Differing Patterns of Human Protooncogene Expression in Peripheral Blood and Bone Marrow Acute Leukemia Cells

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ABSTRACT

The levels of protooncogene RNA in matched bone marrow and peripheral blood cells obtained from patients with newly diagnosed acute myelogenous leukemia were compared. While the absolute amounts of c-myc RNA in the matched specimens are similar, the levels are not correlated. In contrast, while the levels of c-fos RNA in the matched bone marrow and peripheral blood cells are correlated, the absolute levels of c-fos RNA differ substantially. The level of histone H3 RNA is higher in bone marrow cells than in peripheral blood cells. These substantial differences in protooncogene RNA levels between leukemic cells found in the bone marrow and in the peripheral blood make it impossible to accurately "characterize" gene expression in leukemic cells if studies are restricted to the cells in either compartment. Additionally, there appears to be a significant relationship between the levels of c-fos RNA and triose phosphate isomerase RNA and the height of the white blood cell count and between the level of c-fos RNA in marrow cells and the proportion of monocytic cells present.

INTRODUCTION

The possible relationship between protooncogene expression and neoplastic disease is under intensive investigation. For reasons of convenience many studies have utilized the peripheral blood cells of leukemic patients (1–4). The known differences in behavior between the leukemic cells present in the bone marrow and in the peripheral blood (5), however, suggest that the pattern of gene expression may not be the same in leukemic marrow and blood cells. For these reasons, we measured the level of gene activity in matched peripheral blood and marrow cells obtained from the same acute leukemic patient. Substantial differences in the level of protooncogene activity in the matched specimens were found, and the data suggest a relationship between the pattern of gene activity in bone marrow cells and the peripheral blood manifestations of the disease.

MATERIALS AND METHODS

Acquisition of Cells for Study and Preparation of RNA

Only patients with newly diagnosed untreated acute nonlymphocytic leukemia (6) were studied. Informed consent was obtained from each patient. Five ml of bone marrow aspirated from the posterior iliac crest and 10 ml of peripheral blood were placed into separate syringes containing 5 ml of 6% sodium citrate and placed on ice. The cell suspensions were layered over Ficoll-Hypaque (specific gravity, 1.077) and centrifuged at 900 rpm for 20 min in the cold, and the interface cells were removed and washed twice. An aliquot of the cells was removed to make slides for morphological evaluation, and the percent monocytes present.

The remaining cells were dissolved in guanidium isothiocyanate (9). The pelleted RNA was reprecipitated once and dissolved in distilled water.

RNA Electrophoresis, Blotting, and Hybridization

Five μg of whole cell RNA were glyoxalated and electrophoresed through a 1.2% agarose gel by the method of McMaster and Carmichael (10). RNA from the gel was blotted onto a nylon mesh (Zetabind; Cuno, Inc.), and the RNA was cross-linked by exposure to 254-nm UV light for 3 to 5 min. DNA was labeled by the random primer method to a specific activity of 1 to 2 x 10^6 cpm/μg, and 2 to 5 x 10^4 cpm/ml of hybridization buffer were used for hybridization (11). Blots were hybridized at 70°C to 3 probes simultaneously, washed by the method of Church and Gilbert (12), and exposed to X-ray film (XR; Kodak). Autoradiograms were quantitated by densitometric scanning using Quick Scan R & D (Helena Laboratories). Each autoradiogram was scanned 3 times, and the average absorbance was computed. Dilutions of various RNA samples demonstrated that the hybridization signal is proportional to the amount of RNA present in the blot.

Probes

The c-myc RNA probe was kindly provided by J. Battey (13). The fos and fms probes were kindly provided by T. Curran (14) and C. Scherr (15), respectively. The TPI probe was kindly provided by L. Maquat (16, 17). Probes for the c-myb, histone H3, c-fes RNAs, and rRNA were kindly provided by S. Ferrari (18), P. Rothberg (19), G. Stein (20), and J. Sylvester (21), respectively.

Quantitation of mRNA Levels

To avoid potential problems related to between-lane differences in the total amount of RNA which was ultimately transferred to the nylon mesh paper, the amount of mRNA was normalized to the amount of rRNA in each lane. To accomplish this the region of the nylon mesh paper, the amount of mRNA was normalized to the amount of rRNA were kindly provided by S. Ferrari (18), P. Rothberg (19), G. Stein (20), and J. Sylvester (21), respectively.

