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TITLE PAGE

Title

Granulocyte-Colony Stimulating Factor in Chronic Angina to Stimulate Neovascularisation. A placebo-controlled crossover trial.

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ABSTRACT

Background: Experimental studies demonstrate that granulocyte-colony stimulating factor (G-CSF) promotes neovascularisation and confers cardioprotection.

Objective: To assess the efficacy of repeated low dose G-CSF plus exercise on myocardial ischaemia in patients with severe chronic ischaemic heart disease (IHD).

Methods: Eighteen patients with Canadian Cardiovascular Society Class III-IV angina completed a randomised, double-blind crossover study of dose-adjusted G-CSF versus placebo. Exercise was commenced 6 weeks prior and continued for the duration of the study. G-CSF or placebo was administered daily for 5 consecutive days at fortnightly intervals for 3 cycles, followed by crossover after 6 weeks. Primary outcome was myocardial perfusion by cardiac magnetic resonance imaging (CMR). Secondary outcomes were: Seattle Angina and Utility-Based Quality-of-life-Heart Questionnaire (SAQ/UBH-Q), exercise stress test (EST) and quantification of endothelial progenitor cells (EPC) by flow cytometry and angiogenic cytokines by immunoassay.

Results: Compared to placebo, G-CSF had no effect on myocardial ischaemia by CMR, EST or SAQ/UBQ-H, despite effective EPC mobilisation (peak fold increase: $CD34^+$=19, $CD34^+CD133^+$=37, $CD34^+VEGFR\text{-}2^+$=5, $CD34^+CD133^+VEGFR\text{-}2^+$=3, all p<0.050 vs. placebo). Plasma levels of stromal cell-derived factor-1, angiopoietin-1, interleukin-8, and tumor necrosis factor-α decreased after a symptom-limited EST, whilst vascular endothelial- and platelet derived-growth factor remained unchanged. All cytokines were unchanged following G-CSF. Seven troponin I-positive events occurred with G-CSF, compared to three with placebo

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(*P*=0.289). High sensitivity C-reactive protein and N-terminal pro-hormone brain natriuretic peptide increased with G-CSF (both $p<0.010$ vs. placebo).

Conclusion: In patients with chronic IHD, G-CSF mobilises EPCs but does not improve myocardial perfusion or angina. G-CSF increases plasma levels of adverse prognostic cardiac biomarkers.

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MAIN TEXT

INTRODUCTION

Experimental studies demonstrate that granulocyte-colony stimulating factor (G-CSF) has beneficial cardiovascular effects and holds promise for the treatment of myocardial ischaemia.[1] G-CSF mobilizes bone marrow stem cells and has direct angiogenic and cardiomyocyte protective effects. Phase I clinical trials, however, have yielded conflicting results and the safety and efficacy of G-CSF in patients with chronic ischaemic heart disease (IHD) remains unclear.[2, 3, 4, 5, 6, 7]

The aim of the *G*-CSF in *A*ngina patients with *I*schaemic heart disease to stimulate *N*eovascularisation (GAIN II) study was to evaluate the efficacy of repeated low-dose G-CSF therapy and exercise in improving myocardial perfusion in patients with severe angina and chronic IHD. We hypothesised that a multi-G-CSF dosing regimen would promote sustained endothelial progenitor cell (EPC) mobilisation and exert direct cardioprotective effects. Additionally, we hypothesised that exercise would generate an ischaemic stimulus in the myocardium and promote the expression of stem cell homing signals, such as stromal cell-derived factor-1 (SDF-1), thus facilitating the trafficking of G-CSF-mobilised stem cells from the circulation into the myocardium.

METHODS

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GAIN II was conducted between June 2007 and October 2009, under the supervision of an independent Data and Safety Monitoring Board (DSMB), with the approval of the St Vincent's Hospital Human Research Ethics Committee.

Patient Population

Patients were required to have stable Canadian Cardiovascular Society class III or IV angina, angiographically documented coronary artery disease (CAD) and inducible myocardial ischaemia in at least one transmural or two subendocardial myocardial segments on cardiac magnetic resonance imaging (CMR). Key exclusion criteria were unstable angina or myocardial infarction (MI) within 3 months, left ventricular ejection fraction <20%, multi-organ failure, malignancy, diabetic retinopathy, contraindication to CMR and significant renal impairment (GFR <30 ml/min).

Study Design

This was a double-blind, placebo-controlled randomised clinical trial involving a 42-week protocol conducted in four phases: phase I exercise, phase II and III treatments, and phase IV follow-up (Figure 1). During phase I, patients underwent a self-supervised 5-week exercise program involving walking for at least 5 days per week for increasing duration and at an intensity to induce moderate angina. Once established on the exercise program at 6 weeks, the exercise regimen was continued throughout the duration of the trial. A subset of patients underwent blood collection for EPC and angiogenic cytokine quantification at baseline and after five weeks at 0, 0.25, 18, 42 and 90 hours following a symptom-limited EST. At the commencement of the first treatment phase (II), patients were randomised to either G-CSF or

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placebo therapy. During phase II, patients underwent three consecutive 14-day cycles of G-CSF or placebo therapy, followed by 6-weeks monitoring. Each 14-day cycle consisted of five days of subcutaneous G-CSF or placebo, followed by nine days of no therapy. G-CSF (Lenograstim, Granocyte, Mayne Pharma, Melbourne, Australia) was commenced at 4.5 μg/kg/day (maximum dose, 600 μg/day or 7.5 μg/kg per day) with subsequent daily dose titration to target a peripheral leukocyte count of 30-35 x 10^9/L. In phase III, patients crossed over to receive the opposite therapy to that given in phase II. After phase III, all patients were monitored by fortnightly telephone follow-up for 12 weeks.

