Hemostatic balance on the surface of leukemic cells: the role of tissue factor and urokinase plasminogen activator receptor

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Background and Objectives. The frequency of thrombotic complications is increased in patients with acute leukemia. Since coagulation processes take place on cell membranes, we hypothesized that expression of coagulation proteins on blast membrane could determine the hemostatic balance on the surface of leukemic cells and may correlate with thrombotic manifestations.

Design and Methods. Fifty-one consecutive patients with newly diagnosed acute leukemia were enrolled over an 11-month period. Twenty-five of the patients had acute myeloid leukemia (AML)-M_{0-2}, 11 had AML-M_3, 6 had AML-M_{4-5}, and 9 acute lymphocytic leukemia (ALL). Peripheral blood and bone marrow were analyzed by flow-cytometry for tissue factor, protease-activated receptor 1, tissue factor pathway inhibitor, urokinase plasminogen activator receptor, and thrombomodulin.

Results. Regardless of the leukemia subtype, tissue factor was predominantly present on leukemic blast surfaces as compared to protease-activated receptor 1, tissue factor pathway inhibitor, urokinase plasminogen activator receptor and thrombomodulin and it was significantly elevated (mean 63±6%) in AML-M_3 and AML-M_{4-5} as compared to AML-$M0_{-2}$ and ALL (mean 37±4%, $p<0.001$). Likewise, urokinase plasminogen activator receptor expression was greater in AML-M_{4-5} (49±11%) than in in AML- M_{0-2}, M_3 and, ALL (mean 17±3%, $p<0.001$). Thrombotic manifestations were present in 13 out of 51 (26%) patients. The tissue factor to urokinase plasminogen activator receptor ratio was higher in patients with a thrombotic event than in patients without thrombotic events (16±4 vs. 6±2, $p=0.042$).

Interpretation and Conclusions. This study demonstrates that tissue factor predominates on leukemic blast surface, particularly in M3 and M4-5 subtypes, while urokinase plasminogen activator receptor is increased on M4-5 blasts. The hemostatic balance on the blast surface may contribute to thrombotic manifestations in leukemic patients.

Key words: cell surface, thrombophilia, thrombosis, tissue factor, urokinase plasminogen activator receptor.

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Specific features of the surface of cells, including expression of protein receptors and glycosaminoglycans as well as lipid composition of the outer leaflet of plasma membranes, determines the cells' role in hemostasis.[1] Cell surface proteins that can have a procoagulant effect include tissue factor and protease-activated receptor-1. Tissue factor is a transmembrane glycoprotein that binds to factor VII, triggering the cell surface assembly of the coagulation protease cascade and leading to fibrin formation. Protease-activated receptor 1 is expressed on various cell types, including human platelets, endothelial cells, and tumor cells, and is the primary mediator of thrombin-stimulated platelet procoagulant activity.[2] Cell surface proteins that have either an anticoagulant or a profibrinolytic effect include tissue factor pathway inhibitor and urokinase, plasminogen activator receptor. Tissue factor pathway inhibitor is a potent direct inhibitor of factor Xa and factor VIIa-tissue factor complex. Heparan sulfate proteoglycans on the cell surface are directly associated with tissue factor pathway inhibitor enabling the latter's inhibitory effect at the cell surface membrane.[3] Urokinase plasminogen activator receptor is a specific cellular receptor for the urokinase plasminogen activator, resulting in the enhanced activation of plasminogen. The absence of urokinase plasminogen activator may predispose to thrombosis.[4,5] Thrombomodulin is a cell surface glycoprotein that forms a complex with thrombin. A high concentration of thrombomodulin has an anticoagulant effect resulting in the activation of protein C, whereas low concentrations of thrombomodulin stimulate the thrombin acti-

vatable fibrinolysis inhibitor.[6] These coagulation proteins are expressed on endothelial cells, as well as on tumor cells.[7-10]

In most cancer patients the coagulation system is activated. Over-expression of tissue factor, cancer procoagulant, and acquired activated protein C resistance[11] have been argued to be the main factors for the coagulopathy in malignant disorders. While patients with acute leukemia generally exhibit activation of the coagulation system, individuals with acute promyelocytic leukemia are particularly at risk of severe thrombotic and bleeding complications due to hemostatic changes. As coagulation processes take place on cell membranes, we assumed that the expression of coagulation proteins on leukemic blast membranes determine the local hemostatic balance and may correlate with thrombotic manifestations. In the present study, blast surface hemostatic balance was evaluated in 51 consecutive patients with newly diagnosed acute leukemia of different subtypes at their admission to hospital, and was correlated with clinical thrombotic manifestations, both venous and arterial. Various plasma coagulation markers and thrombophilic factors were also studied and correlated with thrombotic manifestations.

