Circulating cells and cytokines in arteriogenesis
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A pilot study on STimulation of ARTeriogenesis using subcutaneous application of granulocyte-macrophage colony-stimulating factor as a new treatment for peripheral vascular disease

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ABSTRACT

Background
GM-CSF was recently shown to increase collateral flow index in patients with coronary artery disease. Experimental models showed beneficial effects of GM-CSF on collateral artery growth in the peripheral circulation. Thus, in the present study we evaluated the effects of GM-CSF in patients with peripheral artery disease.

Methods and Results
A double-blind, randomized, placebo-controlled study was performed in 40 patients with moderate or severe intermittent claudication. Patients were treated with placebo or s.c. (10 μg/kg) for a period of 14 days (total of 7 injections). GM-CSF treatment led to a strong increase in total white blood cell count and CRP. Monocyte fraction initially increased but thereafter decreased significantly as compared to baseline. Both the placebo group as well as the treatment group showed a significant increase in walking distance at day 14 (placebo: 127 ± 67 meters vs. 184 ± 87 meters, P=0.03, GM-CSF: 126 ± 66 meters vs. 189 ± 141 meters, P=0.04) as well as at day 90. Change in walking time, primary endpoint of the study, was not different between groups. No change in ABI was found upon GM-CSF treatment at day 14 or at day 90. LDF measurements showed a significant decrease in microcirculatory flux reserve in the control group (P=0.03) and no change in the GM-CSF group.

Conclusions
The present study does not support the use of GM-CSF for treatment of patients with moderate or severe intermittent claudication. Issues that need to be addressed are dosing, the selection of patients and potential differences between GM-CSF effects in the coronary and the peripheral circulation.
1. INTRODUCTION

Arterial occlusion, either acute or chronic, is a final event in the natural course of atherosclerotic disease. In the coronary arteries, such an occlusion may cause refractory angina pectoris, myocardial infarction or death. However, numerous cases have been documented in which arterial occlusion in the coronary arteries is compensated by augmentation of the capacity of the collateral circulation and subsequent restoration of blood flow to jeopardized myocardial territories [1,2].

In the peripheral circulation, arterial occlusion may cause intermittent claudication and in some instances will lead to critical leg ischemia and/or limb loss. However, the compensatory mechanisms in the peripheral circulation are more efficient than in the coronary circulation. In a large proportion of patients suffering from peripheral arterial disease, the collateral circulation compensates over time almost completely for the impaired tissue perfusion. Nevertheless, a cohort of patients remains in whom symptomatic PAD progresses despite natural compensation, exercise training and risk factor modulation. In these cases interventional therapies like bypass surgery or percutaneous transluminal angioplasty (PTA) are installed. Unfortunately, these interventions show relatively high rates of re-occlusion [3]. Thus a need exists for alternative, preferentially pharmacological, strategies for symptomatic and functional improvement. Cilostazol is the first substance that has been shown to increase walking distance in patients with PAD, although the exact mechanism of action is unknown [4]. Another potential new treatment modality is the stimulation of arteriogenesis, i.e. the development of large collateral conductance arteries [5]. Over the past years several substances were shown to induce arteriogenesis in experimental models [6]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used in the clinical setting for many years now to treat leucopenia in patients that underwent chemotherapy. GM-CSF also showed a strong pro-arteriogenic efficacy in rodents [7]. In humans, Seiler et al reported for the first time the stimulation of arteriogenesis using GM-CSF. In a small randomized study he found that coronary collateral flow index was increased in patients with chronic coronary artery disease, directly upon a 14-day treatment with GM-CSF. In addition, GM-CSF treatment led to a decrease in ST-segment changes and episodes of angina during balloon occlusion at the end of the treatment period [8]. In the present study the effects of GM-CSF were tested for the first time in a group of patients with moderate or severe intermittent claudication (Rutherford grade I, category 2 or 3). Change in walking distance directly after the 14-day treatment served as primary endpoint.

2. METHODS

Detailed information on the design of the START-study has been published. In short, 40 patients with moderate or severe claudication and a walking distance repeatedly below 200 meters were included. Patients were recruited from the Rijnland hospital (n=10) and the Academic Medical Center Amsterdam (n=20) in the Netherlands and from the Uni-
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University Hospital Freiburg (n=10) in Germany. The presence of a severe occlusion had to be documented by Duplex or angiography and all patients were candidates for bypass surgery or PTA. Exclusion criteria were clinical or laboratory signs of chronic or acute inflammation, previous or current history of neoplasm, diabetes, pregnancy or preserved child bearing capabilities and refusal or inability to give informed consent.

