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Original research article

How do megakaryocytic microparticles target and deliver cargo to alter the fate of hematopoietic stem cells?

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ABSTRACT

Megakaryocytic microparticles (MkMPs), the most abundant MPs in circulation, can induce the differentiation of hematopoietic stem and progenitor cells (HSPCs) into functional megakaryocytes. This MkMP capability could be explored for applications in transfusion medicine but also for delivery of nucleic acids and other molecules to HSPCs for targeted molecular therapy. Understanding how MkMPs target, deliver cargo and alter the fate of HSPCs is important for exploring such applications. We show that MkMPs, which are distinct from Mk exosomes (MkExos), target HSPCs with high specificity since they have no effect on other ontologically or physiologically related cells, namely mesenchymal stem cells, endothelial cells or granulocytes. The outcome is also specific: only cells of the megakaryocytic lineage are generated. Observation of intact fluorescently-tagged MkMPs inside HSPCs demonstrates endocytosis as one mechanism of cargo delivery. Fluorescent labeling and scanning electron microscopy (SEM) imaging show that direct fusion of MkMPs into HSPCs is also engaged in cargo delivery. SEM imaging detailed the membrane-fusion process in four stages leading to full adsorption of MkMPs into HSPCs. Furthermore, macropinocytosis and lipid raft-mediated were shown here as mechanisms of MkMP uptake by HSPC. In contrast, the ontologically related platelet-derived MPs (PMPs) cannot be taken up by HSPCs although they bind to and induce HSPC aggregation. We show that platelet-like thrombin activation is apparently responsible for the different biological effects of MkMPs versus PMPs on HSPCs. We show that HSPC uropods are the preferential site for MkMP binding, and that CD54 (ICAM-1), CD11b, CD18 and CD43, localized on HSPC uropods, are involved in MkMP binding to HSPCs. Finally, we show that MkMP RNA is largely responsible for HSPC programming into Mk differentiation.

1. Introduction

Cell-derived microparticles (MPs) are small membrane-enclosed vesicles (0.1 to 1.0 μm in diameter) derived from direct budding off the plasma membrane of mammalian cells and mediate inter-cellular communication in various physiological and pathophysiological processes, including coagulation, inflammation, tumorigenesis, and differentiation [1–3]. MPs are different from the smaller (<0.1 μm) exosomes that derive from the multivesicular endosome compartment of cells and have distinct physical and biological properties [4,5]. Platelet-derived MPs (PMPs), discovered over 40 years ago, participate in many physiological processes, including hemostasis, maintenance of vasculature, immunity and inflammatory responses [6]. The most abundant (70%–90%) circulating MPs express CD41 or CD61 [7]. For a long time, they were thought to originate from platelets and thus possess the biological functions of PMPs. However, it was shown that most circulating $CD41^+$ MPs in healthy subjects derive from megakaryocytes (Mks), and these Mk-derived MPs (MkMPs) are different from PMPs [7,8]. A role for MkMPs has not been known until our recent study, which documented a novel biological function of MkMPs, namely that they can induce differentiation of hematopoietic stem and progenitor cells (HSPCs) towards the Mk lineage without exogenous thrombopoietin (TPO) stimulation [9].

Several studies have examined mechanisms by which MPs interact with target cells. The interaction typically starts with a ligand-receptor mediated binding of MPs to target cells, and, in some cases, this initial interaction is sufficient to alter the fate of target cells [10–16]. Yet, in most cases, MPs exert their biological effect through transfer of signaling molecules (proteins, mRNAs, miRNAs or phospholipids), which requires uptake of MPs by the target cells [4,17,18]. It has been reported that two mechanisms used by target cells to integrate MPs are cell endocytosis and membrane fusion [4,17,18], and that cells use one of these two mechanisms to uptake MPs [19,20]. For example, PMPs were internalized by human brain endothelial cells through active endocytosis as demonstrated by colocalization of MPs with endosomes and lysosomes [19]. Direct fusion of MPs with cells was claimed in other studies [20–

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23], whereby, MPs were first stained with a lipid-membrane or cytosol dye and then cocultured with target cells [20–23]. The evidence to support MP-cell fusion was the observed transfer of dye or MP-specific membrane proteins to target cells as detected by confocal or electron microscopy [20–23]. Yet, it is possible that endocytosis of MPs may contribute partially, if not fully, to the observed transfer of dyes or proteins. Thus, membrane fusion remains to be firmly established as a mechanism of MP uptake by cells.

