Peripheral blood cell preparation from 23 normal subjects and 72 patients with acute and 32 patients with chronic myeloid leukemia were cultured in vitro and released plasminogen activators were analyzed. The quantity of plasminogen activator secreted by leukemic cells varied widely and could not be correlated with the clinical severity of the disease. Immunochemical and electrophoretic techniques have been used to show that normal peripheral blood granulocytes released exclusively urokinase-like plasminogen activator, whereas leukemic cells secreted either urokinase or a tissue activator-like enzyme. The molecular species of enzyme released by acute myeloid leukemic cells may serve as a diagnostic marker of relevance to the management of this disease, since patients with acute myeloid leukemia whose cells released only tissue plasminogen activator did not respond to combination chemotherapy. Tissue plasminogen activators released by leukemic cells may display an unusual electrophoretic pattern that resembles that shown by urokinase. Immunochemical procedures are therefore essential for the correct identification of these enzymes.

Although the chemotherapy of leukemia is based on sound pharmacologic principles, it remains empirical to the extent that individual patients may respond very differently to a particular chemotherapeutic regimen in terms of induction of remission, maintenance of remission, or incidence of undesirable side effects. It would therefore, be of value if additional laboratory criteria were available that could be used in conjunction with clinical and conventional histologic procedures to predict response to therapy.

It has recently been noted that plasminogen activator synthesis and release are inducible cellular functions that are subject to modulation by hormones, drugs, and other agents, many of which affect expression of the transformed phenotype. For this general reason, it seemed to us possible that the study of plasminogen activator synthesis in vitro would prove of value for the further characterization of human leukemic cells. Two recent observations have provided a more particular justification for this investigation. First, it has been demonstrated that normal granulocytes synthesize plasminogen activator; and second, it has been shown that human cells release plasminogen activators of two distinct immunochemical types—one similar to urokinase and the other similar to tissue activator. It was therefore of interest to determine (A) whether leukemic myeloid cells released both types of enzymes and, if so, (B) whether the molecular species of plasminogen activators released by myeloid leukemic cells differed in a way that would be nosologically useful.

In this article we present results of a series of studies showing that peripheral blood cells from patients with myeloid leukemia released plasminogen activators when cultured in vitro. Differences in molecular species of enzyme released by leukemic cells were observed. These differences appeared to have prognostic significance.

Materials and Methods

Subjects

Normal blood samples were obtained from 23 healthy persons working in the laboratory. Leukemic blood samples were obtained from 72 patients with acute myeloblastic leukemia (AML) and 32 patients with chronic myeloid leukemia (CML) who attended the hematologic service at Groote Schuur Hospital.

Diagnoses were based on histologic and histochemical examination of peripheral blood and bone marrow specimens. Romanowsky-stained preparations, cytochemical stains, and ultrastructural features were used to classify each specimen according to the French-American-British recommendations. Fifty-eight patients with acute myeloblastic leukemia were treated with combination chemotherapy that included the epipodophyllotoxin, VP-16-213, cytosine arabinoside, and Adriamycin. Response to therapy was assessed by aspiration and trephine-biopsy of the bone marrow on the tenth day following completion of the cytotoxic chemotherapy. Induction of remission was judged to have been successful if marrow hypocellularity was achieved with subsequent regrowth of blast-free marrow that remained apparently normal for at least 4 wk.

Of the 58 patients treated in this way, 27 went into complete remission, 15 failed to enter remission, and 16 died before response to therapy could be assessed.

The 14 remaining patients that are identified in Table 1 as having palliative or alternate therapy are individuals who, by virtue of their advanced age or associated clinical conditions, were judged to be unsuitable for combination chemotherapy.

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Table 1. Correlation Between Clinical Outcome and Molecular Species of Plasminogen Activator Released by Cultured Cells of 72 Patients With AML

<table>
<thead>
<tr>
<th>Therapy Group</th>
<th>Response</th>
<th>Nature of Plasminogen Activator</th>
<th>TA*</th>
<th>UK†</th>
<th>TA and UK</th>
<th>Unknown</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Combination chemotherapy</td>
<td>A</td>
<td>Assessment completed</td>
<td>0</td>
<td>22</td>
<td>3</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Complete remission</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No remission</td>
<td>(8)</td>
<td>(28)</td>
<td>(3)</td>
<td>(3)</td>
<td>(42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Subtotals)</td>
<td>3</td>
<td>10</td>
<td>1</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Palliative/alternate therapy</td>
<td>B</td>
<td>Died before assessment</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>14</td>
</tr>
<tr>
<td>Totals</td>
<td>15</td>
<td>45</td>
<td>5</td>
<td>7</td>
<td>72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*TA, tissue activator.
†UK, urokinase.

