Peripheral Artery Disease

A Randomized, Controlled Pilot Study of Autologous CD34+ Cell Therapy for Critical Limb Ischemia

Douglas W. Losordo, MD; Melina R. Kibbe, MD; Farrell Mendelsohn, MD; William Marston, MD; Vickie R. Driver, DPM, MS; Melhem Sharafuddin, MD; Victoria Teodorescu, MD; Bret N. Wiechmann, MD; Charles Thompson, MD; Larry Kraiss, MD; Teresa Carman, MD; Suhail Dohad, MD; Paul Huang, MD; Candice E. Junge, PhD; Kenneth Story, MS; Tara Weistroffer, MS; Tina M. Thorne, MS; Meredith Millay, BS; John Paul Runyon, MD; Robert Schainfeld, DO; for the Autologous CD34+ Cell Therapy for Critical Limb Ischemia Investigators

Background—Critical limb ischemia portends a risk of major amputation of 25% to 35% within 1 year of diagnosis. Preclinical studies provide evidence that intramuscular injection of autologous CD34+ cells improves limb perfusion and reduces amputation risk. In this randomized, double-blind, placebo-controlled pilot study, we evaluated the safety and efficacy of intramuscular injections of autologous CD34+ cells in subjects with moderate or high-risk critical limb ischemia, who were poor or noncandidates for surgical or percutaneous revascularization (ACT34-CLI).

Methods and Results—Twenty-eight critical limb ischemia subjects were randomized and treated: 7 to 1×10^5 (low-dose) and 9 to 1×10^6 (high-dose) autologous CD34+ cells/kg; and 12 to placebo (control). Intramuscular injections were distributed into 8 sites within the ischemic lower extremity. At 6 months postinjection, 67% of control subjects experienced a major or minor amputation versus 43% of low-dose and 22% of high-dose cell-treated subjects (P=0.137). This trend continued at 12 months, with 75% of control subjects experiencing any amputation versus 43% of low-dose and 22% of high-dose cell-treated subjects (P=0.058). Amputation incidence was lower in the combined cell-treated groups compared with control group (6 months: P=0.125; 12 months: P=0.054), with the low-dose and high-dose groups individually showing trends toward improved amputation-free survival at 6 months and 12 months. No adverse safety signal was associated with cell administration.

Conclusions—This study provides evidence that intramuscular administration of autologous CD34+ cells was safe in this patient population. Favorable trends toward reduced amputation rates in cell-treated versus control subjects were observed. These findings warrant further exploration in later-phase clinical trials.

Key Words: peripheral vascular disease ■ revascularization ■ reperfusion ■ randomized trial ■ stem cells

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From the Division of Cardiovascular Medicine, Northwestern Memorial Hospital and Feinberg Cardiovascular Research Institute (D.W.L., T.W., T.M.T., M.M.) and Divisions of Cardiology and Vascular Surgery, Northwestern Memorial Hospital, Northwestern University, Chicago, IL (M.R.K.); Center for Therapeutic Angiogenesis, Birmingham, AL (F.M.); Division of Vascular Surgery, University of North Carolina, Chapel Hill, NC (W.M.); Department of Surgery, Boston Medical Center and Boston University School of Medicine, Boston, MA (V.R.D.); Department of Surgery, University of Iowa, Iowa City, IA (M.S.); Division of Vascular Surgery, Mount Sinai School of Medicine, New York, NY (V.T.); Vascular and Interventional Physicians, Gainesville, FL (B.N.W.); Orlando Regional Medical Center Vascular Specialist of Central FL, Orlando, FL (C.T.); Division of Vascular Surgery, University of Utah, Salt Lake City, UT (L.K.); University Hospitals Case Medical Center, Cleveland, OH (T.C.); Cedars-Sinai Medical Center, Los Angeles, CA (S.D.); Swedish Medical Center, Seattle, WA (P.H.); Baxter Healthcare, Deerfield, IL (C.E.J., K.S.); Christ Hospital, Cincinnati, OH (J.P.R.); and Massachusetts General Hospital, Boston, MA (R.S.).
Correspondence to Douglas W. Losordo, MD, Baxter Healthcare Corporation, One Baxter Parkway, Deerfield, IL 60015. E-mail: douglas_losordo@baxter.com
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WHAT IS KNOWN

- Human CD34+ cells are well known as hematopoietic stem cells used for stem-cell transplants in patients who have bone marrow ablation by chemotherapy or radiation therapy.
- Preclinical studies in models of myocardial or limb ischemia show that local delivery of human CD34+ cells improves perfusion and function in ischemic tissue.

