DECREASED HEMATOPOIESIS IN BONE MARROW OF MICE WITH CONGESTIVE HEART FAILURE

PER OLE IVERSEN,1 PER REIDAR WOLDBÆK,2,3 THEIS TÖNNESSEN,2,3 AND GEIR CHRISTENSEN2

1Institute for Nutrition Research, University of Oslo; 2Institute for Experimental Medical Research, and 3Department of Cardiothoracic Surgery, Ullevål University Hospital, 0316 Oslo, Norway

Received 18 June 2001; accepted in final form 31 August 2001

Despite recent therapeutic advances, heart failure carries a poor prognosis, with acute myocardial infarction being a leading cause of this debilitating disease (5). During progression to overt heart failure, reduced cardiac output and concomitant neuroendocrine activation affect the functions of several organs. It has been observed that patients with severe heart failure are prone to infectious diseases and anemia (3). The reason for this apparent depression of the hematopoietic system is not known, but one possibility is that the function of the bone marrow is affected. However, whether a sustained decrease in the hematopoietic activity occurs during development of heart failure has not been detailed.

Recent evidence suggests that the proinflammatory cytokine tumor necrosis factor-α (TNF-α) might play an important pathogenic role in heart failure. It has been shown that the failing myocardium expresses elevated levels of both TNF-α mRNA and TNF-α protein (11, 20, 27, 29). TNF-α also induced apoptosis among cardiomyocytes, and transgenic mice with cardiac-specific overexpression of TNF-α developed cardiomyopathy (13, 24).

TNF-α is a negative regulator of normal hematopoiesis in vitro, at least partly due to induction of the CD95/APO-1/Fas system, known to promote apoptosis among hematopoietic progenitor cells (4, 15, 18, 25). The various inhibitory effects of TNF-α are pronounced in the cachectic state frequently noted among patients with heart failure (14). Hence the increased TNF-α involved in heart failure might also repress normal hematopoiesis, possibly via Fas/Fas ligand activation. Interestingly, recent data suggest that this Fas system might be involved in apoptosis of ischemic cardiomyocytes (10, 28). Therefore this study was conducted to test the hypothesis that bone marrow hematopoiesis is affected during heart failure. To this end we used mice with congestive heart failure induced by acute myocardial infarction and examined function, proliferation, and viability of their leukocytes as well as the local expression of TNF-α and Fas system among bone marrow cells.

MATERIALS AND METHODS

Animal preparation. The protocol was in accordance with the Norwegian National Committee for Animal Welfare Act, which closely conforms to the Guide for the Care and Use of Laboratory Animals [DHEW Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205]. We used 5- to 6-wk-old male BALB/c mice. Anesthesia was induced by injections of 0.2 ml (10 mg/ml) propofol (Diprivan, Zeneca, Macclesfield, Cheshire, UK) into the tail vein. After an anterior cervical midline incision was made, a tracheotomy was performed and a 20-gauge cannula was inserted. The animal was then connected to a rodent ventilator (model 874902, B. Braun, Melsungen, Germany) and given a mixture of 2% isoflurane and 98% oxygen. A left thoracotomy was performed in the third intercostal space, and a silk suture was placed directly.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: P. O. Iversen, Institute for Nutrition Research, PO Box 1046 Blindern, 0316 Oslo, Norway (E-mail: poiversen@hotmail.com).
underneath the left auricle in the interventricular groove. Acute myocardial infarction was induced by ligation of the left coronary artery. Myocardial blanching was taken to signify that this artery was occluded. Perioperative mortality was 40%, comparable to that observed in other studies (6). In the sham-operated animals the left coronary artery was not occluded. The lungs were reexpanded by applying positive end-expiratory pressure (15 cmH₂O), and the thoracotomy and skin incision were closed with silk sutures. The animal was then ventilated with 100% oxygen before it was extubated. Postoperatively the animal was given 0.01 ml of the analgesic buprenorphine (0.3 mg/ml sc). Four to six weeks after primary surgery, the animal was anesthetized and a 1.4-French micromanometer-tipped catheter (model SPR-671, Millar Instruments, Houston, TX) was placed within the left ventricle via the left atrium for pressure recordings. Then a smear was made, stained with May-Grünwald-Giemsa, and differential counting was performed. We isolated granulocytes and mononuclear cells after hemolysis with NH₄Cl by density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway). The mononuclear cell suspension was stained with fluorescent monoclonal antibodies (PharMingen, San Diego, CA) directed against either the CD4, CD19, or pan-natural killer (NK) antigens to identify T, B, and NK cells, respectively, using flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). The positive staining of cell populations was identified using the profiles obtained with an irrelevant control antibody to adjust for nonspecific staining.

