Platelets and platelet-like particles mediate intercellular RNA transfer

Antonina Ristano, Lea M. Beaulieu, Olga Vitseva, and Jane E. Freedman

Platelets are anucleate cells generated from megakaryocytes and released in the bloodstream where they play an active role in hemostasis, inflammation, and immune responses. They are also involved in tumor progression and metastasis, with complex molecular mechanisms that continue to be elucidated. Recent studies have focused on analyzing platelet RNA content including microRNA (miRNA) and messenger RNA (mRNA). Although lacking nuclei, platelets retain megakaryocyte-derived cytoplasmic RNA and may translate small amounts of mRNAs as well as process miRNAs. Platelets express high levels of miRNAs, and profiling studies have shown expression changes. Platelet transcript variability also has been described in a large community-based cohort study with inflammatory RNA significantly correlated with specific cardiovascular risk factors, notably increased body mass index. The platelet transcriptome mirrors the protein expression profile, but only 69% of proteins have been found at the mRNA level leaving open the question about the functionality of the remaining RNA.

Introduction

The role of platelets in hemostasis and thrombosis is clearly established; however, the mechanisms by which platelets mediate inflammatory and immune pathways are less well understood. Platelets interact and modulate the function of blood and vascular cells by releasing bioactive molecules. Although the platelet is anucleate, it contains transcripts that may mirror disease. Platelet mRNA is only associated with low-level protein translation; however, platelets have a unique membrane structure allowing for the passage of small molecules, leading to the possibility that its cytoplasmic RNA may be passed to nucleated cells. To examine this question, platelet-like particles with labeled RNA were cocultured with vascular cells. Coculture of platelet-like particles with activated THP-1, monocytic, and endothelial cells led to visual and functional RNA transfer. Posttransfer microarray gene expression analysis of THP-1 cells showed an increase in HBG1/HBG2 and HBA1/HBA2 expression that was directly related to the transfer. Infusion of wild-type platelets into a TLR2-deficient mouse model established in vivo confirmation of select platelet RNA transfer to leukocytes. By specifically transferring green fluorescent protein, we also observed external RNA was functional in the recipient cells. The observation that platelets possess the capacity to transfer cytosolic RNA suggests a new function for platelets in the regulation of vascular homeostasis.

Methods

Cell cultures

MEG-01 cells and THP-1 cells (ATCC) were grown in suspension in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen). HUVECs were obtained from Lonza Walkersville, cultured in endothelial growth medium (Lonza Walkersville) and used between second and fifth passages.

PLP isolation

PLPs were isolated from MEG-01 cultures treated with 100 ng/mL recombinant human thrombopoietin (TPO; R&D Systems) for 48 hours. Preparations were centrifuged at 150g for 15 minutes, and sediments were discarded and centrifuged again at 750g for 15 minutes. Supernatants thus obtained were centrifuged at 1600g for 15 minutes, and sediments containing PLPs were washed and resuspended in culture medium.

Taken together, these observations suggest that platelet transcript content may play a biologic role beyond being remnant RNA derived from the megakaryocyte. To test the hypothesis that platelet-derived RNA is transferred, we have developed an in vitro transfer model using MEG-01 cells, a human megakaryoblastic cell line known to release platelet-like particles (PLPs) with characteristics similar to human platelets. These PLPs were then examined for potential transfer of nucleic acids to recipient cells. In this study, we show not only that PLPs are able to transfer RNA to THP-1 cells, a monocytic cell line, and human umbilical vein endothelial cells (HUVECs), but also that the transferred RNA is functional.
inhibited with 10 U/mL SUPERase-In RNase (Invitrogen) inhibitor and 0.2M potassium phosphate buffer, respectively. PLPs were washed in culture medium after this treatment.