Data Analysis

Comparison of Peripheral Blood and Bone Marrow Cells. The level of gene expression and the distribution of cell types and tritiated thymidine labeling indices in the density cut matched peripheral blood and bone marrow specimens were compared using the Wilcoxon matched-pairs signed ranks test.

Correlations between Measured Parameters. Correlations were sought between the levels of activity of the various genes being studied and between the level of gene activity and the characteristics of the cells from which the RNA was extracted. For the latter studies the proportion of different cell types present in the cell population from which the RNA was extracted was determined by evaluating slides prepared from the interface cells after Ficoll-Hypaque density cut centrifugation. In contrast, when a relationship was sought between the level of gene expression in bone marrow cells and the characteristics of the peripheral blood in the patient at the time of diagnosis, the distribution of cell types were determined as described above.

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3 The abbreviation used is: TPI, triose phosphate isomerase.
PATTERNS OF PROTOONCOGENE EXPRESSION IN ACUTE LEUKEMIA

Table 1 Characteristics of the bone marrow and peripheral blood cells of eleven patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Characteristics of the peripheral blood and bone marrow in vivo</th>
<th>Characteristics of the specimens from which RNA was extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FAB type</td>
<td>WBC count/μl</td>
</tr>
<tr>
<td>1</td>
<td>M2</td>
<td>7,500</td>
</tr>
<tr>
<td>2</td>
<td>M2</td>
<td>21,500</td>
</tr>
<tr>
<td>3</td>
<td>M2</td>
<td>16,400</td>
</tr>
<tr>
<td>4</td>
<td>M4</td>
<td>79,500</td>
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<tr>
<td>5</td>
<td>M1</td>
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<td>6</td>
<td>M2</td>
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<tr>
<td>7</td>
<td>M2</td>
<td>5,900</td>
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<tr>
<td>8</td>
<td>M2</td>
<td>10,800</td>
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<tr>
<td>9</td>
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<tr>
<td>10</td>
<td>M1</td>
<td>24,700</td>
</tr>
<tr>
<td>11</td>
<td>M2</td>
<td>43,600</td>
</tr>
</tbody>
</table>

* RNA extracted from light density cells after Ficoll-Hypaque centrifugation (see "Materials and Methods" for details).

FAB, French-American-British Cooperative group; LI, labeling index; PMN, NRBC, nucleated RBC; BM, bone marrow; PB, peripheral blood.

RESULTS

Patient and Leukemic Cell Characteristics. Table 1 provides information regarding the characteristics of the bone marrow and peripheral blood cells of the 11 patients whose cells were studied. The percentage of S-phase cells and the number of nucleated RBC are greater in the marrow than in the peripheral blood (P = 0.007 and 0.068, respectively). These characteristics are not, however, correlated with each other. The WBC count ranges from 5.9 to 82.6 x 10^3 cells/μl with the percentage of leukemic cells ranging between 3 and 93%. The median values for the WBC count and the percentage of leukemic cells in the peripheral blood are 21.5 x 10^3/μl and 52%, respectively. Note that the cell differentials presented in Table 1 represent the percentage of cell types in the post-density cut specimen from which RNA was extracted (see "Materials and Methods").

The percentage of leukemic cells in the marrow ranges from 55 to 97% with a median value of 83%. There is a significant inverse relationship between the percentage of S-phase cells in the marrow and the percentage of myeloblasts plus promyelocytes in the peripheral blood (r values for parametric and nonparametric tests range from -0.624 to -0.775 with P values f 0.005 to 0.009) (Fig. 1).

Histone H3 RNA Levels. The level of histone H3 RNA present in the leukemic cells varies widely between specimens, being not detectable in 2 of 9 bone marrow specimens or in 4 of 11 blood specimens. Fig. 2A provides the Northern blot analysis, and Table 2 provides the densitometric scanning data. While the level of histone H3 RNA on occasion is similar in bone marrow and peripheral blood cells, as a group the marrow cells contain significantly higher histone H3 RNA levels (P = 0.043). The level of histone H3 RNA is not correlated with the level of expression of any of the other genes studied. In fact, even the absence of detectable histone H3 RNA does not preclude leukemic cells from containing high levels of c-myc or c-myb RNA (Fig. 2A, Patient 5). On the other hand, the level of histone H3 RNA in marrow cells is correlated with the proportion of nucleated erythroid cells in the marrow (Pearson: r = 0.614, P = 0.078; r = 0.595 and 0.781 with P values of 0.036 and 0.013 for Kendal and Spearman analyses).

