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Laboratory characterization of leukemic cell procoagulants

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Abstract

Background: In acute myeloid leukemias, there is an increased chance to develop thrombotic disorders. We hypothesized that in addition to leukemic promyelocytes, monocytic leukemia cells may also have a higher procoagulant activity.

Methods: Fibrin formation was assessed by a one-stage clotting assay using a magnetic coagulometer. The thrombin generation test (TGT) of magnetically isolated normal human monocytes, intact leukemic cells and their isolated microparticles was performed by a fluorimetric assay. Phosphatidylserine (PS) expression of leukemic cells and microparticle number determinations were carried out by flow cytometry.

Results: All cell lines displayed a significant procoagulant potential compared to isolated normal human monocytes. In the TGT test, the mean of lagtime and the time to peak parameters were significantly shorter in leukemic cells (3.9–4.7 and 9.9–10.3 min) compared to monocytes (14.9 and 26.5 min). The mean of peak thrombin in various monocytic leukemia cell lines was 112.1–132.9 nM vs. 75.1 nM in monocytes; however, no significant difference was observed in the ETP parameter. Factor VII-deficient plasma abolished all procoagulant activity, whereas factor XII-deficient plasma did not affect the speed of fibrin formation and thrombin generation but modulated the amount of thrombin. Factor XI-deficient plasma affected the time to peak values in one leukemic cell line and also attenuated peak thrombin. Leukemia cell-derived microparticles from all three cell lines exerted a procoagulant

effect by significantly shortening the lagtime in TGT; there was a nonsignificant difference in case of ETP parameter.

Conclusions: All investigated monocytic leukemia cell lines exhibited significant thrombin generation. This phenomenon was achieved by the procoagulants on the surface of leukemic cells as well as by their microparticles.

Keywords: monocytic leukemia; procoagulant activity; thrombin generation; tissue factor.

Introduction

Venous thromboembolism, including deep vein thrombosis and pulmonary embolism, is a common complication of malignancy and also occurs in acute leukemias [1]. Numerous factors may be involved in the development of thromboembolic events in leukemias, and several of these cases may be related to therapeutic interventions like the central venous catheter-associated prothrombotic effect [2, 3] or the therapy-associated thrombotic events of L-asparaginase treatment [4, 5]. Aside from these well-documented causes, acute leukemias can develop a hypercoagulable state, which frequently remains subclinical. One type of acute myeloblastic leukemias (AML), the acute promyelocytic leukemia, has long been described to cause activation of blood coagulation and thus can lead to disseminated intravascular coagulation [6, 7]. Other AML types are less investigated in this respect, although systemic coagulation activation has been detected in other types of AML as well [8–11].

The ultimate key player that will integrate all signals and will generate a hypercoagulable state in the systemic circulation is the serine protease thrombin. A global coagulation assay, the thrombin generation test (TGT) provides valuable information about the speed and amount of generated thrombin, as it would ultimately reflect clinically meaningful bleeding or prothrombotic condition [12, 13]. Thus, the characterization of pathological alterations that will result in enhanced thrombin generation in human plasma is important in understanding the mechanisms that contribute to prethrombotic states in leukemias. Plasmas deficient in factors VII (FVII), XI (FXI) or XII (FXII) were used to evaluate the coagulation factor dependence

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of leukemic cell-induced thrombin generation. The initiator of thrombin generation in numerous cancers and several other pathological states is the appearance of tissue factor (TF) in the systemic circulation that is a potent activator of coagulation [14].

In this study, we investigated three monocytic leukemia cell lines, derived from a patient with de novo AML, a case of relapsed AML and from an AML case that developed secondary to myelodysplastic syndrome. We hypothesized that monocytic leukemic cells have a higher procoagulant activity (PCA); thus, we characterized their thrombin generating potential by different assays using coagulation factor-deficient plasmas and TF neutralization. Results were compared to isolated normal human monocytes as negative and to an acute promyelocytic leukemia cell line as positive controls. Because in previous studies microparticles (MPs) from various cells have been shown to differentially modulate clot formation [15, 16], we also tested the thrombin generating potential of the MPs derived from these leukemic cell lines.