Study Endpoints

The primary study endpoint was myocardial perfusion evaluated by change in number of ischaemic myocardial segments and myocardial perfusion reserve index (MPRi) according to adenosine stress CMR. Secondary study endpoints were: (1) angina symptoms and quality of life as assessed by the Seattle Angina and Utility Based Quality of life-Heart Questionnaire (SAQ/UBQ-H),[8, 9] (2) exercise performance on Bruce protocol exercise stress testing, (3) mobilization of $CD34^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ EPCs and (4) plasma levels of angiogenic cytokines SDF-1, angiopoietin-1 (Ang-1), VEGF, interleukin-8 (IL-8), platelet derived growth factor-BB (PDGF-BB) and tumor necrosis factor-α (TNF-α).

Cardiac Magnetic Resonance Imaging

CMR was performed at baseline and at the completion of each phase (I-IV) on a 1.5 T scanner (Philips Intera, Best, The Netherlands) using a five-channel cardiac phased-array coil. Cardiac

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functional imaging was assessed by steady-state free precession cine imaging and myocardial perfusion, using a T1-weighted single shot gradient echo sequence with a saturation recovery pre-pulse. First-pass perfusion was assessed using three 0.05 mmol/kg boluses (flow rate 5 ml/s) of gadolinium (Magnevist, Bayer Schering Pharma), administered at peak stress, rest and after completion of rest imaging. Stress was induced with an intravenous infusion of 140 μg/kg/min adenosine (Adenoscan, Sanofi-Synthelabo), commenced up to four minutes prior to stress image acquisition. Late gadolinium enhancement imaging for infarct evaluation was performed using an inversion recovery gradient-echo sequence with inversion time optimised to null normal myocardium.

Image analysis was performed by two observers who were blinded to the assigned therapy. Segmental analysis was based on an 18-segment model: 6 equal radial segments per basal, mid and apical short axis slice. Segmental resting wall motion was graded as 1=normal, 2=hypokinetic, 3=akinetic and 4=dyskinetic. Wall motion score index was calculated from the summed segmental wall motion score divided by the number of segments analysed. Perfusion defects were graded by their transmural extent (1=normal, 2=subendocardial defect, 3=transmural defect) and degree of hypoenhancement (1=normal (bright), 2=probably abnormal (grey defect), 3=definitely abnormal (black defect)). Transmural and hypoenhancement ischaemia indices, reflecting the degree of reversible ischaemia, were calculated as a ratio of summed segmental stress-to-rest scores, thus resulting in a grading scale of 1 (no ischaemia) to 3 (severe ischaemia). Abnormal ischaemic segments were defined as transmural ischaemia index >1. Myocardial perfusion ratio index (MPRi) was derived from maximal upslopes of stress-to-rest myocardial contrast signal intensity time curves normalised to LV input. Segmental

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infarction was graded as 1=normal, 2=subendocardial infarct and 3=transmural infarct. The infarct index was determined by the summed segmental infarct score divided by the number of segments analysed.

Exercise Stress Testing

Exercise stress testing was performed at baseline and at the completion of each phase (I-IV) by standard Bruce protocol. Standard clinical criteria were used to determine discontinuation of the test.

Seattle Angina and Utility-Based Quality of life-Heart Questionnaire

The SAQ/UBQ-H was completed at baseline and after each phase (I-IV). Standard algorithms were used to score angina frequency, physical limitation, disease perception and quality of life.[8, 9]

Flow Cytometry

EPCs were evaluated on Day 1, 4 and 6 of G-CSF/placebo cycles by fluorescence-activated cell sorting (FACS) (Canto II, Becton-Dickinson (BD), San Jose, CA, USA). Whole blood samples were incubated with fluorophore-conjugated anti-CD45 (BD), anti-CD34 (BD), anti-CD133/1 (Miltenyi BioTech, Bergisch, Gladbach, Germany) and anti-VEGFR-2 (R&D Systems, Minneapolis, MN, USA) antibodies. Samples were lysed in ammonium chloride, (Pharmlyse, BD Pharmingen) before FACS analysis. Data were analysed using DiVa 6.0 software (BD). Absolute $CD34^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ EPC cell

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counts were determined from the percentage of these cells in the $CD34^{+}CD45^{+}$ population and the peripheral leucocyte count.

Angiogenic Cytokines

Plasma cytokines were measured on Day 1 and 6 of G-CSF/placebo cycles. SDF-1 and Ang-1 were measured by enzyme-linked immunoadsorbent assays (ELISA) (R&D Systems) and IL-8, VEGF, PDGF-BB and TNF-α by bead-based multiplex immunoassays (MIA) (Bio-Rad Laboratories and Panomics). Minimum detectable concentrations were: SDF-1=18, Ang-1=3.4, IL-8=0.67, TNF-α=0.44, VEGF=2.55 and PDGF-BB=1.71 (all units, pg/ml). Intra-assay variation was <6% and <10% for, respectively, ELISA and MIA. Inter-assay variation was <17% for ELISA and <25% for MIA.

