Differential effects of cilostazol and pentoxifylline on vascular endothelial growth factor in patients with intermittent claudication

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ABSTRACT

Cilostazol is a new phosphodiesterase inhibitor with anti-platelet and vasodilatory properties. Cilostazol and pentoxifylline are the only two drugs that have been approved for the treatment of patients with intermittent claudication. However, the mechanisms by which exercise tolerance is improved remain unclear. Vascular endothelial growth factor (VEGF) is a potent endothelial mitogen that results in angiogenesis when overexpressed in human subjects. To assess the potential role of VEGF in the improvement in exercise tolerance, we investigated plasma levels of VEGF in 50 patients with intermittent claudication who were allocated randomly to groups receiving cilostazol (n = 17), pentoxifylline (n = 17) or placebo (n = 16). Patients given either cilostazol or pentoxifylline showed a significant improvements in maximal walking distance compared with the placebo group (34 m and 33 m respectively, compared with 5 m; both P < 0.05). Neither cilostazol nor pentoxifylline increased the ankle-brachial index after treatment. Circulating VEGF levels were increased (from 116 ± 29 to 169 ± 45 pg/ml; P = 0.002), and the levels of VEGF were correlated significantly with exercise tolerance in a positive direction (r = 0.88, P = 0.004), in those patients treated with cilostazol that did not have diabetes mellitus. In contrast, VEGF levels remained stable after the administration of pentoxifylline. These findings suggest that VEGF may contribute to the cilostazol-related improvement in exercise tolerance in non-diabetic patients. However, pentoxifylline did not affect VEGF levels, although a similar improvement in maximal walking distance was achieved. Thus the mechanisms involved in the pentoxifylline-treated group were different from those in the cilostazol-treated group, and require further study.

INTRODUCTION

The prevalence of peripheral arterial occlusive disease (PAOD) of the lower extremities increases with age. Haemodynamically significant PAOD manifests as intermittent claudication (IC) in 1–7% of men aged 50–75 years [1]. Numerous drugs have been studied for the treatment of IC, including prostaglandins, vasodilatory agents, anticoagulants and anti-platelet drugs [2]. Cilostazol is a novel phosphodiesterase inhibitor that has been used for treatment of PAOD. Cilostazol consistently improved patients’ walking on treadmill testing [3,4].

Key words: cilostazol, intermittent claudication, pentoxifylline, peripheral vascular disease, phosphodiesterase inhibitor, vascular endothelial growth factor.

Abbreviations: ABI, ankle-brachial index; IC, intermittent claudication; MWD, maximal walking distance; PAOD, peripheral arterial occlusive disease; VEGF, vascular endothelial growth factor.

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However, the mechanisms responsible for this improvement in exercise tolerance remain unclear. Dawson et al. [4] have demonstrated a significant increase in the ankle-brachial index (ABI) after cilostazol treatment, implying an important role for the ABI in the improvement of IC.

The development of collaterals is an important adaptive process following PAOD. Perfusion insufficiency, and the resultant hypoxia, induces a compensatory neovascularization to satisfy the needs of the tissue. Vascular endothelial growth factor (VEGF) is a potent endothelial-specific mitogen [5]. The ability of VEGF to induce the formation of a collateral circulation in ischaemic extremity has been demonstrated in animals [6,7] and in patients [8]. Claudicants may be subjecting their endothelium to repeated damage due to the cycles of ischaemic reperfusion, which are a possible consequence of walking [9]. The cyclical attack of ischaemic reperfusion will induce VEGF release. VEGF can increase vascularity, and improve blood flow and muscle function through angiogenesis in an ischaemic limb [10]. Cilostazol and pentoxifylline are the only two drugs that have been approved by the U.S. Food and Drug Administration for the pharmacological treatment of IC. Thus we tested whether the improvement in maximal walking distance (MWD) after cilostazol treatment is mediated by increased levels of VEGF release, and compared these effects with those induced by pentoxifylline.

METHODS

Patient selection
Eligible patients were more than 40 years old, and had IC with no symptomatic changes in the previous 3 months. The presence of PAOD was defined by a Doppler-measured ABI of < 0.9. Eligible patients had a baseline MWD of between 30 and 200 m.