Design and Methods

Patients

This study was performed on 51 consecutive patients with newly diagnosed acute leukemia enrolled over an 11-month period, between February 2003 and December 2003, at Rambam Medical Center. Twenty-five patients had acute myeloid leukemia (AML)-M_{0-2}, 11 had AML-M_3, 6 had AML-M_{4-5} and 9 had acute lymphocytic leukemia (ALL). The patients were diagnosed according to established morphological, immunophenotypic and molecular criteria.[12] After obtaining informed consent, peripheral blood and/or bone marrow aspirates were taken at the time of diagnosis (prior to chemotherapy treatment). The patients were followed-up prospectively for the occurrence of symptomatic thrombotic manifestations during the first month of hospitalization. Deep vein thrombosis was diagnosed by compression ultrasonography and interpreted by our senior hospital radiologists.

Cells

Twenty milliliters of peripheral blood or 2-4 mL of bone marrow aspirate were analyzed. The mononuclear cells of the samples were separated on Ficoll Hypaque density gradient (Sigma), washed twice with phosphate-buffered saline (PBS), resuspended in 1 mL RPMI 1640 (Beit Haemek, Israel) with 10% dimethyl sulfoxide (Sigma), and frozen in liquid nitrogen. Cells were gradually thawed in 10 mL PBS (Beit Haemek, Israel) containing 10% human albumin (Omrix, Isreal) and then resuspended to 50 mL with the addition of PBS. After centrifugation (300 g) for 10 minutes, the pellet was resuspended in 100 μL of PBS containing 0.02% sodium azide. Cell viability was assessed by trypan blue exclusion. Only samples with more than 80% viable cells were included in the study.

Flow cytometric analysis

Cells (0.5 to 1×10^6) were stained for 15 minutes at 4°C with specific antibodies and subjected to analysis using FACS Calibur (Becton Dickinson). Ten thousand cells were studied in each sample. Pathologic cells were separated according to size and granularity parameters and by using specific antibodies: anti-CD45, anti-CD34, anti-HLA DR, anti-CD10 and anti-CD20 (Becton-Dickinson; 1:10). Tissue factor staining was performed by fluorescein isothiocyanate-conjugated anti-human monoclonal IgG_1 (American-Diagnostica; 1:10). Protease-activated receptor 1 was stained with phycoerythrin-conjugated monoclonal IgG_1 (Santa Cruz Biotechnology; 1:10) and tissue factor pathway inhibitor was stained with unconjugated monoclonal IgG_1 (American-Diagnostica; 1:50). Urokinase plasminogen activator receptor staining was performed using unconjugated monoclonal IgG1 (R&D Systems; 1:50) and thrombomodulin was stained with unconjugated monoclonal IgG_1 (American-Diagnostica; 1:50). Fluoroscein isothiocyanate or phycoerythin-conjugated anti-mouse antibodies (Santa Cruz Biotechnology; 1:100) were used as secondary antibodies and fluoroscein isothiocyanate and/or phycoerythin-conjugated anti-human non-specific monoclonal IgG_1 (American-Diagnostica; 1:10) were employed as controls. Twenty-seven out of the 51 samples were analyzed twice, on two different occasions. No major differences were observed between the two matched samples and the mean values were calculated.

Tissue factor activity assay

Tissue factor procoagulant activity was quantified using the Actichrome® tissue factor activity assay (American -Diagnostica). Eleven samples containing more then 90% pathologic cells were studied. One thousand cells from each sample, in assay reaction buffer, were mixed with human factor VIIa and human factor X. The cells and reagents were incubated at 37°C, allowing for the formation of tissue factor/factor VIIa complex and for the complex to convert the human factor X to factor Xa. The amount of factor Xa genereted was measured by its ability to cleave Spectrozyme® Xa, a highly specific chro-

mogenic substrate for factor Xa, which was added to the reaction solution. The solutions absorbance was read at 405 nm.

