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

Rui Xie,1 Chunyan Gao,1 Wen Li,1 Juxin Zhu,1 Valerie Novakovic,2 Jing Wang,1 Ruishuang Ma,1 Jin Zhou,1 Gary E. Gilbert,2 and Jialan Shi1,2

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 2Veterans Affairs Boston Healthcare System, Brigham and Women’s Hospital, and Harvard Medical School, Boston, MA

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 found that viable and apoptotic APL cells expose PS.8,9 It is conceivable that because of uncontrolled APL blast proliferation and chemotherapy,7 professional macrophages (MΦs) 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 MΦs 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 because of 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 MΦs and ECs.13,14 This protein contains a domain structure of EGF1-EGF2-C1-C2.15 It anchors PS-externalized cells 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 phosphatidylserine-exposed target APL cells was time-dependently decreased after incubation with phagocytes (THP-1–derived macrophages or HUVECs). Thrombin production on target APL cells was reduced by 40%-45% after 2 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 2 hours of phagocytosis and ~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. (Blood. 2012;119(10): 2325-2334)

Methods

Patients

Sixteen patients with newly diagnosed APL 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 the 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, and therapy.
cytogenetics, and molecular biology.8 Cytogenetic analysis indicated the t(15;17) translocation and PML/RARα fusion gene in all cases. The main characteristics of the patients at the moment of BM aspiration were reported on Table 1.

**Reagents**

EC medium was purchased from ScienCell. RPMI 1640 medium, FBS, and 0.25% Trypsin-EDTA were from Gibco. Ficol-Hypaque, daunorubicin (DNR), phorbol 12-myristate 13-acetate, poly-d-lysine, BSA, EDTA, and 0.25% Trypsin-EDTA were from Gibco. Ficoll-Hypaque, daunorubicin. Lactadherin was purified from bovine milk.12,13 Lactadherin and annexin V were obtained from BD PharMingen. Alexa Fluor 488 donkey anti–goat IgG were from Invitrogen. Pro–human annexin II IgG and goat anti–human IgG were from AbD Serotec.

**Cell culture**

Freshly isolated APL blasts were obtained from BM specimens by centrifugation through Ficol-Hypaque. These cells (5 × 10^6/mL) were propagated in complete RPMI 1640 medium supplemented with 20% FBS, 2mM L-glutamine, and 1% penicillin-streptomycin solution at 37°C in a 5% CO2 humidified atmosphere.

Human APL cell line NB4 cells, a gift from Dr James O’Kelly (Los Angeles, CA), and monocytic cell line THP-1 cells from the American Type Culture Collection were maintained under the same conditions aforementioned except that 10% FBS was used. HUVECs from ScienCell used up to passage 42 were cultured in EC medium containing 5% FBS, 1% EC growth supplement, and antibiotics.

**Preparation of MΦs**

As previously described,22 PBMCs from healthy volunteers were isolated by Ficoll-Hypaque density gradient centrifugation. Positive isolation of primary monocytes from mononuclear cells was performed with a MACS separation kit with human CD14+ microbeads (Miltenyi Biotec). Cells were cultured in 10% FBS containing complete RPMI 1640 medium and allowed to differentiate into MΦs for 5 days.

Alternatively, THP-1–derived MΦs were obtained by stimulating THP-1 monocytes with 20 ng/mL phorbol 12-myristate 13-acetate for 72 hours.22

**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 (0.1μM, 0.5μM, 1μM) at 37°C for 24 hours.9 Those cells treated with 1μM DNR were used as target cells. After extensive washing to remove residual DNR,23 a total of 1 × 10^6 target NB4 or APL cells were incubated with 5 × 10^5 MΦs or HUVECs in 12-well culture plates for different times.

Lactadherin-opsonized or annexin V–opsonized targets were obtained as follows: target APL cells were preincubated with 2nM 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 coincubated with MΦ or HUVEC monolayers.