Patients with Buerger’s disease, category II or III chronic lower-extremity ischaemia [11], or arterial surgery/angioplasty or sympathectomy within the previous 3 months were excluded. Patients were instructed to avoid aspirin-containing products, non-steroid anti-inflammatory agents, anticoagulants and ticlopidine.

The patients were not given specific risk factor modification instructions, such as diet control, exercise or smoking cessation. All medications were kept constant throughout the study.

Study design
The study was designed to be a prospective, randomized trial conducted in a single medical centre. After a 2-week placebo run-in phase, eligible patients were assigned randomly to receive cilostazol (n = 17) (100 mg, twice a day), pentoxifylline (n = 17) (400 mg, twice a day) or placebo (n = 16) for 8 weeks in a double-blind manner.

The dose of pentoxifylline was chosen because previous studies have shown that pentoxifylline is effective in improving IC at a dose of 800 mg/day [12]. Each patient received a randomized code number, according to which sponsor supplied the study drug. Special drug packaging was used to maintain the blindedness of the treatment code. A sealed envelope, with information on the treatment allocated, was kept in the clinical file of each patient. In an emergency, the seal could be broken and the treatment code disclosed, at the discretion of the case physician. The study was conducted in accordance with good clinical practices and local regulations. The protocol was approved by an ethical committee, and patients were required to sign an informed consent form before undergoing screening procedures.

Study procedures
Clinical examination, vital signs and blood tests were recorded at baseline and at the end of the study. Treadmill exercise tests were performed on the two screening visits, and at the end of the study. Treadmill exercise tests were performed at 3.2 km/h and with a 12.5% gradient, under the supervision of the same operator and at the same time of day for a given patient throughout the trial. The distance that had been walked when the patient could not proceed was defined as the MWD. Randomization and initiation of study drug treatment were allowed only if treadmill testing at the two screening tests demonstrated variance of ≤ 20% in MWD. The change in MWD was the main outcome.

Determination of plasma VEGF levels
To avoid VEGF release during platelet aggregation [13], venous blood was heparinized. Blood was taken from all subjects in the morning after an overnight fast. The plasma was stored at −70 °C until assayed. Plasma VEGF levels in each sample were measured in duplicate using a human VEGF ELISA kit (Quantikine human VEGF; R&D System, Minneapolis, MN, U.S.A.) according to the manufacturer’s instructions. The detection limit was 9 pg/ml. The interassay variability was < 5%.

Statistics
The analysis was performed on the set of patients who completed the trial and had a valid treadmill test and blood tests at baseline and at the end of the study. All statistical analyses were performed by using SAS software (version 6.12; SAS Institute, Cary, NC, U.S.A.). Continuous variables are expressed as means ± S.D. Because many variables were not normally distributed, non-parametric statistical tests were used throughout. The differences in continuous parameters among the three groups were compared by the Kruskal–Wallis test. For the categoric parameters, differences were compared by
the chi-squared test and the Fisher exact test if the case number was < 5. Linear regression models were used to compute the association between the changes in MWD and VEGF in the cilostazol and pentoxifylline groups. A \( P \) value of < 0.05 was considered statistically significant.

**RESULTS**

Cilostazol was very well tolerated by all patients, and none showed any significant subjective side-effects. Table 1 shows the clinical and biochemical characteristics of the three study groups. There were no differences in baseline characteristics among these three groups. The blood test results, including white cell count, platelet and glucose concentrations, were stable throughout the study (Table 2). There were no significant changes in ABI during treatment in any of the three groups, and ABI values did not differ between groups.

### Table 1  Demographic data of patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cilostazol (n = 17)</th>
<th>Pentoxifylline (n = 17)</th>
<th>Placebo (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>66 ± 9</td>
<td>68 ± 5</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>14/3</td>
<td>14/3</td>
<td>14/2</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>60 ± 7</td>
<td>63 ± 9</td>
<td>64 ± 8</td>
</tr>
<tr>
<td>Duration of IC (years)</td>
<td>2.0 ± 2.1</td>
<td>2.7 ± 2.7</td>
<td>2.5 ± 2.1</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>15 (88%)</td>
<td>11 (65%)</td>
<td>11 (69%)</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
<td>9 (53%)</td>
<td>9 (53%)</td>
<td>9 (56%)</td>
</tr>
<tr>
<td>Smokers (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>4 (24%)</td>
<td>4 (24%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>Previous</td>
<td>3 (18%)</td>
<td>2 (12%)</td>
<td>4 (25%)</td>
</tr>
<tr>
<td>Current</td>
<td>10 (59%)</td>
<td>11 (65%)</td>
<td>8 (50%)</td>
</tr>
<tr>
<td>Lipid profile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>202 ± 28</td>
<td>216 ± 43</td>
<td>212 ± 34</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>43 ± 10</td>
<td>55 ± 31</td>
<td>45 ± 18</td>
</tr>
<tr>
<td>LDL (mg/dl)</td>
<td>126 ± 24</td>
<td>129 ± 39</td>
<td>125 ± 29</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>188 ± 131</td>
<td>163 ± 76</td>
<td>184 ± 79</td>
</tr>
</tbody>
</table>

