Separate mechanisms cause anemia in ischemic vs. nonischemic murine heart failure

Per O. Iversen,1,2 Kristin B. Andersson,3,4 Alexandra V. Finsen,3,4 Ivar Sjaastad,3,4,5
Thomas G. von Lueder,4,6 Ole M. Sejersted,3,4 Håvard Attramadal,4,6 and Geir Christensen3,4
1Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway; 2Department of Hematology and 3Institute for Experimental Medical Research, Oslo University Hospital, Ullevaal, Oslo, Norway; 4Center for Heart Failure Research, University of Oslo, Oslo, Norway; 5Department of Cardiology, Oslo University Hospital, Ullevaal, Oslo, Norway; and 6Institute for Surgical Research, Oslo University Hospital, Rikshospitalet, Oslo, Norway

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Iversen PO, Andersson KB, Finsen AV, Sjaastad I, von Lueder TG, Sejersted OM, Attramadal H, Christensen G. Separate mechanisms cause anemia in ischemic vs. nonischemic murine heart failure. Am J Physiol Regul Integr Comp Physiol 298: R808–R814, 2010. First published December 23, 2009; doi:10.1152/ajpregu.00250.2009.—In ischemic congestive heart failure (CHF), anemia is associated with poor prognosis. Whether anemia develops in nonischemic CHF is uncertain. The hematopoietic inhibitors TNF-α and nitric oxide (NO) are activated in ischemic CHF. We examined whether mice with ischemic or nonischemic CHF develop anemia and whether TNF-α and NO are involved. We studied mice (n = 7–9 per group) with CHF either due to myocardial infarction (MI) or to overexpression of the Ca2+-binding protein calsequestrin (CSQ) or to induced cardiac disruption of the sarcoplasmic reticulum Ca2+-ATPase 2 gene (SERCA2 KO). Hematopoiesis was analyzed by colony formation of CD34+ bone marrow cells. Hemoglobin concentration was 14.0 ± 0.4 g/dl (mean ± SD) in controls, while it was decreased to 10.1 ± 0.4, 9.7 ± 0.4, and 9.6 ± 0.3 g/dl in MI, CSQ, and SERCA2 KO, respectively (P < 0.05). Colony numbers per 100,000 CD34+ cells in the three CHF groups were reduced to 33 ± 3 (MI), 34 ± 3 (CSQ), and 39 ± 3 (SERCA2 KO) compared with 68 ± 4 in controls (P < 0.05). Plasma TNF-α nearly doubled in MI, and addition of anti-TNF-α antibody normalized colony formation. Inhibition of colony formation was completely ablated with blockade of endothelial NO synthase in CSQ and SERCA2 KO, but not in MI. In conclusion, the mechanism of anemia in CHF depends on the etiology of cardiac disease; whereas TNF-α impairs hematopoiesis in CHF following MI, NO inhibits blood cell formation in nonischemic murine CHF.

Patients with heart failure continue to face high mortality rates despite extensive efforts to optimize treatment strategies. Anemia has emerged as an important comorbidity associated with a dismal prognosis. Although most studies report a correlation between poor left ventricular myocardial function and low hemoglobin concentration (1a, 10, 22, 24), the prevalence of anemia in heart failure patients varies considerably (26). This might be partly explained by differences in definitions of anemia and disease severity and that many studies were originally not designed to examine anemia but were primarily clinical trials conducted in selected patients. It may also be possible that the various etiologies of heart failure in the patients studied might lead to anemia through different mechanisms and thus partly explain the variation in prevalence. It is also unclear whether anemia is an independent pathogenic factor in heart failure or only a marker of a deteriorating heart function (17).

Myocardial infarction (MI) remains a leading cause of congestive heart failure (CHF). A low-grade inflammation is often, but not always, associated with ischemic CHF. Such a low-grade inflammation may lead to development of anemia (3). In a murine model of post-MI CHF we have found that anemia was linked to apoptosis of bone marrow progenitor cells via the TNF-α/Fas signaling pathway (16). Activation of this apoptotic machinery was not confined to the erythropoietic compartment alone since subsequent studies in mice revealed that the functions of granulocytes and lymphocytes were also impaired (15). The latter finding was recently corroborated by the observation of depressed erythro- and lymphopoiesis also in human heart failure (5).

