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Elevation of miR-221 and -222 in the Internal Mammary Arteries of Diabetic Subjects and Normalization with Metformin

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Abstract

Diabetes is a major risk factor for cardiovascular disease and is associated with increased intimal thickening and accelerated vascular smooth muscle cell (VSMC) proliferation. We measured the expression of two microRNAs that promote intimal thickening, miR-221/222, and mRNA encoding a downstream target, p27^{Kip1}, in internal mammary artery (IMA) segments collected from 37 subjects undergoing coronary artery bypass grafting. The segments were stratified into three groups: non-diabetic subjects (ND), diabetic subjects not on metformin (DMMet–), and diabetic subjects on metformin (DMMet+). The DMMet– group exhibited a significant increase in miR-221/222 and decrease in p27^{Kip1} mRNA compared to both the ND and DMMet+ groups. miR-221/222 levels inversely correlated with metformin dose. VSMCs isolated from the IMAs of the DMMet– group proliferate at a faster rate than those of the ND and DMMet+ groups. Further studies into the importance of miR-221/222 in the increased intimal thickening observed in diabetic subjects is warranted.

Keywords

miR-221/222; diabetes; metformin; internal mammary artery; vascular smooth muscle cell

1. Introduction

Diabetic subjects have two to four fold greater mortality from cardiovascular diseases (CVD) and exhibit increased intimal thickening, a component of CVD progression [1].

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Intimal thickening involves vascular smooth muscle cells (VSMC) migrating to the intima of an artery and proliferating. VSMCs isolated both from animal models of diabetes and diabetic subjects exhibit increased proliferation and migration [2–4]. Diffuse intimal thickening creates sites prone to fatty streak development, an initial step in CVD lesion formation [5]. Pathologic intimal thickening in response to lesion growth promotes fibrous cap formation and plaque stability [5]. Thus, changes in the molecular mechanisms regulating VSMC proliferation and migration have the potential to greatly impact CVD progression.

Recently, two microRNAs with similar function and expression patterns, miR-221 and miR-222, were shown to promote intimal thickening following arterial injury, at least in part, through down-regulation of the cyclin-dependent kinase inhibitor, p27^{Kip1} [6–8]. Here we report that miR-221/222 are elevated in the internal mammary arteries (IMA) of subjects with type 2 diabetes. This increase in miR-221/222 is accompanied by a decrease in mRNA for p27^{Kip1}. Additionally, we found that those diabetic subjects on metformin therapy had levels of miR-221/222 and mRNA encoding p27^{Kip1} that were similar to non-diabetic subjects. These data demonstrate that diabetes is accompanied by an increase in expression of miR-221/222 in the vasculature, and therefore suggest a novel mechanism for the increased CVD seen in the diabetic population.

2. Material and Methods

2.1 IMA Collection

This cross-sectional, observational study was performed at Ochsner Clinic Foundation and approved by its Institutional Review Board (Protocol # 2007.025.A). Each participant signed the informed consent. A segment of the internal mammary artery was obtained from 37 subjects undergoing coronary artery bypass surgery in the Department of Surgery. Relevant medical histories and current medication were obtained. Inclusion in one of the diabetes group required a previously established clinical diagnosis, and included subjects treated both pharmacologically (n = 33) and by diet (n = 4).

2.2 Real-time PCR

Total RNA was isolated from IMAs using the miRNeasy (miR-221/222) or RNeasy (p27^{Kip1}) Plus Mini kits (Qiagen, Inc., Valencia, CA). For measurement of miR-221/222, cDNA was prepared using the miScript II RT Kit (Qiagen) and the miScript Primer Assays for hsa-miR-221 (MS00009170), hsa-miR-222 (MS00009177), and the loading control, U6 snRNA (MS00033740) were performed using the miScript SYBR Green PCR Kit. For measurement of p27^{Kip1} mRNA, the QuantiTect Primer Assays for human p27 (QT00998445) and the loading control, human α -tubulin (QT01678264) were performed using the QuantiTect SYBR Green One-step RT-PCR (Qiagen). Target expression was normalized to the expression of loading control for each sample and the fold difference between samples was calculated using the $2^{-\Delta\Delta C_t}$ method.

