Platelets Promote Mitochondrial Uncoupling and Resistance to Apoptosis in Leukemia Cells: A Novel Paradigm for the Bone Marrow Microenvironment

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Abstract Here we report that leukemia cell lines and primary CD34+ leukemic blasts exposed to platelet rich plasma (PRP) or platelet lysates (PL) display increased resistance to apoptosis induced by mitochondria-targeted agents ABT-737 and CDDO-Me. Intriguingly, leukemia cells exposed to platelet

components demonstrate a reduction in mitochondrial membrane potential ($\Delta\Psi$ M) and a transient increase in oxygen consumption, suggestive of mitochondrial uncoupling. Accompanying the ranolazine-sensitive increase in oxygen consumption, a reduction in triglyceride content was also observed in leukemia cells cultured with platelet components indicating that lipolysis and fatty acid oxidation may support the molecular reduction of oxygen in these cells. Mechanistically, platelet components antagonized Bax oligomerization in accordance with previous observations supporting an antiapoptotic role for fatty acid oxidation in leukemia cells. Lastly, substantiating the notion that mitochondrial uncoupling reduces oxidative stress, platelet components induced a marked decrease in basal and rotenone-induced superoxide levels in leukemia cells. Taken together, the decrease in $\Delta\Psi$ M, the transient increase in ranolazine-sensitive oxygen consumption, the reduction in triglyceride levels, and the reduced generation of superoxide, all accompanying the increased resistance to mitochondrial apoptosis, substantiate the hypothesis that platelets may contribute to the chemoprotective sanctuary of the bone marrow microenvironment via promotion of mitochondrial uncoupling.

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Introduction

Acute myeloid leukemia remains largely incurable due to relapse mediated by chemoresistant blasts, even though most patients achieve a complete remission after first line induction

and consolidation chemotherapy [1]. It appears likely that the leukemic bone marrow provides a privileged sanctuary that shields quiescent leukemic progenitors from chemotherapy induced cell death, with mesenchymal stromal cells playing a critical role in chemoprotection by inducing complex epigenetic and prosurvival signal transduction alterations in leukemia cells (reviewed in [2]). This hypothesis is also congruent with the clinical observation that peripheral blast responses tend to be more rapid and lasting than bone marrow blast responses, and while other stromal cell types have been implicated – such as adipocytes [3] and osteoblasts [4] – much remains to be understood regarding the precise cellular and molecular mechanisms that orchestrate the chemoresistant phenotype of leukemic cells within the bone marrow.

Recent evidence has demonstrated that mitochondrial uncoupling – a short circuit in the electrochemical gradient of the mitochondrial membrane – promotes resistance to intrinsic apoptosis in leukemia cells via, in part, antagonism of bax/bak oligomerization [5, 6]. Moreover, this metabolic phenotype, originally reported in leukemia cells cultured on mesenchymal stromal feeder layers [5], results in decreased entry of pyruvate into the Krebs cycle and a shift to fatty acid oxidation to support oxygen consumption, presumably permitting utilization of glucose carbon skeletons for the generation of biomass [7]. Nevertheless, it is still unclear what precise molecular or physiological events initiate mitochondrial uncoupling in leukemia cells.

Megakaryopoiesis and thrombopoiesis occur in the bone marrow milieu ([8] and reviewed in [9]), and although blood platelet counts are an important parameter (reviewed in [10, 11]), and the scientific literature supports a solid tumor promoting effect of platelets [12–15], only few studies have investigated the interaction of platelets with leukemia cells in vitro (reviewed by Foss and Bruserud [10]). It is conceivable that multiple platelet-released mediators would promote the growth of leukemia cells – similar to the effects reported for mesenchymal stromal cells and solid tumors [16, 17]. Indeed, Foss B and Bruserud O reported that exogenous platelets promote proliferation of leukemic blasts [18] and normal hematopoietic stem cells [19], and hypothesize that platelet-released mediators played a key role in this effect, although the precise molecular mechanisms were not thoroughly investigated. On the other hand, platelet-derived transforming growth factor β (TGF β) could possibly inhibit the growth of leukemia cells by modulating cell cycle protein expression and/or phosphorylation status as has been reported for synthetic TGF β [20, 21]. Additionally, Foss and Bruserud have also reported that AML blasts from certain patients promote the platelet release of soluble (s) CD62P and platelet-derived growth factor (PDGF) [22], suggesting the possibility that AML blasts could promote platelet activation. However, the underlying mechanisms by which platelet contents modulate leukemia cell survival remain largely

uninvestigated, and it is not known if platelets contribute to chemoresistance.

