Heart failure-associated anemia: bone marrow dysfunction and response to erythropoietin

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Abstract Heart failure (HF)-associated anemia is common and has a poor outcome. Because bone marrow (BM) dysfunction may contribute to HF-associated anemia, we first investigated mechanisms of BM dysfunction in an established model of HF, the transgenic REN2 rat, which is characterized by severe hypertrophy and ventricular dilatation and SD rats as controls. Secondly, we investigated whether stimulation of hematopoiesis with erythropoietin (EPO) could restore anemia and BM dysfunction. After sacrifice, erythropoietic precursors (BFU-E) were isolated from the BM and cultured for 10 days. BFU-E were quantified and transcript abundance of genes involved in erythropoiesis were assayed. Number of BFU-E were severely decreased in BM of REN2 rats compared to SD rats (50±6.2 vs. 6.4±1.7, \( p < 0.01 \)). EPO treatment increased hematocrit in the SD-EPO group (after 6 weeks, 49±1 vs. 58±1%, \( p < 0.01 \)); however, in the mildly anemic REN2 rats, there was no effect (43±1 vs. 44±1%). This was paralleled by a 67% decrease in BFU-E in BM of REN2 rats compared to SD (\( p < 0.01 \)). EPO significantly improved BFU-E in both SD and REN2 but could not restore this to control levels in the REN2 rats. Expression of several genes involved in differentiation (LMO2), mobilization (SDF-1), and iron incorporation (transferrin receptor) of the BM were differentially expressed in REN2 rats compared to SD rats, and EPO did not normalize this. Altogether, these results suggest that BM dysfunction is an important contributor to HF-associated anemia and that EPO is not an effective agent to treat HF-associated anemia.

Keywords Anemia · Heart failure · Bone marrow dysfunction · REN2 · Erythropoietin · Renin · Renin-angiotensin system

Introduction

Anemia as a co-morbidity in heart failure (HF) is associated with a poor outcome [1, 2]. The prevalence of anemia in HF is reported to be over 40% in patients with advanced HF [3]. The causes of anemia in HF are only partially understood, although several mechanisms have been implicated [4], including treatment with ACE-inhibitors [5], a blunted erythropoietin (EPO) production due to renal dysfunction [6], congestion [7], and iron deficiency [8]. However, these causes only partially explain the severity of anemia and are difficult to target.

Recent publications pointed towards bone marrow (BM) dysfunction as a potential contributing factor to anemia in HF. Iversen et al. showed decreased hematopoiesis in BM
in an experimental (murine) model of post-myocardial infarction HF [9]. Our group recently provided the first clinical proof for the existence of BM dysfunction in human ischemic HF [10]. We showed that HF is associated with a profound and general BM dysfunction, simultaneously affecting multiple hematopoietic lineages. Very recently, the group of Iversen provided further evidence that HF is associated with general BM dysfunction by showing that anemia occurs in both ischemic and non-ischemic murine HF models [11]. Apparently, the mechanisms of anemia in HF are dependent of etiology and are possibly caused by general HF factors like overproduction of neurohormones or hypoperfusion [11]. Importantly, data as to whether pro-erythropoietic therapy could restore anemia and BM function in HF is lacking. Therefore, we evaluated the severity and mechanisms of anemia and BM dysfunction in an established experimental model of hypertensive HF, the transgenic TGR(mREN2)27 rat. Furthermore, we investigated if stimulation of hematopoiesis with EPO administration could restore, at least in part, HF-associated anemia and BM function. Finally, we studied in detail well-established factors of erythroid differentiation (LMO2, GATA-1) and mobilization (MMP9, SDF-1) (Fig. 1, adapted from Koury et al. [12]). LMO2 and GATA-1 are both differentiation factors, where MMP9 mobilizes erythroid precursors from the BM into the peripheral blood, and SDF-1 is a negative regulator of erythroid mobilization. Furthermore, we analyzed factors involved in iron incorporation in the BM (transferrin receptor and ferroportin, both involved in maintaining cellular iron homeostasis) and studied if expression of these factors is changed during EPO administration.

