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Usefulness of Clopidogrel to Protect Against Diabetes-Induced Vascular Damage

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Abstract

Clopidogrel enhances the levels of endothelial nitric oxide and prostacyclin in tissue culture. We have previously described a marked increase in circulating endothelial cells (CECs), an ex vivo indicator of vascular injury, in patients with type 2 diabetes mellitus. We hypothesized that clopidogrel treatment would result in a decrease in CEC number and increased activity of endothelial progenitor cell recruitment signaling pathways in diabetic patients. CECs were isolated from the peripheral blood of 9 patients with type 2 diabetes using anti-CD146-coated Dynabeads. The cells were stained with acridine orange and counted by fluorescence microscopy. Endothelial progenitor cells were isolated in a similar fashion using anti-CD34 and anti-CD133 and assayed for expression of phosphorylated Akt and phosphorylated adenosine monophosphate kinase. The patients were then treated with clopidogrel 75 mg/day for 30 days, after which repeat blood specimens were analyzed. As previously observed, diabetic patients had an elevated number of CECs (mean 79 ± 15 cells/ml peripheral blood), which was reduced by clopidogrel treatment (mean 10 ± 4 cells/ml; $p < 0.001$). This was associated with a significant increase in the expression of both phosphorylated Akt and phosphorylated adenosine monophosphate kinase ($p = 0.05$). In conclusion, clopidogrel reduces endothelial cell sloughing and increases expression of endothelial progenitor cell phosphorylated Akt and phosphorylated adenosine monophosphate kinase in the peripheral blood of patients with type 2 diabetes mellitus. This represents a novel mechanism by which this agent can promote improved vascular function, protect against oxidative stress, inhibit apoptosis, and attenuate vascular damage in patients with diabetes mellitus.

Recent work has suggested that, in addition to its primary effect on platelet function, clopidogrel has anti-inflammatory capability¹ and enhances endothelial production of nitric oxide and prostacyclin in tissue culture.² This, in concert with its ability to inhibit CD40 ligand both in vitro and in vivo,³ suggests that clopidogrel possesses the ability to preserve endothelial function by a mechanism that might be independent of its antiplatelet activity.

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Among other effects, CD40 ligand blockade stimulates heme oxygenase 1 (HO-1) expression.⁴ We have previously demonstrated a marked increase in circulating endothelial cells (CECs) in patients with type 2 diabetes mellitus and have documented the beneficial effects of HO-1 on endothelial preservation in diabetes, in both animal and human models.^{5,6} We, therefore, hypothesized that clopidogrel administration would reduce endothelial cell sloughing, as measured by CEC levels, and increase endothelial progenitor cell (EPC) recruitment signaling pathways in patients with type 2 diabetes mellitus.

Methods

The inclusion criteria were a previous diagnosis of type 2 diabetes mellitus, documentation of chronic hyperglycemia with hemoglobin A1c of $\geq 6\%$ at diagnosis, ≥ 18 years old, either gender, and any race. The patients could be treated with metformin, sulfonylureas, acarbose, and/or supplementary insulin but not with thiazolidinediones or thienopyradines. Exclusion criteria included uncontrolled hypertension (blood pressure $>140/90$ mm Hg), history of stroke, creatinine >2 mg/dl, and malignancy.

After enrollment, an initial blood specimen was assayed for mature CECs, after which patients began treatment with clopidogrel, 75 mg/day, taken orally for 1 month. The hemoglobin A1c level, body mass index, age, gender, and coincident medication use were also recorded at entry. Clopidogrel was discontinued after 1 month of therapy, at which time a second blood specimen was obtained. No other medication changes were made during the month of treatment. All participants provided written informed consent, and the Committee for Protection of Human Subjects, the institutional review board of New York Medical College, approved the study. All investigations were performed in accordance with the principles of the Declaration of Helsinki, as revised in 2000.

