A New Procoagulant in Acute Leukemia

By Anna Falanga, Maria Grazia Alessio, Maria Benedetta Donati, and Tiziano Barbiu

To verify whether cancer procoagulant (CP), a cysteine proteinase procoagulant distinct from tissue factor (TF), is associated with leukemic cells, we assayed the procoagulant activity of blast cell extracts from 26 patients with different cytological subtypes of acute nonlymphoid leukemia (ANLL) according to the French-American-British classification. All the samples except two shortened the recalcification time of normal human plasma, the effect being significantly greater in the M3 subgroup. The two criteria used to distinguish between CP and TF, independence from factor VII in initiating blood coagulation and sensitivity to cysteine-proteinase inhibitors, were positive in 19 samples from M1, M2, M3, and M4 cytological subtypes. None of the M5 samples fulfilled these criteria. In addition, M1, M2, M3, and M4 samples immunoreacted with an anti-CP goat polyclonal antibody on an Ouchterlony immunodiffusion plate. This study provides the first evidence for a procoagulant other than TF that is associated with leukemic cells.

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PATIENTS WITH MALIGNANT DISEASE have an elevated incidence of thromboembolic and/or hemorrhagic disorders.1

Hemorrhage is the most common hemostatic disorder in patients with all types of acute leukemias2,3,5 and reaches an incidence of 80% to 90% in acute promyelocytic leukemia.4,5 Consumption of coagulation factors is considered to be an important cause of this complication.

Among the mechanisms that can promote abnormal activation of blood clotting in malignancy, tumor-associated procoagulants are considered to play a prominent role.6 Three procoagulant activities have been described in tumor tissues: (a) tissue factor (TF), a glycoprotein that requires factor VII (VIIa) to activate coagulation and is represented in most normal tissues; (b) a tumor cell membrane structure that triggers the assembly of the prothrombinase complex;7 and (c) cancer procoagulant (CP), a cysteine proteinase that directly activates coagulation factor X and has thus far been described in tumor tissues from solid neoplasms9,11 and in human amnion-chorion tissue.12

In previous studies, the procoagulant activity of leukemic cells has been characterized as only TF.3,13-15 We report here that tissue extracts from leukemic cells of acute nonlymphoid leukemia (ANLL) patients also express a procoagulant activity with enzymatic and immunologic characteristics of CP.

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MATERIALS AND METHODS

Patients. Twenty-six patients with ANLL, eight males and 18 females (age range, 15 to 72 years), were studied at diagnosis before starting chemotherapy. They were classified into five groups according to the following cytological subtypes:16 four, M1; ten, M2; five, M3; three, M4; and four, M5.

In eighteen patients showing 85% to 95% circulating blast cells the study was performed on peripheral blood, in seven patients (one of M1, two of M2, three of M3, and one of M4 groups) it was performed on bone marrow, and in one patient (of the M5 group) both peripheral blood and bone marrow were used.

Ten healthy donors (five males and five females; age range 22 to 78 years) acted as controls for the study on peripheral blood, and four subjects (one male and three females; age range, 24 to 41) with mild iron deficiency anemia were the controls for the study on bone marrow. All patients were advised of procedures and attendant risks, in accordance with institution guidelines, and gave informed consent.

Tissue extracts. Peripheral blood or bone marrow was collected in 3.8% Na-citrate and diluted in phospholipase-buffered saline, pH 7.4. Mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo) density gradient centrifugation17 and consisted of >99% malignant blasts as assessed by phase microscopy after May-Grünwald-Giemsa staining and cytochemical staining (α-naphthyl-butyrate-esterase, Sudan black, PAS, and peroxidases).

After separation, cells were suspended in 1 to 3 mL of 20 mmol/L veronal buffer, pH 7.8, depending on the total cell number. The minimum used was 50 × 10⁶ cells/sample. Tissue extract of fresh cells was made at 4°C in three changes of veronal buffer for two hours each. Extracts were concentrated from 1:4 to 1:6 times on a Centricon 30 (Amicon Corp, Danvers, MA). Final volumes ranged from 0.5 to 2.5 mL.

Protein determination. Proteins were determined by Bradford's method.18

Procoagulant activity assay. The procoagulant activity of extracts was tested by the one-stage plasma recalcification assay1 in a clot timer, model 202 A (Heller Laboratories, Santa Rosa, CA). Briefly, 0.1 mL sample was added to 0.1 mL plasma and prewarmed for one minute at 37°C; the reaction was started by adding 0.1 mL of 0.025 mol/L CaCl₂.

The dependence on coagulation factor VII (F VII), factor IX (F IX), or factor X (F X) was tested by the recalcification assay of congenitally deficient plasmas (Dade Division, Pharmaseal, Trieste, Italy).

