Combined Procoagulant Activity and Proteolytic Activity of Acute Promyelocytic Leukemic Cells: Reversal of the Bleeding Disorder by Cell Differentiation

By P.W. Wijermans, V.I. Rebel, G.J. Ossenkoppele, P.C. Huijgens, and M.M.A.C. Langenhuijsen

In the human promyelocytic cell line HL60, we observed both a strong procoagulant activity (PCA) on the cell membrane and proteolytic activity in the lysate of these cells. Because these cell-line cells are susceptible to differentiation to either a more mature granulocytic or monocytic form, we were able to study the hypothesis that the combination of PCA and proteolytic activity is confined to the promyelocyte. This may explain the severe coagulopathy seen in patients with acute promyelocytic leukemia. Cell differentiation in a myeloid direction induced by retinoic acid or DMSO led to a diminished PCA, while not affecting the fibrinolytic activity. On the other hand, monocytic differentiation obtained by culturing the cells in the presence of 1%; 25 dihydroxy vitamin D3 led to the complete disappearance of the proteolytic activity of the cell lysate, although the procoagulant activity was still present. Furthermore, we found that the elastase activity almost disappeared after monocytic differentiation. We also studied the PCA, proteolytic activity, and elastase activity of blast cells of patients with acute myeloid leukemia. Only in patients with acute promyelocytic leukemia did we observe both a strong PCA and fibrinolytic activity. This supports our hypothesis that the combination of these activities is unique to the promyelocyte and may explain the observed bleeding complications in patients with acute promyelocytic leukemia.

ACUTE PROMYELOCYTIC LEUKEMIA (APL) is a subvariety of acute myeloid leukemia (AML) and is associated with a high incidence of severe hemorrhage. The coagulopathy is attributed to disseminated intravascular coagulation (DIC) with secondary fibrinolysis, probably due to tissue factor-like activity on the cell membrane of the leukemic cells. However, the observed bleeding disorders cannot always be related to DIC, and signs of primary fibrinolysis are found in these cases. We studied the hypothesis that severe coagulopathy is largely confined to the promyelocytic subtype of AML because of the unique interaction of procoagulant activity (PCA) and the fibrinolysis of the promyelocyte. If this combination of activities is unique to the promyelocyte, it also explains the relation between the French American British (FAB) classification and the fibrinolysis or DIC (procoagulant activity) in AML patients.

The HL60 cell-line cells, originally established from a patient with APL, are susceptible to differentiation in vitro to either more mature granulocytic or monocytic cells by various compounds. Monocytic differentiation of HL60 promyelocytes should lead to a decrease of proteolytic activity, because the leucocyte proteases from the azurophilic granules have been shown to be responsible for this activity. Myeloid differentiation should lead to a diminished PCA since this activity is attributed to monocytes and macrophages. In the present study, we investigated the influence of monocytic and granulocytic differentiation on the PCA and proteolytic activity of HL60 promyelocytes. We compared our findings with those obtained with normal human monocytes and granulocytes and tumor cells of patients with AML of different subtypes according to the FAB classification.

MATERIALS AND METHODS

Cell culture. HL60 cells were cultured as previously described. After four days of culturing in the presence or absence of differentiation-inducing agents, cells were harvested. Cell viability was determined by the trypan blue dye exclusion method, and the tests were performed only when cell viability was >85%. The following differentiation-inducing agents were used: dimethylsulphoxide (DMSO) at a concentration of 1.25%; retinoic acid (RA) 10^{-7} M, and 1; 25 dihydroxy vitamin D3 (vit D3) 10^{-6}M.