C-myc RNA Levels. C-myc RNA is detectable in every specimen. While the RNA level varies among the specimens, the range is less than that noted for the other genes studied (Fig. 2A; Table 2). Peripheral blood and marrow cells have similar c-myc RNA levels, and the relationship between the 2.2- and the 2.4-kilobase c-myc transcripts is also similar. Despite the narrow range of c-myc RNA levels, there is no correlation.
Table 2  Protooncogene RNA levels in matched bone marrow and peripheral blood cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Specimen</th>
<th>Mean ± SD*</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histone H3</td>
<td>BM*</td>
<td>0.9 ± 0.93</td>
<td>0.44</td>
<td>0.2-2</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0.1 ± 0.1</td>
<td>0.09</td>
<td>0-0.35</td>
</tr>
<tr>
<td>c-myc</td>
<td>BM</td>
<td>10.2 ± 12.1</td>
<td>5.5</td>
<td>0.34-39.7</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>5.9 ± 8.2</td>
<td>2.7</td>
<td>0.09-27.1</td>
</tr>
<tr>
<td>c-myc</td>
<td>BM</td>
<td>4.5 ± 1.3</td>
<td>4.1</td>
<td>3.3-7.4</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>3.8 ± 1.04</td>
<td>3.6</td>
<td>2.4-5.7</td>
</tr>
<tr>
<td>c-fos</td>
<td>BM</td>
<td>1.1 ± 1.6</td>
<td>0.17</td>
<td>0.02-5.1</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0.9 ± 0.9</td>
<td>0.25</td>
<td>0.02-3.6</td>
</tr>
<tr>
<td>c-fes</td>
<td>BM</td>
<td>1 ± 0.4</td>
<td>1.1</td>
<td>0.39-1.6</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>1.2 ± 0.4</td>
<td>1.1</td>
<td>0.58-1.8</td>
</tr>
<tr>
<td>c-fms</td>
<td>BM</td>
<td>0.5 ± 0.6</td>
<td>0.5</td>
<td>0.02-1.6</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0.8 ± 0.5</td>
<td>1.04</td>
<td>0.07-3.1</td>
</tr>
<tr>
<td>TPI</td>
<td>BM</td>
<td>0.5 ± 0.6</td>
<td>0.2</td>
<td>0.05-1.7</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>0.4 ± 0.35</td>
<td>0.33</td>
<td>0.04-1.2</td>
</tr>
</tbody>
</table>

* To determine relative absorbance values, the absorbance value obtained from densitometric scanning of the RNA band was divided by the absorbance value for the corresponding rRNA band.

BM, bone marrow; PB, peripheral blood.

of S-phase cells in the cell populations from which the RNA was extracted. The relationship between c-myc and histone H3 RNA levels differs for bone marrow and peripheral blood cells. For the former cells, the mean ± SD and the median values for c-myc RNA + histone H3 RNA levels are 102 ± 192 and 8.6. The comparable values for peripheral blood cells are 182 ± 194 and 35.8, respectively. The difference in c-myb RNA + histone H3 RNA levels between bone marrow and peripheral blood leukemic cells is a reflection of the higher histone H3 RNA levels in bone marrow cells. The data also suggest that the level of c-myc RNA in bone marrow cells is inversely related to the level of c-fes RNA (Pearson: r = -0.291, P = 0.485; Kendal: r = -0.618, P = 0.034; Spearman: r = -0.079, P = 0.023).

c-myb RNA Levels. c-myb RNA is detected in every specimen with RNA levels varying substantially between specimens (Fig. 2A, Table 2). For bone marrow cells, there is an inverse relationship between the level of c-myb RNA and the proportion of nucleated erythrocytes in the sample (Pearson: r = -0.281, P = 0.46; Kendal: r = -0.609, P = 0.028; Spearman: r = -0.757, P = 0.02). c-myb RNA levels are not correlated with the RNA levels of the other genes studied nor is there a relationship between the levels of c-myb RNA in bone marrow and in peripheral blood cells.

c-fos RNA Levels. c-fos RNA levels vary widely among specimens, being undetectable or barely detectable in 5 specimens and being highly represented in other specimens. Fig. 2B and Table 2 provide these data. The levels of c-fos RNA in matched bone marrow and peripheral blood cells are highly correlated with each other (r values, 0.778 to 0.912, with P values of 0.001 to 0.004 for Pearson, Kendal, and Spearman tests).

