ORIGINAL ARTICLE

A human model of platelet–leucocyte adhesive interactions during controlled ischaemia in patients with peripheral vascular disease

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Aims: In humans, little is known about the effects of platelet–leucocyte interactions on blood viscosity and microvascular perfusion. This study tested the hypotheses that (1) activation and interactions between platelets and leucocytes may have an impact on microvascular blood viscosity and perfusion in patients with stage II peripheral arterial occlusive disease, and (2) a powerful antiplatelet drug such as Clopidogrel might help to improve microvascular perfusion by reducing platelet–leucocyte activation and blood viscosity.

Methods: Plasma concentrations of certain markers of leucocyte and platelet activation, in addition to low and high shear rate blood viscosity, were measured before and after a repeated exercise treadmill test. Functional parameters including maximum walking time, transcutaneous oxygen pressure, and half recovery time were also measured.

Results: Blocking platelet activation only with a single dose of Clopidogrel (300 mg) did not improve microvascular blood viscosity and perfusion after repeated exercise, but a significant improvement in microvascular perfusion during controlled ischaemia and a lack of post exercise increase in low shear rate blood viscosity was achieved when both platelet and leucocyte activation were suppressed by a relatively longer treatment with Clopidogrel (four days).

Conclusions: Clopidogrel, by inhibiting platelet activation and aggregation, might also block the vicious cycle of leucocyte–platelet activation, thus improving the functioning of the microcirculation.

Recent studies suggest that platelet activity stimulates leucocyte activation and is linked to microvascular dysfunction.[1] Conversely, platelet activation may also be initiated by activated leucocytes, thus triggering a vicious cycle of platelet–leucocyte activation.[2] Specific adhesive interactions between leucocytes and platelets are mediated by P-selectin,[3] a cell surface adhesion molecule, the plasma concentration of which is thought to be a marker of platelet activation.[4] In addition, pseudopodia formation among white blood cells is considered to be a possible marker of leucocyte activation,[5] which is a condition of increased risk for microvascular dysfunction.[5–7] Thus, although other assays are available that look more directly at platelet and white blood cell activation—such as platelet aggregometry and flow cytometry[8 9]—raised plasma concentrations of P-selectin and an increased proportion of white blood cells with pseudopodia might be a measure of impaired microvascular perfusion.

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In humans, little is known about the effects of platelet—leucocyte interactions on whole blood viscosity (WBV), which plays a crucial role in microvascular perfusion.[10–12]

To test the hypothesis that the activation and interactions between platelets and leucocytes might have an impact on microvascular blood viscosity and perfusion in humans, we extended an exercise model used in previous studies,[13 14] in which either leucocyte or platelet behaviour was monitored. Briefly, in our present study, the model of a repeated treadmill exercise test was used in patients with stage II peripheral vascular disease (PVD), with the aim of generating both platelet and leucocyte activation. Blood viscosity at low shear rate and transcutaneous oxygen pressure ($TcPO_2$) in ischaemic limbs were also monitored as measures of microcirculatory function.

Because platelet activation is a potential stimulus of leucocyte activation,[1] we studied whether the use of a powerful antiplatelet drug such as Clopidogrel[15] might help mitigate leucocyte activation, possibly through the inhibition of platelet activity. In addition, the effect of the administration of Clopidogrel was also monitored to investigate whether the simultaneous inhibition of both leucocyte and platelet activation might occur after prolonged treatment with this antiplatelet drug and also whether it can prevent the post exercise increase in WBV and improve microvascular perfusion at the onset of calf pain in patients with stage II PVD.

METHODS

Patients

Thirty patients with stable stage II PVD, who also were smokers, were recruited from those attending the angiology section of the clinical and experimental medicine department, Perugia General Hospital, Italy.

PVD had originally been confirmed by ultrasound screening of the femoral tracts by high resolution ultrasonography Biosound II s.a. (Esaote Biosound, Florence, Italy) equipped with an 8 Mhz probe, combined with Doppler velocimetry (including the treadmill test), and spectral analysis.

