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Platelet-Secreted MicroRNA-223 Promotes Endothelial Cell Apoptosis Induced by Advanced Glycation End Products via Targeting the Insulin-like Growth Factor 1 Receptor

Yi Pan,1 Hongwei Liang,1 Huan Liu, Donghai Li, Xi Chen, Limin Li, Chen-Yu Zhang, and Ke Zen

Platelets play a significant role in atherosclerosis, stroke, and asthma through active interaction with neutrophils, monocytes, and vascular endothelial cells. The mechanism underlying these intercellular interactions, however, is incompletely understood. In this study, we report that platelets can remotely modulate vascular endothelial cell apoptosis through releasing microRNA-223 (miR-223)–containing microvesicles (MVs). First, platelets expressed abundant miRNAs, and miR-223 had the highest level of expression. Platelet miR-223 and other miRNAs can be upregulated by the stimulation with thrombopoietin (TPO) or thrombin. Unlike leukocytes, platelets contained high levels of pre-miRNAs, and upregulation of mature platelet miRNAs by TPO was correlated with decreased pre-miRNAs. Second, under stimulation with TPO, platelets released a large amount of MVs, which also contain higher levels of miR-223. Elevation of miR-223 inside circulating platelet MVs (P-MVs) was also observed in plasma samples from patients with enteritis, hepatitis, nephritis, or atherosclerosis. Third, incubation of P-MVs with HUVECs, which had significantly lower levels of miR-223 than platelets, showed that P-MVs effectively delivered miR-223 into HUVECs. Finally, in HUVECs, exogenous platelet miR-223 decreased the level of insulin-like growth factor 1 receptor and thus promoted HUVEC apoptosis induced by advanced glycation end products. The proapoptotic effect of P-MVs on HUVECs was largely abolished by depletion of cellular miR-223 using anti-miR-223 antisense oligonucleotide. In conclusion, our study presents the first evidence, to our knowledge, that platelet-released miR-223 promotes advanced glycation end product–induced vascular endothelial cell apoptosis via targeting insulin-like growth factor 1 receptor. The Journal of Immunology, 2014, 192: 437–446.
physiological and pathological processes, the mechanism that governs the role of P-MVs in target cells, particularly vascular endothelial cells, remains to be further explored.

MicroRNAs (miRNAs) are a class of small noncoding RNAs that posttranscriptionally regulate gene expression in plants and animals (18). miRNA guides the binding of the RNA-induced silencing complex to complementary sequences in the 3′-untranslated region of the open reading frame of target mRNA molecules, resulting in either mRNA degradation or translational inhibition. Accumulating evidence has demonstrated that miRNAs play a key role in the cellular processes of differentiation, proliferation, maturation, and apoptosis. Our recent studies have demonstrated that miRNAs are stably expressed in animal serum/plasma and that their unique expression patterns can serve as fingerprints of various diseases (19). Mechanistic studies further suggest that cells can selectively secrete miRNAs via MVs in response to various stimuli and that the MV-encapsulated miRNAs are associated with Argonaute 2 (AGO2) complexes (20, 21). Cell-secreted miRNAs in MVs can be efficiently delivered into target cells, in which they silence their target genes and thus affect recipient cell function (20). Therefore, cell-secreted miRNAs in MVs can serve as a novel class of signaling molecules to remotely mediate intercellular communication.

Recent studies have shown that anucleate platelets also contain abundant miRNAs (22–25); however, the biogenesis pathway of miRNAs in anucleate platelets is unclear. In addition, platelets have been shown to express certain miRNA processing machinery including Dicer, RNA-binding protein 2, and AGO2 (22), implying that platelet miRNA may have biological functions and that platelets may be able to process pre-miRNA into mature miRNA.

Diehl et al. (26) reported that miRNAs, including miR-19, miR-21, miR-126, miR-133, miR-146, and miR-223, could be detected in P-MVs, suggesting that platelets can secrete miRNAs through P-MVs. Delivery of functional platelet miRNAs into endothelial cells via P-MVs has also been reported recently (27, 28). Although many miRNAs have been predicted to be targets of these platelet miRNAs, the function of platelet miRNAs, particularly the miRNAs stored in P-MVs, has yet to be shown. The work by Gidlöf and coworkers (28) provided the first evidence that activated platelets could release functional miRNAs, which enter into endothelial cells and regulate endothelial ICAM-1 expression. This finding suggests that delivery of functional platelet miRNAs into vascular endothelial cells by P-MVs can play a critical role in modulating vascular endothelial inflammatory responses.

