level was adjusted as suggested by Cross and Chaffin: $p^* = p \times [k - x + 1]^{-1}$, where $p^*$ is the adjusted significance level for an individual test, p is the nominal significance level for the sequence of tests, k is the total number of the tests, and x is the number of significant tests. As age is a prognostic parameter in CLL, we calculated...

### Table 1 - Distribution of the different AgNOR patterns in peripheral blood lymphocytes from patients with CLL

<table>
<thead>
<tr>
<th>AgNOR pattern (%) of cells</th>
<th>Mean (%)</th>
<th>Minimum (%)</th>
<th>Maximum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusters</td>
<td>14.6</td>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td>One compact nucleolus</td>
<td>63.1</td>
<td>27</td>
<td>85</td>
</tr>
<tr>
<td>Two compact nucleoli</td>
<td>18.0</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>Scattered</td>
<td>4.4</td>
<td>0</td>
<td>22</td>
</tr>
</tbody>
</table>
lated the partial correlations between the former parameters when age was controlled. Third, we correlated age with TTM, the percentage of cells with clusters, DT and LDT, and then with DT and LDT when the variable percentage of cells with clusters was constant. Finally, we did a discriminant analysis with the categories stable/progressive disease as the dependent variables and the values of the four different AgNOR patterns as the independent ones. As the AgNOR parameters had been calculated as percentages, we subjected them to an angular transformation. With this procedure we achieved a good approximation to Gaussian distribution. The goodness-of-fit was tested according to Kolmogorov-Smirnov. The classification matrix was calculated using the jackknife procedure.

Results

According to clinical criteria and an observation period ranging from three to 18 months, 23 patients were assigned to the stable category and 25 in the progressive disease category. The AgNOR pattern of the peripheral lymphocytes from 48 patients revealed four clearly defined types of silver binding (table 1). Of these types, the percentage of cells with clusters correlated best with TTM, DT and LDT (table 2). Therefore, we chose this parameter for further calculations. In the second step there were significant Spearman correlations between the percentage of clusters and age, haemoglobin concentrations, TTM, and LDT. This was not the case for DT and the platelet count. The partial correlation coefficients for haemoglobin concentration, platelet count, LDT and TTM when age was constant did not change substantially. In addition, there was a strong negative correlation between the percentage of clusters and DT, which had been 'hidden' by the influence of age (table 3).

As shown in table 4, age correlated significantly with TTM. When the variable percentage of cells with clusters was constant, there was a strong negative correlation between age and DT (r = -0.88), but not between age and TTM.

Linear discriminant analysis of the percentages of cells with the different AgNOR patterns as independent variables and the categories stable or progressive disease as the dependent ones gave the following standardised coefficients: 1.51 for cells with clusters; 0.04 for cells with a single compact nucleolus; -0.01 for cells with two compact nucleoli; and 0.22 for cells with scattered silver stained dots. The Mahalanobis distance was 2.59 (p < 0.0001).

Jackknife analysis correctly classified 45 (94%) of the 48 patients. Because the standardised coefficients showed clearly that the percentage of clusters was the most important factor for discriminating between patients with stable and progressive disease, we did another discriminant analysis with this parameter as the only independent variable. The jackknife procedure again classified 45 (94%) patients correctly. In the three cases which had been placed in the wrong category, 13% of cells contained clusters.

Discussion

Wachtler et al.26 have shown that normal lymphocytes in peripheral blood are usually quiescent (that is, in G0 phase) and contain one or two compact nucleoli when stained by the AgNOR method. After in vitro stimulation with phytohaemaglutinin (PHA), the cells entering the cell cycle develop AgNOR clusters. A close relation between the presence of AgNOR clusters and the proliferative activity of cells has been demonstrated during haemopoiesis9 and in the small intestine, liver, prostate, salivary glands, and experimental tumours.32 Although Wachtler et al.26 studied normal blood lymphocytes, which are mainly T cells, and most cases of CLL are of B cell origin, it is possible to compare the AgNOR pattern they described with that observed by us, as the metabolic events during the G0/G1 transition are similar in several vertebrate somatic cell systems.37 Thus, in CLL, lymphocytes with clusters may be regarded as belonging to the proliferative cell fraction of the tumour. The AgNOR patterns observed in the present study suggest that most cells are quiescent and contain one to two compact nucleoli; and that there is a smaller, but highly variable, fraction of cells with AgNOR clusters, entering the cell cycle. This hypothesis is confirmed by the observation that the percentage of cells with clusters is correlated with TTM and the tumour progression parameters LDT and DT. Interestingly, the correlation with DT was unmasked only when age was constant. Our finding that the clustered AgNOR pattern correlates best with TTM and LDT strengthens the concept that the cells with clusters represent the proliferative fraction in CLL. It is also interesting to note that TTM, which is a

