Darbepoetin enhances endothelium-dependent vasomotor function in patients with stable coronary artery disease only after preceding ischaemia/reperfusion

Lindsey TILLING*, Joanne HUNT*, Ann DONALD*, Brian CLAPP† and Phil CHOWIENCZYK*
*British Heart Foundation Centre, Cardiovascular Division, Department of Clinical Pharmacology, King's College London, St Thomas' Hospital, London SE1 7EH, U.K., and †Department of Cardiology, St Thomas' Hospital, London SE1 7EH, U.K.

ABSTRACT

Vasoprotective effects of erythropoietin in animal models are mediated by endothelium-derived NO and/or mobilization of EPCs (endothelial progenitor cells) and may be enhanced by ischaemia: whether they are present in humans is unknown. We examined whether the erythropoietin analogue darbepoetin improves FMD (flow-mediated dilatation), a measure of endothelium-derived NO, and whether this is influenced by preceding I/R (ischaemia/reperfusion). A total of 36 patients (50–75 years) with stable coronary artery disease were randomized to receive a single dose of darbepoetin (300 μg) or saline placebo. FMD was measured at the brachial artery using high-resolution ultrasound. CD133+/CD34+/VEGFR2+ (vascular endothelial growth factor receptor 2) circulating EPCs were enumerated by flow cytometry. Measurements were made immediately before darbepoetin/placebo and at 24 h, 72 h and 7 days. At 24 h, FMD was repeated after 20 min of I/R of the upper limb. A further group of 11 patients was studied according to the same protocol, all receiving darbepoetin, with omission of forearm I/R at 24 h. Immunoreactive erythropoietin peaked at 24 h and remained elevated at approximately 50-fold of baseline at 72 h. FMD did not differ significantly between groups at 24 h (before I/R). At 72 h (48 h after I/R), FMD was greater (by 2.3 ± 0.5 % in the darbepoetin compared with the placebo group, a 66 % increase over baseline; P < 0.001) and greater than FMD at the same time point without preceding I/R (P < 0.01). Increases in CD133+/CD34+/VEGFR2+ cells after darbepoetin did not differ according to the presence or absence of preceding I/R. Preceding I/R is required for darbepoetin to enhance endothelial function, possibly by increasing expression of the erythropoietin receptor and by a mechanism likely to involve Akt/NO rather than circulating EPCs.

INTRODUCTION

Animal models demonstrate marked tissue-protective effects of the glycoprotein EPO (erythropoietin) in cardiac and neural tissue [1–3]. These pleiotropic effects, unrelated to erythropoiesis, may relate to activation/up-regulation of eNOS (endothelial nitric oxide synthase) through the Akt pathway to increase the availability

Key words: endothelial function, endothelial progenitor cell, erythropoietin, ischaemia/reperfusion, nitric oxide.

Abbreviations: CAD, coronary artery disease; CI, confidence interval; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; EPO, erythropoietin; EpoR, EPO receptor; FMD, flow-mediated dilatation; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; IQR, interquartile range; I/R, ischaemia/reperfusion; NTG, nitroglycerine; PMA, platelet–monocyte aggregate; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2.

Correspondence: Professor Phil Chowienczyk (email phil.chowienczyk@kcl.ac.uk).
of endothelium-derived NO [4–6]. Mobilization of endothelial reparative cells such as those previously termed ‘EPC’ (endothelial progenitor cell) (but whose progenitor origin is now disputed) may also be involved [7–10]. Since endothelium-derived NO has antiatherogenic actions and NO availability is inversely related to clinical outcome [11], such actions could offer long-term protection even in the absence of tissue injury. However, there are no results available in humans to support increased endothelium-derived NO. Clinical trials of erythropoietin have been disappointing with equivocal evidence of benefit and an increase in thrombotic events with higher haemoglobin targets [12–14].

