Impairment of Wound Healing in Patients With Type 2 Diabetes Mellitus Influences Circulating MicroRNA Patterns via Inflammatory Cytokines

Seema Dangwal,* Bernd Stratmann,* Claudia Bang, Johan M. Lorenzen, Regalla Kumarswamy, Jan Fiedler, Christine S. Falk, Claus J. Scholz, Thomas Thum,* Diethelm Tschoepe*

Objective—MicroRNAs (miRNA/miR) are stably present in body fluids and are increasingly explored as disease biomarkers. Here, we investigated influence of impaired wound healing on the plasma miRNA signature and their functional importance in patients with type 2 diabetes mellitus.

Approach and Results—miRNA array profiling identified 41 miRNAs significantly deregulated in diabetic controls when compared with patients with diabetes mellitus–associated peripheral arterial disease and chronic wounds. Quantitative reverse transcriptase polymerase chain reaction validation confirmed decrease in circulating miR-191 and miR-200b levels in type 2 diabetic versus healthy controls. This was reverted in diabetic subjects with associated peripheral arterial disease and chronic wounds, who also exhibited higher circulating proinflammatory cytokine levels compared with diabetic controls. miR-191 and miR-200b were significantly correlated with proinflammatory cytokine levels in patients with diabetes mellitus. Indeed, proinflammatory stress increased endothelial- or platelet-derived secretion of miR-191 or miR-200b. In addition, dermal cells took up endothelial-derived miR-191 leading to downregulation of the miR-191 target zonula occludens-1. Altered miR-191 expression influenced angiogenesis and migratory capacities of diabetic dermal endothelial cells or fibroblasts, respectively, partly via its target zonula occludens-1.

Conclusions—This study reports that (1) inflammation underlying nonhealing wounds in patients with type 2 diabetes mellitus influences plasma miRNA concentrations and (2) miR-191 modulates cellular migration and angiogenesis via paracrine regulation of zonula occludens-1 to delay the tissue repair process. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.305048.)

Key Words: diabetes mellitus, type 2 ■ inflammation ■ microRNAs ■ peripheral arterial disease ■ zonula occludens-1 protein

Diabetes mellitus–associated impaired wound healing severely affects life quality of patients with diabetes mellitus leading to prolonged hospitalization and lower limb amputations.1,2 Diabetes mellitus causes ≈50% of all nontraumatic amputations of the lower extremities worldwide and >80000 procedures are performed annually.3 The life-time risk of amputation in patients with diabetes mellitus is 10% to 15%, which is 10 to 30× higher in comparison with the general population.4–6 Multifactorial pathways including peripheral arterial disease (PAD), peripheral neuropathy, chronic inflammatory state, and altered cellular functions influence the development of diabetic chronic wounds.7,8 PAD is present in approximately one-half of all patients with foot ulcers and accounts for chronic wounds because of insufficient perfusion.9 Leg amputation because of atherosclerotic PAD corresponds to a mortality rate of ≈30% and a 5-year prognosis with survival rates of <5 years.6,10,11 Persistent inflammation is a hallmark of patients with chronic diabetic wounds as the circulating cytokine levels in patients with diabetes mellitus with nonhealed foot ulcers remain closer to that of the active diabetic foot ulcer patients.7

MicroRNAs (miRNAs/miRs), highly conserved small RNAs (≈22 nucleotides), are master regulators of diverse aspects of cellular functions acting mainly via translational inhibition...
after recognition binding of 3′UTR of mRNA transcripts.12–14 MiRNAs present in body fluids including blood have demonstrated their potential as novel biomarkers of diabetes mellitus and cardiovascular disorders.15–17 These extracellular miRNAs mainly originate by cellular secretion (exosomes, microparticles) or shedding of apoptotic bodies.16 Protective packaging of miRNAs in microvesicles, protein complexes (nucleophosmin, Ago-2), or lipoproteins (low-density lipoprotein or high-density lipoprotein) physically shields circulating miRNAs from endogenous RNases and renders remarkable stability.18 Deregulation of plasma miRNAs in diabetes mellitus has been reported16; however, effects of diabetes mellitus–associated impairment of tissue repair on diabetic miRNA signatures are unknown. We therefore investigated circulating miRNAs patterns in patients with diabetes mellitus–associated PAD with or without chronic wounds compared with patients with diabetes mellitus without PAD or healthy controls. We further explored the mechanism of inflammation-driven secretion of diabetic miRNAs and their potential paracrine role in cellular interactions to control cellular functions essential for tissue repair.