Inclusion criteria were male sex, ankle/arm pressure ratio < 0.80 at rest, stable maximum walking distance of 200 to

Abbreviations: MWT, maximum walking time; PAD, peripheral arterial occlusive disease; PVD, peripheral vascular disease; $TcPo_2$, transcutaneous oxygen pressure; WBV, whole blood viscosity

Table 1 Clinical details of 30 men with stage II peripheral vascular disease after randomisation

	Total	Placebo	Clopidogrel
Number	30	15	15
Age (years)			
Range	44–62	44–61	46–62
Mean (SD)	54.7 (4.8)	54.1 (5.2)	55.2 (4.4)
Mean (SD) body mass index (kg/m^2)	24.78 (0.79)	24.65 (0.94)	24.90 (0.62)
Smoking			
<20 cigarettes/day	26	13	13
>20 cigarettes/day	4	2	2
Time of onset of claudication (months)			
Range	9–24	12–24	9–21
Mean (SD)	16.8 (2.7)	16.6 (2.8)	17.1 (2.6)
Windsor index in the worse leg (at rest)			
Range	0.65–0.73	0.65–0.72	0.65–0.73
Mean (SD)	0.68 (0.01)	0.68 (0.01)	0.68 (0.02)

The smoking data are based on answers at interview; the worse leg is the leg that forced the patient to interrupt the treadmill exercise.

Table 2 Study design

	10.00 am	12.00 noon Clopidogrel	or	12.00 noon Placebo
Day 1	Treadmill	300 mg		Placebo
Day 2	Treadmill	75 mg		Placebo
Day 3		75 mg		Placebo
Day 4		75 mg		Placebo
Day 5	Treadmill			

See text for details of the treadmill excercise. Blood samples were taken before (baseline) and after (peak) each treadmill test (see text).

500 m, and a maximum walking time (MWT) of 180 to 300 seconds at the last two monthly checkup (reconfirmed immediately before enrolment in our study) during a standard treadmill test.

Exclusion criteria were rest pain, diabetes mellitus, hyperlipidaemia (serum cholesterol, > 240 mg%; serum triglycerides, > 160 mg%), and hypertension (systolic blood pressure, >160 mm Hg; diastolic blood pressure, > 90 mm Hg), vascular disease in other regions, any other serious disease, recent infection, history of vascular surgery and/or acute thromboembolism, and chronic alcoholism.

Study plan

One month before randomisation, all 30 patients started a normocaloric diet and began an outpatient physical training programme[16]; one week later they suspended antiplatelet treatment.

Table 1 reports the clinical details of the 30 patients.

Informed consent was obtained from all patients, and our study was approved by the Umbria hospital board ethics committee.

The patients were randomised to two treatment groups in a double blind, placebo controlled design by a predetermined computer code to ensure homogenous distribution of inclusion criteria.

Table 2 illustrates the double blinded, placebo controlled study design.

Biological safety parameters included a clinical checkup, blood pressure measurement, and a complete haematological profile before and after Clopidogrel administration.

Treadmill test model

Each exercise test was composed of two treadmill tests.

$TcPO_2$ was monitored constantly during the two treadmill tests (10% slope, 3 km/hour) and the recovery period between the two walks. A $TcPO_2$ monitor (Ts 3300; Kontron Instruments, Basel, Switzerland) was used, with one electrode being positioned 10 cm below the knee on the anterior tibial muscle. The second electrode was placed on the chest in the subclavicle area for control readings.

When walking was stopped because of pain, the time of recovery to half the original $TcPO_2$ value was monitored. It was calculated according to the following formula: the difference between the basal $TcPO_2$ value and the minimum was divided by two and the results were added to the minimum value. Walking was immediately resumed until calf pain became prohibitive and the walking time was monitored.

Blood measurements

Blood samples were drawn from an antecubital vein at baseline and after the second treadmill test. The following parameters were determined:

- Whole blood viscosity at corrected haematocrit as described previously,[17 18] with an intra-assay coefficient of variation of 1.1%.
- Leucocyte activation, measured as pseudopodia formation (morphological assay of white blood cell activation), as described elsewhere.[19]
- The leucocyte filterability rate after separation and suspension in phosphate buffered saline.
- Plasma rich in white blood cells but relatively few red blood cells and platelets was obtained according to the method of Mikita *et al*.[20]
- The erythrocyte filterability rate after erythrocytes were separated as described elsewhere.[21] All filtrations were carried out at room temperature (25 ± 1°C). The methodology of filtration has been described in detail elsewhere.[22] The filterability rate was expressed as the final filtration pressure generated by the cell suspension relative to the buffer, previously filtered through the same filter (a 5 µm pore filter; nuclepore, batch 54B5E2; Costar Corporation, Cambridge, Massachusetts, USA). The reliability of the filtration procedures was confirmed by the coefficients of variation from five successive filtrations of each suspension sample, which were 2.9% for erythrocytes and 3.1% for leucocytes.
- Platelet activation evaluated as concentrations of soluble P-selectin, measured by a standard enzyme linked immunosorbent assay (Takara Shuzo Ltd, Japan), which showed intra-assay and interassay coefficients of variation of 2.5% and 5.4%, respectively. The minimum sensitivity for the assay was 1.3 ng/ml.
- Total platelet and leucocyte counts assayed by an automatic analyser.