In the current study, we examined the miRNA expression profile of anucleate platelets and their potential role in gene regulation. Our results demonstrated that platelets contained abundant miRNAs, particularly miR-223, and these miRNAs were upregulated by inflammatory factors such as thrombopoietin (TPO) and thrombin. Compared to platelets under resting conditions, platelets stimulated by inflammatory factors released significantly more MVs. Moreover, the concentration of miR-223 and other miRNAs in individual P-MVs from activated platelets was also increased. Finally, we showed that P-MVs could effectively deliver miR-223 into HUVECs, in which platelet miR-223 targeted endothelial insulin-like growth factor 1 receptor (IGF-1R) and promoted HUVEC apoptosis induced by advanced glycation end products (AGEs).

Materials and Methods

Reagents, cells, and Abs

HUVECs were purchased from the China Cell Culture Center (Shanghai, China) and cultured in standard RPMI 1640 medium supplemented with 10% FBS (Life Technologies) and 0.5% ECGF (ReliaTech) in a 5% CO2, water-saturated atmosphere. HUVECs were seeded on gelatin-coated (Difco, Detroit, MI) tissue-culture plates. Anti–IGF-1R and anti-GAPDH Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Synthetic oligonucleotides, including premiR-223, anti–miR-223, and scrambled negative control (pre-ncRNA and anti-ncRNA), were purchased from Ambion (Austin, TX). TPO was purchased from PeproTech (Rocky Hill, NJ). Thrombin was purchased from Novagen (Madison, WI).

Blood collection

Platelets from normal volunteer donors were isolated from venous blood collected on 42 mN trisodium citrate and 4.2 mM EDTA (1 volume anticoagulant for 6 volumes blood). All blood samples were collected from consenting individuals according to protocols approved by the ethics committee of Nanjing University. We included 8 enteritis patients, 9 nephritis patients, 8 hepatitis patients, 6 coronary artery bypass grafting patients, and 10 healthy donors in the study. The patients were treated at Jinling Hospital (Nanjing, China). Healthy donors were recruited from a large pool of individuals seeking a routine health checkup at the Healthy Physical Examination Centre of Jinling Hospital. Whole blood was centrifuged at 150 × g for 15 min at room temperature to obtain platelet-rich plasma. PMNs and monocytes were isolated by centrifuging the lower part on top of Ficoll-Hypaque (Pharmacia, Piscataway, NJ) cushions, allowing sedimentation of the Ficoll-Hypaque pellets in 3% dextran at room temperature, and lysing of residual RBCs by hypotonic saline (29).

Platelet isolation and stimulation

Platelet-rich plasma was diluted in washing buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 25 mM glucose, 4.2 mM EDTA, 4.2 mM trisodium citrate, and 1 mM proteoglandin E1 [pH 6.6]). The platelet suspension was centrifuged at 750 × g for 15 min at 20°C, and the pellet was resuspended in wash buffer without prostaglandin E1 for an additional centrifugation in the same conditions. Finally, platelets were recovered in suspension buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2 mM MgCl2, 25 mM glucose) at the concentration of 4 to 5 × 108 platelets/ml. Aliquots of the platelet suspensions were activated in the presence of 2.5 mM CaCl2, with 1 ng/ml TPO or 0.1 U/ml and 1 U/ml thrombin. The mixtures were incubated at 37°C for 4 h without stirring to avoid major platelet aggregation.

RNA isolation and quantitative RT-PCR of miRNAs and pre-miRNA

Total RNA was extracted using TRIzol Reagent (Invitrogen). Quantitative RT-PCR (qRT-PCR) was carried out using TaqMan miRNA probes (Applied Biosystems, Foster City, CA) or synthesized primer (Invitrogen). Briefly, 5 μl total RNA was reverse transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and a stem-loop RT primer (Applied Biosystems). Real-time PCR was performed using a TaqMan PCR kit on the 7300 Sequence Detection System (Applied Biosystems). All reactions, including no-template controls, were run in triplicate. After the reaction, the threshold cycle values were determined using fixed threshold settings. To calculate the absolute expression levels of target miRNAs, a series of synthetic miRNA oligonucleotides at known concentrations was also reverse transcribed and amplified. The absolute amount of each miRNA was then calculated by referring to the standard curve. In the experiments presented in this study, miRNA expression in HUVECs is normalized to U6 small nuclear RNA. Because platelets are anucleate, miRNA expression in platelet cells is normalized to the total RNA sampling amount. The expression levels of target miRNAs in platelet MVs were directly normalized to the total protein content of MVs.