Western blot analysis of tissue factor and urokinase plasminogen activator receptor

Eighteen samples, containing more than 90% (94%±2) pathologic cells, were evaluated by western blot analysis. Cell extracts were prepared using a lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100 in the presence of protease inhibitors (Roche). Proteins were resolved by SDS-PAGE under reducing conditions using 10% polyacrylamide gels. After electrophoresis, the gels were transferred to a polyvinylidene floride membrane (BioRad). The membrane was first probed with the polyclonal anti-human tissue factor antibody (American-Diagnostica) and monoclonal anti-human urokinase plasminogen activator receptor antibody (R&D Systems) followed by a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). The blot was developed using a chemiluminescence substrate (Pierce).

Coagulation assays and thrombophilia assesment

The plasma coagulation parameters measured included: prothrombin time, activated partial thromboplastin time, D-dimer, prothrombin fragments 1+2, fibrinogen, α_2 antiplasmin, and plasminogen. These plasma coagulation parameters were evaluated at the time of diagnosis. The thrombophilia work-up included assessment of protein C activity, antithrombin activity, protein S level, activated protein C sensitivity ratio, lupus anticoagulant and anticardiolipin antibodies, homocysteine, factor V Leiden, factor II G20210A, and MTHFR C677T mutations. The thrombophilia assessment in patients with AML was performed at least one month after the beginning of the first hospitalization, and prior to the second admission for chemotherapy. In patients with ALL, the thrombophilia evaluation was performed between 6-8 weeks after starting the chemotherapy protocol and at least 2 weeks after completion of treatment with L-asparaginase. Blood samples were collected by venipuncture into 3.2% citrate tubes. The prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer and activated protein C sensitivity ratio were measured in fresh platelet-poor plasma, prepared by centrifugation at 2000 g for 15 min. For other plasma assays, following a second centrifugation at 2000g for 15 min, plasma aliquots were frozen at -35±5°C if they were to be used with 3 months and at -70±5°C if storage was expected to be longer. Plasma aliquots for assays were thawed by placing them in a water bath at 37±0.5°C for 15 min. Genomic DNA was extracted from blood leukocytes by standard methods. The prothrombin time, activated partial thromboplastin time, fibrinogen and D-dimer assays were performed on the STA-R analyzer using recombinant human thromboplastin Dade® Innovin® (Dade Behring Marburg GmbH, Germany) for the prothrombin time assay. STA-PTT®, STA-FIBRINOGEN and STA-LIATEST® D-DI kits were used for the activated partial thromboplastin time, fibrinogen and D-dimer assays, respectively, (Diagnostica STAGO, France). The activated protein C sensitivity ratio was determined by adding activated protein C to the activated partial thromboplastin time assay without factor V deficiency plasma dilution, using a Coatest activated protein C resistance kit (Chromogenix AB, Molendal, Sweden). All samples with an activated protein C sensitivity ratio of 2.5 or less were screened for the factor V Leiden mutation. Activated protein C-resistance was defined as an activated protein C sensitivity ratio < 2.0. Protein C, antithrombin, plasminogen and antiplasmin activities in plasma were measured on the STA-compact analyzer using STA-STACHROM® kits (Diagnostica STAGO, France). Free protein S antigen was determined by immunoturbidimetric assay on the STA-compact analyzer using a STA-Liatest® Free Protein S kit (Diagnostica STAGO, France). Lupus anticoagulant assays were performed using the diluted Russell's viper venom time assay (Screen and Confirm, Gradipore, North Ryde, Australia) according to the recommendations from the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis. Positive samples were further confirmed by the thromboplastin titration index assay. Factor V Leiden, factor II G20210A and MTHFR C677T mutations were determined by polymerase chain reaction followed by digestion with a restriction enzyme, electrophoresis in acrylamide gel and staining with ethidium bromide, as previously described.[13] The level of prothrombin fragments 1+2 in plasma was measured by enzyme immunoassay using Enzygnost® F1+2 micro (Dade Behring Marburg GmbH, Germany). Homocysteine was measured in the plasma by a fluorescence polarization immunoassay on an Abbott IMX analyzer (Abbott Laboratories). Blood samples for homocysteine measurements were drawn after an overnight fast and the concentration of homocysteine measured immediately. Anticardiolipin antibodies were measured using an ELISA kit (Diagnostica STAGO, France).

Statistical analysis

Data were evaluated by SPSS software for Windows version 13.0 (SPSS Inc., Chicago, IL, USA). Pearson's correlation (r) was used to assess the link

Table 1. Thrombotic events in the 13 patients with acute leukemia.