Here we report that leukemia cell lines promote activation of platelets as evidenced by surface expression of CD62p and phosphatidyl serine externalization, and that although no effect on leukemia proliferation was evidenced from this interaction, the contents released from live platelets – or contained in platelet lysates – promote mitochondrial uncoupling and resistance to activation of the intrinsic pathway of apoptosis. Similar effects were reported in leukemia cells cultured with MSC, supporting the novel paradigm that platelets may contribute to the chemoprotective environment of the bone marrow.

Materials and Methods

Cell Lines, Chemicals, and Biochemicals OCI-AML3 (acute myeloid) and MOLT4 (acute T lymphoblastic) leukemia cell lines were purchased from ATCC and maintained in RPMI-1640 supplemented with 10 % fetal calf serum, 1 % glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1 mg/L amphotericin B in a 37 °C incubator containing 5%CO₂. All culture reagents were obtained from Gibco (Life Technologies, Grand Island, NY). Mesenchymal stromal cells (MSC) were derived from lipoaspirates of patients undergoing cosmetic liposuction as previously described [23]. Anti-CD62 conjugated to FITC, anti-CD90 conjugated to APC, and Annexin V conjugated to FITC were obtained from EBiosciences (San Diego, CA). Monodansylcadaverine (MDC), LipidTox Green for neutral lipids, Tetramethyl Rhodamine Methyl Ester (TMRM), dihydroethidine (DHE), and JC-1 were obtained from Invitrogen (Carlsbad, CA).

Platelet Rich Plasma (PRP), Platelet Lysate (PL), and Platelet Supernatant (SP) Preparation PRP was prepared by a single centrifugation step of whole citrated blood as previously described with minor modifications [24]. Briefly, blood was drawn from healthy volunteers under informed consent into ACD (solution A) vacutainer tubes (Beckton Dickinson, Franklin NJ). Tubes were immediately centrifuged at room temperature 800 $\times$ g for 8 min, and the bottom 50 % of plasma volume was removed with a plastic transfer pipette taking care to avoid the plasma/blood interface. PRP routinely contained 2.5–3.5 $\times$ 10⁵ platelets/ μ L, and buffy coat derived platelet bags contained an average of 1 $\times$ 10⁶ platelets/ μ L. PL was obtained by centrifugation (2,000 $\times$ g for 5 min) of pooled units (n =8) of buffy coat derived platelets that had been subjected to 3 cycles of freeze/thaw. Recently (<24 h) expired buffy coat derived platelet bags were a kind gift of the Colombian Red Cross. 2 U/ml sodium enoxaparin (Clexane, Sanofi Aventis, France) were supplemented into the culture medium prior to addition of PL or PRP to prevent clot formation. In some experiments

where it was desired to avoid the use of heparin (which was found to reduce oxygen consumption and $\Delta\Psi M$ in leukemia cells by 10–20 % - data not shown), PRP was exposed to OCI-AML3 cells (1×10^5 /mL in fresh RPMI medium) until clot formation occurred (4–6 h) and culture media was centrifuged, filtered, and used as platelet supernatant (SP) to resuspend fresh OCI-AML3 cells. OCI-AML3 conditioned media in the absence of added PRP was used as a control for SP.

Measurement of CD62P Expression and Annexin V Staining in Live Platelets RPMI medium (300 μ L) was inoculated with 2.5 % PRP and exposed to 1 % Calcium gluconate, or increasing numbers of MOLT4 or OCI-AML3 cells (50, 100, or 250×10^3 /mL) for 1 h under standard culture conditions. Cultures were then stained with CD62P-FITC or Annexin V-FITC (1:100 dilutions) for 30 min on ice. CD62P expression and Annexin staining in platelets was determined by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) using a 488-nm argon ion excitation laser gating on platelets by FSC and SSC parameters.

Measurement of Apoptosis and Mitochondrial Damage by Flow Cytometry After appropriate treatments, cells were washed twice in PBS and then resuspended in 100 μ L Annexin binding buffer containing a 1:100 dilution of Annexin V-FITC and 50 nmol/L tetramethyl-rhodamine methyl ester (TMRM); where appropriate for MSC coculture experiments, a 1:100 dilution of anti-CD90 APC-conjugated antibody was added. CD90 was used to discriminate MSCs (positive) from leukemia cells (negative). Cells were then analyzed by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) using a 488-nm argon ion and 633-nmHeNe excitation lasers. Apoptotic cells were quantified as % Annexin-FITC(+) cells, and mitochondrial damage was quantified as %TMRM(–) cells.