Cells

Blood was taken by sterile venipuncture into tubes containing preservative-free heparin (Thromboliquine, Organon Teknika, Holland) to give a final concentration of 5 U/ml. Cells were fractionated by dilution with RPMI 1640 medium and centrifugation on a layer of Ficoll-Hypaque. Leukemic blast cells were harvested from the plasma-Ficoll interface and contaminating red blood cells removed by incubation for 5 min in 0.83% ammonium chloride (pH 7.4). Cells were washed once by centrifugation and resuspension in RPMI and were then resuspended in RPMI containing 3% fetal calf serum (FCS) to give 4 x 10⁶ cells/ml. One-milliliter samples of this suspension were incubated in 35-mm Falcon plastic tissue culture Petri dishes at 37°C in a humid atmosphere of 5% CO₂ in air. After 24 hr of incubation, the cells were suspended in fresh medium and the incubation was continued for a further 24 hr. At the end of this second period, the medium (harvest fluid) was collected by centrifugation and analyzed qualitatively and quantitatively for plasminogen-dependent proteases as described below.

In some cases, a caseinolytic plaque assay was used to obtain a quantal estimate of the number of individual cells that synthesized plasminogen activators. In this procedure, the cells were washed and mixed with appropriate prewarmed solution to provide a final suspension of 6 x 10⁵ cells/ml in RPMI containing 0.8% agar, 1.3% solution of commercial instant non-fat dry milk powder, and 160 µg/ml purified human plasminogen. This suspension was run into prewarmed moulds fashioned from glass microscope slides separated by short lengths of thin wire. The preparations were then allowed to set at room temperature, when the slides were carefully separated and the gels incubated at 37°C in a humid atmosphere for plaques of lysis to develop (Fig. 1).

Plasminogen Activator Assay

Plasminogen activator in harvest fluid was assayed by measuring the plasminogen-dependent release of soluble radioactive fibrin degradation peptides from insoluble 125I-labeled fibrin-coated Linbro multiwell plates. This was done exactly as previously described, save for the addition of 4 µg of plasminogen to each well instead of 2 µg.

Rabbit Antibodies to Human Urokinase and Tissue Plasminogen Activator

Rabbits were immunized by subcutaneous injection of commercially purified human urokinase or human tissue plasminogen activator secreted into the medium of a human melanoma cell line. The IgG antibody fractions from immune sera were prepared as pre-
Electrophoretic and Immunochemical Analysis of Plasminogen Activators

Molecular species of plasminogen activators present in harvest fluids collected from normal and leukemic cells were analyzed by 3 procedures previously described in detail. 10

1) Harvest fluid samples were electrophoresed in 11% polyacrylamide gel slabs containing 0.1% SDS. The gels were then washed in 2.5% Triton X-100 to remove the SDS, and the bands of plasminogen activator activity were detected by plasminogen-fibrin-agar zymography in which plasminogen-dependent fibrinolysis was evident as clear lysis zones in the opaque fibrin indicator slab (Fig. 4).

2) Harvest fluid samples were assayed for residual plasminogen activator activity after treatment with serial dilutions of rabbit antibody to urokinase or to tissue plasminogen activator (Fig. 5).

3) Plasminogen activator in any given harvest fluid could be analyzed by a combined electrophoretic and immunochemical procedure in which specific antibody was added to a trough cut in the plasminogen-fibrin-agar indicator layer. The polyacrylamide gel slab containing electrophoresed plasminogen activators was then carefully layered over the indicator layer in such a fashion that adjacent electrophoretic tracks lay parallel to and on either side of the antibody-containing trough. Specific inhibition of individual activator bands could be seen in the antibody-rich agar adjacent to the trough (Figs. 6 and 7).