**PLP-vascular cell coincubation**

THP-1 cells treated with 1 μg/mL Pam3CSK4 (PAM; InvivoGen) were coincubated for 24 hours with PLPs isolated from MEG-01 cells cultures at a ratio 1:10. PLPs were stained with 2μM PKH67 (Sigma-Aldrich) according to the manufacturer’s directions or with CD41a, CD42b FITC-conjugated antibodies (eBioscience). PKH67 is a fluorescent green dye attached to long aliphatic tails that is incorporated into the cell membranes. HUVECs were treated with 0.5 μM thrombin (Sigma-Aldrich) for 10 minutes in endothelial basal medium (Lonza Walkersville) without phenol red, washed, and coincubated with PKH67-labeled PLPs for 30 minutes in endothelial basal medium containing 2mM CaCl2 (Sigma-Aldrich) at the same ratio. After coincubation, both cell types were washed in phosphate-buffered saline (PBS; Invitrogen) and analyzed by flow cytometry and confocal microscopy.

**BrUTP transfection and transfer**

MEG-01 cells were transfected with 0.1mM 5-bromouridine-5’-triphosphate (BrUTP; Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s directions. Cells were treated with 100 ng/mL TPO for 24 hours, and then PLPs were collected and cocultured with THP-1 cells, as described in “PLP-vascular cell coincubation,” with time points 0, 3, 6, 12, and 24 hours. HUVECs were coincubated with PLPs for 1 hour in the same conditions described in “PLP-vascular cell coincubation.” HUVECs and THP-1 cells were analyzed for BrUTP positivity by flow cytometry using a BrdU Flow kit (BD Biosciences) according to the manufacturer’s protocol.

**GFP transfection and transfer**

MEG-01 cells were transfected with a plasmid encoding green fluorescent protein (pmaxGFP; Lonza Walkersville) using a Nucleofector II device according to the manufacturer’s suggestions. Cells were allowed to recover after nucleofection and grow for 48 hours with 100 ng/mL TPO treatment. PLP collection and coincubations were performed as described in the preceding paragraphs. THP-1 cells were analyzed after 24 hours and then washed to eliminate residual PLPs and cultured for additional 24 hours. HUVECs were coincubated with PLPs for 1 hour and then washed and allowed to grow for 24 hours. GFP fluorescence was recorded by flow cytometry.

**Animal models and in vivo platelet RNA transfer**

C57BL/6J (WT) and B6.129-Tlr2tm1Kir/J (TLR2−/−) mice were purchased from The Jackson Laboratory and housed in the animal facility at the University of Massachusetts Medical School. All procedures were approved by the Institute Animal Care and Use Committee at University of Massachusetts Medical School.

Platelets were isolated from wild-type (WT) mice by centrifugation approximately 5.5 times, and the supernatant was further diluted with 3 times the volume with platelet wash buffer and centrifuged at 3500g for 10 minutes. The resulting pellet contains peripheral blood mononuclear cells (PBMCs). The supernatant was further diluted with 3 times the volume with platelet wash buffer and centrifuged at 3500g for 10 minutes. The resulting pellet contains platelets. Approximately 5.5 × 10^7 platelets resuspended in citrate-phosphate-dextrose solution were injected into TLR2−/− mice via tail-vein injection (total volume, 165 μL). After 1 hour, TLR2−/− mice were injected with 5 μg/g lipopolysaccharide (LPS; InvivoGen) or saline intraperitoneally. Blood samples were collected into citrate-phosphate-dextrose solution (1:5) at 3 and 6 hours after LPS injection. Whole blood samples were tested for the percentage of heterotypic aggregates (platelet-monocyte aggregates) and TLR2-positive monocytes by flow cytometry. Isolated platelets and PBMCs were also processed for RNA isolation and real-time RT-PCR as described in “Real-time RT-PCR.”

