Circulation of CD34+ progenitor cell populations in patients with idiopathic dilated and ischaemic cardiomyopathy (DCM and ICM)

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Introduction
After myocardial infarction, cytokines like stromal cell-derived factor-1 (SDF-1) are elevated in peripheral blood. These cytokines stimulate the bone marrow resulting in a release of CD34+ progenitor cells into the blood circulation. The progenitor cells travel to ischaemic tissue: due to the expression of homing receptors like CXC-chemokine receptor-4 (CXCR-4), they are incorporated via interaction with the corresponding homing factor SDF-1, which is upregulated immediately after myocardial infarction, and other homing factors like stem cell factor (SCF), HIF-1a, vascular cell adhesion molecule (VCAM), and hepatocyte growth factor (HGF). In the myocardium, they finally contribute to vasculogenesis and prevent apoptosis. In addition, low levels of circulating CD34+ progenitor cells are clinically associated with cardiovascular events and death from cardiovascular causes. It is unknown whether this mechanism of progenitor cell circulation also applies to patients with dilated cardiomyopathy (DCM) and ischaemic cardiomyopathy (ICM) who exhibit an increased number of apoptotic cells as well as an inhibition of endothelial function. Therefore, we analysed several cytokines in peripheral blood and measured different circulating CD34+ cell populations (CD34+CD133+, CD34+CD31+), CD34+CXCR-4+ in patients suffering from DCM and ICM. CD34+CD133+ and CD34+CD31+ cells represent endothelial progenitor cells (EPCs) in early and late stages of development; CD34+CXCR-4+ cells show the expression of the homing receptor CXCR-4 on CD34+ cells in general. Furthermore, we examined the expression of the homing factors SDF-1 (interacts with CXCR-4), SCF, and HGF (responsible for progenitor cell engraftment), VCAM (interacts with VLA-4) as well as hypoxia-inducible factor-1 (HIF-1)—a transcription factor that regulates SDF-1 expression—in explanted DCM, ICM, and control hearts.
Methods

Patients

Thirty patients with idiopathic DCM (mean age 52 ± 11 years; 25 men), 15 control subjects (mean age 52 ± 16 years; 11 men), and 19 patients suffering from ICM (mean age 62 ± 12 years; 15 men) were enrolled into the study. Analysis of peripheral blood was performed in 25 DCM patients, 15 ICM patients, and 10 controls. Explanted heart tissue was analysed in another five DCM patients, four ICM patients, and five donor hearts that proved to be unsuitable for transplantation due to palpable coronary calcifications; the latter served as control.

The diagnosis of DCM was based on established criteria.13 Only patients with an angiographic left ventricular ejection fraction (LVEF) <40% were included. Exclusion criteria were coronary artery disease [i.e. no coronary stenosis >30% in coronary angiography and no previous percutaneous coronary intervention (PCI)], significant valvular heart disease, congenital heart disease, active myocarditis, hypertrophic cardiomyopathy with dilatative course, alcohol ingestion >100 g/day, and a history of exposure to cardio-toxic drugs. DCM patients showed the following characteristics (mean and standard deviation): NYHA-class 2.8 ± 0.4, ejection fraction 28 ± 7%, end-diastolic volume (EDV) 280 ± 118 mL, and BNP 488 ± 471 pg/mL. DCM patients received standard medical treatment which included β-blockers (95%), diuretics (90%), and angiotensin converting enzyme-inhibitors (ACE-inhibitors) (90%), aspirin (90%), statins (17%), and angiotensin II receptor blockers (10%).

ICM patients had at least one vessel disease with previous PCI. Only patients with an angiographic LVEF <40% were included. Exclusion criteria were acute events like myocardial infarction or PTCA less than 3 months ago and other severe concurrent illness (e.g. active infection, malignancy). ICM patients had the following characteristics: NYHA-class 2.7 ± 0.7, ejection fraction 33 ± 6%, EDV 248 ± 70 mL, and BNP 450 ± 533 pg/mL. They were treated with β-blockers (95%), diuretics (80%), ACE-inhibitors (70%), aspirin (95%), statins (100%), and angiotensin II receptor blockers (10%).