Acute leukemia type	*Site of thrombosis type*	*Number of patients*
M0-2	Acute myocardial infarction	1
M0-2	Central line thrombosis	3
M0-2	Superficial vein thrombosis	2
M3	Acute myocardial infarction	1
M3	Pulmonary emboli*	2
M3	Splenic infarction#	2
M3	Central line thrombosis	1
M3	Superficial vein thrombosis	1

**One patient also had pulmonary and central nervous system hemorrhage and the other also had central nervous system and severe vaginal hemorrhage.*
#One of the patients also had superficial vein thrombosis.

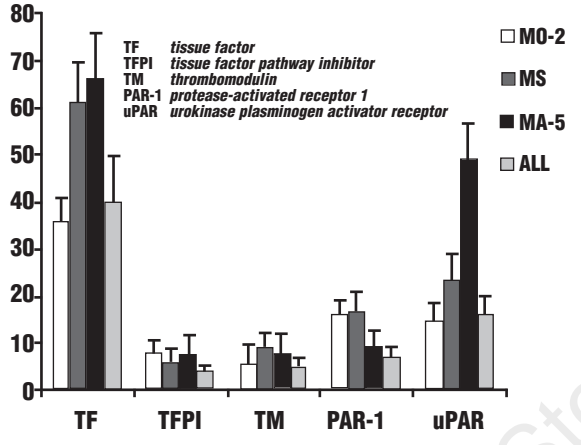

Figure 1. The various hemostatic markers on the blast cells of the different subtypes of leukemia. Of the 51 patients studied, 25 had AML-$M_{0\text{-}2}$, 11 AML-M_3, 6 had AML-$M_{4\text{-}5}$ and 9 had ALL. Data refer to the percentage of total positive cells by flow-cytometry for each of the markers described and represent the mean ± s.e.m. The dominant marker on the cell surface of all leukemia subtypes was tissue factor.

between the relative density of tissue factor protein in the cell extracts and cell surface tissue factor expression; between the relative density of urokinase plasminogen activator receptor protein in the cell extracts and cell surface urokinase plasminogen activator receptor expression; and between tissue factor cell surface expression and tissue factor activity. T-tests for independent samples were used. Values are reported as mean ± s.e.m or mean ± SD. A p value <0.05 is considered statistically significant.

Results

The study included 51 consecutive patients with newly diagnosed acute leukemia (25 with AML-$M_{0\text{-}2}$, 11 with AML-M_3, 6 with AML-$M_{4\text{-}5}$, and 9 with ALL), who were followed-up prospectively for thrombotic

Table 2. Overall characteristics of the 51 patients studied.

Parameters	*All patients (n=51)*	*Without a thrombotic event (n=38)*	*With a thrombotic event (n=13)*
Age (years, mean±SD)	50	50 ± 20	50 ± 17
Female sex, n (%)	19 (37)	17 (45)	2 (15)
Acetylsalicylic acid use at enrollment, n (%)	2 (4)	1 (3)	1 (8)
Concomitant illness at enrollment, n (%)			
Ischemic heart disease	6 (12)	3 (8)	3 (23)
Hypertension	3 (6)	2 (5)	1 (8)
Non-insulin-dependent diabetic mellitus	8 (16)	5 (13)	3 (23)
Cerebrovascular accident	2 (4)	1 (3)	1 (8)
Hyperlipidemia	1 (2)	0 (0)	1 (8)
Chemotherapy drugs*, n (%)			
Ara-C, doxorubicin	38 (75)	25 (66)	13 (100)
Daunorubicin, vincristine, prednisone, L-asparginase	8 (16)	8 (21)	0 (0)
Hydroxyurea	5 (10)	5 (13)	0 (0)

**During the first month of treatment.*

manifestations during the first month after their diagnosis. Thrombotic manifestations occurred in 13 out of the 51 (26%) patients. In detail, thrombotic events occurred in 6 out of 25 patients (24%) with AML-$M_{0\text{-}2}$, in 7 out of 11 patients (64%) with AML-M_3. No thrombotic manifestations were found in patients with AML-$M_{4\text{-}5}$ or ALL (Table 1). The characteristics of the 13 patients who had a thrombotic event and of the 38 patients who did not are listed in Table 2. The two groups of patients differ in the relative representation of females, concomitant illness at diagnosis, and chemotherapy drugs used during the first month of treatment, although the subgroups are too small for a statistical evaluation of the differences. Figure 1 illustrates the hemostatic markers on the blast cells in patients with the different subtypes of leukemia. Regardless of the leukemia subtype, tissue factor was more predominantly present on the surface of leukemic blasts (mean 46±4%) than were protease-activated receptor 1, tissue factor pathway inhibitor, urokinase plasminogen activator receptor and thrombomodulin (mean 14±2%, 7±2%, 21±3%, 9±2%, respectively p<0.001). Tissue factor levels were significantly higher in AML-M_3 (62±8%) than in AML-$M_{0\text{-}2}$ (36±5, p=0.01), higher but not statistically different from those in ALL (40±9, p=0.09) and comparable to those in AML-$M_{4\text{-}5}$ (67±9, p=0.7). Cell surface tissue factor expression was significantly greater in