Animals

Animal maintenance and experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for Use of Experimental Animals and approved by the Medicine Animal Care Committee of Nanjing University (Nanjing, China). Apolipoprotein E-null (ApoE−/−) mice on the C57BL/6 background were maintained from 4 wk of age on a high-fat diet composed of 21% (w/w) adjusted calories from fat and 0.15% (w/w) cholesterol ad libitum. After mice were sacrificed at the end of the experiment, blood samples were collected via cardiac puncture.

Fluorescence labeling of MVs for quantification and confocal microscopy

To analyze the MVs released from platelets under various conditions, platelets were labeled with Dil-C6, and then washed three times with PBS. The cells were resuspended and activated by TPO or thrombin for 4 h. One part of the supernatant was used to analyze the quantity of P-MVs by flow cytometry. The other part of the supernatant was collected and centrifuged to isolate MVs. P-MVs in RPMI 1640 medium were incubated with cultured HUVECs. After incubation for 12 h, HUVECs were washed, fixed, and
observed under confocal microscopy (FV1000; Olympus, Tokyo, Japan). The pictures were taken under the following conditions: objective lens: PLAPON 60×, oil, numerical aperture 1.42; scan mode: XY; excitation wavelength: 405 nm for DAPI and 543 nm for Dil-C14; image size: 1024 × 1024 pixels.

**TaqMan microRNA array and quantitative analysis of pre-miRNA and miRNA**

For the TaqMan microRNA array, RNA was reverse transcribed, and the cDNA was preamplified for 12 cycles. The preamplified product was 4× diluted with buffer, loaded on the TaqMan microarray, and run in the 7900HT instrument (Invitrogen). A total of 768 human miRNAs were tested, and the results were normalized by U6. Reverse transcriptase reactions were performed using designed Primer (Invitrogen) and AMV reverse transcriptase (TaKaRa) according to the manufacturers’ instructions. Real-time PCRs were performed using an Applied Biosystems 7300 Sequence Detection System (Applied Biosystems) and SYBR Green to monitor dsDNA synthesis. The reaction mixture contained 1 μl 20× SYBR Green PCR Master Mix (Applied Biosystems), 1 μl gene-specific primers (200 nM each), and TaqMan PCR kit in a final volume of 20 μl. The following thermal profile was used for all real-time PCRs: 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. After each real-time PCR run, dissociation curve analyses were performed.

**Cell transfection with ncRNA, premiR-223, and small interference RNAs**

HUVECs were seeded on six-well dishes and transfected the following day using Lipofectamine 2000 (Invitrogen). For miR-223 overexpression, 100 pM mimic miR-223 or scrambled negative control (NC) miRNA (mimic-NC) was used (Invitrogen). Cells were harvested 24 h after transfection. IGF-1R small interference RNA (siRNA) was designed to target the coding region of the IGF-1R mRNA (aa 3103–3122; 5′-CCAAGGGUGUGU-GAAAGA-3′). NC siRNAs (si-NC), which do not lead to the specific degradation of any known cellular mRNA, were used as the negative control. The siRNAs were delivered into the cultured HUVECs by Lipofectamine 2000 (Invitrogen). At 24 h posttransfection, RNAs were extracted for qRT-PCR analyses, and cell lysates were prepared for Western blot. Cell apoptosis assays were performed at 24 h posttransfection.

**MV isolation and incubation with HUVECs**

To isolate P-MVs, TPO- or thrombin-stimulated platelets in platelet-rich plasma were centrifuged at 2000 × g for 15 min at 4°C, and the platelet microparticle–enriched plasma was collected. The plasma was centrifuged at 100,000 × g for 1 h at 4°C in a TL-100 ultracentrifuge (Beckman Coulter). P-MVs were collected from the pellet and resuspended in FBS-free RPMI 1640 medium. For incubation of P-MVs with HUVECs, HUVECs were seeded on 12-well dishes the night before, and 200 μg P-MVs isolated from TPO- or thrombin-stimulated platelets was added into each well. After incubation for 24 h, HUVECs were collected for qRT-PCR and the quantitative protein assay. Apoptosis assays were performed at 24 h posttransfection.