RESULTS

Fibrinolytic Activity Released by Cells Cultured In Vitro

When caseinolytic or fibrinolytic activity was released by normal or leukemic cells, this was invariably and completely plasminogen-dependent. This was evident as plaques of proteolysis in the casein-agar system (Fig. 1A) that were not seen when plasminogen was omitted from the indicator gel (Fig. 1B). Similarly, release of 125I-fibrin degradation peptides in the solid-phase radioenzymatic assay system was dependent on the presence of plasminogen.
Normal mononuclear cell preparations that remained on top of the Ficoll-Hypaque layer (i.e., lymphocytes and monocytes) released too little plasminogen activator for accurate quantitation or characterization.

Normal neutrophils that centrifuged through the Ficoll-Hypaque layer released easily measurable amounts of plasminogen-dependent fibrinolytic activity for purposes of molecular identification. Unfortunately, however, these cells survived poorly in culture.

The lack of a stable viable cell population over the duration of the experimental period made it impossible to express the quantity of enzyme released on the basis of cell number for purposes of comparison with leukemic cells.

Leukemic cells fared well in culture and, by the end of the 48 hr, more than 85% were still viable. These cultures therefore provided data that could be used both to determine the amount of enzyme released and the molecular species of enzyme that was synthesized.
Release of Plasminogen Activators by Leukemic Cells

Cell preparations from 61 patients with AML and 32 patients with CML were cultured in vitro, and harvest fluids were taken for measurement of plasminogen activators released during the second 24 hr period. The results are presented in Fig. 3, from which it can be seen that the quantity of enzyme secreted varied widely over a 10,000-fold range from 0.001 U/10^7 cells/24 hr to 22.4 U/10^7 cells/24 hr. In 9 cases (7 AML and 2 CML), leukemic cells secreted too little enzyme to be detected in the fibrin plate assay.

Molecular Species of Plasminogen Activators Released by Normal and Leukemic Cells

Representative results of an electrophoretic analysis of the plasminogen activators released by cultured cells are given in Fig. 4, which shows a fibrin-agar indicator gel that was used to identify the enzymes present in four leukemic cell culture fluids. These were compared with urokinase (A) and tissue activator (F). The enzymes present in leukemic cell harvest fluids, B, D, and E, were electrophoretically similar to urokinase. Leukemic cell harvest fluid, C, contained plasminogen activators of three electrophoretically distinct types—one similar to tissue activator, one similar to urokinase, and a third gave a band of fibrinolysis corresponding to a mol wt of 100,000.

Antibody titrations showed that enzymes secreted by leukemic cells could be immunochemically identified as urokinase or tissue activator (Fig. 5). In this experiment, cells from one leukemic subject secreted urokinase-type activator (Fig. 5C), while cells from a second leukemic subject released enzyme that was exclusively tissue activator in type (Fig. 5D).

The immunochemical identity of the enzymes in cell harvest fluids could be more definitely identified using the combined immunochemical and electrophoretic procedures illustrated in Fig. 6. As shown in this example, one sample of leukemic cell harvest fluid contained activators that were completely inhibited by...
LEUKEMIC CELL PLASMINOGEN ACTIVATORS

antibody to urokinase (tracks A and B), whereas the harvest fluid from a second leukemic cell preparation (tracks C and D) contained one activator species of mol wt 60,000 that was inhibited by the antibody to urokinase, whereas the activators with mol wt of 70,000 and 100,000 were unaffected by the antibody to urokinase.

These procedures were used to show that cells from 15/72 patients with AML secreted tissue activator, cells from 45 patients secreted the urokinase-type enzyme, and cells from 5 patients secreted a mixture of urokinase- and the tissue-type activator. Cells from 7 patients with acute myeloblastic leukemia secreted too little enzyme for the activator to be identified with certainty (Table I).

Cells from 32 patients with chronic myeloid leukemia were studied. Of these, 13 secreted urokinase, 12 secreted tissue activator, 5 secreted a mixture of urokinase and the tissue-type activator, and cells from the remaining 2 patients secreted too little enzyme to identify.

Neutrophils isolated from 23 normal subjects invariably released only the urokinase-type enzyme.

It is of interest to note that, in two cases, a plasminogen activator was observed that migrated electrophoretically as a single enzyme band with a molecular weight corresponding to that of urokinase. It was, however, unaffected by antibody to urokinase and completely inhibited by antibody to the tissue activator (Fig. 7).