It is not known whether anemia is a feature restricted to ischemic CHF or is a more generalized response also present in nonischemic CHF. In ischemic disease, both TNF-α and nitric oxide (NO) are central signaling molecules in the heart failure syndrome (27, 28). However, inflammatory mediators and NO may be activated also in patients with CHF of nonischemic etiologies (21). Endothelial dysfunction associated with heart failure may alter endothelial NO synthase (eNOS) activity, hence further augmenting myocardial dysfunction due to increased oxidative stress (12, 31). Paralleling these cardiodepressive actions, NO might also directly inhibit bone marrow hematopoietic activity (6, 20).

In this study, we asked whether anemia would develop in nonischemic heart failure by examining two different mouse models with targeted alterations of cardiac sarcoplasmic reticulum calcium handling. We also examined whether TNF-α and NO exerted similar effects on hematopoietic activity in these nonischemic CHF mice compared with ischemic CHF mice induced by acute MI.

METHODS

Animals. The study was approved by local institutional ethical committees, and the animals were cared for in accordance with the Norwegian Animal Welfare Act, which conforms to the American Physiological Society’s Guiding Principles in the Care and Use of Animals (1). Health monitoring according to the Federation of European Laboratory Animal Science Associations test panels did not detect any pathogenic microbes in bone marrow, splenic, fecal, or urine samples, nor was any significant amount of endotoxin detected in blood samples from any of the included mice.

Three different models of heart failure were included in the study. Acute MI was induced in eight C57B6/J mice by ligation of the left...
coronary artery, while it was left unligated in nine sham-operated animals (sham) as described (16). In short, the mice were anesthetized with 0.2 ml (10 mg/ml) propofol and mechanically ventilated with a mixture of 1.5–2% isoflurane and 98% oxygen. Following left-sided thoracotomy, the left coronary artery was ligated before the chest was closed. Postoperatively the mice were given 0.01 ml (0.3 mg/ml sc) buprenorphine as analgesia. Six weeks later, cardiac function was analyzed.

Adult mice with a tamoxifen-inducible cardiomyocyte-specific deletion of the sarcoplasmic reticulum Ca2+ ATPase 2 gene (SERCA2 KO) develop nonischemic heart failure within 7 wk (2). These mice were backcrossed onto the C57B6 J background. Eight Serca2flox/floxTg(MCM) (SERCA2 KO) and seven control Serca2flox/flox (SERCA2 FF) (Serca2flox/flox) mice aged 9 wk were used. Disruption of the Serca2 gene in the heart was induced with tamoxifen as described (2). Cardiac function was analyzed after 7 wk.

Transgenic mice with cardiac-restricted overexpression of the sarcoplasmic reticulum-resident Ca2+-binding protein calsequestrin (CSQ) on a DBA background, develop nonischemic heart failure with 50% mortality within 4–5 mo (13, 14). Seven of these mice were compared with eight age-matched nontransgenic DBA controls. After 12–14 wk, cardiac function was analyzed.

Echocardiography. Transthoracic echocardiographic examination was performed on all mice using the fully digital Vivid 7 System (GE Vingmed Ultrasound, Horten, Norway) and a 13 MHz linear array transducer (11). All measurements were made off-line using the Echopac software (GE Vingmed Ultrasound). Short-axis dimensions were recorded at the level of the tip of the papillary muscle. After gain settings were optimized, M-mode tracings were recorded at the same level. Left ventricle internal dimensions were recorded at end-diastole and at end-systole. For MI mice the left ventricle internal dimensions were recorded as the largest anteroposterior diameter outside the infarcted area. In the MI and sham mice, as well as in the SERCA2 KO and SERCA2 FF mice, the recordings were made on spontaneously breathing mice given anesthesia (2% isoflurane and 98% oxygen) whereas in the CSQ mice, recordings were obtained in mice sedated with midazolam (6.25 mg/kg sc; Dormicum, Roche, Basel, Switzerland).

Collection of organs, blood, and bone marrow samples. Following echocardiography, anesthesia depth was increased with 2% isoflurane and blood from the inferior caval vein was collected from all mice before excision of the heart. The lungs were removed and weighed, and bone marrow was isolated from femoral bones, as described (4).

