Phagocytosis by macrophages and endothelial cells inhibits procoagulant and fibrinolytic activity of acute promyelocytic leukemia cells

Short title for running head: Engulfment and coagulation in APL

Rui Xie, Chunyan Gao, Wen Li, Jiuxin Zhu, Valerie Novakovic, Jing Wang, Ruishuang Ma, Jin Zhou, Gary E. Gilbert, and Jialan Shi

1Health Ministry Key Laboratory of Cell Transplantation, Heilongjiang Institute of Hematology and Oncology, Department of Hematology, The First Affiliated Hospital, Harbin Medical University, Harbin, China; and 2Medicine Departments of VA Boston Healthcare System, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts, USA

Correspondence:
Jialan Shi
1400 VFW Parkway, VA Boston Healthcare System, Brigham and Women’s Hospital, Harvard Medical School, West Roxbury, MA 02132
Tel.: +1 857 203 5140
Fax: +1 857 203 5592
E-mail: jialan_shi@hms.harvard.edu

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Abstract

The coagulopathy of acute promyelocytic leukemia (APL) is mainly related to procoagulant substances and fibrinolytic activators of APL blasts, but the fate of these leukemic cells is unknown. The aim of this study was to investigate the removal of APL blasts by macrophages and endothelial cells in vitro and consequent procoagulant and fibrinolytic activity of APL cells. We found that human umbilical vein endothelial cells as well as THP-1 and monocyte-derived macrophages bound, engulfed and subsequently degraded immortalized APL cell line NB4 and primary APL cells. Lactadherin promoted phagocytosis of APL cells in a time-dependent fashion. Furthermore, factor Xa and prothrombinase activity of PS-exposed target APL cells was time-dependently decreased after incubation with phagocytes (THP-1-derived macrophages or human umbilical vein endothelial cells). Thrombin production on target APL cells was reduced by 40-45% after two hours of coincubation with phagocytes, and 80% by a combination of lactadherin and phagocytes. Moreover, plasmin generation of target APL cells was inhibited 30% by two hours of phagocytosis, and approximately 50% by lactadherin-mediated engulfment. These results suggest that engulfment by macrophages and endothelial cells reduce procoagulant and fibrinolytic activity of APL blasts. Lactadherin and phagocytosis could cooperatively ameliorate the clotting disorders in APL.

Keywords:

Acute promyelocytic leukemia, phosphatidylserine, phagocytosis, lactadherin, macrophages, endothelial cells, procoagulant activity, fibrinolytic activity
Introduction

Acute promyelocytic leukemia (APL) is characterized by the dual phenomenon of life-threatening thrombosis and bleeding\(^1,2\) as well as the accumulation of immature promyelocytes.\(^3\) Although this disease represents a paradigm for successful target treatment with remarkable advances at both the clinical and laboratory levels,\(^4,5\) the removal process of APL cells remains to be investigated.

Phosphatidylserine (PS) is a critical ‘eat me’ signal for phagocytes.\(^6,7\) Our and other prior reports have demonstrated that viable and apoptotic APL cells expose PS.\(^8,9\) It is conceivable that due to uncontrolled APL blast proliferation and chemotherapy,\(^9\) professional macrophages (MΦ) may become overwhelmed by the excessive amounts of PS-exposed APL blasts. This may lead to the clearance of these cell corpses by amateur phagocytes, such as ubiquitously distributed endothelial cells (ECs). Hence, we speculate that both MΦ and ECs contribute to the removal of APL cells.

In addition, exposed PS on cells provides a catalytic surface for the assembly of tenase and prothrombinase complexes.\(^10\) Our previous study indicated that PS exposure is a major mechanism through which APL blasts enhance procoagulant activity (PCA).\(^8\) Furthermore, clinical hemorrhage in patients with APL is thought to be due to disseminated intravascular coagulation, abnormal fibrinolysis, or both.\(^11\) However, the contribution of phagocytosis by scavengers to the PCA and fibrinolytic activity (FLA) of APL cells is still unclear. In view of this fact, we infer that changes in clearance of APL blasts may influence the established clinical patterns when coagulation abnormalities worsen or improve depending on the type of treatment.

Lactadherin, a milk fat globule membrane glycoprotein,\(^12\) is secreted by MΦ and ECs.\(^13,14\) This protein contains a domain structure of EGF1-EGF2-C1-C2.\(^15\) It anchors PS-externalized cells through its C-terminus to phagocyte α\(_5\)β\(_{3/5}\) integrins for engulfment via its RGD motif in the EGF2 domain.\(^16\) Recently, lactadherin has been shown to promote elimination of platelet-derived microparticles by MΦ,\(^17\) and to mediate phagocytosis of microparticles\(^18\) and melanoma cells\(^19\) by angiogenic ECs. Moreover, lactadherin binds to PS-containing membranes, competing for membrane sites recognized by factor V and factor VIII.\(^15,20\) We previously found that it reduces the PCA of APL cells.\(^8\) Considering these properties, we have further investigated the cooperative effect of lactadherin and phagocytosis on the PCA and FLA of APL cells.

In the present study, we explored the phagocytosis of APL cells by MΦ and ECs \(\textit{in vitro}\) and its relevance to the PCA and FLA of APL cells. In addition, we investigated the role of lactadherin in
phagocytosis and determined the contribution of lactadherin and phagocytosis cooperation to the modulation of coagulation in APL.

