Introduction

Recent reports have suggested that the injection of bone marrow-derived stem cells (BMSCs) into ischemic myocardial territories may improve perfusion and symptoms in patients with chronic ischemia refractory to conventional treatments [1, 4]. These initial clinical studies have followed a consistent basic and preclinical body of evidence [5 –8] showing that BMSCs can improve myocardial vascularization in the ischemic heart. The angiogenic response was attributed to the ability of BMSC both to differentiate into fully mature endothelial cells [9,10] and to exert a supportive paracrine effect on tissue-resident endothelial cells by secreting a number of angiogenic factors [11].

The aim of the present pilot study was to evaluate the hypothesis that direct minimally invasive inoculation of autologous BMSCs into the ischemic myocardium of patients with refractory angina pectoris may safely reduce anginal symptoms and improve myocardial perfusion.

Material and Methods

Patients

Five patients were enrolled from December 2001 to June 2006. The patients suffered from severe chronic angina (Canadian Class III or IV) with evidence of anterior, anterolateral, or inferior ischemia on nuclear imaging (gated technetium-99m single-photon emission computed tomography [SPECT]) ([Table 1]). Before enrolment they were required to have attempted the “best” medical therapy, including long-acting nitrates, maximal use of β-blockers and calcium channel agents, without control of symptoms, and to be taking at least 2 antianginal medications. Baseline char-
acteristics of the study group included a history of hypertension in 3 patients, smoking history in 2, peripheral vasculopathy, diabetes mellitus and rheumatoid arthritis in 1 case. None of the patients underwent prior automated implantable cardioverter-defibrillator implantation. Two patients had had prior percutaneous transmyocardial laser revascularizations or prior surgical coronary intervention. All patients but one underwent prior percutaneous and/or surgical revascularization procedures (Table 1).

The patients were judged ineligible for percutaneous or surgical revascularization as assessed by coronary angiography. A committee comprising 3 cardiovascular surgeons and 2 cardiologists determined the ineligibility for percutaneous or surgical revascularization. The exclusion criteria were: age greater than 75 years, an acute myocardial infarction in the 6 months of enrolment in the study, LV ejection fraction lower than 40%, a history of malignant ventricular arrhythmias, or serious concomitant diseases, such as diabetes mellitus and rheumatoid arthritis in 1 case. None of the patients had had prior cardiovascular procedures.

Study design
The severity of angina was graded according to the Canadian Cardiovascular Society Score. The Angina Seattle Questionnaire was used to assess patients’ quality of life. The following laboratory exams were obtained within 1 week before cell injection: 24-hour Holter monitoring to assess ventricular arrhythmias, 2-dimensional echocardiography to assess LV function, volume, and scar tissue, and gated SPECT to assess perfusion and ischemia. The follow-up evaluations performed at 1, 3, 6, 12 and, when applicable, 24 months after the injection procedure included a clinical evaluation, a laboratory evaluation, echocardiography and 24-hour Holter monitoring to assess ventricular arrhythmias. At 6 and 12 months of follow-up, a gated exercise SPECT and at 6 months a coronary angiography were performed.

Bone marrow aspiration and isolation of CD133+ cells
Two different procedures were used for cell harvesting, according to site preferences. We selected CD133+ stem cells, because they represent a population of early precursors highly enriched for multipotent stem cells with potent angiogenic capacity [12]. In the first 3 patients (2001 – 2003), stem cells were mobilized [13,14] as follows: 10 mg/kg die of lenograstim (Granocyte 34; Aventis Pharma, Milan, Italy) were administered s.c. for 4 consecutive days in order to mobilize stem cells from the bone marrow to the peripheral blood. At the end of the mobilization therapy, the WBC count peaked to 40 800/mm³ with neutrophil prevalence, and then peripheral blood stem cells (PBSC) were collected by means of apheresis, using the COBE Spectra Apheresis System (Gambro BCT Inc., Stockholm, Sweden). This automated procedure includes blood collection, blood processing by centrifugation to collect white cells in the size range of stem cells, and blood return to the patient. The duration of the procedure ranges from 3 to 4 h. The product of apheresis collection was processed by a CliniMacs device (Miltenyi Biotech, Bergisch Gladbach, Germany) in order to obtain CD133+ cell fractions.