Materials and Methods
Detailed Materials and Methods are available in the online-only Data Supplement.

Study Population
The study was confirmed by the local Ethical Committee and all patients received/signed written informed consent. The study cohort included 61 patients, diagnosed with type 2 diabetes mellitus (T2DM) according to the current ADA/EASD guidelines, from inpatients at the Heart and Diabetes Center North Rhine-Westphalia in Bad Oeynhausen and 20 body mass index–matched healthy controls (characteristics in Table 1). Patients with PAD history or current PAD were considered as PAD positive and wounds were defined as chronic if they persisted >2 weeks without healing tendency on standard treatment.

All antidiabetic drugs, antiplatelet agents, antihypertensive, and lipid-lowering agents were allowed to resemble real hospital/life situation (Table 1). Standardized examinations included clinical biochemistry and detailed investigation of comorbidity following current medical guidelines.

Plasma Total RNA Isolation, miRNA Profiling, and Expression Validation
Total RNA was isolated from plasma and wound fluid samples using miRNeasy kit (Qiagen, Hilden). Affymetrix miRNA array was performed on total RNA pooled from 23 diabetic controls or 17 patients with diabetes mellitus with PAD and chronic wounds, respectively, and data were analyzed as described.15 The microarray data were

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**Table 1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>T2DM+PAD+Wounds (n=26 cases)</th>
<th>T2DM+PAD (n=12 cases)</th>
<th>T2DM Control (n=23 cases)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>64.1±1.8</td>
<td>66.6±2.0</td>
<td>61.0±2.1</td>
<td>ns</td>
</tr>
<tr>
<td>Sex</td>
<td>20 m/6 f</td>
<td>7 m/5 f</td>
<td>18 m/5 f</td>
<td>ns</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>30.4±0.7</td>
<td>31.0±1.0</td>
<td>29.1±0.9</td>
<td>ns</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>141.7±10.0</td>
<td>145.9±14.7</td>
<td>147±9.1</td>
<td>ns</td>
</tr>
<tr>
<td>Duration of diabetes mellitus, y</td>
<td>15.7±2.0</td>
<td>19.4±3.4</td>
<td>9.8±2.1</td>
<td>ns</td>
</tr>
<tr>
<td>Insulin therapy†</td>
<td>17/26 cases</td>
<td>5/12 cases</td>
<td>12/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>Metformin therapy</td>
<td>10/26 cases</td>
<td>5/12 cases</td>
<td>16/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>Lipid-lowering therapy‡</td>
<td>11/26 cases</td>
<td>8/12 cases</td>
<td>14/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>Antihypertensive therapy§</td>
<td>19/26 cases</td>
<td>11/12 cases</td>
<td>18/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>Anticoagulation therapy¶</td>
<td>23/26 cases</td>
<td>9/12 cases</td>
<td>11/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>CAD</td>
<td>4/26 cases</td>
<td>3/12 cases</td>
<td>5/23 cases</td>
<td>ns</td>
</tr>
<tr>
<td>Wound size, cm²</td>
<td>16.2±3.6</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Wound infection</td>
<td>17/26 cases</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>HbA1c, % (mmol/mol)</td>
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<td>9.0±0.4</td>
<td>8.6±0.4</td>
<td>ns</td>
</tr>
<tr>
<td>Lp(a), mg/dL</td>
<td>22.0±4.8</td>
<td>17.3±8.5</td>
<td>10.5±2.1</td>
<td>ns</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>40.2±2.2</td>
<td>46.9±4.8</td>
<td>46.5±2.7</td>
<td>ns</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>101.6±8.1</td>
<td>84.2±10.7</td>
<td>116.2±7.3</td>
<td>ns</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>4.2±1.13</td>
<td>0.50±0.20</td>
<td>0.49±0.11</td>
<td>ns</td>
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<tr>
<td>Leukocyte count, 10E9/L</td>
<td>10.1±0.5</td>
<td>8.6±0.4</td>
<td>7.4±0.5</td>
<td>#</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; CAD, coronary artery disease; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; PAD, peripheral arterial disease; na, not available; ns, nonsignificant; and T2DM, type 2 diabetes mellitus.

