Concise report

Mobilization of endothelial progenitor cells by intravenous cyclophosphamide in patients with systemic sclerosis

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Abstract

Objective. To evaluate the effects of i.v. CYC on the number of circulating endothelial progenitor cells (EPCs) in patients with SSc, and the potential association of the EPC response with CYC’s effect for treating interstitial lung disease (ILD).

Methods. This open-label, prospective study involved 12 patients with SSc and alveolitis (CYC group). All patients received six courses of i.v. CYC (0.5 g/m²) at 4-week intervals in combination with low-dose prednisolone. Ten patients were followed for 24 months. Seven SSc patients treated with low-dose prednisolone alone were used as a control for the EPC measurement (control group). Five patients with non-SSc CTD who received i.v. CYC and prednisolone also served as disease controls. EPCs were quantified by the partial enrichment of CD34+ cells followed by three-colour flow cytometry. The circulating levels of vascular injury markers were measured by immunoassay.

Results. The EPC count was significantly increased at 2 weeks after treatment in the CYC group (P = 0.02), but not in the control group, while CYC increased EPC count in all disease controls. The SSc patients in the CYC group were divided into five EPC responders and seven EPC non-responders. Circulating vascular injury markers were reduced in the responders, but not in the non-responders. During the 24-month follow-up, 3 of 10 patients developed end-stage lung disease, and all of them were EPC non-responders.

Conclusion. A low-dose i.v. CYC induces EPC mobilization, which may contribute to the efficacy for treating SSc-associated ILD.

Key words: Scleroderma, Respiratory, Biomarkers, Immunosuppressants, Outcome measures.

Introduction

Interstitial lung disease (ILD) is the leading cause of mortality in patients with SSc [1]. The pathogenesis of SSc-associated ILD is thought to be chronic inflammation in the lung parenchyma, which causes lung injury and resultant fibrosis. Based on this theory, immunosuppressive agents, such as CYC, are used for treating SSc-associated ILD. In a placebo-controlled, double-blind, randomized trial in SSc patients with ILD and alveolitis, 1 year of treatment with oral CYC resulted in significant beneficial effects on lung function, although a significant proportion of CYC-treated patients showed a deterioration of lung function [2]. Another clinical trial, in which six courses of i.v. CYC (0.6 g/m²) were followed by AZA in combination with low-dose CSs, demonstrated a potential effect on stabilizing lung function; a statistical trend towards preventing a decline of forced vital capacity [3]. Therefore, the beneficial effects of CYC on SSc-associated ILD seem limited, with only a subset of patients obtaining a substantial treatment benefit [4].

Recently, accumulating lines of evidence have indicated that bone marrow-derived progenitors contribute to tissue...
repair and remodelling of the lung [5]. Such progenitors include endothelial progenitor cells (EPCs), which play an important role in vascular formation and healing in response to vascular injury, by homing to the site of injury and working in concert with existing endothelial cells [6]. Human EPCs, also termed circulating endothelial precursors, are identified as non-haematopoietic cells with a characteristic phenotype positive for CD34, CD133 and VEGF receptor type 2 (VEGFR2) [7]. We recently reported that EPCs in SSc patients are reduced in number and deficient in their capacity to mature into endothelial cells compared with those of healthy individuals [8], although whether the number of EPCs is reduced is a matter of debate [9]. In haematopoietic stem cell transplantation (HSCT), mobilization and conditioning regimens that include high-dose CYC (≥ 4 g/m²) mobilize a variety of bone marrow-derived progenitors [10]. Thus, we hypothesised that EPCs are also mobilized, to some extent, even by the low-dose CYC regimen used to treat SSc-associated ILD, and that increasing the circulating EPCs would contribute to CYC’s clinical benefits.

To test this hypothesis, we conducted a pilot study to evaluate the effect of low-dose CYC regimen on the EPC count in circulation, and its association with clinical efficacy for SSc-associated ILD.

Materials and methods

Study design

This open-label, prospective study was conducted at Keio University, Nagasaki University and Kanazawa University, Japan, during the period from June 2004 to June 2010. Twelve consecutive patients with SSc who received i.v. CYC for ILD were enrolled. All the patients fulfilled the ACR preliminary classification criteria for SSc [11], and had ILD with alveolitis confirmed by high-resolution CT and/or analysis of bronchoalveolar lavage fluid [2]. Exclusion criteria included end-stage lung disease (ESLD), which was defined by a per cent vital capacity (%VC) < 50% or a requirement for continuous oxygen supplementation [12]. The i.v. CYC protocol consisted of six courses of the i.v. infusion of 0.5 g/m² CYC at 4-week intervals. Prednisolone at a dose of < 30 mg daily was simultaneously started, and then tapered gradually. After completion of the CYC regimen, two patients received AZA as maintenance therapy, but the others did not. As a control for the evaluation of EPC number, we enrolled seven SSc patients who received prednisolone alone (< 30 mg daily) for progressive skin thickening (n = 4) or inflammatory conditions such as arthritis (n = 3) during the study period. In addition, five patients with non-SSc CTD who received i.v. CYC (0.5 g/m²) and moderate- to high-dose prednisolone (> 30 mg daily) served as disease controls. These included three patients with SLE, and one each with DM or microscopic polyangiitis (MPA). Peripheral blood samples were obtained at pre-treatment and 2 weeks after the first i.v. CYC course (CYC group) or after the initiation of prednisolone (control group). The study was approved by individual institutional review boards (IRBs) (Keio University IRB, Kanazawa University IRB and Nagasaki University IRB), and written informed consent was obtained from each patient.