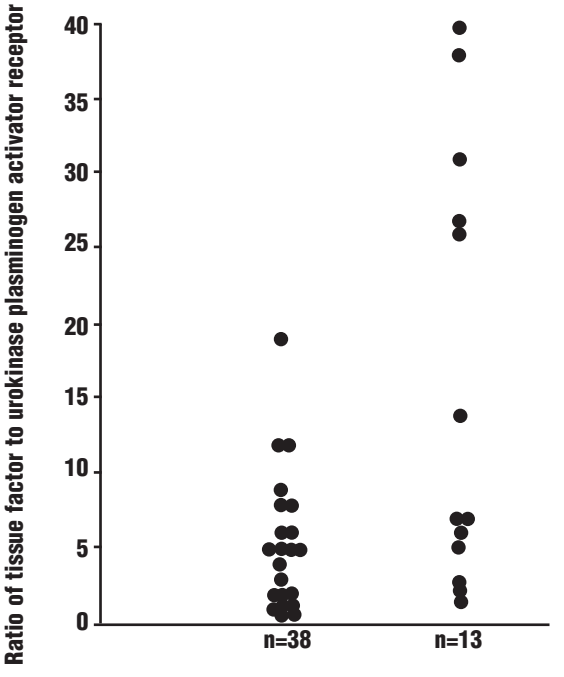

Figure 2. The ratio of tissue factor to urokinase plasminogen activator receptor in patients without (n=38) and with a thrombotic event (n=13). Since the two dominant hemostatic proteins were tissue factor and urokinase plasminogen activator receptor, the ratio between these two parameters was calculated in the 38 patients without a thrombotic event and compared to that in the 13 patients with a thrombotic event (6±2 vs.16±4). A statistically significant difference was observed in the t-test for independent samples (p=0.042).

AML-M$_3$ and AML-M$_{4-5}$ (mean 63±6%) than in that in AML-M$_{0-2}$ and ALL (mean 37±4%, p<0.001). In addition, urokinase plasminogen activator receptor was significantly higher in AML-M$_{4-5}$ (mean 49±11%) than in AML- M$_{0-2}$, ALL, and also AML-M$_3$ (mean 17±3%, p<0.001). As tissue factor expression was found to be high in all subtypes of leukemia, especially in AML-M$_3$ and AML-M$_{4-5}$, whereas urokinase plasminogen activator receptor exhibited a different pattern, being dominantly elevated in AML-M$_{4-5}$, we hypothesized that the raised level of urokinase plasminogen activator receptor may have a protective, anti-thrombotic role. In order to verify this assumption the ratio between tissue factor and urokinase plasminogen activator receptor was calculated in the 13 patients with a thrombotic event and in the 38 patients without such an event (6±2 vs. 16±4, p=0.042) (Figure 2). When the tissue factor to urokinase plasminogen activator receptor ratio was multiplied by the total white blood cell count and the means of these two groups of patients (783±455 vs. 153±55) were compared by a t-test for independent samples, the difference remained statistically significant (p=0.044). In order to validate the results using the flow-cytometry method, 11 samples were studied for tissue factor activity and 18 samples were evaluated by western blot analysis for the expression of tissue factor and urokinase plasminogen activator receptor in the cell extracts. A positive correlation was found between cell surface tissue factor and tis-