**Flow cytometry**

THP-1 cells were stained with anti-human CD11b–PE-Cy7 and CD11b–APC, and HUVECs were stained with CD54–PE-Cy5. Anti-human CD41a–FITC and CD42b–FITC (eBioscience) antibodies were used for PLPs, and anti-human BrdU–FITC (BD Bioscience) was used to detect BrUTP-labeled RNA. Mouse platelets and monocytes were stained with anti-mouse CD41-PE and CD11b-APC (eBioscience), respectively, whereas CD282(TLR2)–FITC (eBioscience) was used for TLR2 detection. All the stainings were performed along with the corresponding isotype controls. The experiments were run on a FACScan analyzer (BD Biosciences) using CellQuest Pro Version 5.2 software (BD Biosciences).

**Confocal microscopy**

Samples from the experiments described thus far were analyzed by confocal microscopy. Imaging was performed on LSM 510 (Carl Zeiss) and TCS SP2 (Leica Microsystems) confocal microscopes. Images were captured using MetaMorph Version 6.1 software (Molecular Devices) and Leica Confocal Version 2.5 software.

**Microarray gene expression analysis**

Total RNA was isolated from Pam3CSK4-treated THP-1 cells cultured in presence and absence of PLPs using the miRNeasy Mini kit (QIAGEN) according to the manufacturer’s directions. Microarray gene expression profile was determined using a Human Genome U133 2.0 Plus chip, human transcriptome complete (Affymetrix). The Gene Expression Omnibus accession number for the data is GSE38027.

**Real-time RT-PCR**

Total RNA was isolated as described in “Microarray gene expression analysis.” RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Invitrogen). Gene expression of human GAPDH and HBO1/HBG2, mouse GAPDH and TLR2 (primers and probes from Invitrogen), using the TaqMan Gene Expression Master Mix (Invitrogen), was assessed with real-time PCR (Applied Biosystems 7900 HT Fast Real-Time PCR System with SDS Version 2.2.2 software; Invitrogen).

**SOD2 activity and ROS measurement**

THP-1 cells were cocultured for 24 hours with PLPs as described in “PLP-vascular cell coincubation” and then tested for superoxide dismutase 2 (SOD2) activity using EpifQuik Superoxide Dismutase kit (Epigentek) according to the manufacturer’s protocol. Reactive oxygen species (ROS) generation was measured using 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA; Invitrogen) as described previously.26 In brief, cells were washed in PBS, treated with 5μM 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate for 30 minutes at 37°C, and washed twice with PBS. The resulting fluorescence was analyzed by flow cytometry.

**Data analysis**

All values are expressed as the average ± SD of at least 3 different experiments. Statistical analysis was performed using Student t test and significance of P less than or equal to .05. Prism Version 5 software (GraphPad) was used for all analyses.

**Results**

PLPs coincubation with HUVECs and THP-1 cells

In culture, MEG-01 cells have the ability to spontaneously produce PLPs with size, structures, and characteristics very similar to...
human platelets. In coincubation experiments, THP-1 cells were stimulated with Pam3CSK4, a synthetic lipoprotein and TLR2 ligand, to induce interaction with PLPs. As a previous study showed, the concentration used for all experiments (1 μg/mL) does not induce any platelet aggregation. PAM-treated THP-1 cells, after 24 hour coincubation with PLPs, were stained with CD41a and CD42b, specific markers for human platelets (Figure 1A). Flow cytometry analysis showed that ~20% of THP-1 cells were positive for the double staining, confirming the PLP binding. This result was further supported by confocal microscopy (Figure 1B–C). Untreated THP-1 cells did not significantly bind PLPs (data not shown). As shown in Figure 1B (PLP), there is colocalization

![Image](image-url)
between a THP-1 cell and a PLP followed by the PLP internalization, an observation confirmed using a complementary staining method (Figure 1C). Changes in THP-1 CD11b staining also were measured during coincubation experiments; as seen in Figure 1D, the staining of the adhesion molecule CD11b, in presence of PLPs, was decreased, suggesting its involvement in the binding process. Similar results were obtained with HUVECs over a shorter period of coincubation. HUVECs were activated with 0.5 U/mL thrombin to promote the adhesion to PLPs, in fact, 19% of activated HUVECs were able to bind and internalized PLPs, although less than ~4% of resting cells were fluorescently positive (Figure 1E). Binding also was seen with HUVECs (Figure 1Fii), as was the progressive increase of cytosol staining from PLP internalization (Figure 1Fiii-iv).

