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Effects of Clopidogrel Therapy on Oxidative Stress, Inflammation, Vascular Function and Progenitor Cells in Stable Coronary Artery Disease

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Abstract

Background—Traditional cardiovascular risk factors lead to endothelial injury and activation of leucocytes and platelets that initiate and propagate atherosclerosis. We proposed that clopidogrel therapy in patients with stable CAD imparts a pleiotropic effect that extends beyond anti-platelet aggregation to other athero-protective processes.

Methods—Forty-one subjects were randomized in a double-blind, placebo-controlled crossover study to either clopidogrel 75 mg daily or placebo for 6-weeks, and then transitioned immediately to the other treatment for an additional 6 weeks. We assessed 1) endothelial function as flow-mediated dilation of the brachial artery, 2) arterial stiffness and central augmentation index using applanation tonometry, 3) vascular function as fingertip reactive hyperemia index, 4) inflammation by measuring plasma CD40 ligand and serum high-sensitivity c-reactive protein levels, 5) oxidative stress by measuring plasma aminothiols, and 6) circulating progenitor cells, at baseline and at the end of each 6-week treatment period.

Results—Clopidogrel therapy resulted in a significant reduction in soluble CD40 ligand (p=0.03), a pro-thrombotic and pro-inflammatory molecule derived mainly from activated platelets. However, clopidogrel therapy had no effect on endothelial function, arterial stiffness, inflammatory and oxidative stress markers, or progenitor cells.

Conclusions—Our findings suggest a solitary anti-platelet effect of clopidogrel therapy in patients with stable CAD, with no effect on other sub-clinical markers of cardiovascular disease risk.

Keywords

Anti-platelet; Atherosclerosis; Endothelial function; Oxidative stress; Progenitor cells

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Introduction

Coronary artery disease (CAD) is the leading cause of mortality and morbidity in the Western world[1]. Traditional CAD risk factors such age, hypertension, diabetes, hyperlipidemia, and smoking lead to endothelial injury and activation of leucocytes and platelets, which initiate and propagate development of atherosclerosis[2]. Platelet activation leads to release of adenosine diphosphate, fibrinogen binding, and subsequent bridging that results in platelet aggregation[3]. Although the role of clopidogrel, an inhibitor of ADP-mediated platelet activation, in the treatment of acute coronary syndrome and prevention of stent thrombosis after percutaneous coronary intervention is well established[4-7], its role in the management and treatment of stable CAD is less clear[8]. Notably, the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial found that dual anti-platelet therapy with aspirin and clopidogrel resulted in a significant reduction in cardiovascular events in a pre-specified subgroup of patients with established cardiovascular disease[9]. However, the mechanisms underlying such benefit remain unclear.

Platelets, upon activation, release CD40 ligand and P-selectin (CD62P) leading to a localized inflammatory response that has been linked to progression of atherosclerosis[10-12]. Levels of CD40 ligand and CD62P on platelets were attenuated and endothelial function improved after clopidogrel in previous studies, suggesting that there might be a favorable link between effectiveness of clopidogrel therapy and reduction of pro-atherogenic stimuli[13-16]. It is well accepted that other processes including oxidative stress, inflammation, and impaired reparative potential mediated by progenitor cells contribute to endothelial dysfunction and adverse cardiovascular outcomes[17-19]. However, whether platelet inhibition by thienopyridine therapy influences these pathways in patients with clinically stable atherosclerosis, and therefore impacts global atherosclerotic risk has not been well investigated.

Our central hypothesis was that the mechanisms underlying the benefits of dual-antiplatelet therapy in CAD extend beyond anti-platelet aggregating effects to other atheroprotective processes. Specifically, we proposed that clopidogrel's pleiotropic effects include reduction of inflammation, improvement in oxidative stress, amelioration of endothelial dysfunction, and mobilization of progenitor cells. Our primary hypotheses were that addition of clopidogrel therapy in patients with stable CAD would 1) increase circulating numbers of progenitor cells, and 2) improve endothelial dysfunction. Our secondary hypotheses were that clopidogrel would 1) improve arterial elasticity indices and microvascular function, 2) reduce inflammation, and 3) reduce oxidative stress.