### Table 2  Effects of different treatments on exercise capacity, VEGF, ABI and blood parameters

Data are means ± S.D. \( P < 0.05 \) compared with respective baseline value; \( P < 0.05 \) compared with cilostazol group at 8 weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cilostazol (n = 17)</th>
<th>Pentoxifylline (n = 17)</th>
<th>Placebo (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWD (m)</td>
<td>111 ± 30</td>
<td>145 ± 53*</td>
<td>114 ± 51</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>126 ± 45</td>
<td>236 ± 106*</td>
<td>139 ± 50</td>
</tr>
<tr>
<td>ABI</td>
<td>0.73 ± 0.12</td>
<td>0.69 ± 0.11</td>
<td>0.66 ± 0.13</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4368 ± 589</td>
<td>4415 ± 545</td>
<td>4755 ± 773</td>
</tr>
<tr>
<td>Monocytes</td>
<td>449 ± 126</td>
<td>484 ± 121</td>
<td>443 ± 116</td>
</tr>
<tr>
<td>Platelets (× 10^4)</td>
<td>224 ± 47</td>
<td>251 ± 47</td>
<td>227 ± 74</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>123 ± 52</td>
<td>133 ± 50</td>
<td>114 ± 34</td>
</tr>
</tbody>
</table>

The cilostazol- and pentoxifylline-treated patients walked significantly further distances than the placebo-treated patients (Table 2). The mean changes from baseline after 8 weeks of treatment were 34, 33 and 5 m for patients who had received cilostazol, pentoxifylline and placebo respectively.

Patients treated with cilostazol showed a significant increase in VEGF levels compared with baseline (236 ± 106 compared with 126 ± 65 pg/ml; \( P = 0.001 \)). In the subgroup analysis in the cilostazol-treated group (Table 3), there was a significantly greater improvement in MWD in patients without diabetes mellitus compared with that in those with this condition [from 116 ± 29 m to 169 ± 45 m in the non-diabetic patients (\( P = 0.002 \)); from 108 ± 33 to 124 ± 54 m in the diabetic patients (not significant)]. However, the non-diabetic and diabetic patients showed similar increases in VEGF levels after cilostazol administration. Linear regression analysis for the non-diabetic patients treated with cilostazol showed
Table 3  Effects of diabetes mellitus on exercise, VEGF and ABI in the cilostazol-treated group

Data are means ± S.D. Significance of differences: * P < 0.05 compared with the respective baseline value.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diabetes mellitus (n = 9)</th>
<th>Non-diabetes mellitus (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>8 weeks</td>
</tr>
<tr>
<td>MWD (m)</td>
<td>108 ± 33</td>
<td>124 ± 54</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>125 ± 57</td>
<td>194 ± 83*</td>
</tr>
<tr>
<td>ABI</td>
<td>0.75 ± 0.10</td>
<td>0.70 ± 0.12</td>
</tr>
</tbody>
</table>

Figure 1  Correlation of changes in VEGF and MWD in cilostazol-treated patients presenting with IC

There was a significant correlation between the changes in VEGF and MWD in non-diabetic patients (left panel), but not in diabetic patients (right panel).

that changes in VEGF were correlated with MWD (change in MWD (%) = 0.240 × change in VEGF (%) + 7.825 (r = 0.88, P = 0.004) (Figure 1A). The relationship between these two parameters remained significant (P = 0.04) when the patient who experienced a +132% change in MWD and a +467% change in VEGF levels was excluded. However, the correlation between the changes between MWD and VEGF was not significant in diabetic patients treated with cilostazol (P = 0.76) (Figure 1B), suggesting that other factors play a role in the improvement in MWD. Changes in ABI, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triacylglycerols were not predictive of the change in MWD (P values of 0.53, 0.31, 0.27, 0.26 and 0.61 respectively).