Patients were randomized to treatment with either placebo or subcutaneously applied rhGM-CSF in a dosage of 10 μg/kg every other day for a period of 14 days. rhGM-CSF was given either as Leukine (Berlex, Montville, NJ) or as Leucomax (co-distributed by Schering-Plough, Kenilworth, NJ and Novartis, Basel, Switzerland). The use of different suppliers of rhGM-CSF was an unplanned deviation from the protocol, caused by a sudden termination of distribution of Leucomax by Novartis and Schering-Plough per December 1, 2002 throughout Europe. Randomization was performed by telephone from a central randomization list.

At day 0, day 14 and day 90 walking distance and ABI were assessed. At day 0, 2, 4, 6, 8, 10, 12, 14 and 90 blood samples were taken to determine total leukocytes, differentiated blood count, creatinine, C-reactive protein, SGOT, SGPT, albumin, triglycerides, total cholesterol, VLDL, LDL and HDL.

During the course of our study it was reported by several groups that stem cells potentially are involved in arteriogenesis. We therefore decided to perform CD34+ stem cell measurements in the remaining GM-CSF treated patients. This resulted in a subset of 7 patients in which the number of CD34+ cells in peripheral blood was determined by flow cytometric analysis. A sample of 100 μl blood in EDTA was stained with 5 μl of PE-conjugated mouse anti-CD34 MoAb (HPCA-2; Becton Dickinson, San Jose, CA). In addition, cells were stained with 5 μl of mouse anti-CD16 and anti-CD66 MoAb (Becton Dickinson). PE-conjugated mouse IgG1 MoAb (BD) was used as isotypic control. After incubation at 4° C for 15 minutes in a light-protected area, RBCs were lysed with lysing solution (FACS, BD) containing 0.83 percent ammonium chloride at room temperature for 10 minutes in a light-protected area, and washed twice with PBS containing 0.1 percent azide. Analysis was performed on a fluorescence-activated cell sorter (FACScan, Becton Dickinson). A gate was established to include all nucleated cells and to exclude PLT’s and RBCs by use of the forward and 90° light scatter. A second gate was established to include only CD45+ cells with the side scatter. The number of events counted was 100,000. The number of bright CD34+ cells with low side scatter was then determined. The percentage of CD34+ cells was calculated by subtracting the number of cells stained with the isotypic control from the number of cells stained with the anti-CD34 antibody and dividing by the number of nucleated cells counted in the first window. WBC counts were obtained with an electronic cell counter (Cell Dyn 4000, Abbott, Illinois, USA). The number of CD34+ cells was calculated from the total number of WBC and the percentage of CD34+ cells.

In 30 out of 40 patients (those included in the Rijnland hospital and the AMC), Laser Doppler fluxmetry was performed. This method to non-invasively measure local skin perfusion has proven to be useful as a diagnostic tool in patients with peripheral arterial dis-
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ease.10,11 The device emits laser light with a wavelength of 780 nm, which penetrates the skin to about 1.5 mm. When reflected by moving particles (mainly erythrocytes), the light undergoes a frequency shift that is proportional to the number and velocity of the moving particles. This ‘flux’ is expressed in Volts. Patients were investigated in the supine position after acclimatization for 15 min. in a temperature-controlled room. The laser Doppler (Periflux 5000, Perimed, Stockholm, Sweden) probes were attached to the plantar side of the big toes of both legs. A data-acquisition system was used (AcqKnowledge III and MP 100WSW, Biopac System Inc., Santa Barbara, Ca, USA) for the recording and off-line analysis of the data. Firstly, measurements were performed at rest (in V), which takes 5-10 minutes to reach a stable value. Subsequently, a 3-minute arterial occlusion was induced by means of suprasystolic (200 mm Hg) inflation of a cuff around the ankle. During this period the laser Doppler value reached a nearly-zero value, also known as the biological zero. This value was subtracted from the other flux parameters measured, as it does not represent blood flow. After release of the cuff, the post-occlusive reactive hyperemia response was recorded, which yields the Peak Flux (in V) and Time to Peak Flux (in s) parameters. The difference between PF and rest flux (‘P-RF’) can then be calculated, which gives information about the reserve capacity of the local microcirculatory vessels. This parameter is independent of the baseline flux, which is known to be rather variable.