Measurement of $\Delta\Psi M$ and Fluorometric Oxygen Consumption A solution of 5 mg/mL of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) in DMSO was diluted 1:100 in complete prewarmed RPMI-1640 medium with vigorous vortexing and further diluted 1:100 directly on cell cultures 15 min before harvesting. After collection, cells were washed once in PBS and $\Delta\Psi M$ was then quantitated by flow cytometry as previously described [25]. For oxygen consumption experiments, OCI-AML3 or MOLT4 cells (2×10^5 cells in 100 μ L) were seeded in 96-well BD oxygen biosensor plates (BD Biosciences, Bedford MA) and treated with PRP or PL for 30 min. Wells were then covered with 2 drops of mineral oil and incubated at 37 °C for 4 h prior to recording fluorescence (Ex485, Em590; bottom optic) in a Fluostar Optima plate reader (BMG, Germany). Samples were corrected for extra-mitochondrial (background) oxygen consumption by subtracting the signal

intensity from wells treated with 2 mM NaCN. Results were expressed as arbitrary fluorescent units (AU).

Measurement of Superoxide Production After appropriate treatments, OCI-AML3 cells were loaded with dihydroethidine (DHE; 500 nmol/L) for 30 min in a 37 °C incubator containing 5%CO₂, followed by addition, or not, of 1 μ mol/L rotenone, and further incubated an additional 30 min. Cells were then washed twice in PBS, and fluorescent emission at 590 nm was analyzed by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) using a 488-nm argon ion excitation laser. Results were expressed as mean fluorescent intensity (MFI).

Measurement of Neutral Lipid Content After appropriate treatments, cells were loaded with LipidTox neutral green stain for 45 min in a 37 °C incubator containing 5%CO₂, followed by two washes in PBS. Fluorescent emission at 520 nm was analyzed by flow cytometry in a FACS Calibur flow cytometer (BD Biosciences) using a 488-nm argon ion excitation laser. Results were expressed as mean fluorescent intensity (MFI).

Bax Crosslinks Bax crosslinks were investigated as previously described [6]. Briefly, after exposure of OCI-AML3 cells to PL and/or ABT-737, mitochondrial extracts generated by hypotonic lysis were resuspended in 150 mM NaCl, 10 mM HEPES (pH 7.4), and 1 % CHAPS at 1 mg/ml of protein and treated with 0.4 mM bismaleimidoethane (Thermo Scientific) for 1 h at room temperature. Lysates (12.5 μ g of protein per well) were then subjected to SDS-polyacrylamide gel electrophoresis in 12 % polyacrylamide gels followed by protein transfer to polyvinylidene difluoride (PVDF) membrane (Thermo Scientific) and immunoblotted with Bax antibody (clone D2E11; Cell Signaling Technology). Signals were detected by chemiluminescence using HRP conjugated anti-rabbit secondary antibodies (Cell Signaling Technology) and high sensitivity X-ray film (Thermo Scientific).

Primary Samples Bone marrow or peripheral blood samples were obtained for in vitro studies from patients with AML or ALL. Samples were collected during routine diagnostic procedures after informed consent was obtained, and within 2 h after sample collection, mononuclear cells were separated by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation and used immediately. Table 1 summarizes the clinical characteristics of the samples used. All experiments with primary samples were performed in triplicate.

Statistics Unless otherwise indicated, results are expressed as mean $\pm$ SD of 3 independent experiments. *P* values were determined by 1-way ANOVA followed by *F* statistics. A *P* value less than 0.05 was considered significant.

Table 1 Characteristics of leukemia patient samples

Figure	DX	Source	WBC	Blast	Monocyte	Cytogenetics	Age	Sex	FLT3	NPM	IDH2	p53	NOTCH1	CEBPA	Karyotype
A	ALL	PB	17.2	79 %	4 %	Normal	23	Male	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation	Normal 46, XY
B	B-ALL	PB	30	91 %	4 %	Normal	25	Male	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation	Normal 46, XY
C	AML	PB	19	84 %	2 %	Normal	75	Female	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation	Normal 46, XY
D	AML	PB	5.5	96 %	3 %	Normal	75	Male	Two internal tandem duplication (ITDS)	Duplication exon (11)	SNV (Exon 4)	No mutation	SNV (Exon 34)	No mutation	Normal 46, XY
E	B-ALL	PB	24.6	86 %	2 %	Normal	38	Female	No mutation	No mutation	No mutation	No mutation	No mutation	No mutation	Normal 46, XY
F	B-ALL	PB	5.5	86 %	1 %	Complex	24	Male	No mutation	No mutation	No mutation	No mutation	No mutation	Isolated mutation	47,XY,+X[3] 44,Y,-X-6[1] 47,XX,+mar[1] 46,XX[10]

