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Beneficial effects of Angiotensin-(1-7) on CD34⁺ cells from heart failure patients

Colleen T. Cole-Jeffrey, PhD¹, Carl J. Pepine, MD², Michael J. Katovich, PhD³, Maria B. Grant, MD⁴, Mohan K. Raizada, PhD¹, and Sugata Hazra, PhD¹

¹Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL, USA

²Department of Medicine, University of Florida, Gainesville, FL, USA

³Department of Pharmacodynamics, University of Florida, Gainesville, FL, USA

⁴Department of Ophthalmology, University of Alabama at Birmingham, Birmingham, AL, USA

Abstract

The dysfunctional nature of CD34⁺ cells from heart failure (HF) patients may make them unsuitable for autologous stem cell therapy. In view of evidence that the vasoprotective axis of the renin-angiotensin system (RAS) improves CD34⁺ cell functions, we hypothesized that CD34⁺ cells from HF patients will be dysfunctional and that Angiotensin-(1-7) [Ang-(1-7)] would improve their function. Peripheral blood was collected from NYHA class II–IV HF patients (n=31) and reference subjects (n=16). CD34⁺ cell numbers from HF patients were reduced by 47% (p<0.05) and also displayed 76% reduction in migratory capacity and 56% (p<0.05) lower production of nitric oxide (NO). These alterations were associated with increases in RAS genes ACE and AT2R (595%, p<0.05) mRNA levels and 80% and 85% decreases in ACE2 and Mas mRNA levels, respectively. Treatment with Ang-(1-7) enhanced CD34⁺ cell function via increased migratory potential and NO production, and reduced ROS generation. These data show that HF CD34⁺ cells are dysfunctional and Ang-(1-7) improves their functions. This suggests that activation of the vasoprotective axis of the RAS may hold therapeutic potential for autologous stem cell therapy in patients with HF.

Keywords

CD34⁺ cells; Renin-Angiotensin System; Angiotensin-(1-7); Heart failure

Introduction

Heart failure (HF) remains a major cause of morbidity and mortality despite recent advances in the treatment of acute and chronic cardiac events¹. Consequently, development of novel therapies for HF is critical. Recently, the benefits of autologous stem cell therapy for

Address Correspondence to: Mohan K. Raizada, Ph.D., Dept. of Physiology & Functional Genomics, University of Florida, Gainesville, FL 32610-0267, Phone: 352-846-0978, mraizada@ufl.edu. Sugata Hazra, Ph.D., Indian Institute of Technology Kanpur, Kanpur 208016, India, Phone: 91-907-318-4570, sugata.hazra@gmail.com.

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treatment of HF have been investigated². Thus far, the clinical trial results have been inconsistent³, but these moderate results may be attributed to technological hurdles^{4, 5} or dysfunctional autologous progenitor cells⁶.

CD34⁺ hematopoietic stem cells have been studied in regards to their role in cardiac repair during HF. CD34⁺ cells play a vital role in vascular maintenance⁷ and cardiac repair⁸. In humans, CD34⁺ cell numbers have been shown to decline with increasing HF severity⁹ and correlate with cardiovascular risk¹⁰. Importantly, previous studies have reported impaired function of CD34⁺ cells in patients with ischemic cardiomyopathy¹¹ or vascular complications from diabetes^{12–14}. Collectively, this data suggests that CD34⁺ cells contribute to cardiovascular health and that their dysfunction during disease may compromise their utility in autologous stem cell transfer.

In this regard, the recent discovery of the vasoprotective axis of the renin-angiotensin system (RAS) is relevant. It is composed of Angiotensin Converting Enzyme 2 (ACE2), Angiotensin 1-7 (Ang 1-7), and Mas receptor which acts as a balance for the vasodeleterious RAS axis [ACE/Angiotensin II (Ang II)/Angiotensin Type 1 Receptor (AT1R)]. Whereas the vasodeleterious axis has long been implicated in cardiovascular disease, studies have demonstrated that a shift towards the vasoprotective axis shows benefits to the heart¹⁵. The beneficial effects of Ang-(1-7), the effector peptide of the vasoprotective RAS, on CD34⁺ cells are currently under investigation, and the outcomes to this point have been promising^{16, 17}. Thus, we propose to study the ability of Ang-(1-7) to enhance the function of CD34⁺ cells from HF patients.