Materials and methods

Isolation of human monocytes

One hundred sixty milliliters of EDTA-anticoagulated (K_2 EDTA, Becton Dickinson vacutainer tube, San Jose, CA, USA) peripheral blood was drawn from healthy volunteers, and peripheral blood mononuclear cells (PBMC) were separated on Ficoll Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). Normal human monocytes were isolated from PBMC by a magnetic monocyte separation, negative isolation kit (Dynabeads® Untouched Human Monocytes, Invitrogen by Life Technologies) using beads in combination with an antibody mix [contains biotinylated mouse IgG antibodies for CD3, CD7, CD16 (specific for CD16a and CD16b), CD19, CD56, CDw123 and CD235a (glycophorin A)] to remove all unwanted cells from the sample, leaving only the monocytes behind. This technique – unlike other column based methods – provides a 95% viability that is important from the point of view of functional tests like thrombin generation. The cell count was adjusted to 5×10^6 /mL in phosphate-buffered saline (PBS) for thrombin generation measurements and 10^7 /mL for flow cytometric analysis.

Cell cultures

THP-1 is a monocytic leukemia cell line derived from a patient at relapse, MOLM-13 is a secondary monocytic leukemia developed from myelodysplasia RAEB and MV4-11 is a de novo monocytic leukemia cell line. The THP-1 cell line was a gift of Prof. Dr. György Vereb (Department of Biophysics and Cell Biology, University of Debrecen, Hungary). MOLM-13 and MV4-11 cell lines were purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Based on the WHO 2008 criteria for the

stratification of AML, all these cell lines belong to the unfavorable prognostic group with multiple chromosomal abnormalities. As a positive control an acute promyelocytic leukemia cell line, NB4 (DSMZ, Braunschweig, Germany) was used, which was a gift of Prof. Dr. Zoltán Balajthy, Department of Biochemistry and Molecular Biology, University of Debrecen, Hungary. All cell lines were maintained in suspension cultures, in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 15% fetal bovine serum (heat inactivated, Sigma-Aldrich, St. Louis, MO, USA) were grown in a cell incubator at 37 °C in a humidified atmosphere of 5% CO₂ in air. The duplication times of monocytic leukemia cells were 35–48 h which was investigated by cell counting at 48 h and cell cycle analysis at 0, 24 and 48 h. The medium was changed three times a week, when maximum cell density was set to 0.5×10^6 /mL in case of monocytic leukemia cell lines and to 0.3×10^6 /mL in case of the NB4 cell line. Cell cultures were subcultured by total medium replacement by using centrifugation every 7 days. Cell viability was >90%, evaluated by trypan blue exclusion before each experiment. Upon centrifugation (190 g, 25 °C, 10 min), the leukemic cells were resuspended in PBS at a concentration of 5×10^6 /mL for the functional thrombin generation test and to 10^7 /mL for flow cytometric analysis.

Isolation of MPs

First, 3.0×10^6 of cells were centrifuged at 190 g, 25 °C for 10 min, and the supernatant was discharged, cells were resuspended in fresh RPMI-1640 supplemented with 15% fetal bovine serum to adjust their cell count to 0.5×10^6 /mL in case of monocytic leukemia cell lines and to 0.3×10^6 /mL in case of NB4 cell line. The cells in suspension were cultured for 48 h and the cell count was set to 5×10^6 /mL. Cells were removed from the suspension by centrifugation (190 g, 25 °C for 10 min) followed by a brief removal of residual intact cells from the supernatant by spinning at 13,000 g for 2 min at room temperature (RT). Subsequently, MPs were isolated by a high speed centrifugation (16,100 g, 30 min, RT), washed with PBS and centrifuged (16,100 g, 30 min, RT). MPs were resuspended in 1 mL PBS. Prior to MP measurements, counting calibration beads were used for cytometer settings. Megamix-Plus SSC (Biocytex, Marseille, France) was used for this purpose, which is a mix of fluorescent beads of variable diameter selected to cover a major part of the MP size range, using SSC as a size-related parameter. The flow cytometer has been calibrated by these Megamix beads, and we found that by the suggested marker (SSC), the FACS Canto II (Becton Dickinson, San Jose, CA, USA) was capable of detecting beads in the range of 0.16–0.5 µm on the SSC channel. According to the manufacturer's recommendation, the 0.16 µm beads need to be excluded and the remaining beads (0.2–0.5 µm on the SSC signal) were considered as MP. We analyzed the MP of the leukemic cells by using this suggested setting. The SSC signal is a similar range to 0.3–1.0 µm on the FSC channel, and we found that the FSC signal of the beads was the same as that of the MP. MP number was evaluated by phosphatidylserine (PS) positivity as measured on the cytometer.

PCA measured by a one-stage clotting assay

For each experiments, peripheral blood was drawn from five healthy volunteers in tubes containing 0.105 M sodium citrate. Platelet poor

plasma was separated by centrifugation at 1220 g for 15 min at room temperature according to the protocol that used in our routine diagnostic laboratory. Pooled platelet poor plasma was prepared by adding 1 mL of platelet poor plasma of each individual. Platelet poor plasma is called hereafter normal plasma.