Occurrence of side-effects was documented every other day during the treatment period as well as at day 90. At day 0, 14 and 90, 4-field fundus photography was performed including the posterior pole and midperiphery of each eye. An experienced retinal specialist read the fundus photographs in a masked fashion.

For the power calculation we estimated the placebo-effect at 40%. We aimed for a 2.5 fold larger increase in walking distance in the GM-CSF treated group (100%). This 2.5 fold increase as compared to the placebo-group was based on earlier published data from the TRAFFIC trial12. Based on own data from 3.500 patients that visited the vascular laboratory in the AMC we expected a baseline walking distance of 87 meters with a standard deviation of 54 meters. In order to have an 80% power, we calculated a total sample size of 36 patients. With an expected 10% drop-out rate we decided to include 40 patients. For primary analysis, an independent samples t-test was used. The primary analysis excluded patients that were not available for follow-up at day 14. A one-sample t-test was applied to test whether the change in walking distance was statistically significant within each treatment group. Statistical significance was assumed at p < 0.05. Analyses involving the secondary endpoints were carried out as subsidiary analyses. For comparison of endpoints at day 90 between the GM-CSF-treated and the placebo group, patients that received a revascularization procedure before day 90 were excluded. For LDF measurements a non-parametric Friedman test was performed. Differences between baseline and 14 days, and between baseline and 90 days were compared by means of the Wilcoxon test. Differences between patients receiving placebo and those receiving GMCSF were tested using the Mann Whitney U test.
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3. RESULTS

The distribution of the forty patients over the three centers was well balanced: 10 vs. 10 at the AMC, 4 vs. 6 at the Rijnland hospital and 6 vs. 4 at the Freiburg University hospital.

Of the 20 patients randomized to the GM-CSF group, in three patients treatment was discontinued due to severe side-effects. In one patient, sensations of chest pain, shortness of breath and hypotension occurred approximately one hour after the first GM-CSF injection. Such anaphylactic reactions have been described occasionally for GM-CSF. This patient was withdrawn from the study. Two other patients decided to discontinue treatment because of the occurrence of severe chest and/or muscular pain. ECG and laboratory testing did not reveal cardiac ischemia. No long-term effects were observed after abrogation of treatment in these patients.

One patient in the placebo group was withdrawn from the study because of a pulmonary embolism at day 6. These patients were excluded from further analysis. The baseline characteristics of the remaining 36 patients show that the two groups were well matched (table 1). Baseline walking distance, rest ABI and post-exercise ABI were comparable between the treatment groups (figure 1). Follow-up thru day 90 was complete in all 36 patients.

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=19)</th>
<th>rhGM-CSF (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>67.6 ± 8.4 y</td>
<td>65.1 ± 10.8 y</td>
</tr>
<tr>
<td>Male sex</td>
<td>80%</td>
<td>60%</td>
</tr>
<tr>
<td>Current smoking</td>
<td>40%</td>
<td>38%</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>60%</td>
<td>72%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Bilateral PAD</td>
<td>60%</td>
<td>50%</td>
</tr>
</tbody>
</table>

Table 1: Baseline characteristics. No statistical significant differences were found for any of the baseline characteristics.
3.1. Walking distance

Both in the placebo as well as the GM-CSF treatment group, a significant increase in walking distance from day 0 to day 14 was observed (placebo: 127 ± 67 meters vs. 184 ± 87 meters, P=0.03, GM-CSF: 126 ± 66 meters vs. 189 ± 141 meters, P=0.04). The increase in walking distance at day 14 was similar between the two treatment groups (placebo 57 ± 113 meters vs. GM-CSF 63 ± 111 meters, p=0.87). Painfree walking distance increased significantly in both the placebo group (62 ± 30 meters vs. 92 ± 36 meters, P=0.04) as well as the GM-CSF treatment group (75 ± 45 meters vs. 103 ± 70 meters, P=0.01). No significant difference in change in painfree walking distance at day 14 was observed between the placebo group and the GM-CSF treatment group (placebo 30 ± 43 meters vs. GM-CSF 28 ± 49 meters, p=0.89). No side-effects of GM-CSF like nausea or muscle pain were reported as specific cause to abrogate exercise at day 14. All patients abrogated exercise because of occurrence of claudication in the most affected leg.