Table 3 Haemorheological behaviour during repeated treadmill exercise test in 15 patients with stage II peripheral vascular disease compared with 15 matched placebo controls before and after Clopidogrel treatment

	1st day		2nd day		5th day	
	Clopidogrel	Placebo	Clopidogrel	Placebo	Clopidogrel	Placebo
Whole blood viscosity (cP) (shear rate 0.94/s)						
Baseline	26.5 (1.0)	26.3 (1.1)	26.6 (1.0)	26.7 (1.6)	26.4 (1.0)	26.4 (1.0)
Peak	29.4 (1.1)*	29.2 (1.3)*	29.5 (1.1)*	29.6 (1.2)*	26.4 (1.1)†‡§	29.5 (1.3)*
Leucoyte activation (%)						
Baseline	10.5 (1.8)	11.1 (2.1)	10.7 (1.2)	10.7 (2.2)	11.1 (1.9)	10.9 (2.0)
Peak	15.9 (2.5)*	16.7 (1.9)*	16.1 (2.5)*	16.3 (2.2)*	11.6 (1.9)†‡§	16.5 (2.1)*
Leucoyte filterability rate (P/P)						
Baseline	6.1 (0.5)	6.0 (0.4)	6.0 (0.4)	6.1 (0.4)	6.2 (0.4)	6.1 (0.5)
Peak	7.8 (0.5)*	7.9 (0.4)*	7.6 (0.8)*	7.9 (0.3)*	6.2 (0.6)†‡§	7.7 (0.3)*
Soluble P-selectin (ng/ml)						
Baseline	366 (12)	366 (19)	362 (11)	371 (14)	359 (16)	359 (16)
Peak	405 (14)*	404 (15)*	356 (25)†§	411 (11)*	353 (23)†‡§	401 (13)*
Platelet count (×10°/l)						
Baseline	248 (10)	235 (17)	253 (10)	242 (15)	244 (23)	239 (22)
Peak	278 (15)*	270 (14)*	281 (16)*	273 (13)*	271 (20)*	275 (19)*
Total leucocyte count (×10°/l)						
Baseline	7.8 (0.5)	7.6 (0.4)	8.0 (0.6)	7.8 (0.5)	7.9 (0.5)	7.9 (0.4)
Peak	9.4 (0.8)*	9.2 (0.8)*	9.7 (0.8)*	9.5 (1.0)*	9.7 (1.0)*	9.6 (0.8)*

Whole blood viscosity refers to the ratio of low shear blood viscosity at haematocrit corrected to 45% to plasma viscosity; leucocyte activation refers to leucocytes with pseudopodia and/or cytoplasmic irregularities; leucocyte filterability rate refers to the pressure ratio of cell suspension to buffer after 6 minutes of filtration.
Intragroup analysis: *p<0.01 *v* baseline; †p<0.01 *v* 1st day, ‡p<0.01 *v* 2nd day; intergroup analysis: §p<0.01 *v* placebo group. Values are expressed as mean (SD).

- Plasma concentrations of fibrinogen were assayed by radial immunodiffusion on Partigen plates (NOR Partigen plates, Behringwerke, Germany).
- The haematocrit was measured by microcentrifugation at 12.500 ×*g* for five minutes and results were expressed as the percentage of erythrocytes in the total blood volume.

Statistical analysis

Comparisons between groups were performed using the Student's *t* test for parametric variables and Wilcoxon rank sum test for non-parametric variables. Repeated measures analysis of variance was used for testing intragroup differences at different times. Correlations were tested with Pearson's and Spearman's rank tests. The data were analysed longitudinally (comparing baseline values with those at the MWT and at the $TcPO_2$ half recovery times and comparing data before and after treatment) and cross sectionally (comparing data in the Clopidogrel group with data in the placebo group). Only p values < 0.05 were taken as significant and are reported here.

RESULTS

The clinical details of 30 male subjects with peripheral arterial occlusive disease (PAD) are reported in table 1. Age, cardiovascular risk factors, and mean Windsor index were evenly distributed in the two groups of patients with PVD.

Table 2 illustrates the double blinded, placebo controlled study design. Briefly, 15 patients with PAD, assigned to antiplatelet drug treatment, took 300 mg of Clopidogrel after a repeated treadmill test on day 1 and 75 mg on days 2 to 4, completing their last treadmill test on day 5. Fifteen matched subjects completed the same study protocol by taking placebo instead of Clopidogrel (table 2). Blood samples were collected for all patients before and after the treadmill tests.