Since MkMPs can be effectively frozen without loss of their biological activity (data not shown), it is possible that they can be used in transfusion medicine to enhance thrombopoiesis or for cargo delivery to HSPCs. Also, it is possible to engineer MkMPs to carry exogenous molecules for delivery to HSPCs, and/or identify key surface molecules that mediate the recognition of HSPCs for engineering synthetic or semisynthetic macro- or nano-particles for targeted drug delivery to HSPCs. Detailed understanding of how MkMPs recognize, target and deliver cargo to various HSPCs to alter their fate would be necessary prior to exploring such applications, and this is the main goal of this report. Here we first show that MkMP target the most primitive hematopoietic stem cells (HSCs) with higher effectiveness than more differentiated HSPCs. We show that both endocytosis and membrane fusion are responsible for delivery of MkMP cargo to HSPCs, and that MkMP RNA is partially, at least, responsible for changing the fate of HSPCs. Our data support the hypothesis that the platelet activation process necessary for PMP biogenesis explains the different biological effects of MkMP vs PMPs, and that MkMPs attach to and enter HSPCs preferentially through their uropods, with CD54, CD11b, CD18 and CD43 being involved in target-cell recognition.

2. Materials and methods

2.1. Materials and antibodies

All chemicals and protein reagents were obtained from Sigma-Aldrich or otherwise indicated. Recombinant human interleukin 3 (rhIL-3), rhIL-6, rhIL-9, rhIL-11, stem cell factor (rhSCF), thrombopoietin (rhTPO), Granulocyte colony-stimulating factor (rhG-CSF) were purchased from PeproTech Inc. Size standard fluorescent beads (0.22, 0.45, 0.88 and 1.34 μm) and AccuCount fluorescent particles (~5.0 μm) were from SpheroTech. Fluorescein isothiocyanate (FITC)—or phycoerythrin (PE)-conjugated *anti*-CD41 (GPαIIb), PE-conjugated anti-CD62P (P-selectin), allophycocyanin (APC)-conjugated *anti*-CD34, PE-conjugated *anti*-CD11b, APC-conjugated anti-CD235a, FITC-conjugated CD63, APC-conjugated CD81 and purified *anti*-CD41, anti-CD42b, anti-CD43, anti-CD50 antibodies as well as corresponding IgG were all from BD Bioscience. APC-conjugated *anti*-CD133 antibody was obtained from Miltenyi Biotec. Purified anti-CD54 (ICAM-1) antibody was from Abcam. Anti-filamin A was from Santa Cruz Biotechnology.

2.2. Megakaryocytic (Mk) culture and kinetics of Mk microparticle (MkMP) formation

Frozen G-SCF mobilized human peripheral blood $CD34^+$ cells were obtained from the Fred Hutchinson Cancer Research Center. $CD34^+$ cells were cultured to Mks as described [24]. At d7, $CD61^+$ cells (Mks) were enriched using *anti*-CD61 magnetic microbeads (Miltenyi Biotec) and were cultured as described [24]. From d8 to d12, CD41 and CD62P expression and concentration of MPs in the cell culture were measured by a flow cytometer (FACSAria II, BD Biosciences) using AccuCount fluorescent particles (0.88 μm and 1.34 μm) as internal control.