Coagulation standard controls were tissue thromboplastin (rabbit brain thromboplastin; Sigma Chemical Co, St Louis) and Russell’s viper venom (RVV, X activating enzyme; Sigma).

The procoagulant activity of samples or standard controls was
expressed as seconds or as the percent decrease in the clotting time compared with the corresponding buffer blank [(blank time – sample clotting time)/blank time]. The specific activity of samples was expressed as units of RVV per milligram protein. Units were calculated on a standard curve prepared with different concentrations (from 10^{-1} to 10^{-4}) of RVV according to Gordon and Lewis. The activity (expressed as the percent decrease of clotting time compared with the buffer blank) was plotted against the corresponding RVV concentration. Because the standard curve in normal plasma was the same as that obtained in F VII-deficient plasma, we consider 1 unit to be equal to the activity of 1 mEq/mL of RVV in the single-stage clotting assay of normal plasma as we have done previously for other tumor extracts.

Direct activation of F X was tested by a two-stage clotting assay. One microgram of purified bovine F X (kindly provided by Dr C. Vermeer, University of Maastricht, The Netherlands) in 0.1 mol/L Tris-buffered saline (pH 7.5) containing 5 mmol/L CaCl_2 and 3 μg phospholipids (Sigma RBC) was incubated at 37°C with RVV (0.5 ng) or an appropriate dilution of samples. At different intervals, 100 μL of the first-stage incubation mixture was tested in a single-stage recalcification assay of F VII- and F X-deficient bovine plasma (Sigma).

Inhibition studies. To determine whether the procoagulant activity of leukemic cell extracts was sensitive (like CP) to cysteine proteinase inhibitors, samples were preincubated for 30 minutes at 25°C with iodoacetamide (2 mmol/L) or HgCl_2 (0.1 mmol/L) before the plasma recalcification test. RVV, a serine proteinase, and papain (Sigma), a cysteine proteinase, were the standard controls for this assay.

To assess whether the samples were sensitive (like TF) to the inhibitory effect of concanavalin A (Con A, Sigma), they were incubated with the inhibitor (100 μg/mL, final concentration) for one hour at 37°C and then assayed for procoagulant activity. Prevention of the Con A inhibition by α-methyl-D-glucoside (Sigma) was also performed.

Immunodiffusion study. The immunoreactivity of the samples to a goat antiserum raised against purified CP (kindly provided by Dr S.G. Gordon, University of Colorado, Denver) was tested by Ouchterlony immunodiffusion in agar gel (1% agar in low-salt veronal buffer; ionic strength, 0.025; pH 8.2). Samples were left to react for 48 hours at 4°C. The plate was then washed, dried, and stained with crocein scarlet and Coomassie blue.

Statistical analysis. The statistical evaluations were performed by the one-way analysis of variance, by Tuckey’s test for multiple comparisons, and by the paired Student’s t test.

RESULTS

Tissue extracts from leukemic cells obtained from peripheral blood or bone marrow of patients with five different subtypes (M1 to M5) of ANLL were tested. All types of samples (except two, one of the M2 and one of the M5 group) significantly shortened the recalcification time of normal plasma. Figure 1 (left panel) shows the specific activity (RVV units per milligram protein) of each sample in normal human plasma. Results are expressed as means of at least two determinations. A one-way analysis of variance showed a significant difference between the groups. Multiple comparisons by Tuckey’s test indicated that the five extracts from M3 subtype cells were significantly more active than all the other samples (M3 v M1, P < .01; M3 v M2, P < .05; M3 v M4, P < .01; M3 v M5, P < .01). There were no significant differences between the activity of samples from peripheral blood and those from bone marrow within the same cytological subgroup. In addition, both bone marrow and peripheral blood samples from the same patient of the M5 group showed comparable procoagulant activity (3.9 and 3.6 RVV U/mg, respectively) in this test.

To characterize the procoagulant activity of the samples, we first attempted to define whether it required F VII to trigger blood coagulation. We therefore measured their capacity to shorten the recalcification time of F VII-deficient human plasma. As shown in Fig 1 (right panel), all the samples from the M1 to M4 groups were active in this test, with the exception of one of four M1 and two of ten M2, whereas none of the samples of the M5 group had activity under the same conditions. Statistical evaluation of these results by the analysis of variance and by Tuckey’s test demonstrated that there was a difference between the groups and also showed that in the absence of F VII the activity of the M3 samples was significantly greater than that of all the others (M3 v M1, P < .05; M3 v M2, P < .05; M3 v M4, P < .01; M3 v M5, P < .05). Samples from bone marrow and peripheral blood also had a comparable amount of procoagulant activity in the absence of F VII within the corresponding group.