Diagnosis, Source, and clinical/molecular characteristics of patient samples used in Fig. 6 (a–f)

Results

Leukemia Cells Promote Platelet Activation

We initially prepared platelet rich plasma (PRP) via a single centrifugation step as previously described [24] and added increasing doses of this blood fraction (%v/v), alone or in combination with calcium gluconate (1 %v/v) to cultures of OCI-AML3 (acute myeloid) and MOLT4 (acute T lymphoblastic) leukemia cell lines. Interestingly, we observed clot formation independent of the addition of calcium gluconate in OCI-AML3 and MOLT4 cells (data not shown). In addition, flow cytometric analysis demonstrated that in the presence of heparin – to inhibit clot formation – MOLT4 cells significantly ($p<0.05$) promoted the expression of CD62P in live platelets (Fig. 1a) and OCI-AML3 promoted externalization of phosphatidyl serine in live platelets (Fig. 1b) in a cell dose-dependent manner supporting the notion originally suggested by Foss and Bruserud [22] that cell-platelet contact and/or paracrine factors in leukemia cell cultures promote platelet activation. Subsequent experiments included heparin or used filtered activated platelet supernatants (SP) to avoid interference from the clot during cell counting and flow cytometry.

To then determine if platelet derived factors could impact on the proliferation of leukemia cells, we exposed OCI-AML3 and MOLT4 leukemia cells to increasing doses of PRP (1, 2.5, and 5 %) or platelet lysate (PL; 1, 2.5, and 5 %) in the presence of heparin for 96 h and counted viable cells in a Neubauer

Fig. 1 Leukemia cells promote platelet activation. **a** RPMI medium was inoculated with 2.5 % PRP and exposed to 1 % Calcium gluconate, or increasing numbers of MOLT4 cells (50, 100, or 250×10³/mL) for 1 h under standard culture conditions. Expression of CD62P on platelets was then analyzed by flow cytometry as described in the materials and methods. **c** RPMI medium was inoculated with 2.5 % PRP and exposed to 1 % Calcium gluconate, or increasing numbers of OCI-AML3 cells (50, 100, or 250×10³/mL) for 1 h under standard culture conditions. Externalization of phosphatidyl serine on platelets was then analyzed by flow cytometry as described in the materials and methods. * = $p<0.05$; ** = $p<0.001$

chamber. In contrast to the effects of platelet lysates reported in MSC and solid tumors [16, 17], and the effects of live platelets on leukemic blasts reported by Foss and Bruserud [18] neither PRP (from 3 healthy individuals) nor PL significantly affected the viability of OCI-AML3 or MOLT4 cells (Data not shown).

Platelet Contents Promote Resistance to Intrinsic Apoptosis in Leukemia Cells and Primary Samples

Although PRP or PL did not have an effect on viable leukemia cell numbers, we questioned if platelet derived factors may impart chemoresistance. To test this hypothesis we exposed leukemia cells to increasing doses of the traditional leukemia chemotherapeutics doxorubicin and AraC, or the mitochondriotoxic agents CDDO-Me or ABT-737, alone or in the presence of 2.5 % PL. Various doses of PL did not promote resistance to doxorubicin or AraC induced cell death in MOLT4 or OCI-AML3 cells (data not shown). Curiously, however, 2.5 % PL promoted significant ($p < 0.05$) resistance to apoptosis (Fig. 2a and b) and mitochondrial damage (TMRM-negative cells; Fig. 2c and d) induced by ABT-737 in both MOLT4 and OCI-AML3 cells suggesting the notion that platelet contents specifically oppose activation of the intrinsic apoptotic pathway in leukemia cells. Similar observations were made in OCI-AML3 cells exposed to 500 nmol/L ABT-737 and increasing doses of PRP (Fig. 2e) or increasing doses PL (data not shown). Moreover, as evidenced in Fig. 3a and c, in MOLT4 cells 2.5 % PL also promoted significant ($p < 0.0005$) resistance to apoptosis and mitochondrial damage induced by CDDO-Me – a synthetic triterpenoid that directly permeabilizes the inner mitochondrial membrane to induce swelling and rupture of the outer membrane with subsequent release of apoptogenic factors [26] – supporting the hypothesis that platelet derived factors also antagonize mitochondrial permeability transition-induced apoptosis. Comparable antiapoptotic and mitoprotective effects were observed in OCI-AML3 cells exposed to increasing doses of CDDO-Me in combination with 2.5 % PL (Fig. 3b and d), or exposed to 500 nmol/L CDDO-Me in combination with increasing doses of PRP (Fig. 3e) or increasing doses of PL (data not shown). Importantly, it was observed that PL also promoted resistance to apoptosis induction in primary leukemia blasts from 4 ALL and CD34(+) blasts from 2 AML patients in response to ABT-737 or CDDO-Me (Fig. 4a–f) suggesting that the prosurvival effects of platelet lysates are not constrained to immortalized cell lines. Additionally, Table 1 summarizes the clinical characteristics of the samples analyzed, which suggest that the observed effects are not specific for particular phenotypic, molecular or cytogenetic characteristics of leukemia cells. We hypothesize that the less impressive effects observed on all primary samples – compared to the clear survival advantage seen in cell lines –