**Patients, materials, and methods**

**Patients**

Sixteen newly diagnosed APL patients admitted to the First and Second Affiliated Hospital of Harbin Medical University between May 2010 and October 2011 were studied after informed consent. This study was approved by Ethics Committee of Harbin Medical University and conducted in accordance with the Declaration of Helsinki. The diagnosis was based on clinical data, morphology, cytochemistry, immunology, cytogenetics and molecular biology.\(^5\) Cytogenetic analysis indicated the t(15;17) translocation and PML/RAR\(\alpha\) fusion gene in all cases. The main characteristics of the patients at the moment of bone marrow aspiration were reported on Table 1.

**Reagents**

Endothelial cell medium was purchased from ScienCell (San Diego, CA, USA). RPMI1640 medium, Fetal bovine serum (FBS) and 0.25% Trypsin–EDTA were from Gibco (Grand Island, NY, USA). Ficoll-Hypaque, daunorubicin (DNR), phorbol 12-myristate 13-acetate, poly-d-lysine, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA) and Triton X-100 were obtained from Sigma-Aldrich (St Louis, MO, USA). FluoReporter FITC protein labeling kit, CellTracker Green CMFDA, CellTracker Red CMTPX and Alexa Fluor 488 donkey anti-goat IgG were from Invitrogen (Carlsbad, CA, USA). Propidium iodide (PI) and annexin V were obtained from BD Pharmingen (San Jose, CA, USA). Lactadherin was purified from bovine milk.\(^12\)\(^13\) Lactadherin and annexin V were coupled to FITC according to the manufacturer’s instructions. Goat anti human annexin II IgG and goat anti human IgG were from AbD serotec (Kidlington, Oxford, UK). Recombinant human factor VIII was from American Diagnostica Inc. (Stamford, CT, USA). Human prothrombin, thrombin, factors Va, VIIa, IXa, X, Xa and glu-plasminogen were obtained from Haematologic Technologies Inc. (Burlington, VT, USA). Plasmin was from Enzyme Research Laboratories (Southbend, IN, USA). Tissue plasminogen activator (t-PA) was obtained from Abcam (Cambridge, MA, USA). The Chromogenix substrate S-2238, S-2765 and S2251 were from DiaPharma Group (West Chester, OH, USA).
Cell culture

Freshly isolated APL blasts were obtained from bone marrow specimens by centrifugation through Ficoll-Hypaque. These cells (5×10^5/mL) were propagated in complete RPMI 1640 medium supplemented with 20 % FBS, 2 mM L-glutamine and 1% penicillin-streptomycin solution at 37 °C in a 5% CO2 humidified atmosphere.

Human APL cell line NB4 cells and monocytic cell line THP-1 cells from the American Type Culture Collection (Manassas, VA, USA) were maintained under the same conditions aforementioned except that 10% FBS was used. Human umbilical vein endothelial cells (HUVECs) from ScienCell used up to passage 4 were cultured in endothelial cell medium containing 5 % FBS, 1% endothelial cell growth supplement and antibiotics.

Preparation of MΦ

As previously described, peripheral blood mononuclear cells from healthy volunteers were isolated by Ficoll-Hypaque density gradient centrifugation. Positive isolation of primary monocytes from mononuclear cells was performed using a magnetic activated cell sorting starting kit with human CD14+ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured in 10% FBS-containing complete RPMI1640 medium and allowed to differentiate into MΦ for 5 days.

Alternatively, THP-1-derived MΦ were obtained by stimulating THP-1 monocytes with 20 ng/mL phorbol 12-myristate 13-acetate for 72 hours.

Coincubation assay

Viable APL cells from each patient with APL or NB4 cells at a density of 5×10^5/mL were exposed to various concentrations of DNR (0.1, 0.5, 1 μM) at 37 °C for 24 hours. Those cells treated with 1 μM DNR were used as target cells. After extensive washing to remove residual DNR, a total of 1×10^6 target NB4 or APL cells were incubated with 5×10^5 MΦ or HUVECs in 12-well culture plates for different time points.

Lactadherin-opsonized or annexin V-opsonized targets were obtained as follows: target APL cells were preincubated with 2 nM lactadherin or annexin V for 10 minutes at room temperature, followed by washing and removal of free proteins-containing supernatant solution. In some instances, these opsonized cells were co-incubated with MΦ or HUVECs monolayers.

Flow cytometry
To quantify PS exposure, NB4 or APL cells suspended in Tyrode’s buffer (for lactadherin-binding test, 137 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO₃, 0.42 mM NaH₂PO₄, 1 mM MgCl₂, 2 mM CaCl₂, 5.5 mM glucose, 5 mM HEPES and 0.35 % BSA, pH 7.4) or annexin V binding buffer (for annexin V-binding assay, 10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) were adjusted to 1×10⁶ cells/mL.

FITC-annexin V or FITC-lactadherin at a final concentration of 2 nM was incubated with the cells for 10 minutes in the dark. After addition of 1 μg/mL PI, cells were analyzed on a flow cytometer (FACSAria, Becton Dickinson, USA). Data acquisition was carried out using FCS express V3 (De Novo Software, Los Angeles, CA, USA).