In the last two patients (2005 – 2006), CD133+ cells were obtained from the iliac crest, as follows [15]: 1 day before surgery, a total of 150 to 200 ml of bone marrow was aspirated from the iliac crest. The procedure was performed within a qualified operating room. Mononuclear cells were isolated by Ficoll density centrifugation. These cells were then incubated with a ferritin-conjugated monoclonal antibody against the human stem-cell marker AC133 for 30 min. AC133+ cells include a non-hemopoietic (CD34–) subpopulation of bone-marrow stem cells that have a high potential to induce angiogenesis. We used the CliniMacs magnetic cell separation device to separate AC133+ cells from the mass of mononuclear bone marrow cells. To avoid competitive binding of the labelling antibody with the AC133 antibody used for cell separation, we labelled cells for counting using CD34 monoclonal antibody (clone 8G12, Becton-Dickinson, Heidelberg, Germany).

Irrespective of the procedure used, the purity of the stem-cell suspension was > 80% in all patients. CD133+ positive cells were stored at 4°C and resuspended in a CE approved medium until intramyocardial injection the next day. The number of cells injected varied from 4 × 10⁶ to 12 × 10⁶ (Table 1).

Direct intramyocardial injection of CD133+ cells
We elected to perform minimally invasive surgical approaches to minimize the surgical trauma (Table 1). In 4 cases with anterior or anterolateral myocardial ischemia, a left anterior mini-thoracotomy was performed; the chest was opened via an approximately 10-cm anterior or anterolateral incision, the pericardium was opened and the myocardial regions to be injected with cells were identified. Then the solution containing the autologous CD133+ stem cells was injected into the target myocardial areas of the beating heart by means of gentle hand injection through a 22-gauge butterfly needle, as previously described [13]. The plastic cover around the needle was left in place and shortened by approximately 3 mm to control the injection of PBSC into the myocardium at a constant depth, avoiding insufficient or excessive penetration. Fifteen to 20 injections of 0.5 of the solution containing BMSC were performed in the target myocardial regions.

In 1 patient with inferior wall ischemia, the heart was visualized via a transdiaphragmatic minilaparotomy approach. The abdo-

Table 1  Selected characteristics of treated patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site of ischemia</th>
<th>Approach</th>
<th>Number of injected cells (CD133+)</th>
<th>EF</th>
<th>Prior procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lateral</td>
<td>Mith</td>
<td>9 × 10⁶</td>
<td>45%</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>inferior</td>
<td>SubDia (redo)</td>
<td>12 × 10⁶</td>
<td>50%</td>
<td>CABG; SCS</td>
</tr>
<tr>
<td>3</td>
<td>anterior</td>
<td>Mith</td>
<td>8 × 10⁶</td>
<td>35%</td>
<td>CABG; PCl; TMR</td>
</tr>
<tr>
<td>4</td>
<td>anterior</td>
<td>Mith (redo)</td>
<td>5 × 10⁶</td>
<td>55%</td>
<td>CABG; PCl; SCS</td>
</tr>
<tr>
<td>5</td>
<td>anterolateral</td>
<td>Mith (redo)</td>
<td>4 × 10⁶</td>
<td>60%</td>
<td>CABG; PCl; TMR</td>
</tr>
</tbody>
</table>

Mith = left mini-thoracotomy; SubDia = subdiaphragmatic approach; EF = ejection fraction; CAB = coronary artery bypass grafting; SCS = spinal cord stimulator; PCI = percutaneous coronary intervention; TMR = transmyocardial laser revascularization
stress scores yielded the summed score at rest and the summed was observed on the images at rest, segments were classified as were considered present when the tracer activity was < 75% of 4 = tracer activity < 25%. Perfusion defects on the stress images tracer activity 50% to 75%; 3 = tracer activity 25% to 50%; and 2 = normal tracer activity > 75%; 1 = minimal blush; 2 = moderate blush but less than obtained during angiography of contralateral or ipsilateral artery; 3 = normal myocardial blush.