Statistical Analyses

We determined that a sample size of 6 and 15 patients in a crossover trial design would have greater than 90% power to detect, respectively, a two-segment change in abnormal perfusion segments and a 0.20 change in MPRi by adenosine stress CMR, assuming two-sided tests and an alpha value of 0.05.[10] The study sample size was adjusted to 20 for an estimated 10% loss of follow-up. Except where noted, continuous data are expressed as mean ± SD and categorical data as numbers (percentage). Between group differences are represented as means and 95% confidence intervals (CI). Comparisons between placebo and G-CSF groups and between 'post-exercise baseline' (at completion of phase I) and 'final' (at completion of phase IV) were performed using paired Student's t-tests. Statistical significance was inferred at a 2-sided *P*-value

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<0.05. No adjustments were made for multiple comparisons. All data were analyzed on PRISM V (GraphPad Software Inc., San Diego, California).

RESULTS

Patient Characteristics

Twenty patients were enrolled with two patients subsequently withdrawn: one patient required percutaneous coronary intervention for in-stent restenosis and another was unable to fulfill trial commitments. The clinical characteristics of the 18 patients who completed the entire trial protocol were typical of a severe chronic-angina IHD population (Table 1).

Phase I Exercise

Following five weeks of exercise training, EST improved from 317±76 to 355±78 seconds (p<0.001), but there was no improvement in myocardial perfusion by CMR (Table 2, 3, 4). There was a small increase in circulating EPC numbers after exercise training, but this did not reach statistical significance for all EPC phenotypes: $CD34^+$=1.7 fold (p=0.058), $CD34^+CD133^+$=1.6 fold (p=0.126), $CD34^+VEGFR\text{-}2^+$=1.9 fold (p=0.018) and $CD34^+CD133^+VEGFR\text{-}2^+$=1.7 fold ($p$=0.064). There was a trend towards increased EPC numbers 15 minutes after a single bout of exercise, which subsequently decreased to baseline. Plasma concentrations of angiogenic cytokines were unchanged following exercise training, but SDF-1, Ang-1, IL-8 and TNF-α decreased significantly 90 hours following a symptom-limited EST (Figure 2).

G-CSF Dosing

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Each patient received 15 doses of G-CSF. With progressive dosing cycles, lower doses of G-CSF were required to reach the target leucocyte count of 30–35 x 10^9/L (p<0.001 for all cycles) (Figure 3A). The mean difference in G-CSF dose on the final day (5) between cycle C and cycle A was -0.91 µg/kg (95% CI, -0.28 to -1.53, p<0.050). As expected, total peripheral leucocyte count did not change during placebo administration but rose dramatically during G-CSF administration by 4- to 6- fold (Figure 3B). This was predominantly due to an increase in neutrophil count, however, all other leucocyte subsets, except for basophils, increased significantly in comparison with placebo (Supplemental Figure 1). EPC mobilisation varied widely between individuals. All four EPC phenotypes increased significantly after G-CSF in comparison to placebo (Figure 3C). An up to 18- 37-, 5- and 3-fold increase was observed in $CD34^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ EPC numbers, respectively after G-CSF compared to placebo therapy.

Primary Outcome: Myocardial perfusion

There were no significant differences in any parameter of myocardial perfusion, LV size or function between G-CSF and placebo or at baseline and final assessments (Tables 2 and 3, Figure 4). On post-hoc analysis, peak $CD34^+CD45^+$ and $CD34^+CD133^+$ EPC numbers correlated positively with MPRi ($CD34^+CD45^+$: r=0.47, p=0.046 and $CD34^+CD133^+$: r=0.61, p=0.007), and negatively with the number of ischaemic segments ($CD34^+CD45^+$: r=-0.55, p=0.020 and $CD34^+CD133^+$: r=-0.48, p=0.042 for $CD34^+CD133^+$). However, no significant correlations were observed between perfusion CMR endpoints and $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$ EPC phenotypes.

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Secondary Outcomes

Subjective measures of myocardial ischaemia

Subjective measures of angina on SAQ-UBQ-H questionnaire improved significantly at final assessment compared with baseline, but did not differ between G-CSF and placebo (Table 4). There were no significant differences in EST times between G-CSF and placebo or between final and baseline assessments (Table 4).

Angiogenic Cytokines

Plasma cytokine concentrations of SDF-1, Ang-1, VEGF, IL-8, PDGF-BB and TNF-α varied widely between individuals, but mean values did not differ between G-CSF and placebo therapy (Supplemental Figure 2). On post-hoc analysis, MPRi was negatively correlated with baseline plasma SDF-1 concentration (r=-0.19, p=0.457) and positively correlated with plasma concentrations of VEGF (r=0.41, p=0.091) and, PDGF-BB (r=0.34, p=0.169), but correlations were statistically significant only for TNF-α (r=0.54, p=0.021), IL-8 (r=0.61, p=0.007), and Ang-1 (r=0.72, p=0.001). A significant negative correlation was observed only between baseline plasma IL-8 concentration and the number of ischaemic segments: r=-0.58. p=0.012.

Adverse Events

Musculoskeletal pain was reported by 94% of patients during G-CSF therapy compared to 11% of patients during placebo therapy (p<0.001). These symptoms were adequately managed with acetaminophen and treatment injections were withheld in only one patient, in whom this was necessary during both G-CSF and placebo. Worsening angina (either in severity or frequency) and/or increasing nitroglycerin usage was reported by 94% and 22% of patients during G-CSF

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and placebo treatment cycles, respectively (p<0.001). Seven troponin-I positive events (>0.06 μg/L) occurred during G-CSF compared to three events during placebo therapy (p=0.289) (Table 5). The majority of these events were not accompanied by a rise in creatinine kinase or ECG changes. An additional five cardiovascular events fulfilling pre-specified criteria for adverse events were recorded: 4 unstable angina (1 during G-CSF) and 1 cardiac failure. No deaths occurred over the duration of the trial.