Therapeutic Correlations

Cells from 72 patients with AML were studied with a view to defining the molecular species of plasminogen activator they released and correlating these with responses to therapy. The combined results are presented in Table 1, from which it can be seen that, taken overall, cells from 15 cases released tissue activator and those from 45 cases released the urokinase-type enzyme. In 5 instances, a mixture of tissue activators and urokinase was released, and in 7 cases the amount of enzyme released was too low for reliable identification.

This general tendency for approximately 20% of AML patients to have cells that released tissue activator was apparent in each of the three major therapeutic subdivisions. Thus, blasts from 4/14 patients who received palliative therapy, 3/16 patients who were treated with standard combination chemotherapy but who died before evaluation could be completed, and 8/42 patients in whom results of therapy could be assessed released tissue activator.

If, however, one considers only the 42 patients in whom the therapeutic response could be determined, it can be seen that in 81% of (25/31) patients whose cells released the urokinase-type enzyme, a remission was satisfactorily induced. In contradistinction, all 8 patients whose cells released tissue activator alone failed to enter remission.

In this limited series, therefore, there was a significant correlation ($\chi^2 = 17.8, p < 0.001$) between the release of tissue activator alone and a poor response to the cytotoxic regimen that was used.

Since it has been reported by others$^{19,20}$ that age and white blood cell (WBC) count at the time of presentation are adversely related to prognosis, patients whose cells released tissue activator and urokinase type enzymes were compared with respect to these parameters. Although differences were found, in no case were these statistically significant. Mean values ±SE of mean for patients whose cells released tissue activator were: age, 47 ± 3.9; total WBC, 33 ± 14.2. Corresponding values for those whose cells released urokinase were: age 37 ±2.8; total WBC, 46 ± 9.1.

DISCUSSION

As previously observed with fibrinolysins released by other human cell types cultured in vitro,$^{10,18,21,22}$ we have found that when fibrinolytic or caseinolytic activity was released by human peripheral blood leukocytes of the myeloid series, this was invariably plasminogen dependent. One is therefore justified in regarding these enzymes as plasminogen activators. This was true for both leukemic and normal cells and confirms the observations made by Granelli-Piperno et al.$^7$ for normal human polymorphonuclear leukocytes.

The amount of plasminogen activator secreted by leukemic cells in culture varied widely and could not be correlated with the clinical severity of the disease. Although blasts isolated from the peripheral blood of patients with AML tended to release more plasminogen activator than did CML cells, there was a considerable overlap between these groups.

Using antibodies to urokinase and tissue activator in combination with electrophoretic analysis, we have shown that normal peripheral blood granulocytes released exclusively urokinase-like plasminogen activator, whereas leukemic cells secreted either a urokinase-like enzyme or a tissue-activator-like enzyme. The data therefore allow the tentative conclusion that the detection of tissue plasminogen activator release by peripheral blood cells is indicative of the leukemic state.

In most cases, cells isolated from any given leukemic blood sample generally secreted only one molecular species of plasminogen activator. This observation merits additional comment in the following three respects.
First, patients with AML whose peripheral blood leukocytes released exclusively activator of the tissue type tended to have a worse prognosis in terms of their susceptibility in induction of remission than did patients whose cells released enzyme of the urokinase type. If more extensive studies confirm this correlation, the molecular species of plasminogen activator released by AML blast cells may serve as a diagnostic marker of relevance to the management of this disease.

Second, we have isolated cells from 5 patients with AML that released both the urokinase-type and the tissue-type of plasminogen activator. In these cases, the cells appeared to be exclusively blastic and of leukemic type, suggesting that certain leukemic clones may develop that synthesize and release both forms of plasminogen activators. The report23 of a cloned cell line that produces two immunochemically unrelated plasminogen activators provides precedent for this suggestion.

Third, our previous experience with plasminogen activators released by human cells has led us to believe that the electrophoretic finding of a 60,000 mol wt plasminogen activator, and the absence of a 70,000 mol wt enzyme in the same track, was sufficient to identify the enzyme as being of the urokinase type. The results we have obtained with two leukemic cell isolates have provided the exception that invalidates the generality of this assumption. We can now say with confidence that it is possible to observe tissue plasminogen activators that cannot be distinguished by electrophoresis from the urokinase type of enzyme. Immunochemochemical procedures are therefore essential for the correct identification of these enzymes.

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The secretion of plasminogen activators by human myeloid leukemic cells in vitro

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