RNA transfer from MEG-01 to HUVECs and THP-1 cells

PLPs from transfected BrUTP MEG-01 cells were coincubated with THP-1 cells. After 6 hours of coincubation, labeled THP-1 cells demonstrated RNA transfer that continued gradually up to 24 hours (Figure 2A). The percentage of BrUTP-positive THP-1 cells after 24 hours was 4% to 5%. Previous studies reported MEG-01 as a difficult cell line to transfect; consequently, the amount of labeled-RNA was limited.32 Confocal microscopy showed that labeled RNA was localized outside and inside the
recipient cells, indicating an ongoing transfer after 24-hour coinoculation (Figure 2Biii-vi). RNA transfer also was observed using HUVECs as recipient cells; ~20% of activated HUVECs were positive for labeled RNA after 1-hour coculture (Figure 2C).

**GFP transfer from MEG-01 to HUVECs and THP-1 cells**

To test mRNA functionality in the recipient cells, we transfected MEG-01 cells with a GFP vector by nucleofection. By flow cytometry, we observed that GFP was expressed in MEG-01 cells and almost 25% of PLPs collected from cultures expressed mature GFP, carried mature GFP, or both. During a coinoculation experiment, both mRNA and proteins can be transferred; therefore, we examined THP-1 cells after 24-hour fusion with PLPs treated or not with RNase. To verify that PLP RNA was effectively degraded by the RNase treatment, we compared the RNA content of RNase-treated PLPs with untreated PLPs; RNase-treated PLPs contained ~48% less RNA. Interestingly, more than 6% of THP-1 cells without RNase treatment were found positive for GFP, a number reduced to 3% in presence of RNase. This suggests that THP-1 cells received GFP both as a mature protein and mRNA that was then translated. When THP-1 cells cocultured with untreated PLPs were washed after 24 hours, to eliminate PLPs, and cultured for additional 24 hours, fluorescence from GFP was still detectable as a result of 2 ongoing processes: GFP-mRNA expression and transferred GFP mature protein degradation (Figure 2D). Six percent of HUVECs were fluorescent 1 hour after coinoculation due to the GFP protein transfer. After 24 hours in absence of PLPs, 16.5% of cells expressed the GFP messenger, a figure assumed to include the percent- 

**THP-1 cell microarray gene expression analysis**

Although transfer of RNA is important, it is of greater interest if found to be functional. To investigate the possible effect of the newly transferred RNAs in the recipient cells, we carried out a gene expression analysis of THP-1 cells in presence and absence of PLPs. Microarray analysis showed that the most up-regulated genes were γ globins (HGB1/HGB2), up to 2-fold, followed by α globins (HBA1/HBA2) and ε globin (HBE1). Two mitochondrial genes (SOD2, MRPS15) and genes involved in transport and exocytosis pathways (STXBP5L, Rab7a, GLPD1), 2 types of matrix metallopeptidase (Table 1). The most up- and down-regulated genes in the control cells (PAM-activated THP-1), noted in Table 2, also were not altered by the presence of the imported RNA. Using the γ globins (the most expressed genes), the microarray results were validated by real-time PCR verifying whether the up-regulation was due to RNA transfer. PLPs were treated with 2 different types of RNases before a coincubation and RT-PCR confirmed that the increased RNA was from outside the cells (Figure 3). The small percentage of residual RNA after RNase treatment depends on the length of treatment: a complete degradation can be achieved with longer treatments but this would have affected PLP vitality and integrity. As a negative control TPO/Pam3CSK4-treated THP-1 cells were examined for HGB1/HGB2 expression to exclude any TPO carryover effect from the previous culture. CD11b did not seem to be down-regulated, confirming that the adhesion molecule was directly involved in the PLP binding, and for this reason not accessible for antibody staining (Figure 1D).