**Flow cytometry**

To quantify PS exposure,24 NB4 or APL cells suspended in Tyrode buffer (for lactadherin-binding test, 137mM NaCl, 2.7mM KCl, 11.9mM NaHCO3, 0.42mM NaH2PO4, 1mM MgCl2, 2mM CaCl2, 5.5mM glucose, 5mM HEPES, and 0.35% BSA, pH 7.4) or annexin V binding buffer (for annexin V–binding assay, 10mM HEPES/NaOH, 140mM NaCl, 2.5mM CaCl2, pH 7.4) were adjusted to 1 × 10^6 cells/mL. FITC–annexin V or FITC–lactadherin at a final concentration of 2nM was incubated with the cells for 10 minutes in the dark. After adding 1 μg/mL PI, cells were analyzed on a flow cytometer (FACSaria; Becton Dickinson). Data acquisition was performed with FCS express V3 (De Novo Software).

Annexin II was analyzed as described before with modifications.25

Leukemic promyelocytes or THP-1 macrophages or HUVECs were stained and analyzed on a FACSAria instrument (Becton Dickinson). Analysis was performed with FCS express V3 (De Novo Software).

**Table 1. Characteristics of patients with APL**

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Reference 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 patients with newly diagnosed APL at the moment of BM aspiration were reported. Hemorrhage was manifested as mucosal bleeding, spontaneous ecchymoses, petechiae, hematemesis, hematuria, melena, or menorrhagia.

WBC indicates white blood cells; Hb, hemoglobin; Pt, platelet; Blasts, promyelocytes + blasts; PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time; Fbg, fibrinogen; and bcr, breakpoint cluster region (bcr1 = intron 6, bcr2 = exon 6, bcr3 = intron 3).
extent of annexin II binding was measured by FASCalibur flow cytometry. CellQuest Pro Version 4 software (BD Biosciences) 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\(^{26}\) with some modifications. Target APL cells stained with 2µM CMFDA (excitation 492/ emission 516, green) were incubated with MΦs or ECs labeled with 1µM CMTPX (excitation 586/emission 613, red) as described in “Coincubation assay.” Mixed cells (target cells and phagocytes) were harvested with a cell scraper (MΦs) 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.\(^{24}\) 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 used to restrict emission wavelength overlap. Images were captured with Zeiss LSM 510 Meta confocal microscope.

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 Ab as described in “Flow cytometry.” Then, cells were counterstained with PI, and the annexin II expression was observed with the confocal microscope.

Engulfment was detected by a modified method as previously described.\(^{21}\) Briefly, 1µM CMTPX-stained MΦs or ECs was seeded on glass coverslips coated with poly-t-lysine in 12-well culture plates, and they were subsequently cocultured with 2µM 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 microscopy assays, target APL cells were cocultured with MΦs or ECs on coverslips. Samples were fixed in 2.5% glutaraldehyde–phosphate fixative and stored at 4°C until processed. After several rinses in 0.1M 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, ~10 nm thick, was sprayed on the samples. All images were viewed with a S-3400N Scanning Electron Microscope (Hitachi Ltd) with an ultra-high-resolution mode.

In transmission electron microscopy 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 Ultratcut Ultramicrotome (Leica). Images were observed with a H7650 transmission electron microscope (Hitachi Ltd).

Coagulation time

Target APL cells with or without opsonization by lactadherin or annexin V were cocultured with MΦs or ECs in 12-well culture plates as described in the “Coincubation assay.” The mixed cells were harvested and resuspended in 100 µL of Tyrode buffer. Moreover, 1 × 10\(^6\) target APL cells with or without opsonization were suspended in 100 µL Tyrode buffer. PCA was determined by a modified prothrombin time test.\(^{22}\) Briefly, 100 µL of the cell suspension was mixed 1:1 with citrated platelet-poor plasma (3.8% sodium citrate, 1:9, vol/vol) from healthy volunteers. After incubation for 180 seconds at 37°C, 100 µL of preheated 25mM CaCl\(_2\) was added. The time to fibrin strand formation was immediately recorded by an Amelung KCA4 coagulometer (Labcon).