Methods

Patient Selection and Characterization

Peripheral blood was collected from New York Heart Association (NYHA) class II–IV HF patients as well as from reference subjects (controls). This study was conducted under approval from the Institutional Review Board of the University of Florida. Participants gave written informed consent to participate in this study, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Patients were consented to participate if they met the following criteria: older than 18 years of age, significant coronary artery disease (70% luminal diameter narrowing of at least one major coronary artery), left ventricular (LV) ejection fraction < 45% limiting angina (class II to IV) and/or congestive HF (NYHA class II–IV), on maximal medical therapy for angina symptoms, and hemodynamically stable as defined by a systolic blood pressure greater than 80 mmHg. Patients were excluded from this study if they had atrial fibrillation, atrial flutter and/or uncontrolled arrhythmias, LV thrombus, platelet count M <100 k/mm³, white blood cells <2 k/mm³, stroke within 60 days of study enrollment, history of malignancy in the last five years, history of HIV, Hepatitis B or C, or hepatic dysfunction as defined by AST (aspartate aminotransferase) and/or ALT (alanine aminotransferase) B 1.5 times the upper limit of normal. Pertinent characteristics of the patients are described in Table 1. All patients were dosed to the highest tolerated or maximal approved therapies as recommended in the ACC/AHA Heart Failure Guidelines. Control subjects were ages 39±13.

Isolation of Human CD34⁺ Cells

Peripheral blood was collected from HF patients (n=30) or reference subjects (n=16) using blood collection tubes with EDTA (Thermo Fisher Scientific, Waltham, MA), and CD34⁺ cells were isolated as described previously¹⁴. It is critical to note that the amount of blood, and thus CD34⁺ cells, from each patient was highly variable and that the number of CD34⁺ cells from many patients was severely limited. As a result, we were not able to perform all experiments with all patient samples.

Quantification of CD34⁺ Cells from Whole Blood

Within 24 h of blood collection, 100 μ L of blood from each donor was used to count CD34⁺ cells by BD Procount progenitor cell enumeration kit (BD Biosciences, San Jose, CA) per manufacturer instructions followed by analysis on a LSR II flow cytometer (BD Biosciences) with FCS Express (BD Biosciences).

Cell Migration Assay

CD34⁺ cell migration toward 100 nM stromal cell-derived factor 1 α (SDF-1 α , R&D Systems, Minneapolis, MN) or PBS for 4 h at 37°C was measured using the QCM Chemotaxis 5 μ m Migration Chamber (Millipore, Billerica, MA) according to manufacturer instructions and as previously described¹⁸. For determination of the effects of Ang-(1-7) on migration, freshly isolated CD34⁺ cells were pretreated with 100 nM Ang-(1-7) (Bachem, Bubendorf, Switzerland) for 1 h before following protocol above. This concentration of Ang-(1-7) was selected based on a preliminary dose-response experiment and previous experience with treatment of CD34⁺ cells with Ang-(1-7) *in vitro*¹⁶.

Measurement of Nitric Oxide Production

NO levels in CD34⁺ cells were measured by fluorescence intensity of DAF-FM (Thermo Fisher) as described previously¹⁹. Briefly, 20,000 CD34⁺ cells were incubated with 20 μ M DAF-FM for 30 minutes in the dark followed by the addition of 1 mM L-arginine and stimulant [100 nM SDF-1 α or 100 nM Ang-(1-7)] for 30 min and fluorescence was measured at 485 nm (excitation) and 528 nm (emission).

Measurement of Reactive Oxygen Species

Basal levels of reactive oxygen species (ROS) production were measured using dihydroethidium (DHE, Sigma-Aldrich, St. Louis, MO). Briefly, 20,000 freshly isolated CD34⁺ cells (in triplicate) were incubated with 5 μ M DHE in Dulbecco's PBS with Ca²⁺ and Mg²⁺ supplemented with 1 mg/mL glucose for 15 min in the dark. For some cells, Ang-(1-7) was subsequently added at a final concentration of 100 nM for 30 min, followed by measurement of fluorescence at 488 nm (excitation) and 585 nm (emission).

Gene Expression by Real Time RT-PCR

Total RNA was extracted from CD34⁺ cells and quantitative RT-PCR was performed as described previously¹⁴. FAM-labeled primers (Applied Biosystems, Foster City, CA) were used: ACE (Hs01104600_m1), ACE2 (Hs01085333_m1), Mas (Hs00267157_s1), AT2R

(Hs00169126_m1). All samples were run in duplicate and normalized to 18s (Hs99999901_s1) cDNA.

Statistical Analyses

All results are presented as mean $\pm$ standard error of the mean (SEM). To compare between two groups, student's t-test was done. For comparison between three or more groups, one way ANOVA followed by Tukey's post-hoc test was performed. The statistical analyses were performed using GraphPad Prism5 (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered as significant.