In the present study, we examined whether the long-acting analogue of EPO darbepoetin can enhance eNOS-dependent vasomotor function or protect against immediate I/R (ischaemia/reperfusion) injury of the forearm in patients with stable coronary artery disease (in whom responsiveness to eNOS-derived NO is chronically impaired). We measured circulating EPCs as characterized by conventional surface markers {CD34/CD133/VEGFR2 [VEGF (vascular endothelial growth factor) receptor 2]}. Since thrombotic events might be driven through an increase in platelet activation independent of haematocrit, we also measured markers of inflammation, platelet activation and formation of PMAs (platelet–monocyte aggregates). A surprising finding was an effect of darbepoetin on eNOS-mediated vasomotor function evident after but not before I/R. We therefore performed studies in the presence and absence of a period of I/R.

MATERIALS AND METHODS

Subjects

Subjects (n = 47) were patients aged between 50 and 75 years with stable CAD (coronary artery disease). Coronary angiography had been performed in all within the past 3 years and demonstrated occlusion of ≥70% of at least one major epicardial coronary artery, treated by medical therapy and/or revascularization. Exclusion criteria included acute coronary syndrome or percutaneous/surgical intervention in the past 3 months, myocardial infarction or stroke in the past 1 year, NYHA (New York Heart Association) class III/IV heart failure, systolic blood pressure >150 mmHg, diastolic blood pressure >95 mmHg, serum creatinine >140 μmol/l, bleeding or coagulation disorders, haemoglobin >15 or <11.5 g/dl, previous treatment with erythropoiesis-stimulating agents, blood transfusion <12 weeks previously, malignancy in the last 10 years, active inflammation, severe chronic obstructive pulmonary disease and hepatic failure. The study was approved by the local research ethics committee and all patients provided written informed consent before randomization. Safety was monitored by the incidence of any adverse events and laboratory parameters (including haemoglobin and haematocrit). Protocol

Study 1

A total of 36 of the eligible patients were assigned in a 1:1 single-blind randomization scheme to receive a single dose of darbepoetin alfa (300 μg intravenous; Amgen), a long-acting synthetic analogue of erythropoietin, or saline placebo. The dose of darbepoetin was based on safety and efficacy reports in previous pilot studies in patients with acute myocardial infarction [15]. Subjects attended a quiet temperature-controlled vascular laboratory in the morning at baseline (before darbepoetin/placebo) and at 24 h, 72 h and 7 days after drug administration. The 24 and 72 h time points were chosen to span the period of maximal bioactivity of darbepoetin, in accordance with previous reports. At each time point, seated blood pressure was measured using an oscillometric method (Omron 705CP) according to the British Hypertension Society guidelines, blood was taken for laboratory studies and endothelial function was measured in the forearm as described below. At 24 h, endothelial function was repeated after a 20-min period of upper-limb ischaemia and 15 min reperfusion (upper limb I/R injury).

Study 2

On completion of the randomized study (n = 36), a further 11 subjects with similar characteristics were studied, all of whom received darbepoetin, using an identical protocol but with an omission of I/R injury at 24 h.

Laboratory studies

Venous blood was collected for laboratory tests including full blood count, lipid profiles and renal function. Commercially available ELISAs (R&D Systems) were used to determine serum immunoreactive EPO, hsCRP (high-sensitivity C-reactive protein), IL-6 (interleukin-6), VEGF, plasma CD40L (CD40 ligand) and P-selectin.

EPC analysis was performed using flow cytometry as previously described [16]. Whole blood collected in EDTA-containing tubes was spun down and the plasma was discarded. Fc receptor blocker (MACS Miltenyi Biotech) was added to each sample, before incubation on ice. Antibodies to peridinin chlorophyll protein-conjugated CD34 (BD Biosciences), phycoerythrin-conjugated CD133 (MACS Miltenyi Biotech) and FITC-conjugated VEGFR2 (R&D Systems) were added to identify EPCs. Antibodies to CD14 and CD41a (BD Pharmingen) were added to identify PMAs. Following red cell lysis with lysis buffer (Ebioscience),
the samples underwent further centrifugation before re-
suspension of the pellet in PBS with 0.5 % newborn
calf serum. Cells (1×10^6) were acquired using a
Becton Dickinson FACSCanto II flow cytometer, and
were analysed using Diva software (BD Biosciences)
and expressed as a fraction of the mononuclear cell
population. All cell population measurements were
carried out in duplicate to assess reproducibility.