In the PCA tests, 100 μ L of normal or factor VII (FVII) deficient (Siemens, Marburg, Germany) or factor XII (FXII)-deficient plasma (Siemens, Marburg, Germany) was incubated with 100 μ L of PBS, monocytes or leukemic cells at 37 °C for 3 min. We used anti-human TF antibody (R&D Systems, Minneapolis, MN, USA), which is a polyclonal goat IgG for blocking. Leukemic cells were incubated with anti-human TF antibody (at final concentration of 25 μ g/mL) for 15 min at 37°C. We used Goat serum (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 45 μ g/mL as negative control for the blocked sample, and 100 μ L of the blocked sample was incubated with normal plasma. Then, 100 μ L of 20 mM CaCl_2 was added to initiate clotting. Fibrin formation in seconds was measured by a KC-1 mechanical coagulometer. A 1:10 dilution of recombinant thromboplastin (Dade Innovin, Siemens, Marburg, Germany) was arbitrarily taken as 12,800 mU, and a serial dilution was used to generate the thromboplastin calibration curve on a double logarithmic scale. The clotting times were converted to PCA expressed as mU/ 10^5 cells.

Thrombin generation assay

Eighty microliters of normal plasma or FVII, FXI (Siemens, Marburg, Germany) or FXII-deficient plasma was incubated with 20 μ L of PBS, monocytes or leukemic cells (5×10^6 /mL) for 10 min at 37 °C in round-bottomed 96-well black microplates. In case of the thrombin generation of MPs, 20 μ L of MPs was added to normal plasma. For each plasma sample, a simultaneously run calibrator – a stable complex of α_2 -macroglobulin and thrombin (Calibrator-Reagent, Thromboscope BV, Maastricht, The Netherlands) – was used to eliminate the differences between samples. Thrombin generation was initiated by the addition of 20 μ L of a mixture of Fluorogenic substrate and Fluo-Buffer that contained CaCl_2 (Thromboscope BV, Maastricht, The Netherlands). Fluorescence was detected by a Fluoroskan Ascent® fluorimeter (Thermo Fischer Scientific, Waltham, MA, USA), and the thrombin generation curves were analyzed by the Thromboscope software (Thromboscope BV, Maastricht, The Netherlands).

Flow cytometric studies

For the analysis of PS exposure of monocytes and cell lines, we used FITC-labeled annexin V and annexin V binding buffer from Becton Dickinson Biosciences, San José, CA, USA.

Cells were labeled for 30 min at RT in dark. Then they were washed twice with PBS, and cells were resuspended in 500 μ L annexin V binding buffer. In each experiment, 30,000 events were collected by an FC500 flow cytometer, and results were analyzed with the Kaluza software (Beckman Coulter, Miami, FL, USA).

For immunophenotyping, surface staining of cells was carried out according to standard 8-color procedures. Fifty microliters of cell suspension adjusted to cell count of 10×10^9 /L by PBS was incubated by saturating concentrations of directly conjugated antibodies for

15 min at room temperature in the dark with antibodies against different cell surface and cytoplasmic epitopes. Labeling was carried out with CD14-FITC, CD11b-PE, HLA-DR-PerCPy5.5, CD4-PB, CD15-FITC, CD34-PerCPy5.5, CD71-FITC, CD117-PE, CD38-APCH7 (Becton Dickinson Biosciences, San José, CA, USA), CD34-APC, CD13-PECy7, CD64-APCAF750, CD33-PECy5.5, CD56-PECy7 (Beckman Coulter, Marseille, France), CD45-PO (Invitrogen, Thermo Scientific Inc., Waltham, MA, USA), MPO-PE (Dako Cytomation, Glostrup, Denmark) and FXIII-A-FITC. PFA-fixed samples were kept at 4 °C for maximum 24 h. Flow cytometric measurements were carried out on a FACSCanto II (Becton Dickinson Biosciences, San Jose, CA, USA) flow cytometer using the same setting for all investigated samples. Data obtained on 50,000 cells were acquired and stored in list-mode data files. Data were analyzed by FACSDiva software (Becton Dickinson Biosciences, San José, CA, USA).