Hematopoietic assays. CD34+ hematopoietic progenitor cells were isolated from the bone marrow cells by density gradient centrifugation and positive selection with CD34+ magnetic beads (MACS; Miltenyi Biotech, Bergish Gladbach, Germany) (16). The fraction of long-term colony initiating cells was determined by culturing CD34+ cells in liquid medium (MethoCult; Stemcell Technologies, Vancouver, BC, Canada) and then in semisolid methylcellulose medium (MyeloCult; StemCells Technologies), as previously detailed (16). In separate experiments CD34+ cells were collected from untreated, healthy C57B6/J and DBA mice. Colony formation was also determined in the presence of a neutralizing anti-TNF-α antibody, or after blockade of eNOS with 1-Nω-(1-iminoethyl)ornithine hydrochloride (MIP, Ann Arbor, MI) (29). A SOD mimetic [4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL); Sigma, St. Louis, MO] was added to inhibit the generation of reactive oxygen species (25).

Apoptosis of CD34+ cells was quantified by detection of surface expression of phosphatidylserine with annexin V (ApopTosis Detection Kit; R&D Systems, Minneapolis, MN) using a flow cytometer (FACSscan; Becton Dickinson, Mountain View, CA).

Measurements of inflammatory mediators. The serum concentration of TNF-α was measured with an ELISA kit (Quantikine; R&D Systems; lower limit of detection 5 pg/ml), and an ELISA kit from Life Diagnostics (West Chester, PA) was used to measure the serum concentration of C-reactive protein (CRP; lower level of detection 5 ng/ml).

Statistical analyses. Each assay was performed in triplicate, and the corresponding median value was used to calculate the group means and SD. Differences between the experimental mice and their respective control groups were evaluated with the unpaired Mann-Whitney U-test. A P value < 0.05 was considered statistically significant.

RESULTS

Mouse models of heart failure. The pooled results of lung weight measurements and echocardiographic recordings given in Table 1 show that the MI, SERCA2 KO, and CSQ mice developed pulmonary congestion and cardiac dysfunction, hence indicating CHF. Representative echocardiographic recordings in the three experimental groups are shown in Fig. 1. Since the increase in lung weight was similar in the three groups, the degree of cardiac failure with subsequent pulmonary congestion seemed to be comparable despite differences in etiology and disease duration (11). However, the degree of cardiac dysfunction was different in the three models, and this was reflected in the echocardiographic measurements.

Both ischemic and nonischemic CHF mice develop anemia. At the time of analysis, the hemoglobin concentrations were markedly reduced (to ~70% of control values) in all mice with a heart failure phenotype (Fig. 2). Interestingly, the reduction in hemoglobin concentrations was similar among the three CHF groups.

Increased plasma concentration of CRP and TNF-α in MI, but not in SERCA2 KO or CSQ mice. In view of the low-grade inflammatory activity that usually accompanies ischemic CHF, we expected that the MI-mice, but not the nonischemic CHF mice, would display enhanced systemic levels of inflammatory mediators. We found that the plasma concentrations of both CRP and TNF-α were increased (P < 0.05) in the MI mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>MI</th>
<th>DBA</th>
<th>CSQ</th>
<th>SERCA2 FF</th>
<th>SERCA2 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>No., rats/group</td>
<td>9</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>26.6 ± 1.3</td>
<td>26.9 ± 1.7</td>
<td>24.7 ± 2.3</td>
<td>26.4 ± 2.1</td>
<td>29.0 ± 1.7</td>
<td>28.4 ± 4.3</td>
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<tr>
<td>Lung weight, g</td>
<td>148 ± 5</td>
<td>230 ± 32*</td>
<td>167 ± 12</td>
<td>218 ± 21*</td>
<td>157 ± 6</td>
<td>218 ± 17*</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>32 ± 2</td>
<td>8 ± 1*</td>
<td>39 ± 2</td>
<td>17 ± 4*</td>
<td>30 ± 3</td>
<td>20 ± 2*</td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.9 ± 0.1</td>
<td>6.1 ± 0.4*</td>
<td>3.1 ± 0.1</td>
<td>4.9 ± 0.7*</td>
<td>4.3 ± 0.1</td>
<td>3.8 ± 0.2*</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>2.7 ± 0.2</td>
<td>5.6 ± 0.4*</td>
<td>1.9 ± 0.1</td>
<td>4.1 ± 0.7*</td>
<td>3.0 ± 0.2</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Left atrial diameter, mm</td>
<td>1.8 ± 0.1</td>
<td>2.7 ± 0.1*</td>
<td>ND</td>
<td>ND</td>
<td>1.8 ± 0.2</td>
<td>3.0 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SD. MI, myocardial infarction; DBA, Dolichos biflorus agglutinin; CSQ, calsequestrin; SERCA2 FF, Serca2flox/flox; SERCA2 KO, Serca2flox/floxTg(MCM); LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; ND, not determined. *P < 0.05 compared with appropriate control group.