Control subjects attended the hospital for angina pectoris and were included after exclusion of coronary artery disease by heart catheterization (i.e. no coronary stenosis >30%). They had no other severe concurrent illnesses (e.g. active infection, malignancy, etc.); 70% of controls suffered from arterial hypertension, and 40% from hypercholesterolemia receiving β-blockers (70%), ACE-inhibitors, angiotensin II receptor blockers (20%), diuretics (10%), and statins (7%).

Initially 48 DCM patients, 27 ICM patients, and 19 controls were assessed for the study. Of these, 14 DCM patients, seven ICM patients, and two controls did not fulfill inclusion criteria or proved to have exclusion criteria. Four DCM patients, one ICM patient, and two controls refused participation in the study.

The study protocol was approved by the institutional committee on human research, and informed consent was obtained from all patients and controls.

Quantification of CD34+ cells

Cytometric analysis was performed using a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany) according to ISHAGE guidelines and according to a standard protocol.14 Each analysis included 100,000 events. For immunophenotyping, we used monoclonal antibodies directed against CD31, CD34, CD133, CXCR4, conjugated with fluorescein isothiocyanate, phycoerythrin, or phycoerythrin cyanine-5 (BD PharMingen/Coulter Immunotech, Hamburg, Germany).

Biochemical measurements

Complete blood count was performed on an automated laboratory cell counter. C-reactive protein was measured by turbidimetry (Roche Diagnostics, Mannheim, Germany). Serum levels of interleukin-6 (IL-6), BNP, and tumour necrosis factor-α (TNFα) (Biosource Diagnostics, Bruxelles, Belgium) as well as SDF-1, SCF, and VEGF (R&D Systems, Wiesbaden, Germany) were assessed using enzyme-linked immunosorbent assay (ELISA).

Real time polymerase chain reaction

Explanted hearts were selected, if their ‘donors’ fulfilled the criteria for DCM and ICM described above. Only tissue of the left ventricle was used. In ICM heart, the left descending artery showed the leading stenosis as was confirmed by coronary angiography. The quantification of mRNA transcripts of explanted hearts was performed via quantitative real-time reverse transcriptase-polymerase chain reaction (RT–PCR) method. The RNA was prepared with Trizol Reagent (Gibco BRL) from homogenized deep-frozen tissue samples following the TRIZOL standard protocol as described by the manufacturer (GibcoBRL, Eggenstein, Germany). Double-stranded cDNA was synthesized using the superscript double stranded cDNA synthesis kit (Invitrogen, Karlsruhe, Germany). cDNA was amplified and quantified by SYBR Green detection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. Thermal cycling and SYBR Green fluorescence detection were done using the Stratagene, MX4000, Multiplex Quantitative PCR System.

Statistical analysis

Results

Clinical characteristics

Table 1 reflects the clinical characteristics of our study population. ICM patients were older than DCM patients and controls. However, age showed no significant correlation to the number of progenitor cells in our study population. Gender distribution and the occurrence of atrial fibrillation were similar in all three groups. In comparison to controls, DCM and ICM patients had a significantly lower LVEF and a significantly increased EDV, and they were in a similar NYHA class (2.8 ± 0.4 and 2.7 ± 0.7). ICM patients had more cardiovascular risk factors (CRFs) than DCM patients and controls, and the number of patients on acetylsalicylic acid and statins was significantly higher in this group; statins had no significant impact on progenitor cell levels, however. Medication concerning β-blockers, diuretics, ACE-inhibitors, and AT-II-blockers was similar in DCM and ICM patients.

CD34+ cell populations in peripheral blood

Figures 1 and 2A show the amount of circulating CD34+ cell populations measured by flow cytometry. In patients suffering from DCM, CD34+CD31+ (3.6 ± 2.1 vs. 1.9 ± 1.0 cells/μL, P = 0.013), CD34+CD133+ (2.9 ± 1.8 vs. 1.2 ± 0.4 cells/μL, P = 0.009), and CD34+CXCR4+ cells (1.6 ± 1.1 vs. 0.5 ± 0.3 cells/μL, P = 0.011) were increased in peripheral blood when compared with the control group and with ICM patients (2.2 ± 0.9 cells/μL, P = 0.063; 1.7 ± 0.7 cells/μL, P = 0.022; 0.9 ± 0.3 cells/μL, P = 0.048). CD34+ cells showed no difference between the ICM and control groups.
ICM patients, and five control patients were analysed for plasma levels of cytokines.