**Endothelial function**

Endothelial NO-dependent vasomotor function was
assessed by measuring FMD (flow-mediated vasodila-
tion) of the brachial artery according to the current
guidelines. High-resolution ultrasound (Siemens Aspen
with 7 MHz linear array transducer, positioned by a
stereotactic manipulator) was used to scan the brachial
artery in a longitudinal section. After optimal positioning
of the transducer a baseline scan was recorded. An
increase in flow was then induced by inflation of a
pneumatic tourniquet placed around the arm (distal
to the arterial segment being scanned) to a pressure
of 250 mmHg for 5 min, followed by release. A
second scan commenced 10 s before release of the
cuff and was continued for 3 min after cuff deflation.
After 10 min of vessel recovery, another resting scan
was taken. Sublingual NTG (nitroglycerine; 25 μg)
was then administered (as an endothelium-independent
control), and a final scan was performed 3–4 min later.
Images were digitized for subsequent blinded analysis
using automated edge detection software (Brachial
Analyser, Medical Imaging Applications, LCC). FMD
was expressed as the percentage increase in brachial
artery diameter from baseline to maximal dilation which
occurred 30–90 s after release of the cuff. Dilation in
response to NTG was expressed as the percentage
increase in brachial artery diameter from baseline to
maximal dilation after NTG. The within-subject S.D. of
between visit differences in FMD in our laboratory is
less than 2 %.

At 24 h after darbepoetin/placebo in study 1, FMD was
repeated after a period of 20 min ischaemia (generated by
inflation of a blood pressure cuff proximal to the arterial
segment being scanned, to a suprasystolic pressure)
and 15 min reperfusion of the upper limb. In healthy
volunteers, this technique results in transient endothelial
dysfunction that can be abrogated by pre-conditioning,
and thus provides a human model of I/R injury that can
be used to study protective mechanisms [17,18].

**Statistical analysis and sample size calculations**

In study 1, a sample size of 36 was calculated to allow a
clinically significant difference in FMD of 1 % (≈ 25 %
change from baseline) to be detected with > 90 % power
for \( P < 0.05 \). In the subsequent follow-up study, the study
was powered (> 90 % power for \( P < 0.05 \)) to detect the
difference seen in the first study between FMD at 72 h in
the darbepoetin and placebo groups, giving a sample size
of \( n = 10 \).

Subject characteristics are expressed as means ± S.D.
and results as means ± S.E.M. [or as medians and
IQR (interquartile range)]. Subject characteristics in
the darbepoetin and placebo groups were compared by
Student’s \( t \) test (continuous variables) or \( \chi^2 \) test
categorical variables). Outcome measures (change from
baseline) in the darbepoetin and placebo groups were
compared using ANOVA for repeated measures. Pre-
specified contrasts were used to test for differences at
specific time points. All tests were two-tailed and \( P < 0.05 \)
taken as significant. SPSS version 16 was used for all
tests.

**RESULTS**

Forty-seven patients (4 female) aged 63 ± 6.7 years were
enrolled. Characteristics of subjects in studies 1 and
2 are shown in Table 1 and were similar as were
baseline brachial artery diameters (4.1 ± 0.17 compared
with 4.0 ± 0.17 mm; \( P \) value not significant). In study
1, groups randomized to darbepoetin and placebo
were also comparable in terms of medical history, use
of medications, atherosclerotic risk factors and renal
function. Mean baseline FMD was impaired (3.5 ± 0.9 %),
consistent with their known CAD. All subjects enrolled
completed the study, and no adverse events were
recorded.

**Immunoreactive erythropoietin and haematological responses**

Serum immunoreactive EPO concentration peaked at
24 h in the darbepoetin group with a median (IQR) value
of 724 (576–733) units/l compared with 12 (9–21) units/l
at the same time point in the placebo group, and remained
elevated at approximately 50-fold above baseline at 72 h
(\( P < 0.0001 \) for increase relative to baseline and placebo;
Figure 1). After 1 week of darbepoetin, haemoglobin,
haematocrit and reticulocyte count increased compared
with baseline values and compared with placebo
(Figure 1). Haemoglobin increased from 14.4 ± 1.3 at
baseline to 15.0 ± 1.29 g/dl (\( P < 0.05 \)), haematocrit from
0.42 ± 0.04 to 0.45 ± 0.04/l (\( P < 0.01 \)) and reticulocytes
from (71.1 ± 36.5) to (166.5 ± 59.6) × 10^9/l (\( P < 0.001 \)).