A 15-ml sample of peripheral blood was obtained, and the first 7.5 ml was discarded. Of the remaining sample, 2 ml of blood was diluted with 2 ml of phosphate-buffered saline, 0.1% bovine serum albumin sodium azide, and 20 μ l FcR blocking agent (Miltenyi, Gladbach, Germany) and incubated with 100 μ l anti-CD146-coated, 45- μ m Dynabeads (1.4×10^8 coated beads/ml) for 30 minutes at 4°C in a Dynal mixer (Dynal, Lake Success, New York) at 50 rpm. Cells bound to anti-CD146-coupled beads were separated from blood in a Dynal magnet, washed (3 washings using phosphate-buffered saline and 0.1% bovine serum albumin and repetitive mixing for 5 minutes in the Dynal mixer at 4°C), and dissolved in 100 μ l buffer. Side-by-side assays were performed with Dynabeads coated with human antibodies against mouse IgG but without anti-endothelial antibody to check for nonspecific binding to the Dynabeads. The cells were mixed with acridine and incubated with 10 μ l *Ulex* (1:1,000) for 2 hours at room temperature, visualized by light and fluorescence microscopy, respectively, and counted in a Nageotte chamber (Brand, Wertheim, Germany). The cells were also stained with anti-von Willebrand factor VIII antibody. CECs were quantitated by counting performed in parallel using both methods (Figure 1).

Nonspecific binding of leukocytes to Dynabeads, presumably by interaction with Fc receptors, has been well documented, and the following steps were taken to avoid it. The cell suspension was flushed vigorously through a 20- μ L pipette tip, and citrate was added to the

buffer with F_c blocking agent to prevent this problem. Side-by-side assays with M-450 Dynabeads coated with human antibodies against mouse IgG but without anti-endothelial antibody were also performed to check for nonspecific binding to Dynabeads. Isolated cells were stained with antibodies to von Willebrand factor (Boehringer, Mannheim, Germany), CD31 (Binding Site, Heidelberg, Germany), and *Ulex europaeus* lectin 1 (Linaris, Wertheim, Germany) and with acridine.

Concurrently, EPCs were isolated in a similar fashion using anti-CD34 and, subsequently, anti-CD133. The cells were detached from the beads, suspended in endothelial medium (endothelial growth medium-2), and plated in collagen-coated 6-multiwell plates. The growth medium was changed after 3 days, and the colonies were assessed by light microscopy at 5 days. The lack of hematopoietic contaminants was tested using a fluorescence-activated cell sorter and was negative for CD45.

EPCs were suspended in buffer and centrifuged at 27,000g for 10 minutes at 4°C. The supernatant was isolated, and the protein levels were determined by immunoblotting with antibodies against phosphorylated Akt and phosphorylated adenosine monophosphate kinase (Cell Signaling Technology, Beverly, Massachusetts) prepared in a 1:5,000 dilution. In brief, 20 µg of supernatant was separated by 12% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Immunoblotting was performed as previously described.⁵ Chemiluminescence detection was performed with the Amersham ECL detection kit (Amersham, Piscataway, New Jersey), according to the manufacturer's instructions.

Patients functioned as their own controls, with the first specimen obtained at entry compared to the second specimen obtained after 1 month of treatment. We have previously documented a mean of 70 cells/ml of whole blood in patients with type 2 diabetes mellitus compared to 10 cells/ml in normal controls.⁵ Assuming a minimum of a 60% decrease in CECs/ml in the experimental group, it was determined that a minimum of 8 subjects would need to be studied to reject the null hypothesis with a power of 0.8. Unpaired Mann-Whitney *U*-testing (2-sided) was used for comparison of cell numbers between blood specimens after the Kruskal-Wallis test had been applied to show significant differences between specimens. Statistical significance between the groups was determined by analysis of variance analysis. *p* Values 0.05 were considered statistically significant. The data are presented as the mean ± SE.

Results

A total of 9 subjects (5 men and 4 women, mean age 55.2 years) were enrolled in the present study. The subjects were recruited from the general medical and endocrine clinics of the Westchester Medical Center (Valhalla, New York), and each had been diagnosed with type 2 diabetes mellitus >2 years previously. The subject characteristics are summarized in Table 1. Most patients were medicated with metformin, aspirin, and either an angiotensin-converting enzyme inhibitor or an AT₁ blocker.

Figure 1 depicts the endothelial cells isolated by immunomagnetic beads and stained with acridine and endothelial cells in whole blood identified by *Ulex* staining. As seen in Figure 2, patients with diabetes mellitus had an elevated number of CECs (mean 79 ± 15 cells/ml of peripheral blood, range 20 to 110). After 4 weeks of therapy with clopidogrel, 75 mg/day, a marked reduction in the number of CECs was observed in all subjects to within the normal range (mean 10 ± 4 cells/ml, range 0 to 40; $p < 0.001$).