WHAT THE STUDY ADDS

- In a double-blind, randomized, placebo-controlled, pilot clinical trial in patients with Rutherford class 4 and 5 critical limb ischemia, direct intramuscular injection of autologous CD34+ cells was associated with reductions in the frequency of amputation.
- The strategy of mobilizing and collecting autologous CD34+ cells in critical limb ischemia patients was shown to be feasible and was not associated with an adverse safety signal. Further study is warranted.

Methods

Study Design

The ACT34-CLI study was a prospective, double-blind, randomized, placebo-controlled clinical pilot study conducted at 14 centers in the United States. A total of 28 subjects were randomized 1:1:1 to 3 treatment groups: low-dose (1×10⁵ Auto-CD34+ cells/kg, n=7), high-dose (1×10⁶ Auto-CD34+ cells/kg, n=9), and control (placebo, n=12). The objectives of this phase II/IIa clinical trial were to evaluate the safety and bioactivity of intramuscular injection of autologous CD34+ cells in subjects with moderate or high-risk CLI, who were poor or noncandidates for surgical or percutaneous revascularization (ACT34-CLI).

Study Population

Male or female patients aged ≥21 years with Rutherford categories 4 or 5 CLI and no suitable revascularization options (determined by independent vascular surgeons and vascular interventionists) were eligible for this study. In addition, demonstrated infrainguinal atherosclerosis with a stenosis (>70%) or occlusion (100%) of a major vessel and an absolute ankle pressure in the affected limb of <60 mm Hg or a reduced toe pressure of <40 mm Hg or abnormal plethysmography, diagnostic of microvascular insufficiency (flat waveforms) were required.

Candidates were excluded (thromboangiitis obliterans [Buerger disease] was allowed) if arterial insufficiency in the lower extremity was the result of a nonatherosclerotic disorder, including but not limited to, advanced scleroderma (CREST syndrome). Additional exclusion criteria included patients with advanced CLI (Rutherford category 6), expected amputation within 4 weeks of screening, clinical evidence of sepsis, advanced AV block or New York Heart Association class III or class IV heart failure, myocardial infarction within 3 months, or clinically successful aortic or lower-extremity arterial surgery, percutaneous revascularization, or lumbar sympathectomy within 3 months preceding screening.

Auto-CD34+ Cell Mobilization, Collection, and Preparation

To maintain the double-blind design, all subjects underwent cell mobilization with 5 μg/kg per day doses of granulocyte colony stimulating factor (Filgrastim/Neupogen, Amgen, Thousand Oaks, CA) administered subcutaneously for 4 or 5 days followed by leukapheresis on the fifth day. The next day, the leukapheresis product was enriched for CD34+ cells using the ISOLEX 300i Magnetic Cell Selection System (Baxter Healthcare, Deerfield, IL). Lot release testing was performed on the final cell preparation to document sterility (gram stain and subsequent culture), viability (7-Aminoactinomycin D apoptosis staining) and purity (fluorescence activated cell sorting for CD34+ cells). Auto-CD34+ cells were suspended in 4 mL of 0.9% NaCl (saline) plus 5% autologous plasma and provided to the investigator in 8 syringes.

Randomization and Blinding

Once the cell product passed all lot release criteria, the subject was randomized to 1 of the 3 treatment arms. Subjects were prospectively stratified centrally for Rutherford category 4 or 5, presence or absence of diabetes mellitus, and smoker or nonsmoker. The investigator, subject, study-site personnel, core laboratory(ies), blinded study statistician, and all sponsor and clinical research organization personnel remained blinded to all subject treatment.

Cell Injection Procedure

On the day of randomization, the total cell dose was delivered via intramuscular injection into 8 distinct sites (0.5 mL/site) in the ischemic lower extremity using a 1-mL syringe fitted with a 27-gauge needle. In the majority of subjects in which ischemia was most prominently manifested in the distal lower extremity (below knee), the 8 injections were distributed in the proximal, mid, and distal calf, according to the subject’s clinical status and vascular anatomy, targeting ischemic muscle supplied by occluded or stenotic arteries.