**Blood pressure and endothelial function**

Systolic and diastolic blood pressure did not change
significantly following administration of darbepoetin,
and there was no significant change in heart rate (Table 2).
Resting brachial artery diameter remained similar during
the intervention period throughout both studies (mean
baseline diameter ranging from 4.01 to 4.10 mm in study
1 and from 3.84 to 3.96 mm in study 2; \( P \) value was not
significant). FMD was similar in darbepoetin and placebo

© The Authors Journal compilation © 2012 Biochemical Society
Table 1  Baseline characteristics for studies 1 and 2
Values are means ± S.D. or numbers (percentage). BMI, body mass index; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study 1 (Placebo n = 18)</th>
<th>Darbepoetin (n = 18)</th>
<th>Study 2 (Darbepoetin n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>63 ± 7.3</td>
<td>63.1 ± 6.3</td>
<td>60.8 ± 8.9</td>
</tr>
<tr>
<td>Male gender (n)</td>
<td>16 (89 %)</td>
<td>17 (94 %)</td>
<td>10 (91 %)</td>
</tr>
<tr>
<td>Ethnicity (white) (n)</td>
<td>15 (83 %)</td>
<td>14 (78 %)</td>
<td>10 (91 %)</td>
</tr>
<tr>
<td>BMI &gt; 30 kg/m² (n)</td>
<td>7 (39 %)</td>
<td>7 (39 %)</td>
<td>2 (18 %)</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>13 (72 %)</td>
<td>10 (56 %)</td>
<td>10 (91 %)</td>
</tr>
<tr>
<td>Diabetes (n)</td>
<td>7 (39 %)</td>
<td>8 (44 %)</td>
<td>2 (18 %)</td>
</tr>
<tr>
<td>Current smoker (n)</td>
<td>3 (17 %)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Treatment (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-platelet</td>
<td>16 (89 %)</td>
<td>18 (100 %)</td>
<td>11 (100 %)</td>
</tr>
<tr>
<td>ACEI/ARB</td>
<td>11 (61 %)</td>
<td>14 (78 %)</td>
<td>10 (91 %)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>12 (67 %)</td>
<td>15 (83 %)</td>
<td>7 (64 %)</td>
</tr>
<tr>
<td>Statin</td>
<td>18 (100 %)</td>
<td>17 (94 %)</td>
<td>11 (100 %)</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>14.0 ± 1.0</td>
<td>14.4 ± 1.3</td>
<td>14.3 ± 1.4</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.41 ± 0.03</td>
<td>0.42 ± 0.04</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>Reticulocytes (× 10⁹/l)</td>
<td>55.4 ± 32.5</td>
<td>71.1 ± 36.5</td>
<td>59.2 ± 48.6</td>
</tr>
<tr>
<td>Platelets (× 10⁹/l)</td>
<td>205 ± 56</td>
<td>227 ± 54</td>
<td>213.9 ± 50.4</td>
</tr>
</tbody>
</table>

Figure 1  Immunoreactive EPO (top panel), reticulocytes (middle panel) and haematocrit (lower panel) after placebo (broken line) and darbepoetin (solid line) in study 1

Inflammatory markers, cytokines, markers of platelet activation and EPCs
hsCRP, IL-6, VEGF, P-selectin, soluble CD40L and PMAs did not change significantly after darbepoetin/placebo and there were no significant differences between treatment groups (Table 2). There was a trend towards an increase in CD133⁺/VEGFR2⁺ and CD133⁺/CD34⁺ EPCs after darbepoetin compared with placebo. This did not differ significantly in studies.
Table 2  Blood pressure, endothelial function, EPCs, and markers of inflammatory and platelet activation (study 1)