**Apoptosis assays**

Apoptosis of cells and in tissues was detected using an Annexin V–FITC/propidium iodide (PI) staining assay. For induced apoptosis of HUVECs, the cells were cultured in 12-well plates with 5 μg/ml BSA–AGEs. BSA–AGEs were prepared as described previously (30). Flow cytometric analysis of apoptotic cells was carried out using an Annexin V–FITC/PI staining kit (Invitrogen). After washes with cold PBS, the cells were resuspended in binding buffer (100 mM HEPES, 100 mM NaCl, and 25 mM CaCl2 [pH 7.4]) and stained with Annexin V–FITC/PI at room temperature in darkness for 15 min. Apoptotic cells were then evaluated by gating PI and Annexin V–positive cells on an FACSCalibur (BD Biosciences). All experiments were performed in triplicate.

**Western blot**

IGF-1R protein levels were quantified by Western blot analysis of whole-cell extracts using Abs against IGF-1R. Normalization was performed by blotting the same samples with an Ab against α-tubulin. Protein bands were analyzed using Bandscan software (ImageJ; National Institutes of Health).

**Statistical analysis**

All images of Western blots and flow cytometry are representative of at least three independent experiments. For each experiment, qRT-PCR assays were performed in triplicate. The data are presented as the mean ± SD for three or more independent experiments. Differences are considered statistically significant at p < 0.05, analyzed using Student t test.

**Results**

**Platelets contain abundant miRNAs that can be upregulated by TPO stimulation**

By miRNA microarray analysis, we obtained the genome-wide profiling of miRNAs in isolated human platelets. The 15 miRNAs with the highest expression in normal human platelets detected by microarray are listed in Table I. The miRNA expression profile was detailed in Supplemental Table I (Gene Expression Omnibus database [http://www.ncbi.nlm.nih.gov/geo/] under accession number GSE51453). We next selectively validated these miRNAs with high expression in our microarray analysis (miR-223, miR-191, miR-146, miR-126, miR-150, miR-24, and miR-16) using the TaqMan probe-based qRT-PCR assay. As recent reports by other groups showed that miR-21, miR-22, miR-23a/b, and miR-26a/b are enriched in platelets (23, 31, 32), we also determined their levels by qRT-PCR assay. To test whether these miRNAs are unique for platelets, we selected miR-155, a leukocyte/lymphocyte–specific miRNA, as an internal control. As shown in Fig. 1A, these miRNAs were highly expressed in platelets, and miR-223, a myeloid cell–specific miRNA, was the most abundant miRNA in platelets. We further compared the levels of these 14 highly expressed miRNAs in platelets with those in monocytes and neutrophils, two major leukocytes in peripheral blood. Platelets, monocytes, and neutrophils were isolated from peripheral blood of five different healthy donors, and the absolute levels of miRNAs in these cells were detected by qRT-PCR. The comparison was made in three ways: the same blood volume (Fig. 1B), the same cell number (Fig. 1C), and the same total RNA quantity (Fig. 1D). Although individual platelets contain less miRNA due to their small size (Fig. 1C), total platelets have significantly more miRNAs than neutrophils and monocytes in the same volume of peripheral blood because platelet number in blood is much higher (Fig. 1B). This is true for most miRNAs, except miR-150, which shows a significantly higher level in monocytes, and neutrophils. The concentration of most miRNAs in platelets, normalized against total RNA, was also higher than the concentration in neutrophils and monocytes (Fig. 1D).

TPO is a glycoprotein hormone produced mainly by the liver and kidney that stimulates the differentiation of megakaryocytes, which fragment into a large number of platelets (33). The level of TPO in plasma is generally increased under various inflammatory conditions, suggesting the involvement of TPO-stimulated platelets in inflammatory diseases. Through screening miRNA expression profiles using low-density miRNA microarray, we found that TPO treatment resulted in a striking alteration in platelet miRNA expression (Supplemental Table I). To validate the results from

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microarray analysis, we determined the levels of several top expressed miRNAs, including miR-223, miR-26a/b, let-7f, and miR-191, using qRT-PCR assay. As shown in Fig. 2A, miRNAs in isolated platelets were indeed upregulated by TPO. To exclude the possible contamination by other isolated cells, particularly monocytes, we also purified monocytes from the peripheral blood of healthy volunteers and then treated the isolated cells with TPO. In contrast, the levels of miRNAs in isolated monocytes were not affected by TPO. These results are consistent with the fact that platelets, not monocytes, express the TPO receptor (34, 35). The alteration of miRNA levels in isolated platelets but not monocytes under the stimulation of TPO indicates that platelet miRNAs have a unique regulatory mechanism compared with leukocytes and also excludes the possibility that presence of miRNAs in platelets is due to contamination by leukocytes during cell isolation.