Bone marrow cells were collected postmortem from the femoral bone as previously described (2). Mononuclear cells were isolated as described above and stained with fluorescent monoclonal antibodies (PharMingen) directed against the lymphocyte antigens listed above and the pan-leukocyte marker CD45, the monocyte/macrophage antigen CD14, and the progenitor cell antigen CD34 and a panel of lineage (Lin) marker antibodies (anti-Gr-1, anti-Thy1.2, anti-Mac-1, anti-CD4, anti-CD8a, anti-TER119) before flow cytometry. T and NK cells were isolated from the bone marrow mononuclear cell suspension by magnetic sorting using the MACS system (Miltenyi Biotech, Bergish Gladbach, Germany).

**Measurement of oxygen consumption rate.** The oxygen consumption rate of blood granulocytes was estimated from the changes in oxygen tension in a cell suspension (21). Briefly, after separation the granulocytes were suspended in Fisher’s medium containing 0.5% bovine albumin and adjusted to pH 7.35 with 10 mmol/l HEPES buffer. One milliliter of the cell suspension (10 million cells) was enclosed in a chamber while being stirred. The oxygen tension was then measured continuously with a polarographic electrode (MSE).

**Assessment of colony formation by hematopoietic progenitor cells.** CD34+ bone marrow cells were isolated from the bone marrow mononuclear cell suspension using the MACS system. To assay for colony (>40 cells) formation, we plated these cells (100,000 cells per plate) in either semi-solid methylcellulose medium and/or liquid medium (MethoCult/MyeloCult; StemCell Technologies, Vancouver, BC, Canada) according to the instructions of the manufacturer. The cells grew for either 10 days (short-term colony formation) or 6–8 wk (long-term colony formation) (9). Subtypes of colonies were identified after supravital staining. In a separate set of experiments we harvested cells from the 10-day colonies and assayed their clonogenic properties by reculturing them for another 10 days. Briefly, cells were harvested from 10–12 different primary colonies, resuspended, and then plated in the semi-solid medium to develop into secondary colonies, as described (17).

**Quantification of apoptosis.** The fraction of apoptotic CD34+ cells was examined with both flow cytometric assessment of hypodiploid DNA stained with propidium iodide (19) and flow cytometric detection of surface expression of phosphatidylserine on apoptotic cells with annexin-V (Apoptosis Detection Kit, R&D Systems, Minneapolis, MN).

**Cytokine measurement, RNase protection assay, and Western blotting.** The concentration of circulating TNF-α was determined in plasma using and ELISA kit according to the instructions of the manufacturer (sensitivity >5 pg/ml; Quantikine, R&D Systems). We determined mRNA for TNF-α, interleukin (IL)-1β, and IL-6 among sorted (MACS system) populations of T, NK, and CD34+ cells using RNase protection assays after extraction of total RNA, as detailed (12). GAPDH-mRNA was used as an internal control. Western blotting with an anti-Fas ligand antibody (PharMingen) was used to detect the Fas ligand protein in these cell populations (8).