Cardiac Biomarkers

In the first two treatment cycles, hs-CRP levels increased after G-CSF compared to placebo (Figure 5A). NT-proBNP levels varied between individuals, but mean values also increased significantly during all three G-CSF cycles compared to placebo cycles (Figure 5B).

DISCUSSION

This study demonstrates that in patients with chronic IHD, G-CSF therapy in combination with exercise effectively mobilises EPCs, but does not improve angina or myocardial perfusion. Experimental studies have shown that G-CSF has beneficial effects post MI, reducing infarct size and adverse remodeling, and improving LV function.[11, 12, 13] The underlying mechanisms identified include direct and indirect G-CSF mediated neovascularisation[14, 15] and cardioprotection.[11] A lack of clinical benefit despite effective EPC mobilisation in this study may have been due to mobilisation of an incorrect EPC phenotype, suboptimal G-CSF dosing, inefficient EPC homing due to dysfunctional cells, and/or insufficient expression of stem cell homing signals in the myocardium.

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EPCs were quantified by FACS and identified by co-expression of cell surface markers CD34, CD133 or VEGFR-2 and due to persisting debate over the precise identity of EPCs, we examined four commonly reported phenotypes derived from the $CD45^+$ subfraction: $CD34^+$, $CD34^+CD133^+$, $CD34^+VEGFR\text{-}2^+$ and $CD34^+CD133^+VEGFR\text{-}2^+$. These subpopulations may not, however, represent the true EPCs. There is no cell surface marker that is unique for an EPC or that is able to differentiate EPCs from HSCs and mature endothelial cells. Both CD133 and VEGFR-2 are expressed on HSCs and EPCs, and VEGFR-2 is also expressed on endothelial cells. Furthermore, there is no definitive data showing that $CD34^+CD133^+VEGFR\text{-}2^+$ cells directly form endothelial colonies. Case et al.[16] demonstrated that $CD34^+CD133^+VEGFR\text{-}2^+$ cells belonged to a HSC subpopulation with no ability to form endothelial colonies in clonogenic assay or vessels in Matrigel assay. They also demonstrated that endothelial cell forming colony activity was increased in the $CD34^+CD45^-$ cell subpopulation.[16] Similarly, Mills et al. showed that patients post percutaneous coronary intervention had a threefold increase in the number of endothelial colonies on functional assay but no significant increase in CD34+$VEGFR\text{-}2^+$ EPCs as quantified by FACS.[17] Thus, the G-CSF mobilised cells quantified in our study may not represent true EPCs.

Clinical studies have shown reduced migratory activity[18] and increased senescence[19] of EPCs in patients with coronary artery disease (CAD). Heeschen et al.[20] demonstrated that in comparison to healthy controls, bone marrow mononuclear cells from patients with ischaemic cardiomyopathy had a lower number of granulocyte-macrophage colony forming units, reduced migratory response to SDF-1 and VEGF and, when injected into mice after hindlimb ischaemia had reduced neovascularisation capacity. Moreover, G-CSF mobilized EPCs have also been shown to have reduced migratory and associated neovascularisation function.[21] Stem cell

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mobilisation and homing are mirror processes. Homing requires stem cell expression of the SDF-1 receptor, CXCR4, whilst G-CSF-induced egress of bone marrow stem cells is mediated by a reduction in stem cell CXCR4 expression.[22] Honold et al.[21] showed that the CXCR4 receptor 6H8 epitope, which is important for functional activity, is reduced on G-CSF mobilised EPCs. Hence, although G-CSF induces EPC-mobilisation, the concurrent impairment of EPC function may attenuate neovascularisation capacity.

An effective adjunctive strategy for augmenting stem cell homing is crucial in strategies such as ours that target stem cell mobilisation. The chemokine SDF-1 is pivotal for stem cell mobilisation and homing. We demonstrated unchanged plasma concentrations of SDF-1, as well as Ang-1, VEGF, PDGF-BB, IL-8 and TNF-α with G-CSF therapy compared to placebo. Brunner et al.[23] showed that G-CSF reduced the expression of SDF-1 and stem cell factor, which resulted in inefficient stem cell homing. Moreover, we combined exercise with G-CSF therapy with the aim of generating an ischaemic myocardial stimulus to upregulate the expression of homing cytokines. Instead, we found no change in a panel of angiogenic cytokines after exercise training, and a significant reduction in plasma SDF-1, Ang-1 and IL-8 concentrations after a single symptom-limited episode of exercise. The importance of angiogenic cytokine expression was highlighted in a rat model of ischaemic cardiomyopathy, which demonstrated increased LV functional improvement when G-CSF was co-administered with SDF-1 compared to G-CSF alone.[24]

The absence of any improvement in ischaemia despite a multi-dose G-CSF regimen over several weeks suggests that a clinically meaningful direct cardiovascular G-CSF effect may not be

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achievable. Although the delivery of higher G-CSF doses or more prolonged therapy may produce the desired direct stimulatory cardiovascular effects as described in experimental models,[11, 25] this strategy may be limited by potential adverse outcomes.