Platelets contain considerably high levels of pre-miRNAs that can be processed into mature miRNAs in response to proinflammatory stimuli

As anucleate platelets have no nuclear microprocessor components such as Drosha and DGC8 (22), the de novo biogenesis of miRNA should not be considered as a mechanism underlying the upregulation of miRNAs in platelets. However, Landry et al. (22) have shown that platelets have Dicer and AGO2 and are capable of processing pre-miRNAs into mature miRNAs. The upregulation of miRNAs in platelets by proinflammatory stimuli may be due to the enhanced maturation of pre-miRNAs. We thus assayed the levels of pre-miRNAs in platelets and their changes during the stimulation of platelets by TPO or thrombin. The cellular levels of pre-miRNAs were detected by qRT-PCR. As shown in Fig. 2C, the levels of pre-miRNAs in the platelets, such as pre–miR-223, pre–miR-191, and pre–miR-26b, were rapidly decreased under TPO stimulation compared with platelets without TPO treatment, which is inversely correlated to the upregulation of miR-223, miR-191, and miR-26b in TPO-stimulated platelets. Interestingly, comparing the ratio of pre-miRNAs/miRNAs in the platelets, monocytes, and neutrophils, we found that platelets generally had a higher ratio of pre-miRNAs/miRNAs (Supplemental Fig. 1). Taken together, these results implicate that platelets contain relatively higher levels of pre-miRNAs, and these pre-miRNAs can be rapidly processed into mature miRNAs when platelets respond to various challenges.

Platelets release large amounts of miR-223-containing MVs under inflammatory stimulation

It has been widely reported that activated platelets can release P-MVs, especially after stimulation with agonists including thrombin or exposure to high-stress shear forces (1, 13, 36). In fact, previous studies suggest that the majority of MVs in peripheral bloodstream
The ratio of relative levels of pre-miRNA/miRNA in the platelets stimulated with TPO and thrombin were 2.53 ∼0.82-fold higher than nontreated platelets, respectively. We next measured the levels of miRNAs in P-MVs and found not only miR-223, miR-26a, miR-191, and miR-126, but also the levels of miRNAs in P-MVs was significantly increased following the stimulation of platelets by TPO (Fig. 3C) or thrombin (Fig. 3D), compared with that in P-MVs released from nonstimulated platelets. Presence of various miRNAs in P-MVs was in agreement with the previous report that P-MVs contain miRNAs in addition to several platelet-endothelium attachment receptors (8). Furthermore, when platelets were stimulated with TPO for 4 h, ∼0.1% of total miR-223 in platelets was released through P-MVs (Fig. 3E, Supplemental Fig. 2). By feeding ApoE−/− mice with a high-fat diet for 12 wk, we successfully established a mouse model of atherosclerotic lesions and found that the concentration of miR-223 in circulating MVs from mouse peripheral bloodstream was significantly elevated after atherosclerotic lesions were induced (Fig. 3F). Because P-MVs generally account for two-thirds of circulating MVs in peripheral bloodstream, this result suggests that P-MVs from ApoE−/− mice with atherosclerotic lesions contain a higher level of miR-223 than control mice without atherosclerotic lesions. More importantly, a significantly increased level of miR-223 in circulating MVs was also observed in the plasma samples from patients under various systemic inflammatory conditions. As shown in Fig. 3G, we assessed miR-223 level in the plasma samples from patients with enteritis, hepatitis, nephritis, or coronary artery bypass grafting by TaqMan probe-based qRT-PCR assay. Compared to the platelets or P-MVs from healthy donors, the platelets or P-MVs from patients had a significantly higher level of miR-223. This result suggests that circulating P-MVs from patients under various inflammatory conditions contain higher levels of miR-223 than circulating P-MVs from healthy donors.