Fig. 1 Overview of different factors involved in differentiation and mobilization during different stages of erythropoiesis, which expression was measured (results shown in Fig. 5). Adapted from Koury et al. [12], according to Hanson et al. [29], Ohneda et al. [31], and Jin et al. [32]. Factors highlighted in green are positive regulators, factor highlighted in red is a negative regulator. HSC hematopoietic stem cell, BFU-E burst forming unit-erythroid, CFU-E colony forming unit-erythroid, RET reticulocyte, RBC red blood cell, LMO2 LIM domain only 2 protein, GATA GATA binding protein, FOG friend of GATA, MMP matrix metallopeptidase, SDF stromal cell-derived factor.

| Stages of erythroid differentiation |
|-------------------------------------|
| HSC | BFU-E | CFU-E | Erythroblast | RET | RBC |
| ![EPO](image) |

1. Factors involved in differentiation of erythroid bone marrow cells

   - LMO2
   - GATA-1
   - RET
   - RBC

2. Factors for mobilization of erythroid bone marrow cells

   - SDF-1
   - MMP9

Fig. 1

Materials and methods

Animal model

We employed 6-week-old, male, homozygous TGR (mREN2)27 rats [13]. See “Electronic supplementary material” for detailed description. Rats were fed with ad libitum regular chow that contains sufficient amounts of vitamin B and iron (RMH-B 2181, AB Diets, The Netherlands). Over the course of the experiment, the mortality rate of REN2 rats was 20% (as reported; mortality was 0% for control (Sprague–Dawley, SD) rats). The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen, The Netherlands, and conducted in accordance with existing guidelines for the care and use of laboratory animals.

Experimental protocol

To stimulate erythropoiesis, rats were randomized to treatment with the long-acting EPO analogue darbepoetin alpha (40 μg/kg, Aranesp®, Amgen Inc., Thousand Oaks, CA, USA) administered subcutaneously every 3 weeks (baseline, 3 and 6 weeks) or saline (used as control). The dose of EPO was based on our extensive experience with this compound and previous data that this dose effectively raises hematocrit in SD rats [14–19]. Rats were sacrificed at week 7 of the experimental protocol (when aged 13 weeks). In total, four groups were studied: SD-PL (placebo; N=12), REN2-PL (N=12), SD-EPO (N=12), REN2-EPO (N=15). At baseline, 3 and 6 weeks, blood was drawn from the tail vein for a full blood count. At sacrifice, we analyzed serum...
iron status. Blood pressure via tail cuff and hemodynamic data at sacrifice were measured as described [20, 21]. Furthermore, to monitor the development of HF, echocardiography was performed at baseline, 3 and 6 weeks. For details, see “Electronic supplementary material”.

Bone marrow isolation and MACs sorting

BM was isolated from tibia and femurs as previously described [22]. C-kit positive (c-kit+) cells were isolated as described in the “Electronic supplementary material”. Briefly, the cell pellet was washed twice with PBS to remove platelets. Cells were labeled with polyclonal rabbit anti c-kit antibody, followed by goat anti-rabbit IgG microbeads. Anti c-kit-labeled cells were sorted by using mini-magnetic activated cell sorting columns in a magnetic field. Effluent was collected in chilled IMEM medium. C-kit+ cells were collected with 1 mL chilled IMEM medium, and cell number was counted.

Cell proliferation assay

After MACs column sorting, $1 \times 10^4$ c-kit+ BM cells were plated in 1 mL semi-solid medium (MethoCult, Stem Cell Technologies, Köln, Germany) in 35 mm Petri dishes in duplicate. The dishes were incubated at 37 C with 5% CO₂ and 95% humidity for 2 weeks. Colonies were scored using an inverted phase-controlled microscope (Leica 090-135002, Leica Microsystems, CMS GmbH, Germany). Burst-forming units-erythroid (BFU-E) were defined as consisting of 30 or more cells. Colony-forming units (CFU) were defined as consisting of 50 or more cells.