EPC colony formation after 5 days was noted to be more robust after clopidogrel treatment (Figure 3). Treatment was also associated with a statistically significant increase in the expression of both phosphorylated Akt (from optical density 0.54 ± 0.01 vs β actin to optical density 0.9 ± 0.01) and phosphorylated adenosine monophosphate kinase (from optical density 0.42 ± 0.04 vs β actin to 1.04 ± 0.03 ; $p = 0.05$; Figure 4).

Discussion

The present study is the first in vivo demonstration of the ability of clopidogrel to reduce the number of CECs to normal or near-normal levels in patients with type 2 diabetes mellitus. It is also the first to demonstrate a clopidogrel-facilitated increase in EPC recruitment associated with a reprogramming of EPC signaling components, as manifested by the restoration of the activities of phosphorylated adenosine monophosphate kinase and phosphorylated Akt.

Endothelial damage reflects an imbalance between the magnitude of vascular injury and the capacity for repair. A variety of evidence has suggested that oxidative stress induces endothelial injury and that impaired endothelial function reflects this ongoing injury. Oxidative stress-mediated endothelial cell injury, and a concurrent increase in CECs are seen in many cardiovascular diseases, including myocardial infarction, unstable angina,⁷ coronary artery disease,⁸ and pulmonary hypertension.⁹ Several reports^{7,8} have suggested that a close correlation exists between cardiovascular disease activity and the extent to which endothelial cell debris is released from lesions associated with ongoing recent inflammation. We have previously reported a marked increase in the number of CECs in patients with type 2 diabetes mellitus compared to controls.⁵

The mechanism by which endothelial cells detach from the vessel wall remains unknown; however, a number of potential stimuli have been postulated, including oxidative stress and the release of proteases and cytokines.¹⁰ In vitro studies have suggested that CECs actively inhibit proliferation, migration, and endothelial nitric oxide synthase (eNOS) expression in the EPCs of patients with vasculitis.¹¹

Activation of Akt promotes the survival of endothelial cells by inhibiting endothelial apoptosis,¹² stimulating nitric oxide synthesis,¹³ and mediating vascular endothelial growth factor-induced cell migration.¹⁴ The enhanced differentiation and augmentation of EPCs by statins is mediated by the phosphoinositide 3-kinase/Akt pathway.¹⁵ Endothelial activation of adenosine monophosphate kinase inhibits hyperglycemia-induced apoptosis.¹⁶ In contrast, adenosine monophosphate kinase deficiency is associated with an increase in myocardial reperfusion injury.¹⁷ Adenosine monophosphate kinase has recently been

identified as a target for pharmacologic intervention to improve endothelial function in patients with diabetes mellitus.¹⁸

Activation of both of these signaling pathways enhances the activity of eNOS.^{19,20} Uncoupling of eNOS, with resultant superoxide anion formation, is associated with a reduction in the number and function of EPCs in patients with diabetes mellitus.²¹ In contrast, eNOS activation is essential for EPC recruitment in an eNOS-deficient mouse model.²² Our laboratory has recently reported that EPC recruitment was inhibited in a rat model of type 2 diabetes mellitus and that this was reversed by an increase in adiponectin and phosphorylated adenosine monophosphate kinase with resultant increases in both eNOS and phosphorylated eNOS.²³

Clopidogrel stimulates nitric oxide-dependent coronary vasodilation in a guinea pig model.²⁴ A single dose of clopidogrel has been reported to enhance brachial artery flow-mediated vasodilation in patients with stable coronary disease.²⁵ In patients with type 2 diabetes mellitus, clopidogrel therapy has been associated with a reduction in the expression of platelet surface p-selectin, platelet-monocyte binding, and platelet-neutrophil binding, a concurrent reduction of the expression of CD40 and CD11b on the monocyte surface, and a reduction in plasma RANTES.²⁶ In contrast, withdrawal of clopidogrel in patients with diabetes has been associated with an increase in high-sensitivity C-reactive protein and p-selectin in platelets both at rest and adenosine diphosphate-stimulated platelets.²⁷