Results

HF Patients Exhibit Reduced Number and Impaired Function of CD34⁺ Cells

We demonstrated that the absolute number of CD34⁺ cells in whole blood was reduced by 47% in HF patients (2.98 ± 1.5 cells/ μ L blood vs controls: 5.59 ± 0.5 cells/ μ L of blood, $p < 0.05$, Fig. 1A). We then determined if there were differences in the function of HF CD34⁺ cells. HF patient CD34⁺ cells demonstrated 76% reduction in migratory response to SDF-1 α compared to CD34⁺ cells from reference donors (9.71 ± 8.6 % increase towards SDF-1 α in HF vs 40 ± 15.9 % increase towards SDF-1 α in controls; $p < 0.05$; Fig. 1B). Basal NO levels in CD34⁺ cells from HF patients were 34% lower than in control CD34⁺ cells (5192 ± 406.3 RFU in control vs 3448.4 ± 184.1 RFU in HF; $p < 0.001$, Fig. 1C). In addition, NO bioavailability in response to SDF-1 α was reduced by 56% in HF patient CD34⁺ cells (47.7 ± 4.5 RFU in control vs 21.1 ± 12.5 RFU in HF; $p < 0.05$, Fig. 1D). Further, basal levels of ROS tended to be 69% higher in CD34⁺ cells from HF patients (1036.3 ± 184.6 RFU in control vs 1755.9 ± 874.7 RFU in HF; Fig. 1E).

CD34⁺ Cells from HF Patients Display Altered Renin-Angiotensin System Gene Expression

Given the involvement of the RAS in the development of cardiovascular disease, we measured the mRNA levels of key members of the RAS. ACE was undetectable in control cells, but trended toward an increase in HF CD34⁺ cells ($2^{-\Delta\text{Ct}}$: 1.66×10^{-4} , Fig. 2A). In contrast, mRNA levels of ACE2 (Control $2^{-\Delta\text{Ct}}$: $4.21 \times 10^{-3} \pm 1.8 \times 10^{-3}$ vs HF $2^{-\Delta\text{Ct}}$: $8.26 \times 10^{-4} \pm 5.3 \times 10^{-4}$, Fig. 2B) and Mas (Control $2^{-\Delta\text{Ct}}$: $6.97 \times 10^{-5} \pm 3.6 \times 10^{-5}$ vs HF $2^{-\Delta\text{Ct}}$: $1.07 \times 10^{-5} \pm 6.9 \times 10^{-6}$, Fig. 2C) were significantly reduced in HF CD34⁺ cells compared with references. In addition, expression of AT2R, a receptor for Ang II, was significantly upregulated in HF CD34⁺ cells (Control $2^{-\Delta\text{Ct}}$: $4.03 \times 10^{-6} \pm 2.2 \times 10^{-6}$ vs HF $2^{-\Delta\text{Ct}}$: $2.41 \times 10^{-3} \pm 7.4 \times 10^{-4}$, Fig. 2D).

Ang-(1-7) Improves Migratory Ability, NO Bioavailability, and ROS Production of CD34⁺ Cells

Next, we were interested if incubation with Ang-(1-7) would restore the reparative capabilities of CD34⁺ cells. Pretreatment of CD34⁺ cells with Ang-(1-7) improved the SDF-1 α -induced migratory abilities of CD34⁺ cells from both controls (147% increase, Fig. 2E) and HF patients (89% increase, Fig. 2F). In addition, pretreatment of CD34⁺ cells with Ang-(1-7) increased NO production by 6.8% in CD34⁺ cells from controls and 15.3% in (Fig. 2G) in those from HF patients. It is interesting to note that although basal NO levels

were lower in HF patient CD34⁺ cells than controls (Fig. 1C), CD34⁺ cells from HF patients exhibited a more robust response to Ang-(1-7) treatment. Lastly, we found that Ang-(1-7) treatment reduced ROS production by 12% in CD34⁺ cells from both HF patients and controls (Fig. 2H).

Discussion

This study demonstrates that pretreatment of CD34⁺ cells with Ang-(1-7) enhances crucial functional capabilities of the CD34⁺ cells isolated from both HF patients and reference individuals. In particular, our study highlights the molecular changes in the RAS of CD34⁺ cells from HF patients that may underlie some of the pathologies induced during HF. Previous studies have shown imbalance in RAS components in the heart and brain during HF^{20–23}. Our group has also demonstrated that diabetic patients have dysfunctional CD34⁺ cells with reduced ACE2 mRNA expression¹⁶. However, to our knowledge, we are the first to report alterations in the vasoprotective and vasodeleterious axes of the RAS in CD34⁺ cells from HF patients. Specifically, HF CD34⁺ cells exhibited increases in ACE and AT2R mRNA expression and decreases in the expression of ACE2 and Mas. Whether these changes are causative of CD34⁺ cell dysfunction remains to be investigated.