Isolation of blood cells and cell lysis. Human blood was obtained from healthy volunteers. Monocytes and polymorphonuclear leukocytes (PMN) were isolated as previously described. Leukemic blast cells were obtained from either peripheral blood or bone marrow and isolated by density-gradient centrifugation on Ficoll Isopaque. Cell number was determined by flow cytometric technique on an AI Cellcounter 134 (Analyse Instruments). Cells were used only when purity was >95% for granulocytes and >85% for monocytes. Fibrinolytic activity, fibrinogenolytic activity, and elastase activity were tested in the cell lysate of HL60 cells and human blood cells. To obtain cell lysate, the cells were resuspended in Hanks balanced salt solution with 1% Triton X 100 for the determination of proteolytic activity. For elastase activity, the cells were resuspended in a buffer containing 0.1 mM/I Tris, and 0.96 M NaCl, pH 8.3. Cells were lysed by freezing them three times in liquid nitrogen. Cell fragments were spun down at 700 g for four minutes, and the supernatant was collected. Procoagulant activity. Procoagulant activity of the cell lysate was determined as the fibrinogenolysis and fibrinogenolytic activity. Fibrinogen lysis was tested using fibrin plates prepared from plasminogen-free bovine fibrinogen (Sigma F4753, St. Louis). Then 0.5 mL of thrombin (50 NIH U/mL) (Hoffmann-La Roche, Basel, Switzerland) was added to 7 mL of fibrinogen (10 g/L). Fibrinogenolytic activity was measured as the area of lysis produced by 10 L of cell lysate ( average of duplicate determinations) after 36 hours of incubation at 37°C. To exclude plasminogen activation, control experiments were performed using both streptokinase (1,000/UL) as well as plasminogen- (100 μg/mL) (Kabi Vitrum, Stockholm, Sweden) rich fibrin plates. Plasmin (10 μL of 2.2 nkat/mL) (Kabi Vitrum) was used as a positive control. Fibrinogenolytic activity was tested by mixing 1.4 mL of fibrinogen (10 g/L) with cell lysate. After

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incubation for 18 hours at 37°C. 0.1 mL of thrombin was added. When proteolytic activity was present in the cell lysate, no clotting was observed. Semi-quantitative experiments were performed using different dilutions of cell lysate. To determine the specificity of the proteolysis inhibition, experiments were performed using α 2 macro-globulin (Sigma M4514, St Louis, a calpain, and elastatin (Boehringer Mannheim, West Germany). Fibrin degradation products (FDPs) were determined with a Latex agglutination technique (Wellcotest, Wellcome Diagnostics, Dartford, England).

Elastase activity (EC 3.4.21.27). Elastase activity was measured using L-phenylalanine-1-carboxy-1-thio-7-ascorbic acid (Sigma P8 139) to enhance the expression of the following clotting factors: VII, VIII, X (General Diagnostics, Morris Plains, NY), V, and II (Merz-Dade, Dillingen, Switzerland). HL60 cells were incubated with elastatinal (Boehninger Wellcome Diagnostics, Dartford, England).

Procoagulant activity. The procoagulant activity was measured by the procoagulant activity using a Depex coagulometer. 0.1 mL of pooled citrated normal human plasma was incubated with 0.1 mL of cell suspension (resuspended in Michaeause buffer pH 7.4) for four minutes at 37°C. Then 0.1 mL of calcium chloride (30 mM) was added to the plasma, and the clotting time was recorded. PCA was also assayed using plasma from patients congenitally deficient in one of the following clotting factors: VII, VIII, X (General Diagnostics, Morris Plains, NY), V, and II (Merz-Dade, Dillingen, Switzerland). HL60 cells were incubated with 2-tetradeacanoyl-phorbol 13 acetate (TPA, Sigma P8139) to enhance the expression of the procoagulant activity, as described by Lyberg and Prydz. The chromogenic substrate was used. To determine the specificity of the procoagulant activity, we tested if the lysate contained elastase activity (Fig 1). Furthermore, we found that elastase activity (Fig 2) was significantly lower (p < 0.001) activity (Fig 2). Furthermore, we determined whether procoagulant activity could be inhibited by several potent inhibitors of fibrinolysis (Table 4). No inhibi-

<p>| Table 1. Fibrinolytic Activity of HL60 Cells |
|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Fibrin Plate (mm²)</th>
<th>Fibrin + Plasminogen (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>0</td>
</tr>
<tr>
<td>HL60 cells (2 x 10⁶ cells)</td>
<td>189 ± 17</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>0</td>
</tr>
<tr>
<td>Plasmin</td>
<td>65 ± 10</td>
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</tbody>
</table>

Fibrinolytic activity of lysate of HL60 cells measured as area of lysis (mm²). The results are means ± SD of three to eight experiments.