**Statistics.** For each mouse, the individual assays were performed in triplicate, and the corresponding median value from each mouse was used to calculate the means and SE for either the mice with heart failure (n = 8) or the sham-operated mice (n = 8). Differences were evaluated with twotailed Wilcoxon’s test for unpaired samples and considered statistically significant for P < 0.05.

**RESULTS**

**Development of congestive heart failure.** Figure 1, bottom, shows fibrosis as well as a pronounced dilatation of the left ventricular myocardium of a mouse studied 6 wk after ligation of the left coronary artery, indicating a large infarcted area. Furthermore, at this time point, lung weight-to-body weight ratio was significantly higher in mice with myocardial infarction compared with the sham-operated mice: 7.9 vs. 6.2 g/100 ml (P < 0.05). Moreover, left ventricular end-diastolic pressure in mice with myocardial infarction and the sham-operated mice averaged 5.3 and 3.2 mmHg (P < 0.05), respectively.

**Hematological changes in mice with heart failure.** The concentrations of blood leukocytes and their subsets in mice with heart failure were not significantly different from those observed in the sham-operated animals (data not shown). The hemoglobin concentration was significantly lower in mice with heart failure compared with sham-operated mice, the hemoglobin concentrations of blood leukocytes and their sub-

**Statistics.** For each mouse, the individual assays were performed in triplicate, and the corresponding median value from each mouse was used to calculate the means and SE for either the mice with heart failure (n = 8) or the sham-operated mice (n = 8). Differences were evaluated with two-tailed Wilcoxon’s test for unpaired samples and considered statistically significant for P < 0.05.

**RESULTS**

**Development of congestive heart failure.** Figure 1, bottom, shows fibrosis as well as a pronounced dilatation of the left ventricular myocardium of a mouse studied 6 wk after ligation of the left coronary artery, indicating a large infarcted area. Furthermore, at this time point, lung weight-to-body weight ratio was significantly higher in mice with myocardial infarction compared with the sham-operated mice: 7.9 vs. 6.2 mg/g (P < 0.05). Moreover, left ventricular end-diastolic pressure in mice with myocardial infarction and the sham-operated mice averaged 5.3 and 3.2 mmHg (P < 0.05), respectively.

**Hematological changes in mice with heart failure.** The concentrations of blood leukocytes and their subsets in mice with heart failure were not significantly different from those observed in the sham-operated animals (data not shown). The hemoglobin concentration was significantly lower in mice with heart failure compared with the sham-operated animals: 10.4 vs. 13.6 g/100 ml (P < 0.05), respectively. Although we could not find any differences in the numbers of mature neutrophils sampled from the bone marrow of mice with heart failure compared with sham-operated mice, the numbers of progenitor cells, detected as either CD34+ cells or Sca-1+/Lin– cells, were markedly reduced in mice with heart failure (Table 1).

We also studied the function of blood granulocytes by measuring their oxygen consumption rate. The function of granulocytes sampled from mice with heart failure was impaired, their oxygen consumption rate being 22.4 compared with 39.6 nmol·1 O₂·1·min⁻¹·g⁻¹ (P < 0.05) in granulocytes from sham-operated mice.
Reduced long-term colony formation by bone marrow progenitor cells of mice with heart failure. To examine the proliferative capacity of early bone marrow progenitor cells, we plated sorted CD34+/H11001 cells in semi-solid medium and assayed their growth as formation of colonies during either a 10-day or a 6- to 8-wk period. There were no significant differences in colony numbers obtained after 10 days between the two animal groups (Table 2). After 6–8 wk of growth, a substantial reduction of all subsets of colonies occurred when mice with heart failure were compared with sham-operated mice (Table 2). In line with this, a lower number of long-term colony initiating cells (i.e., early, noncommitted progenitor cells) was detected in mice with heart failure than in sham-operated mice: 10 vs. 58 per 10⁶ inoculated CD34+ cells (P < 0.05). Also corroborating these findings were the results of the recloning experiments. Here the number of secondary colonies of mice with heart failure was much smaller than those of the sham-operated mice when assayed after another 10 days in methylcellulose culture (Fig. 2).