An increase in hs-CRP with G-CSF has been reported by others[4, 26] and was confirmed in this study. hs-CRP is a predictor of adverse vascular outcomes[27, 28, 29] and CRP is associated with deleterious pro-atherosclerotic effects on endothelial cells and EPCs.[30, 31, 32, 33] Recently, however, in a study of patients with prior MI, Kim et al.[26] demonstrated no increase in endothelial dysfunction despite a significant increase in hs-CRP following G-CSF. This suggests that the negative pro-inflammatory effects of CRP and G-CSF may be counteracted by the direct beneficial effects of both EPCs and G-CSF on endothelial cells.[34] We also observed a two-fold increase in mean NT-proBNP with G-CSF compared to placebo. The underlying mechanism and significance of this finding is unclear. NT-proBNP is the biologically inactive N-terminal fragment of the prohormone proBNP that is principally secreted by ventricular myocytes in response to increased myocardial wall stress.[35] Cardiac ischaemia without LV dysfunction has also been shown to increase cardiac proBNP gene expression.[36] Thus, our finding of an elevated NT-proBNP may reflect subclinical myocardial ischaemia, ventricular dysfunction and/or hemodynamic alterations with G-CSF. Additionally, NT-proBNP is an established prognostic marker of adverse cardiovascular outcomes in patients with heart failure[37, 38] acute coronary syndromes[39, 40, 41] and CAD.[42, 43]

The significant increase in hs-CRP and NT-proBNP suggests the potential for adverse outcomes with G-CSF. We observed a trend towards an increase in troponin-I events during G-CSF

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compared to placebo therapy: 7 versus 3 events for G-CSF and placebo, respectively (p=0.289). Previous studies have reported an up to 20% incidence of MI and troponin positive events following G-CSF therapy.[2, 3, 4] Although these studies had small patient numbers and the frequency of troponin elevations in patients with severe CAD is unknown, thus far, the reported rate of myocardial ischaemia in chronic IHD trials of G-CSF appears significant and raises safety concerns for the use of G-CSF in this patient population. Based on the findings from this study and others, caution should be exercised in future trials of G-CSF in chronic IHD.

In the only other placebo-controlled study of G-CSF in chronic IHD patients, Meier et al.[5] reported G-CSF induced improvements in coronary collateral flow index by coronary angiography and disappearance of ST segment elevation during a 1 minute coronary balloon occlusion after a two week course of G-CSF (10 µg/kg). The authors concluded that G-CSF may be beneficial for patients with chronic angina based on coronary collateralisation with potential myocardial salvage.[5] The clinical significance of these endpoints measured at two weeks post treatment without longer-term follow-up is uncertain. In our study, although we cannot exclude an acute effect of G-CSF on myocardial perfusion, patients were followed for up to 36 weeks after commencement of G-CSF with no long-term effect observed. Hence, any potential improvement in myocardial perfusion after G-CSF therapy is short-term and not sustained. Earlier open-label or non-placebo-controlled studies suggested efficacy based predominantly on improvements in angina, quality of life and exercise stress testing times.[2, 3, 6, 7, 44] Similarly, we demonstrated improvements in SAQ/UBQ-H at final assessment compared to baseline, but no improvement in myocardial ischaemia between G-CSF and placebo across all subjective and objective clinical endpoints. A placebo effect may have accounted for the observed benefits in

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earlier trials, in particular given the refractory angina patient cohort being examined and the subjective nature of the selected endpoints.

We demonstrate that a G-CSF based strategy is ineffective and potentially hazardous in patients with chronic IHD. G-CSF application was assessed in a clinically relevant manner and using a dosing regimen that should have optimised both G-CSF direct cardiac and indirect stem-cell mediated effects. Our results are in contrast to experimental and early clinical studies that suggested significant promise for G-CSF in these patients. Additionally, we observed an increased rate of clinical myocardial ischaemic events during G-CSF therapy. Thus, our study sounds a very important warning regarding the use of G-CSF in patients with chronic angina and we believe provides an important counter-balance to the existing literature regarding the use of G-CSF in chronic IHD.

Limitations

This study has several limitations. (1) *In vivo* stem cell tracking techniques were not employed to confirm inefficient EPC myocardial homing. (2) The examined EPC phenotypes were not corroborated by ex-vivo functional studies. (3) Exercise, and thus the level of myocardial ischaemia generated, was non-standardised and may have been insufficient to stimulate angiogenic cytokine expression. (4) Small patient numbers may have limited the power to detect changes in EPCs numbers and cytokine levels.

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Conclusion

In patients with chronic IHD, G-CSF effectively mobilises EPCs but does not improve angina or myocardial perfusion. The homing of stem cells to the myocardium may have been impaired, which may in part be due to an unfavorable cytokine milieu. Furthermore, lack of improvement despite prolonged G-CSF administration suggests that a clinically beneficial direct myocardial G-CSF effect is unlikely. Our results do not support a therapeutic role for G-CSF as monotherapy in chronic IHD, particularly given its potential to cause adverse cardiovascular outcomes.

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TABLES

TABLE 1. Patient Demographics.