The percent ratio between the activity in F VII-deficient...
plasma and the total activity in normal plasma for each sample is presented in Table 1.

The requirements of other coagulation factors were measured by using F IX– or F X–deficient plasmas only for pooled samples of each group. These experiments (Table 2) confirmed previous observations indicating an independence of the activity from the intrinsic pathway (the samples were active even in the absence of F IX) and a dependence on coagulation F X.

Extracts from normal cells failed to show any procoagulant activity in either normal or deficient plasmas.

To confirm that the procoagulant from the first four subtypes of leukemic cells triggered blood coagulation by directly activating F X, control RVV and pooled extracts of M2 or M3 samples were incubated with pure F X, and the generation of F Xa was measured in a second step by the shortening of recalcification time of F X– and F VII–deficient plasma. As shown in Fig 2, both extracts were able to promote a time-dependent activation of F X to the same extent as standard RVV.

An F VII–independent/F X–dependent procoagulant with cysteine proteinase characteristics has been isolated from extracts of V2 carcinoma of the rabbit and characterized enzymatically and immunologically in extracts of human melanoma; we therefore tested whether the procoagulant activity of leukemic cells of the first four groups also had cysteine proteinase activity. Active samples of each group were incubated for 30 minutes at 25°C with either iodoacetamide (2 mmol/L) or HgCl2 (0.1 mmol/L) and then tested in the one-stage clotting assay. Figure 3 reports the susceptibility to cysteine proteinase inhibitors (iodoacetamide and HgCl2) of samples representative of each of the five groups of patients. Both iodoacetamide and HgCl2 produced a significant inhibition (P < .01) of activity in M1, M2, M3, and M4 extracts. Neither inhibitor affected the M5 sample. As a reference for the system, the inhibitors were also tested against the activity of two standard control proteinases as already described: papain, a cysteine proteinase, was signifi-

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<th>Table 1. Estimation of the Percent F VII–Independent Activity in ANLL Cell Extracts</th>
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Abbreviations: PB, peripheral blood; BM, bone marrow.  
*Numbers are specific activity in F VII–deficient plasma/specific activity in normal plasma x 100.
Fig 3. Effect of cysteine proteinase inhibitors on the procoagulant activity of cell extracts from five patients with different ANLL cytological types (M1 to M5). The procoagulant activity of samples or standards (RVV and papain) was assayed before and after incubation for 30 minutes at 25°C with 2 mmol/L iodoacetamide (left panel) or 0.1 mmol/L HgCl₂ (right panel). Results are the means of three measurements and are expressed as RVV units per milligram protein (as in Fig 1). The M3 extract was diluted to obtain an activity in the same range as that of the other samples.

Fig 4. Effect of Con A on the procoagulant activity of ANLL cell extracts. Blast extracts of M2 (pool of three samples), M4 (pool of four samples), M5 (from a single patient), and two standard controls (rabbit brain thromboplastin [TF] and RVV) were incubated with Con A (100 μg/mL) for one hour at 37°C. Aliquots of samples were incubated with α-methyl-D-glucoside for 15 minutes at 37°C before the treatment with Con A. After incubations, control (white bars), Con A-treated (striped bars), and α-methyl-D-glucoside plus Con A-treated (black bars) samples were tested for clotting activity in the one-stage clotting assay of normal human plasma. Results are expressed as seconds normalized to a blank clotting time of 250 seconds and are the means ± SE of three determinations.

The procoagulant activity of leukemic cells can play a role in the pathogenesis of the severe coagulopathy often occurring in acute leukemia. Thus far TF had been characterized in cells from ANLL and acute lymphoid leukemia. This study shows that in different types of leukemic cells, another procoagulant can be demonstrated, ie, a direct activator of coagulation F X with cysteine protease activity (CP). Our experiments were conducted on leukemic cell extracts because CP had been purified to homogeneity from extracts of rabbit V2 carcinoma and also because, in human melanoma, CP activity showed much higher stability in extracts than in isolated cells.

We were able to identify a procoagulant activity with enzymatic characteristics of CP in all leukemic samples with the exception of four with the M5 subtype and three from the other groups. This is borne out by four findings: first, the samples' ability to activate coagulation F X in the absence of F VII; second, their sensitivity to cysteine proteinase inhibitors; third, the activation of pure F X in a two-stage clotting assay; the level of decrease of TF activity by Con A represented complete inhibition. Two M5 blast extracts were inhibited by Con A to the same extent as the TF control (only one is shown in the figure), which suggests that the procoagulant activity was TF. Inhibition of the M4 sample (pool of three different extracts) reduced the activity to about 30% of that of the TF control. The M2 extract (pool of four different extracts) was the least inhibited by Con A, its activity being reduced to about 20% of the TF control. Pretreatment of the samples with α-methyl-D-glucoside abolished the effect of Con A in the Con A-sensitive samples.