Methods

Study Population and Study Design

Forty-eight subjects with stable CAD documented by an abnormal non-invasive stress test, previous myocardial infarction, or coronary angiography were recruited from the patient population at the various Emory healthcare sites and 41 subjects completed the study

(Clinicaltrial.gov/NCT01283282). Exclusion criteria included 1) treatment with clopidogrel in the preceding 3 months; 2) acute coronary syndrome or percutaneous coronary intervention in the previous 3 months; 3) premenopausal women with potential for pregnancy; 4) initiation or change in the dose of any concomitant medical therapy within the previous 2 months; 5) ongoing treatment with coumadin; 6) chronic renal or liver failure; 7) cancer or chronic inflammatory diseases requiring anti-inflammatory therapies; 8) recent acute infections; 9) history of substance abuse; 10) significant valvular heart disease; or 11) > NYHA Class II heart failure.

Risk factors including age, gender, race, hypertension (defined as systolic blood pressure ≥140 mmHg or diastolic blood pressure of ≥90 mm Hg, or treatment with antihypertensive medications), diabetes (both insulin and non-insulin dependent), current or prior tobacco smoking, serum total cholesterol levels, triglyceride levels, high density lipoprotein (HDL) levels, and low density lipoprotein (LDL) levels were recorded. Written informed consent was obtained and the study was approved by the Emory University Institutional Review Board.

Forty-one study subjects were randomized in a double-blind, placebo-controlled crossover study to receive either clopidogrel 75 mg daily or identical placebo for 6 weeks, in addition to a standard stable medical regimen for CAD including aspirin and statins. After the initial 6-week treatment period, all recruited subjects were crossed-over immediately, without a washout period, to the opposite arm for another 6 weeks, thus serving as their own controls. Measurements were made at the end of each 6-week treatment period.

Measurement of Endothelial Function

Endothelium–dependent and –independent vascular function was estimated using brachial artery flow–mediated vasodilation (FMD) and nitroglycerin–mediated vasodilation, respectively, as described previously[20]. In our laboratory, the mean difference in FMD between assessments performed in 11 subjects on consecutive days was 1.26±0.76%, with a correlation coefficient of 0.75. The mean difference in the FMD between 2 readings was 0.82±0.48% (r=0.97).

Measurement of Arterial Stiffness

Arterial stiffness was estimated by measuring pulse wave velocity (PWV) and pulse wave analysis using the Sphygmocor device (Atcor Medical, Australia)[21]. PWV was measured between carotid and femoral arteries and provided a regional assessment of aortic stiffness. Briefly, pressure waveforms at the carotid and femoral arterial were acquired using EKG gating. Velocity [distance/time in m/s] was calculated using the “foot-to-foot” method and the distance between the sites was measured manually.

Pulse wave analysis was performed on the pulse waveform acquired from the radial artery, which estimates central (proximal) aortic pressures and the degree of pressure augmentation secondary to reflected waves from the periphery. This permits derivation of an augmentation index (AIX) (augmentation index = augmented pressure/total central pulse pressure), which is considered a composite marker of arterial stiffness and wave reflections or *systemic*

stiffness. Due to its sensitivity to heart rate, a standardized value to 75 bpm (AIX75) was employed in this study[22]. Quality control indices were evaluated at the time of study and non-acceptable readings were discarded and repeated. Reproducibility studies in our laboratory on 9 subjects on consecutive days have demonstrated a coefficient of variation of 3.8% and 20.3% for PWV and AIX, respectively.

Peripheral Arterial Tonometry (PAT)

During the FMD testing above, digital vascular function was also assessed simultaneously as fingertip reactive hyperemia (Endo-PAT2000, Itamar Medical, Israel) as described previously[23]. Briefly, pneumatic plethysmographs apply uniform pressure at both index fingers, which allow for measurement of minute pulsatile volume changes by generating a pulse wave tracing. Reactive hyperemia index (RHI) is defined as the ratio of post-deflation to baseline pulse amplitude in the hyperemic finger divided by that in the contra-lateral finger.