The most critical aspect of our study is the demonstration that treatment of CD34⁺ cells with Ang-(1-7) enhances CD34⁺ cell function. It is well known that Ang-(1-7) has anti-inflammatory, antifibrotic, and vasodilatory properties, and it is also associated with NO release and attenuation of oxidative stress²⁴. Further, Ang-(1-7) improves cardiac function in different animal models of HF^{25, 26}. In this study, our data suggest that these cardioprotective effects of Ang-(1-7) may be, in part, a result of its actions on CD34⁺ cells.

This study is a proof-of-concept that manipulation of the vasoprotective RAS may enhance the function of CD34⁺ cells for treatment of HF. However, there are caveats to this study that need to be addressed before such treatment may be possible. First, low patient and cell numbers prohibited us from performing every test with cells from each donor and from reporting results separately for each NYHA class. Having more patients in each category would have allowed us to determine if these measures of dysfunction are progressive, and if dysfunction in any of these parameters precedes the dysfunction of the others. Second, we have shown here that CD34⁺ cell function can be improved by incubation with Ang-(1-7). However, the amount of time cells spent incubated with Ang-(1-7) here was relatively short, and the parameters tested were *in vitro*. It remains to be seen what *in vivo* effects, if any, CD34⁺ cells treated *in vitro* would have and how this may change with longer treatment with Ang-(1-7). Nevertheless, the discoveries made here show strong potential for solving one of the major limitations for autologous stem cell therapy through the treatment of CD34⁺ cells with Ang-(1-7).

Conclusions

Incubation of CD34⁺ cells with Ang-(1-7) enhances crucial functional capabilities of CD34⁺ cells isolated from both HF patients and reference individuals. The use of Ang-(1-7)

pretreatment of CD34⁺ cells may be a valuable strategy before autologous cell therapy in HF patients.

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Figure 1.

CD34⁺ cells from HF patients are dysfunctional. A) FACS analysis of the absolute number of CD34⁺ cells per μL of whole blood (n=4–9). B) Migratory response of CD34⁺ cells towards stromal cell derived factor-1 (SDF-1α) (n=7–13). C) Basal (n=3–4) and D) Stromal cell-derived factor 1 α (SDF-1α)-induced nitric oxide (n=8–9) production assessed by DAF-FM fluorescence. E) Basal ROS levels measured by DHE fluorescence (n=4–5). Data are presented as mean ± SEM. *p<0.05. RFU: relative fluorescence units.

Figure 2.

CD34⁺ cells from HF patients display imbalance in the RAS, and restoration of RAS balance by overexpression of Ang-(1-7) improves CD34⁺ function. Real time RT-PCR analysis of gene expression of A) ACE, B) ACE2, C) Mas, and D) AT2R in CD34⁺ cells from HF patients and reference individuals (control). Pre-treatment of CD34⁺ cells with Ang-(1-7) enhances migratory response of CD34⁺ cells from E) reference individuals and F) HF patients (n=3) towards SDF-1α. Pre-treatment of CD34⁺ cells with Ang-(1-7) also G) improves NO bioavailability (n=6–10) and H) reduces ROS generation (n=5–8). Data are presented as mean ± SEM. *p<0.05.

Table 1**Baseline Characteristics of Study Participants**

Heart Failure Patients (n=30)	
Demographics	
Age, mean	60.0 ± 1.9 (30)
Gender, n male/female (male %/female %)	27/3 (90/10)
New York Heart Association class II, n (%)	17 (56.7)
New York Heart Association class III–IV, n (%)	13 (43.3)
Physical Examination	
Weight, mean, kg (n)	89.0 ± 3.8 (28)
Pulse (n)	70.9 ± 1.8 (29)
Body mass index, mean (n)	29.3 ± 1.2 (28)
Systolic blood pressure, mean, mm Hg (n)	112.8 ± 3.1 (29)
Diastolic blood pressure, mean, mm Hg (n)	69.0 ± 2.2 (29)
Medical Risk Factors or Comorbidities	
Coronary artery disease, n (%)	30 (100)
Ischemic cardiomyopathy, n (%)	23 (76.7)
Hypertension, n (%)	20 (66.7)
Diabetes mellitus, n (%)	8 (26.7)
Myocardial infarction, n (%)	5 (16.7)
Hyperlipidemia, n (%)	20 (66.7)
Atrial fibrillation, n (%)	9 (30.0)
Type of Treatment	
ACE inhibitor or angiotensin receptor blocker, n (%)	22 (73.3)
β-Blocker, n (%)	28 (93.3)
Mineralocorticoid receptor antagonist, n (%)	5 (16.7)
Diuretic, n (%)	23 (76.7)
Statin, n (%)	26 (86.7)
Cardiac glycoside, n (%)	12 (40.0)
Implantable cardioverter-defibrillator, n (%)	11 (36.7)

Data presented as mean ± SEM, and n represents the number of donors used. ACE: Angiotensin-converting enzyme. Percentage of patients within each category is noted in parentheses.