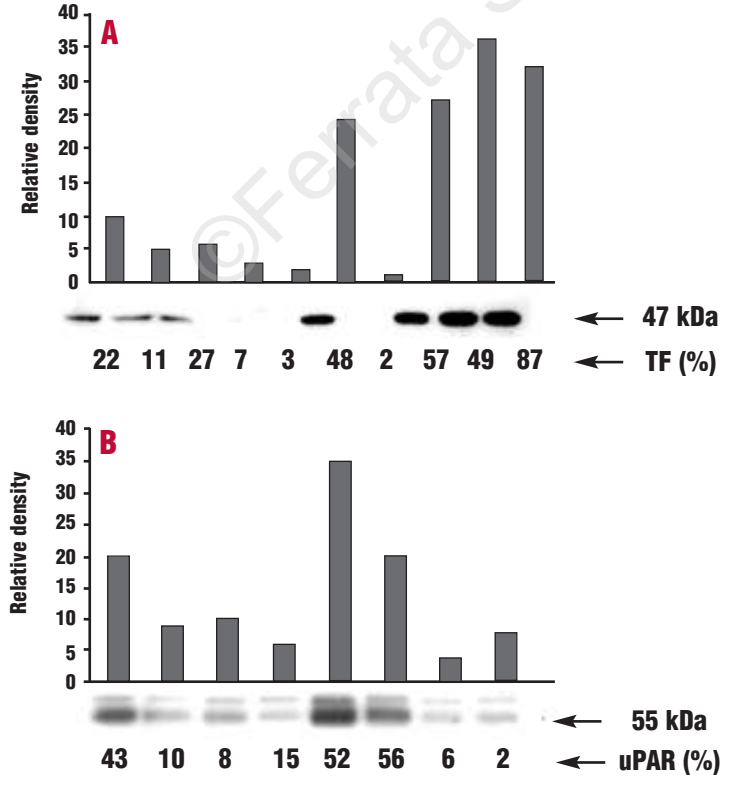

Figure 3. A. Correlation between protein and cell surface tissue factor expression in leukemia cells illustrated by immunoblotting and the relative density of tissue factor protein (47 kDa) from leukemia cells of 10 patients (upper panel) and the cell surface tissue factor expression (%) by flow-cytometry of the same patients' cells (lower panel). A strong positive correlation was found between the relative density of total tissue factor in the cell extracts and the cell surface tissue factor expression (r=0.91, p=0.001). B. Correlation between protein and cell surface urokinase plasminogen activator receptor expression in leukemia cells of 8 patients. A strong positive correlation was observed between the relative density of total urokinase plasminogen activator receptor protein in the cell extracts and the cell surface urokinase plasminogen activator receptor expression (r=0.87, p=0.002).

Table 3. Coagulation parameters in the leukemia patients at the time of diagnosis.

Leukemia type	*PT (60-120%)*	*APTT (27-40 seconds)*	*D-dimer (0.0-0.5 mg/L)*	*PT1+2 (0.4-1.1 nmol/L)*	*Fibrinogen (160-400 mg/dL)*	*α_2AP (70-130%)*	*Plasminogen (70-130%)*
Patients evaluated (n)	51	51	43	39	47	29	29
M_{0-2}	80±20	36±7	3.2±1.9	3.3±2.6	453±155	100±24	90±26
M_3	60±14	33±5	7.6±6.1	5±3.5	252±132	71±22	79±25
M_{4-5}	68±21	38±7	4.1±3	0.74±0.08	476±106	98±15	90±11
ALL	82±19	28±4	4±2.8	4.2±2.4	331±208	91±25	89±38

PT: prothrombin time; APTT: activated partial thromboplastin time; PT1+2: prothrombin fragments 1+2; α_2AP: $\alpha_2$antiplasmin. Values are mean ± SD.

Table 4. Thrombophilia in the various leukemia subtypes.

Leukemia type	*Thrombophila*	*Hyperhomocysteinemia**	*LAC/ACL*
$M0_{-2}$	12/15 (80%)	5/8 (63%)	4/15 (27%)
M_3	7/9 (77%)	3/9 (33%)	3/9 (33%)
M_{4-5}	3/4 (75%)	1/4 (25%)	0/4 (0%)
ALL	5/6 (83%)	3/6 (50%)	1/6 (17%)
All patients	27/34 (79%)	15/34 (44%)	8/34 (24%)

LAC/ACL: lupus anticoagulant/anticardiolipin antibodies.
**Normal homocysteine levels: females < 12 μmol/L, males < 14 μmol/L.*
Most patients with hyperhomocysteinemia (12/15, 80%) had only mild elevation of homocysteine levels (<20 μmol/L).

sue factor activity (r=0.95, p=0.03). Similarly, a good correlation was found between cell surface levels and protein expression in the cell lysate for tissue factor (r=0.91, p=0.001) and urokinase plasminogen activator receptor (r=0.87, p=0.002) (Figure 3).