In contrast, there were no significant changes in VEGF levels in patients treated with pentoxifylline compared with baseline (178 ± 91 and 139 ± 50 pg/ml respectively). Patients treated with pentoxifylline did not show a significant correlation between the change in MWD and VEGF levels (r = 0.32, not significant).

DISCUSSION

There were significant improvements in MWD, compared with baseline and placebo results, after 8 weeks of treatment with either cilostazol or pentoxifylline: 30% in the cilostazol group and 29% in the pentoxifylline group (compared with 4% in the placebo group). Concomitant measurements of ABI showed no significant changes, despite clinical walking improvements. Plasma levels of VEGF were increased significantly in non-diabetic patients treated with cilostazol, and were positively correlated with the improvement in MWD. These results indicate that cilostazol modulates exercise tolerance through a VEGF-dependent mechanism in such patients. In contrast, although a similar degree of improvement in MWD was noted in the pentoxifylline group, VEGF levels were not increased significantly, which indicates that the two drugs act via different mechanisms. This study is, to our knowledge, the first to describe a correlation between VEGF levels and exercise tolerance in patients treated with either cilostazol or pentoxifylline.

The improvement in MWD after either cilostazol or pentoxifylline treatment was significant, without a concomitant increase in ABI, suggesting no improvements in blood flow through the large peripheral arteries after treatment. The improvement may have been confined to the microcirculation, which will not be reflected by the ABI. The effects of these drugs on ABI were not consistent with the finding of Dawson et al. [4], who demonstrated a significant increase in ABI after administration of cilostazol. An improvement in the microcirculation is compatible with our proposed mechanisms.

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Increased VEGF levels were associated with increases in arteriole and capillary density [14] at the level of the microcirculation. Thus ABI is not a suitable method for detecting flow changes attributable to the mechanisms of actions of these drugs.

The degree of improvement in MWD that occurred after administration of cilostazol or pentoxifylline was lower (34 m/30% for cilostazol; 33 m/29% for pentoxifylline) than in previous reports. Previous studies recorded differences of 48 m between pentoxifylline and placebo groups and of 106 m between cilostazol and placebo groups [4] for the increase in MWD [15]. A likely explanation is the high incidence of diabetes mellitus among patients in the present study (53%). Waltenberger et al. [16] demonstrated that the angiogenesis response to VEGF is attenuated in patients with diabetes, as a consequence of impaired VEGF-induced and Flt-1-mediated signal transduction. Although previous studies have shown that the serum level of VEGF is elevated in diabetic patients [16], collateral vessel development is impaired in such patients, including those with myocardial [17] as well as lower-extremity [17] ischaemia. Furthermore, recent evidence indicates that nitric oxide mediates cellular VEGF signalling, and that the VEGF-induced angiogenesis effect is inhibited by antagonists of nitric oxide synthase [18]. The requirement for nitric oxide for the induction of collateral vessel development by VEGF also explains, at least in part, the lack of improvement in MWD in diabetic patients, who show impairment of nitric oxide production [19]. Taken together, the cellular response to VEGF is attenuated in diabetic patients due to a signal transduction defect. Thus we postulate that the VEGF-induced process of collateral formation is severely impaired in patients with diabetes. This conclusion is compatible with our results, showing that, although serum levels of VEGF after cilostazol administration were elevated in diabetic patients, MWDs did not show parallel improvements in these subjects.