**Platelet TLR2 RNA transfer in vivo**

Mouse studies also were performed to test whether a specific mRNA could be transferred by platelets to monocytes in vivo. Platelets were isolated from WT mice (TLR2 RNA present) and

**Table 1. Coincubation: THP-1 + PLPs gene expression analysis**

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>Down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGB1/HGB2</td>
<td>HBE1</td>
</tr>
<tr>
<td>HBA1/HBA2</td>
<td>MMP13</td>
</tr>
<tr>
<td>HBP1</td>
<td>MP2</td>
</tr>
<tr>
<td>HBE1</td>
<td>CALB1</td>
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<tr>
<td>HPGD</td>
<td>NPYA</td>
</tr>
<tr>
<td>DNAJC12</td>
<td>STXBP5L</td>
</tr>
<tr>
<td>TRAJ17/TRAV20</td>
<td>SOD2</td>
</tr>
<tr>
<td>MRPS15</td>
<td>XDH</td>
</tr>
<tr>
<td></td>
<td>TUNFRS11A</td>
</tr>
<tr>
<td></td>
<td>GPA</td>
</tr>
</tbody>
</table>

**Table 2. Pam3CSK4-treated THP-1 gene expression analysis**

<table>
<thead>
<tr>
<th>Up-regulated genes</th>
<th>Down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM1</td>
<td>EPB41L3</td>
</tr>
<tr>
<td>VCAM1</td>
<td>THBS1</td>
</tr>
<tr>
<td>CCL2</td>
<td>GFPBP3</td>
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<tr>
<td>CCL14</td>
<td>NEXN</td>
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<tr>
<td>BCL2A1</td>
<td>RTN1</td>
</tr>
<tr>
<td>PLA2G7</td>
<td>IFI44L</td>
</tr>
<tr>
<td>LUM</td>
<td>S100A12</td>
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<tr>
<td></td>
<td>WDR49</td>
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<td>SERPNI2</td>
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* Fold change of 2.
† Fold change of 1.5.
infused into TLR2-deficient animals. Complementary to the coincubation experiments, we induced the formation of heterotypic aggregates using LPS, a bacteria LPS and TLR4 agonist. Flow cytometry results from platelet transfusion experiments performed on TLR2 knockout mice showed the presence of platelet-monocyte heterotypic aggregates 3 hours after transfusion with an increase in TLR2-positive monocytes (83.60% ± 2.24%) compared with saline treatment (33.6% ± 0.33%). LPS treatment produced a modest increase in platelet-positive monocytes (12.76% ± 1.46) compared with the relative saline treatment (9.56% ± 3.46). Because coculture experiments showed RNA transfer 6 hours after coincubation, we collected platelets and PBMCs 6 hours after platelet transfusion and analyzed RNA to verify whether TLR2 mRNA was transferred to the recipient cells. A 6-fold increase in TLR2 mRNA clearly demonstrated that during the stage of heterotypic aggregates formation platelet-monocyte RNA transfer had occurred (Figure 4). This was particularly important because it established that the process occurs in vivo and with platelets as well as PLPs.

Relationship between SOD2 activity and ROS production in THP-1 cells

Microarray results showed a modest increase in SOD2 expression that was confirmed by assaying directly the activity in the cells (Figure 5A); there was a 13% increase in SOD2 activity in presence of PLPs compared with the control (PAM). ROS production also was increased by the presence of PLPs. PAM-activated THP-1 cells produced ~ 30% more ROS compared with resting cells; however, in presences of PLPs, there was a further 12% increase that was consistent with the amount of SOD2 activity recorded (Figure 5B).

