Short Communication

Circulating Endothelial Precursor Cells (EPC) in Patients Undergoing Allogeneic Haematopoietic Progenitor Cell Transplantation

(endothelial precursor cells / allogeneic stem cell transplantation)

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Abstract. We have studied the number of endothelial precursor cells in eighteen patients undergoing allogeneic haematopoietic stem cell transplantation. Endothelial precursor cells were evaluated by colony-forming assay and compared to healthy controls. Patients undergoing allogeneic haematopoietic stem cell transplantation had significantly lower numbers of endothelial precursor cells before the procedure than healthy controls. The numbers of endothelial precursor cells were even lower in the first year after the treatment and seemed to recover partially after twelve months, but even then, they were lower than in healthy volunteers. On the other hand, the number of circulating CD146⁺CD31⁺ mature endothelial cells were higher than in healthy controls after more than a one-year follow-up. We hypothesize that lower numbers of endothelial precursor cells and higher numbers of endothelial cells in patients undergoing allogeneic haematopoietic stem cell transplantation reflect ongoing endothelial damage, probably caused by immunological mechanisms, and that this long-term damage may explain the higher risk of cardiovascular events in allogeneic haematopoietic stem cell transplant survivors.

Introduction

Endothelial precursor cells (EPC) circulate in small numbers in peripheral blood. They are characterized by combination of surface markers such as CD34, CD133, or VEGFR2 (Dome et al., 2008), but the best method for their enumeration is yet to be found. Today, EPC numbers can be evaluated by fluorescence-activated cell sorters (Dome et al., 2007; Van Craenenbroeck et al., 2008), or in colony-forming assays (Hill et al., 2003). Numbers of EPC are elevated in patients with cancer, where they reflect ongoing tumour vasculogenesis (Igreja et al., 2007; Gora-Tybor et al., 2009). On the other hand, EPC numbers are lower in patients with vasculitis or end-stage renal failure (Závada et al., 2008), in advanced stages of heart failure (Valgimigli et al., 2004), or in rheumatoid arthritis (Grisar et al, 2005) and other inflammatory conditions (Verma et al., 2004).

Allogeneic haematopoietic progenitor cell (HPC) transplant patients suffer from widespread endothelial damage during conditioning chemoradiotherapy before progenitor cell infusion (Woywodt et al., 2004a). After engraftment, recipient vessels may be attacked by the immune cells of the donor (Biedermann et al., 2002). These mechanisms are known to elevate markers of endothelial damage, as are circulating mature endothelial cells (EC) and endothelial microparticles (Nomura et al., 2008). However, there is no data about the ability of damaged endothelium to regenerate. Therefore, we have studied the numbers of EPC in allogeneic transplant patients before and after transplantation to evaluate the potential for repair of damaged endothelium in these patients.
**Material and Methods**

**Patients**

Eighteen patients treated with allogeneic stem cell transplantation were studied at the Institute for Haematology and Blood Transfusion, Prague. The characteristics of these patients are given in Table 1. Numbers of EPC were compared with numbers of EPC of 86 healthy volunteers.

**EPC enumeration**

EPC enumeration was performed with a colony-forming assay (CFU-En) (Hill et al., 2003). Briefly, 20 ml of blood anticoagulated with heparin was taken by venepuncture and centrifuged with Ficoll-Hypaque (Amersham, Uppsala, Sweden) to obtain peripheral blood mononuclear cells (PBMC). PBMC were washed twice in phosphate-buffered saline, resuspended in the EndocultTM medium (StemCell Technologies, Vancouver, Canada), counted with a haematological analyser (AcTDiff2, Beckman-Coulter, Fullerton, CA) and adjusted to the final number of 2.5 × 10³ cells per ml. Four milliliters of resulting cell suspension were divided in two wells of the BD BioCoatTM Gelatin 6-well plate (BD Biosciences, Franklin Lakes, NJ) and cultivated for 48 h at 37 °C in an atmosphere with 5 % CO₂. Non-adherent cells were then removed together with the medium, counted, and reseeded in the BD BioCoat™ Fibronectin 24-well plate. After another 72 h, endothelial colonies (CFU-Hill) were scored under an inverted microscope. Only colonies with at least 20 cells, containing rounded cells in the middle and elongated cells at the periphery, were considered as CFU-Hill colonies.

In seven patients, evaluation of circulating mature EC was performed by flow cytometry. Briefly, anticoagulated whole blood samples were labelled with antiCD146 FITC and antiCD31 PE antibodies as positive discriminating events. Unwanted events were excluded with dump channel, containing lineage-specific (Lin) antibodies antiCD3, antiCD14, antiCD15 and antiCD19, all labelled with PE-Cy5. Gating strategy was employed to exclude debris and nonspecific fluorescent events. Cells in lymphocyte gate staining CD146 CD31 Lin were counted as mature EC. At least 500,000 events were acquired during each analysis.

Chimerism of allogeneic transplant patients was evaluated by variable number tandem repeat (VNTR) and short tandem repeat (STR) polymorphism studies. Except for one case of syngeneic transplantation, all the donor-recipient pairs were informative.