suggest an increased sensitivity of cell lines to platelet derived components and/or result from primary samples already being exposed to platelet components in the in vivo setting.

Platelet Contents Promote Mitochondrial Uncoupling and Antagonize Bax Oligomerization in Leukemia Cells

In order to probe the mechanisms by which platelet contents promote resistance to activation of the intrinsic apoptotic pathway, we monitored mitochondrial membrane potential ($\Delta\Psi$ M) by flow cytometry utilizing the ratiometric potential sensitive dye JC1 as previously described [5]. Intriguingly, as shown in Fig. 5a, 2 h exposure to 2.5 % PL reduced $\Delta\Psi$ M by ~80 % in both MOLT4 and OCI-AML3 cells, reminiscent of the decrease observed in OCI-AML3 cells cultured in MSC feeder layers (Fig. 5b), suggesting that platelet contents – like MSC feeder layers – directly and profoundly modulate mitochondrial metabolism. Additional experiments demonstrated that PL induced a significant ($p < 0.001$), dose-dependent decrease in $\Delta\Psi$ M (Fig. 5c), and as observed for OCI-AML3 cells cultured on MSC feeder layers [5], the observed loss of $\Delta\Psi$ M occurred rapidly, within 30 min of exposure to platelet supernatants (SP; $p < 0.001$), inducing highly significant ($p < 0.0001$) and profound mitochondrial depolarization at 18 h (Fig. 5d). Similar dose-dependent effects on $\Delta\Psi$ M were observed in MOLT4 cells treated with increasing doses of PL for 4 h (Fig. 5e). Although platelets have been shown to release significant amounts of TGF β 1 [27], the observed effects of SP on $\Delta\Psi$ M were insensitive to pharmacological treatment with the potent and specific inhibitor of the TGF β receptor superfamily, SB-431542 (Fig. 5f). It is also noteworthy that SP that had been filtered (0.2 μ m) or heat inactivated (65 °C for 1 h) maintained a $\Delta\Psi$ M reducing effect indicating that platelets contain a small, heat insensitive, mitochondrial depolarizing activity (Fig. 5f). To then investigate if the loss in $\Delta\Psi$ M is a consequence of reduced electron transport, we monitored oxygen consumption in OCI-AML3 cells treated with increasing doses of PL for 30 min. Intriguingly, as shown in Fig. 6a, OCI-AML3 cells significantly ($p < 0.005$) increase their oxygen consumption capacity in response to PL, supporting the notion that rather than damaging the oxidative capacity of leukemia cell mitochondria, platelet contents may instead be promoting mitochondrial uncoupling. Similar observations were made in MOLT4 cells (data not shown). Moreover, as shown in Fig. 6b, the increase in oxygen reducing capacity in OCI-AML3 cells exposed to PL is largely dependent on the oxidation of fatty acids, as the fatty acid inhibitor ranolazine (200 μ M) significantly ($p < 0.005$) reduced oxygen consumption capacity to control levels. Consistent with the notion that uncoupled mitochondria depend on fatty acids to support oxygen reduction capacity, 2.5 % PL induced a 50 % reduction ($p < 0.005$) in triglyceride levels in OCI-AML3 cells as monitored by flow cytometry (Fig. 6c).

Fig. 2 Platelet contents promote resistance to apoptosis induced by ABT-737 in leukemia cells. **a** and **c** MOLT4 and **(b and d)** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed, or not, to 2.5 % PL for 30 min prior to the addition of increasing doses of ABT-737 for 16 h. Apoptosis (**a** and **b**) and mitochondrial damage (**c** and **d**) were determined as described in the materials and methods. * = $p < 0.005$ from untreated RPMI without platelet components; # = $p < 0.05$ from ABT-737 treated RPMI without platelet components. **e** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed to increasing doses of PRP (0, 0.5, 1, and 2.5 %) 30 min prior to the addition of 500 nmoles/L ABT-737 for 16 h. Apoptotic cells were quantified as above. * = $p < 0.0005$ from untreated RPMI without platelet components; ** = $p < 0.005$ from ABT-737 treated RPMI without platelet components