**Mechanism of action of cilostazol**

Our data confirm previous findings that cilostazol treatment results in a consistent improvement in MWD in patients with IC. The mechanism by which cilostazol improves exercise tolerance in non-diabetic patients appears to involve increasing plasma levels of VEGF. VEGF-induced angiogenesis is correlated with improvements in exercise tolerance [20]. However, the mechanisms responsible for increased VEGF protein expression in patients treated with cilostazol remain unknown. VEGF is produced by a great variety of normal cell types [21]. VEGF expression has been shown to be dramatically up-regulated under hypoxic conditions, induced by critical atherosclerotic lesions [22]. An imbalance between O₂ supply and O₂ demand induces the formation of adenosine [23]. Previous studies have shown that adenosine can stimulate angiogenesis directly by increasing VEGF production in endothelial cells [24]. In addition, analysis of the VEGF promoter has revealed the existence of several potential binding sites for transcription factors, such as AP-1 (activator protein-1), AP-2 and SP1 [25]. Consistent with the presence of these elements, VEGF expression is up-regulated by cAMP. Cilostazol is a phosphodiesterase inhibitor type III, which increases intracellular cAMP levels, hence leading to increased VEGF production. Furthermore, cilostazol increases nitric oxide synthesis in vascular smooth muscle cells through a cAMP-dependent pathway [26]. Nitric oxide has been implicated in the regulation of expression of VEGF [27].

Because skeletal muscle in the placebo group would have been much more ischaemic than in the treated groups, it might be expected that VEGF would be more highly expressed in the placebo-treated subjects. However, we found that VEGF levels were greater after treatment with cilostazol. This can be explained by our contention that hypoxia is not the only factor to stimulate VEGF. Other factors, such as increased intracellular cAMP and nitric oxide, also play a crucial role in stimulating VEGF release. High blood glucose is also associated with raised VEGF levels [28]. However, this is unlikely to have affected the present results, because glucose concentrations were similar before and after treatment with cilostazol.

**Mechanism of action of pentoxifylline**

Pentoxifylline is the most extensively evaluated and widely used drug for the treatment of IC. Porter et al. [29] demonstrated that, after 24 weeks of pentoxifylline treatment, the geometric mean of the percentage change from baseline for MWD was 38%. Although similar clinical improvements in MWD were noted for cilostazol and pentoxifylline in the present study, VEGF levels were not increased in patients treated with pentoxifylline. This result is compatible with previous findings that pentoxifylline inhibited PMA- and hypoxia-induced VEGF secretion [30,31]. The mechanism of action of pentoxifylline is to improve haemorrhheological properties by increasing red blood cell flexibility and blood filterability, and by reducing plasma viscosity, serum fibrinogen levels and erythrocyte aggregation [32,33]. Rheological factors play a key role in predisposing a subject to leg ischaemia in the presence of a given ABI [34]. Improved haemorrhheological properties in the microcirculation can thus have positive effects in conditions of ischaemia.

**Study limitations**

The main limitation of the present study is that our data were obtained from a limited number of carefully selected patients. Hence caution is recommended until other studies confirm these results. Secondly, we did not assess the localization of expression of VEGF protein. VEGF
mRNA is labile under normal oxygen concentrations. However, the expression of VEGF is amplified under cellular hypoxia. Thus it is believed that VEGF could be produced in sites distal to atherosclerotic lesions. Monocytes [35], neutrophils [30] and platelets [36] may be regarded as the main sources of VEGF. However, it is unlikely that a change in the number of monocytes, neutrophils and platelets was responsible for the observed effects. There were no significant changes in differential blood cell counts and platelet numbers after administration of either cilostazol or pentoxifylline. A third limitation is that we focused the study on VEGF as the likely major growth factor in the non-diabetic patients treated with cilostazol. Our study does not, however, exclude the involvement of other factors, such as acidic and basic fibroblast growth factors, hypoxia-inducible factor, platelet-derived growth factor, angiopoietin-1 and local shear stress, in the regulation of angiogenesis. Fourthly, histological and morphometric analysis of capillary density in skeletal muscle, in order to evaluate the angiogenic effects of increased VEGF levels, cannot be carried out due to ethical considerations. Finally, in the present study the diagnosis of IC was based on clinical history and an ABI value of ≤ 0.9. Peripheral angiography was not routinely performed, because of ethical considerations. In comparisons with angiographic studies of the lower extremities, an ABI of ≤ 0.9 has been demonstrated to have a sensitivity and specificity of 96% for occlusive lesions [37].

Conclusions

Treatment with cilostazol may induce increased levels of VEGF in non-diabetic patients, and this effect may lead to improved exercise tolerance. In contrast, although patients treated with pentoxifylline showed similar improvements in MWD, levels of VEGF were unchanged, implying that different mechanisms are responsible for the effects of these two drugs on exercise tolerance.

REFERENCES


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