Original article

Effect of clopidogrel on circulating biomarkers of angiogenesis and endothelial activation

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Summary  Angiogenic cytokines have been shown to influence vessel injury, and platelets represent a disposable circulating pool of angiogenic molecules. In the present study, objectives were to determine whether clopidogrel could have a potential effect on levels of circulating biomarkers of angiogenesis and endothelial activation.

We explored 28 healthy white male volunteers treated for 7 days with clopidogrel 75 mg/day. We quantified angiogenic growth factors that have been shown to be correlated to cardiovascular risk or endothelial progenitor cell mobilization such as vascular endothelial growth factor (VEGF)-A and its soluble receptor forms VEGFR1 and VEGFR2, placenta growth factor, and stromal cell-derived factor-1. We also quantified soluble E-selectin and von Willebrand factor to evaluate endothelial activation. Blood samples were drawn just before the first clopidogrel intake on day 1, and after the last dosing (day 7).

As expected, we observed a decrease in platelet reactivity in response to clopidogrel, confirmed by vasodilator-stimulated phosphoprotein phosphorylation assay. However, the 7-day intake of clopidogrel did not significantly modify the levels of the selected angiogenic factors or biomarkers of endothelial activation.

KEYWORDS
Angiogenic factors; Endothelial activation; Clopidogrel; Platelet; P2Y12

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Introduction

Platelet adhesion, activation, and aggregation are considered as the initial steps in thrombus formation. The key role of platelet activation in atherothrombosis and restenosis after percutaneous coronary intervention (PCI) [1,2] is underlined by the efficacy of antiplatelet therapy with aspirin and clopidogrel in these indications [3,4]. Once recruited to the proximity of the vessel wall, platelets may release large amounts of either proinflammatory molecules or angiogenic growth factors, such as vascular endothelial growth factor (VEGF) or stromal cell-derived factor (SDF)-1 [5,6]. Moreover, the treatment of human platelets with different agonists results in the release of pro- or anti-angiogenic containing granules [7]. Thus, separate packaging of angiogenesis regulators might be differently modulated by antiplatelet agents. These pharmacological molecules targeting platelet activation pathways might modify the mechanisms by which platelets can locally stimulate or inhibit angiogenesis [8]. Beside their role in limiting platelet activation, there is increasing evidence that antiplatelet agents also exert anti-inflammatory actions. Indeed, reduced levels of CD40 ligand, C-reactive protein or P-selectin have been found in patients taking antiplatelet agents, either aspirin or clopidogrel [9,10].

Clopidogrel targets the ADP P2Y12 receptor on platelets, responsible for platelet irreversible aggregation in response to ADP. However, recent data suggest further roles for this receptor. First, platelet P2Y12 receptor has been shown to influence vessel wall responses to injury and thrombosis [11,12]. Second, P2Y12 has been evidenced on other cell types, including smooth muscle cells [13] and endothelial cells [14,15]. A role for P2Y12 has been recently suggested in endothelial function with the demonstration of an improvement of endothelial dysfunction in patients with stable coronary artery disease after a loading dose of clopidogrel [16] and an improvement in endothelial nitric oxide bioavailability in patients with symptomatic coronary artery disease, suggesting that P2Y12 blockade may have vasoprotective effects [17]. Finally, P2Y12 blockade by clopidogrel has been recently found to decrease circulating endothelial cells (CEC) levels in type 2 diabetes [18] and to prevent their release after PCI [19].

Angiogenic cytokines have been shown to influence vessel injury, and platelets represent a disposable circulating pool of angiogenic molecules. We hypothesized that clopidogrel could have a potential effect on physiological levels of circulating biomarkers of angiogenesis and endothelial activation.