Markers of Oxidative Stress and Inflammation

Oxidative stress was estimated by measuring plasma cysteine and glutathione, their oxidized disulfides, cystine and GSSG, and their ratios. Detailed descriptions of procedures and techniques for measuring these and other plasma aminothiols have been reported previously[24]. Briefly, samples were collected directly into specially prepared tubes containing a preservative to retard auto-oxidation, centrifuged, and the resultant supernatant frozen at -80°C. Analyses by high performance liquid chromatography were performed after dansyl derivatization on a 3-aminopropyl column with fluorescence detection. Metabolites were identified by co-elution with standards and quantified by integration relative to the internal standard, with validation relative to external standards. Issues of sample collection, stability, analysis, and standardization have been extensively studied and this method has been used in several clinical studies [25].

CD40 ligand and high-sensitivity C-reactive protein (hs-CRP) were measured on plasma and serum, respectively. Blood samples were centrifuged for 15 minutes at 1000g, followed by another centrifugation at 10000g for 10 minutes for complete platelet removal. Samples were then stored at -80°C for future batch analysis. CD40 ligand was measured using the Fluorokine Multi Analyte Profiling (MAP) Human Base Kit B from R&D Systems (Minneapolis, USA) on a Luminex 100 Bio-Plex platform (Bio-Rad, CA USA). The calculated inter-assay variability for this assay was 1.5%. Hs-CRP was measured by Dade Behring nephelometry (Deerfield, Illinois).

Measurement of Circulating Progenitor cells

Circulating progenitor-enriched populations were estimated by measuring the expression of surface antigens using direct flow cytometry analysis (BD FACS Canto II Flow Cytometer). Approximately 300 microliter of venous blood (anticoagulant: EDTA) was incubated with fluorochrome-labeled monoclonal mouse anti-human antibodies, namely, FITC-CD34 (BD Biosciences), PerCP-CD45 (BD Biosciences), PE-VEGF2R (R&D system - also known as “Kinase insert Domain Receptor-KDR”), and APC-CD133 (Miltenyi) for 15 minutes. Red blood cells were removed by lysis in 1.5 milliliter of ammonium chloride lysing buffer,

which was added to the sample and incubated for an additional 10 minutes. The lysis process was stopped by adding 1.5 milliliter of staining medium (PBS with 3% heat-inactivated serum and 0.1% sodium azide). Five million events were acquired from the Cytometer with Flowjo software (Treestar, Inc.) used for subsequent analysis of accumulated data. Absolute cell counts for target cell subsets were determined using an indirect cell estimation method. The total absolute white cell count and absolute counts for lymphocytes, monocytes, and granulocytes was determined using a Coulter ACT/Diff cell counter (Beckman Coulter). The absolute mononuclear cell count was then estimated as the sum of lymphocytes and monocytes. The frequency of our target cell populations was reported as a subset of the mononuclear cell compartment (MNCs). This frequency (% of MNCs) was then multiplied by the absolute count of mononuclear cells to calculate the absolute count of our target cells, which are a component of the mononuclear cell compartment and reported as cell counts/μl.

Circulating cell populations commonly considered to be enriched for hematopoietic and endothelial progenitors include those expressing CD34+, CD133+, and VEGF2R+ surface markers among a CD45 medium population, either singly or in combination. The CD34+ population represents hematopoietic progenitors from which subsequent lineages including endothelial progenitors are derived[26]. The CD133+ subset of CD34+ cells are believed to include immature or early progenitors as the marker subsequently disappears as they mature, while CD34 positivity with VEGF2R markers are widely considered to be a population enriched for endothelial progenitors[27-29]. We thus examined the frequency in the peripheral circulation of CD34+, dual positive CD34+/CD133+, dual positive CD34+/VEGF2R+, and triple positive CD34+/CD133+/VEGF2R+ cell populations among the CD45 medium population. Twenty samples were repeatedly analyzed on two occasions by two technicians. The percent repeatability coefficients (%) were calculated as SD of differences between pairs of measurements/mean of measurements*100. The repeatability coefficients for the various cell types were: CD34+: 7.4%, CD133+: 7.0%, CD34+/CD133+: 4.4%, CD34+/VEGF2R+ 16.3%, and CD34+/CD133+/VEGF+: 19.2%.