Apoptosis of bone marrow progenitor cells is enhanced in mice with heart failure. To further study the mechanism underlying the reduced numbers of progenitor cells in mice with heart failure, we examined the apoptotic fraction among sorted bone marrow-derived CD34+ cells using propidium iodide labeling and annexin-V staining. With these two independent assays we could clearly demonstrate increased apoptosis of CD34+ cells collected from mice with heart failure (Fig. 3). We could not detect any significant difference in the percentage of cells stained with either propidium iodide or annexin-V in either mice with heart failure or in the sham-operated mice.

We examined the cytotoxic ability of bone marrow T and NK cells in vitro by coculturing these cells from sham-operated mice or from mice with heart failure with CD34 progenitor cells collected from bone marrow of intact, syngenic mice. Figure 4 shows that T cells (Fig. 4A) and NK cells (Fig. 4B) from mice with heart failure induced apoptosis of CD34 progenitor cells.

### Table 1. Bone marrow cytograms

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cellularity (×10⁶ per femur)</td>
<td>22.0 ± 3.1</td>
<td>21.7 ± 2.3</td>
</tr>
<tr>
<td>CD45</td>
<td>98.0 ± 2.9</td>
<td>97.8 ± 3.2</td>
</tr>
<tr>
<td>CD4</td>
<td>69.2 ± 8.5</td>
<td>71.4 ± 9.9</td>
</tr>
<tr>
<td>CD19</td>
<td>21.7 ± 2.9</td>
<td>19.7 ± 3.5</td>
</tr>
<tr>
<td>Pan-NK</td>
<td>0.21 ± 0.11</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>CD14</td>
<td>2.1 ± 0.6</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>CD34</td>
<td>1.3 ± 0.2</td>
<td>0.55 ± 0.12*</td>
</tr>
<tr>
<td>Sca-1+/Lin−</td>
<td>0.87 ± 0.21</td>
<td>0.34 ± 0.11*</td>
</tr>
</tbody>
</table>

All values, except for total cellularity, are given as percentages of total nucleated cells and expressed as means ± SE, n = 8. *P < 0.05 for mice with heart failure compared with sham-operated mice.

### Table 2. Formation of CFU by bone marrow progenitor cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Heart Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short-term CFUs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocyte</td>
<td>42 ± 5</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Granulocyte/macrophage</td>
<td>20 ± 6</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Macrophage</td>
<td>16 ± 3</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>37 ± 5</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Dendritic</td>
<td>12 ± 3</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>Long-term CFUs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Granulocyte</td>
<td>24 ± 3</td>
<td>11 ± 3*</td>
</tr>
<tr>
<td>Granulocyte/macrophage</td>
<td>13 ± 3</td>
<td>6 ± 3*</td>
</tr>
<tr>
<td>Macrophage</td>
<td>11 ± 2</td>
<td>4 ± 2*</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>21 ± 2</td>
<td>11 ± 3*</td>
</tr>
<tr>
<td>Dendritic</td>
<td>6 ± 3</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 8, ND, not detectable; CFU, colony-forming units. *P < 0.05 for mice with heart failure compared with sham-operated mice.
failure more efficiently induced apoptosis among normal CD34 hematopoietic progenitor cells than lymphocytes from sham-operated mice.