Variable	All Patients (n = 18)
Age, years	62±7 (47-74)
Male	16 (89)
Vascular Risk Factors	
Body mass index, kg/m^2	31.7±3.6 (25.7–46.7)
Diabetes	4 (22)
Hypertension	14 (78)
Dyslipidaemia*	15 (83)
Family history of ischaemic heart disease†	9 (50)
Previous smoking	13 (72)
Cardiac History and Status	
Number of diseased native vessels	2.8±0.4 (2–3)
Number of coronary artery bypass graft operations	1.5±0.8 (0–3)
Number of percutaneous coronary interventions	1.1±1.0 (0–4)
Left ventricular ejection fraction, %	62±13 (38-82)
Medications	
Total number of cardiac medications per day	8.3±1.2 (7–11)
Aspirin only	4 (22)
Clopidogrel only	7 (39)
Aspirin and Clopidogrel	7 (39)
β-Blocker	13 (72)
Calcium channel blocker	11 (61)
Long-acting nitrate	14 (78)
Angiotensin converting enzyme inhibitor or receptor blocker	17 (94)
HMG-CoA reductase inhibitor	17 (94)

Data are presented as number (%) or mean ± 1 SD (range).
*Presence of hyperlipidaemia or lipid-lowering therapy.
†Ischaemic heart disease in first-degree male relative <55 or female <65 years of age.

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TABLE 2. Myocardial perfusion cardiac magnetic resonance imaging.

Parameter	Pre-exercise	Post-exercise Baseline	Placebo	G-CSF	Final	Treatment Effect G-CSF - Placebo		Overall Effect Final -Baseline	
						Mean Difference	*P*	Mean Difference	*P*
Resting wall motion index[*]	1.39±0.39	1.33±0.27	1.38±0.36	1.38±0.33	1.44±0.53	0.00 (-0.09, 0.08)	0.968	0.11 (-0.07, 0.29)	0.212
Infarct index[†]	1.30±0.31	1.29±0.26	1.34±0.36	1.33±0.26	1.27±0.23	-0.01 (-0.10, 0.07)	0.744	-0.02 (-0.09, 0.04)	0.446
Number of ischaemic segments[‡]	7.16±3.35	8.28±3.36	7.44±2.96	8.28±2.47	8.89±3.78	0.83 (-0.56, 2.23)	0.226	0.61 (-1.50, 2.72)	0.549
Transmural ischaemia index[§]	1.49±0.22	1.51±0.23	1.48±0.23	1.55±0.20	1.52±0.39	0.08 (-0.05, 0.20)	0.208	0.01 (-0.24, 0.26)	0.933
Hypoenhancement ischaemia index[‖]	1.73±0.23	1.67±0.21	1.60±0.30	1.64±0.27	1.63±0.39	0.04 (-0.11, 0.20)	0.574	-0.04 (-0.25, 0.17)	0.708
MPRi[#]	1.29±0.39	1.39±0.39	1.37±0.39	1.32±0.45	1.51±0.52	-0.05 (-0.27, 0.16)	0.601	0.12 (-0.14,0.38)	0.334

Values are mean ± SD, except for between group differences (95% CI). n=18 for all groups except pre-exercise (n=17). MPRi, myocardial perfusion reserve index.

[*]Summed segmental wall motion score divided by number of segments analysed (1=normal, 2=hypokinetic, 3=akinetic, 4=dyskinetic).

[†]Summed segmental infarct score divided by number of segments analysed (1=normal, 2=subendocardial infarct, 3=transmural infarct).

[‡]Total number of myocardial segments with reversible ischaemia by qualitative analysis.

[§]Summed segmental transmural stress:rest score (1=normal, 2=subendocardial defect, 3=transmural defect).

[‖]Summed segmental hypoenhancement stress:rest score (1=normal, 2=grey defect, 3=black defect).

[#]Averaged segmental stress:rest maximal normalised upslopes.

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TABLE 3. Left ventricular volumes, function and mass on cardiac magnetic resonance imaging.

Parameter	Pre-exercise	Post-exercise Baseline	Placebo	G-CSF	Final	Treatment Effect G-CSF - Placebo		Overall Effect Final -Baseline	
						Mean Difference	*P*	Mean Difference	*P*
Ejection fraction, %	62±13	61±13	60±14	61±14	59±14	0.33 (-1.76, 2.42)	0.740	-2.00 (-4.51, 0.52)	0.111
End diastolic volume, ml	170±40	167±44	165±42	165±38	167±39	0.11 (-8.54, 8.76)	0.979	-0.39 (-10.52, 9.74)	0.936
End systolic volume, ml	67±37	67±34	69±38	68±34	71±37	-1.22 (-6.66, 4.21)	0.641	3.44 (-4.14, 11.03)	0.351
Stroke volume, ml	103±25	101±25	96±24	97±23	96±26	1.11 (-4.45, 6.67)	0.678	-4.89 (-11.65, 1.88)	0.146
Mass, g	151±42	154±39	153±39	153±40	155±41	0.44 (-4.00, 4.89)	0.835	0.50 (-6.30, 7.30)	0.878

Values are mean ± SD, except for between group differences (95% CI). n=18 for all groups except pre-exercise (n=17).

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TABLE 4. Seattle Angina and Utility Based Quality of Life-Heart Questionnaire and Exercise Stress Test.