FACS analysis

BM mononuclear cells were harvested from tibia and femur of experimental animals and frozen in liquid nitrogen with 10% DMSO in medium in assay. FACS analysis was performed on an LSRII cytometer (BD Biosciences, San Jose, CA, USA). For phenotypic analysis by flow cytometry, $2–5 \times 10^7$ cells were dissolved in 10 mL cold DMEM medium with 10% PBS. Cell pellet were resuspended in 80 μL PBS/0.5% BSA. Samples were subsequently stained with rabbit polyclonal anti c-kit antibody (1 μg/10⁶ cells, Santa Cruz Biotechnology, Heidelberg, Germany) on ice for 30 min. After washing twice with 2 mL PBS/0.5% BSA, cells were incubated with Alexa Fluor® 488 (1 μg/10⁶ cells, Invitrogen, OR, USA) and FITC rat anti-mouse IgG (1 μg/10⁶ cells, BD Biosciences Pharmingen, Breda, The Netherlands) as secondary antibodies. Cells were then suspended in 0.5 mL PBS/0.5% BSA and analyzed within 1 h. Unstained cells and cells stained with second antibody only were used as a negative control. Data were analyzed by FlowJo software (V7.5.5, Ashland, OR, USA).

Quantitative real-time PCR

Total RNA from c-kit+ cells and left ventricular (LV) tissue was extracted using the Nucleospin RNA II kit, according to manufacturer’s protocol (Machery-Nagel, Düren, Germany). cDNA synthesis and quantitative real-time PCR (RT-qPCR) were performed as previously described with using 0.5 μg total RNA [23]. mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalize expression data, multiple reference genes were used as described [24]. Please see “Electronic supplementary material” for full protocol and list of primers.

Statistical analyses

Results are reported as means±standard errors of the mean. Number of BFU-E, CFU, and c-kit+ cells were normalized to SD-PL group. Statistical analysis among groups was performed by ANOVA with the Bonferroni post hoc test if distributed normally or with the Kruskall–Wallis test followed by Mann–Whitney U test when skewed distributed. All p values are two-tailed, and a p value of less than 0.05 was considered significant. All analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL, USA).

Results

Hypertensive REN2 rats develop heart failure

REN2 and SD rats were treated with or without EPO and were monitored for 6 weeks and sacrificed at an age of 13 weeks. As expected, REN2 rats suffered from severe hypertension (Fig. 2), providing the substrate for HF development. Heart weight (adjusted for tibia length), LV end diastolic pressure (LVEDP), and atrial natriuretic peptide (ANP) mRNA expression were all significantly increased in the REN2-PL and REN2-EPO groups, indicative for HF (Fig. 2). Table 1 shows the hemodynamic data at sacrifice. Indices of maximal contraction and relaxation of the LV (dP/dtmax and dP/dtmin) were both significantly decreased in the REN2 groups, compared to the SD groups, indicating development of HF. Cardiac output (CO), assessed with echo Doppler prior to sacrifice, is decreased in both REN2 groups compared to the SD groups. Echocardiographic data during the experiment confirmed development of LV hypertrophy and LV dilatation in REN2 rats (shown in “Electronic supplementary material”).
REN2 rats are anemic and have a limited response to EPO

Table 2 shows the full blood count for all experimental groups at sacrifice. As hypothesized, REN2 rats were mildly anemic and had mild to moderate reductions of other hematologic parameters as well (except platelet and reticulocyte count, which were increased). Administration of EPO increased hematocrit (Ht) levels significantly in the SD-EPO group compared to the SD-PL group (57.8±1.1% vs. 49.3±0.8%, p<0.01); however, EPO administration did only marginally increase Ht in the REN2-EPO group (44.0±0.6% vs. 42.7±0.5%, compared to REN2-PL, p=NS, Fig. 3). An identical pattern was observed for hemoglobin levels. Furthermore, RBC, MCV, and MCH were all significantly decreased in both the REN2-PL and the REN2-EPO groups, providing further evidence of anemia. We observed an increased reticulocyte count in the REN2 groups, which is commonly seen in anemia. EPO plasma levels increased significantly in all EPO treated groups (measured with ELISA as before [17], see “Electronic supplementary material”). Deficiencies of iron, vitamin B12, and folic acid were ruled out and did not differ between SD and REN2 groups (see “Electronic supplementary material”).