FXa and prothrombinase assays

The activation of intrinsic FXa in the presence of cells was performed as follows.\(^{27}\) Cells were incubated with 1nM factor IXa, 5nM factor VIII, 0.2nM thrombin, 130nM factor X, and 5mM CaCl\(_2\) in FXa buffer (200 µL of 10% BSA, 1 mL od 10× TBS, and 8.8 mL of ddH\(_2\)O). The reaction was stopped by EDTA at a final concentration of 7nM. FXa generation was determined immediately at 405 nm on a SpectraMax M5 Microplate Reader (Molecular Devices) in kinetic mode after incubation with 10 µL of S-2765 (0.8mM). Measurement of extrinsic FXa formation was analogous to that for intrinsic FXa except that cells were mixed with 1nM factor VIIa, 130mM factor X, and 5mM CaCl\(_2\). For the prothrombinase assay, the samples were incubated with 1nM factor Va, 0.05nM FXa, 1µM prothrombin, and 5mM CaCl\(_2\) in prothrombinase buffer (50µL of 10% BSA, 1 mL of 10× TBS, and 8.95 mL of ddH\(_2\)O) for 5 minutes at room temperature. Thrombin production was evaluated immediately at 405 nm in the kinetic microplate reader with S-2238 (0.8mM) after the addition of EDTA.

Plasmin production tests

A previously described method with minor modification was used to measure plasmin formation.\(^{25-28}\) In brief, cells were preincubated with 10mM-1 PA for 10 minutes and washed twice with PBS. After adding 200nM glu-plasminogen for 10 minutes at room temperature in 96-well culture plates, 200µM plasmin substrate S2251 was added to each well. Generation of plasmin was assayed at 405 nm with 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 ± SD for ≥3 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 with the use of flow cytometry (Figure 1A). Untreated cells had a low percentage of protein binding. DNR dose dependently enhanced the lactadherin-binding or annexin V–binding percentage of NB4 cells. After treatment with 1μM DNR for 24 hours, ~75% of NB4 cells and 80% of APL cells were positive for lactadherin compared with 8% of NB4 and 11% of 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, whereas 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 used as PS-exposed target cells for all subsequent experiments.

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

Two sources of MΦs were used as in vitro models of phagocytic clearance. Monocytes isolated from human blood were allowed to differentiate into MΦs. Alternatively, THP-1–derived MΦs, which have similar properties to monocyte-derived MΦs, were obtained by stimulation with phorbol 12-myristate 13-acetate. The target NB4 or APL cells showed structural features of apoptosis, including loss of most microvilli, karyopyknosis, and chromatin aggregation (Figure 2). As expected, both MΦs swiftly bound and engulfed these targets. After 30 minutes of coincubation, most target cells were adhered to MΦs (Figure 2A-B). Within 1 hour, MΦs spread pseudopodia to grasp targets, which were internalized (Figure 2C-D). After 2 hours of coincubation, uptake of target NB4 and APL cells by MΦs 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 MΦs (data not shown).

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

Because ECs have been reported to behave as amateur phagocytes for dying cells, we used 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 EC 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, nearly all phagocytosed intracellular materials had disappeared, suggesting that degradation was virtually complete (Figure 3F). These results showed 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. To explore the contribution of lactadherin to the uptake of APL cells, the amount of phagocytosis was further measured by flow cytometry. On the basis of the microscopic observations as described in Figure 2, 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 times, in a time-dependent manner (Figure 4A). In the absence of lactadherin, the level of uptake was ~ 10% by either THP-1–derived MΦs or...
supernatant fluid. These cells were added to 5 nM annexin V for 10 minutes at room temperature, followed by removal of free protein—binding protein, annexin V, markedly inhibited the uptake. However, lactadherin opsonization, with lactadherin and annexin V separately. Lactadherin enhanced phagocytosis, whereas annexin V decreased engulfment; with statistical significance. Thrombin formation of target APL cells was inhibited by lactadherin opsonization, ~40%-45% by phagocytes, and ~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 relation between engulfment and fibrinolysis. A plasminogen activation test was used to measure the generation of cell-surface plasmin. Treatment with 1μM DNR for 24 hours decreased plasmin generation of APL cells from 15nM to 8nM (Figure 7B). Other chemotherapy drugs cytosine arabinoside and etoposide also reduced plasmin formation of promyelocytes (data not shown). In contrast, THP-1–derived MΦs 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 ~30% after a 2-hour incubation with THP-1–derived MΦs or HUVECs. We further examined the effect of lactadherin-mediated phagocytosis on plasmin generation of APL targets (Figure 7B). At 2 hours, lactadherin and phagocyte cooperation reduced ~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 reported 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. We assessed the annexin II expression of promyelocytes and phagocytes (THP-1 macrophages and HUVECs). Consistent with the prior observation that used a fluorescein-tagged Ab, green fluorescence showed that the permeabilized t(15;17)–positive APL cell line NB4 cells without drug 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, ~40%-50% of the thrombin production was inhibited by coincubation with either THP-1–derived MΦs 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 because of phagocytosis by MΦs 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 (MΦs 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.