The height of the WBC count is correlated with the level of c-fos RNA in both peripheral blood and bone marrow cells (Fig. 3A). For the bone marrow, the r values for 0.634, 0.511, and 0.552 for Pearson, Kendal, and Spearman analyses with corresponding P values of 0.049, 0.004, and 0.008, respectively. For blood cells, the corresponding values are r = 0.595 (P = 0.069), 0.422 (P = 0.089), and 0.585 (r = 0.074). There is also a highly significant correlation between the level of c-fos RNA in bone marrow cells and the number of monocytes present in the marrow (Pearson: r = 0.893, P = 0.001; Kendal: r = 0.548, P = 0.045; Spearman: r = 0.644, P = 0.036). Further, the levels of c-fos RNA and TPI are correlated in both bone marrow and peripheral blood cells.

With respect to the marrow cells, the r and the P values for Pearson, Kendal, and Spearman correlations for the relationship between c-fos and TPI RNA are 0.896 (P < 0.001), 0.422 (P = 0.089), and 0.612 (P = 0.06), respectively. The parallel
patterns of protooncogene expression in acute leukemia

values for peripheral blood cells are 0.799 (P = 0.006), 0.584 (P = 0.02), and 0.736 (P = 0.015), respectively.

c-fes RNA Levels. c-fes RNA levels vary widely among specimens, being undetectable in 4 of 20 specimens (Fig. 2B; Table 2). In 2 of the 4 specimens with undetectable c-fes RNA (Patients 6 and 8), however, the amount of RNA blotted is low. The level of c-fes RNA in peripheral blood cells is highly correlated with the number of mature granulocytes in the blood with r values for Pearson, Kendal, and Spearman analyses of 0.722, 0.781, and 0.865, respectively, with P values of 0.067, 0.015, and 0.01. No relationship was noted between c-fes RNA levels in bone marrow cells and the number of mature granulocytes present in the marrow with r and P values for Pearson, Kendal, and Spearman analyses of 0.734 (P = 0.015), 0.674 (P = 0.007), and 0.863 (P = 0.001), respectively.

TPI RNA Levels. TPI RNA is detectable in every specimen with a wide range of values being noted (Fig. 2B; Table 2). The TPI RNA levels in marrow and peripheral blood cells are not correlated. On the other hand, the height of the WBC count is correlated with the TPI RNA levels in both marrow and blood cells (Fig. 3B). For the marrow cells, the correlation values are: Pearson (r = 0.703, P = 0.024); Kendal (r = 0.407, P = 0.06); and Spearman (r = 0.552, P = 0.098). With respect to peripheral blood cells, the correlations of TPI RNA levels with WBC count are Pearson (r = 0.747, P = 0.013); Kendal (r = 0.539, P = 0.031); and Spearman (r = 0.705, P = 0.023). The level of TPI RNA in bone marrow cells is also correlated with the proportion of monocytes present in the marrow (Pearson: r = 0.655, P = 0.04; Kendal: r = 0.548, P = 0.045; and Spearman: r = 0.664, P = 0.036). TPI RNA levels in peripheral blood cells are not correlated with the percentage of monocytes present in the blood.

As noted earlier, TPI RNA levels in the marrow and blood are correlated with c-fos RNA levels. In marrow cells TPI RNA levels are also correlated with c-fms RNA levels.

DISCUSSION

Leukemic cells in the peripheral blood have a lower thymidine labeling index than leukemic marrow cells (5, 7). This behavioral difference between blood and marrow leukemic cells presents strongly suggests that there are differences in the levels of gene expression of well. If this is the case, then studies of gene expression in peripheral blood cells cannot be used to define the patterns of gene expression in “leukemia” per se. The studies described here demonstrate that there are indeed substantial differences in the levels and patterns of gene expression present among blood and marrow leukemic cells of the same patient. The only apparent relationships found are the significant correlation between c-fos RNA levels in marrow and blood leukemic cells and in the close absolute values of c-myc RNA levels in blood and marrow leukemic cells. These apparent relationships are not strong; however, since the absolute levels of c-fos RNA in matched marrow and blood specimens are substantially different, while despite the similar absolute values for c-myc RNA levels, the levels in matched blood and marrow cells are not correlated.