Methods

Subjects and study protocol

To be free from hormonal status or of any interference of angiogenic cytokines released by circulating activated platelets in patients with cardiovascular diseases, we explored only healthy male volunteers. Twenty eight subjects aged 18–35 years were enrolled, based on the following criteria: nonsmokers, normal physical and laboratory results (including white and red blood cell count, liver enzymes, blood glucose, serum creatinine, prothrombin time, activated partial thromboplastin time, and plasma fibrinogen, results reported in Table 1). The subjects were asked to come to the Clinical Investigations Center every day at 9 AM, where they were given a standard dose of clopidogrel 75 mg once daily for 7 consecutive days in the presence of medical staff. Blood samples were drawn just before the first clopidogrel intake on day 1, and after the last dosing (day 7). The objective of this study was to evaluate the response to clopidogrel according to P2Y12 genotype (H1H1 and H1H2 haplotypes) as detailed elsewhere [20,21]. The sample size was calculated with the hypothesis of a 25% difference in aggregation between groups with a risk alpha of 5% and beta 20%. The present study was further performed given that no difference in platelet response to clopidogrel was noticed according to the genotype and that baseline angiogenic growth factor levels did not differ between groups.

The study was approved by the "Comité de Protection des Personnes se Prêtant à la Recherche Biomedicale" (Paris-Cochin, France), and all the subjects gave their written informed consent to participate, in accordance with the Declaration of Helsinki.

Pharmacodynamic evaluation

We assessed the level of phosphorylated vasodilator-stimulated phosphoprotein (VASP), a good index of P2Y12 activity [21,22]. P2Y12 is a G1-coupled receptor whose activation reduces the platelet cyclic adenosine monophosphate (cAMP) level by inhibiting adenylyl cyclase. The decrease in cAMP production leads to a reduction in the activation of specific protein kinases, which can no longer phosphorylate VASP. The level of phosphorylated VASP, which can be measured by flow cytometry, is thus a good index of inhibition of the ADP-P2Y12 interaction by clopidogrel. VASP was measured in whole blood on days 1 (baseline) and 7 of clopidogrel intake, using a flow cytometric assay (Platelet VASP; Diagnostica Stago, Biocytex, Asnières, France) adapted to a Cy-Flow apparatus (Partec, Münster, Germany). Results were expressed as a platelet reactivity index (PRI, %) calculated from the mean fluorescence intensity (MFI) of samples incubated with prostaglandin (PGE1) alone or with both PGE and ADP simultaneously, using the following formula: \((\text{MFI}_{\text{PGE1+PGE}} - \text{MFI}_{\text{PGE1}})/\text{MFI}_{\text{PGE1}} \times 100\).

ELISA assays

We quantified angiogenic growth factors that have been shown to be correlated to cardiovascular risk or endothelial...
Table 1 Biological characteristics of the subjects.

<table>
<thead>
<tr>
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<th>N = 28</th>
</tr>
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<tbody>
<tr>
<td>Blood leukocyte count (g/µL)</td>
<td>5.3 (3.2–8.1)</td>
</tr>
<tr>
<td>Red blood cell count (tera/µL)</td>
<td>4.7 (4.2–5.3)</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.2 (13.2–15.4)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>42 (39–45)</td>
</tr>
<tr>
<td>Blood platelet count (giga/µL)</td>
<td>216 (153–321)</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>4.8 (3.4–5.4)</td>
</tr>
<tr>
<td>Aspartate transaminase (IU/L)</td>
<td>19 (11–44)</td>
</tr>
<tr>
<td>Alanine transaminase (IU/L)</td>
<td>21 (12–50)</td>
</tr>
<tr>
<td>Alkaline phosphatase (IU/L)</td>
<td>52 (31–82)</td>
</tr>
<tr>
<td>Gamma-glutamyltransferase (IU/L)</td>
<td>19 (5–40)</td>
</tr>
<tr>
<td>Serum creatinine (µmol/L)</td>
<td>83 (63–111)</td>
</tr>
<tr>
<td>Plasma total protein (g/L)</td>
<td>90 (73–100)</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (APTT ratio)</td>
<td>1.08 (0.9–1.2)</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.2 (1.9–3.5)</td>
</tr>
</tbody>
</table>

Data are presented as median values (range).

progenitor cell mobilization such as VEGF-A and its soluble receptor forms, placenta growth factor (PIGF), and SDF-1. We also quantified soluble E-selectin and von Willebrand factor (VWF) as a reflection of endothelial activation. All markers but VWF were quantified in citrated plasma with Elisa Quantikine test (R&D Systems, Lille, France). VWF was quantified with Asserachrom® VWF Antigen kit (Stago, Courtaboeuf, France).

