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New paradigms in thrombosis: novel mediators and biomarkers platelet RNA transfer

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Abstract

Platelets, anucleated cells with a central role in hemostasis and inflammation, contain messenger RNAs and microRNAs of unknown functionality and clinical relevance. Historically, platelet RNA was viewed as merely a remnant of platelet biogenesis; however, several studies now refute this assumption. Studies have shown that platelets can actively translate RNA to protein and that specific RNA profiles correlate with select human clinical phenotypes. These studies support a more fluid role for platelet RNA in platelet function and disease development. Our lab and others have recently studied the platelet's unique ability to transfer RNA to recipient cells and the effect this transfer has on the recipient cells' functions. This transfer may represent a previously unknown form of vascular cell communication and modulation. Unlike the well-characterized thrombotic properties of platelets, the nature and purpose of platelet RNA transfer has not been determined, partly due to limitations in techniques used to manipulate platelet RNA profiles. Defining the mechanism of RNA transfer and its role in the vascular system will allow for the better understanding of how platelets function in both their traditional thrombotic role and non-traditional functions, potentially having widespread implications in several fields.

Keywords

Platelet; Thrombosis; Transcriptomics; Inflammation

Introduction

Platelets, small anucleated cells of the vascular system, play key roles in hemostasis and inflammation. In their traditional role, circulating platelets respond to sites of vascular injury through receptor recognition of exposed subendothelium [1]. Upon recognition, activation occurs through receptor binding and release of platelet granules [1]. Granule secretion results in increased thrombotic response and helps regulate clot formation [1]. Granule secretion and the platelet's role in thrombosis have long been evaluated; however, recent studies have focused on the non-granular/non-protein content of platelets and how this content may affect platelet function. Despite their lack of nuclei, platelets contain all the

components necessary to perform translation in a signal-dependent fashion [2]. Though these studies found that platelet RNA translation resulted in sporadic protein production, they initiated interest in platelet RNA content [2]. This has led to the characterization of platelet RNA and the identification of specific messenger RNAs (mRNAs) and microRNAs (miRNAs). Though initial serial analysis of gene expression and microarray hybridization studies only identified approximately 1,500 specific RNA transcripts in healthy donor platelets [3, 4], the development of deep sequencing techniques revealed an extended profile of approximately 9,500 transcripts [5, 6]. Subsequent analyses, using both microarray analysis and RNA sequencing, have focused on non-healthy individuals and correlated RNA profiles to specific human diseases [7–12]. This includes correlating inflammatory transcript levels with body mass index [7] and upregulated type 1 interferon system transcripts with systemic lupus erythematosus [10]. Several additional studies have identified distinct RNA profiles that correlate with thrombocytosis [8, 9, 11].

Although several studies had identified a large number of platelet transcripts and platelets had been shown to translate a select number of targets, the primary function of these transcripts remained unclear. Several observations suggested a larger role for platelet RNA. Since platelets are anucleate, their RNA pool is relatively fixed, and in different clinical settings in specific populations, there are specific platelet RNA transcripts differentially expressed, suggesting a connection with phenotype or disease. Thus, the identification of rich RNA profiles specific to human diseases supports a role for platelet RNA in how platelets function and effect disease development.

Platelets transfer RNA to vascular cells

Platelets have a distinct cannicular membrane system that allows passage of small molecules out of the cell [13]. Platelets also release microvesicles and exosomes, both structures previously implicated in cell–cell communication [14–18]. The presence of these methods of cell–cell communication in platelets led to the thought that platelet RNA may be involved in platelet–cell communication (Fig. 1). This hypothesis has been investigated by several different labs, resulting in 3 separate publications on the topic (Table 1). Initially, in order to investigate the possibility of platelet RNA transfer, we created an in vitro modeling system using cultured cell lines to mimic the vascular environment [13]. By treating MEG-01 cells, a human megakaryocyte cell line, with thrombopoietin (TPO), a megakaryocyte maturation hormone that induces thrombopoiesis in vivo [19], we created platelet-like particles (PLPs). PLPs are structures similar to platelets but with some phenotypic differences [20]. These PLPs allowed us to observe how platelets interact with vascular cells in vitro and to monitor platelet RNA during these interactions. In this study, PLPs containing fluorescent RNA were created by nucleofecting MEG-01 cells before TPO stimulation [13]. Upon incubation of these fluorescently labeled PLPs with either human umbilical vein endothelial cells (HUVEC), or a monocyte cell line, THP-1, we observed RNA transfer to both types of target cells, using both flow cytometry and fluorescence microscopy for analysis [13]. Additionally, using microarray analysis, cells treated with non-labeled PLPs had specific transcript expression increases, the most prevalent of which were globins [13]. Upon infusion of wild type platelets into *TLR2*^{−/−} mice and aggregation induced by lipopolysaccharide, the *TLR2* transcript was observed in the monocytes of *TLR2*^{−/−} host

