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Disease dynamics in patients with acute myeloid leukemia: New biomarkers

Inna Tzoran[a,b,c], Annie Rebibo-Sabbah[a,c], Benjamin Brenner[a,c], and Anat Aharon[a,c]

[a]*Department of Hematology and Bone Marrow Transplantation, Rambam Health Care Campus, Haifa, Israel;* [b]*Internal Medicine C, Rambam Health Care Campus, Haifa, Israel;* [c]*Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel*

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Acute myeloid leukemia (AML) is characterized by rapid growth of leukemic blast cells. Extracellular vesicles (EVs), shedding from various cells, express antigens, reflecting their cellular origin. The current study was designed to explore the role of circulating EVs as potential biomarkers of AML activity and predictors of thrombogenicity in patients with this malignancy. Blood samples were collected from healthy controls and patients with newly diagnosed AML at three time points: diagnosis, nadir, and remission. EV concentration, cell origin, and expression of coagulation proteins were characterized using fluorescence-activated cell sorting. EV cytokine contents were evaluated by protein array. Procoagulant activity was assessed using Factor Xa chromogenic assay. Forty-two AML patients were enrolled in the study. Total EV numbers were higher in patients in first remission compared with controls, whereas blast EV counts were higher in patients at diagnosis compared with controls and patients in remission. Blast EV levels were significantly lower in patients who achieved remission and were alive at 3-year follow up compared with their succumbed counterparts. At all three time points, percentage of endothelial EVs was higher in patients compared with controls. EV procoagulant activity was elevated at diagnosis and in remission, and, unlike controls' EVs, patients' EVs increased endothelial cell thrombogenicity. EVs of AML patients express membrane proteins of blast cells and might serve as biomarkers of leukemia dynamics and presence of minimal residual disease. Increased levels of endothelial EVs and their procoagulant activity may indicate a vascular injury associated with a hypercoagulable state in AML.

Acute myeloid leukemia (AML) is characterized by intense proliferation of abnormal blast cells that accumulate in the bone marrow (BM) and interfere with the production of normal blood cells [1]. AML patients in remission, despite the normalization of their blood counts, still often harbor leukemic burden. This fact emphasizes the need for diagnostic tools for the identification of minimal residual disease (MRD) [2]. In addition, up to 5% of AML patients can develop venous thromboembolism (VTE) despite thrombocytopenia, which is often observed in these individuals [3].

Extracellular vesicles (EVs) include microparticles (<1 micron), released from the surface of cell membranes, and exosomes (30–100 nm), originating from the intracellular compartment. EVs bear antigens, which reflect their cell origin, growth factors, cytokines, coagulation proteins, and RNAs [4–6]. Cell stimulation, such as exposure to chemotherapy or radiation leading to cell apoptosis, results in EV shedding from a variety of cells, including tumor cells, into the blood. Accumulating evidence suggests that EVs participate in promoting and maintaining cancer dissemination and progression [7]. They are also involved in coagulation initiation and thrombus formation [8]. Furthermore, EVs bearing tissue factor (TF), the main initiator of the coagulation cascade, play a major role in the pathogenesis of the prothrombotic state observed in patients with malignancies [9–11]. Although increased procoagulant activity was reported in EVs obtained from patients with solid tumors at diagnosis [12,13], information on the role of EVs in patients with hematological malignancies, particularly AML, is limited [14].

Our previous studies showed that the ratio between TF and tissue factor pathway inhibitor (TFPI) reflected EV thrombogenicity [15,16]. In addition, the exposure of endothelial cells (ECs) to high-dose or long-term chemotherapy,

Offprint requests to: Dr. Inna Tzoran, Department of Hematology and Bone Marrow Transplantation, Rambam Health Care Campus, Haifa 31096, Israel; E-mail: i_tzoran@rambam.health.gov.il

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as well as to monocyte EVs, was reported to result in the release of procoagulant endothelial EVs [17,18].

We hypothesized that circulating EVs serve as malignancy biomarkers, capable of reflecting changes in various cell populations and also predicting the thrombogenic state in AML patients at diagnosis and in remission.

To that end, the aims of the current study were to assess whether the presence of blast EVs could indicate AML activity at diagnosis, postchemotherapy nadir, and in remission and whether EVs could be used for the identification of prothrombotic states.

Material and methods

The study was conducted between the years 2011 and 2014 and was approved by the Institutional Review Board of the Rambam Health Care Campus, Haifa, Israel (Approval no. 0351-10). All participants signed the informed consent form.

Blood sample collection and EV isolation

Blood samples (12 mL) were collected in tubes with sodium citrate (1:10) and centrifuged twice for 15 min at 1,500 *g*. The poor platelet plasma (PPP) was immediately frozen in aliquots at −80°C.