Statistical Analysis

Continuous variables are summarized as mean ± standard error (SE), and categorical variables as counts and proportions. The study design required a paired analysis in which continuous variables were compared using the *t* test if they appeared normally distributed or after logarithmic transformation for heavily skewed distributions. Data analyses were performed using SAS statistical software version 9.2. P values are two-sided with a significance level of ≤0.05. Based on previous studies, we expected that a sample size of 41 subjects would have the power to observe a 70% change in CD34+ cell count and a 1.5% change in FMD[30].

Results

Baseline characteristics of subjects are summarized in Table 1. Subjects were mainly male, white, with a history of hypertension, and on a stable medical regimen that included aspirin and statins in the majority.

Effect of clopidogrel on markers of inflammation

There was a significant reduction in the level of soluble CD40 ligand with clopidogrel therapy (p=0.03, Table 2). In comparison, HsCRP levels remained statistically unchanged.

Effect of clopidogrel on aminothiol markers of oxidative stress

Glutathione and its oxidized metabolite (GSSG), cysteine and its oxidized metabolite (cystine), and their ratios remained unchanged with clopidogrel therapy as compared to placebo (Table 2).

Effect of clopidogrel on vascular function

Three different functional vascular indices were measured. These included brachial artery endothelium-dependent and -independent function, microvascular function using PAT-derived RHI, and arterial elasticity indices including AIX75 and PWV. None of these measures changed significantly with clopidogrel therapy compared to placebo (Table 2).

Effect of clopidogrel on circulating progenitor cell populations

We measured several circulating progenitor cell-enriched populations. The hematopoietic progenitors expressing CD34+ and those enriched for endothelial progenitor subsets including CD34+/CD133+, CD34+/VEGF2R+, and CD34+/CD133+/VEGF2R+ were all unchanged during clopidogrel therapy as compared to the placebo treatment period (Table 2, Supplementary Figure 1).

In subgroup analyses, we also investigated whether the biomarkers and vascular function tests were significantly improved in the subgroup where soluble CD40 ligand levels were significantly reduced (>median), but found no differences.

Discussion

In this randomized, double blind, placebo-controlled crossover study, we demonstrate that clopidogrel therapy in patients with stable CAD significantly lowers soluble CD40 ligand levels. However, when a wide spectrum of biomarkers from other pathophysiological pathways involved in the genesis and progression of atherosclerosis were tested, no significant effects of clopidogrel were observed after 6 weeks of therapy as compared to placebo. Specifically, we observed no changes in systemic inflammation estimated as HsCRP levels, aminothiol measures of oxidative stress, endothelium-dependent flow-mediated vasodilation and endothelium-independent nitroglycerin-mediated dilation of the brachial artery, digital reactive hyperemia, and arterial elasticity measures with clopidogrel therapy. Finally, circulating populations of cells enriched for hematopoietic and endothelial progenitors also remained unchanged.

CD40 ligand is expressed on the membrane of activated platelets, immune cells, and certain vascular cells. It is cleaved to generate the water soluble fragment called soluble CD40 ligand, and the majority of the circulating soluble CD40 ligand is derived from platelets[10]. Experimental studies highlight the pro-thrombotic and pro-inflammatory activities of soluble CD40 ligand molecule, which may play an important role in the pathogenesis of

atherosclerosis and acute coronary syndrome[31, 32]. Despite significant reduction in soluble CD40 ligand levels by clopidogrel therapy in this study of a stable CAD population, we did not find a reduction in any other sub-clinical measures of atherosclerosis.

HsCRP is frequently employed as a marker of inflammation, which predicts future CAD or its adverse events[33, 34]. Studies suggest that clopidogrel is most effective in patients with the highest level of CRP and increases in CRP induced by percutaneous coronary interventions may be attenuated with clopidogrel[35, 36]. However, previous investigations also failed to demonstrate any significant change in hsCRP levels in stable CAD patients with clopidogrel[13, 14].