Statistical analysis

Student’s paired t test was used to compare endothelial biomarkers before and after clopidogrel treatment. StatView software was used for all statistical analyses, and p-values below 0.05 were considered to denote significant differences.

Results

Subjects were given clopidogrel 75 mg once daily during 7 days, with no loading dose. Pharmacodynamic response to clopidogrel was assessed by the VASP assay (as PRI). On day 7, the PRI had significantly decreased compared to baseline, from 72 ± 6% to 45 ± 17% (p < 0.001, Fig. 1).

Baseline plasma levels of VEGF, PIGF, SDF1, sVEGFR1, sVEGFR2, E-selectin, and VWF were all within the normal range reported by our group and others [23]. As shown in Fig. 2, a 7-day intake of clopidogrel did not significantly modify the levels of the selected angiogenic factors (VEGF, p = 0.15; PIGF, p = 0.78; SDF1, p = 0.30), angiogenic receptor soluble forms (sVEGFR1, p = 0.66; sVEGFR2, p = 0.88), nor biomarkers of endothelial activation (E-selectin, p = 0.65; VWF, p = 0.64; Fig. 3). Moreover, no correlation was found between the difference between day 7 and baseline value of each parameter and VASP PRI.

These results show that: (1) circulating angiogenic factor level is not driven by P2Y12 platelet receptor-induced

Figure 1 Vasodilator-stimulated phosphoprotein (VASP) phosphorylation level (platelet reactivity index, %) at baseline and after a 7-day clopidogrel 75 mg intake.

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Figure 2 Comparison of vascular endothelial growth factor (VEGF), stromal cell-derived factor (SDF)-1, placenta growth factor (PIGF), and soluble receptors of VEGF (sVEGFR-1 and sVEGFR-2) levels in 28 healthy male volunteers after 7 days of clopidogrel. The median VEGF value (range) was 12.1 pg/mL [3.3–207.5] at day 1 and 10.6 pg/mL [2.3–23.3] after 7 days of clopidogrel. The median SDF-1 value (range) was 2015 pg/mL [1373–2690] at day 1 and 1910 pg/mL [1435–2690] after 7 days of clopidogrel. The median PIGF value (range) was 4.6 pg/mL [1.5–9.9] at day 1 and 5.2 pg/mL [2.6–8.4] after 7 days of clopidogrel. The median sVEGFR-1 value (range) was 84.7 pg/mL [49.9–1334] at day 1 and 92.4 pg/mL [50.6–237.4] after 7 days of clopidogrel. The median sVEGFR-2 value (range) was 1673.5 pg/mL [1074–2394] at day 1 and 1595 pg/mL [1311–2080] after 7 days of clopidogrel.
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Figure 3 Comparison of sE-selectin and von Willebrand factor (VWF) levels in 28 healthy male volunteers after 7 days of clopidogrel. The median sE-selectin value (range) was 26.3 ng/mL [12.1 – 44.8] at day 1 and 268.6 ng/mL [100 – 45.6] after 7 days of clopidogrel. The median VWF antigen value (range) was 69.2% [28.9 – 134.9] at day 1 and 70.0% [35.6 – 117.2] after 7 days of clopidogrel.

Discussion

To our knowledge, this is the first study exploring the effect of clopidogrel on angiogenic and endothelial activation soluble biomarkers. Clopidogrel is widely used in patients with coronary artery disease (CAD) and/or PCI to reduce the risk of thrombotic events [24–29]. The VASP index is the most specific platelet assay to evaluate P2Y12 ADP receptor blockade and is not influenced by other antiplatelet agents or anticoagulants [22]. VASP index was shown to have a high negative predictive value for major adverse cardiovascular events [30,31]. Despite the large well-known inter-individual variability in the response to clopidogrel, we did not find any modification in angiogenic molecule levels upon clopidogrel treatment and no correlation according to P2Y12 inhibition, as assessed by the VASP index. Identical results were obtained with the level of 10 μM ADP-induced platelet aggregation inhibition (data not shown).