Annexin II was analyzed as described before with modifications. Leukemic promyelocytes or THP-1 macrophages or HUVECs (1×10⁶/mL) were fixed in 2% paraformaldehyde at room temperature for 10 minutes, washed three times with phosphate buffered saline (PBS), and were incubated with 5 μg/mL goat anti human annexin II IgG or goat anti human IgG at room temperature for 1 hour. The samples were washed twice using PBS, and then exposed to Alexa Fluor 488 donkey anti-goat IgG for 30 minutes. After washing, the extent of annexin II binding was measured by FASCalibur flow cytometry. CellQuest software was used for data acquisition and analysis. In some experiments, fixed cells were permeabilized with 0.1% Triton X-100.

Phagocytosis was evaluated by a previous method with some modifications. Target APL cells stained with 2 μM CMFDA (excitation 492/emission 516, green) were incubated with MΦ or ECs labeled with 1 μM CMTPX (excitation 586/emission 613, red) as described in “Coincubation assay”. Mixed cells (target cells and phagocytes) were harvested with a cell scraper (MΦ) or Trypsin-EDTA solution (ECs). Phagocytosis was quantified by measuring the percentage of green fluorescence (CMFDA)-positive CMTPX (red) phagocytes by FACSAria flow cytometry.

Confocal microscopy

To locate PS, NB4 or APL cells were incubated with the indicated concentrations of PI and fluorescein-labeled lactadherin or annexin V. Cells were washed to remove unbound proteins and analyzed immediately. The samples were excited with 488 nm emission line of a krypton-argon laser, and narrow bandpass filters were utilized to restrict emission wavelength overlap. Images were captured using Zeiss LSM 510 Meta confocal microscope (Carl Zeiss Jena GmbH, Jena, Germany).

NB4 or APL cells on coverslips were fixed with 3.7% formaldehyde at room temperature for 10 minutes and then washed with PBS. Samples with or without permeabilization were exposed to goat anti
human annexin II IgG and labeled with Alexa Fluor 488-conjugated secondary antibody as described in “Flow cytometry”. Then, cells were counterstained with PI and the annexin II expression was observed using the confocal microscope.

Engulfment was detected by modified method as previously described. Briefly, 1 μM CMTPX-stained MΦ or ECs were seeded on glass coverslips coated with poly-d-lysine in 12-well culture plates, they were subsequently co-cultured with 2 μM CMFDA-labeled target NB4 or APL cells as described in “Coincubation assay”. The mixed cells were then fixed in 3.7% formaldehyde and identified under the confocal microscope.

**Electron microscopy**

In scanning electron microscope assays, target APL cells were co-cultured with MΦ or ECs on coverslips. Samples were fixed by immersion in 2.5% glutaraldehyde-phosphate fixative and stored at 4°C until processed. Following several rinses in 0.1 M Na-cacodylate HCl buffer, co-cultivations were postfixed in 1% OsO₄ and dehydrated in a graded series of ethanol (30, 50, 70, 90 and 100%; twice in 5 minutes). After critical drying, a layer of platinum, approximately 10 nm thick, was sprayed on the samples. All images were viewed with a S-3400N Scanning Electron Microscope (Hitachi Ltd., Tokyo, Japan) using an ultra-high-resolution mode.

In transmission electron microscope experiments, the mixed cells were collected and double fixed in 2.5% glutaraldehyde and 1% OsO₄. After dehydration and embedding, ultrathin sections were prepared with Reichert-Jung Ultracut Ultramicrotome (Leica, Vienna, Austria). Images were observed under a H7650 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

**Coagulation time**

Target APL cells with or without opsonization by lactadherin or annexin V were co-cultured with MΦ or ECs in 12-well culture plates as described in the “Coincubation assay”, the mixed cells were harvested and resuspended in 100 μL Tyrode’s buffer. Moreover, 1×10⁶ target APL cells with or without opsonization were suspended in 100 μL Tyrode’s buffer. PCA was determined by a modified prothrombin time test. Briefly, 100 μL of the cell suspension was mixed 1:1 with citrated platelet-poor plasma (3.8% sodium citrate, 1:9, v/v) from healthy volunteers. After incubation for 180 seconds at 37 °C, 100 μL of preheated 25 mM CaCl₂ was added. The time to fibrin strand formation was immediately recorded by an Amelung KC4A coagulometer (Labcon, Heppenheim, Germany).
Factor Xa and prothrombinase assays
The activation of intrinsic factor Xa in the presence of cells was carried out as follows. Cells were incubated with 1 nM factor IXa, 5 nM factor VIII, 0.2 nM thrombin, 130 nM factor X and 5 mM CaCl₂ in factor Xa buffer (200 μL 10% BSA, 1 mL 10×TBS, 8.8 mL ddH₂O). The reaction was stopped by EDTA at a final concentration of 7 mM. Factor Xa generation was determined immediately at 405 nm on a SpectraMax M5 Microplate Reader (Molecular Devices, USA) in kinetic mode after incubation with 10 μL of S-2765 (0.8 mM). Measurement of extrinsic factor Xa formation was analogous to that for intrinsic factor Xa except that cells were mixed with 1 nM factor VIIa, 130 nM factor X and 5 mM CaCl₂. For the prothrombinase assay, the samples were incubated with 1 nM factor Va, 0.05 nM factor Xa, 1 μM prothrombin and 5 mM CaCl₂ in prothrombinase buffer (50 μL 10% BSA, 1 mL 10×TBS, 8.95 mL ddH₂O) for 5 minutes at room temperature. Thrombin production was evaluated immediately at 405 nm in the kinetic microplate reader using S-2238 (0.8 mM) after the addition of EDTA.