Statistical analysis

The statistical analysis was performed with GraphPad Prism 5.0 version. Normality of the data was evaluated by the Shapiro-Wilk normality test. Elements outside mean $\pm$ 2SD were removed as outliers. Predetermined pair-wise differences were analyzed by paired t-test, and in case of a nonnormal distribution, the Wilcoxon matched paired test was used. For nonpairwise comparison, unpaired t-test (Gaussian distribution) and Mann-Whitney test (non-Gaussian) were used. ANOVA with Bonferroni post test (Gaussian distribution) or Kruskal-Wallis test with Dunn's post test (non-Gaussian distribution) was used for multiple group comparison. *p*-Values less than 0.05 were considered as statistically significant.

Results

PCA measured by a one-stage clotting assay

The clotting time of normal plasma contained PBS, but no cells was over range (>200 s, data not shown), and similar values were observed using isolated normal human monocytes, both resulting in a very low PCA. Using the same cell concentrations, all three cell lines displayed significantly shorter clotting times and consequently higher PCA. The neglectable PCA of isolated normal human monocytes was unaffected by FVII-deficient plasma. We observed that the FVII deficient plasma and a polyclonal anti-human tissue factor (ahuTF) antibody pretreatment completely abolished the fibrin forming capacity of all monocytic leukemia cell lines resulting in very low PCA, whereas FXII-deficient plasma had no significant blocking effect (Figure 1). Although, the PCA of the acute monocytic leukemia cell lines was significantly increased compared to the buffer control or monocytes, there was

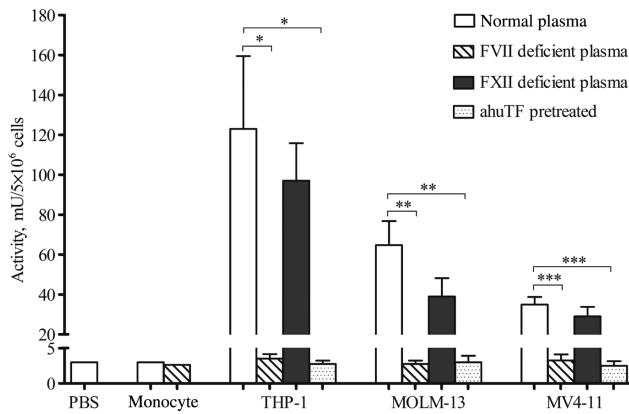


Figure 1: Procoagulant activity (PCA) of monocytic leukemia cells and controls.

White bars indicate normal plasma, striped bars represent the PCA in FVII-deficient plasma and black bars correspond to results in FXII-deficient plasma. Dotted bars indicate anti-TF antibody pretreated cells. The results are the mean and standard error of the mean (SEM) of five different experiments. Statistical significance was assessed by unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PBS, phosphate-buffered saline; FVII, factor VII; FXII, factor XII; ahuTF, anti-human tissue factor.

no significant difference between the three cell lines in normal plasma.

Thrombin generation assay

Another way to evaluate the PCA of cells was done by the TGT. A representative overlay curve of the investigated cell lines displays the differences of steepness and the maximum amount of thrombin generated. The positive control NB4 cell line resulted in an abrupt increase in thrombin and reached the highest peak thrombin value. The monocytic leukemia cell lines (THP-1, MOLM-13 and MV4-11) displayed rather similar thrombin generation profiles, and thrombin was formed much faster than in case of normal human monocytes (Figure 2).

There was no significant difference between the thrombin generation of normal monocytes compared to PBS in normal plasma (Figure 3A–C). Intact leukemic cells of all three cell lines significantly shortened the lagtime and time to peak, the shortest times were observed in the positive control NB4 cells. FVII-deficient plasma and anti-TF pretreatment completely eliminated the shortenings in the monocytic leukemia cells, whereas FXI- and FXII-deficient plasma had no such effect. In case of the time to peak parameter the TF-blocked and FVII-deficient samples exerted the same effect on THP-1 cells, but attenuated neutralizing effects were observed

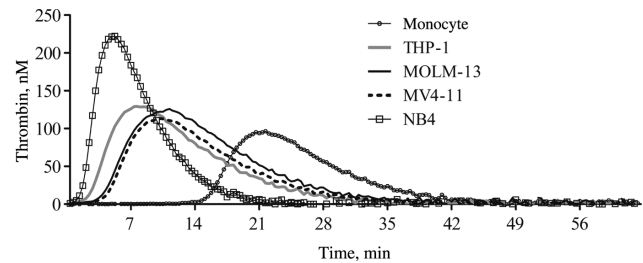


Figure 2: Representative thrombin generation curves of monocytes and leukemic cell lines.