In a subset of the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial, clopidogrel therapy was independently associated with a decrease in vascular death, myocardial infarction, stroke, and rehospitalization for ischemia or bleeding compared to aspirin use in patients with diabetes.²⁸ In vitro exposure of hepatocytes to ticlopidine, a similar thienopyridine, resulted in induction of HO-1.²⁹ We have previously demonstrated the salutary effect of HO-1 on glucose-mediated endothelial injury and repair.⁶ However, the identification of the P2Y₁₂ receptor in human coronary artery and umbilical vein endothelial cells suggests the possibility that endothelial function might be enhanced by a clopidogrel-specific mechanism rather than through the activity of an intermediary such as HO-1.³⁰ This would represent a novel additional mechanism through which clopidogrel promotes improved vascular function, protects against oxidative stress, inhibits apoptosis, and attenuates vascular damage in patients with diabetes.

Our study had several limitations. Although prospective, it was a pilot trial. Thus, the study population was small, and the outcome measures were confined to a limited number of elements. Notwithstanding, the differences measured were robust and serve to generate hypotheses for additional study.

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Figure 1. Morphology of CECs with Dynabeads attached in blood from patients with type 2 diabetes mellitus. Typical CECs captured by Dynabeads coated with anti-CD146 under light (*a*) or fluorescent microscopy (*b*). CECs stained with acridine orange, isolated by Dynabeads, and stained with *Ulex europaeus* agglutinin 1 lectin.

Figure 2.

CECs in 9 patients with diabetes mellitus before and after 1 month of treatment with clopidogrel 75 mg/day (mean $\pm$ SE). Numbers of CECs were measured as described in the “Methods” section. Number of CECs decreased significantly in patients with diabetes to a mean of 10 ± 4 CEC/ml of pooled blood ($p < 0.001$) after 1 month of clopidogrel therapy. Previous studies in our laboratory have reported a mean of 10 ± 5 CEC/ml in nondiabetic control subjects.

Figure 3. Representative EPC colonies on day 5 from human peripheral blood harvested before (*a*) and after (*b*) 1 month of clopidogrel treatment. Colony formation was markedly more robust after treatment.

Figure 4.

Western blot of EPC supernatant (a) with antibodies against phosphorylated Akt (pAkt) and phosphorylated adenosine monophosphate kinase (pAMPK) from peripheral blood harvested before and after clopidogrel treatment. Densitometry analysis of (b) phosphorylated Akt and (c) phosphorylated adenosine monophosphate kinase versus actin eluted from EPCs in peripheral blood of subjects with diabetes harvested before and after clopidogrel treatment. Statistically significant increase in both phosphorylated Akt and phosphorylated adenosine monophosphate kinase expression ($p < 0.05$) after clopidogrel treatment.

Table 1

Patient characteristics

Pt.No.	Age (years)	Gender	Risk		Medications																		
			D	SH	A	ACEI	AT ₁	St	F	E	βB	αB	Su	D4-I	M	I	IM	T	W	T	PPI	S	
1	31	Female	0	0	+	+	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
2	42	Female	+	0	+	+	0	+	0	0	0	0	0	0	0	0	+	0	0	0	0	0	0
3	50	Male	+	+	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0	+	+
4	52	Female	+	+	+	0	+	+	0	0	0	0	0	0	0	0	+	0	+	0	0	0	0
5	59	Male	+	+	+	+	0	+	+	0	+	0	+	0	+	0	0	0	0	0	0	0	0
6	60	Male	+	+	+	0	0	+	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0
7	61	Male	+	+	+	+	0	+	0	+	0	+	0	+	0	0	0	0	0	0	0	0	0
8	65	Male	+	+	+	+	0	+	0	+	0	0	+	0	0	0	+	0	0	0	0	0	0
9	75	Female	+	+	+	+	0	+	0	+	0	0	+	0	0	0	+	0	0	+	+	0	0

A = aspirin; α B = alpha blocker; ACEI = angiotensin-converting enzyme inhibitor; AT₁ = AT₁ blocker; β B = beta blocker; D = dyslipidemia; D4-I = dipeptidyl peptidase-4 inhibitor; E = ezetimibe; F = fibrate; I = insulin; IM = incretin mimetic; M = metformin; PPI = proton pump inhibitor; S = sildenafil; SH = systemic hypertension; St = statin; Su = sulfonylurea; T = thiazide; W = warfarin.