Plasmin production tests
A previously described method with minor modification was used to measure plasmin formation. In brief, cells were preincubated with 10 nM t-PA for 10 minutes and washed twice with PBS. After addition of 200 nM glu-plasminogen for 10 minutes at room temperature in 96-well culture plates, 200 μM plasmin substrate S2251 was added to each well. Generation of plasmin was assayed at 405 nm using a SpectraMax M5 Microplate Reader, and was then calculated according to a calibration curve for standard plasmin.

Statistical analysis
All values were presented as mean ± standard deviation for at least three replicates. Statistical analysis was analyzed with Student t-test. P < .05 was considered statistically significant.

Results
PS exposure on NB4 and APL cells
DNR was used to induce PS exposure on NB4 and APL cells. The extent of PS exposure was measured through either annexin V or lactadherin binding using flow cytometry (Figure 1A). Untreated cells had a low percentage of proteins binding. DNR dose-dependently enhanced the lactadherin-binding or annexin V-binding percent of NB4 cells. After treatment with 1 μM DNR for 24 hours, about 75% of NB4 cells and
80% of APL cells were positive for lactadherin versus 8% of NB4 and 11% APL cells without DNR. Confocal microscopy was used to directly locate PS on APL cells treated with 1 μM DNR (Figure 1B). Early apoptotic cells showed rings of green fluorescence (FITC-annexin V or FITC-lactadherin) only, while late apoptotic cells without intact cell membranes double labeled with both green and red (PI). Thus, NB4 or APL cells treated with 1 μM DNR for 24 hours were utilized as PS-exposed target cells for all subsequent experiments.

**Clearance of NB4 and APL cells by MΦ**

Two sources of primary macrophages (MΦ) were used as *in vitro* models of phagocytic clearance. Monocytes isolated from human blood were allowed to differentiate into MΦ. Alternatively, THP-1-derived MΦ, which have similar properties to monocyte-derived MΦ, were obtained by stimulation with phorbol 12-myristate 13-acetate. The target NB4 or APL cells showed morphological features of apoptosis, including loss of most microvilli, karyopyknosis and chromatin aggregation (Figure 2). As expected, both MΦ swiftly bound and engulfed these targets. After 30 minutes of coincubation, most target cells were adhered to MΦ (Figure 2A-B). Within 1 hour, MΦ spread pseudopodia to grasp targets, which were internalized (Figure 2C-D). After 2 hours of coincubation, uptake of target NB4 and APL cells by MΦ was present (Figure 2E-F). After 3 hours of coincubation, large vacuoles filled with fragments were formed within the MΦ, indicating the engulfed APL apoptotic bodies were destroyed by degradation (Figure 2G). Untreated NB4 cells and viable APL blasts had numerous slender microvilli and did not display apoptotic features. However, some of the cells were still taken up by MΦ (data not shown).

**Elimination of NB4 and APL cells by ECs**

Since ECs have been reported to behave as amateur phagocytes for dying cells, we utilized the αv-expressing model HUVECs to study the fate of NB4 and APL cells after coincubation with ECs. Within 1 hour of incubation, target NB4 and APL cells bound to the ECs surface (Figure 3A-B). Targets with apoptotic bodies were incorporated into ECs after 1.5 hours (Figure 3C). Separated apoptotic bodies of a target APL cell were phagocytosed by the EC after 2 hours (Figure 3D). At 3 hours, target APL cells were digested within the ECs (Figure 3E). By this time point, nearly all phagocytosed intracellular materials had disappeared, suggesting degradation was virtually complete (Figure 3F). These results demonstrated that phagocytosis of PS-exposed APL cells by ECs was indeed occurring. In addition, ECs engulfed some viable APL cells (data not shown).
Lactadherin promoted the phagocytosis of APL cells

Lactadherin acts as a bridge between PS-exposed cells and phagocytes. In order to explore the contribution of lactadherin to the uptake of APL cells, the amount of phagocytosis was further measured by flow cytometry. Based on the microscopic observations as described above, the extent of phagocytosis before 2 hours was first evaluated. We found that compared with PS-exposed APL cells without lactadherin opsonization, target APL cells pretreated with lactadherin showed a greater enhanced phagocytosis by both kinds of phagocytes at all time points, in a time-dependent manner (Figure 4A). In the absence of lactadherin, the level of uptake was about 10% by either THP-1-derived MΦ or HUVECs at 2 hours (Figure 4B). However, lactadherin opsonization enhanced the phagocytic percentage to approximately 23% by THP-1-derived MΦ and 19% by HUVECs. In contrast, another PS-binding protein, annexin V, markedly inhibited the uptake. However, the stimulatory effect of lactadherin was also seen for the engulfment of viable APL cells (data not shown), indicating that patient viable APL blasts also presented a recognition signal to lactadherin.