Values are means ± S.E.M. †P < 0.001.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n = 18)</th>
<th>Darbepoetin (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 24 h 72 h 7 days</td>
<td>Baseline 24 h 72 h 7 days</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140 ± 4.8 138 ± 5 135 ± 3.4 140 ± 3.2 129 ± 3.7 126 ± 3.1 128 ± 3.1 126 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79 ± 2.7 79 ± 1.3 75 ± 2.4 78 ± 2.4 75 ± 2.5 73 ± 1.4 73 ± 1.4 73 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Endothelial function</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMD (%)</td>
<td>3.5 ± 0.80 3.4 ± 0.73 2.9 ± 0.63 3.4 ± 0.75 3.5 ± 0.92 4.4 ± 0.97 5.2 ± 0.97† 3.7 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>NTG (%)</td>
<td>8.5 ± 1.1 10.0 ± 1.1 8.3 ± 1.0 9.8 ± 0.8 9.2 ± 1.4 10.4 ± 1.2 9.9 ± 0.9 10.7 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>Progenitor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD133+/VEGFR2+ (n)</td>
<td>505 ± 114 400 ± 77 396 ± 84 499 ± 121 426 ± 68 482 ± 74 482 ± 71 477 ± 71</td>
<td></td>
</tr>
<tr>
<td>CD34+/VEGFR2+ (n)</td>
<td>12.9 ± 2.7 13.4 ± 3.5 10.3 ± 3.5 12 ± 2.7 23.9 ± 10 27.4 ± 9.9 23.5 ± 6.9 33.5 ± 10.4</td>
<td></td>
</tr>
<tr>
<td>CD133+/CD34+ (n)</td>
<td>51.3 ± 7.8 47.9 ± 7.1 47.4 ± 8.7 58.5 ± 10.4 66.5 ± 8.5 68.6 ± 10.2 78.9 ± 12.2 77.2 ± 12.8</td>
<td></td>
</tr>
<tr>
<td>Platelet markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD40L (pg/ml)</td>
<td>131 ± 21 124 ± 31 144 ± 46 121 ± 18 120 ± 25 94 ± 11 116 ± 18 83 ± 12</td>
<td></td>
</tr>
<tr>
<td>P-selectin (ng/ml)</td>
<td>54 ± 5.0 56 ± 7.3 55 ± 6.3 58 ± 7.2 71 ± 7.4 72 ± 9.0 79 ± 13.9 75 ± 7.8</td>
<td></td>
</tr>
<tr>
<td>PMA (% monocytes)</td>
<td>14.5 ± 2.5 11.7 ± 1.3 12.1 ± 2.1 14.5 ± 1.9 12.4 ± 1.4 9.8 ± 1.8 10.7 ± 1.2 9.7 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Inflammatory markers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsCRP (ng/ml)</td>
<td>75 ± 24 66 ± 15 81 ± 18 83 ± 12 90 ± 30 86 ± 26 67 ± 17 52 ± 10</td>
<td></td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>324 ± 73 337 ± 79 314 ± 71 398 ± 95 364 ± 86 354 ± 78 370 ± 80 375 ± 89</td>
<td></td>
</tr>
<tr>
<td>IL-6 (µg/ml)</td>
<td>3.2 ± 0.6 4.1 ± 1.1 4.3 ± 1.1 3.5 ± 0.8 8.6 ± 3.3 6.2 ± 2.1 6.5 ± 1.7 9.1 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2  Change from baseline in FMD at 72 h, 48 h after I/R (+ IR), after placebo and darbepoetin (study 1) and after darbepoetin without preceding I/R (− IR, study 2)

Black bar, darbepoetin; white bar, placebo.

DICUSION

The present study investigated for the first time the ability of darbepoetin to improve endothelium-dependent NO-dependent vasomotor function as assessed by FMD in patients with established atherosclerotic disease. FMD is mediated almost exclusively through eNOS [19], and is regarded as the most reliable non-invasive clinical test of endothelial function [20]. It relates to coronary endothelial vasomotor function, is predictive of cardiovascular events in patients with and without obstructive coronary disease and responds to interventions that influence cardiovascular outcomes [20,21]. We also examined effects of darbepoetin to protect against the immediate effects of I/R injury in a well-established model of upper-limb I/R injury [17]. We found no significant effect of darbepoetin to augment FMD at 24 h when levels of immunoreactive EPO were maximal, nor any significant effect of darbepoetin to protect against endothelial dysfunction induced by immediate I/R injury. However, at 72 h, 48 h after I/R, darbepoetin resulted in a marked improvement in FMD compared with the placebo group. The fact that this was not simply the result of a time-course effect with a delay in actions of darbepoetin was confirmed by the lack of effect of darbepoetin at 72 h in subjects who did not undergo forearm I/R injury.