The vertical axis represents the amount of formed thrombin by 60 min using Thromboscope software.

on the two other cell lines. Importantly, on the MOLM-13 cell line and to a smaller extent in case of MV4-11 cells, FXI deficiency also prolonged the time to peak similarly to TF blockage, whereas FXII-deficient plasma did not significantly affect this parameter (Figure 3A, B). We found that healthy monocytes generated the same low amount of thrombin as normal plasma without any cells, whereas all monocytic leukemia cell lines induced considerable thrombin generation as observed in the peak thrombin parameter. However, it did not reach the level of the promyelocytic leukemia NB4 cells. The elevation in the quantity of produced thrombin was nonsignificant in case of THP-1 cells compared to normal monocytes but peak thrombin values were significantly higher than the monocyte control in case of the MOLM-13 and MV4-11 cell lines. The peak thrombin parameter was dependent on factors VII, XI and XII in case of all cell lines; however, a nonsignificant FVII dependence was observed in case of MOLM-13 cells. FVII deficiency affected nearly all investigated parameters, while eliminating FXI from the system caused a considerable reduction in thrombin generation that was particularly striking in case of MOLM-13 and MV4-11 cells (Table 1). Interestingly, anti-TF pretreatment – with the antibody that effectively blocked the shortening of lagtime – was ineffective in modulating the formed thrombin quantity (Figure 3C).

Thrombin generation of MPs

By using the same cellular density for culturing during a 48-h period, all three cell lines generated considerable amount of MP. We isolated these MPs and investigated their PCA by TGT. We found that the THP-1 cell line produced twice as much MP as the other two cell lines. The mean MP number was $4.7 \times 10^7 / 5 \times 10^6$ cells vs. 2.2 and $2.6 \times 10^7 / 5 \times 10^6$ cells in the MOLM-13 and MV4-11 cell lines,