Quantification of EPCs

The absolute number of EPCs in circulation was determined using a method described previously [13]. Briefly, a CD34⁺-cell-enriched fraction was prepared from peripheral blood mononuclear cells using a magnetic-activated cell sorter immunomagnetic technique (Miltenyi Biotech, Bergisch Gladbach, Germany) with Fc receptor blocking reagent, according to the manufacturer’s protocol. The CD34⁺CD133⁺VEGFR2⁺ cells were detected by flow cytometric analysis on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Finally, the number of EPCs in 20 ml of peripheral blood was calculated based on the ratio of CD34⁺ cells to FlowCount microbeads (Beckman-Coulter, Fullerton, CA). All procedures were performed by the same experienced operator (Y.O.), who was blinded to the sample identity. This procedure met all the recommendations proposed by the EULAR Scleroderma Trials and Research group [9], except the use of a viability marker. Instead, we excluded dead cells by gating for lymphocytes in the scatter analysis before examining cell surface markers. Our preliminary analysis comparing the use of viability marker 7-AAD with the use of gating strategy in SSc patients and healthy controls revealed a strong correlation between the results obtained from these strategies (r = 0.96) [Kuwana M. (data not published)]. In SSc patients, an EPC responder was defined as a patient meeting both of the following criteria 2 weeks after treatment: (i) 50% increase in the pre-treatment EPC level; this was shown to result in significant improvement in RP variables and reductions in the up-regulated vascular endothelial injury markers during the atorvastatin treatment [13], and (ii) more than 600 EPCs/20 ml peripheral blood; this is the lower limit of EPC distribution in healthy individuals [8]. The remaining patients were classified as EPC non-responder, although there is currently no definition of the EPC responder in literature.

Circulating levels of vascular injury markers

The level of VEGF and soluble E-selectin in heparinized plasma samples was measured using ELISA kits (Quantikine; R&D Systems, Minneapolis, MN).

Statistical analysis

All continuous variables are shown as the mean (± S.D.). Changes in serial variables were analysed by the paired t-test. The cumulative rates for no ESLD in two groups were compared by the log-rank test.

Results

Baseline characteristics of the patients

Table 1 shows the baseline clinical characteristics of SSc patients in the CYC and control groups. There was no difference in the disease duration, modified Rodnan...
Effects of i.v. CYC on the EPC count

All patients in the CYC group completed six courses of i.v. CYC, and 10 of them completed a 24-month follow-up. Flow cytometric analyses for stained CD133 and VEGFR2 on the gated CD34+ cells in a representative SSc patient, in whom the EPCs were increased 2 weeks after i.v. CYC, are shown in Fig. 1A. The 2-week time point was chosen because preliminary serial analyses revealed that the number of EPCs started to increase 1 week after i.v. CYC, and reached the peak at 2 weeks, which lasted for at least a week (data not shown). EPCs were significantly increased from the baseline at 2 weeks [243 (153) to 476 (287), \(P = 0.02\)], but only five patients (42%) were classified as EPC responders (Fig. 1B). In one responder, the EPC response following i.v. CYC treatment was reproduced in all six consecutive courses. In contrast, no increase in EPC number was observed in the control group treated with prednisolone alone [207 (116) to 162 (74)]. Interestingly, all disease controls experienced an increase in EPC number after CYC treatment, and this change was statistically significant [685 (277) to 1229 (406), \(P = 0.003\); Fig. 1C]. Within the CYC group of SSc patients, there was no difference in the baseline characteristics, including the disease duration, mTSS, %VC and initial prednisolone dosage, between five EPC responders and seven non-responders.

Effects of i.v. CYC on vascular injury markers

In SSc patients, EPC responders in the CYC group showed reduction in the VEGF level and a trend towards the reduced soluble E-selectin level at 2 weeks (\(P = 0.04\) and 0.07, respectively), while these trends were not observed in EPC non-responders of the CYC group or in the control group (Fig. 1D).

Association between the EPC response and efficacy of i.v. CYC on ILD

In 10 SSc patients who completed the 24-month follow-up period, none of the 4 responders, but 3 (50%) of 6 non-responders developed ESLD. However, life-table analysis to compare the probability of no ESLD showed that this difference did not reach statistical significance (\(P = 0.1\)). Four patients in the CYC group had digital ulcers at the time of entry. In two EPC responders, the number of new digital ulcers prior to the enrolment was 2 and 6/year, but these decreased to 1 and 1.5/year after the CYC treatment. In contrast, in two EPC non-responders, the number of new digital ulcers did not change before and after the CYC treatment.