**P-MVs effectively deliver miR-223 into the recipient HUVECs**

Recently, the studies by us (20) and others (27, 28, 39) have demonstrated that cell-secreted MVs, including the shedding vesicles and exosomes, can deliver miRNAs into the recipient cells to modulate the expression of their target genes. To determine the potential biological functions of the miRNA-containing P-MVs, we studied the delivery of miR-223 into HUVECs via P-MVs. First, the comparison of miR-223 levels in HUVECs, platelets, and P-MVs by TaqMan probe-based qRT-PCR assay clearly showed that miR-223 level in platelets (Fig. 4A) and P-MVs (Fig. 4B) was much higher than that in HUVECs. Second, we collected P-MVs released from platelets and incubated the P-MVs with cultured HUVECs, as depicted in Fig. 4C. For tracing the P-MVs in HUVECs, we labeled the P-MVs with DiI-C16, and then detected the localization of fluorescent P-MVs in HUVECs after incubation at different temperatures. As shown in Fig. 4D, we collected P-MVs released from platelets and incubated the P-MVs in HUVECs at 4˚C. The negative fluorescent labeling in HUVECs at 4˚C indicated that the adherence of fluorescent P-MVs to HUVECs was blocked at 4˚C. The similar low level of adherence of fluorescent P-MVs to HUVECs was observed when we depleted ATP level to block the internalization of P-MVs (data not shown). The result showed that internalization of P-MVs into recipient cells is an active process. In agreement with this observation, incubation of HUVECs with P-MVs at 37˚C strongly increased miR-223 level (∼10-fold), and this elevation could be largely abolished by cotransfecting HUVECs with anti–miR-223 antisense oligonucleotide (ASO) (Fig. 4E). In contrast, the pre–miR-223 level in the recipient HUVECs was not altered by incubation with P-MVs (Fig. 4F), suggesting that the elevation of miR-223 level in HUVECs is not due to de novo miRNA biosynthesis but directly derived from the delivery by P-MVs.
Platelet miR-223 promotes HUVEC apoptosis induced by AGEs via targeting endothelial IGF-1R

To determine whether exogenous miR-223 delivered by P-MVs has a biological function in HUVECs, we analyzed the potential target genes of miR-223 using three computer-aided algorithms: TargetScan, miRanda, and PicTar. As shown in Fig. 5A, we predicted IGF-1R as a target gene of platelet miR-223. Overexpression of miR-223 in HUVECs via transfection of miR-223 mimic (Fig. 5B) clearly showed that miR-223 strongly reduced protein level (Fig. 5C, 5D) but not mRNA level (Fig. 5E) of IGF-1R, suggesting that miR-223 indeed targets IGF-1R in HUVECs.

We next determined whether the exogenous miR-223 delivered by P-MVs could affect the level of IGF-1R in the recipient HUVECs. Western blot analysis demonstrated that incubation of HUVECs with P-MVs significantly decreased the levels of cellular IGF-1R protein but not mRNA, and this reduction of IGF-1R protein levels by P-MVs was largely abolished by cotransfection with the anti–miR-223 ASO (Fig. 5F–H). As a positive control, both the IGF-1R protein and mRNA levels in HUVECs were strongly reduced by the siRNA against IGF-1R (Supplemental Fig. 3). The reduction of protein level of endothelial IGF-1R by P-MVs was comparable to that by directly transfecting HUVECs with siRNA against IGF-1R (Supplemental Fig. 3). Together, these results confirmed the role of platelet miR-223 in reducing IGF-1R levels in the HUVECs.

As a member of the largest class of the tyrosine kinase receptor family, IGF-1R is activated by IGF-1, a polypeptide protein hormone similar in molecular structure to insulin. The IGF-1/IGF-1R system has been implicated in the pathogenesis of insulin resistance and cardiovascular diseases (40). We determined the possible role...
of miR-223 targeting IGF-1R in modulating the HUVEC apoptotic process in the presence of AGEs. Previous studies have shown that AGEs and the receptor for AGEs can effectively induce cell apoptosis and promote inflammation (41, 42). In our study, BSA-AGEs were prepared as previously described (1). Cultured HUVECs were then treated with 5 μg/ml BSA-AGEs to