RESULTS

Proteolytic activity. With the fibrin plate technique, a high fibrinolytic activity (FA) was found in the lysate of HL60 cells (Table 1). This activity could not be attributed to plasminogen activation. A higher FA was found in the lysate of PMN (P < 0.00001), and a significantly (P < 0.0001) lower activity was found in the lysate of monocytes (Fig 1). Monocytic differentiation (vit D3) led to a complete disap-

Fig 1. Fibrinolytic activity of cell lysate of human monocytes, granulocytes, and HL60 cells. Results are expressed as mean area of lysis ± SD (mm²) of five to eight experiments.

Table 2. Influence of Differentiation on Fibrinolytic Activity

| Fibranolytic Activity (mm²) of HL60 Cells |
|-----------------------------|-----------------------------|
| Control | VIT D3 | Retinoic Acid | DMSO |
|-----------------------------|-----------------------------|
| 1 x 10⁶ cells | 210 ± 45 | 0 | 207 ± 52 | 47 ± 20 |
| P | NS | <0.0001 |
| 2 x 10⁶ cells | 267 ± 64 | 0 | 258 ± 50 | 108 ± 125 |
| P | NS | <0.02 |

Influence of cell differentiation on the fibrinolytic activity of HL60 cells (mm²). The results are means ± SD of eight experiments. No activity was found any more after monocytic differentiation (vit D3). Results are compared with control HL60 cells.
tion was observed with tranexamic acid or α 2 macroglobulin. Also, incubation was elastatinal, claimed to be a specific inhibitor of elastase.20 did not show an inhibition of FA or elastase activity. With alpha 1 antitrypsin, a complete inhibition of both FA and elastase activity was found. With human plasma, we also found an inhibition of both FA and elastase activity. Plasma obtained from a patient with an alpha 1 antitrypsin deficiency gave a partial inhibition of the FA and elastase activity. Fig 3 shows that the plasma concentration necessary to inhibit the FA was significantly higher than the concentration necessary to block the elastase activity. The influence of cell differentiation on the inhibition of elastase activity with human plasma is shown in Fig 4 (representative experiment). In accordance with the decreased elastase activity observed after differentiation induction with vit D3, plasma concentrations necessary to inhibit the elastase activity were lower than for control HL60 cells. Although we observed a lower elastase activity after culturing HL60 cells in the presence of DMSO (Fig 2), the difference with control cells did not reach statistical significance. However, lower plasma concentrations could inhibit the elastase activity, indicating lower elastase concentrations after differentiation induction with DMSO (P < 0.01) (Fig 4).

### Table 3. Influence of Cell Differentiation on Fibrinogenolytic Activity and FDPs

<table>
<thead>
<tr>
<th></th>
<th>PMN</th>
<th>MONO</th>
<th>Control</th>
<th>Vit. D3</th>
<th>Ret. DMSO</th>
<th>Plasmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogenolysis</td>
<td>+++</td>
<td>++</td>
<td>ND</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>FDPs</td>
<td>+++</td>
<td>++</td>
<td>0</td>
<td>+++</td>
<td>+</td>
<td>ND</td>
</tr>
</tbody>
</table>

Influence of cell differentiation of HL60 cells on fibrinogenolytic activity and the determination of fibrin degradation products (FDPs) in the lysate obtained from the fibrin plate. Semi-quantitative differences were found by performing dilution experiments.

Fig 2. Elastase activity expressed as mU per mg protein in control HL60 cells compared with those cultured in the presence of DMSO (ns), retinoic acid (ns), or vitamin D3 (P < 0.01). Activity is compared with normal human granulocytes and monocytes. Results are expressed as mean mU ± SD of three experiments.

### Table 4. Inhibition of Fibrinolytic Activity and Elastase Activity by Lysate of HL60 Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Fibrinolytic Activity</th>
<th>Elastase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tranexamic acid (100 μM-1.6μM)</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Elastatinal (1 mg/mL) (1:1-1:64)</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Alpha-2 macroglobulin</td>
<td>No inhibition</td>
<td>No inhibition</td>
</tr>
<tr>
<td>Alpha-1 antitrypsin (100 μg/mL)</td>
<td>Complete inhibition</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>Human plasma</td>
<td>Complete inhibition</td>
<td>Complete inhibition</td>
</tr>
<tr>
<td>Alpha-1-antitrypsin deficient plasma</td>
<td>Partial inhibition</td>
<td>Partial inhibition</td>
</tr>
</tbody>
</table>

Inhibition of fibrinolytic activity was determined by adding alpha-2 macroglobulin or alpha-1 antitrypsin to fibrinogen before forming fibrin plates or by incubating the cell lysate together with tranexamic acid, elastatinal, or human plasma before adding on the fibrin plate. Inhibition of elastase activity was determined by incubating the cell lysate with the different drugs or human plasma before measuring the elastase activity.