Endpoints

Safety

The primary end point of this exploratory study was the safety of intramuscular injection of Auto-CD34+ cells. Adverse events, vital signs, and laboratory assessments (clinical chemistry, hematology, cardiac biomarkers, and urinalysis) were assessed during the treatment period (granulocyte colony stimulating factor cell mobilization, apheresis and intramuscular injection) and during the follow-up period at weeks 2, 4, 6, 8, and 12 weeks and 6 and 12 months.

Efficacy

To assess limb salvage, the occurrence of amputation, nature of amputation (toe or transmetatarsal, below or above knee, preserving or not preserving function), and time to amputation were recorded during the 12-month follow-up period.
A subject diary was used to record rest pain. Subjects began recording rest pain in their diaries 7 days before each follow-up visit. Changes from baseline in the duration, frequency, and intensity (numerical rating pain scale from 1 [least pain] to 10 [greatest pain]) of rest pain, analgesic use, and sleep history were assessed. The Six Minute Walk test was performed at baseline, week 12, and months 6 and 12 to assess functional improvement in subjects. The Modified Borg scale was used to measure fatigue and a baseline score was determined before beginning the test. All symptoms, walking distance, and time to onset of leg cramping/pain were recorded. A core laboratory (Canfield) was used for wound assessment. Assessment included ulcer tracing and photography of the wound. Acetate tracings of the wound and digital planimetry were used to assess changes from baseline in ulcer size (area). Time to complete healing or change to a state of potentially successful surgical closure or skin grafting was recorded. Quality of life was assessed using the Social Functioning-36 Quality of Life questionnaire (version 1). Disease severity was assessed by changes from baseline in the Rutherford Clinical Severity score, absolute ankle and toe pressure, and ankle brachial index (ABI) and toe brachial index, respectively.

**Statistical Analysis**

This study was designed to help determine the selection of end points, time points, and the appropriate sample sizes for subsequent clinical studies of Auto-CD34+ cells for subjects with CLI. All analyses performed were based on intent to treat. Efficacy analyses were exploratory in nature and no corrections for multiple comparisons or formal sample size calculations were performed. Baseline characteristics were summarized. One-way ANOVA was used to test for differences in the treatment groups for continuous variables and Fisher exact test was used for categorical variables. Adverse events were summarized. Fisher exact test was used to test for differences between treatment groups in percent of subjects with amputations. Log-rank tests were used to test for differences in the distributions of time to first amputation. The adjusted amputation rates were calculated assuming that amputations have a negative binomial distribution. Changes in function and disease severity over time are presented descriptively; no statistical analysis was performed.

**Results**

**Subject Disposition and Baseline Characteristics**

Between November 2007 and April 2010, 14 centers across the United States screened 43 subjects; 28 subjects met the entry criteria for this study and underwent granulocyte colony stimulating factor cell mobilization, apheresis to collect total mononuclear cells, randomization and intramuscular injections of Auto-CD34+ cells or placebo (Figure 1). In total, 20 subjects completed the 1-year study follow-up period. There were no statistically significant differences in subject baseline demographics, medical history, and disease characteristics among treatment groups (Table 1). The study population included 9 women and 19 men with a mean age of 67 years. Previous lower-extremity bypass surgery or percutaneous coronary intervention had been performed in all subjects.

**Safety of Auto-CD34+ Cell Therapy During Treatment and Follow-up Period**

A total of 60 serious adverse events in 22 subjects (79%) occurred during the study of which 59 occurred after intramuscular injection and 1 occurred during mobilization. The majority of serious adverse events were considered unrelated.
to study treatment by the investigator, with the exception of 2 serious adverse events that were considered possibly study related: 1 subject experienced moderate hypotension during mobilization, which required prolonged hospitalization, and 1 subject experienced severe worsening of CLI in the target leg after injection, which required prolonged hospitalization. Only 1 serious adverse event was cardiac related: 1 subject in the control group experienced an acute non-ST segment elevation myocardial infarction ≈4.5 months postinjection. There were 2 deaths during the study, which were not considered study related and these were the only subjects to discontinue because of an adverse event.