Association of thrombotic events with plasma coagulation markers

A range of plasma coagulation parameters were evaluated at the time of diagnosis (Table 3). The rationale was to assess whether the level of the coagulation markers could predict thrombotic manifestations and therefore render evaluation of surface coagulation markers redundant. T-tests for independent samples, performed for each coagulation marker in patients with and without a thrombotic event, showed that the two groups were comparable for the following markers: prothrombin time (p=0.85), activated partial thromboplastin time (p=0.59), D-dimer (p=0.12), prothrombin fragments 1+2 (p=0.77), fibrinogen (p=0.065), antiplasmin (p=0.49), and plasminogen (p=0.69). Interestingly, in contrast to the other plasma parameters evaluated, D-dimer levels were raised in all patients in whom they were measured.

Association of thrombotic events with thrombophilia

Twenty-seven out of 34 (79%) patients had one or more pathologic thrombophilia marker (Table 4). The most common types of thrombophilia were hyperhomocysteinemia (15 out of 34, 44%) and lupus anticoagulant/anticardiolipin antibodies (8 out of 34, 24%). Three out of 34 patients had both hyperhomocysteinemia and lupus anticoagulant/anticardiolipin antibodies. Of the 8 patients with lupus anticoagulant/anticardiolipin antibodies 6 had lupus anticoagulant, 2 had anticardiolipin antibodies, and one had both. Five patients had combined thrombophilia. The prevalence of thrombophilia was similar in patients with and without a thrombotic event, reaching 72% (8 out of 11). Among the patients with thrombosis, four had lupus anticoagulant antibodies, four had hyperhomocysteinemia and one had both. Two patients with a thrombotic event had combined thrombophilia.

Discussion

Coagulation cell surface receptors are important in controlling hemostasis. In the present study we explored the hemostatic balance on the surface of leukemic cells, with a special focus on assessing the range of coagulation markers. Specific cell surface hemostaic markers have been analyzed in a number of previous studies. Several groups evaluated cell surface expression of tissue factor alone on leukemic cells.[14,15] Lopez-Pedrera *et al.*[16] used flow cytometry to study the cell surface expression of both tissue factor and urokinase plasminogen activator receptor in 26 patients with acute leukemia. In that study, tissue factor level was found to be high (>68%) in two out of four patients with AML-M_3 and in all the three patients with AML-M_5, whereas in patients with other subtypes of leukemia the tissue factor value was less than 16%. Urokinase plasminogen activator

receptor expression was high (92%) in one of the four AML-M_3 patients and in one of the AML-M_5 patients (70%); the other two AML-M_5 patients were not evaluated.

In the rest of the patients the urokinase plasminogen activator receptor expression was less than 17%. The results of Lopez-Pedrera *et al.*, collected in a very small number of patients, are confirmed by our study. In the present study, the cell surface markers studied were two procoagulant receptors, (tissue factor and protease-activated receptor 1), one anticoagulant marker, tissue factor pathway inhibitor, and one profibrinolytic receptor (urokinase plasminogen activator receptor). At high concentrations thrombomodulin has an anticoagulant effect, whereas at low concentrations it has an antifibrinolytic effect.[4] Tissue factor was found to be the dominant marker on the cell surface, prevailing over protease-activated receptor 1, tissue factor pathway inhibitor, urokinase plasminogen activator receptor, and thrombomodulin. These findings, in accordance with the good correlation found between tissue factor cell surface expression and tissue factor activity, support the assumption that the surface of leukemic cells is procoagulant and may contribute to thrombotic manifestations. We found that virtually all patients with acute leukemia expressed tissue factor on the cell surface of their blasts, in contrast to older data from Carson *et al.*,[17] who claimed that the cell surfaces in acute promyelocytic leukemia (AML-M_3) generally do not express tissue factor. The difference between these findings may be the consequence of the more sensitive antibodies used in our study. Among the various leukemia subtypes, tissue factor expression was significantly higher in AML-M_3 and AML-M_{4-5} than in AML-M_{0-2} and ALL. Thrombotic manifestations are well-established complications of AML-M_3,[18] whereas they are less common in AML-M_{4-5}. Surface expression of the other hemostatic factors was low, except for urokinase plasminogen activator receptor. The expression of urokinase plasminogen activator receptor was significantly higher in AML-M_{4-5} than in the other subtypes of leukemia, including AML-M_3, indicating its potential, protective antithrombotic effect.