Table 1 Data at sacrifice

|                | SD-PL (N=12) | REN2-PL (N=12) | SD-EPO (N=12) | REN2-EPO (N=15) |
|----------------|--------------|----------------|--------------|-----------------|
| LV weight (mg) | 841±15       | 1,105±44*      | 809±17       | 1,157±36**      |
| TL (mm)        | 38.3±0.3     | 38.1±0.2       | 38.6±0.2     | 38.2±0.3        |
| Body weight (g) | 367±6        | 309±7*         | 360±8        | 316±9**         |
| Heart rate (bpm) | 352±17    | 347±12         | 352±12       | 350±10          |
| dPdmax (mmHg/s) | 7,993±290   | 5,625±356*     | 8,676±618    | 5,785±295**     |
| dPdmin (mmHg/s) | −9,211±666  | −5,730±335*    | −10,249±711  | −6,010±367**    |
| CO (mL/min)    | 145±4        | 112±10*        | 138±6        | 121±11**        |

*p<0.05 vs. SD-PL; **p<0.05 vs. SD-EPO

Fig. 2 Shows that the used animal model is a model of hypertensive heart failure. a Mean arterial pressure (MAP) during the experiment. MAP is significantly higher at all time points for REN2 groups compared to SD groups. b Heart weight normalized to tibia length. c Left ventricular end diastolic pressure (LVEDP) at sacrifice. d mRNA expression of atrial natriuretic peptide (ANP) in left ventricular tissue. ANP is expressed as fold change. HW heart weight, Base baseline, Wk week, PL placebo.
Bone marrow dysfunction in the REN2 rats

To provide mechanistic insight into the causes of anemia, we studied the number of BFU-E and CFU colonies with a cell proliferation assay (Fig. 3b, c). REN2 rats (treated with placebo) had a severely decreased number of BFU-E colonies, compared to SD-PL (0.33±0.11-fold, normalized to SD-PL, \( p < 0.05 \)). This indicates either a reduced number or a limited proliferation capacity of erythroid precursors in the BM. EPO administration increased the number of BFU-E colonies, both in the SD-EPO group (1.92±0.33-fold vs. SD-PL, \( p < 0.05 \)) as well as in the REN2-EPO group, albeit much less (0.88±0.21-fold vs. SD-PL, \( p < 0.05 \)).

To dissect abnormalities in the erythroid precursors only from general abnormalities in the BM, we also investigated number of CFU colonies. Number of CFU is only slightly, but significantly decreased in the REN2-PL group compared to SD-PL group.

Low levels of hematopoietic stem cells in REN2 rats

To confirm the findings in the cell proliferation assay, we performed FACS analysis of c-kit+ cells in BM (Fig. 4). Compared to SD-PL, the number of c-kit+ cells is reduced in REN2-PL (with 43%, \( p < 0.05 \)) in line with the BFU-E cell proliferation assay number. EPO treatment increased the number of c-kit+ cells measured by FACS in both the SD-EPO group (1.92±0.33-fold vs. SD-PL, \( p < 0.05 \)) as well as in the REN2-EPO group, albeit much less (0.88±0.21-fold vs. SD-PL, \( p < 0.05 \)).

mRNA expression of factors involved in erythroid differentiation, mobilization, and iron incorporation

LMO2 was significantly reduced in the REN2-PL group (34% vs. SD-PL, \( p < 0.05 \)). EPO treatment reduced the
LMO2 expression in the SD-EPO group but did not increase the LMO2 expression in the REN2-EPO group. Vice versa, expression of GATA-1 was 2.3-fold increased in the REN2-PL group compared to the SD-PL group ($p < 0.01$). Treatment with EPO increased the GATA-1 expression in the SD-EPO group, but did not affect the GATA-1 expression in the REN2-EPO group. These data suggest that differentiation of erythrocyte-like BM precursors is inhibited in HF and that EPO treatment partially restores this (Fig. 5).