Engulfment decreased PCA of target APL cells

The implication that MΦ and ECs were able to phagocytose APL cells motivated us to explore the contribution of phagocytosis to coagulation. Target APL cells were added to THP-1-derived MΦ and HUVECs separately for various time points. We first evaluated the PCA of the mixed cells. A modified prothrombin time assay, in which cells provided the thromboplastin, was used (Figure 5A). Reduced PCA was exhibited by increased clotting time. The PCA of the mixed cells time-dependently decreased, with statistical significance at 2 hours. We next tested the capacity of the mixed cells to support individual enzyme complexes that contribute to PCA. The support of cells for enzyme complexes was similar to support for PCA. The decrease of FXa complex generation (Figure 5B-C) and thrombin formation (Figure 4D) paralleled the increasing incubation time. By 2 hours, approximately 40-50% of the thrombin production was inhibited by coincubation with either THP-1-derived MΦ or HUVECs (Figure 5D). Moreover, at every time point, PCA of single phagocytes (data not shown) or target APL cells had no significant changes. The data suggested that declined PCA of the mixed cells was due to phagocytosis by MΦ and ECs.

Lactadherin and phagocytes cooperatively reduced PCA of APL cells

In the subsequent experiment, we investigated the possibilities of cooperative effect of lactadherin and
phagocytes on PCA of target APL cells. We found that lactadherin opsonization statistically increased coagulation time (Figure 6A) and reduced generation of intrinsic FXa (Figure 6B), extrinsic FXa (Figure 6C), or thrombin (Figure 6D) of target APL cells. Compared with incubating phagocytes (MΦ or ECs) with targets for 2 hours, a greater amount of enhanced clotting time (Figure 6A) and decreased coagulation complexes (Figure 6B-D) were observed by combination of lactadherin and phagocytes, with statistical significance. Thrombin formation of target APL cells was inhibited by lactadherin opsonization, about 40-45% by phagocytes, and approximately 80% by lactadherin and phagocytes together. Moreover, annexin V or cooperation of annexin V and phagocytes had little effect on thrombin formation. These results indicate that lactadherin and phagocytes could decrease PCA of target APL cells in a cooperative manner.

**Phagocytosis with or without lactadherin decreased FLA of APL cells**

Major determinants of coagulation disorder in APL are related to not only PCA but also fibrinolytic properties of APL cells. We explored the relationship between engulfment and fibrinolysis. A plasminogen activation test was used to measure the generation of cell-surface plasmin. Treatment with 1 μM DNR for 24 hours decreased plasmin generation of APL cells from 15 nM to 8 nM (Figure 7B). Other chemotherapy drugs Ara-c and etoposide also reduced plasmin formation of promyelocytes (data not shown). In contrast, THP-1-derived MΦ or HUVECs showed little plasmin generation (Figure 7A). When we added target APL cells to either phagocytes, the plasmin production of the mixed cells was reduced in a time-dependent fashion. Plasmin production was inhibited about 30% after a 2-hour incubation with THP-1-derived MΦ or HUVECs. We further examined the effect of lactadherin-mediated phagocytosis on plasmin generation of APL targets (Figure 7B). At 2 hours, lactadherin and phagocytes cooperation reduced approximately 50% plasmin production of APL target blasts. Annexin V had little effect on plasmin production. Taken together, our data indicated that engulfment of target APL blasts attenuated plasmin generation and decreased more with lactadherin-opsonized phagocytosis.

Previous studies revealed that abnormally high levels of annexin II in promyelocytes promote plasmin generation, perhaps accounting for the relatively high incidence of hyperfibrinolysis-related bleeding in APL. Subsequently, we assessed the annexin II expression of promyelocytes and phagocytes (THP-1 macrophages and HUVECs). Consistent with the prior observation using a fluorescein-tagged antibody, green fluorescence showed that the permeabilized t(15;17) positive APL cell line NB4 cells without drug treatment reacted with the antibody against annexin II (Figure 7C, left), indicating both cell membrane and cytoplasmic annexin II were highly expressed in untreated NB4 cells. However, after exposure to 1 μM...
DNR for 24 hours, most PI-counterstained nuclei (red) became condensed or fragmented, and DNR downregulated annexin II of permeabilized NB4 cells (Figure 7C, right) and permeabilized blast cells from patients with APL (Figure 7D). For non-permeabilized APL cells, cell surface annexin II was also reduced by DNR. Flow cytometry indicated that with permeabilization, the extent of annexin II expression from one APL patient was 98.3%, but treatment with 1 μM DNR for 24 hours decreased annexin II expression to 35.1% (Figure 7E, left). We also found annexin II expression both on the membrane and in the cytoplasm of THP-1 Mφ (Figure 7E, middle). Though the amount of annexin II expression on HUVECs surface was low, cytoplasmic annexin II was accessible (Figure 7E, right).

Discussion

Our results showed that both cultured Mφ and ECs phagocytosed APL blasts and that lactadherin promoted this engulfment in a time-dependent manner. Phagocytosis inhibited both the PCA and FLA of APL cells. Lactadherin and phagocytosis could cooperatively improve the coagulation disturbance in APL.

Mφ throughout the body clear apoptotic cells rapidly and efficiently. PS works as a recognition cue for phagocytosis. Previous studies have also shown that activated Mφ destroy tumor cells. We found that Mφ were able to recognize, engulf and ultimately dispose of PS-exposed NB4 and APL cells. That two human Mφ subsets from different origin (primary and cell line-derived Mφ) both ingested APL cells indicates that it is a general property of Mφ, not related to one particular cell type. Here, orchestrated elimination of apoptotic APL cells is important to avoid leakage of cell contents and to limit inflammatory or immunogenic responses. Clinical trials of systemic Mφ activation for therapy of disseminated metastases progress successfully in some carcinomas. Therefore, the phagocytic capacity of Mφ toward APL cells presents an attractive possibility for APL therapy.