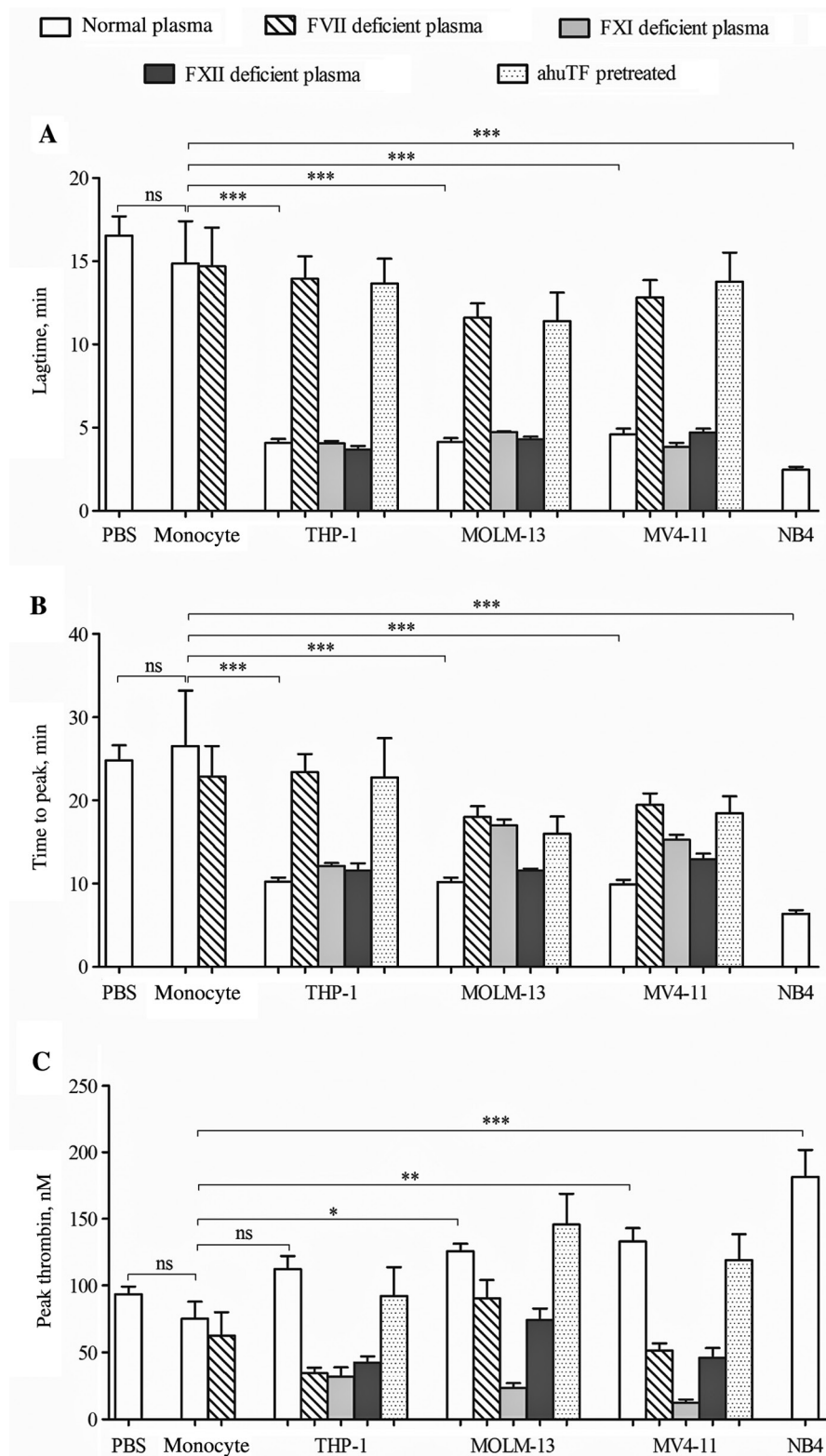


Figure 3: Thrombin generation of leukemic cells in normal and factor-deficient plasmas. Measurements were carried out in normal plasma (white bars), FVII-deficient plasma (striped bars), FXI-deficient plasma (gray bars) and FXII-deficient plasma (black bars), and dotted bars indicate anti-TF antibody pretreated cells. The results are the mean and standard error of the mean (SEM) of at least six different experiments except for FXI-deficient measurements where three assays have been done. Statistical significance was assessed by ANOVA and Bonferroni's multiple comparison test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. PBS, phosphate-buffered saline; FVII, factor VII; FXII, factor XII; ahuTF, anti-human tissue factor; ns, nonsignificant.

Table 1: Differences in the thrombin generation parameters of leukemic cell lines in normal and factor-deficient plasmas.

	Lagtime	Time to peak	Peak
Normal vs. FVII-deficient plasma			
THP-1	$p < 0.001$	$p < 0.001$	$p < 0.001$
MOLM-13	$p < 0.001$	$p < 0.001$	ns
MV4-11	$p < 0.001$	$p < 0.001$	$p < 0.001$
Normal vs. FXI-deficient plasma			
THP-1	ns	ns	$p < 0.001$
MOLM-13	ns	$p < 0.01$	$p < 0.001$
MV4-11	ns	ns	$p < 0.001$
Normal vs. FXII-deficient plasma			
THP-1	ns	ns	$p < 0.001$
MOLM-13	ns	ns	$p < 0.05$
MV4-11	ns	ns	$p < 0.001$

Statistical significance was assessed by ANOVA and Bonferroni's multiple comparison test. ^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$. FVII, factor VII; FXI, factor XI; FXII, factor XII; ahuTF, anti-human tissue factor; ns, nonsignificant.

respectively. The MP number of the positive control NB4 cell line was between these values ($3.3 \times 10^7 / 5 \times 10^6$ cells). These MP have strong thrombin inducing ability as demonstrated by the significantly reduced lagtime (panel A) and time to peak (panel B) values, compared to a buffer control (PBS). No significant differences were observed in peak thrombin and Velocity Index parameters (Figure 4C and D).

Flow cytometric studies for PS expression and cell lineage

Among the three monocytic leukemia cell lines, the lowest PS expression was observed on THP-1 cells (mean $\pm$ SD: $6.5\% \pm 2.3\%$) and was somewhat higher on the MOLM-13 (mean $\pm$ SD: $12.1\% \pm 3.3\%$) as well as on the MV4-11 cell line (mean $\pm$ SD: $21.8\% \pm 8.4\%$), although differences were