Parameter	Pre-exercise Baseline	Post-exercise	Placebo	G-CSF	Final	Treatment Effect G-CSF - Placebo		Overall Effect Final -Baseline	
						Mean Difference	*P*	Mean Difference	*P*
Angina frequency score*	38.0±21.9	37.2±19.9	56.7±28.7	54.4±26.8	63.3±27.4	-2.22 (-14.23, 9.79)	0.701	26.11 (13.10, 39.12)	0.001
Physical limitation score*	42.9±16.3	50.5±18.5	55.4±15.4	55.4±16.1	60.0±16.1	0.00 (-6.05, 6.05)	0.999	9.57 (1.62, 17.51)	0.021
Disease perception score*	36.3±24.7	41.7±19.8	52.78±24.1	52.5±21.3	55.6±21.4	-0.28 (-6.60, 6.04)	0.928	13.89 (5.85, 21.93)	0.002
Quality of life score†	0.91±0.05	0.92±0.06	0.95±0.04	0.95±0.05	0.96±0.04	-0.01 (-0.02, 0.01)	0.307	0.04 (0.02, 0.07)	0.003
Exercise time, seconds	318±76	354±71	377±144	403±133	383±142	26 (-7, 58)	0.115	29 (-22, 79)	0.245
Duke treadmill score	-7.33±2.55	-5.69±3.87	-3.96±5.11	-4.97±5.58	-3.63±5.49	-1.01 (-2.04, 0.01)	0.053	2.06 (-0.10, 4.21)	0.060

Values are mean ± 1 SD, except for between group differences (95% CI). n=18 for all groups.

*Arbitrary units on a scale of 0 (severe symptoms) to 100 (no symptoms).

†Arbitrary units on a scale of 0 (extremely poor quality of life) to 1 (excellent quality of life).

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TABLE 5. Troponin-I positive events.

Patient Number	Trial Stage	Drug	Peak Troponin-I (μg/L)	Leucocyte Count ($x10^9$/L)	Comments
1	Week 22, phase III Cycle C, day 6	G-CSF	0.13	30.1	Angina unchanged
2*	Week 19, phase III Cycle A, day 3	G-CSF	0.09	21.5	Angina unchanged
6	Week 11, phase II Cycle C, day 4	G-CSF	0.12	48.5	Angina unchanged
11	Week 9, phase II Cycle B, day 3	Placebo	0.85	8.1	Myocardial infarction CK 542 U/L
13*	Week 11, phase II Cycle C, day 1	Placebo	0.10	7.0	Myocardial infarction PCI 7 months prior
13*	Week 15, phase II	Placebo	0.16	5.6	Cardiac failure 90% instent restenosis
16	Week 19, phase III Cycle A, day 4	G-CSF	0.14	28.2	Myocardial infarction
17	Week 11, phase II Cycle C, day 4	G-CSF	0.35	40.7	Myocardial infarction New AF, spontaneously reverted
18	Week 7, phase II Cycle A, day 6	G-CSF	0.11	30.0	Myocardial infarction
18	Week 10, phase II Post cycle B	G-CSF	0.39	13.9	Myocardial infarction

G-CSF, granulocyte-colony stimulating factor; CK, creatinine kinase; AF, atrial fibrillation; PCI, percutaneous coronary intervention.
*Patients subsequently withdrawn.

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CONFLICT OF INTEREST

None declared.

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FIGURE LEGENDS

Figure 1. Overview of study protocol. Cardiac assessment comprised Seattle Angina and Utility Based Quality of Life-Heart Questionnaire, exercise stress test and cardiac magnetic resonance imaging.

Figure 2. Plasma concentration of SDF-1, Ang-1, PDGF-BB, VEGF, IL-8 and TNF-α post symptom-limited EST. $^{*}p<0.05$ versus baseline, multiple comparisons with Bonferroni adjustment. Error bars indicate ± 1 SEM. n = 10 for all, except IL-8, n = 7.

Figure 3. A, Mean G-CSF dose administered. **B**, Mean leucocyte count. **C**, Mean endothelial progenitor cell count according to phenotype. A, B and C refers to cycle. 1 to 6 refers to day within each cycle. $*p<0.05$ by paired t-test comparison for G-CSF versus placebo. Error bars indicate ± 1 SEM.

Figure 4. A, Total number of ischaemic myocardial segments. **B**, Mean myocardial perfusion reserve index. Error bars indicate 1 SEM.

Figure 5. A, Mean hs-CRP. **B**, Mean N-terminal pro-hormone brain natriuretic peptide. Error bars indicate ± 1 SEM.

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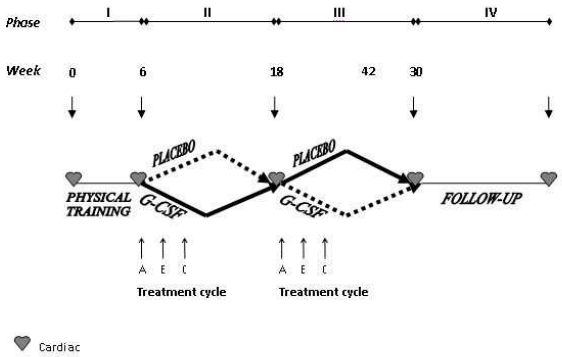

Figure 1

Overview of study protocol. Cardiac assessment comprised Seattle Angina and Utility Based Quality of Life-Heart Questionnaire, exercise stress test and cardiac magnetic resonance imaging.
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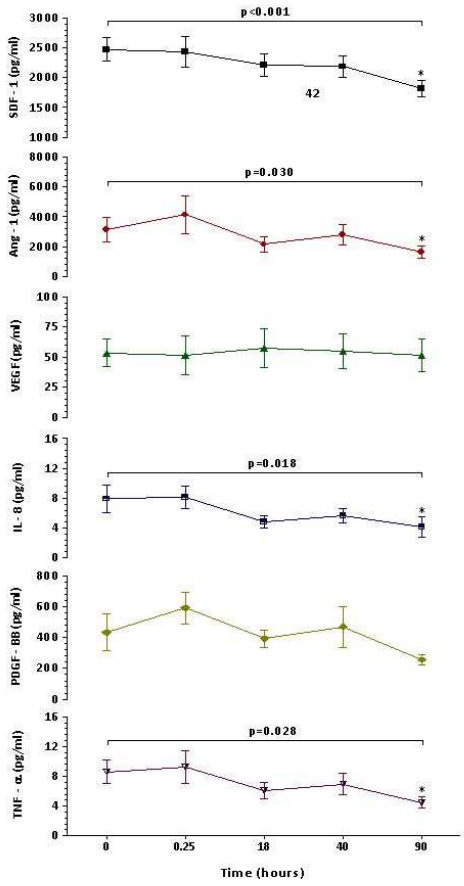