In APL, due to uncontrolled leukemia proliferation and chemotherapy, Mφ may be overwhelmed by large numbers of PS-exposed APL cells. The burden for phagocytes may be highest after cytotoxic chemotherapy when a large number of circulating APL cells enter apoptosis. It is therefore not surprising that non-professional ECs, the cells that line blood vessels in every organ system, may contribute adjunctive phagocytic activity. In our study, HUVECs bound, engulfed and subsequently digested APL blasts. As we clarify that APL blasts can be engulfed by a cell type beyond the mononuclear phagocyte system, our results seems to provide a novel strategy to study uptake of leukemic cells in vitro. Nevertheless,
engulfment is an active and highly regulated process, it comprises several separate but linked events including adhesion, internalization and digestion of the phagocytosed substances.\textsuperscript{34} An array of apoptotic cell-associated ligands, intermediates and phagocytic receptors has been proposed to be involved in the removal of apoptotic cells.\textsuperscript{7} Ongoing research is warranted to study the effect of these materials on phagocytosis by ECs.

Our current data indicated that phagocytosis by MΦ and ECs decreased PCA of PS-exposed APL cells with time. We previously reported that the major PCA of APL cells is PS-dependent, and the active tissue factor on APL blasts is most likely localized in the membrane areas where PS and tissue factor overlap.\textsuperscript{8} Prior studies showed that excessive PCA of APL is associated with formation of factor Xa through a cysteine protease, named cancer procoagulant\textsuperscript{35,36} as well as by expression of tissue factor.\textsuperscript{1,37} PCA of cancer procoagulant is not dependent upon exposed PS and appears to account for about 20\% of factor Xa generation by NB4 cells as well as by leukemic blasts from patients with APL.\textsuperscript{8} The degree of inhibition of both intrinsic and extrinsic factor Xa complexes by PS-blockade in this study is consistent with the expected residual contribution from cancer procoagulant. Thus, we speculate that internalization of APL blasts by phagocytes decreases PCA due to both tissue factor and cancer procoagulant. As a result, the progression of pathologic coagulation process may be prevented.

Annexin II, the tissue plasminogen activator (t-PA) and plasminogen binding receptor, is overexpressed on leukemic promyelocytes.\textsuperscript{25} Annexin II plays an important role in plasmin generation resulting in increased fibrinolysis and offers a link to the hemorrhagic diathesis in APL.\textsuperscript{25} In agreement with a recent study,\textsuperscript{38} we found that DNR lowered the amount of annexin II on NB4 or APL cells. Consequently, this drug also reduced plasmin generation of APL cells. We speculated that due to reduced markers of fibrinolytic activation, phagocytosis by phagocytes for 2 hours statistically decreased FLA of APL targets.

Lactadherin exists in normal plasma\textsuperscript{39} and on the outer surface of human MΦ\textsuperscript{13} and ECs.\textsuperscript{14,40} This opsonin serves as a bridge between PS on apoptotic cells and integrins on phagocytes.\textsuperscript{16} Lactadherin-deficient mice show defective clearance of apoptotic cells.\textsuperscript{41} Addition of recombinant lactadherin can correct this defect.\textsuperscript{42} In this study, the inhibition of phagocytosis in the presence of annexin V, a protein that has a PS-binding domain but no integrin binding domain, indicated a major role for PS in this process. Furthermore, we found that in a time-dependent manner, lactadherin promoted the phagocytosis of APL cells by both MΦ and ECs. While, α\textsubscript{v}-integrins are present on phagocytes,\textsuperscript{6} neither α\textsubscript{v}β\textsubscript{3} nor α\textsubscript{v}β\textsubscript{5} integrin can bind PS.\textsuperscript{13} Lactadherin resolves this dilemma by linking PS on APL cells to α\textsubscript{v}-integrins on
phagocytes to mediate uptake. In addition, lactadherin C2 domain has sequence homology to the C2 domains of factors V and VIII.\textsuperscript{43} This allows lactadherin, through binding to PS-containing membranes, to act as an anticoagulant.\textsuperscript{20,44} Our results indicated that compared with the anticoagulant effect of lactadherin or phagocytosis alone, lactadherin and phagocytes might cooperatively block exposed PS in combination with other procoagulants and thus together inhibit more PCA of target APL cells. Compared to phagocytosis without lactadherin, lactadherin-opsonized engulfment might block higher amounts of cell-surface annexin II and consequently decrease more FLA of APL targets. Therefore, lactadherin and phagocytes cooperation can be utilized for modulating coagulation in APL.

In conclusion, we demonstrate that phagocytosis of APL cells by MΦ and ECs \textit{in vitro} results in prevention of APL coagulation disorder. Lactadherin-enhanced engulfment of APL cells may be used to decrease the amount of leukemic cells. The capacity of lactadherin and phagocytes to cooperatively decrease clot-promoting activity may be an attractive strategy for treatment of PS-related coagulopathy. Furthermore, lactadherin-mediated engulfment may be useful to improve the hyperfibrinolytic states in APL.