**Increased expression of TNF-α and Fas ligand in bone marrow lymphocytes from mice with heart failure.** The increase in apoptosis of the bone marrow progenitor cells could result from increased TNF-α/Fas pathway locally within the bone marrow. We therefore studied whether the expression of these molecules was increased in bone marrow lymphocytes. Figure 5A shows that, among the mice with heart failure, TNF-α mRNA synthesis was substantially increased in sorted NK and T cells. The relative TNF-α mRNA level in the T cells was significantly higher than in the NK cells in mice with heart failure, whereas no difference was detected in the sham-operated mice. The concentrations of circulating TNF-α were 19.5 and 7.7 pg/ml ($P < 0.05$) in the mice with heart failure and in the sham-operated group, respectively. We could not detect either IL-1β- or IL-6 mRNA in any cell type, and no TNF-α mRNA was observed in CD34+ cells (data not shown). Enhanced levels of the Fas ligand protein were present within NK and T cells of the mice with heart failure (Fig. 5B). Flow cytometric analysis did not reveal any differences in surface-bound Fas ligand among either NK or T cells when mice with heart failure were compared with sham-operated mice (data not shown). This indicates that the increased level of Fas ligand protein (Fig. 5B) was primarily localized within the cytoplasmic compartment. Fas ligand was not detectable within the CD34 population (data not shown), and the expression of Fas receptor on these cells was apparently unchanged when mice with heart failure were compared with sham-operated mice (Fig. 6).

**DISCUSSION**

In this study we demonstrated anemia and impaired function among blood granulocytes as well as reduced numbers and decreased viability among immature bone marrow progenitor cells in mice with congestive heart failure after acute myocardial infarction. In addition to these novel findings, we showed that the TNF-α/Fas death molecules were upregulated in bone marrow-derived lymphocytes of affected animals.

Congestive heart failure after myocardial infarction in the mouse is characterized by left ventricular dila-
In the present study the infarcted area was examined immediately postmortem to ensure that only mice with uniformly sized infarcted hearts were included. Furthermore, mice with myocardial infarction had pleural effusion and pulmonary edema with a consistent increase in lung weight, indicating that these mice had developed a marked congestive heart failure. Moreover, the observed increase in left ventricular end-diastolic pressure in mice with myocardial infarction was in line with other studies (6, 23).

Heart failure is a serious condition that affects the functions of several organs. However, whether the bone marrow activity can be affected has not yet been systematically investigated. In the present study we observed a marked reduction in the number of hematopoietic progenitor cells recorded with three independent methods: 1) flow cytometric assessment of bone marrow cells labeled with specific monoclonal antibodies to enumerate CD34+ or Sca-1+/Lin− cells; 2) proliferation assays with reculturing of colony-forming cells; and 3) hematopoietic progenitor cells cultured for as long as 6–8 wk. Notably, the observed hematopoietic defect must have occurred at an early stage of blood cell formation, because all tested cell lineages were affected. The blood concentrations of leukocytes did not change with the establishment of heart failure. This is probably not the result of an increased differentiation capacity among the progenitor cells, because the number of cells within the individual colonies did not differ between sham-operated mice and mice with heart failure (data not shown). Alternatively, the unchanged blood concentration of leukocytes in mice with heart failure might reflect a changed distribution of circulating and margined leukocytes and/or that most mature leukocytes never leave the bone marrow, as has been shown for neutrophilic granulocytes (16). Possibly, the pools of circulating and margined mature leukocytes suffice as immune defense against minor infections in heart failure, but they may become too small to mount a sufficiently vigorous response to combat a prolonged infection efficiently. In addition, we found a reduced oxygen consumption rate among blood granulocytes of mice with heart failure, indicating an impaired ability to form, for example, reactive oxygen compounds necessary for mounting an adequate inflammatory response.

With the use of two independent markers of apoptosis using flow cytometry, we consistently demonstrated an increased apoptotic rate in situ among bone marrow progenitor cells from mice with heart failure. This finding was confirmed with purified effector lymphocytes and CD34 cells in vitro. Our findings suggest that...
the hematopoietic activity of the bone marrow is markedly suppressed in heart failure, possibly due to increased activity of the TNF-α/Fas death machinery in T and NK cells locally in the bone marrow. How the TNF-α/Fas system may interact in this context is unknown. Our data, showing an upregulation of both cytoplasmic Fas ligand and TNF-α in lymphocytes and unaltered expression levels in CD34+ cells, suggest an increased activation of bone marrow-derived lymphocytes and not necessarily any change in the progenitor cell population itself. The discrepancy between cytoplasmic and surface levels of Fas remains to be fully explained. Possibly the increased cytoplasmic level reflects increased synthesis of Fas ligand molecules that can be secreted into the bone marrow microenvironment to reach the target cells. Presently it is not possible to measure local interstitial concentrations of Fas ligand in situ in the bone marrow.