Discussion

CYC is an alkylating agent that exerts its immunosuppressive effect mainly through a rapid cytotoxic effect on activated lymphocytes. On the other hand, immature progenitor cells including EPCs are relatively resistant to this drug, and thus are recruited to the circulation in the recovery phase after CYC exposure [14]. This pilot study demonstrated that low-dose i.v. CYC (0.5 g/m²) plus CSs, but not CSs alone, increased the EPC count in a subset of SSc patients, although CYC-induced EPC recruitment was less efficient in patients with SSc compared with those with other CTDs. This may be explained by impaired differentiation potential of bone marrow progenitors in SSc patients [15]. We found that EPC responders showed trends towards reduced levels of circulating vascular injury markers, and a low probability of developing ESLD, whereas non-responders did not. These findings suggest that EPC mobilization may contribute to the efficacy of i.v. CYC for treating SSc-associated ILD. In this regard, in a recent study of high-dose CYC (50 mg/kg for 4 consecutive days) without HSCT rescue, patients with active dcSSc showed clinically significant improvement in lung function [16]. It is likely that treatment with a higher dose of CYC would result in more prominent EPC

<table>
<thead>
<tr>
<th>Demographic and clinical findings</th>
<th>CYC group (n = 12)</th>
<th>Control group (n = 7)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (s.d.), years</td>
<td>56.6 (10.3)</td>
<td>47.7 (19.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Female, %</td>
<td>67</td>
<td>43</td>
<td>NS</td>
</tr>
<tr>
<td>SSc duration, mean (s.d.), years</td>
<td>2.4 (2.8)</td>
<td>3.8 (8.9)</td>
<td>NS</td>
</tr>
<tr>
<td>dcSSc, %</td>
<td>83</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>mTSS, mean (s.d.)</td>
<td>21.1 (9.6)</td>
<td>24.3 (10.7)</td>
<td>NS</td>
</tr>
<tr>
<td>ILD, %</td>
<td>100</td>
<td>71</td>
<td>NS</td>
</tr>
<tr>
<td>VC, mean (s.d.), %</td>
<td>80.8 (13.7)</td>
<td>103.0 (13.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Anti-topo I antibody, %</td>
<td>58</td>
<td>57</td>
<td>NS</td>
</tr>
<tr>
<td>Anti-RNA polymerase III antibody, %</td>
<td>17</td>
<td>14</td>
<td>NS</td>
</tr>
<tr>
<td>Initial prednisolone dosage, mean (s.d.), mg/day</td>
<td>19.0 (6.2)</td>
<td>22.1 (5.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS: not significant.
mobilization in many patients than the low-dose treatment we used in the present study.

It has been reported that EPCs are mobilized during massive tissue damage and recruited to the lung of patients with acute lung injury [17, 18]. Moreover, in a rat model of pulmonary hypertension, therapeutic application of bone marrow progenitors containing EPCs resulted in remodelling of the lung and heart [19]. In these studies,
it has been hypothesized that bone marrow EPCs contribute to the repair of the damaged tissue by homing to the site of injury, replacing the injured vascular endothelium and promoting regeneration processes. The pathogenesis of SSc-associated ILD mainly involves the excessive fibrotic process in response to the alveolar epithelial injury [20], and one of the histological characteristics is prominent reduction of pulmonary vascular density [21]. Since failure of re-endothelialization and re-epithelialization of the alveolar-capillary barrier leads to destroyed lung architecture and fibrosis [22], EPCs recruited to the damaged lung may suppress this ongoing pathogenic process. Therefore, it is likely that the clinical benefit of i.v. CYC observed in a subset of SSc patients might have resulted, in part, from repair or remodelling of the lung parenchyma through EPC mobilization. This mechanism is probably unique for the i.v. CYC regimen, since no increase in EPC number was observed during oral CYC treatment (our unpublished observation).

In a retrospective study of i.v. CYC (0.6 g/m²) followed by AZA in 27 patients with SSc-associated ILD, 22% had improved, 30% were stable and 48% had worsened [23]. This finding suggests that a clinical response to the i.v. CYC was observed only in a subset of patients. In this regard, we found that mobilization of EPCs after the first i.v. CYC administration may predict beneficial effects of the CYC regimen for ILD. Therefore, EPC measurement may be useful for deciding whether a potentially harmful CYC regimen should be continued or discontinued.

A major limitation of this study is a small number of the patients analysed. Thus, it still remains unanswered if increasing the circulating EPCs would contribute to CYC’s clinical benefits. Since this is a preliminary study, further prospective studies involving a large number of patients are necessary to confirm our hypothesis.

**Rheumatology key messages**

- The i.v. CYC used for treatment of SSc-associated ILD increased circulating EPC count in some patients.
- EPC mobilization after i.v. CYC may predict the beneficial effects for ILD.

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