**Statistical analysis**

Data are reported as means ± SDs. Quantitative data were compared using a paired, 2-tailed Student’s t-test. Categorical data were compared using the Wilcoxon signed rank test. A p value < 0.05 was considered significant.

**Results**

### Early and mid-term outcome

No deaths or major complications were observed postoperatively. One patient presented with pleural effusion. Periprocedural laboratory evaluations demonstrated an absence of inflammation or myocardial injury as indicated by a peak C-reactive protein of 28 ± 19 mg/L and peak troponin T level of 0.33 ± 0.17 μg/L. No ventricular arrhythmias were recorded during hospitalization. Post-procedural pericardial effusion was excluded in all cases by 2-D echocardiography.

All patients experienced a significant improvement of angina class at a mean of 3.8 weeks after cell injection, and the mean Canadian Cardiovascular Society Score improved from 3.8 at baseline to 1.8 after 6 months (Fig. 1). The frequency of angina episodes per day decreased from 3.1 ± 4 at baseline to 0.3 ± 0.9 at 6 months (p < 0.01). The quality of life improved from 46 ± 8% to 74 ± 12% at 6 months (p < 0.01). It is worth noting that improvements were found in all 5 scales of the Seattle Angina Questionnaire at 6 months compared with baseline. The 24-hour Holter recordings at 1, 3, and 6 months after injection did not reveal ventricular arrhythmias.

At 6 months’ follow-up, the number of segments with stress-inducible ischemia per patient decreased from 5.6 ± 2.8 to 2.4 ± 2 (p < 0.01). Rest and stress scores improved in the injected segments from 1.3 to 0.9 and from 1.8 to 1.3, respectively (Fig. 2). The summed stress score per patient improved from 25.2 ± 5.0 to 21.1 ± 3.2 (p < 0.01). Perfusion at rest also improved with an increase in the summed score at rest from 20.3 ± 3.1 to 18.2 ± 3.1 (p < 0.05). The mean ejection fraction did not change significantly from baseline (49% preoperatively vs. 51.3% at 6 months). Coronary reinvestigation at 6 months showed an improvement of myocardial blush into the injected area in 3 out of 5 patients (mean blush score variation: from 0.6 at baseline to 1.1 at 6 months).

### Late outcome

No deaths, myocardial infarction, malignant ventricular arrhythmias, or new ventricular masses were observed after an average of 36.5 (range 8 – 62) months of follow-up. The improvements in

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**Fig. 1 Individual CCS class at baseline (pre) and at 6 and 12 months (postoperatively) after autologous BMSC inoculation.**

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**Fig. 2**
the angina status noted at 6 months were found to be unchanged in 4 patients. One patient with rheumatoid arthritis and chronic steroid therapy required one rehospitalization for recurrence of angina 10 months from the operation, and experienced a worsening of his angina class (from Canadian Class 2 at 6 months to 4 at 1 year postoperatively) (Fig. 1). The mean Canadian Cardi-ovascular Society Score increased from 1.8 at 6 months to 2.2 one year after cell injection. No significant differences were noted in the mean left ventricular ejection fraction (EF) 12 months post-operatively when compared to the 6-month controls. The number of segments with stress-inducible ischemia per patient changed very little from 2.4 ± 2 at 6 months to 2.5 ± 1 at 1 year. An example of SPECT polar maps before and 12 months after autologous BMSC injections is shown in Fig. 3.