**Statistical analysis**

Statistical analysis was performed using STATISTICA v.8 software (StatSoft Inc., Tulsa, OK). Categorical variables were compared with Yates corrected test. As CFU-En numbers did not follow normal distribution, non-parametric tests were used for comparison of numerical variables. The Mann-Whitney U test was used for comparison of independent samples and the Wilcoxon paired test for comparison of paired samples. Correlations were performed with non-parametric Spearmann test. Results are given as median and ranges. Values of P < 0.05 were considered statistically significant.

**Results and Discussion**

Patients undergoing allogeneic HPC transplantation had lower numbers of CFU-En colonies (median, 1.15 per ml of peripheral blood, range 0–39.1/ml) than healthy volunteers (median 27.7/ml, range 0–267.1/ml, P < 0.00001). There were more men among alloHPC patients than among healthy controls (P = 0.01), but the age was comparable (median patient age, 45 years vs. median volunteer age 41 years, P = 0.80).

General kinetics of CFU-En numbers in allogeneic transplant patients is shown in Fig. 1. When the differences between CFU-En numbers before and after transplantation were evaluated with the Wilcoxon paired test, the differences were not significant. However, when the Mann-Whitney U test was used, there was a borderline significant difference (P = 0.053) between CFU-En before transplantation and in the first 12 months after transplantation, and there was a significant difference (P = 0.038) between CFU-En <12 months after transplantation and >12 months after transplantation. The difference between CFU-En before transplantation and >12 months after transplantation was not statistically significant. At all time-points, numbers of CFU-En were lower in alloHPC recipients than in healthy volunteers. The numbers of CFU-En colonies in transplanted patients did not depend on the diagnosis, type of conditioning chemoradiotherapy, immunosuppression at the time of sampling (yes vs. no), or on the fact whether patients had the graft-versus-host disease (GvHD). All evaluated patients have eventually reached full chimerism, i.e. 100% donor haematopoiesis, at 2.5–6 months.
after transplantation. When samples collected before achievement of full chimerism were compared with samples collected from 100% chimeric patients, there was a higher number of CFU-En colonies in the latter group (1.7/ml vs. 0.4/ml), but this did not reach statistical significance (P = 0.3).

We were interested whether the low numbers of CFU-En were the result of the haematological disease or of the treatment before transplantation. Therefore, we compared the numbers of CFU-En with a group of eighteen newly diagnosed patients with haematological malignancies (16 lymphomas and 2 myelomas). There was no difference in CFU-En colonies between newly diagnosed patients and alloHPC patients, who were in most cases already heavily pre-treated before sampling (median, 3.5 CFU-En/ml v. 1.15/ml, P = 0.48). Therefore, we conclude that haematological disease itself may cause low numbers of EPC.

It is known that patients shortly after alloHPC transplantation have higher numbers of mature endothelial cells (EC) and endothelial microparticles (Woywodt et al., 2004a; Nomura et al., 2008). However, it was not studied whether higher numbers of EC persist also after a longer follow-up. Therefore, we evaluated CD146+CD31+Lin- cells in seven patients who were >12 months after transplantation (median, 26.4, range 15.6–30.4 months). The numbers of EC were significantly elevated in patients >12 months after autologous stem cell transplantation (ASCT) compared to healthy controls (median, 289 cells/ml vs. 84 cells/ml, P = 0.01). Five of these seven patients were available for second examination 8–9 months after the first sampling. No differences between the first and second group of samples were found in paired tests (P = 0.50) and additionally, there were more EC in transplanted patients than in healthy controls (P = 0.009). Therefore, we conclude that elevated numbers of endothelial cells as a marker of ongoing endothelial damage persist in allogeneic patients for more than one year after HPC transplantation. As our cohort was very small, we could not search for correlations of the numbers of EPC with the presence of graft-versus-host disease or with the use of cyclosporin (Woywodt et al., 2003).

Endothelial damage after allogeneic HPC transplantation results in a number of well-recognized clinical entities, namely venooclusive disease, capillary leak syndrome, or thrombotic microangiopathy (Woywodt et al., 2004b). Late complications in alloHPC transplantation survivors also include increased cardiovascular risk when compared to the general population (Tichelli et al., 2007). Evaluation of the number of endothelial progenitors or mature endothelial cells shortly after transplantation is difficult, if not impossible, because of the generally low numbers of nucleated cells in peripheral blood. In this situation, other markers of endothelial damage should be employed, and several studies have investigated the diagnostic and prognostic potential of soluble molecules such as thrombomodulin, soluble von Willdebrandt factor, or soluble plasminogen activator inhibitor type-1 (PAI-1) (Bland and Seigneur, 1997; Salat et al., 1997; Nürberger et al., 1998). However, during long-term follow-up, EPC and EC measurements may be useful for prediction of the risk of cardiovascular complications in alloHPC transplantation patients. If the results of larger studies confirm our data, then also therapeutic measures to reduce the EC number and/or to elevate the number of EPCs should be sought.
References