†Long acting insulin and short acting insulin; ‡fibrates or statins; §at least 1 antihypertensive medication; and ¶asacilic acid, clopidogrel, heparin therapy, and marcumar therapy.

*P<0.01 vs T2DM control; †P<0.001 vs T2DM control; and #P<0.01 vs T2DM+PAD.
submitted to GEO and referenced with accession no. GSE49885. Selected miRNAs were validated by quantitative real-time polymerase chain reaction using Taqman assays. Because of low abundance of conventional ribonucleic acid controls in body fluids, spiked in Caenorhabditis elegans miR-39 was used as a control for normalization as described.19

Statistical Analysis

Data are presented as mean±SEM and statistical analysis was performed using SPSS-17 (SPSS, Inc, Chicago, IL) or GraphPad Prism software-6 (GraphPad Prism Software, Inc, San Diego, CA). Variables were analyzed for normal distribution using the Kolmogorov–Smirnov test. Correlation analysis for normally distributed variables was performed using the Pearson correlation coefficient. Non-normally distributed variables were assessed by Spearman correlation analysis. Continuous variables were compared using the Mann–Whitney rank sum test or the Kruskal–Wallis 1-way ANOVA. Categorical variables were compared using the $\chi^2$ test. MiRNA or gene expression data and cytokine levels analyzed using Student t test. $P$ values ≤0.05 were considered statistically significant for all statistical procedures used.

Results

Circulating miRNA Levels Are Regulated in Patients With T2DM With Chronic Wounds

Levels of 41 miRNAs were significantly altered in miRNA profiling of pooled plasma of 20 diabetic controls versus 17 patients with diabetes mellitus with PAD and associated chronic wounds (Figure 1A). To analyze whether changes in miRNA concentrations were because of underlying PAD or wound-associated inflammatory conditions, levels of miRNAs were further validated in individual plasma samples from an entire cohort comprising 26 patients with PAD and associated impaired wound healing, 12 patients with PAD alone, 23 diabetic control subjects without PAD or chronic wounds, and 20 healthy individual control subjects. Patient’s characteristics are given in Table 1. Based on the level of regulation and their reported functional roles, 2 miRNAs were selected for further validation: (1) miR-191: the top most deregulated candidate
derived from miRNA screens, which is expressed in endothelial cells and platelets and (2) miR-200b: an inflammation-regulated modulator of diabetic skin wound healing.\textsuperscript{20,21} Indeed, in the candidate validation experiments, plasma levels of circulating miR-191 and miR-200b were significantly decreased ≤3- to 6-fold in type 2 diabetic subjects (n=23) versus healthy controls (P<0.05; n=20; Figure 1B). Presence of PAD alone with diabetes mellitus did not affect plasma levels of these miRNAs. In contrast, an increase ≤2-to 3-fold was observed in miR-191 and miR-200b levels in patients with the presence of PAD and chronic wounds (P<0.05; n=26; Figure 1B).

#### Diabetes-Associated Circulating MiRNA Pattern Correlates With Inflammatory Markers and Wound Size

Patients with diabetes mellitus with impaired wound healing are prone to systemic inflammation. Indeed, among patients with diabetes mellitus, significantly higher plasma levels of C-reactive protein were observed in the presence of chronic wounds (4.21±1.13 mg/dL; n=26) compared with patients without wounds (0.49±0.11 mg/dL; n=23; P=0.0002) or with PAD only (0.50±0.20 mg/dL; n=12; P=0.006; Table 1). In addition, elevated blood leukocyte counts in patients with diabetes mellitus with chronic wounds compared with other groups (P<0.05; Table 1) indicated an aggravated inflammatory environment. Circulating miR-191 levels significantly correlated with C-reactive protein (r=0.33; P=0.009), monocyte counts (r=0.342; P=0.009), and wound size (r=0.416; P=0.035), whereas miR-200b positively correlated with C-reactive protein levels (r=0.329; P=0.011) in the complete patient cohort (Table 2). Circulating levels of miR-191 and miR-200b were also strongly correlated with each other (Tables 2 and 3).