Levels of myocardial homing factors

Samples from explanted hearts of five DCM patients, four ICM patients, and five control patients were analysed for SDF-1, SCF, HIF-1α, VCAM, HGF, and GAPDH expression on mRNA level (using quantitative Real Time PCR). In ICM heart, the level of SDF-1 (1.4 × 10^4 ± 1.4 × 10^4 relative units vs. 1.4 ± 0.9; P = 0.006), SCF (7.7 × 10^2 ± 2.2 × 10^2 vs. 1.1 ± 0.7; P = 0.01), HIF-1α (7.7 × 10^3 ± 1.6 × 10^3 vs. 5.4 ± 9.5; P = 0.017), and VCAM (8.1 × 10^4 ± 3.0 × 10^4 vs. 32 ± 35; P = 0.006) mRNA—in relation to GAPDH—was significantly higher than in the DCM group (Figure 4). HGF was upregulated in trend (61 ± 41 vs. 2.5 ± 1.7; P = 0.114). Further, the mRNA level of SDF-1, SCF, HIF-1α, and HGF tended to be lower in DCM hearts in comparison to control hearts (4.0 ± 2.4, P = 0.073; 1.4 ± 1.1, P = 0.76; 11 ± 11, P = 0.073; 2.5 ± 1.7, P = 0.19; Figure 4).

Discussion

SDF-1 is known as a potent stimulus of the bone marrow and our data demonstrate that its concentration is elevated and positively correlated to BNP in patients with dilated and ischaemic cardiomyopathy. Furthermore, we found an increased number of circulating cells with stem cell markers in DCM patients—but not in ICM patients and controls. In contrast, essential homing factors like SDF-1, SCF, HIF-1α, and VCAM were significantly upregulated on mRNA level in ICM hearts but not in DCM myocardium and controls. These findings lead us to the hypothesis that in DCM patients endogenous myocardial regeneration via circulating CD34+ cells is impaired. The decreased levels of homing factors in DCM patients may be explained by an impaired mobilisation of progenitor cells from bone marrow to ischaemic heart tissue.
cell populations may be impaired by a lack of upregulation of important myocardial homing factors.

There may be several reasons for the increased number of circulating progenitor cells in DCM patients. Today, several cytokines like G-CSF, SCF, VEGF, and SDF-1 are known to stimulate bone marrow in response to myocardial ischaemia resulting in a release of progenitor cells into the circulation. In DCM patients, endothelial dysfunction and

Figure 1 CD34+ populations in peripheral blood measured by flow cytometry. Histograms show that circulating CD34+CD31+, CD34+133+, and CD34+CXCR-4+ are increased in patients with dilated cardiomyopathy compared with ischaemic cardiomyopathy patients and controls.

Figure 2 CD34+ populations in peripheral blood measured by flow cytometry. (A) FACS data demonstrate an increased level of CD34+ progenitor cell populations in peripheral blood of dilated cardiomyopathy patients. (B) Histogram shows that number of CD34+CD31+ cells in peripheral blood of dilated cardiomyopathy patients declines after heart transplantation.
a reduced number of capillaries might trigger the release of progenitor cells from bone marrow via cytokine cascades in a similar way. We showed an increase of circulating SDF-1 (in significant correlation to BNP), and there may be other unidentified circulating factors that lead to a progenitor cell release from bone marrow. There was no significant difference between DCM patients and controls concerning atrial flutter (AF) and CRF known to influence progenitor cell circulation. Probably, a combination of an elevated SDF-1 in peripheral blood, a lower age in the DCM group compared to ICM patients and a diminution of progenitor cell recruitment to the damaged heart may be responsible for increased circulating progenitor cells in DCM patients.

After heart transplantation for DCM, circulating CD34+ cells show a trend for a decrease in number. As a limitation of the study, we cannot exclude an influence of immunosuppression on this decrease. But our data show that donor hearts express a higher level of several homing factors. It is tempting to speculate that they have a higher capacity to incorporate circulating progenitors because of their slightly increased homing capacity—which may lead to lower progenitor cell levels after heart transplantation. This could indirectly indicate a cause-effect relationship between progenitor cell circulation and expression of myocardial homing factors. To directly prove a cause-effect relationship between the increased concentration of EPCs and reduced homing factor expression, we would need blood and tissue samples of the same patients.