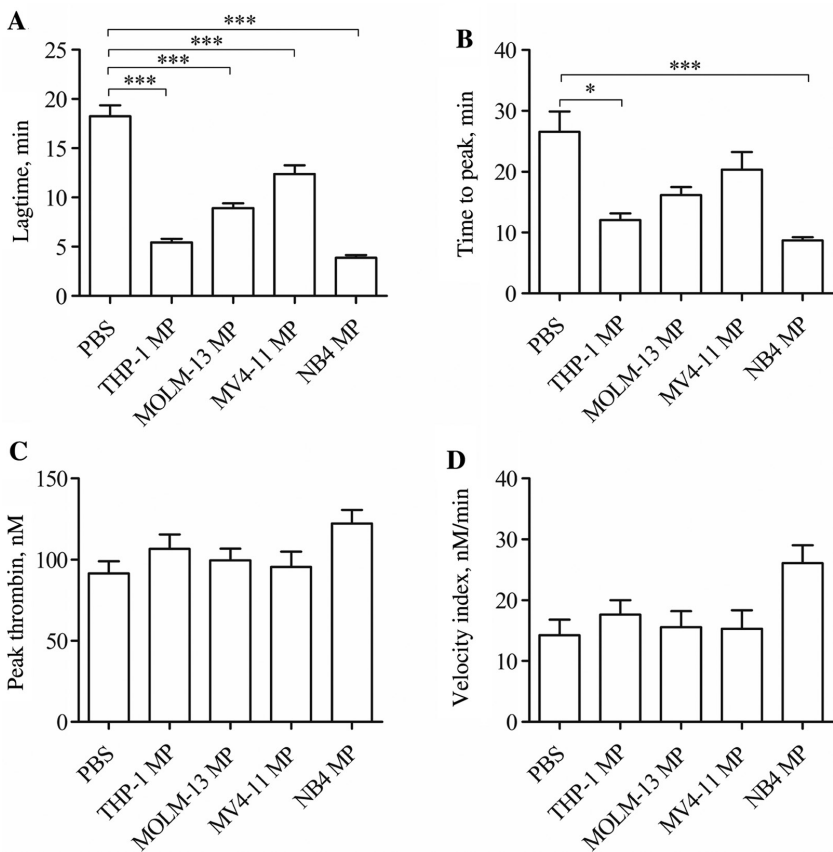


Figure 4: Thrombin generation of cell-derived microparticles (MP) compared to PBS in normal plasma. The results are the mean and standard error of the mean (SEM) of six different experiments. In case of parametric data, the statistical significance was assessed by ANOVA and Bonferroni's multiple comparison test; in case of nonparametric data, the statistical analysis was performed with Kruskal-Wallis and Dunn's multiple comparison test. * $p < 0.05$; *** $p < 0.001$. PBS, phosphate-buffered saline; MP, microparticle.

nonsignificant. There was, however, a significant difference in the intensity of PS expression as characterized by the ratio of mean fluorescence intensity (MFI) values of the PS positive population divided by the MFI value of the unlabeled cells. The mean±SD of the MFI ratio from five experiments was 14.12±3.9 for THP-1 cells that was nonsignificantly different from MV4-11 cells (mean±SD: 20.23±12.12), but in case of MOLM-13 cells, this ratio was significantly higher than in the other two cell lines (mean±SD: 75.91±30.22). This means that although MV4-11 cells expressed the highest percentage of PS positivity, the intensity of expressed PS was highest on the MOLM-13 cell line.

The myeloid marker CD33 was the only antigen that was uniformly expressed on the surface of all cell lines, whereas the other pan-myeloid marker CD13 as well as various monocytic markers displayed a variable expression. The three cell lines showed different immunophenotype with incongruent expression of monocytic and myeloid markers; however, THP-1 seemed to be the most mature with no CD34 and small CD117 expression. Complex cytogenetic abnormalities are present in all cell lines that are responsible for their poor prognosis, irrespective of the FLT3-ITD mutation status (Table 2).

Discussion

In the initiation of coagulation, the key surface protein is TF that binds factor VIIa and rapidly leads to thrombin generation. The majority of the evidence in the literature point out that exposure of anionic phospholipids, such as PS, on the outer leaflet of plasma membrane is a key regulator of TF PCA at the cell surface [17]. In earlier studies, we have observed a considerable difference in TF activity of activated intact and lysed monocytes, as the latter displayed a much higher PCA [18], and similar differences between intact and lysed cells have been described more recently for PBMC samples derived from patients suffering from AML [9]. Using lysed cancer cell lines, some authors found FVII dependence of the promyelocytic NB4 cell line and the MCF7 breast cancer cell line, but they detected very low thrombin generation, with no TF activity and no FVII dependence in other leukemia cell lysate like the K562 chronic myeloid leukemia and the HEL cell erythro-leukemia cell lines [19].

There are important differences when the procoagulant activity is detected by fibrin formation or thrombin generation. In a one-stage clotting assay, very little thrombin is sufficient to form a clot in the coagulometer within

Table 2: Characteristics of the acute monocytic leukemia cell lines.