A total of 7 patients (placebo: n=4, GM-CSF: n=3) underwent interventional therapy between day 14 and day 90. These patients were excluded from walking distance and ABI analysis at day 90. For patients that received no interventional therapy between day 14 and day 90, the increase in walking distance prevailed at day 90 in both the placebo as well as the GM-CSF treated group (placebo: 127 ± 67 meters vs. 196 ± 99 meters, P=0.03, GM-CSF: 120 ± 64 meters vs. 209 ± 36 meters, P=0.01). No significant difference in change in walking time was found between the placebo and the GM-CSF treated group at day 90 (figure 2).
3.2. ABI

Depending on whether claudication was unilateral or bilateral, one or two values of ABI per patient were available for analysis. Only ABI’s with a value at day 0 of less then 0.95 were included for repeat analysis. At day 14 this resulted in a total of 22 repeat ABI measurements from the GM-CSF treatment group and a total of 27 repeat ABI measurements from the placebo group. A small but significant increase in ABI from day 0 to day 14 was found in the placebo group (day 0: 0.59 ± 0.16 vs. day 14: 0.62 ± 0.18, no unit, P=0.04). No significant changes in ABI were found upon GM-CSF treatment (day 0: 0.65 ± 0.18 vs. day 14: 0.60 ± 0.20, no unit, P=0.27). At day 90 a total of 17 repeat ABI measurements were available from the GM-CSF treatment group and a total of 20 from the placebo group. No significant changes in ABI from day 0 to day 90 were found either in the placebo group (day 0: 0.58 ± 0.14 vs. day 90: 0.62 ± 0.17, no unit, P=0.17) or in the GM-CSF treatment group (day 0: 0.61 ± 0.18 vs. day 14: 0.63 ± 0.17, no unit, P=0.42). Also no statistical significant difference was found at day 90 for the direct comparison between the GM-CSF treatment and the placebo group.
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3.3. LDF measurements

The results of the LDF parameters at the three different time-points are shown in table 2.

<table>
<thead>
<tr>
<th></th>
<th>Rest Flux (V)</th>
<th>Peak Flux (V)</th>
<th>Peak – Rest Flux (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo (n=16)</td>
<td>GMCSF (n=11)</td>
<td>Placebo (n=16)</td>
</tr>
<tr>
<td>Baseline</td>
<td>0.28</td>
<td>0.09</td>
<td>0.93</td>
</tr>
<tr>
<td>14 days</td>
<td>0.17</td>
<td>0.22</td>
<td>0.66</td>
</tr>
<tr>
<td>90 days</td>
<td>0.21</td>
<td>0.06</td>
<td>0.59*</td>
</tr>
</tbody>
</table>

Table 2: LDF. In placebo patients, peak flux as well as peak minus rest flux decrease over time. In the GM-CSF treatment group these values remain stable (and even show a tendency to increase at day 14). * indicates a P-value < 0.05 as compared to baseline.

LDF was performed in 30 patients. Three patients in which GM-CSF was discontinued because of side-effects were excluded from analysis, leaving a total of 11 GM-CSF treated patients as well as 16 placebo treated patients available for analysis. Three patients from the placebo group as well as 3 patients from the treatment group received bypass surgery between day 14 and day 90.

At baseline, RF and PF were significantly higher in the placebo group than in those treated with GMCSF (P=0.003 and P=0.015, respectively). This difference disappeared during both follow-up moments. In the patients treated with GMCSF, no significant differences in time were observed in any of the LDF parameters. In the patients treated with placebo, the TtPF increased significantly (P=0.030) while the P-RF decreased significantly over time (P=0.024) indicating a decrease in microcirculatory reserve capacity over time, as opposed to the GMCSF-treated group. No differences for any of the parameters were observed over time in asymptomatic legs.

3.4. Side-effects

Less severe but frequently occurring side-effects in the treatment group were skin-rash, fever, head-ache and perspiration (table 3). The repeated eye examinations by fundus photography showed no induction of retinopathy or other ophthalmologic disorders in any of the patients. No acute cardiac events were reported in any of the patients during the 90-day follow up period.
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<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=20)</th>
<th>rh-GM-CSF (n=20)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin rash</td>
<td>0%</td>
<td>25.2%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Muscle pain</td>
<td>0%</td>
<td>21%</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fever</td>
<td>0%</td>
<td>14.3%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Headache</td>
<td>9.8%</td>
<td>14.3%</td>
<td>NS</td>
</tr>
<tr>
<td>Loss of appetite</td>
<td>0%</td>
<td>5.9%</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Perspiration</td>
<td>1.5%</td>
<td>6.7%</td>
<td>NS</td>
</tr>
<tr>
<td>Nausea</td>
<td>5.3%</td>
<td>1.7%</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3: Side effects. The use of GM-CSF led to a large percentage of observed side-effects during treatment time. Most reported side effects were skin rash, muscle pain and fever.