Table 3 shows the haemorheological behaviour of all the participants during the repeated treadmill exercise tests. At baseline (first day), all markers of platelet and leucocyte activation, in addition to low shear rate WBV, rose significantly after the treadmill exercise both in subjects assigned to Clopidogrel and in control patients. Thus, for instance, in the placebo group, 51% and 11% increases in WBC activation and P-selectin levels, respectively, were accompanied by a significant 11% increase in WBV after exercise. Similar changes were seen in those patients with PAD who were assigned to Clopidogrel (table 3).

On day 2, similar haemorheological post exercise patterns to those seen on day 1 were documented in the placebo and Clopidogrel groups, except for the soluble P-selectin plasma values in the Clopidogrel group, which did not change after repeated exercise (1.6% decrease after exercise; p = NS (not significant)). Conversely, despite the use of Clopidogrel, the exercise test still induced increases in WBC activation (from 10.7% (SD, 1.2%) to 16.1% (SD, 2.5%); $p < 0.01$) and WBV (from 26.6 cP (1.0) to 29.5 cP (1.1); $p < 0.01$).

On day 5, in the Clopidogrel group, soluble P-selectin plasma values continued to be unaffected by exercise (1.6% reduction after exercise; p = NS). Interestingly, markers of leucocyte activation and WBV remained stable after the treadmill test; thus, post exercise variations in leucocyte activation (4.5% increase), leucocyte filterability (0% variation), and WBV (0% variation) were not significantly different (p = NS for all). In the placebo group, changes after exercise in the haemorheological profile were not significantly different from those seen on day 1 and day 2 (increases in WBV, WBC activation and filterability, soluble P-selectin values, platelet and WBC counts; table 3).

When analysing the longitudinal behaviour of blood parameters (day 5 *v* day 1), we found that post exercise WBV (−10%; $p < 0.01$), WBC activation (−27%; $p < 0.01$), and filterability of unfractionated leucocytes (−20%; $p < 0.01$), in addition to plasma soluble P-selectin values (−13%; $p < 0.01$), were all reduced on day 5 compared with day 1 in the Clopidogrel group.

Table 4 reports $TcPO_2$ half recovery times and MWT in those patients assigned to either Clopidogrel or placebo during the study programme. The $TcPO_2$ half recovery times and MWT were not dissimilar between the two groups on days 1 and 2. However, a significant difference between the groups emerged on day 5, with $TcPO_2$ half recovery times being lower (Clopidogrel group, 183 seconds (SD, 13) *v* placebo group: 221 seconds (SD, 17); $p < 0.01$) and MWT higher (Clopidogrel group, 320 seconds (SD, 62) *v* placebo group 241 seconds (SD, 45); $p < 0.01$) in patients with PAD taking Clopidogrel. Both the $TcPO_2$ half recovery times and MWT were significantly ameliorated by Clopidogrel treatment on day 5 (183 seconds (SD, 13) and 320 seconds (SD, 62), respectively) compared

Table 4 Transcutaneous oxygen pressure half recovery time ($TcPO_2$ hrt) after first walk and maximum walking time (MWT) (both measured in seconds) during repeated treadmill exercise tests in 15 patients with stage II peripheral vascular disease compared with 15 matched placebo controls before and after Clopidogrel treatment

	1st day		2nd day		5th day	
	Clopidogrel	Placebo	Clopidogrel	Placebo	Clopidogrel	Placebo
$TcPO_2$ hrt	222 (14)	228 (15)	209 (14)	223 (16)	183 (13)*†‡	221 (17)
MWT	236 (44)	242 (34)	241 (43)	247 (37)	320 (62)*†‡	241 (45)

Intragroup analysis: *p<0.01 *v* 1st day; †p<0.01 *v* 2nd day; intergroup analysis: ‡p<0.01 *v* placebo group. Values are mean (SD).

with day 1 (222 seconds (SD, 14) and 236 seconds (SD, 44), respectively). No variation of these functional parameters was present in patients with PAD assigned to placebo.

Thus, improvement of indicators of vascular perfusion—namely, $TcPO_2$ half recovery time and MWT—parallelled the improvement in the haemorheological profile only in patients taking Clopidogrel for the duration of the study (four consecutive days).

At no stage of the study were significant changes seen in fibrinogen, haematocrit, or the erythrocyte filterability rate (results not shown). No patients dropped out of our study and no side effects were reported to, or observed by, the physician.