Predominantly modest and exclusively asymptomatic elevations in cardiac enzyme levels were observed during the mobilization (granulocyte colony stimulating factor) and injection period (Table 2). Of the subjects with cardiac enzyme measurements, elevated levels of troponin, creatine kinase myocardial band fraction and CK (>1×ULN) were observed in 9 (56.3%), 13 (54.2%), and 4 (15.4%) subjects, respectively, during the mobilization and injection period. During the follow-up period, elevated levels of troponin, creatine kinase myocardial band fraction, and CK (>1×ULN) were observed in 5 (27.8%), 8 (30.8%), and 5 (17.9%) subjects, respectively.
All efficacy analyses were exploratory in nature as the study was not powered to detect differences among treatment groups in efficacy parameters. At 6 months postinjection, 8 subjects (66.7%) in the control group, 3 (42.9%) in the low-dose group, and 2 (22.2%) in the high-dose group experienced an amputation ($P=0.137$, Table 3). Major amputations occurred in 4 subjects in the control group, in 3 subjects in the low-dose group and 2 subjects in the high-dose group ($P=0.780$). At 12 months postinjection there was no increase in the incidence of amputations in the cell-treated groups from the 6-month postinjection time point, but the incidence increased slightly in the control group (9 subjects [75.0%]; $P=0.058$). The incidence of major amputations was slightly higher in the control group (n=6 [50%]) compared with the cell-treated groups, but this difference was not statistically significant ($P=0.488$). Trends toward lower amputation rates in the cell-treated groups versus the control group were observed at 6 months ($P=0.187$) and 12 months postinjection ($P=0.121$). Statistically significant differences in major amputation rates among the control and cell-treated groups at 6 months ($P=0.303$) and 12 months ($P=0.430$) postinjection were not detected.

When subjects in the cell-treated groups are combined, the incidence of total amputations at 6 months and 12 months postinjection was 66.7% in control versus 31.3% in cell-treated subjects ($P=0.125$) and 75.0% in control versus 31.3% in cell-treated subjects ($P=0.054$), respectively.

### Table 3. Summary and Analysis of All Amputations by Treatment Group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>6 Mos</th>
<th>12 Mos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>$1\times10^5$ c/kg</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=7)</td>
</tr>
<tr>
<td>% with amputation (N)</td>
<td>66.7% (8)</td>
<td>42.9% (3)</td>
</tr>
<tr>
<td>% with major amputation (N)</td>
<td>33.3% (4)</td>
<td>42.9% (3)</td>
</tr>
<tr>
<td>Total number of amputations</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Adjusted amputation rate* (lower, upper 95% CI)</td>
<td>1.50 (0.78, 2.87)</td>
<td>1.42 (0.59, 3.42)</td>
</tr>
<tr>
<td>Adjusted major amputation rate* (lower, upper 95% CI)</td>
<td>0.66 (0.25, 1.77)</td>
<td>1.42 (0.59, 3.42)</td>
</tr>
<tr>
<td>% with amputation (N)</td>
<td>75.0% (9)</td>
<td>42.9% (3)</td>
</tr>
<tr>
<td>% with major amputation (N)</td>
<td>50.0% (6)</td>
<td>42.9% (3)</td>
</tr>
<tr>
<td>Total number of amputations</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>Adjusted amputation rate* (lower, upper 95% CI)</td>
<td>1.00 (0.57, 1.76)</td>
<td>0.72 (0.30, 1.73)</td>
</tr>
<tr>
<td>Adjusted major amputation rate* (lower, upper 95% CI)</td>
<td>0.50 (0.22, 1.11)</td>
<td>0.72 (0.30, 1.73)</td>
</tr>
</tbody>
</table>

* Amputations per year. Adjusted for different rates for each subject using negative binomial model.
months postinjection was 33.3% in control versus 31.3% in cell-treated subjects ($P=1.000$) and 50.0% in control versus 31.3% in cell-treated subjects ($P=0.441$), respectively.

There were trends toward an increased probability of amputation-free survival in the low-dose and high-dose groups compared with the control group during the 12-month postinjection follow-up period ($P=0.35$, log-rank test, Figure 2A). When the cell-treated groups are combined, the probability of amputation-free survival was significantly increased in the cell-treated group compared with the control group.