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compared with the sham mice (Table 2). In the SERCA2 KO and the CSQ models, the plasma concentrations of CRP and TNF-α were not significantly altered compared with the relevant control groups.

Impaired growth of immature hematopoietic progenitor cells in CHF: role of TNF-α and NO. Growth of hematopoietic cells was determined in a colony assay designed to select for the very immature CD34+ bone marrow progenitor cells. In all three heart failure groups, the colony numbers of CD34+ cells were reduced by 50% (P < 0.05) compared with their respective control groups (Fig. 3A). When bone marrow cells from MI animals were treated with a neutralizing anti-TNF-α antibody, CD34+ colony formation was almost completely restored and reached values similar to the control group. In the SERCA2 KO and CSQ models, there were no significant changes (Fig. 3B). Blockade of eNOS almost completely restored CD34+ colony formation in both SERCA2 KO and the CSQ mice (Fig. 3C), but did not have any effect (P > 0.05) in MI mice.

TNF-α and NO induce apoptosis of hematopoietic progenitor cells in CHF. We next asked if the reduction of colony formation in CHF could be due to increased apoptosis of hematopoietic cells (Fig. 4A). Indeed, apoptosis occurred more frequently in all three heart failure groups compared with the respective control mice, as assessed by quantification of surface annexin V (Fig. 4B). To test whether TNF-α and NO production affected apoptosis of CD34+ cells, a neutralizing antibody against TNF-α or a selective blocker of eNOS was added to the cell cultures. Consistent with the findings from the colony assays, the fraction of apoptotic CD34+ cells from the MI mice decreased markedly (P < 0.05) in the presence of the anti-TNF-α antibody, whereas there was no effect on cells from SERCA2 KO and CSQ mice (P > 0.05) (Fig. 4C). Blockade of the eNOS decreased apoptosis in CD34+ cells from both SERCA2 KO and CSQ mice (P < 0.05), but not in cells from MI mice (Fig. 4D).

NO inhibits hematopoiesis in CHF via reactive oxygen species. We have previously found that a decrease in hematopoiesis is observed in postinfarction CHF and may be linked to activation of the TNF-α/Fas pathway (16). NO can exert detrimental effects via the generation of reactive oxygen species (29). To test this in our CHF models, we added the SOD mimetic TEMPO to reduce the formation of reactive oxygen species.
species. Figure 5 shows that the colony numbers from both the SERCA2 KO and the CSQ mice were partly restored upon addition of TEMPO, while no significant effect was detected in the post-MI group.

![Hemoglobin (Hb) concentration in the CHF mice and control groups.](image)

![Hb (g/100mL) concentration in the CHF mice and control groups.](image)

![Hb (g/100mL) concentration in the post-MI group.](image)

**Table 2. Plasma concentrations of TNF-α and CRP**

<table>
<thead>
<tr>
<th>Group</th>
<th>No., rats/group</th>
<th>TNF-α, pg/ml</th>
<th>CRP, ng/ml</th>
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<tbody>
<tr>
<td>MI</td>
<td>8</td>
<td>46 ± 6*</td>
<td>22 ± 3*</td>
</tr>
<tr>
<td>Sham</td>
<td>9</td>
<td>25 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>SERCA2 KO</td>
<td>8</td>
<td>28 ± 2</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>SERCA2 FF</td>
<td>7</td>
<td>24 ± 4</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>CSQ</td>
<td>7</td>
<td>32 ± 4</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>DBA</td>
<td>8</td>
<td>25 ± 2</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SD. CRP, C-reactive protein. *P < 0.05 compared with appropriate control group.