The proinflammatory state in patients with T2DM with impaired wound healing was further characterized by multiplex protein assays to screen plasma cytokine and chemokine levels. This approach identified plasma levels of 13 of tipplex protein assays to screen plasma cytokine and chemokines (IL-6, CXCL8 [IL-8], hepatocyte growth factor, VEGF, tumor necrosis factor-\(\alpha\), IL-18; IL-6, IL-10, interferon-\(\gamma\)) as well as the growth factors FGF, VEGF, and hepatocyte growth factor to be significantly increased in patients with T2DM having abnormal wound healing compared with no wound patients (Figure 2A). Significant correlations were observed between circulating miR-191 levels and IL-1\(\beta\), platelet-derived growth factor-bb and MIF, whereas miR-200b levels correlated with MIF and platelet-derived growth factor-bb in an overall diabetic cohort (Table 2). A potential reason of increased plasma miRNA levels in patients with chronic wounds could be a local overproduction and release of miRNAs within the wounds. To test this hypothesis, we measured inflammatory cytokines and miRNAs directly in wound fluid versus peripheral blood obtained from the same patients. Patients with diabetes mellitus with chronic wounds exhibited extremely high extracellular cytokine levels in wound fluids, indicating an inflammatory environment of the wounds (Figure 2B). In contrast, levels of miR-191 in wound fluids were lower (almost 2 Ct cycles, P=0.06) in comparison with corresponding plasma samples (Figure 2C). Hence, the inflammatory cells accumulated in the local wound site might not be serving as an active source to release miR-191 and to elevate the circulating miR-191 concentrations.

#### Endothelial and Platelet Secretions of T2DM-Associated MiRNAs Increase Under Inflammatory Settings

To explain inflammation-mediated increase in plasma miRNA concentrations in patients with T2DM with chronic wounds, we hypothesized that various inflammatory cytokines could trigger direct miRNA release from vascular endothelial cells or platelets. We thus mimicked a proinflammatory environment as observed in the plasma samples of patients with diabetes mellitus with chronic wounds by stimulating endothelial cells or isolated human platelets with inflammatory cytokines/chemokines which were significantly elevated in type 2 diabetes mellitus+PAD+chronic wounds group. Indeed, exposure of endothelial cells to the various concentrations of cytokines/chemokines (IL-6, CXCL8 [IL-8], hepatocyte growth factor, VEGF, or tumor necrosis factor-\(\alpha\)) alone (data not shown) or in combination increased endothelial-secreted miR-191 levels ≤3-fold (P<0.05) as measured in cell culture supernatants (Figure 3A). Likewise, miR-200b release was also increased from washed human platelets on cytokine stimulation (Figure 3B).

#### Paracrine Functional Effects of Circulating Diabetic miRNAs

Further studies were performed to investigate the influence of chosen miRNAs on cellular functions involved in dermal
wound healing. We performed either gain or loss of functional studies by transfection of miRNA precursors or inhibitors to dermal diabetic microvascular endothelial cells and dermal fibroblasts under hyperglycemic settings (Figures 4 and 5).

In dermal microvascular endothelial cells isolated from type 2 diabetic subjects, both miRNAs inhibited tube formation capacity and cellular migration (Figure 4A and 4B; Figure IA and IB in the online-only Data Supplement) without influencing apoptosis or cell proliferation (data not shown). Zonula occludens-1 (ZO-1), a putative angiogenic target of miR-191 predicted by 3 different bioinformatic tools (TargetScan, miRwalk, and Diana tools), was downregulated in cells overexpressing miR-191 (Figure 4C–4E). ZO-1 is a multiple domain scaffold protein with a reported role in cell to cell adhesion, maintenance of tissue structure and hence modulates angiogenesis and cellular migration during the tissue remodeling and repair. The binding of miR-191 to 3′ UTR of ZO-1 transcript was confirmed by reduced luciferase reporter activity after miR-191 overexpression (Figure 4D). In line, ZO-1 knockdown showed a similar phenotype as that of miR-191 overexpression in dermal cells. ZO-1 silencing rescued the angiogenic effect of miR-191 inhibitor treatments of dermal endothelial cells (Figure 4A and 4B). This suggests miR-191 to function at least in part through its direct target ZO-1.