Additionally, since mitochondrial uncoupling has been reported to reduce the production of reactive oxygen species [28, 29], we monitored superoxide production in OCI-AML3 cells by flow cytometry. As evidenced in Fig. 6d, PL dose-dependently promoted a decrease in basal (maximal reduction of 36 %, $p < 0.005$) and rotenone-induced (maximal reduction of 27 %, $p < 0.05$) superoxide levels, lending additional support to the hypothesis that platelet contents promote mitochondrial uncoupling. Based on a recent report demonstrating that

mitochondrial uncoupling promotes autophagy [30], we questioned whether PL could induce programmed cell death type II as a mechanism to antagonize apoptosis, and found that neither MOLT4 nor OCI-AML3 cells exposed to PL for 48 h increased the number of monodansylcadaverine (MDC) containing vesicles, suggesting that autophagy may not be playing a role in PL-mediated protection against apoptotic stimuli (data not shown). Instead, it was observed in OCI-AML3 cells that PL reduced the formation of higher

Fig. 3 Platelet contents promote resistance to apoptosis induced by CDDO-Me in leukemia cells. **a** and **c** MOLT4 and **(b and d)** OCI-AML3 cells (1×10^5 /mL) were exposed, or not, to 2.5 % PL for 30 min prior to the addition of increasing doses of CDDO-Me for 16 h. Apoptosis (**a** and **b**) and mitochondrial damage (**c** and **d**) were determined as described in the materials and methods. **= $p < 0.0005$ from untreated RPMI without platelet components; #= $p < 0.0005$ from CDDO-Me treated RPMI without platelet components. **e** OCI-AML3 cells (1×10^5 /mL) were exposed to increasing doses of PRP (0, 0.5, 1, and 2.5 %) 30 min prior to the addition of 500 nmoles/L CDDO-Me for 16 h. Apoptotic cells were quantified as above. **= $p < 0.0005$ from untreated RPMI without platelet components; *= $p < 0.0005$ from CDDO-Me treated RPMI without platelet components. **f** OCI-AML3 cells (1×10^5 /mL) were seeded on MSC feeder layers (1×10^4 /cm²) and exposed to increasing doses of ABT-737 or CDDO-Me for 16 h and apoptotic cells were quantified as above. *= $p < 0.05$ from RPMI only control

molecular weight Bax immunoreactive bands in response to ABT-737, in agreement with our previous observations demonstrating that fatty acid oxidation and mitochondrial uncoupling antagonized oligomerization of Bax in leukemia cells (Fig. 6e). Taken together, our results suggest that platelet contents promote resistance to activation of the intrinsic apoptotic pathway via mitochondrial uncoupling, independent of the induction of autophagy.

Discussion

Although clinically relevant for leukemia treatment and diagnosis, the in vitro effects of platelets and their contents on leukemia cell biology have remained largely uninvestigated. In contrast, in vitro and in vivo evidence supports the hypothesis that platelets promote solid tumor progression and dissemination [12–15], and may contribute to chemoresistance

Fig. 4 Platelet contents promote resistance to apoptosis induced by CDDO-Me or ABT-737 in leukemic blasts. **a–f** Peripheral blood mononuclear cells (1×10^6 /mL) from AML or ALL patients were exposed, or not, to 2.5 % PL for 30 min prior to the addition of increasing doses of CDDO-Me or ABT-737 for 16 h. Cells were harvested, stained with CD34-FITC and propidium iodide, and viable cell numbers were determined by flow cytometry as described in the materials and methods. **= $p < 0.0005$ from untreated RPMI without platelet components; #= $p < 0.0005$ from CDDO-Me treated RPMI without platelet components. *= $p < 0.05$ from RPMI only control

[14]. The results presented here, demonstrate that leukemia cell lines promote platelet activation, supporting the notion that the leukemic bone marrow may possibly activate newly formed platelets, perhaps contributing to leukemic thrombocytopenia [31]. This notion is also congruent with the observation by Foss B and colleagues [32] that platelets can adhere to acute myeloid leukemia blasts, and their hypothesis that platelets and leukemic blasts share in vivo microenvironments. In addition, contrary to what has been reported for solid tumor cell lines, MSC [16, 17] and leukemic blasts [18], platelet contents did not affect the proliferation of OCI-AML3 or MOLT cells, suggesting that the proliferative and the chemoprotective effects of platelets may be mediated by

different mechanisms in a cell context dependent manner. More experiments, beyond the scope of this work, will be required to validate the relevance of our findings in additional leukemia cell lines and primary samples.