Using VASP index, optimal clopidogrel responsiveness was recently found associated with reduced CEC elevation following PCI [19]. Increased levels of CEC have been identified across a broad spectrum of cardiovascular [32] and pulmonary diseases [33,34] and have been linked to the clinical severity, as well as to the clinical outcomes of CAD [32]. Several studies have highlighted the interest of CEC count in the early evaluation of endothelial dysfunction in patients undergoing PCI [35–37]. ADP receptor P2Y12 blockade was shown to independently predict CEC level, demonstrating a protective effect of clopidogrel-dependent inhibition of platelet reactivity on endothelial damage during PCI. The mechanisms underlying the protection of the endothelium by clopidogrel remain to be fully elucidated, in particular by distinguishing specific endothelial protection in PCI or angiogenic modification whatever the clinical context. Moreover, one of the main questions remains: is the endothelial protection dependent on endothelium–platelet interactions?

Indeed, findings from animal models and in vitro studies have identified platelet-independent effects of thienopyridines on vascular cells. A modulation of vasoconstrictors after intravenous administration of clopidogrel [38] and an increase in nitric oxide production in endothelial cells incubated with thienopyridines have been previously described [39]. Moreover, previous work showed that ADP increased human endothelial cell migration, thus potentially contributing to reendothelialization and angiogenesis after vascular injury, by activating P2Y1 receptor mediated mitogen-activate protein kinase pathway, but not via P2Y12 [40]. In platelets, P2Y1 is known to be involved in shape change and in reversible platelet aggregation, contrasting with its potential important function in endothelial cells. Our results could add relevance to the non significant implication of P2Y12 in endothelial cell biology, in line with clopidogrel specificity against P2Y12. In addition, platelet release generated by activation with ADP has been recently shown to promote migration and formation of capillary structures by human umbilical vein endothelial cells in in vitro angiogenesis models [41]. Thus, the inhibition of P2Y12 activation by clopidogrel might interfere with circulating VEGF or other growth factors released by platelets. However, no modification was observed in our study.

One limitation of this work is the small sample size of our population that may not allow detection of minor differences. However, the absence of any trend whatever the biomarker tested argues against a major effect. The effect of clopidogrel on endothelial function remains to be explored in cardiovascular patients, in which atherothrombosis mediators, such as nicotine, may increase P2Y12 endothelial expression level [14]. Moreover, short or long-term clopidogrel treatment has been proposed to act like an anti-inflammatory drug [42–44], and P2Y12 receptor has been shown to be required for proinflammatory actions [45]. Because inflammation and angiogenic markers have been found linked in different clinical settings and in endothelial progenitor cell mobilization, a modulation of angiogenic markers could be one of the effects of clopidogrel. In our setting, i.e. physiological situation, we showed that P2Y12 is not involved in angiogenesis homeostasis, with the limitation of the parameters studied.

Another limitation of our study is the dose of clopidogrel that could be insufficient to influence the level of angiogenic soluble factors. However, the antiplatelet effect of clopidogrel has been demonstrated at the early beginning to prevent ischemic events in patients with atherosclerosis [46] at a recommended dose of 75 mg/day. The significant decrease of VASP index shown in Fig. 1 confirms that clopidogrel 75 mg/day modifies platelet functions but we have no idea of the required dose to modify platelet-independent functions of clopidogrel. Thereby, angiogenic and endothelial markers should also be explored following clopidogrel loading dose or with maintenance doses higher than 75 mg/day, and particularly with the new more potent antiplatelet agents (prasugrel, ticagrelor, anti-PAR1).
In conclusion, the receptor P2Y12 inhibition by clopidogrel is not associated with a modification of physiological levels of endothelial/angiogenesis biomarkers in healthy subjects. It remains to be determined how clopidogrel P2Y12 blockade on cells other than platelets could have consequences in atherothrombosis and/or vessel injury and remodeling.

Acknowledgments

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