2.3 Cell Culture

VSMCs were isolated from the IMA as described [9]. VSMCs were seeded in 96-well plates and incubated in Smooth Muscle Basal Medium (Lonza Group Ltd.) supplemented with 0.5% fetal bovine serum for 24 hours. Proliferation was stimulated with Smooth Muscle Growth Medium-2 (Lonza) and incorporation of bromodeoxyuridine was measured using an enzyme linked immunoassay (Roche Diagnostics). Inhibition of miR-221/222 was achieved using a previously reported antisense oligonucleotide [7] and elevation of miR-221/222 in VSMCs was achieved using miScript miRNA Mimics (Qiagen) for miR-221 and miR-222. Transfection of VSMCs was performed as previously described [10]. Anti-miR miRNA

Inhibitors Negative Control #1 (Qiagen) was used as a non-targeting control oligonucleotide.

2.4 Statistics

All data are expressed as the mean $\pm$ standard error of the mean (SEM). For comparisons of categorical variables Chi-square analysis was used. Student's t-test was used to compare the means of two samples. Statistical analysis between multiple groups was performed using one way ANOVA, and Tukey's HSD test was used to compare the individual mean values.

3. Results

3.1 Population characteristics

IMAs were collected from a total of 37 subjects undergoing coronary artery bypass grafting and stratified according to the donor's diabetic status. Initial analysis indicated that those diabetic subjects on metformin therapy may exhibit different miR-221/222 levels than the other diabetic subjects. Therefore, the samples were further stratified in three groups; non-diabetic subjects (n=18, ND), diabetic subjects whose current medications include metformin (n=10, DMMet+), and diabetics whose current medications do not include metformin (n=9, DMMet-). The demographics of these groups were similar for gender, race, smoking, presence of hypertension, and statin use (Table 1). Both diabetic groups exhibited a higher average body mass index than the ND group, and the DMMet+ group was significantly younger than the ND and DMMet- groups. Lipid profiles were not significantly different across the groups, although a trend toward lower high density lipoprotein and increased triglycerides was seen in the DMMet+ group. Between the two diabetic groups, there was not a significant difference in haemoglobin A1c, duration of diabetes, or the distribution of subjects on insulin, sulfonylureas, or thiazolidinediones. No subjects were currently receiving dipeptidyl peptidase-4 inhibitors or exenatide.

3.2 miR-221/222 are elevated in the arteries of subjects with type 2 diabetes

The levels of miR-221/222 were measured in whole tissue homogenates of the IMAs. The DMMet- group exhibited significantly higher levels of both miR-221/222 compared to the ND group (Fig. 1a). Unexpectedly, we found that the DMMet+ group exhibited levels of miR-221/222 that were comparable to the ND group. The levels of miR-221/222 did not correlate with age, body mass index, or serum lipid concentrations, and remained significantly associated with the subject stratification after adjustment for age, body mass index, and serum lipids ($p < 0.05$). There was also a significant inverse correlation between the dose of metformin and the level of miR-221/222 (Fig. 1b, $R^2 = 0.3577$, $p < 0.01$; $R^2 = 0.245$, $p < 0.05$, respectively). The use of insulin or sulfonylureas was not associated with a significant difference in miR-221/222.

3.3 Downstream effects of elevated miR-221/222 are present in the IMAs of subjects with type 2 diabetes

Down-regulation of the cyclin-dependent kinase inhibitor, p27^{Kip1}, is a key mechanism in the promotion of VSMC proliferation and intimal thickening by miR-221/222 [7]. The DMMet- group exhibited significantly decreased mRNA for p27^{Kip1} compared to the ND and DMMet+ groups (Fig 2a). Additionally, VSMCs isolated from the IMAs of DMMet- group exhibited an increased proliferation rate compared with the ND and DMMet+ groups (Fig. 2b). Together these data describe a potential molecular and functional effect of increased miR-221/222.