Figure 2

Plasma concentration of SDF-1, Ang-1, PDGF-BB, VEGF, IL-8 and TNF-α post symptom-limited EST. *p<0.05 versus baseline, multiple comparisons with Bonferroni adjustment. Error bars indicate ± 1 SEM. n = 10 for all, except IL-8, n = 7.
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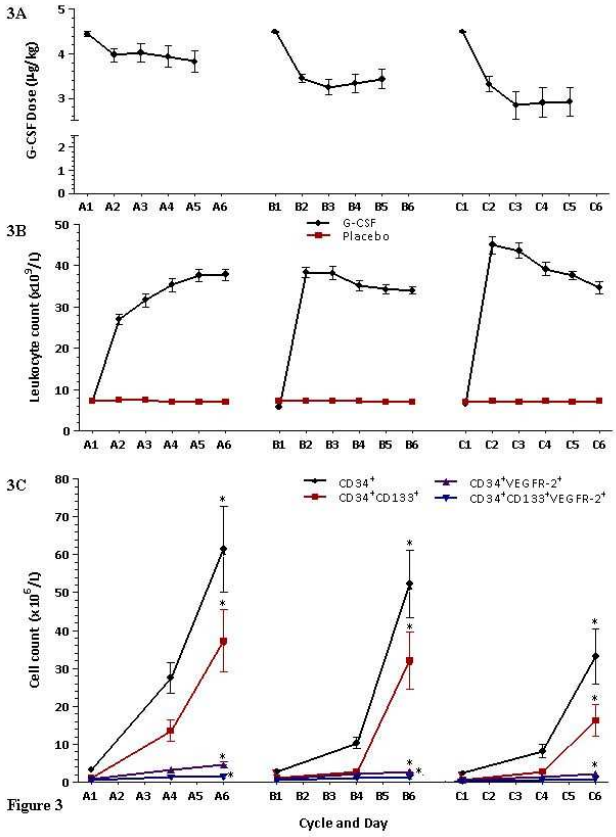

Figure 3

A, Mean G-CSF dose administered. B, Mean leucocyte count. C, Mean endothelial progenitor cell count according to phenotype. A, B and C refers to cycle. 1 to 6 refers to day within each cycle. *p<0.05 by paired t-test comparison for G-CSF versus placebo. Error bars indicate ± 1 SEM.
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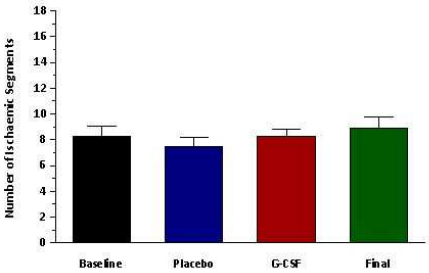
4A



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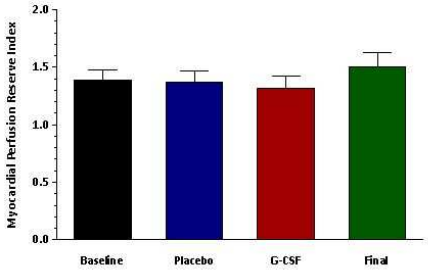

Figure 4

A, Total number of ischaemic myocardial segments. B, Mean myocardial perfusion reserve index. Error bars indicate 1 SEM.
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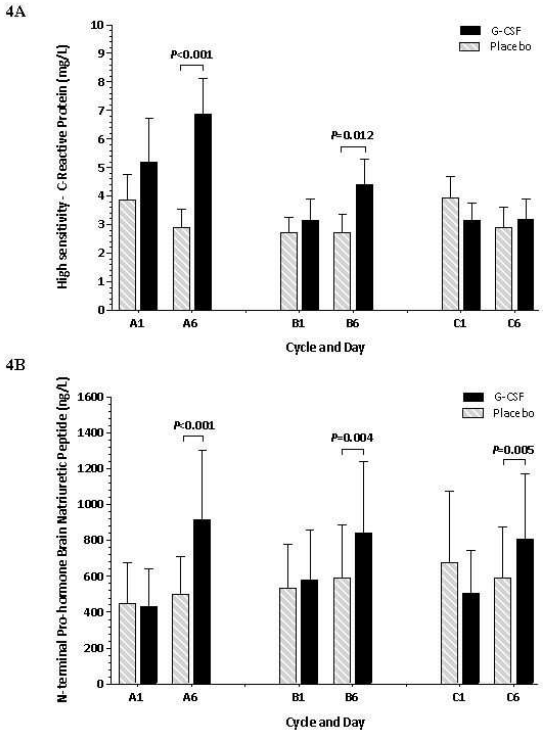

Figure 5

A, Mean hs-CRP. B, Mean N-terminal pro-hormone brain natriuretic peptide. Error bars indicate ± 1 SEM.
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