Our results also demonstrate that platelet contents impart resistance to apoptosis induced by mitochondriotoxic agents like ABT-737 and CDDO-Me, but not to AraC or doxorubicin, supporting the intriguing notion that platelets selectively impair the intrinsic apoptotic pathway. It is important to point out that albeit both CDDO-Me and ABT-737 target the mitochondria, they do so by different mechanisms. While ABT-737 promotes Bax/Bak oligomerization and outer membrane permeability [33], CDDO-Me directly permeabilizes the inner

Fig. 5 Platelet contents promote loss of $\Delta\Psi$ in leukemia cells. **a** OCI-AML3 and MOLT4 cells ($1 \times 10^5/\text{mL}$) were treated with 2.5 % PL for 2 h and analyzed for $\Delta\Psi$ as described in the materials and methods. Shown are representative flow cytometry plots of JC1 aggregate fluorescence (FL2:H) vs. JC1 monomer fluorescence (FL1:H). **b** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were seeded on MSC feeder layers ($1 \times 10^4/\text{cm}^2$) for 16 h and $\Delta\Psi$ was quantified as above gating on CD90-negative cells. **c** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed to increasing doses of PL (0, 0.5, 1, and 2.5 %) for 18 h. $\Delta\Psi$ was quantified by flow cytometry as described in the materials and methods. **d** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed 2.5 % of PL for 0, 0.5, 4, and 18 h, and $\Delta\Psi$ was quantified as above. **e** $\Delta\Psi$ was quantified in MOLT4 cells ($1 \times 10^5/\text{mL}$) that were exposed to increasing doses of PL (0, 0.5, 1, and 2.5 %) for 4 h. **f** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed to SB-431542 ($1 \mu\text{mol/L}$) for 30 min, prior to the addition of 5 % SP for 3 h. In parallel, cells were also exposed to SP that had been heated to 65°C for 1 h (SP HI) or filtered through a $0.2 \mu\text{m}$ mesh (SP F). $\Delta\Psi$ was quantified by flow cytometry as described in the materials and methods. * = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$ from untreated controls

mitochondrial membrane, inducing matrix swelling and rupture of the outer mitochondrial membrane [26]. Mechanistically, we demonstrate that platelets antagonize Bax/Bak oligomerization, although it is clear that this may not be the only mechanism since the expression of these proteins is not required for CDDO-Me cytotoxicity [26]. Moreover, our results evidence a greater antiapoptotic effect of platelet contents against CDDO-Me than ABT-737, perhaps indicating a direct protective effect over the inner mitochondrial membrane that may in turn antagonize Bax/Bak oligomerization on the outer mitochondrial membrane.

Interestingly, we observed that leukemia cells exposed to PRP or PL significantly reduced their $\Delta\Psi$, while, paradoxically, increasing their oxygen consumption. Additionally, we

observed a marked decrease in triglyceride content, suggestive of lipolysis, and coincidentally we showed that the increase in oxygen consumption induced by platelet components was sensitive to the fatty acid oxidation inhibitor ranolazine. This finding is consistent with the phenomenon of mitochondrial uncoupling – a short circuit of the electrochemical gradient in mitochondria that results in $\Delta\Psi$ -independent, fatty acid oxidation- and lipolysis-dependent oxygen consumption [7, 34, 35]. Mechanistically, our results substantiate the hypothesis that mitochondrial uncoupling is associated with the increased stability, and intrinsic apoptotic resistance, of the mitochondrial membranes in leukemia cells exposed to platelet components. This is intriguing considering that we have reported a similar behavior in leukemia cells cultured on MSC

Fig. 6 Leukemia cells exposed to platelet contents increase lipolysis-dependent oxygen consumption, reduce generation of superoxide anion, and antagonize bax oligomerization. **a** OCI-AML3 cells ($100\ \mu\text{l}$; 2×10^5 cells in $100\ \mu\text{l}$) were seeded in Oxygen Biosensor plates and exposed to increasing doses of PL for 4 h. Oxygen consumption was monitored as described in the materials and methods. **b** OCI-AML3 cells ($100\ \mu\text{l}$; 2×10^5 cells in $100\ \mu\text{l}$) were seeded in Oxygen Biosensor plates and exposed, or not, to $200\ \mu\text{mol/L}$ ranolazine (RAN) for 30 min prior to addition of 2.5 % PL for 4 h, oxygen consumption was monitored as above. **c** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed to increasing doses of PL (0, 0.5, 1, and 2.5 %) for 18 h and phospholipid and triglyceride content was quantitated by flow cytometry as described in the materials and methods. **d** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed to increasing doses of PL (0, 0.5, 1, and 2.5 %) for 3 h followed by the addition, or not, of $1\ \mu\text{mol/L}$ rotenone. Superoxide levels were quantified as described in the materials