animals, confirming the in vitro phenomenon in vivo [13]. Taken together, these studies support the transfer of platelet RNA from platelets to vascular cells, specifically endothelial cells and monocytes.

Platelets transfer miRNA to vascular cells

In addition to mRNAs, platelets contain a wide array of miRNAs [21]. MiRNAs, 19 to 24-nucleotide non-coding RNAs, regulate mRNA translation through sequence-specific recognition of non-coding regions in the 3' untranslated region (UTR) of target mRNAs [22]. miRNAs can regulate several targets at once and thus can have a profound effect on cellular environments with minimal material. The transfer of platelet miRNA to recipient cells presents a unique opportunity for platelets to have a large and broad effect on other cells of the vascular system. Gidlöf et al. looked at the role miRNAs play in platelets during myocardial infarction [23]. During myocardial infarction, blood flow is disturbed by thrombosis and oxygen supply to cardiac tissue is blocked, resulting in damage or death to the myocardium. In this setting, there is activation of platelets at the site of injury, thus, Gidlöf et al. hypothesized that miRNA content of platelets and the release and subsequent uptake of these miRNA played a role in the evolution of myocardial injury [23]. Initial studies focused on identifying if ST elevation myocardial infarction (STEMI) patients possessed a unique miRNA profile as compared to healthy subjects [23]. In this study, STEMI patients had 9 miRNAs with significantly different expression levels as compared to controls [23]. In addition, 8 miRNAs were significantly downregulated in patients while miR-320a was upregulated [23]. Since the majority of differentially expressed miRNAs decreased in platelets in STEMI patients, Gidlöf hypothesized that this was due to platelet release of miRNAs upon aggregation [23]. They choose 4 candidate miRNAs from the 8 described above (based on the relevance of their possible targets to cardiovascular disease) and showed that post thrombin stimulation of platelets, these miRNAs significantly increased in the supernatant [23]. This insight was confirmed through further analysis of healthy donor platelets, STEMI patient peripheral blood platelet and STEMI patient platelets from the site of occlusion [23]. Platelets from the actual thrombus showed decreased levels of these 4 miRNAs as compared to both healthy individuals and STEMI peripheral platelets [23]. They further investigated whether these miRNAs released from platelets during aggregation transferred to endothelial cells [23]. By transfecting platelets with a synthetic exogenous miRNA, as well as a synthetic fluorescently labeled miRNA, they showed transfer from platelets to HMEC-1 cells in a time and activation dependent manner [23].

miRNA transfer via microparticles

Several reports have shown microparticle dependent miRNA transfer between cells [14, 16–18, 24]. Platelets have also been shown to release microparticles into the bloodstream upon activation [15]. Based on these reports, both Gidlöf and Laffont investigated the role microparticles potentially play in platelet RNA transfer. Preliminary studies by Gidlöf focused on monitoring fluorescent synthetic miRNA transfer to platelet microparticles as well as blocking microparticle production using Brefeldin A [23]. Gidlöf demonstrated significant enrichment of the fluorescent miRNA in platelet derived microparticles post thrombin activation [23]. This enrichment was abated with pretreatment with Brefeldin A to

block microparticle formation [23]. He additionally showed that blocking platelet microparticle production significantly decreased transfer of a fluorescently labeled miRNA from platelets to HMEC-1 cells upon cocubation [23].