**Procoagulant activity (PCA).** PCA could be assessed with the recalification time when whole cells were mixed with normal human pool plasma. Cell lysate supernatant did not have PCA, whereas cell fragments obtained from the pellet fraction after ultracentrifugation of sonicated HL60 cells showed PCA values identical to or even higher than whole HL60 cells (data not shown). Fig 5 shows the PCA of HL60 cells compared with normal human PMN and (unstimulated) monocytes. As shown, HL60 cells had a significantly higher PCA than human monocytes (P < 0.01) and PMN (P < 0.001). Granulocytic differentiation induced by DMSO led to a significant (P < 0.001) increase in the recalification time: control versus DMSO with 0.3 × 10⁶ cells 96.0 ± 17.0 seconds versus 136.1 ± 22.8 seconds, thus indicating a decrease in PCA (Fig 5). Only a small increase in the recalification time was found for retinoic acid (115 ± 15.9 seconds, P < 0.05) (Fig. 5). A positive time-response relationship was found in cultures with DMSO and in cultures with retinoic acid (data not shown). This excludes a direct influence of the differentiating agents on the PCA. No differences were found for the PCA after culturing the
cells in the presence of vit D3 (91.0 ± 13.6 seconds not significant). Since human monocytes exhibit their PCA mainly after “stimulation,”21 we stimulated the HL60 cells shortly with TPA. After only one hour, an increase in PCA activity was observed (Fig 6). No signs of cell differentiation could be detected after such a short incubation period, as measured by cytochemistry, cell function parameters (Nitro Blue Tetrazolium [NBT] test and phagocytosis), and quantitative enzyme determinations (data not shown).16

Characterization of the PCA. Using the recalcification test with plasma deficient in different clotting factors, we tried to characterize the way HL60 cells generate the PCA (Table 5). With factor VIII-deficient plasma, no change in the recalcification time was observed. However, with factor VII–deficient plasma, an increase was found (P < 0.001) but clotting was still observed. No clotting was seen when factor II, factor V, or factor X–deficient plasma was used.

Acute myeloid leukemia patients. From 13 patients with acute myeloid leukemia, we were able to test the PCA activity of the leukemic cells together with the fibrinolytic and elastase activity of the cell lysate. As can be seen in Table 6, a very high PCA for the (unstimulated) blast cells together with a high fibrinolytic and elastase activity was found only in patients with the promyelocytic (M3) subtype of acute myeloid leukemia.

DISCUSSION

In this study, we investigated the hypothesis that in APL the promyelocytes have a combination of strong PCA and proteolytic activity that may be the cause of the coagulation disorders. We observed a strong PCA as well as a proteolytic activity in whole HL60 cells (promyelocytes) and cell lysate, respectively. Furthermore, our findings that cell differentiation along the monocytic or granulocytic lineage leads to a decrease of proteolytic activity or to a diminished PCA respectively indicate that it is probably the specific maturation state of the promyelocyte that is unique in exhibiting the combination of strong PCA and proteolytic activity.

Although we were able to show a decrease in FA and

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**Table 5. Characterization of Procoagulant Activity of HL60 Cells**

<table>
<thead>
<tr>
<th>Recalcification Time (S) for 0.3 × 10⁶ Cells</th>
<th>Normal Plasma</th>
<th>F.VIII Def. Plasma</th>
<th>F.VII Def. Plasma</th>
<th>F.II Def. Plasma</th>
<th>F.V. Def. Plasma</th>
<th>F.X Def. Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 ± 17</td>
<td>101 ± 12</td>
<td>161 ± 20</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td>&gt;300</td>
<td></td>
</tr>
<tr>
<td>P NS</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
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</table>