Mechanism of protection

The prerequisite for preceding I/R to reveal beneficial effects of darbepoetin may provide insight into the lack of effect of EPO in clinical trials of neuro- and cardio-protection [14,15,22]. Although EpoRs (EPO receptors) are found on the surface of mature endothelial receptors) are found on the surface of mature endothelial
cells, vascular smooth muscle cells, neuronal cells and ventricular myocytes [23], mRNA for EpoR is expressed at low levels in human neuronal, cardiac and endothelial cells compared with cells in the erythroid lineage [24]. Furthermore, initial results using an antibody thought to be highly specific for EpoR show little or no detectable EpoR protein on the cell surface, or evidence of EpoR downstream signalling on exposure to high concentrations of EPO [24]. However, hypoxia induces expression of EpoR and up-regulates eNOS and the combination of EPO and hypoxia markedly enhances expression of EpoR- and eNOS-derived NO in human umbilical endothelial cells and bone marrow microvasculature endothelial cells [6]. It is notable that, in animal studies, protective effects of EPO on I/R injury are usually manifest later rather than earlier in the reperfusion phase-consistent with a stress-induced expression of the EpoR [1,3]. Our in vivo findings in humans are in line with low/absent EpoR expression and signalling in non-erythropoietic tissue in the absence of a stress stimulus. I/R injury may increase EpoR expression and play a permissive role in subsequent protective actions involving up-regulation of eNOS through the EpoR. Although the increase in FMD seen in the present study was modest in absolute terms, it represents an approximately doubling of the response to eNOS-derived NO, an effect that could be of potential clinical significance in terms of limiting ischaemia, endothelial cell dysfunction and apoptosis. In combination with previous cellular and animal studies, the present study suggests that it may be worth examining the response to EPO therapies in conditions in which there is chronic or repetitive ischaemia that may up-regulate the EpoR. Under these conditions, actions of EPO to increase eNOS-derived NO might enhance tissue perfusion through the vasorelaxant effect of NO, protect against subsequent ischaemia and promote angiogenesis. It is notable that in a model of chronic hind limb ischaemia, sustained delivery of low-dose EPO up-regulated the EpoR, activated Akt and eNOS, and improved capillary and arteriolar density [25].

Role of EPCs

Previous studies have reported an increase in circulating EPCs in response to EPO and have implicated EPCs in cardioprotective effects [9,10]. However, cardioprotective effects attributed to Akt/NO signalling have been observed in the absence of effects on EPCs [25]. There are relatively few data on effects of EPO on EPCs in humans. In patients with chronic kidney disease and anaemia, darbepoetin increased EPC number and function assessed using a tube formation assay [8]. However, chronic EPO therapy for heart failure did not increase circulating haemopoietic and endothelial stem cells, defined by various combinations of CD34, CD45, CD133 and VEGFR2. In the present study, there was a trend towards an increase in CD133+/CD34+/VEGFR2+ cells in the treated group which just reached significance when results from the two studies were combined. However, unlike FMD, the increase in EPCs did not differ significantly in the presence(absence of upper-limb I/R. Thus effects of darbepoetin to enhance FMD after I/R were likely to be mediated by a direct effect on eNOS rather than by EPCs.