How TNF-α exerts its effects on cardiomyocytes and hematopoietic progenitor cells remains to be fully explained. There is strong evidence that TNF-α is a major inducer of the Fas/Fas ligand known to inhibit hematopoietic activity in various settings (4, 15, 18, 25), and recent data also indicate that the TNF-α/Fas pathway can be activated in the ischemic myocardium (10, 28). Others have suggested that the negative inotropic effects of TNF-α might be due to activation of the sphingomyelinase pathway (22). Whether this inhibitory mechanism operates within the hematopoietic system during increased TNF-α activity is, however, not known. Moreover, TNF-α reportedly acts as a repressor of cardiomyocyte function via blunting of adrenergic stimulation (7). In line with this, murine bone marrow progenitor cells reportedly express adrenergic receptors, indicating a modulating role of catecholamines in hematopoiesis (1). To this end one cannot exclude the possibility that the activation of the autonomous nervous system that accompanies the failing heart could repress hematopoietic activity. Although the bone marrow innervation apparently plays no regulatory role during baseline hematopoiesis in intact animals (2), we found that patients with a complete spinal cord injury had impaired hematopoiesis in decentralized bone marrow where the input from the autonomic nervous system is altered (9).

In addition to TNF-α, the vasoactive cytokine endothelin and the proinflammatory cytokines IL-1β and IL-6 may be involved in the pathogenesis of myocardial dysfunction (20, 26). It is, however, not likely that locally derived IL-1β or IL-6 will have an impact on hematopoiesis in heart failure, because they were not expressed in bone marrow NK and T cells from our mice with heart failure.

Finally, altered local hemodynamics within the bone marrow microenvironment could perturb hematopoiesis. We have, however, been unable to demonstrate any changes in either bone marrow blood flow, interstitial fluid pressure, or cellular oxygenation in rats with a similar postinfarction congestive heart failure (unpublished observations).

The present study showing a depressed hematopoietic activity during the development of heart failure might help explain why patients with heart failure are predisposed to infections and anemia.

Perspectives

The heart failure syndrome represents a serious disorder that affects the function of several vital organs, such as the heart itself and the kidneys. The present investigation using an in vivo model indicates that heart failure also affects bone marrow function. How this dysregulation comes about remains to be fully established. Our study shows that the TNF-α/Fas pathway is activated in the bone marrow in mice with heart failure. This finding suggests that local production of mediators in the bone marrow induces changes in hematopoiesis in a condition that is considered to be primarily a hemodynamic disorder. It is possible that alterations in the autonomous nervous system or circulating factors will emerge as important links between circulatory disturbances in heart failure and local factors affecting bone marrow function. Further elucidation of the mechanisms by which heart failure influences bone marrow function may allow development of novel therapeutic strategies. If studies in patients with heart failure reveal that the TNF-α/Fas pathway plays an important role in depression of the hematopoietic system observed in this disease (3), novel drugs targeted to interact with this pathway may prove beneficial in these patients.

We thank H. B. Benestad for critical reading of the manuscript. This study was financed by The Throne Holst Foundation, The Norwegian Cancer Society, Anders Jahre’s Fund for the Promotion of Science, and The Norwegian Research Council.

REFERENCES

8. Iversen PO, Hercus TR, Zacharakis B, Woodcock JM, Stomski FC, Kumar S, Nelson BH, Miyajima A, and Lopez AF. The apoptosis-inducing granulocyte-macrophage colony-stimulating factor (GM-CSF) analog E21R functions through specific regions of the heterodimeric GM-CSF receptor, and re-