3.5. Blood sampling

Treatment with GM-CSF led to a gradual increase in total number of leukocytes over the treatment period, resulting in significant differences as compared to day 0 starting at day 10. The percentage of monocytes was significantly increased at day 4 (7.8% ± 1.8% vs. 11.0% ± 5.0%, P=0.02). Over time the percentage of monocytes decreased, resulting in a significantly lower percentage of monocytes at day 12 and 14 as compared to day 0 (7.8% ± 1.8% vs. 5.6% ± 2.7% and 5.7% ± 2.5% respectively, P=0.01 for both comparisons). A slight but significant decrease in the percentage of basophiles was found at day 12 and 14. The percentage of granulocytes remained unchanged. Strong changes were found for the percentage of eosinophiles. Their percentage increased from 2.4% ± 1.4% at day 0 to 9.6% ± 4.9% at day 14 (P=0.000002) (figure 3a). The number of CD34 positive stem cells was significantly increased at day 6 and 8 after initiation of GM-CSF treatment. Thereafter, their number decreased again, returning to baseline values at day 14 (figure 3b). A rapid increase was found for CRP. At day 2, CRP levels were increased from 6.1 ± 3.3 to 43.9 ± 32.6 (P=0.00002). Over time, CRP levels decreased but remained elevated over the complete treatment period as compared to day 0 (figure 3c). At day 90 all of the above mentioned parameters had returned to baseline levels. In the placebo-group, all above-mentioned parameters remained unchanged during treatment time as well as at follow-up.
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Figure 3: Results of blood sampling. Serum levels of leukocytes during the 14-day GM-CSF treatment. Monocytes initially increased but thereafter gradually decreased again (A, n=17). A small but significant increase was observed for CD34+ stem cells at day 4 and day 6 (B, n=7). CRP increased and remained elevated during the complete treatment period (C, n=17).

* indicates a significant increase as compared to day 0 measurements, † a significant decrease.
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4. Discussion

In the present study we determined the effects of subcutaneously applied GM-CSF on maximal walking distance in patients suffering from moderate to severe claudication. This is the first randomized, placebo-controlled study aiming specifically at the stimulation of arteriogenesis in PAD patients. A 14-day treatment schedule did not lead to an increase in maximum walking distance, neither directly after treatment nor at 90 days follow-up. Also other parameters like ankle-brachial index showed no increase upon GM-CSF treatment.

The only observed significant effects of treatment were a tendency to an increase in the peak flux and the peak minus rest flux in the GM-CSF group as compared to a significant decrease in the control group. This might indicate a beneficial effect of GM-CSF on the microcirculation, probably secondary to improved endothelial function. However, the clinical relevance of these findings in the present patient population is not clear.

The observed placebo effect was large. In the placebo group, an approximate 50% increase in walking distance was observed at day 14. At day 90, the mean walking distance in the placebo-group was still about 50% larger as compared to day 0. Since no change of ABI was observed between day 0 and day 90, it can be concluded that this increase in walking distance is a real placebo-effect and not secondary to actual macrocirculatory changes, due to for example training effect or natural arteriogenesis. Similar placebo effects have been observed in most trials on stimulation of collateral artery growth.

Monocytes are target cells of pro-arteriogenic strategies since these cells provide growing collateral arteries with the necessary growth factors and cytokines. It is also claimed that the often debated endothelial progenitor cells are derived from circulating monocytes. Somewhat to our surprise, GM-CSF led to only a temporary increase in monocytes. After day 4 a gradual decrease was observed, finally leading to a level below baseline level both in absolute numbers as well as in percentage of total leukocytes. A similar trend was seen in CD34+ stem cells, indicating that one of the potential explanations for failure of the current treatment strategy is a sub-optimal dosing scheme. Potentially, repetitive short periods of treatment are more effective in raising the number of circulating monocytes and stem cells.