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Authorship

Contribution: R.X. designed the research, performed experiments, analyzed results, made the figures and wrote the paper; J.S. obtained funding, designed the study, performed experiments, analyzed results, made the figures, and revised the manuscript; J. Zhou provided partial funding support; C.G., W.L., V.N., J.W., R.M. performed some experiments; G.G. analyzed data and revised the manuscript; J. Zhu made the figures
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Correspondence: Jialan Shi, Department of Hematology, The First Affiliated Hospital, Harbin Medical University, Harbin 150001, China; or VA Boston Healthcare System, Brigham and Women’s Hospital, Harvard Medical School, West Roxbury, MA 02132, USA; e-mail: jialan_shi@hms.harvard.edu.

References


### Table 1. APL patients’ characteristics

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<th>WBC (×10^9/L)</th>
<th>Hb (g/L)</th>
<th>Plts (×10^9/L)</th>
<th>Blasts (BM %)</th>
<th>PT (sec)</th>
<th>APTT (sec)</th>
<th>TT (sec)</th>
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| Ref. range | 4-10 | 110-170 | 100-300 | 0-0.4 | 10-15 | 20-40 | 11-20 | 2-4 | 0-0.3 |

The main clinical and laboratory features of 16 newly diagnosed APL patients at the moment of bone marrow aspiration were reported. WBC indicates white blood cells; Hb, hemoglobin; Plts, platelets; Blasts, promyelocytes + blasts; BM, bone marrow; bcr, breakpoint cluster region (bcr1=intron 6, bcr2=exon 6, bcr3=intron3); and hemorrhage was manifested as mucosal bleeding, spontaneous ecchymoses, petechiae, hematemesis, hematuria, melena, or menorrhagia.
Figure Legends

**Figure 1. PS exposure of NB4 and APL cells.** Cells were co-stained with PI and either FITC-annexin V or FITC-lactadherin. (A) The cells were incubated with indicated concentrations of DNR for 24 hours. Percent of annexin V/lactadherin-binding cells was analyzed by flow cytometry. After treatment with 1 μM DNR, about 75% of NB4 cells and 80% of APL cells were positive for lactadherin. (B) The plasma membrane of APL cells displayed green fluorescence when stained by FITC-annexin V (left) or FITC-lactadherin (right). Cell nuclei displayed red fluorescence when labeled by PI. Co-stained areas appeared yellow. Bars represent 10 μm.

**Figure 2. Association of target NB4 and APL cells with MΦ.** NB4 and APL cells treated with 1 μM DNR for 24 hours were used as PS-exposed target cells for all subsequent coculture assays. MΦ were differentiated from monocytes or THP-1 cells. Target cells incubated with MΦ at a 2:1 ratio at 37 °C for different time points were analyzed. (A) Confocal microscopy image of CMTPX-stained THP-1-derived MΦ (red) with bound CMFDA-labeled target NB4 cells (green, arrows) after 30 minutes of incubation. A scattered target NB4 cell (green, arrowhead). (B) Scanning (left) and transmission (right) electron microscopic examination of anchored (square) and adhered (stars) target APL cells to the surface of THP-1-derived MΦ (triangles) after 30 minutes. (C) After 1-hour incubation, scanning electron microscopy of grasped (left, star) and internalized (right, square) target NB4 cells by THP-1-derived MΦ (triangles). (D) Scanning electron microscopy of a THP-1-derived MΦ (triangle) extending pseudopodia over a target APL cell (star) after 1-hour incubation. (E) After 2 hours of incubation, transmission microscopy of NB4 material (left, square) and a morphologically apoptotic target NB4 cell with karyopyknosis (right, star) engulfed by THP-1-derived MΦ (triangles). (F) Transmission micrograph showing phagocytosed target APL material (square) in a monocyte-derived MΦ (triangle) after 2 hours of incubation. (G) Transmission microscopy image of a THP-1-derived MΦ (triangle) with digested APL apoptotic bodies (arrows) after 3 hours of incubation. Bars represent 10 μm (A) or 4 μm (B-G).

**Figure 3. Contact of target NB4 and APL cells with HUVECs.** Two-fold target NB4 or APL cells were added to HUVECs at 37 °C for various time points before the mixed cells were imaged. (A) Confocal image of free (arrowhead) and bound (arrows) CMFDA-labeled target NB4 cells (green) to the membrane of a CMTPX-stained EC (red) after 30 minutes of incubation. (B) After 1-hour coculture, attached target APL
cell (left, star, scanning electron microscopy) and target APL cell with apoptotic bodies (right, square, transmission electron microscopy) to the surface of ECs (triangles). (C) A target NB4 cell with apoptotic bodies (left, square, transmission electron microscopy) and a target APL cell (right, star, scanning electron microscopy) trapped by ECs (triangles) after 1.5 hours of incubation. (D) Transmission microscopy image of several separated apoptotic bodies of APL cells (square) in an EC (triangle) and another target APL cell with apoptotic bodies (star) bound to this EC after 2-hour incubation. (E-F) Transmission micrographs showed target APL cells (stars) undergoing degradation in ECs (triangles) after 3 hours. Bars represent 10 μm (A) or 4 μm (B-F).