Decreased levels of nitric oxide in the presence of endothelial dysfunction or atherosclerosis promote platelet aggregation, inflammation, and release of growth factors[2, 37]. Conversely, inhibition of platelet aggregation also improves nitric oxide bioavailability in the microcirculation as we have shown with aspirin, and Heitzer and colleagues have found with clopidogrel using acetylcholine infusions[15, 38]. In a study of patients with stable CAD that also employed ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery, a single loading dose of clopidogrel 300mg or 600mg resulted in improvement in flow-mediated dilation[16]. However, our study failed to show a sustained effect of clopidogrel therapy on ultrasound measured brachial artery flow-mediated vasodilation after 6 weeks. Moreover, arterial stiffness and wave reflections are known to contribute to cardiovascular morbidity and mortality, but we found no changes in these parameters with clopidogrel therapy[39].

We also investigated the effect of clopidogrel on circulating progenitor cell populations. Several recent studies including ours have shown a reduction in the number and migratory activity of circulating progenitor cells in patients with cardiovascular disease (CVD), with a negative correlation between cell counts or function of progenitor cells and endothelial function, CVD risk factors, and adverse CVD outcomes[18, 40-42]. We found no change in any of the circulating subsets of progenitor-enriched cell populations with clopidogrel therapy. Whether this was because of concomitant therapy with statins and angiotensin antagonists, that are known to improve circulating progenitor cells, remains to be investigated.

Major aminothiol compounds constitute indices of non-free radical oxidant stress and can be quantified in plasma to assess oxidative stress burden in vivo[43]. Of these, cysteine constitutes the major thiol pool *extracellularly* that reacts readily with oxidants to form its oxidized disulfide cystine. *Intracellularly*, glutathione is a major antioxidant that helps eliminate peroxides and maintain cellular redox, and its oxidized form is GSSG[44]. Increased oxidative stress, measured as lower levels of glutathione, higher levels of cystine, or altered ratios of reduced to oxidized thiols, is associated with risk factors for CVD, subclinical vascular disease, CVD itself, and adverse cardiovascular outcomes[43, 45, 46]. We found no reduction in oxidative stress with clopidogrel, which is in agreement with the lack of improvement in endothelial function in this cohort. Even the subgroup of subjects with the highest level of oxidative stress did not have significant reduction in their levels with clopidogrel therapy.

Limitations

There are some strengths and limitations to our study. The strength is that it is the most comprehensive investigation of the effects of clopidogrel on markers of CVD risk and vascular function in patients with stable CAD to date. The lack of effects on markers of inflammation, oxidative stress, and vascular function may have been secondary to our limited sample size of 41 subjects. Furthermore, it is possible that patients with acute coronary syndrome and post coronary intervention may continue to experience benefits in these markers which are activated in these clinical situations, and thus our findings cannot be extended to these subsets. It is also plausible that a longer treatment period may have beneficial effects on these markers, which warrants further investigation.

Conclusions

In summary, our study shows that 6 weeks of oral clopidogrel therapy in patients with stable CAD reduces soluble CD40 ligand levels, but none of the other sub-clinical markers of CVD risk. These findings suggest a solitary anti-platelet effect of clopidogrel in this clinical setting. The lack of any substantial effects on other sub-clinical risk factors of atherosclerosis progression is in agreement with clinical trial data demonstrating few benefits of clopidogrel in a broad population of patients at high risk for atherothrombotic events[8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1
Baseline Characteristics of Study Population