Probably PAD patients are more difficult to treat than CAD patients. In PAD, about 60% of patients compensate adequately for vascular obstruction by mechanisms of natural arteriogenesis and changes in metabolism. Moreover, the time between first presentation and interventional therapy is generally very long. Thus, patients that were eligible for the present study represent a cohort of patients that have a deficiency in their innate response to vascular obstruction and suffer from atherosclerotic disease for a prolonged period of time. In contrast, in CAD patients severe coronary stenoses are left unnoticed only in a minority of patients (although several cases are reported in the literature) and time between first presentation and interventional therapy is generally much shorter than in PAD. This might explain the difference in outcome between the present study and the
study by Seiler et al. [8]. Another important difference between our study and the study by Seiler is the method to detect collateral artery growth. Seiler used a very sensitive method of intracoronary derived pressure measurements. Unfortunately, such techniques are not validated for the peripheral circulation and therefore in our protocol we were bound to the presumably less sensitive and less objective endpoint of walking distance. Potentially more sensitive measurements of collateral flow in the peripheral circulation like MRI-flow measurements or invasive pressure and flow measurements would be of great value for future trials in PAD patients.

Several clinical trials on stimulation of vascular growth, either angiogenesis or arteriogenesis, were conducted in the last few years. Most of these trials included patients with coronary artery disease. A minority of studies focused on peripheral arterial disease (for review see also Schirmer et al. [14]). The first published large randomized placebo-controlled trial on stimulation of collateral artery growth in PAD patients was the TRAFFIC trial [12]. In this trial fibroblast growth factor-2 (FGF-2) protein was intra-arterially infused into the lower extremities of 190 PAD patients with moderate or severe intermittent claudication. FGF-2 is known for its angiogenic properties but also displays pro-arteriogenic properties. Patients were randomized to either placebo, single-dose (30 μg/kg) or double-dose (60 μg/kg) treatment. An increase was found in peak walking time at 90 days (primary endpoint) in the single-dose group only after secondary intention-to-treat analysis. In the double-dose group, surprisingly no increase was detected. Also the subsequent RAVE-trial, assessing the effects of VEGF in PAD patients with intermittent claudication, did not meet the high expectations [15]. VEGF mainly stimulates angiogenesis and has only a weak pro-arteriogenic potential. In this trial a total of 105 patients with intermittent claudication was treated with intramuscular injections of adenoviral VEGF 121. Apart from enhanced peripheral edema, no significant increase in walking time, ankle-brachial index or quality of life could be observed. Thus, the present study is the third randomized, placebo-controlled study in PAD patients in which no increase in walking capacity was found upon growth factor therapy.

Nevertheless, growth factor therapy still constitutes a promising therapeutic strategy to treat patients with atherosclerotic disease. A large body of pre-clinical data is available that unequivocally shows that arteriogenesis alleviates the consequences of arterial obstruction. Several factors involved have been identified and cellular and molecular mechanisms underlying this process have been unraveled not completely but to a large extent [16-22]. The biggest challenge will be the identification of the most optimal factor or combination of factors, as well as delivery platforms (gene therapy vs. protein therapy). Also, choice of endpoints and choice of target population will decide upon failure or success of future clinical studies.
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5. Limitations of the Study

Inadequate sample size is a potential problem which might mask smaller beneficial effects of GM-CSF treatment. It should be noted though that the observed differences between the two groups in either walking distance or ABI are practically zero. Although the current data do not exclude the possibility of a small treatment benefit, they certainly do not support the hypothesis that GM-CSF is beneficial to patients with intermittent claudication.

The change of GM-CSF supplier was an unwanted deviation from the protocol. Data on pharmacokinetics as provided by the manufacturers are comparable. In a subsidiary analysis no significant differences were found for walking distance, ABI or laboratory parameters as derived from patients treated with either Leucomax (Novartis/Schering-Plough) or Leukine (Berlex).

The blinding process in the present study was hampered by the strong side-effects. In our study we have sought to prevent observer bias by having an observer without knowledge of the side-effects performing the exercise test and the ABI and LDF measurements. Obviously, the patient cannot be prevented from observing his or her own side-effects. On the other hand, it can also be advocated that the strong side-effects had a negative impact on training exercise between day 0 and day 14. Placebo patients potentially did perform more exercise between day 0 and day 14. It should be noted though that patients were not instructed to perform extra exercise in this period and even more importantly, all these patients had been refractory to structured training programs in the past.

In any case, such problems with blinding and also the previously mentioned large placebo-effect underscore the strong demand for reliable objective endpoints.

6. Acknowledgements

The present study was supported by Netherlands Heart Foundation, grant number 2002B076b.
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7. REFERENCES


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