Characterization of procoagulant activity of HL60 cells using human plasma deficient for different coagulation factors. Recalcification time (seconds) was determined using 0.3 × 10⁶ cells. Results are expressed as mean ± SD of five experiments and are compared with the clotting time observed in normal human plasma (pooled plasma obtained from 20 donors).
elastolytic activity. Comparing the HL6O cell line with normal human PMN, one expects to find an increase in proteolytic activity with further granulocytic maturation. However, the fact that the values obtained with retinoic acid did not increase with myeloid differentiation might be explained by the leukemic nature of the HL6O cell line. On the other hand, it was shown both with cytochemical techniques and with quantitative enzyme analysis that cell differentiation leads to a decrease of lysozomal enzymes, probably as a result of a diminished azurophilic granule constituent. This explains the lower FA and elastase activity found in the cell lysate of cells cultured in the presence of DMSO and is in agreement with our findings for other enzymes such as myeloperoxidase. The differences found between DMSO and retinoic acid might be explained by the different ways of action of these two compounds.

Elastase and cathepsin G are two major proteases of PMN granules, and both can degrade fibrinogen. These two proteases are said to account almost entirely for the fibrinolytic activity of PMN. We demonstrated changes in elastase activity with cell differentiation in monocytic direction. Surprisingly, we observed residual elastase activity after differentiation with vit D3, whereas no fibrinolytic activity was observed anymore. This phenomenon is hard to explain since the pH optimal for the reaction and the inhibition experiments of FA indicate that elastase is at least partially responsible for the fibrinolysis; the substrate is said to be specific for granulocyte elastase, and the fibrin plate test is very sensitive in measuring proteolysis. Inhibition experiments with human plasma show that elastase is probably not the only proteolytic enzyme responsible for the observed fibrinolysis because plasma concentrations necessary to inhibit fibrinolysis were significantly higher than those to inhibit elastase activity.

Both FA and elastase activity were completely inhibited by alpha 1 antitrypsin, but no inhibition was obtained with alpha 2 macroglobulin. This can be explained by the fact that chromogenic substrate assays also measure elastase in alpha 2 macroglobulin-elastase complex by not blocking its active site. It is known that this complex changes gradually at 37°C to lower molecular weight forms possessing proteolytic activity also for fibrinogen. Although elastatinal has been described as an inhibitor of elastase, our studies confirm those of Nagamutsu et al., who showed that elastatinal inhibits only pancreatic elastase and not granulocytic elastase.

The PCA observed in HL60 cells was higher than that of monocytes. Aspecific stimulating of monocytes by adherence to glass or with phorbol esters leads to an increase of PCA. We found the same phenomenon for HL60 cells. A further increase of PCA with monocytic differentiation did not occur. Already higher values were found for HL60 cells as for monocytes. Nevertheless, HL60 cells can express a higher PCA, as was shown after stimulation with TPA. Granulocytic differentiation did lead to a diminished PCA as was expected. Kornberg et al. observed an increase in PCA in HL60 cells. This might, however, be due to aspecific stimulation since he used phorbol esters. The HL60 cells shortened the clotting time of plasma only when adequate amounts of factor V, VII, X, and II were present. Factor VIII was not a prerequisite for PCA, suggesting that the procoagulant material stimulates coagulation through the extrinsic pathway and resembles the activity of thromboplastin. This was previously found for APL cells and for monocytes. The hypothesis that the combination of PCA and fibrinolysis-inducing capacity is unique to the promyelocyte is attractive not only to explain the coagulopathy in APL, but to explain the findings of others who observed either PCA or proteolytic activity in leukemic cells in relation to the FAB classification. Although we studied only a small group of patients, the results we obtained in our AML patients support the hypothesis.

It has been shown that PMN undergo a secretory response in association with blood coagulation. Thrombin is a likely candidate to mediate the activation of these cells, leading to secretion of lysozomal enzymes, including elastase. It will be of interest to see whether coagulation induced by the PCA of promyelocytes can lead to thrombin formation and subsequently to a release reaction of lysozomal enzymes, thus explaining the severe coagulation disorders seen in APL.

The combination of proteolysis and coagulation activation may have implications for the treatment of APL patients. For this reason, we combine chemotherapy with heparin, tranexamic acid, and fresh frozen plasma to prevent severe bleeding complications. Furthermore, the influence of cell differentiation–inducing agents on PCA and proteolytic activity may have interesting consequences for the management of patients with APL.

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