2.3. Isolation of MkMPs and MkExos

MkMPs were isolated from d12 Mk culture as described [9]. Briefly, cells were removed from d12 culture medium at 150 × *g* centrifugation for 10 min. Next, platelet-like particles (PLPs) and cell debris were removed by centrifugation at 1000 × *g* for 10 min [9]. Following that, MkMPs were enriched from the supernatant by ultracentrifugation at 25,000 rpm (38,000 × *g*) for 1 h at 4 °C using an Optima Max Ultracentrifuge and a TLA-55 rotor (Beckman Coulter). To isolate MkExos, we used a standard protocol [25], whereby the supernatant from the previous step was filtered through 0.22 μm filters (CellTreat), and untracentrifuged at 100,000 × *g* for 90 min at 4 °C using an Optima L-90 K Ultracentrifuge and a SW41 Ti Rotor (Beckman Coulter). MkMPs or MkExos were resuspended in IMDM or PBS and stored at −80 °C for further use.

2.4. Characterization of MkMPs and MkExos with dynamic light scattering (DLS)

For these experiments, MkMPs or MkExos isolated as above were suspended in PBS that had been filtered with 0.22 μm filters twice before being used. This filtered PBS contains no particles as confirmed by DLS measurement. DLS measurement of suspensions of MkMPs or MkExos were performed at 25 °C using a Zetasizer Nano ZS (Malvern Instruments) with 633 nm He—Ne laser, fixed angle of 173°, and automatic attenuator. Size distribution by number was collected with at least 3 measurements for each sample.

2.5. Characterization of MkMPs and MkExos with flow cytometry

To detect MkExos with flow cytometry, isolated MkExos were first captured with Dynabeads magnetic beads (4.5 μm, Thermo Fisher) coated with *anti*-CD63 antibody at 4 °C overnight, following the manufacturing protocol in Exosome - Human CD63 Isolation/Detection kit (Invitrogen). MkMPs or bead-bound MkExos were then incubated with isotype IgG, FITC *anti*-CD63, or APC anti-CD81 antibodies for 15 min at room temperature before flow cytometry analysis. The latter two antibodies target the common surface antigens of endosomal origin expressed by human exosomes [26], and which are not expressed on MkMPs.

2.6. Preparation of human platelets and PMPs

Blood for isolation of human platelets was collected by venipuncture from adult human volunteers after providing written informed consent as approved by the Institutional Review Board at the University of Delaware (IRB protocol # 622751-1). Blood was collected from healthy donors and PMPs were prepared as described [9]. Briefly, 50 mL of blood was collected into syringe with ACD buffer (trisodium citrate, 65 mM; citric acid, 70 mM; dextrose, 100 mM; pH 4.4) at a volume ratio of 1:6 (ACD:blood). Following that, blood was centrifuged at 250 × *g* for 10 min and the platelet rich plasma was isolated from the supernatant. Platelets were then pelleted at 750 × *g* for 10 min, followed by 1 wash with HEN buffer (10 mM HEPES, pH 6.5, 1 mM EDTA, 150 mM NaCl) containing 0.05 U/mL apyrase. After that, platelets were resuspended in HEPES-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1 g/L BSA, 1 mM $MgCl_2$, 2.7 mM KCl, 3.3 mM NaH_2PO_4). To generate PMPs, platelets were activated by 2 U/mL human thrombin (Sigma), and removed by centrifugation at 1000 × *g* for 10 min. Lastly, PMPs were isolation by ultracentrifugation at 25,000 rpm for 1 h at 4 °C, resuspended in IMDM medium, and stored at −80 °C.

2.7. Human umbilical vascular endothelial cells (HUVECs), mesenchymal stem cells (MSCs) and granulocytic cultures

Primary HUVECs were obtained from ATCC and cultured according to ATCC recommendation (growth medium: vascular cell basal medium (ATCC) supplemented with endothelial cell growth kit-VEGF (ATCC)). Passages 3–5 of HUVECs were used for following coculture experiment. Human MSCs (passages 2–4) were courtesy from Prof. Xinqiao Jia at the University of Delaware and cultured according to Lonza