Characteristic	All Patients (N=41)
Age (years, Mean ± SE)	63 ± 1.5
Male sex N(%)	26 (63)
Caucasian race N(%)	34 (83)
Hypertension N(%)	32 (78)
Diabetes Mellitus N(%)	9 (22)
Current and previous tobacco smoking (%)	22 (54)
History of Myocardial Infarction N(%)	22 (54)
History of Percutaneous Coronary Intervention N(%)	38 (93%)
History of Coronary Artery Bypass Graft Surgery N(%)	2 (5%)
Medications	
Aspirin N(%)	39 (95)
Statins N(%)	34 (83)
Beta-blockers N(%)	27 (66)
Angiotensin converting enzymes inhibitors N(%)	21 (51)
Calcium channel blockers N(%)	9 (22)
Fasting Lipid Profile (Mean ± SE)	
Total cholesterol (mg/dL)	163 ± 7
Triglycerides (mg/dL)	128 ± 11
High density lipoprotein (mg/dL)	53 ± 3
Low density lipoprotein (mg/dL)	87 ± 6
Oxidative Stress Markers (Mean ± SE)	
Cystine (μM)	84.11 ± 6.12
Cysteine (μM)	12.89 ± 1.13
Eh Cysteine/Cystine Ratio	-75.37 ± 1.88
Glutathione (μM)	1.56 ± 0.12
Oxidized glutathione (μM)	0.28 ± 0.11
Eh Glutathione/Oxidized Glutathione Ratio	-129.89 ± 3.25
Inflammatory Markers (Mean ± SE)	
Hs-CRP (mg/L)	2.44 ± 0.43
CD40 Ligand (pg/ml)	1558.01 ± 360.41
Vascular Function (Mean ± SE)	
Flow Mediated Vasodilation (%)	4.90 ± 0.59
Nitroglycerin Mediated Vasodilation (%)	18.54 ± 1.58
Reactive Hyperemia Index	2.40 ± 0.80
Augmentation Index at 75 beats per minute	25.67 ± 1.47
Pulse Wave Velocity (m/s)	8.75 ± 0.37
Endothelial Progenitor Cells (Mean ± SE)	
CD34+ (cells/μl)	1.22 ± 0.13
CD34+/133+ (cells/μl)	0.69 ± 0.07

Characteristic	All Patients (N=41)
CD34+/VEGF2R+ (cells/μl)	0.08 ± 0.02
CD34+/CD133+/VEGF2R+ (cells/μl)	0.07 ± 0.02

Table 2

Comparison of oxidative stress markers, inflammatory markers, vascular function measures, and endothelial progenitor cells after the clopidogrel and placebo treatment periods (n=41)

Characteristics	Clopidogrel (Mean ± SE)	Placebo (Mean ± SE)	p-value
Oxidative Stress Markers (Mean ± SE)			
Cystine (μM)	105.25 ± 7.36	102.39 ± 7.29	0.71
Cysteine (μM)	13.71 ± 0.90	14.75 ± 0.89	0.39
Eh Cysteine/Cystine Ratio	-77.06 ± 1.76	-78.72 ± 1.74	0.73
Glutathione (μM)	1.52 ± 0.17	1.71 ± 0.17	0.28
Oxidized Glutathione (μM)	0.08 ± 0.06	0.19 ± 0.06	0.19
Eh Glutathione/Oxidized Glutathione Ratio	-130.62 ± 2.30	-128.47 ± 2.27	0.47
Inflammatory Markers (Mean ± SE)			
Hs-CRP (mg/l)	5.98 ± 1.95	4.31 ± 1.95	0.55
CD40 Ligand (pg/ml)	**1202.21 ± 318.35**	**2169.32 ± 318.35**	**0.03***
Vascular Function (Mean ± SE)			
Flow-mediated vasodilation (%)	4.89 ± 0.59	4.81 ± 0.59	0.89
Nitroglycerin-mediated vasodilation (%)	19.31 ± 1.49	17.10 ± 1.51	0.17
Reactive Hyperemia Index	2.50 ± 0.80	2.20 ± 0.70	0.29
Augmentation Index at 75 beats per minute	25.04 ± 1.63	23.64 ± 1.62	0.26
Pulse Wave Velocity (m/s)	8.77 ± 0.45	9.044 ± 0.45	0.46
Endothelial Progenitor Cells (Mean ± SE)			
CD34+ (cells/μl)	1.46 ± 0.16	1.54 ± 0.21	0.77
CD34+/CD133+ (cells/μl)	0.68 ± 0.07	0.75 ± 0.16	0.71
CD34+/VEGF2R+ (cells/μl)	0.08 ± 0.03	0.09 ± 0.02	0.70
CD34+/CD133+/VEGF2R+ (cells/μl)	0.03 ± 0.009	0.03± 0.008	0.96