![Fig. 3. Colony formation of bone marrow CD34⁺ cells. A: CD34⁺ colony numbers in the 3 CHF mouse models and respective control groups. B: CD34⁺ colony numbers in cell cultures added a neutralizing anti-TNF-α monoclonal antibody. C: CD34⁺ colony numbers in cell cultures added a blocker of endothelial nitric oxide synthase (eNOS). Symbols and number of mice/group as in Fig. 2. LTC-IC, long-term colony-initiating cells. Values are the means and SD. *P < 0.05 for CHF vs. controls.](image)
In the present study, we show that mice with either ischemic (MI group) or nonischemic (SERCA2 KO and CSQ groups) CHF developed marked anemia with a similar reduction of hemoglobin concentrations and impaired bone marrow hematopoietic activity of CD34<sup>+</sup>/H11001 cells. This is in line with the decline in bone marrow hematopoiesis observed in patients with coronary artery disease (18). We also showed that inhibition of TNF-α almost completely restored CD34<sup>+</sup>/H11001 colony formation and decreased the fraction of apoptotic CD34<sup>+</sup> cells in the ischemic CHF mice but not in nonischemic CHF mice. Blockade of eNOS inhibited apoptosis of CD34<sup>+</sup> cells from mice with nonischemic CHF, whereas this effect was absent among the ischemic CHF mice.

Despite growing epidemiological evidence for an association between CHF and anemia, the mechanism underlying impaired erythropoiesis in this condition has not been identified. It is uncertain whether low levels of erythropoietin may be a direct cause of anemia in CHF, as CHF is not always accompanied by low erythropoietin levels (28). Postinfarction CHF is accompanied by a low-grade inflammatory state and increased levels of several proinflammatory compounds such as TNF-α. In addition to direct cardiodepressive effects, TNF-α inhibits the normal production of blood cells partly by promoting apoptosis of hematopoietic CD34<sup>+</sup> cells via activation of Fas/Fas ligand (19, 23). We have previously shown that the TNF-α/Fas pathway was activated in lymphocytes resident in the bone marrow of MI mice and suggested this as a mechanism explaining the development of anemia in CHF (16). In the present study, we extend these observations by showing that antibody-mediated neutralization of TNF-α improved colony formation and reduced apoptosis of bone marrow cells from MI mice. These findings strongly indicate that TNF-α may be an important local signaling molecule in the

**DISCUSSION**

Fig. 4. Apoptosis of bone marrow CD34<sup>+</sup> cells. A: original flow cytometric profiles showing less apoptosis, i.e., lower number of Annex V-labeled bone marrow CD34<sup>+</sup> cells, harvested from a sham mouse (top) compared with an MI mouse (bottom). B: %apoptotic cells in the 3 CHF mouse models and respective control groups. C: %apoptotic cells in cultures added a neutralizing anti-TNF-α monoclonal antibody. D: %apoptotic cells in cultures added an eNOS blocker. Symbols and number of mice/group as in Fig. 2. Values are the means and SD. *P < 0.05 for CHF vs. controls.
bone marrow for increased apoptosis and subsequent development of anemia in ischemic CHF after MI.

To further examine whether anemia is a more general feature independent of heart failure etiology, we also examined two nonischemic murine CHF models. Anemia was evident in both the SERCA2 KO and the CSQ mice. Importantly, TNF-α played no role in these two nonischemic CHF models since the TNF-α concentrations were unchanged and there was no positive effect of a neutralizing anti-TNF-α antibody on bone marrow hematopoiesis or on apoptosis of CD34+ cells.