Expression of MMP9 was reduced with 60% in the REN2-PL group compared to the SD-PL group ($p < 0.05$). Treatment with EPO did not exert substantial effects on MMP9 expression in neither SD nor REN-2 rats. In contrast, SDF-1 expression was significantly increased in the REN2-PL group (3.5-fold vs. SD-PL, $p < 0.05$). Treat-
Fig. 5 mRNA expression of different factors involved in differentiation (a, b) and mobilization (c, d) of c-kit⁺ cells. mRNA expression of different factors involved in iron metabolism (e, f). *LMO2 LIM domain only 2 protein, **GATA-1 GATA binding protein, *MMP9 matrix metalloproteinase, SDF-1 stromal cell-derived factor, PL placebo. *p<0.05; **p<0.01
ment with EPO further increased the expression of SDF-1 (5.6-fold vs. SD-EPO, \( p < 0.01 \)). These data suggest that mobilization of erythrocyte-like BM precursors is inhibited in HF and that this cannot be restored by EPO treatment.

mRNA expression of ferroportin was not statistically different between the four groups, albeit that ferroportin is slightly less expressed in both REN2 groups. mRNA expression of transferrin receptor was comparable between the SD-PL vs. REN2-PL groups (1.6±0.2-fold vs. SD-PL, \( p = \text{NS} \)). However, after treatment with EPO, the expression of transferrin receptor was increased in the SD-EPO group (2.8±0.7-fold vs. SD-PL, \( p < 0.05 \)), where the expression was not increased in the REN2-EPO group, indicating a reduction in iron incorporation in the erythroid cells in REN2 rats after EPO administration.

Discussion

In hypertensive REN2 rats with progressive HF, we observed mild anemia and a profound dysfunction of the erythroid lineage in the BM. Treatment with EPO with the aim to rescue this anemia did not diminish the anemia. Apparently, HF-associated BM dysfunction is a rather complex phenomenon that may require other treatment options. In concert with this, we observed that along the entire line of maturation of the erythroid lineage, multiple genes associated with differentiation, mobilization, and iron incorporation were abnormally expressed in BM from HF rats. Figure 6 provides an overview of the mechanisms of BM dysfunction involved in HF-associated anemia.

We used a monogenetic rat model with overexpression of the renin gene, resulting in hypertension and concomitant HF development. These rats also develop mild anemia, which is in line with the observations of Iversen et al., who described for the first time the existence of HF-associated anemia in mice [9]. Our results extend this observation of Iversen to another species (rat) and moreover show that this also occurs in animals in which hypertension is the sole source for HF development.

![Fig. 6 Schematic overview of the mechanisms of bone marrow dysfunction involved in HF-associated anemia. Different triggers of anemia in heart failure such as insensitivity of EPO, iron deficiency, and neurohormonal activation have a negative effect on the hematopoietic stem cells in the bone marrow. Furthermore, impaired proliferation, differentiation, mobilization, and iron incorporation in hematopoietic stem cells contribute to the bone marrow dysfunction, causing anemia and adding to the heart failure syndrome, which in turn triggers anemia in heart failure, etc. EPO treatment does not rescue the dysfunction of the erythroid lineage and does not improve heart failure-associated anemia. SNS sympathetic nervous system, RAS renin–angiotensin system](image)
Since HF-associated anemia has a dramatic impact on prognosis in humans [1–3, 25], we tried to restore this by EPO treatment. As expected, EPO treatment resulted in a clear increase in Ht in the control rats. Surprisingly, however, EPO did not rescue anemia in the REN2 rats and only showed marginal effects on Ht values. Since the main action of EPO is the regulation of erythropoiesis, this is apparently not the single culprit in HF-associated anemia. Several clinical observations from our group suggested that EPO levels in HF are discordant with the (expected) levels of hemoglobin [26–28], and our current observations that EPO cannot rescue HF-associated anemia are in concert with this. This misbalance between EPO and hemoglobin levels is related to an increased mortality risk. Mechanistically, this may be explained by a diminished response of the BM to high plasma EPO levels in HF, a phenomenon we confirm in our present study. Together, this indicates that in HF-associated anemia other treatment options will be needed.

To obtain further insight in the cause of this anemia, we analyzed the BM. We observed a profound (43%) decrease in BM hematopoietic progenitor cells (c-kit+ cells) in REN2 rats as compared to control animals. This is in line with the observation of Iversen et al., who observed a reduction of up to 60% of similar CD34+ cells in mice with HF [9]. Although not investigated in our rat model, they suggested that the mechanisms of HF-associated anemia may depend on the etiology of the cardiac disease, where inflammation seems to be dominant in ischemic HF, while neurohormonal factors seem to be the player in non-ischemic HF [11].