Cell line	THP-1	MOLM-13	MV4-11
Clinical characteristic	Relapsed monocytic AML	AML transformed from MDS	De novo monocytic AML
Duplication time	35–50 h	50 h	50 h
Phenotype (antigen)			
Myeloid			
CD33	100%	100%	100%
CD13	100%	7%	20%
CD15	100%	100%	100%
cyMPO	0%	0%	34%
CD11b	100%	15%	100%
Monocytic			
CD64	50%	11%	100%
CD14	0%	5%	14%
CD4	20%	100%	100%
HLA-DR	12%	3%	18%
cyFXIII-A	0%	11%	60%
Other			
CD38	100%	100%	20%
CD71	100%	11%	100%
CD56	100%	100%	18%
CD34	0%	7%	14%
CD117	9%	5%	20%
Cytogenetic	Complex cytogenetic abnormalities		
Molecular genetics	MLL-AF9	MLL-AF9	MLL-AF4
FLT3-ITD mutation	wt/wt	wt/FLT3-ITD ⁺	FLT3-ITD ⁺ /FLT3-ITD ⁺

AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; wt, wild type; FLT3-ITD, fms-like tyrosine kinase 3-internal tandem duplication.

a few minutes as a result of the formed fibrin mesh. This process seems to be totally FVII dependent as was exemplified by the parallel abrogating effect of VII-deficient plasma and TF blocking antibody. In the thrombin generation assay, however, the reaction is followed up to 1 h, well beyond that of fibrin formation, enabling one to better characterize the contribution of individual coagulation factors by using factor-deficient plasmas. Based on our observations, we propose that FVII is important in triggering the procoagulant effect evidenced by the fibrin formation and lagtime in the TGT. Subsequent thrombin generation as determined by the time to peak and peak thrombin, however, is very much dependent on factor XI and partially on FXII as these proteases seem to be necessary in the propagation of the coagulant reaction. The particular role of FXI in peak thrombin for the MOLM-13 and to some degree the MV4-11 cells suggests that the thrombin generation is mediated by a feedback activation of FXI by thrombin at least partially independently of FVII and FXII. This is in agreement with the role of FXI in affecting the time to peak by MOLM-13 and partially for MV4-11 cells. The discrepancy between the effect of using FVII-deficient plasma or anti-TF antibody in peak thrombin measurement for THP-1, MOLM-13 and MV4-11 cells might stem from a TF-independent activation of FX/FIX by FVII and PS exposed on the cells and a resultant feedback activation of FXII by thrombin.

In clinical cases of AML, other aspects should be taken into consideration as PS expression is an inducible event that was found more pronounced if the patient was treated by chemotherapy [20].

The overall contribution to the generated thrombin depends on the surface of the particle and its potentially procoagulant receptors. If we consider leukemic monocytes and their MPs as spheres, their surface can be calculated. Taking into consideration the detection limit of the Canto-II flow cytometer that was used for the analysis of microvesicles and monocytic cells, we can estimate the radii of the MP and cells at 0.3 μm and 10 μm , respectively. Based on the formula for the calculation of the surface for spheres ($A = 4r^2\pi$), the intact cells possess about a 1000-fold larger surface than that of the microparticles. Since during a 2-day culturing 4–10 MPs were released from one intact leukemic cell, this would imply that per a given surface, MPs derived from these monocytic leukemia cells are a 100-fold more procoagulant than their parent cells.

The PCA of MP is related also to their count, although even by a state-of-the-art flow cytometric technique, we may underestimate MP count as the refractive index of the vesicles appear lower than that of the polystyrene

beads, and the results may also be hampered by swarming as described more recently [21]. All MP contribute to thrombin generation mostly by reducing the lagtime and to a much lesser extent the time to peak parameters, whereas the peak thrombin is determined by the leukemic cells that can support thrombin formation supposedly by their much larger surface. This means that MP derived from these monocytic cell lines rather affect the initiation – and not the propagation phase – of thrombin formation. Thrombin generation can also be modified by components of the pooled plasma like the soluble tissue factor pathway inhibitor (TFPI) that is the only serine protease inhibitor of FVII/TF-induced coagulation. An earlier report established that TFPI can postpone the initiation phase as it affected the lagtime but does not influence the propagation phase measured by the endogenous thrombin potential [22].

We have observed larger cell counts and a higher proliferation phase during culturing of the MV4-11 and MOLM-13 cell lines compared to the THP-1 cells. This phenomenon was also translated to a larger percentage of cells that became necrotic in the medium that lead to enhanced annexin V binding and contributed to the observed procoagulant activity.

In these experiments, we have found that monocytic leukemia cell lines and MP generated by these cells exhibit significantly higher PCA compared to normal monocytes and that this effect is primarily TF dependent; however, other factors may modulate this effect. Our results demonstrate that functional assays may provide a useful tool to identify cell induced thrombin generation in acute myeloid leukemias.

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