The samples were collected from healthy volunteers (once) and from AML patients who were treated with the standard chemotherapy regimen commonly known as 3+7 (daunorubicin at a dose of 60 mg/m^2 daily for 3 days and cytarabine [ara-C] at a dose of 100 mg/m^2 daily for 7 days, given as a continuous infusion) [19]. Samples from AML patients were collected at 6:00 a.m., after overnight fasting, at three time points: (1) diagnosis before chemotherapy, (2) 2 weeks after chemotherapy initiation (nadir), and (3) after achievement of the first complete remission (CR). To minimize the freezing/thawing effect on EVs, the PPP quick freezing method was used.

EV pellets were isolated from thawed PPP by centrifugation (1 hour, 18,000 *g*) [15,20,21].

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords obtained at term of normal pregnancy, as previously described [18].

EV characterization

EVs were characterized using flow cytometry (FACS-CyAn ADP analyzer, Beckman Coulter). To rule out potential platelet ($\sim$3 μm in diameter) contamination [22] in the EV ($<$1 μm) population, the size and granularity of EVs were evaluated using the standard Megamix beads (0.5 μm, 0.9 μm, and 3 μm; Biocytex, Marseille, France) and 0.78-μm beads (BD Biosciences); gates for the EV analysis were set at $<$1.5 μm. Forward-scatter and side-scatter parameters were set on logarithmic scales [20]. EV concentrations were measured with 7.5-μm count beads [5,20]. EVs were labeled with fluorescein isothiocyanate–Annexin V (Bender MedSystems, Austria) and with specific fluorescent antibodies: phycoerythrin (PE)-CD41, allophycocyanine (APC)-CD62E, APC-CD144 (Biolegend), antirabbit immunoglobulin G (IgG)-PE, antimouse IgM-PE (Jackson), FITC-CD14 (IQ Products, Netherlands), PE-CD11a, PE-CD62p, PE-CD34, APC-CD117, FITC-CD33, PE–human leukocyte antigen D-region related (HLA-DR) and PE-IgG1κ isotype controls (BD Biosciences).

EV protein contents

EV pellets, obtained from 2 mL of the pool of eight specimens (250 μL of PPP of every patient) in each study group (healthy, AML I, AML II, and AML III), were resuspended in the lysis buffer (Ray Biotech) combined with the protease inhibitor cocktail (Sigma-Aldrich) [18]. The EV protein extract was evaluated using the bicinchoninic acid (BCA) protein quantification kit (Thermo Fisher Scientific). We assessed cytokine contents using the Human Angiogenesis Protein Antibody Array (Ray Biotech) and performed the assay according to the manufacturer's instructions. Briefly, following the slide washing and blocking steps, EV protein extracts were added to each well in the slides, which were then incubated at the room temperature for 2 hours ending with three washings. After that, the slides were incubated with biotin-conjugated anti-cytokines for another 2 hours with gentle rocking, then washed. Subsequently, the slides were completely dried. Then, we scanned them at 5-micron resolution on the Agilent G2565BA Microarray Scanner (Agilent Technologies, Santa Clara, CA) and analyzed using the TotalLab software (UK). Findings were normalized to those of the healthy group.

EV thrombogenicity

To determine the procoagulant potential of the EVs, each sample was labeled with florescent antibodies against TF, TFPI (America Diagnostica), and endothelial protein C receptor (EPCR; Santa Cruz Biotechnology). The TF/TFPI ratio was calculated. Procoagulant activity of EVs isolated from 1 mL of PPP was evaluated using the Factor Xa (FXa) chromogenic assay [18]. Results were converted to TF arbitrary units (AU).

EVs' effect on endothelial cells

HUVECs were seeded on 96-well plates and stimulated for 20 hours with an EV pellet, isolated from 0.5 mL of PPP. Subsequently, cells were washed and collected; their thrombogenic effect was evaluated by the FXa chromogenic assay. Fluorescence-activated cell sorting (FACS) analysis was used to measure the levels of TF and TFPI antigen. In addition, cell injury was evaluated using Annexin V labeling.

The effects of EVs on HUVEC gene expression were evaluated by quantitative polymerase chain reaction (PCR) after 6 hours of coincubation of HUVECs in 24-well plates with EV pellet, isolated from 2 mL of PPP. Total RNA was extracted from HUVECs using TRI-reagent (Sigma-Aldrich). The purity and concentration of RNA were determined by Nano Drop. We constructed cDNA using a cDNA synthesis kit (Applied Biosystems). Quantitative mRNA expression of TF and TFPI was assessed by real-time PCR (Applied Biosystems) using Luminaris Color Probe High ROx qPCR master mix (Thermo Fisher Scientific) with specific TaqMan probes (Hs00175225_m1 and Hs00196731_m1, respectively; Applied Biosystems) that were normalized to human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; Applied Biosystems).

Statistical analysis

Data were analyzed using GraphPad Prism 5 software. Results were assessed by one-way analysis of variance with a Bonferroni multiple comparisons test. The Fisher exact test was used for the 2 × 2 contingency table analysis.