DISCUSSION

Our experimental data showed that repeated exercise in patients with stage II PVD was associated with platelet activation, as documented by post exercise increased soluble P-selectin values and platelet count, in addition to white blood cell activation and increased blood viscosity. This is in agreement with our previous report and those of others reporting either the activation of leucocytes or platelets after repeated treadmill tests.[13 14] Because exercise did not produce changes in other parameters that are known to contribute to blood viscosity (that is, fibrinogen, red blood cell deformability, and haematocrit), changes in leucocytes and platelet behaviour might have had an influence on the observed post exercise increase in blood viscosity. This is supported by our results showing the lack of an increase in post exercise low shear rate blood viscosity when both post exercise platelet and white blood cell activation were suppressed in subjects taking Clopidogrel for the entire duration of our study. In our present study, we monitored both platelet and white blood cell characteristics, in addition to blood viscosity at low and high shear rate. Moreover, by using an antiplatelet agent like Clopidogrel, we investigated the effects of platelet inhibition on microvascular blood viscosity. Our results showed that an interaction between leucocytes and platelets occurred in patients with PVD at the onset of calf pain. In particular, our data indicated that a significant post exercise reduction in blood viscosity at low shear rate was reached only when platelet activation was inhibited and white blood cell rheology improved. Indeed, the observed post exercise increase of low shear rate blood viscosity on days 1 and 2 was not seen in the Clopidogrel group on day 5, when post exercise soluble P-selectin concentrations, WBC activation, and filterability remained unchanged after the treadmill test. Thus, the observed antiplatelet effects of Clopidogrel might be accompanied by effects on WBC activation, probably as a consequence of an interaction between platelets and white blood cells.

> "A significant post exercise reduction in blood viscosity at low shear rate was reached only when platelet activation was inhibited and white blood cell rheology improved"

Clopidogrel had no effect on plasma fibrinogen concentrations or red blood cell deformability, which remained substantially unaltered throughout the study. This discrepancy with other reports[21 22] can be explained by our short term administration of the drug (four days). A previous study showed that ticlopidine derivatives (including Clopidogrel) improved red blood cell deformability by reducing erythrocyte membrane microviscosity.[21] This effect seems to depend on lipid–lipid and cholesterol–phospholipid bond variations and appears to be common to cell substances which, like Clopidogrel, are amphiphiles.[23] A recent study of ours[24] showed that Clopidogrel improved red blood cell deformability after three weeks in subjects with subclinical atherosclerosis. Interestingly, in the same study we found that whole blood viscosity improved after seven days of treatment with 75 mg Clopidogrel daily.[24]

Another interesting point to emerge from our study is that, although platelet activation was inhibited by the loading dose of Clopidogrel, on day 2 the single large dose had no significant effect on MWT, $TcPO_2$ half recovery time, or other functional measurements. Half recovery time improved only when white blood cell rheology improved and platelet activation was inhibited. Thus, a concerted platelet–leucocyte action is undoubtedly present at the onset of calf pain, and might have an important influence on microvascular perfusion. Interestingly, the shortening in half recovery time correlated with the improvement in low shear rate blood viscosity (results not shown).

In conclusion, our study provides evidence that platelet–leucocyte adhesive interactions occur at the onset of calf pain in claudicants and that the suppression of the post exercise activation of both cell populations does not allow low shear rate whole blood viscosity to increase after exercise, thereby improving the half recovery time after the treadmill test. Clopidogrel, by inhibiting platelet activation and aggregation, might also blocks the vicious cycle of leucocyte entrapment, plugging, and activation, thus improving the functioning of the microcirculation.

Take home messages

- In patients with peripheral vascular disease (PVD), microvascular blood viscosity and perfusion after repeated exercise did not improve when platelet activation alone was blocked with a single dose of Clopidogrel
- However, there was a significant improvement in microvascular perfusion during controlled ischaemia and a lack of post exercise increase in low shear rate blood viscosity was achieved when both platelet and leucocyte activation were suppressed by a relatively longer treatment with Clopidogrel (four days).
- Thus, platelet–leucocyte adhesive interactions occur at the onset of calf pain in patients with PVP and Clopidogrel, by inhibiting platelet activation and aggregation, might also block the vicious cycle of leucocyte–platelet activation, thus improving the microcirculation
- Clinical trials should be undertaken to determine whether Clopidogrel would be useful in the pharmacological management of PVD

Clinical trials should be started to determine whether Clopidogrel has a place in the pharmacological management of peripheral arterial occlusive disease.

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