To study whether miRNAs secreted from endothelial cells under proinflammatory settings would affect dermal fibroblast biology, we performed coculture assays (Figure 5A, scheme). Indeed, coculturing dermal fibroblasts with miRNA-secreting endothelial cells (under inflammatory stimuli conditions) increased intracellular fibroblast concentration of miR-191 compared with fibroblasts cultured without endothelial cells (Figure 5B). In addition, fibroblasts cocultured with endothelial cells under inflammatory settings showed inverse correlation between expression levels of miR-191 and its target ZO-1 suggesting functional effects of miRNA exchange (Figure 5B). Selective transfer of miR-191 from vascular endothelial cells to dermal fibroblasts or dermal endothelial cells was further demonstrated by uptake assays; indeed, on transfection with Cy3-labeled miR-191, vascular endothelial cells secreted labeled miR-191 carrying vesicles into the cell supernatant which were taken up by the dermal microvascular endothelial cells or fibroblasts within 24 hours (Figure 5C, arrows). This suggests an effective cellular uptake of extracellular miRNAs released from endothelial cells rather than direct transcriptional effect of proinflammatory mediators on dermal fibroblasts. An increase in miR-191 expression along with attenuated ZO-1 expression is seen in fibroblasts when cocultured with miR-191–overexpressing endothelial cells compared with the scrambled control–transfected cells (Figure 5D). The functional roles of miR-191 on dermal fibroblasts were investigated in cells overexpressing miRNA-191 on transient transfection with miR-191 precursor. Indeed, increased expression of miR-191 delayed the wound closure in a scratch wound assay (Figure 5E) and induced apoptosis (Figure 5C in the online-only Data Supplement) without affecting the cellular proliferation and was also correlated to parallel downregulation of ZO-1 in these cells (data not shown). Similar to angiogenesis, miR-191 inhibitor (anti–miR-191)–mediated migratory response was also

Table 3. Correlation Analysis of Variables Within the Group of Patients With Diabetes Mellitus With Peripheral Arterial Disease and Chronic Wounds (n=26)

<table>
<thead>
<tr>
<th>Variables</th>
<th>miR_191</th>
<th>miR_200b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P Value</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>-0.35</td>
<td>0.077</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>0.403*</td>
<td>0.046</td>
</tr>
<tr>
<td>Wound size, cm²</td>
<td>0.416*</td>
<td>0.035</td>
</tr>
<tr>
<td>C-peptide, mg/dL</td>
<td>-0.59†</td>
<td>0.002</td>
</tr>
<tr>
<td>Proinsulin, mg/dL</td>
<td>-0.28</td>
<td>0.308</td>
</tr>
<tr>
<td>Platelet count, 10E9/L</td>
<td>0.475*</td>
<td>0.014</td>
</tr>
<tr>
<td>Eosinophile count, 10E9/L</td>
<td>-0.46*</td>
<td>0.029</td>
</tr>
<tr>
<td>miR-191</td>
<td>1.00</td>
<td>…</td>
</tr>
<tr>
<td>miR-200b</td>
<td>0.719†</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*BMI indicates body-mass index; miR, microRNA; and r, correlation coefficient. *P<0.05; and †P<0.01.

Figure 2. A, Cytokine profiling heatmap presentation of circulating plasma cytokines screened in the type 2 diabetic cohort with (type 2 diabetes mellitus [T2DM]+peripheral arterial disease [PAD]+wounds [W]; n=26) or without (T2DM+PAD; n=12) chronic wounds. Comparison of (B) cytokine profiles and (C) microRNA-191 (miR-191) expression levels in plasma vs wound fluid of 6 subjects from T2DM+PAD+W group (n=6; P<0.06).
rescued by ZO-1 silencing in dermal fibroblasts (Figure 5E).

The schematic representation of miRNA secretion from vascular endothelial cells and subsequent paracrine functions of miRNA in dermal cells on their uptake is depicted in Figure 6.

Discussion

Altered levels of circulating miRNAs, which can be detected in body fluids, for example, blood and urine, have been linked to various pathophysiological situations including diabetes mellitus. A recent clinical study demonstrated biomarker potential of circulating miR-126 in T2DM. However, plasma miRNA regulation in diabetes mellitus–associated impaired wound healing was not addressed previously. Our study reports a differential signature pattern of circulating miRNAs in patients with T2DM depending on the presence or absence of chronic wounds. A thorough investigation of circulating cytokines

Figure 3. Secretion of (A) microRNA (miR)-191 and (B) miR-200b to the cellular supernatants on cytokines or LPS (1 μg/mL) stimulation of endothelial cell or washed human platelet preparations in vitro (n=4–5; *P<0.05).