SERCA2 KO mice and CSQ mice both displayed reduced cardiac function resulting from targeted alterations in sarcoplasmic reticulum proteins and Ca2+ homeostasis. There are differences between these two nonischemic CHF models that might be relevant with regard to data interpretation. Adult SERCA2 KO mice are phenotypically normal until induction of Serca2 gene disruption, which then leads to a rapid reduction in SERCA2 and subsequent CHF within 7 wk (2). The SERCA2 KO mouse model has been carefully studied by us in a large number of mice by using both echocardiography and invasive pressure measurements (2). In this mouse model, isovolumetric pressure decay time constant (tau) is fivefold increased compared with control mice and no left ventricular dilatation, indicating that diastolic dysfunction is a predominant feature. CSQ mice have increased protein amounts of CSQ and altered sarcoplasmic reticulum function from the embryonic stage. These animals develop CHF more slowly (13, 14). We examined the latter mice at 12–14 wk of age.

In contrast to the ischemic CHF mice, there was no increase in CRP or TNF-α in the two nonischemic CHF models. A detailed investigation of possible alterations in inflammatory mediators during development of CHF in SERCA2 KO or CSQ mice would require a comprehensive screening of a wide range of molecules both at the protein and gene level, and is thus outside the scope of the present study.

NO exerts negative effects on both myocardial function and hematopoiesis. We therefore tested the possible role of NO as a hematopoietic inhibitor in our CHF mouse models. Whereas blockade of eNOS had no effect on colony formation of CD34+ cells from bone marrow of MI mice, colony formation was almost completely normalized in both SERCA2 KO and CSQ mice. In further search of the mechanisms of NO-mediated inhibition of bone marrow hematopoiesis, we attempted to reduce the formation of reactive oxygen species by neutralizing oxygen radicals with a SOD analog. Addition of TEMPOL partially normalized colony formation of CD34+ cells sampled from bone marrow of SERCA2 KO and CSQ mice, showing that NO, at least partly, inhibits hematopoiesis in nonischemic CHF via production of reactive oxygen species, possibly originating from bone marrow resident immunocompetent cells. Another possible explanation for our findings in the SERCA2 KO and CSQ mice involves the small population of endothelial progenitor cells (EPCs). Reportedly, such cells may stimulate bone marrow progenitor cells (7). A decrease in EPCs in CHF may therefore inhibit hematopoiesis. Whether the number of EPCs are reduced in our murine CHF models, remains to be examined.

The pulmonary congestion in CHF might induce the production and/or secretion of factors, e.g., from the pulmonary vascular bed with antihematopoietic activities and thereby inducing anemia (30). However, since pulmonary congestion was a common feature in all three CHF models, this cannot explain the differential effects of TNF-α and NO in ischemic vs. nonischemic CHF. How the NO system is regulated in response to various types of reduction in heart function is not well known. It has been shown that plasma NO levels are elevated in patients with isolated diastolic heart failure and that in patients with left ventricular systolic failure, the severity of left ventricular diastolic dysfunction determines the amount of NO production (9).

Perspectives and Significance

This study demonstrates that different mechanisms may account for impaired hematopoiesis and anemia in ischemic and nonischemic heart failure. However, our mouse models may not mimic human heart failure in all aspects. For example, in patients, other factors might also be involved in development of anemia, such as concomitant illness and the impact of various therapeutic regimens used to treat heart failure. Our study suggests that novel treatment modalities for anemia in heart failure may be needed based on the etiology of the heart disease, although exercise training may inhibit both TNF-α and oxidative stress (8). TNF-α activation of the Fas/Fas ligand death pathway seems to be important for development of anemia in ischemic CHF. With regard to the role of NO in suppressing hematopoiesis in nonischemic heart failure, further studies are necessary to identify the participating cell types and the signals leading to alterations in eNOS with subsequent production of reactive oxygen species.

In conclusion, we show here for the first time that a marked anemia developed in two different genetically engineered mouse models of nonischemic heart failure. Moreover, mice with CHF of both ischemic and nonischemic origin had a similar reduction in bone marrow hematopoietic activity. Inhibition of TNF-α almost completely restored colony formation and decreased the fraction of apoptotic CD34+ cells in ischemic, but not in mice with CHF of nonischemic etiology. Blockade of eNOS inhibited apoptosis among CD34+ cells from mice with nonischemic CHF, but had no such effect in mice with ischemic CHF. Thus, we suggest that the mechanism leading to anemia in CHF is dependent on the etiology of the cardiac disease.
GRANTS

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DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


R814 ANEMIA IN CONGESTIVE HEART FAILURE

No conflicts of interest are declared by the authors.