Figure 4. Effect of lactadherin on phagocytosis of target APL cells. A total of 1×10⁶ CMFDA-labeled target APL cells were preincubated with 2 nM lactadherin or annexin V for 10 minutes at room temperature, followed by removal of free proteins-containing supernatant. These cells were added to 5×10⁵ CMTPX-stained THP-1-derived MΦ or HUVECs which were seeded in 12-well culture plates before analyses by flow cytometry. Phagocytosis was quantified by measuring the percentage of CMFDA (green)-positive red fluorescence (CMTPX) phagocytes. (A) Phagocytic index was calculated in the absence or presence of lactadherin at indicated time points before 2 hours. Lactadherin enhanced the extent of phagocytosis in a time-dependent manner. * and # indicate $P < .05$ from phagocytosis by MΦ and ECs without lactadherin, respectively. (B) Phagocytic percent of 2 hour-incubation was assayed after pretreatment of target APL cells with lactadherin and annexin V seperately. Lactadherin enhanced phagocytosis, whereas annexin V decreased engulfment. * $P < .05$.

Figure 5. Change in PCA at various time points after incubation of target APL cells with phagocytes. A total of 1×10⁶ target APL cells were incubated with 5×10⁵ THP-1-derived MΦ and HUVECs separately in 12-well culture plates for indicated time points. Target APL cells were used as control. In a time-dependent manner, clotting time of the mixed cells increased (A), while generation of intrinsic FXa (B), extrinsic FXa (C), or thrombin (D) decreased. * $P < .05$ compared to 0 hour time point of each group.

Figure 6. Effect of lactadherin on PCA of coincubated target APL cells and phagocytes. Target APL cells were preincubated with 2 nM lactadherin or annexin V for 10 minutes at room temperature. Clotting time (A), intrinsic FXa (B), extrinsic FXa (C), and thrombin (D) of 1×10⁶ target APL cells, or 1×10⁶ target APL cells opsonized by annexin V or lactadherin, or incubation of 1×10⁶ target cells with phagocytes
(THP-1-derived MΦ or HUVECs) for 2 hours, or incubation of 1×10⁶ annexin V-opsonized or lactadherin-opsonized target cells with phagocytes for 2 hours were determined. Lactadherin and phagocytes cooperatively increased coagulation time and reduced enzyme complexes of target APL cells. * P < .05 compared with single target APL cells. + P < .05; # P < .01; and ** P < .001 compared to the mixture of APL targets and phagocytes (MΦ and ECs separately).

**Figure 7. Effect of phagocytosis on plasmin formation and annexin II expression.** (A) Plasmin generation of 1×10⁶ target APL cells, 5×10⁵ phagocytes (THP-1-derived MΦ or HUVECs), or incubation of 1×10⁶ target APL cells with 5×10⁵ phagocytes was evaluated at the given time points. Plasmin production of the coincubated cells was time-dependently reduced. * P < .05 compared to 0 hour time point of each group. (B) Plasmin formation of 1×10⁶ target APL cells, with or without 2 nM annexin V or lactadherin, and with or without incubation with 5×10⁵ phagocytes (THP-1-derived MΦ or HUVECs) after 2 hours was measured. Plasmin formation of 1×10⁶ viable APL cells is also shown. * P < .05 compared with the mixture of target APL cells and phagocytes (MΦ and ECs separately). (C) NB4 cells were first labeled with goat anti human annexin II IgG, and then with an Alexa Fluor 488-conjugated secondary antibody. Annexin II expression on permeabilized untreated NB4 cells (left) and 1 μM DNR treated NB4 cells (right) was viewed using confocal microscopy. The cell nuclei were counterlabeled with PI (red) and bars represent 10 μm. (D) Non-permeabilized or permeabilized APL cells with and without 1 μM DNR treatment were stained as in (C) and analyzed by flow cytometry. Annexin II expression of DNR treated cells decreased compared with untreated viable APL cells. (E) Flow cytometry was used to quantitate annexin II expression on cells that were treated as in (C). Cells stained with goat anti human IgG and Alexa Fluor 488-conjugated secondary antibody were used as control (black). The percentage of annexin II-positive viable APL cells (green) and target APL cells (pink) from one patient with permeabilization were 98.3% and 35.1%, respectively (left panel). Middle panel showed that compared with controls, annexin II was expressed on the surface of non-permeabilized THP-1-derived MΦ (pink) and more so on permeabilized cells (green). Permeabilized HUVECs showed an increase in annexin II (green), but compared with controls, non-permeabilized HUVECs (pink) showed no increase in annexin II (right panel).
Figure 1

A

[Bar chart showing the percentage of cells in NB4 and APL treated with Annexin V and Lactadherin at different concentrations of DNR (μM).]

B

[Images showing Annexin V + PI and Lactadherin + PI staining of cells.]

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Figure 7

A

Plasmin (nM)

Target APL +
MΦ -
ECs -

0 h
1 h
2 h

B

Viable APL+
Target APL
Phagocytes
Annexin V
Lactadherin

MΦ
ECs

C

D

Non-permeabilized
Permeabilized

Annexin II (%)

E

Black: APL+IgG
Green: Viable APL
Pink: Target APL

Black: MΦ+IgG
Green: Permeabilized
Pink: Non-permeabilized

Black: ECs+IgG
Green: Permeabilized
Pink: Non-permeabilized

Cells/channel

Annexin II

Annexin II

Annexin II
Phagocytosis by macrophages and endothelial cells inhibits procoagulant and fibrinolytic activity of acute promyelocytic leukemia cells

Rui Xie, Chunyan Gao, Wen Li, Jiuxin Zhu, Valerie Novakovic, Jing Wang, Ruishuang Ma, Jin Zhou, Gary E. Gilbert and Jialan Shi