Thrombotic manifestations were present in 13 out of 51 (26%) patients: in 6 out of 25 (24%) patients with AML-M_{0-2} and in 7 out of 11 (64%) patients with AML-M_3, but in none of the patients with AML-M_{4-5} or ALL. According to the ALL chemotherapy protocol used at our department, patients receive L-asparaginase (10,000 U/m^2), a potentially prothrombotic drug, and prophylactic enoxaparin (40 mg/day) for the last two weeks of the first month of treatment.[19]

This strategy may have influenced the thrombotic manifestations in the ALL subgroup.[20] The use of different chemotherapy protocols in AML and ALL and the use of all-trans retinoic acid in AML-M_3 may also influence thrombosis. Bleeding manifestations in leukemic patients may perhaps represent the result of a consumption coagulopathy, which is basically a thrombotic event. However, since other reasons for bleeding may coexist, for instance thrombocytopenia and primary fibrinolysis, the bleeding episodes in our patients were not evaluated in this study.

Tissue factor and urokinase plasminogen activator receptor were found to be the two dominant hemostatic proteins and a statistically significant difference in the tissue factor to urokinase plasminogen activator receptor ratio was revealed between the 13 patients with a thrombotic event and the 38 patients without such an event. Large scale studies are needed to verify this ratio in leukemia patients and in other prothrombotic states. It is conceivable that the number of white blood cells may have contributed to the thrombotic potential of the cell surfaces. Although we did not find a significant difference in white cell counts between patients with or without thrombotic manifestations, when the tissue factor to urokinase plasminogen activator receptor ratio was multiplied by the white cell count, the difference between these two groups of patients became significant.

Plasma coagulation parameters were evaluated at the time of diagnosis. The assumption was that activation of the coagulation system may predict thrombotic manifestations in patients with acute leukemia during the first month of hospitalization. The markers included prothrombin time, activated partial thromboplastin time, fibrinogen, D-dimer, prothrombin fragments 1+2, antiplasmin and plasminogen. However, the coagulation markers of the 13 patients with a thrombotic event were comparable to those of the 38 patients without a thrombotic event and none of the evaluated parameters was statistically different between the two groups. D-dimer levels were high in all the patients in whom they were measured, suggesting that D-dimer is a very sensitive but non-specific marker. Hence, these results indicate that plasma parameters at diagnosis cannot be used as predictors of thrombotic events.

Various inherited and acquired thrombophilia parameters were also studied. Thrombophilia was found to be common among patients with acute leukemia, the most frequent thrombophilic states being hyperhomocysteinemia and lupus anticoagulant/anticardiolipin antibodies. Twenty-seven out of 34 (79%) patients had one or more pathologic marker and five patients had combined thrombophilia. The incidence of thrombophilia was comparable in patients with and without a thrombotic

event (8 out of 11, 72%). The most common pathologic markers revealed in the patients with a thrombotic event were hyperhomocysteinemia and lupus anticoagulant antibodies. Two patients had combined thrombophilia. Larger scale studies are needed to determine whether combined thrombophilia may predict thrombotic complications in leukemic patients.

The present study has some limitations. First, the number of patients assessed is relatively small, although it is the largest group that has been reported so far. Second, the samples used were first frozen in liquid nitrogen and then gradually thawed. This procedure may have some effect on the cell surface expression of the coagulation markers. Third, thrombotic manifestations are very common in AML-M3 and the high blast surface tissue factor expression is probably only part of the explanation for the procoagulant state. Other cell surface coagulation markers, such as endothelial protein C receptor and annexin II, may contribute to the membrane local hemostatic balance.[21,22] It is possible that the procoagulant profile in leukemic patients is determined by a combination of the plasma thrombophilic state and the cellular procoagulant state on leukemic cells. Endothelial cells and microparticles derived from platelets, endothelial cells and potentially from tumor cells may also contribute to the hemostatic balance. Nevertheless, this study indicates that the hemostatic balance on the blast surface is procoagulant. Plasma coagulation parameters and thrombophilia markers could not be used to predict thrombotic manifestations. One reason for this finding is that these factors may contribute to but do not determine the thrombotic state. Other parameters, such as the cell surface procoagulant balance, may have a more powerful impact.

All authors participated sufficiently in the work to take public responsibility for the content. The authors contributed to conception and design, or analysis and interpretation of data; and to drafting the article or revising it critically for important intellectual content. All authors approved the final version to be published. The authors declare that they have no potential conflicts of interest.

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