Safety concerns of darbepoetin therapy

Long-term safety of treatment with erythropoiesis-stimulating agents in chronic kidney disease has recently been questioned. Several trials have demonstrated that therapy targeted to achieve a higher rather than lower level of haematocrit/haemoglobin shows a trend to an increase in adverse cardiovascular events. Most recently, the TREAT (Trial to Reduce Cardiovascular Events with Aranesp Therapy) reported no evidence of benefit, an increase in stroke and a trend towards overall harm with darbepoetin [12]. One interpretation of these findings is that adverse cardiovascular events are not related to haemoglobin concentration but an off-target pro-thrombotic adverse effect of erythropoiesis-stimulating agents [13]. An additional aim of our study was to seek potential mechanisms for such an effect, examining sensitive markers of platelet activation: circulating soluble CD40L, P-selectin and PMAs. CD40L, a protein primarily expressed on T-cells, is stored in α-granules in unstimulated platelets, but is expressed on the platelet surface within seconds of the platelet being activated and released in soluble form [26]. Ligation of CD40L with the CD40 receptor leads to destabilization of plaque through up-regulation of pro-inflammatory and pro-thrombotic genes, expression of adhesion molecules, release of cytokines and recruitment of inflammatory leucocytes [27,28]. Formation of PMAs is another potentially negative consequence of EPO treatment. Like homotypic aggregation, platelets bind monocytes through activation of the platelet membrane protein P-selectin (circulating levels of which are increased in atherosclerotic disease) [29]. Degranulated platelets rapidly lose P-selectin, hence PMAs, which are themselves increased in CAD, have been proposed to be a more sensitive marker of platelet activation [30,31]. Once bound to the platelet the monocyte undergoes morphological changes [32], and an inflammatory cascade is unleashed, similar to that initiated by CD40L. In the present study, we found no significant effect of darbepoetin on CD40L, PMAs, IL-6 or hsCRP. Thus our present study does not support increased activation of platelets as an explanation for increased thrombotic events.

Limitations

A number of important limitations to our present study should be noted. The number of patients studied was relatively small and although the effect of darbepoetin...
to enhance FMD was significantly greater in the presence rather than absence of I/R we cannot exclude a small effect of darbepoetin in the absence of I/R due to a type 2 error. Similarly, we cannot exclude a small effect of darbepoetin to protect against the immediate effects of I/R. We studied patients with coronary artery disease, and our results may not be relevant to subjects with endothelial dysfunction resulting from other causes. There are limitations in the use of FMD as a measure of endothelial function. In particular, the relationship of FMD to the hyperaemic flow stimulus is complex [33] and the effects of darbepoetin to enhance FMD could have been due to an alteration in post-hypoaemic flow or the relationship between the flow stimulus and FMD. Although we have attributed the effects of darbepoetin on FMD to a direct or indirect action on eNOS, they could be mediated and/or influenced by other vasoactive mediators such as endothelin-1 and brain natriuretic peptide (both of which are influenced by EPO [34,35]). The immediate blunting of FMD by I/R in the present study was less than that observed in healthy volunteers [17] and it is possible that this also contributed to the negative finding with respect to protection from the immediate effects of I/R. The effects of chronic EPO administration may differ from acute effects. Finally, we studied ‘EPCs’ characterized by CD34+/133+/VEGFR2+ surface markers. We chose these antigens as they have been accepted as defining an EPC for many years, although they were recently suggested to be an enriched population of haemopoietic precursors incapable of endothelial cell formation [36]. Despite this, CD34+/133+/VEGFR2+ cells have been repeatedly demonstrated to correlate with cardiovascular risk factors and outcomes, and it is still possible that these cells are mobilized to areas of tissue injury and function in a paracrine manner to facilitate repair by other local cells.

Conclusions

In conclusion, the present study suggests that effects of EPO to enhance NO-dependent vasomotor function are contingent on preceding tissue I/R. Future studies should examine tissue protective effects of EPO therapies in conditions characterized by repetitive or sustained ischaemia.

AUTHOR CONTRIBUTION

Lindsey Tilling contributed to the design of the study, recruited the patients, performed the vascular measures, performed the statistical analysis and drafted the paper. Joanne Hunt performed the FACS and biochemical assays. Ann Donald oversaw the FMD measurements. Brian Clapp and Phil Chowienczyk contributed to the conception of the study, its design and to the final paper.

FUNDING

This work was supported by the Guy’s and St Thomas’ Charity, London, U.K. [grant number R070722]. We also acknowledge the financial support from the UK Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy’s & St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust.

REFERENCES


© The Authors Journal compilation © 2012 Biochemical Society

Published as Immediate Publication 3 October 2011, doi:10.1042/CS20110369