and methods. **e** OCI-AML3 cells ($1 \times 10^5/\text{mL}$) were exposed, or not, to 2.5 % PL and treated with $500\ \text{nmol/L}$ ABT-737 for 16 h. Mitochondrial extracts were then exposed to bismaleimidoethane (BMH), and Bax crosslinking was determined by Western blot as described in the materials and methods. **f** Proposed model of platelet mediated antiapoptotic effects in leukemia cells. Leukemia cells promote platelet activation that results in liberation of a mitochondrial depolarizing/uncoupling activity – possibly cationic antimicrobial peptides (CAPs) – that promote permeability of the inner mitochondrial membrane, loss of $\Delta\Psi$, increased oxygen consumption, decreased superoxide generation, and increased resistance to apoptotic stimuli. **a–c** * = $p < 0.05$; ** = $p < 0.005$; from untreated control; # = $p < 0.005$ from RAN control. **d** * = $p < 0.05$; ** = $p < 0.005$; from rotenone control without PL; # = $p < 0.005$ from untreated control without PL

feeder layers [5], and supports a broad role for mitochondrial metabolic alterations in leukemia cell chemoresistance. It is tempting to speculate that perhaps the reduced negative electric potential within the matrix of uncoupled mitochondria prevents cardiolipin from efficiently externalizing on the inner

membrane to orchestrate mitoptosis, as would be predicted by the model put forth by Garcia-Fernandez and colleagues [36], although this remains to be examined. Could the bone marrow microenvironment as a whole – MSC and newly formed platelets – promote fatty acid oxidation dependent

mitochondrial uncoupling as a means of maintaining a privileged niche? If so, our observations are in agreement with previous work demonstrating that fatty acid oxidation inhibitors in combination with ABT-737 display therapeutic efficacy in a murine model of human leukemia [6]. Nevertheless, additional experiments, not germane to this study, will be required to determine 1) the exact *in vivo* contribution of platelets and MSC to leukemia progression and chemoresistance, and 2) the precise mechanisms by which mitochondrial uncoupling imparts resistance to apoptosis.

Notably, our results utilizing the autophagic vacuole marker MDC do not support a role for autophagy in platelet-mediated chemoprotection. This was surprising as several recent reports have suggested that mitochondrial uncoupling results in autophagy/mitophagy [30, 37]. Nevertheless, it could be conceivable that uncoupling in leukemia cells results in low levels of mitophagy that are undetectable utilizing MDC, and this is currently being investigated. Additionally, although determining the identity of the platelet component(s) that cause mitochondrial uncoupling in leukemia cells was beyond the scope of this work, the results presented here indicate that it is unlikely to be TGF β , and that regardless of the precise molecular identity, it must be smaller than 0.2 μ m (possibly ruling out microvesicles) and must be heat-resistant. Moreover, it is tempting to speculate that the molecular agent ought to be cationic – thus being tropic for the negatively charged mitochondrial matrix/inner membrane – and that this agent perhaps contributes positive charges and/or promotes proton conductance resulting in loss of $\Delta\Psi$ M. The above description may fit a number of cationic antimicrobial peptides (CAPs) that are released from thrombin-stimulated human platelets, including classical microbicidal chemokines (“kinocidins”) such as platelet factor 4, RANTES, and CTAP-3, together with fibrinopeptide B and thymosin β -4 [38–40]. Recent reports demonstrate that the bactericidal activity of related CAPs depends on the permeabilization of bacterial membranes [41, 42], and that this permeabilizing effect is strongly dependent on the presence of cardiolipin [41], suggesting the possibility that mitochondria may be *ipso facto* targets of this peptides. Figure 6f illustrates the proposed mechanism of platelet mediated antiapoptotic effects in leukemia cells. However, it remains to be determined if these kinocidins could mediate mitochondrial uncoupling in leukemia cells exposed to platelet components.

In summary, our observations substantiate the hypothesis that leukemia cells may promote deregulated activation of platelets resulting in the release of an uncoupling agent(s) that may promote leukemic resistance to mitochondrial toxic chemotherapeutics in the bone marrow microenvironment. These results support a potential role of platelets as components of the leukemic bone marrow niche, and call for the inclusion of platelet components *in vitro* to conduct drug efficacy studies in leukemia cells.

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Conflict of Interest The authors declare that they have no conflict of interest.

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