Table 4. Summary of Change from Baseline in Function, Wound Healing and Pain

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment Group</th>
<th>Baseline (N)</th>
<th>Change From Baseline (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mo 6</td>
<td>Mo 12</td>
</tr>
<tr>
<td><strong>Six Minute Walk</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to leg pain (s), mean±SD</td>
<td>Control</td>
<td>175.4±88.7 (7)</td>
<td>66.5±263.8 (2)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>212.2±89.4 (5)</td>
<td>−158.0±264.5 (2)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>142.4±34.7 (5)</td>
<td>122.7±78.0 (2)</td>
</tr>
<tr>
<td>Distance to leg pain (ft), mean±SD</td>
<td>Control</td>
<td>307.1±234.6 (7)</td>
<td>355.0±629.3 (2)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>356.8±172.6 (5)</td>
<td>−55.0±304.1 (2)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>306.7±135.9 (4)</td>
<td>321.5±454.7 (2)</td>
</tr>
<tr>
<td>Total distance walked (ft), mean±SD</td>
<td>Control</td>
<td>717.2±402.0 (9)</td>
<td>493.3±510.5 (3)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>527.3±371.9 (6)</td>
<td>315.0±438.5 (3)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>704.0±334.3 (7)</td>
<td>53.5±421.5 (4)</td>
</tr>
<tr>
<td>Leg ulcer</td>
<td>Control</td>
<td>5.7±6.2 (7)</td>
<td>2.8±11.2 (4)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>1.7±2.0 (3)</td>
<td>12.7±0.7 (2)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>8.7±5.8 (4)</td>
<td>0.1±10.4 (3)</td>
</tr>
<tr>
<td>Rest pain</td>
<td>Control</td>
<td>33.0 [3,123] (12)</td>
<td>−19.5 [−112, 18] (10)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>10.0 [4.2,7] (7)</td>
<td>−8.5 [−13.0] (4)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>17.5 [7,125] (8)</td>
<td>−11.0 [−15.0] (5)</td>
</tr>
<tr>
<td>Pain-intensity score, mean±SD</td>
<td>Control</td>
<td>5.4±1.3 (12)</td>
<td>−1.1±2.2 (10)</td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>6.7±3.0 (7)</td>
<td>−2.8±0.8 (4)</td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>6.0±2.0 (8)</td>
<td>−0.6±1.4 (5)</td>
</tr>
</tbody>
</table>
A trend toward improved major amputation-free survival was observed in the individual cell-treated groups (Figure 2C) and combined cell-treated group (Figure 2D) compared with the control group ($P=0.013$ and $P=0.294$, respectively).

**Functional Improvement, Wound Healing, and Rest Pain**

Of the 28 subjects enrolled in the study, 22 completed the Six Minute Walk test at baseline (Table 4). A total of 11 subjects completed the Six Minute Walk test at the 6- or 12-month postinjection time points (Figure 3). In the control group (n=4), the distance walked increased for 2 subjects and decreased for 2 subjects. In the cell-treated groups (n=7), the distance walked increased for 6 subjects and decreased slightly for 1 subject. Eleven subjects did not complete the test at 6 months and 12 months postinjection for the following reasons: amputation (n=7), withdrawal from study (n=3), and unknown (n=1).

A total of 14 subjects had leg ulcers at baseline (Table 4). At 6 months’ postinjection, wound area measurements were not reported in 5 of the 14 subjects with ulcers at baseline because of amputation (Figure 3). At 12 months postinjection, wound area measurements were not reported in an additional 4 subjects because of subject withdrawal (n=2) or assessment not performed (n=2). There were no treatment-related trends in terms of wound healing observed postinjection at 6 or 12 months.

A total of 27 subjects completed the pain diary at baseline. Overall, decreases in the median number of pain episodes per week and the average pain-intensity scores were observed in all groups postinjection at 6 and 12 months (Table 4).

**Disease Severity**

There were minor fluctuations in the ABI and toe brachial index among subjects in all treatment groups with preserved limbs and measurements at 6 months and 12 months (Table 5). Of the subjects with Rutherford score data at 6 months and 12 months postinjection (n=13 and n=11, respectively), the mean Rutherford score decreased from baseline in all treatment groups postinjection at 6 months and postinjection in the control and high-dose groups at 12 months. The mean Rutherford score in the low-dose group remained unchanged from baseline at 12 months’ postinjection.