To further characterize BM dysfunction and the irresponsiveness to EPO in the REN2 rat model of HF, we performed RT-qPCR analysis to measure the expression of markers of differentiation (LMO2, GATA-1) and mobilization (MMP9, SDF-1) of the erythroid lineage and incorporation of iron in these cells (ferroportin, transferrin receptor) (Fig. 5). LMO2, a positive regulator of erythroid differentiation [29], was down-regulated in the REN2-PL group, which may contribute to the decreased number of BFU-E. GATA-1 is a transcription factor playing a key role in gene regulation during erythroid differentiation [30]. This factor is strictly controlled during development and differentiation of erythroid cells and essential for the survival of erythroid progenitors [31]. GATA-1 mRNA expression is 2.3-fold increased in our experiments in the REN2-PL group, indicating that survival of the erythroid progenitors is promoted in this weakened erythropoietic system.

Furthermore, impaired mobilization of erythroid progenitors from the BM could contribute to the lack of increase in Ht upon EPO stimulation. Degradation of BM SDF-1 by MMP9 contributes to increased mobilization of erythroid progenitors [32]. So, decreased levels of MMP9, which result in increased levels of BM SDF-1, could in turn lead to impaired mobilization of erythroid progenitors from the BM in REN2 rats.

Analysis of factors involved in iron transportation yielded some interesting results. mRNA expression of ferroportin, involved in iron transportation from inside of a cell to the outer surface, was not different between any group. mRNA expression of transferrin receptor, involved in maintaining cellular iron homeostasis, was comparable between the SD-PL vs. REN2-PL groups. However, after treatment with EPO, expression was increased in the SD-EPO group, where expression was not increased in the REN2-EPO group. We speculate that the lower expression of transferrin receptor in REN2 rats treated with EPO is associated with lesser iron incorporation in erythroid cells, which may explain in part the lesser response to EPO. In general, we observed a changed expression profile of genes involved in differentiation, mobilization, and incorporation of iron in BM.

Besides defects in the erythropoietic lineage of the BM (reduction in c-kit+ cells and BFU-E), we also observed a reduction in number of CFU (with 27%) compared to control animals. EPO treatment did not improve these values in the REN2 rats. We recently showed that human HF is associated with a profound and general BM dysfunction, simultaneously affecting multiple hematopoietic lineages [10]. Since all these patients obtained optimal HF-treatment and were obviously more heterogeneous, our confirmation in this monogenetic rat model further confirms these observations in a human population and show that it is treatment independent. Moreover, HF-associated anemia appears to be a general phenomenon in mammals.

dPdtdmax, dPdtmin, and CO were all significantly decreased in the REN2 groups compared to the SD groups, indicating development of HF. Hypoperfusion of the bone marrow by reduced CO could potentially affect erythropoiesis in HF [8]. However, we believe that hypoperfusion is not (yet) a critical factor, as we have sacrificed the REN2 rats before overt (decompensated) HF had ensued.

Because the REN2 transgene is expressed ubiquitously and thus also in the hematopoietic cells, we cannot exclude that the effects of the transgene mediate the phenotype at least to some extent. However, the sequel of events in the development of HF in the REN2 rat (hypertrophy, diastolic dysfunction, systolic dysfunction, overt HF, death) are very much alike the human situation. Furthermore, the mild anemia and bone marrow dysfunction associated with HF in the REN2 rat are also comparable to what we observed in earlier human studies. So, although this model (like any rodent model) has limitations, the phenotype we study herein is, at least to a large extent, comparable to the human situation.

In conclusion, in REN2 rats with progressive HF, there is mild anemia and a profound dysfunction of the erythroid
lineage of the BM. Response to EPO is diminished in REN2 rats and does not rescue the dysfunction of the erythroid lineage. Multiple genes appear to be involved in this process. This does, however, not exclude EPO as a treatment option in HF, because we and others have shown that EPO has also direct positive effects on the heart, including anti-apoptotic, pro-angiogenic, and anti-ischemic actions [14–19]. Therefore, EPO can be beneficial in HF but does not improve HF-associated anemia. The latter probably needs a more complex treatment regimen.

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