Engulfment decreased PCA of target APL cells

The implication that MΦs 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 MΦs and HUVECs separately for various times. 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 HUVECs at 2 hours (Figure 4B). However, lactadherin opsonization enhanced the phagocytic percentage to ~23% by THP-1–derived MΦs 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 blasts also presented a recognition signal to lactadherin.

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 relation between engulfment and fibrinolysis. A plasminogen activation test was used to measure the generation of cell-surface plasmin. Treatment with 1μM DNR for 24 hours decreased plasmin generation of APL cells from 15nM to 8nM (Figure 7B). Other chemotherapy drugs cytosine arabinoside and etoposide also reduced plasmin formation of promyelocytes (data not shown). In contrast, THP-1–derived MΦs 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 ~30% after a 2-hour incubation with THP-1–derived MΦs or HUVECs. We further examined the effect of lactadherin-mediated phagocytosis on plasmin generation of APL targets (Figure 7B). At 2 hours, lactadherin and phagocyte cooperation reduced ~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 reported 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. We assessed the annexin II expression of promyelocytes and phagocytes (THP-1 macrophages and HUVECs). Consistent with the prior observation that used a fluorescein-tagged Ab, green fluorescence showed that the permeabilized t(15;17)–positive APL cell line NB4 cells without drug 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, ~40%-50% of the thrombin production was inhibited by coincubation with either THP-1–derived MΦs 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 because of phagocytosis by MΦs 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 (MΦs 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, ~40%-45% by phagocytes, and ~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.

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treatment reacted with the Ab 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 $\frac{1}{H}9262^M$ DNR for 24 hours, most PI-counterstained nuclei (red) became condensed or fragmented, and DNR down-regulated annexin II of permeabilized NB4 cells (Figure 7C right) and permeabilized blast cells from patients with APL (Figure 7D). For nonpermeabilized 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 patient with APL was 98.3%, but treatment with $\frac{1}{H}9262^M$ 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 Ms (Figure 7E middle). Although the amount of annexin II expression on HUVEC surface was low, cytoplasmic annexin II was accessible (Figure 7E right).

**Discussion**

Our results showed that both cultured Ms 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.

Ms throughout the body clear apoptotic cells rapidly and efficiently. PS works as a recognition cue for phagocytosis. Previous studies have also shown that activated Ms destroy tumor cells. We found that Ms were able to recognize, engulf, and ultimately dispose of PS-exposed NB4 and APL cells. That 2 human Ms subsets from different origin (primary and cell line-derived Ms) both ingested APL cells indicates that it is a general property of Ms, 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 Ms activation for therapy of disseminated metastases progress successfully in some carcinomas. Therefore, the phagocytic capacity of Ms toward APL cells presents an attractive possibility for APL therapy.

In APL, because of uncontrolled leukemia proliferation and chemotherapy, Ms 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 nonprofessional 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. Because 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. An array of apoptotic cell-associated ligands, intermediates, and phagocytic receptors has been proposed to be involved in the removal of apoptotic cells. Ongoing research is warranted to study the effect of these materials on phagocytosis by ECs.
Our current data indicated that phagocytosis by MΦs 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 probably localized in the membrane areas where PS and tissue factor overlap. Priory studies showed that excessive PCA of APL is associated with formation of FXa through a cysteine protease, named cancer procoagulant. As well as by expression of tissue factor.

In conclusion, we demonstrate that phagocytosis of APL cells by both MΦs and ECs in vitro results in the 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 block exposed PS in combination with other procoagulants and thus together inhibit more PCA of target APL cells. Compared with phagocytosis without lactadherin, lactadherin-opsonized engulfment might block higher amounts of cell-surface annexin II and consequently decrease more FLA of APL targets.
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Authorship

Contribution: R.X. designed the research, performed experiments, analyzed results, made the figures, and wrote the paper; C.G., W.L., V.N., J.W., and R.M. performed some experiments; J. Zhou provided partial funding support; J. Zhu made the figures and...
analyzed data; G.E.G. analyzed data and revised the manuscript; and J.S. obtained funding, designed the study, performed experiments, analyzed results, made the figures, and revised the manuscript.

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References


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

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