FIGURE 4. Effective delivery of platelet miR-223 into HUVECs by P-MVs. (A and B) Comparison of the absolute levels of miR-223 in platelets, P-MVs, and HUVECs. The level of miR-223 was assessed by qRT-PCR and normalized against the amount of total RNA. Note that both platelets (A) and P-MVs (B) have significantly higher levels of miR-223 than HUVECs have. (C) A flow chart of the experimental design. (D) Fluorescently labeled P-MVs entering HUVECs. Fluorescently labeled P-MVs were incubated with HUVEC cells for 1 h at 4˚C or 37˚C. HUVECs were then washed, fixed, and observed under confocal microscopy. Original magnification ×100. Note that P-MVs rapidly enter into HUVECs (red) at 37˚C but not at 4˚C. Levels of miR-223 (E) and pre-miR-223 (F) in HUVECs following the incubation with or without P-MVs or with P-MVs plus transfection with anti-miR-223 ASO or control oligonucleotide (anti-miR-NC) for 2 h. Data are presented as the mean ± SD of five independent experiments. ***p < 0.001.

FIGURE 5. Exogenous miR-223 delivered by P-MVs reduced IGF-1R protein expression in HUVECs. (A) Schematic illustration of IGF-1R 3′-untranslated region (utr) as the putative target for miR-223. Paired bases are indicated by a black oval, and G:U pairs are indicated by two dots. Sequence alignment of the putative miR-223 binding sites across species showed that the seed sequence is highly conserved. (B–D) The inverse relationship between miR-223 and IGF-1R in HUVECs. Compared to HUVECs transfected with control oligonucleotide (pre-miR-NC), transfection of HUVECs with pre-miR-223 strongly increased miR-223 levels (B) but decreased the protein levels of IGF-1R (C, D). (E) IGF-1R mRNA levels in HUVECs after transfection with pre-miR-223 or pre-miR-NC. IGF-1R protein levels (F, G) and mRNA levels (H) in HUVECs with or without incubation with P-MVs or P-MVs plus transfection with anti-miR-223 ASO or control oligonucleotide (anti-miR-NC). Data are presented as the mean ± SD of five independent experiments. **p < 0.01, ***p < 0.001.
initiate cell apoptosis. As shown in Fig. 6, incubation of HUVECs with P-MVs strongly increased the BSA-AGE–induced apoptosis of HUVECs. The promotion of AGE-induced HUVEC apoptosis by P-MVs was largely abolished by depleting miR-223 using anti–miR-223 ASO, indicating that the effect of P-MVs on HUVEC apoptosis is due to the delivery of platelet miR-223 by the P-MVs.

To confirm the role of IGF-1R, a target of miR-223, in HUVEC apoptosis, we directly knocked down IGF-1R levels in HUVECs using IGF-1R siRNA or transfecting HUVECs with pre–miR-223 to increase miR-223 levels. As can be seen, both IGF-1R siRNA and pre–miR-223 transfection promoted the HUVEC apoptosis induced by BSA-AGEs (Fig. 6). Interestingly, when HUVECs were treated with both IGF-1R siRNA and P-MVs prior to the induction of cell death by BSA-AGEs, we observed no additional cell apoptosis compared with IGF1R siRNA group or P-MV-treated group, which is in line with our conclusion that P-MVs are increasing cell death by reducing IGF-1R expression. These results collectively showed that platelet-secreted miR-223 via P-MVs could be effectively delivered into HUVECs, in which it targets endothelial cell IGF-1R, and thus promotes AGE-induced cell apoptosis.

Discussion

Recent studies have shown that anucleate platelets contain miRNAs and platelet miRNAs that are clinically and biologically relevant as potential regulators of megakaryocyte/platelet protein translation and expression (43, 44) and can be used as biomarkers for hematologic diseases and platelet reactivity; however, the regulation and potential function of these miRNAs are not completely understood. In the current study, we showed that platelet miRNAs, including miR-223, could be upregulated during platelet inflammatory responses, and this upregulation of miRNAs in platelets was derived from maturation of pre-miRNAs. In addition, we found that platelets could remotely modulate vascular endothelial cell function via secreting miR-223–containing P-MVs. Specifi-

![FIGURE 6. Platelet miR-223 delivered by P-MVs promotes HUVEC apoptosis induced by BSA-AGEs. (A) HUVEC apoptosis assessed by Annexin V/PI staining and flow cytometry. (B) Analysis of flow cytometry data from five independent experiments. The percentage of HUVEC apoptosis detected by flow cytometry was normalized to total applied cells. HUVECs directly treated with siRNA against IGF-1R (si–IGF-1R) or control siRNA (si-NC) served as positive or negative control. Data are presented as the mean ± SD. **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)

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ually, P-MVs effectively delivered miR-223 into endothelial cells, in which platelet miR-223 inhibited the protein expression of endothelial IGF-1R and promoted the AGE-induced endothelial cell apoptosis.