**Quality of Life**

At 6 months postinjection, improvements from baseline in the majority of health domain scores were observed in all treatment groups (Figure 4). At 12 months postinjection, improvements in the mean scores were observed in 8, 5, and 3 of...
the health domains in the low-dose, high-dose, and control groups, respectively.

Discussion

The results from this phase I/IIa pilot study provide initial evidence that intramuscular injection of Auto-CD34+ cells is safe and well tolerated in patients with moderate or high-risk CLI, who are poor or noncandidates for surgical or percutaneous revascularization.

Trends toward decreased amputation in Auto-CD34+ cell-treated subjects compared with control subjects demonstrate the potential efficacy of Auto-CD34+ cell therapy in this population. One must use caution in interpreting these results, however, because a higher percentage of subjects experienced amputations during this study relative to other recent clinical studies of CLI.

Several surrogate markers (ABI, toe brachial index, leg pain, walking distance, and wound healing) of limb perfusion were explored and no differences were detected between the cell-treated and control groups; however, this study was not powered to detect differences in efficacy end points. In addition, the high rate of amputation observed in this study resulted

Table 5. Summary of Change From Baseline in Disease Severity

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment Group</th>
<th>Baseline (N)</th>
<th>Change From Baseline (N)</th>
<th>Mo 6</th>
<th>Mo 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ankle and toe pressure</td>
<td>Control</td>
<td>0.3 [0.0, 0.8] (11)</td>
<td>0.1 [−0.1, 0.4] (6)</td>
<td>0.1 [−0.1, 0.6] (5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>0.5 [0.0, 0.9] (7)</td>
<td>0.2 [−0.5, 1.1] (4)</td>
<td>0.2 [−0.3, 0.6] (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>0.5 [0.0, 0.7] (9)</td>
<td>0 [−0.2, 0.4] (6)</td>
<td>0.1 [−0.1, 0.1] (5)</td>
<td></td>
</tr>
<tr>
<td>TBI (treated leg), median [min, max]</td>
<td>Control</td>
<td>0 [0, 0.3] (9)</td>
<td>0 [0, 0] (2)</td>
<td>−0.1 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low dose</td>
<td>0.1 [0, 1.3] (7)</td>
<td>0 [−0.5, 0.2] (4)</td>
<td>0 [−0.4, 0.3] (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High dose</td>
<td>0.1 [0, 1.1] (8)</td>
<td>0 [0, 0.1] (5)</td>
<td>0.1 [0, 0.2] (4)</td>
<td></td>
</tr>
<tr>
<td>Rutherford score</td>
<td>Control</td>
<td>4.6±0.5 (12)</td>
<td>−1.7±2.0 (6)</td>
<td>−1.5±1.9 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low Dose</td>
<td>4.4±0.5 (7)</td>
<td>−0.7±1.2 (3)</td>
<td>0.0±2.0 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>High Dose</td>
<td>4.4±0.5 (9)</td>
<td>−1.0±2.4 (4)</td>
<td>−0.8±2.2 (4)</td>
<td></td>
</tr>
</tbody>
</table>

ABI indicates ankle brachial index; and TBI, toe brachial index.
In our study we chose to isolate and administer CD34+ cells for 2 principal reasons: (1) because of their demonstrated proangiogenic potential in vivo;5,6 and (2) because an available, approved technology permitted the manufacturing of CD34+ cell preparations by standardized methods. The advantage of this approach is that selection of CD34+ cells results in a higher concentration of endothelial progenitor cells in each dose compared with unselected MNCs resulting in greater therapeutic potency in preclinical models. There are no known disadvantages of this approach other than the added step in cell processing. A theoretical disadvantage of this approach is the possibility that other cell types, which may exert proangiogenic or reparative functions, are removed; however, the evidence from preclinical models does not support this concept. Similar to the studies described above, we observed favorable trends in efficacy, including reduced amputation rates and improved amputation-free survival in the cell-treated groups compared with the control group. The high rate of amputations observed in our study, however, limited the interpretation of other efficacy end points.

In conclusion, the overall positive safety profile of collecting and administering autologous CD34+ cells in this patient population and the potential efficacy of preventing amputations warrant larger scale studies to verify these findings, and to further refine the methods for collecting and administering Auto-CD34+ cells to patients with disabling CLI.

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Disclosures
Drs Losordo, Junge, and Story are employed by Baxter Healthcare Corporation. The other authors have no conflicts to report.

References


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