Figure 4. Tube formation capacity is influenced by microRNA (miR)-191 modulation and rescued by zo-1 knockdown of dermal endothelial cells. A, Tube formation capacity after miR-191 precursor (pre), anti-miR (anti) with or without zo-1 small interfering RNA (siRNA) compared with corresponding scrambled controls (-scr) and (B) the representative pictures. C, mRNA expression levels, (D) luciferase reporter activity, and (E) protein expression levels of miR-191–target gene ZO-1 are reduced on miR-191 overexpression in type 2 diabetes mellitus dermal microvascular endothelial cells in at least 3 replicates. *P<0.05.
Figure 5. Uptake of microRNA (miR)-191 by dermal cells affects their miR-191 expression and cell function. A, Schematic representation of endothelial cells and dermal fibroblasts coculture assay. B, miR-191 and the target ZO-1 expression in dermal fibroblasts in the absence or presence of cocultured endothelial cells under inflammatory stimuli—Cytokines or LPS. (C) Vesicular uptake of miR-191 by either dermal endothelial cells or fibroblasts (cy-3–labeled pre–miR-191 in red shown by arrows, alexa-488–labeled CD-31 or WGA-agglutinin in green and 4',6-diamidino-2-phenylindole in blue) after 24-hour stimulation with endothelial cell–derived vesicles. D, Levels of miR-191 and its target zo-1 in fibroblasts cocultured with miR-191–overexpressing vascular endothelial cells. E, Migration of dermal fibroblasts on miR-191 modulation is rescued by zo-1 knockdown. (n=3–6; *P<0.05). Scr indicates scrambled controls; and siRNA, small interfering RNA.

suggests that inflammation underlying abnormal wound healing is a key factor to influence plasma miRNA levels.

miR-191 is a stress-sensitive miRNA that has been extensively studied in cancer pathologies and reduced levels of this miRNA are shown in connection with diabetes mellitus.16,23 Modulation in miR-191 expression regulates apoptosis, cell proliferation, migration, and cell cycle under various pathological settings.23 Interestingly, altered plasma levels of endothelial-rich miRNAs, for example, miR-191 or miR-126 is reported to reflect underlying endothelial dysfunction in diabetes mellitus.16 miR-191 is coexpressed with the miR-191/425 cluster which is highly conserved in higher eukaryotes24 and our plasma miRNA profiles also confirm a unidirectional changes in circulating plasma levels of both clustered miRNAs from patients with diabetes mellitus with impaired wound healing compared with diabetics with no chronic wound. miR-200b is a known hypoxia-sensitive miRNA that induces angiogenesis in hypoxic dermal cells.20,21 Under inflammatory stress, miR-200b is upregulated in endothelial cells and targets angiogenic genes VEGF.21 In diabetic skin wounds, the expression of miR-200b remains higher compared with nondiabetic wounds and the silencing of miR-200b supports wound angiogenesis.21 In line, our study not only confirms inflammatory regulation of miR-200b in vitro and in plasma of patients with diabetes mellitus but also extends to the regulation of another miRNA candidate miR-191 in similar pathological settings. Positive correlations between plasma levels of both miR-191 and miR-200b to plasma C-reactive protein and cytokine levels confirm underlying inflammatory conditions to influence plasma miRNA levels.