Discussion

The present study is, to our knowledge, the first evidence that myocardial therapeutic angiogenesis in a setting of refractory myocardial ischemia can be induced by direct BMSC injection using a minimally invasive surgical approach. Klein and co-workers [19] have recently proposed a less invasive surgical approach (left thoracotomy) for sole stem cell therapy in patients with severe post-infarctual cardiomyopathy (10 patients with EF < 22%) ineligible for bypass surgery, irrespectively of the amount of regional ischemia. A mid-term significant improvement of EF in all patients was reported. Direct stand-alone inoculation of CD34+ enriched stem cells has been previously proposed by Archundia and co-workers in patients with a severely scarred and dysfunctional myocardium [20]. Our data support the hypothesis that epicardial BMSC inoculation into ischemic territories may enhance perfusion and lead to significant and long-term enhancement of angina class. These findings agree with previous reports on intramyocardial endocavitary BMSC inoculation in patients with refractory angina and stress-inducible ischemia, in which cell injection was accomplished through a percutaneous approach by means of left ventricular electromechanical mapping with the NOGA system and endoventricular injection catheter [1 –4,21]. Taken together, these studies suggest that stem cell delivery into the ischemic myocardium can reduce angina and improve exercise capacity without the occurrence of ventricular arrhythmias. These clinical benefits were associated with enhanced perfusion in the injected segments, while the extent of myocardial scar tissue was unchanged [4,22]. Perfusion improvements have been documented with different techniques, such as scintigraphy or magnetic resonance [2,22]. Interestingly, an increase in left ventric-
ular function after 3 months' follow-up was also noted [23], concomitantly with a reduction of systolic volumes [3]. It has also been shown that stem cell transplantation can induce a long-term improvement in angina class and quality of life [4,14,22]. Very recently, Losordo and co-workers [24] published a phase I/II randomized placebo-controlled trial of intramyocardial endocavitary inoculation of stem cells isolated from circulating blood (CD34+) in patients with intractable angina. They provided evidence that this strategy may improve perfusion and reduce symptoms in patients with advanced coronary disease who have exhausted the currently available therapeutic armamentarium. Our preliminary results suggest that the aforementioned clinical and perfusion improvements may be achieved with an epicardial approach and this strategy may be an alternative to endocardial injection under NOGA guidance. The more pronounced surgical trauma, even if reduced by the less invasive approach, may be compensated by a greater precision of cell delivery into the target areas [25]. Moreover, direct inoculation may be useful when a percutaneous approach is not advisable, for instance in cases with concomitant severe peripheral arteriopathy.

Cell therapy is a novel and promising potential treatment for patients suffering from angina pectoris chronically resistant to conventional treatments [26]. The clinical profile of these patients is often difficult to objectify, as is the success of potential treatments [27]. According to Kornowski and co-workers [26], the present and previous results of BMSC intramyocardial delivery seem to satisfy all the proposed major criteria for efficacy (improved perfusion, lowered ischemia and level of chest pain symptoms) in a setting of refractory ischemia. In particular, we observed an improvement of at least 4 of the 6 points to be fulfilled in order to accept a treatment effect [27]: reduced myocardial perfusion defects; improved symptomatic angina (minimal requirement ≥ 1 CCS class but ideally ≥ 2 CCS classes); improvement in standard quality of life questionnaire (e.g., Seattle Angina Survey) parameters; improved regional myocardial blush score using selective coronary angiogram. In the past, other potential treatments of refractory ischemia have failed to parallel subjective with objective improvements [28].

The limitations of the present study need to be acknowledged. This preliminary pilot report is aimed at providing a proof of the concept, without any claim for a demonstration of its efficacy, due to the very low number of patients and the absence of a control group. However, a case-controlled study is not advisable because of ethical issues related to the use of a surgical approach in the control group. Magnetic resonance was not included as non-invasive imaging modality. However, since the main goal of the study was to document perfusion improvements in viable tissue, we believe that SPECT may provide sufficient information. Many issues still remain to be elucidated. It will be critically important to establish criteria to select patients who are likely to be the best responders to cell therapy as well as those patients who are less likely to respond because of risk factors severely impairing cell activity, such as age, diabetes, etc. [29].

We also believe that further opportunities to possibly enhance the therapeutic potential of cell therapy will be offered by basic research. For instance, engineered human EPCs transduced with vascular endothelial growth factor (VEGF164) have been shown to have a 30-times higher angiogenic activity compared to native cells [30].

In conclusion, our study has explored the feasibility of stem cell inoculation into ischemic cardiac territories to induce therapeutic angiogenesis using a minimally invasive surgical approach. Our preliminary results agree with current data indicating a possible durable benefit in terms of ischemia relief. On this basis, we believe that this treatment option deserves to be investigated in more depth in the future with larger and controlled trials.

References