Laffont et al. further investigated the role of platelet miRNA in transfer and the role microparticles may play [25]. Laffont was able to show that miR-223, a miRNA highly expressed in platelets, was shed post platelet thrombin activation [25]. Thrombin induced activation results in over a 60 fold increase in miR-223 expression in platelet derived microvesicles [25]. The shed miR-223 is complexed with Ago2 protein in microvesicles and is functional in RISC activity assays [25]. Laffont also showed platelet-derived microparticle uptake into HUVEC cells, with the microparticle persisting for 48 h within cells before slowly diffusing [25].

Transferred platelet RNA is functional in recipient cells

The relevance of platelet RNA transfer strongly depends on the functionality of the transferred RNA in the target cells. Gidlöf, Risitano and Laffont all demonstrated the functional relevance of platelet RNA transfer. Risitano et al. demonstrated RNA functionality through transfer of a GFP labeled vector from platelets to recipient HUVEC and THP-1 cells [13]. Gidlöf demonstrated transfer of functional miRNAs from platelets to endothelial cells [23]. Gidlöf introduced luciferase reporter constructs designed with the 3'-UTRs of the predicted targets of their 4 miR-NAs of interest (miRNAs 22, 185, 320b and 423-5p, mRNA targets ICAM-1, eNOS, VEGFA and VEGFB, respectively) into target HEK293 cells in culture [23]. After incubation with thrombin-activated platelets, the ICAM1 reporter signal was quenched compared to a scrambled pre-miRNA introduced supporting the transfer of functional miR-22 and miR-320b [23]. Additionally, HMEC-1 cells incubated with platelet releasate after thrombin activation showed ICAM1 downregulation by 30 %, confirming the construct results [23]. Upon knockdown of endogenous miR-320b, platelet releasate could rescue miR-320b induced ICAM1 degradation [23]. These experiments were confirmed with direct miRNA overexpression of miR-320b in HMEC-1 cells [23]. Similarly, Laffont et al. showed miRNA-Ago2 complexes present in platelets were functional post transfer from thrombin activated platelets to HUVEC cells [25]. Transfected reporter constructs in HUVEC cells showed 44 % degradation post platelet cocubation and thrombin activation [25]. Additionally, endogenous mRNA targets of miR-223 in HUVECs showed >50% degradation post platelet microparticle incubation [25]. All these studies support the hypothesis that platelet RNA may affect vascular cell function through cell-cell communication and RNA regulation.

Unanswered questions

The ability of platelets to transfer RNA to recipient cells presents a novel way for platelets to interact with the vascular environment (Fig. 1). Though some understanding of the mechanism of transfer has come to light, there are still many unanswered questions. The limited studies have focused solely on specific miRNAs as well as utilized highly manipulated systems to evaluate how and why platelets transfer RNA to other cells. It is likely that many transcripts are transferred in unison and this will alter the recipient cell in

unclear and unknown ways. Whether the platelet can transfer a group of RNAs or only participate in nonspecific/nonselective transfer remains unclear. In addition, the mechanism of transfer remains unclear and may be variable in different environments and between different cells. A thorough examination of how much RNA is transferred, to which cells and how this affects recipient cells still needs to be undertaken to fully understand the impact of platelet RNA transfer on the vascular system.

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Fig. 1.

Megakaryocytes release platelets with specific RNA/miRNA profiles that may be dependent upon the specific risk factors and disease state of the individual. This platelet RNA can be transferred to other vascular cells altering their phenotype and influencing vascular reactions

Table 1
Summary of studies demonstrating definitive platelet RNA transfer

Title	Journal information	Transfer shown	Cell effect	Authors
Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression	Blood, 2013 May 9; 121(19): 3908–3917	miRNA	miRNA released from platelets during myocardial infarction can regulate ICAM1 expression in endothelial cells	Gidlöf, O. et al. [23]
Platelets and platelet-like particles mediate intercellular RNA transfer	Blood, 2012 Jun 28; 119(26): 6288–6295.	mRNA	Platelet-like particles can transfer active mRNA to endothelial cells in vitro and in vivo	Risitano, A. et al. [13]
Activated platelets can deliver mRNA regulatory Ago2-microRNA complexes to endothelial cells via microparticles	Blood, 2013 May 7 [Epub ahead of print]	miRNA	miR-223 is shed from activated platelets into microparticles in Ago2-miR-223 complexes. These microparticles are taken up by endothelial cells and actively affect gene expression	Laffont, B. et al. [25]