Table 2 Spearman rank order correlation of the different AgNOR patterns (p value)

<table>
<thead>
<tr>
<th>AgNOR pattern (% of cells)</th>
<th>TTM</th>
<th>DT</th>
<th>LDT</th>
<th>p value</th>
<th>r (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusters</td>
<td>0.72 (&lt;0.0001)</td>
<td>-0.18 (0.29)</td>
<td>-0.74 (0.0004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>One compact nucleolus</td>
<td>-0.30 (0.02)</td>
<td>-0.01 (0.48)</td>
<td>-0.08 (0.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two compact nucleoli</td>
<td>-0.07 (0.31)</td>
<td>0.11 (0.37)</td>
<td>0.50 (0.02)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scattered</td>
<td>-0.18 (0.11)</td>
<td>-0.01 (0.48)</td>
<td>0.45 (0.034)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Spearman rank order correlation of percentage of cells with clusters

<table>
<thead>
<tr>
<th>AgNOR pattern (% of cells)</th>
<th>Age</th>
<th>Platelet count</th>
<th>Haemoglobin concentration</th>
<th>LDT</th>
<th>p value</th>
<th>r (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clusters</td>
<td>-0.573</td>
<td>-0.15</td>
<td>-0.32</td>
<td>-0.74</td>
<td>-0.18</td>
<td>0.72</td>
</tr>
<tr>
<td>p value</td>
<td>0.15</td>
<td>0.012*</td>
<td>0.0004*</td>
<td>0.29</td>
<td>&lt;0.0001*</td>
<td></td>
</tr>
<tr>
<td>Partial correlation coefficient for percentage of cells with clusters constant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clusters</td>
<td>-0.09</td>
<td>-0.27</td>
<td>-0.73</td>
<td>-0.81</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.30</td>
<td>0.03</td>
<td>&lt;0.0001†</td>
<td>0.01†</td>
<td>&lt;0.0001†</td>
<td></td>
</tr>
</tbody>
</table>

*p < p* = 0.0167; †p < p* = 0.025.

Table 4 Spearman rank order correlation for age

<table>
<thead>
<tr>
<th>Platelet count</th>
<th>Haemoglobin concentration</th>
<th>LDT</th>
<th>DT</th>
<th>TTM</th>
<th>p value</th>
<th>r (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.127</td>
<td>0.18</td>
<td>0.31</td>
<td>-0.60</td>
<td>-0.50</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.195</td>
<td>0.109</td>
<td>0.118</td>
<td>0.024</td>
<td>0.0002*</td>
<td></td>
</tr>
<tr>
<td>Partial correlation coefficient for age with percentage of cells with clusters constant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.05</td>
<td>-0.005</td>
<td>0.03</td>
<td>-0.88</td>
<td>-0.14</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>0.48</td>
<td>&gt;0.50</td>
<td>&gt;0.50</td>
<td>&gt;0.003†</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

*p < p* = 0.0125; †p < p* = 0.001.
clinical parameter and may underlie errors of measurement, especially of spleen size, showed the same strong correlation as LDT, which can be calculated readily. However, TTM can be obtained for every patient, as can the AgNOR pattern, whereas LDT can be measured only in those patients who have been followed for some time.

Although some authors regard age as a prognostic factor in CLL, reports are conflicting. In our investigation a partial correlation analysis showed that there is a strong negative correlation between age and DT, but not between age and LDT, when the influence of the cluster values had been eliminated. We assume that the negative correlation between age and DT indicates a more benign course of CLL in older patients. The impact on survival is not easy to evaluate because CLL occurs predominantly in elderly patients who may eventually die of a variety of diseases unrelated to CLL. Therefore, survival studies for these patients are difficult to interpret as they must consider overall mortality data for the same age group.

The clinical course of CLL is highly variable. Some patients have an indolent course, with a rather small tumour mass and a normal haemoglobin concentration and platelet count. Other patients present with systemic symptoms, bulky disease or signs of bone marrow insufficiency (progressive disease). These patients usually have a worse prognosis and require chemotherapy. Several staging systems and prognostic factors have been studied in CLL. Of those, these based on tumour mass and the kinetics of peripheral lymphocytes or tumour burden are the best predictors of prognosis.

AgNOR staining is quick and easy to use in a clinical setting. Our investigation showed that the percentage of cells with AgNOR clusters in the peripheral blood is a good parameter of tumour kinetics, correctly classifying 94% of patients in the present study. For practical purposes, a value of more than 13% of cells with AgNOR clusters is suggestive of progressive disease.

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