3.4 Proliferation of VSMCs isolated from IMAs is sensitive to alterations of miR-221/222 levels

To confirm that miR-221/222 mediate the increased proliferation of VSMCs isolated from the IMAs of the DMMet- group, we measured proliferation of VSMCs isolated from both the DMMet- and ND groups in response to growth medium following transfection with an antisense oligonucleotide that inhibits both miR-221 and -222. While there was little change in the proliferation of VSMCs from the ND group, the VSMCs from the DMMet- IMAs had significantly reduced proliferation following inhibition of miR-221/222 (Fig 3a). Similarly, transfection with miR-221 or miR-222 mimics produced increased proliferation in VSMCs from ND IMAs (Fig 3b). These data support a role for miR-221/222 in the increased proliferation seen in the VSMCs from IMAs of the DMMet- group.

Discussion

The data presented here demonstrate that diabetes is associated with increased expression of miR-221/222 in the vasculature that is coupled with a reduction of p27^{Kip1} mRNA and an increase in VSMC proliferation rate. Previously, miR-222 was found to be elevated in adipose tissue of the Gyoto-Kakizaki rat model of type 2 diabetes [11] and incubation in high glucose medium promoted an increase in miR-221 in human umbilical vein endothelial cells [12]. In contrast, miR-222 is decreased in the serum of subjects with gestational diabetes mellitus [13]. In morbidly obese subjects, serum levels of miR-222 is increased, while miR-221 is decreased [14]. Thus, expression of these microRNAs in response to diabetic factors may be tissue specific and dynamic.

Increased miR-221/222 expression in VSMCs leads to reduced levels of p27^{Kip1}, increased VSMC proliferation and migration, and increased intimal thickening following arterial injury [6–8]. Inhibition of miR-221/222 reduces proliferation of VSMCs isolated from DMMet- group while having minimal effect on VSMCs from the ND group. It is possible that given a more robust stimulus than the growth medium used here, a difference in the VSMCs of the ND group might be observed, as has been reported in normal VSMCs [7]. We also confirmed that elevation of miR-221/222 leads to increased proliferation in VSMCs from IMAs of ND subjects. In both cases the magnitude of change is similar to the increase observed directly between the VSMCs of the IMAs from the ND and DMMet- groups.

Loss of p27^{Kip1} serves to accelerate atherosclerosis [15,16] and increased p27^{Kip1} retards atherosclerotic plaque formation [17]. Given the nature of the study, we could not confirm a dependence of the reduced p27^{Kip1} levels and the increased VSMC proliferation on the elevation in miR-221/222 seen in the DMMet- group. However, under normal conditions, p27^{Kip1} is regulated at the post-translational level, so elevation of miR-221/222 represents an earlier stage repression of p27^{Kip1}. Together these data suggest a potential new mechanism for the increased intimal thickening associated with diabetes [1].

The fact that the subjects on metformin exhibit lower levels of miR-221/222 was an unexpected finding of this study. Metformin is biguanide derivative that is effective as an oral hypoglycaemic agent, best described as an activator of 5' adenosine monophosphate-activated protein kinase (AMPK), but additional AMPK-independent mechanisms of action of metformin exist. In the vasculature, metformin inhibits inflammation, reduces reactive oxygen species production, and blocks VSMC proliferation [18,19]. Clinically, metformin therapy is associated with reductions in carotid intima-media thickness in subjects with type 2 diabetes [20].

This is a cross-sectional observational study with limitations that should be noted. All IMA samples were obtained from subjects undergoing coronary artery bypass grafting and

therefore all groups represent subjects with coronary artery disease. The IMA is not traditionally prone to atherosclerosis and may not represent the state of the coronary or other arteries prone to atherosclerosis. In our laboratory, we have observed a similar increase of miR-221/222 in the carotid artery plaques obtained from diabetic subjects undergoing carotid endarterectomy (unpublished data). However, this analysis is complicated by the presence of the plaque and its impact on miR-221/222 levels. Measuring miR-221/222 levels in IMAs provides an assessment of the effects of diabetes on the vasculature in the absence of the effects of arterial disease.

In summary, the IMAs of diabetics exhibit elevated levels of miR-221/222, which are known to promote intimal thickening in animal models of vascular injury. This increase in miR-221/222 was accompanied by a decrease in p27^{Kip1} mRNA and an increase in the proliferation rate of VSMCs isolated from these IMAs. Interestingly, IMAs collected from subjects currently on metformin therapy exhibited a reversal of each of these factors. These studies provide a basis for future studies into whether diabetes promotes an increase in intimal thickening through an up-regulation of miR-221/222, as well as studies examining the ability of metformin to reverse the increase in miR-221/222.