**High pre-miRNA/miRNA ratio in platelets and upregulation of platelet miRNA under inflammatory conditions**

Because of their anucleate nature, platelets do not express the nuclear microprocessor components Drosha and DGC8; therefore, the de novo synthesis of new miRNAs in platelets is minimal. As miR-223 and other mature miRNAs were upregulated by TPO and thrombin (Fig. 2A), we explored the potential mechanism underlying the modulation of miRNA in platelets. Our data showed that platelets contained a relatively high ratio of pre-miRNA/miRNA compared with other blood cells such as monocytes and neutrophils (Supplemental Fig. 1). Interestingly, under the stimulation by TPO or thrombin, the levels of pre-miRNAs in platelets were decreased, which was negatively correlated to the upregulation of miRNAs (Fig. 2C). These results collectively suggest that the high level of pre-miRNAs may be the source of the upregulation of miRNAs in platelets.

**Regulation and pathophysiologic role of miR-223–containing P-MVs in modulating vascular endothelial dysfunction under vascular inflammatory conditions**

It has been well known that platelets release large numbers of P-MVs particularly under inflammatory conditions, and these P-MVs form the majority of circulating MVs in the peripheral bloodstream. P-MVs are physiologically active and can mediate intensive interaction with other recipient cells, including neutrophils, monocytes/macrophages, and vascular endothelial cells. In the current study, our results confirmed that platelets released more P-MVs in response to TPO, thrombin, and other inflammatory stimulation. Furthermore, we showed that P-MVs contained abundant miRNAs, and the concentration of many miRNAs in P-MVs such as miR-223 was upregulated by the stimulation of TPO or thrombin. In other words, stimulation by TPO or other inflammatory factors could enhance not only the release of P-MVs by platelets but also the concentration of selective miRNAs in individual P-MVs.

To our knowledge, our data provided the first evidence that P-MVs could effectively deliver platelet miR-223 into HUVECs to promote AGE-induced cell apoptosis via targeting recipient cell IGF-1R. This result is in agreement with the recent studies showing that cell-secreted MVs, particularly exosomes, serve as physiological carriers of miRNAs to exchange genetic materials and signaling molecules between cells (45, 46). As a hematopoietically specific miRNA with crucial functions in myeloid lineage development (47, 48), miR-223 has been shown to target a transcription factor MeF2c (47), CEBP-β (49), glutamate receptors (GluR2 and NR2B) (50), and IGF-1R (51) in other cell types. By the luciferase reporter assay and experimental validation, we confirmed IGF-1R as a target of miR-223 in vascular endothelial cells. To support the role of miR-223–targeting IGF-1R to promote AGE-induced HUVEC apoptosis, direct silencing of IGF-1R expression by IGF-1R siRNA showed an increased AGE-induced apoptosis of HUVECs (Fig. 6). Through directly comparing eosinophil progenitor cultures derived from miR-223 knockout mice and miR-223 wild-type littersmates, Cocucci et al. (52) recently showed that miR-223 deficiency increased eosinophil progenitor proliferation. Although the effect of factors other than platelet miR-223 on the reduction of IGF-1R and promotion of HUVEC apoptosis cannot be excluded at this stage, depleting miR-223 in HUVEC by anti–miR-223 ASO largely reversed the enhancement of HUVEC apoptosis by P-MVs, implicating that platelet miR-223 in P-MVs plays a major role in modulating HUVEC apoptosis.

In summary, our results demonstrate the first evidence, to our knowledge, that platelets contain abundant miRNAs, particularly miR-223, and that through release of miR-223–containing P-MVs, platelets can remotely modulate the apoptosis of vascular endothelial cells induced by pathophysiologic factors such as AGES. In addition, as depletion of miR-223 by anti–miR-223 ASO treatment can effectively decrease AGE-induced HUVEC apoptosis, our study also provides a potential miRNA-based therapeutic strategy for atherosclerosis.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