Discussion

Intercellular nucleic acid transfer is known to be an important mechanism regulating metastasis and stem cell communication networks. Platelets are modestly active in translating their own mRNA, but few studies have examined the function of the platelet transcriptome. In this study, we explored the possibility that platelets influence peripheral blood and vascular cells by transferring RNA to these cells. RNA horizontal transfer was investigated by in vitro and in vivo models, shedding new light on the relationship between platelets and other vascular cells.

This study demonstrates that there is successful transfer of RNA from MEG-01 cells to HUVECs and THP-1 cells using PLPs as mediators of the transfer (Figure 6). Vascular cells bound and internalized PLPs with mechanisms similar to those already described for platelets and microvesicles. For example, MEG-01 cells stimulated with TPO produced PLPs carrying mainly fetal hemoglobin transcripts that were then transferred to the recipient cells having almost no baseline expression of these genes. As demonstrated in different cell lines, human TPO induces predominantly fetal and adult hemoglobin synthesis and, for the purpose of this study, were an excellent marker to validate an RNA transfer. Interestingly, these data also suggest that RNA was not randomly transferred because MEG-01 cells may have distributed transcripts in the newly formed PLPs. This observation is supported by a recent study describing how megakaryocytes selectively transfer matrix metalloproteases and their tissue inhibitors into platelets. PLP RNase treatments abrogated up to 75% of HGB1/HGB2 transfer after 24-hour coincubation, confirming that the mRNA...
recovered in THP-1 cells was from an outside source. The hemoglobin transfer was detectable after 12-hour coincubation (result not shown) in accordance with the slow process observed in THP-1 cells with the bromine-labeled RNA transfer. Many of the other gene expression changes were related to exo- and endocytosis.

In addition, the functionality of the PLP transcripts in the recipient cells was demonstrated by the GFP transfer; both the mature protein and mRNA presence were highlighted. PLPs actively interacted and transferred their RNA and GFP messenger to activated HUVECs as well; the process was faster and more efficient compared with THP-1 cells. After the fusion in THP-1 cells, we also observed an increase in ROS content that may be the result of the modest \textit{SOD2} overexpression, metabolic activity increase, or both.

Results obtained from in vitro experiments were clearly supported by the first mouse model showing that platelets can transfer \textit{TLR2} mRNA to PBMCs after an inflammatory stimulus. Considering the significant level of transcript detected 6 hours after transfection in leukocytes, we conclude that RNA transfer in vivo is relatively fast and more efficient compared with the in vitro model. However, there are limitations to this experiment as transfer of only 1 transcript was studied.

Taken together, our observations suggest that platelets may have an interesting additional role in intercellular communication by acting as a source of transcripts. The transfer mechanism seems to require the direct contact between the cells, although the specific mechanism is not yet known. Cocultured cells have been shown to transfer short interfering RNAs with a mechanism that involves gap junctions, mRNAs instead were mainly identified in microparticles released from cells, indicating that size and structure prevent larger nucleic acids from being transferred through membrane channels. Our findings are unique in showing PLP internalization and GFP mRNA transfer, suggesting that platelets have a more complex regulatory role compared with microparticles.

Human platelets cannot be produced in vitro or cultured but PLPs, except for the lack of thrombotic activity, are very similar to human platelets. The RNA transfer model developed for this study suggests an intriguing in vivo scenario. The study shows that RNA was transferred and expressed in the recipient cells, and these results add a new role for the platelet transcriptome. Thus, platelet expression profiles analyzed in cancer and cardiovascular diseases reveal transcript variations that may affect not only platelet function but also influence platelet-interacting cells and vascular homeostasis by direct transfer of genetic information.

**Acknowledgments**

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**Authorship**

Contribution: A.R. designed and performed experiments, analyzed data, and edited the manuscript; L.M.B. designed and performed animal studies, analyzed related data, and edited the manuscript; O.V. performed confocal microscopy shown in Figures 1B and 2B; and J.E.F. designed experiments and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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