An immunologic identification of CP in the extracts was performed by Ouchterlony double immunodiffusion. A precipitin line was present on the immunodiffusion plate between the anti-CP antibody and M1, M2, M3, and M4 extracts (Fig 5). No precipitation was observed against M5 and the control bone marrow extract.

DISCUSSION

The procoagulant activity of leukemic cells can play a role in the pathogenesis of the severe coagulopathy often occurring in acute leukemia. Thus far TF had been characterized in cells from ANLL and acute lymphoid leukemia. This study shows that in different types of leukemic cells, another procoagulant can be demonstrated, ie, a direct activator of coagulation F X with cysteine protease activity (CP). Our experiments were conducted on leukemic cell extracts because CP had been purified to homogeneity from extracts of rabbit V2 carcinoma and also because, in human melanoma, CP activity showed much higher stability in extracts than in isolated cells.

We were able to identify a procoagulant activity with enzymatic characteristics of CP in all leukemic samples with the exception of four with the M5 subtype and three from the other groups. This is borne out by four findings: first, the samples' ability to activate coagulation in the absence of F VII; second, their sensitivity to cysteine proteinase inhibitors; third, the activation of pure F X in a two-stage clotting assay;
and fourth, the cross-reactivity to a polyclonal antibody raised against purified CP.

Three tumor cell procoagulant activities have been described. TF is a 53,000-molecular weight (mol wt) membrane glycoprotein that is found in normal and malignant cells; it requires F VII for activity and is inhibited by Con A. The tumor cell membrane structure described by Van de Water et al facilitates the assembly of the prothrombinase complex on tumor cells and accelerates the activation of prothrombin; it does not activate F X, nor is it inhibited by Hg²⁺ or iodoacetamide. CP is a cysteine proteinase that directly activates F X and is inhibited by Hg²⁺ and iodoacetamide but not by Con A.

Although other properties of the purified procoagulant could not be defined (eg, mol wt, pl) because of the very small size of the samples and absolute characterization of any procoagulant needs purification to homogeneity, we have found that each of the four criteria tested in this study are consistent with the properties of only one of the known procoagulants, CP, and are not compatible with the others.

The question may arise whether other cells such as contaminating monocyte-macrophages might be responsible for this new type of procoagulant activity. This was ruled out by the homogeneity of the cell suspensions (>99% blasts) from which extracts were made, by the fact that to our knowledge mono- and peripheral blood mononuclear cells from normal donors did not show any kind of procoagulant activity. The last observation confirms other findings demonstrating (a) that peripheral blood mononuclear cells do not produce TF if not challenged by the appropriate stimuli (ie, endotoxin, phorbol esters, immuno-complexes, etc) and (b) that human myeloid precursor cells do not produce procoagulant activity.

This therefore appears to be the first report on a procoagulant different from the TF associated with leukemic cells.

These experiments were designed to extract and analyze CP activity, so there was not necessarily an efficient and quantitative recovery of TF in the extracts. Our data, however, suggest that both F VII-dependent (TF) and F VII-independent (CP) activities were present in the ANLL extracted samples as in other malignant tissues. Because it was not possible, under the described experimental conditions, to determine the specific activities of both proteins, we cannot quantify the amount of each procoagulant in the samples. A rough estimation of our data seems to suggest that there are different proportions of CP and TF activity in each cell type: the M5 had no measurable F VII-independent activity and was almost totally inhibited by Con A, whereas the M4 subtype had about 60% F VII-independent activity and 30% Con A-sensitive activity.

Other investigators have demonstrated TF activity in promyelocytic and other leukemic cell preparations and have demonstrated the immunologic cross-reactivity with human brain TF.

Most of those studies were conducted on a cell suspension, which is a type of sample preparation very different from that used in this work. They all describe the presence in acute leukemia of an F VII-dependent procoagulant activity, although some of the oldest characterizations have probably been done on nonhomogeneous cell populations and the immunologic study performed with a not well characterized anti-TF polyclonal antibody.

In conclusion, this report describes the expression, in a different experimental condition, of a new procoagulant activity in cells from ANLL; its relative role in the pathogenesis of severe coagulopathy remains to be established.

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PROCOAGULANT ACTIVITY OF LEUKEMIC CELLS


A new procoagulant in acute leukemia

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