The most consistent difference between marrow and blood leukemic cells is in the level of histone H3 RNA with levels in marrow cells being significantly higher than in peripheral blood cells. Histone H3 RNA levels in the marrow are significantly correlated with the number of nucleated erythroid cells present in the marrow but are not directly correlated with the percentage of S-phase cells. The most likely explanation for the lack of correlation between the percentage of S-phase cells and histone H3 RNA levels is that the histone H3 RNA level in a cell population is probably determined by both the rate of DNA synthesis and the percentage of cells synthesizing DNA. The labeling index, unfortunately, provides information only regarding the latter. Hence, these observations are not necessarily
indicative of an uncoupling of DNA synthesis and the transcription of histone mRNA.

Even though the genes for histone H3 and the c-myc and c-myb protooncogenes have all been defined as being proliferation related (22), there were no discernible relationships among the RNA levels for these three genes. The most likely explanation for this observation is that histone H3 RNA levels are directly related to DNA synthesis per se, while c-myc and perhaps c-myb RNA levels are related to proliferative potential (23) and not necessarily to the actual proliferative state of the cells. The data, therefore, suggest that, while the proliferative status of bone marrow and peripheral blood leukemic cells differs, their proliferative potentials are similar. In contrast, histone H3 RNA levels in marrow cells exceed those present in blood cells, since a higher proportion of marrow cells are in cycle (5, 7). Given these data, one must be very circumspect about generalizing data obtained from studies of peripheral blood cells. In fact, the report that acute leukemia is characterized by a high ratio of c-myc RNA to histone H3 RNA (24) failed to take into account these differences between marrow and blood leukemic cells. All of the conclusions found in this report are based upon studies of peripheral blood cells. As demonstrated above, when the histone H3 RNA levels in leukemic marrow cells are used to compute the ratio of c-myc RNA to histone H3 RNA the values obtained are much lower than that obtained when the histone H3 RNA levels in blood cells are used to calculate the ratio, since the H3 RNA levels in bone marrow cells are higher.

One additional observation regarding c-myc RNA levels warrants discussion. The c-myc RNA levels in the blood and marrow cells of Patient 5 were very similar despite the fact that the blood contained few immature cells. This is unusual, since c-myc expression appears to be related to cellular immaturity and to proliferative potential (23). We have observed this once before when a remission bone marrow contained the same c-myc RNA levels as did the marrow upon subsequent leukemic relapse. Both of these unusual observations perhaps represent the inappropriate expression of c-myc in normal-appearing cells.

The height of the WBC count at diagnosis is significantly correlated with the level of c-fos and TPI RNAs. With respect to TPI, one might postulate that the activity of this gene is a reflection of the level of cellular metabolism which, in turn, is related to the proliferative rate. Since TPI RNA levels vary among patients depending upon the WBC count and on the degree of monocytic differentiation, RNA levels for this “housekeeping enzyme” and perhaps for others as well have only limited utility as internal standards in Northern blot analyses (25). The relationship between c-fos RNA levels and the height of the WBC count is perhaps a reflection of the relationship of this gene to monocytic differentiation patients, since patients with very high WBC counts at diagnosis often have monocytic leukemia (26). The fact that a cell does not present the morphological appearance of the monocytic cell may not mean that it is not of the monocytic lineage. The same reasoning may account for the apparent lack of relationship between c-fos RNA levels and the proportion of monocytic cells present in a specimen.

In summary, leukemic cells in the bone marrow and peripheral blood differ from each other in their pattern of gene expression. The differences are manifested not only in the different levels of expression but also in the different relationships between gene expression and the characteristics of the peripheral blood. These differences in RNA patterns together with the differences in histone H3 RNA levels suggest that leukemic cells in the marrow and blood differ substantially in their pattern of gene expression and in their biological characteristics. Therefore the data obtained from the study of leukemic cells in either compartment cannot be taken to be representative of expressions in leukemic cells per se.

ACKNOWLEDGMENTS

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REFERENCES

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