We also analysed the progenitor cell concentration in ICM patients. They showed a comparable NYHA class, ejection fraction, and EDV of the left ventricle in comparison to the DCM patients. However, ICM patients had normal levels of circulating progenitor cells despite the fact that they showed elevated levels of SDF-1 in peripheral blood. One could argue that ICM patients were older and had more statin therapy than DCM patients. However, age and statin therapy had no significant impact on our results. As statin therapy has been described to increase progenitor cell

Figure 3  Stromal cell-derived factor-1 and brain natriuretic peptide in serum measured by enzyme-linked immunosorbent assay. (A) Histogram shows the upregulation of Stromal cell-derived factor-1 in serum of dilated cardiomyopathy and ischaemic cardiomyopathy patients. (B) Diagram demonstrates the significant correlation of stromal cell-derived factor-1 and BNP in serum of dilated cardiomyopathy (n = 10) and ischaemic cardiomyopathy (n = 9) patients.

Figure 4  Relative amounts of mRNA of stromal cell-derived factor-1, stem cell factor, hypoxia-inducible factor-1a, vascular cell adhesion molecule, and hepatocyte growth factor in heart tissue. Histogram shows the upregulation of myocardial mRNA of stromal cell-derived factor-1, stem cell factor, hypoxia-inducible factor-1a, vascular cell adhesion molecule, and hepatocyte growth factor in relation to glyceraldehyde-3-phosphate dehydrogenase in ischaemic cardiomyopathy patients when compared with dilated cardiomyopathy patients and controls measured by reverse transcriptase-polymerase chain reaction.
levels, the ‘real’ difference between DCM and ICM may even be higher without influence of statins. Therefore, the normal progenitor level in peripheral blood of ICM patients might be due to the increased expression of homing factors in ICM hearts.

In contrast to a recent publication by Valgimigli et al., we did not observe a correlation between mobilization of CD34+ cells, levels of TNFα, and NYHA classification. Furthermore, these authors found no role for the aetiology of cardiomyopathy. This may be due to the fact that our study population is more homogenous. We included DCM and ICM patients in NYHA classes II or III only, and excluded heart disease due to hypertension, valvular disorders, myocarditis, and congenital disorder. Whatever the reason may be, there exist some obvious contradictions between the two publications that have to be resolved in further studies. Nevertheless, we believe that the level of myocardial homing factors has important influence on circulating progenitor cell levels.

In contrast to DCM and controls, important homing factors like SDF-1, SCF, HIF-1α, and VCAM were significantly upregulated on mRNA level in ICM hearts. We measured all homing factors in relation to GAPDH, which is expressed ubiquitously in all cells because we expected the architecture of DCM, ICM, and control hearts to be different in regard to concentration of collagen, myocytes, and non-myocytes. Possibly, this downregulation of several homing factors impairs the migration of circulating progenitor cells into the DCM heart and thus contributes besides other factors like elevated SDF-1 to an increased amount of circulating progenitor cells in these patients. An inefficient incorporation of progenitor cells into DCM hearts could finally cause an increased apoptosis of local cardiomyocytes. A knock-out animal model and cardiac FACS will be used in order to study trafficking of circulating progenitor cells to finally prove our hypothesis of impaired homing.

Our study is limited by a small sample size as well as differences in the study population (see Table 1). Furthermore, there are limitations regarding the analysis of the myocardial homing factors utilizing a random sample of tissue from a heterogeneous myocardium. Nevertheless, our findings may have immediate consequences on progenitor cell therapy in patients suffering from heart failure. Intracoronary infusion of progenitor cells seems to improve outcome of ICM patients, maybe because of the increased myocardial homing capacity. Possibly, DCM hearts may respond less to progenitor cell mobilization or transfusion as suggested by a recent pilot study. Therefore, new therapeutic options providing homing factors to the apoptotic myocardium may become necessary for the treatment of DCM patients. Tomita et al. showed in a mouse model that G-CSF administration leads to increased incorporation of bone-marrow cells into doxorubicin-induced cardiomyopathic hearts. It is conceivable that G-CSF administration might enhance the homing capacity of cardiomyopathic hearts in addition to progenitor cell mobilization. Furthermore, transcription factors such as HIF-1 could be an interesting target to eventually increase the expression of homing factors in DCM hearts. Providing a conductive environment for efficient homing of circulating CD34+ cells may therefore become a promising new treatment option for patients suffering from DCM.

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References