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Abbreviations

AMPK	5' adenosine monophosphate-activated protein kinase
CVD	cardiovascular disease
IMA	internal mammary artery
VSMC	vascular smooth muscle cell

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Fig. 1. miR-221/222 is elevated in the IMAs of the DMMet- group in a manner sensitive to metformin therapy. (A), miR-221/222 levels in the IMAs of the ND, DMMet-, DMMet+ groups. (B), X-Y Scatterplot of miR-221/222 levels versus metformin dose for the diabetic patients in the study. Data are expressed as the ΔC_t value for miR-221/222 for each sample. Data represent the mean $\pm$ SEM. Brackets indicate $p < 0.05$.

Fig. 2.

The DMMet- group exhibits decreased p27^{Kip1} mRNA levels and VSMCs isolated from these IMAs proliferate at an increased rate. (A), Levels of p27^{Kip1} mRNA in the IMAs of the ND, DMMet-, DMMet+ groups. (B), Proliferation of VSMCs isolated from IMAs of the ND, DMMet-, DMMet+ groups. Data are normalized to unstimulated VSMCs from each group. Data represent the mean $\pm$ SEM. Brackets indicate $p < 0.05$.

Fig. 3.

Proliferation of VSMCs isolated from the IMAs are sensitive to changes in miR-221/222 levels. (A), Proliferation of VSMCs isolated from IMAs of the ND and DMMet- groups following transfection with either non-targeting control oligonucleotide (NT) or an antisense to miR-221/222 (Anti-miR). (B), Proliferation of VSMCs isolated from IMAs of the ND group following transfection with either non-targeting control oligonucleotide (NT) or mimics of miR-221 and miR-222. Data are normalized to VSMCs from the ND group transfected with non-targeting control oligonucleotide. Data represent the mean $\pm$ SEM. Brackets indicate $p < 0.05$.

Table 1

Characteristics of the sample population

Characteristic	ND	DMMet–	DMMet+	<i>p</i> value*
<i>n</i> (%)	18 (49%)	10 (27%)	9 (24%)	
Demographics				
Male (%)	15 (83%)	5 (50%)	6 (67%)	0.17
African-American (%)	0 (0%)	2 (20%)	1 (11%)	0.22
Smoker (%)	9 (50%)	3 (30%)	5 (56%)	0.34
Hypertension (%)	17 (94%)	9 (90%)	8 (89%)	0.85
Statin use (%)	15 (83%)	9 (90%)	9 (100%)	0.42
Age (yrs)	65.9 ± 2.3	68.7 ± 3.7	55.4 ± 2.7	0.02
Body Mass Index (kg/m ²)	27.6 ± 1.2	34.8 ± 1.4	31.9 ± 1.4	>0.01
Lipid Panel				
HDL (mg/dL)	43.1 ± 3.7	36.3 ± 2.7	29.5 ± 2.7	0.05
LDL (mg/dL)	103.6 ± 9.1	89.7 ± 9.4	80.8 ± 14.8	0.35
Total (mg/dL)	172.2 ± 12.5	158.9 ± 10.7	154.5 ± 18.9	0.63
TriG (mg/dL)	124.8 ± 16.1	164.2 ± 16.1	241.8 ± 79.0	0.07
Diabetes				
Hemoglobin A1c (%)	5.5 ± 0.4	7.0 ± 0.5	9.6 ± 0.8	0.78
Duration of DM (yrs)		10.4 ± 2.9	9.3 ± 4.0	0.75
Insulin use (%)		4 (40%)	4 (44%)	1.00
Sulfonylurea use (%)		2 (20%)	3 (33%)	0.63
Thiazolidinediones use (%)		1 (10%)	1 (11%)	1.00

HDL: Serum High Density Lipoprotein; LDL: Serum Low Density Lipoprotein; Total: Total Serum Cholesterol; Trig: Serum Triglycerides:

Data are presented as *n* (%) or means ± SEM

* χ^2 test for categorical variables; ANOVA with Tukey's HSD test for continuous variables.