miRNAs circulating in blood plasma represent a pool of miRNAs secreted by vascular cells, mainly endothelial cells, platelets, and challenged immune cells in the form of vesicles, for example, exosomes or apoptotic bodies.25–27 The miRNA patterns in body fluids including plasma therefore depend on several factors of cellular origin, presence of stressors, pathological conditions, or drug treatment.16,28 In our study, differences in miR-191 levels were also observed between wound exudates versus plasma as the local composition of cells secreting these miRNAs varies; for example, the local wound environment is rich in leukocytes and cellular debris. Thus, the low levels of miR-191 in wound fluid indicates a minor role of wound accumulated inflammatory cells; however, inflammation-sensitive endothelial cells or platelets might be the major contributors to the systemic miRNA increase in patients with diabetes mellitus with abnormal wound healing. Vascular endothelial cells secrete miRNAs in the form of exosomes or apoptotic bodies enabling their transport to the distant pathological targets, as evident form transport of miR-126 to the atherosclerotic lesions which indicates to a unique paracrine-signaling function of miRNAs during atherosclerosis.27 Differences in circulating miRNAs in patients with diabetes mellitus with impaired wound healing may arise because of altered secretion of miRNAs from inflammation-stimulated endothelial cells and apparently our in vitro observations prove miR-191 release from endothelial cells under
proinflammatory stress. Pertaining to the miRNA secretory capacity of platelets and increased atherothrombotic events in patients with diabetes mellitus, platelets might be another prominent source of circulating miRNAs in plasma. In fact, platelets exposed to proinflammatory conditions release significantly higher amount of miR-200b compared with unstimulated platelets. Most of the advanced patients with diabetes mellitus with PAD and abnormal wound healing were prescribed antiplatelet treatment to lower the risk of atherosclerotic and atherothrombotic events, which could suppress the miRNA secretion from platelets. However, an increased proportion of reticulated platelets with more secretory capacity despite of high platelet turnover rate suggests a potential involvement of platelet-secreted miRNAs in the diabetic plasma pool.

Current evidences in the literature support the existence of extracellular miRNAs-mediated cross-talks between different types of cells under various pathological conditions that may exert paracrine effects on cellular signaling and functions. In line with previous reports, we observed that dermal fibroblasts and dermal microvascular endothelial cells can take up vascular endothelial-secreted miR-191 leading to high miRNA levels in these cells with detrimental cellular effects such as impaired migratory or angiogenic responses and increased apoptosis. Nevertheless, miR-191 target gene ZO-1 was inversely regulated in cocultured fibroblasts as well as in dermal endothelial cells or fibroblasts on miR-191 overexpression. The expression of ZO-1 is reportedly increased on the locomotive surface of cells during wound healing. Reduced expression of ZO-1 parallel to miR-191 upregulation as seen in LPS or cytokine challenged dermal fibroblasts when cocultured with endothelial cells therefore could attenuates cellular migration. This was evident by delayed wound closure on miR-191 overexpression not only in fibroblasts but also in dermal microvascular endothelial cell. In endothelial cells, ZO-1 is a mediator of angiogenesis and its deficiency leads to defects in vascular development with impaired formation of vascular trees important for tissue organization and remodeling. The paracrine regulation of ZO-1 in dermal cells mediated via miR-191 uptake potentially contributes in slowing down of the tissue repair process commonly observed in patients with diabetes mellitus.

Conclusions

Our study presents novel data on differential patterns of circulating plasma miRNAs in patients with diabetes mellitus with associated impaired wound healing. We show for the first time that inflammation underlying nonhealing wounds mediate endothelial miR-191 secretion which is also correlated with the clinical findings of increased plasma levels of miR-191 in diabetic foot ulcer patients. Furthermore, our results suggest a subsequent paracrine mechanism involving miR-191 uptake and modulation of target genes ZO-1 in recipient dermal cells to compromise the cellular functions essential for tissue repair.

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Disclosures

None.

References

tissue repair associated with diabetes mellitus. Similarly in dermal endothelial cells, miR-191 overexpression or ZO-1 knockdown inhibits angiogenic functions to compromise expression of miR-191 leads to attenuation in wound closure because of reduced fibroblast migration and increased apoptosis in dermal


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Significance

Our study demonstrates for the first time that circulating plasma miRNA signature of patients with diabetes mellitus having impaired wound healing and peripheral artery disease significantly differs from that of patients with no such complications. Among 41 significantly deregulated plasma miRNA candidates, miR-191 was regulated to the highest degree and was further identified as a potential paracrine modulator of dermal wound healing under inflammatory diabetic settings. miR-191, secreted from vascular endothelial cells challenged with inflammatory mediators, is taken up by dermal fibroblasts or recipient endothelial cells to suppress its target gene ZO-1 expression. Increased expression of miR-191 leads to attenuation in wound closure because of reduced fibroblast migration and increased apoptosis in dermal fibroblasts. Similarly in dermal endothelial cells, miR-191 overexpression or ZO-1 knockdown inhibits angiogenic functions to compromise tissue repair associated with diabetes mellitus.
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