Changes in cytokine production and impaired hematopoiesis in patients with anorexia nervosa: the effect of refeeding

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Abstract

The changes in cytokines and hormones involved in hematopoiesis were studied in the serum of 7 girls with anorexia nervosa, 15–24 yr old, on admission and after 5% and 10% weight gain. Hematopoiesis was studied by in-vitro culturing of circulating granulocyte–macrophage colony forming cells and erythroid burst forming cells. Nutritional status was studied by anthropometric measurements and resting energy expenditure. On admission, granulocyte–macrophage colony forming cells and erythroid burst forming cells were significantly lower than in age-matched controls and increased significantly along weight gain. Blood leptin and erythropoietin levels increased significantly with weight gain. TNF-α levels tended to decrease while IL-1β levels were lower than in the controls on admission (p < 0.05) and did not change significantly during weight gain. IL-3, GM-CSF and IL-6 were undetected on admission or along weight gain. The changes in granulocyte–macrophage colony forming cells and erythroid burst forming cells positively correlated with changes in resting energy expenditure and fat free mass. These results may suggest that undernutrition affects hematopoiesis as indicated by the reduction of hematopoietic progenitor cells before treatment and the significant increase with weight gain. The changes in the levels of hormones and cytokines known to be involved in hematopoiesis along refeeding may suggest a role for these factors in anorexia nervosa.

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1. Introduction

The production of mature blood cells is dependent on a small pool of stem cells with high proliferative capacity, which develop gradually from pluripotent to unipotent committed progenitor cells [1]. This development depends on interactions with specific hematopoietic growth factors which regulate proliferation, differentiation and cellular functions [2]. Our present knowledge sustains the notion that IL-3, and to a lesser extent granulocyte–macrophage colony stimulating factor (GM-CSF), play a major role in multipotent hematopoiesis stem cell survival, proliferation and differentiation, whereas erythropoietin, granulocyte-CSF (G-CSF), macrophage-CSF (M-CSF) and IL-5 affect the proliferation and maturation of their specific lineages into their end stage products. Cytokines such as IL-1β, IL-4 and IL-6 are cofactors in this process [2].

Anorexia nervosa (AN) is an example of undernutrition with no superimposed infection, and as such can serve as a model for studying the different effects of semi-starvation on metabolic paths. Different metabolic disarrangements are involved in AN including changes in glucose availability, reduction in energy metabolism and changes in protein turnover [3–5]. Mild anemia and moderate leukopenia are frequent findings in patients with AN [6–8]. Recently we have shown defective in-vitro granulopoiesis in AN patients, during their active

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disease. This was manifested by lower numbers of granulocyte–macrophage colony forming cells (GM-CFC) and a lower concentration of GM-CSF in the conditioned medium of circulating mononuclear cells of these patients [9]. As anemia is also a feature of severe undernutrition, it is not unreasonable to presume that decreased circulating erythroid burst forming cells (BFU-E) will be found. These cells describe the earliest known erythroid precursor cells that eventually differentiate into erythrocytes and can be assayed in a colony assay in vitro. BFU-E colony formation reflect erythroid bone marrow functionality.

The involvement of cytokines in eating disorders has been studied in the last years and a number of studies indicated that proinflammatory cytokines may play a role [10]. Cytokines as well as different hormones such as leptin and cortisol were considered to play a role in the adaptation processes occurring in these patients [11]. Moreover, several cytokines such as IL-1β and TNF-α were found to regulate neuropeptide expression which may indicate a possible link with the eating habits of these patients [12]. Cytokines affecting hematopoiesis (IL-1β, TNF-α, IL-3 and IL-5) may well be affected by impaired nutritional status and undernourished states [13]. Different hormones, such as leptin [14] and insulin growth factor-1 (IGF-1) [15], were suggested to play a role in hematopoiesis along with erythropoietin [16], and thus may also be affected by undernutrition.

In the present study, assessment of GM-CFC, BFU-E and several cytokines (IL-1β, TNF-α, GM-CSF, IL-3 and IL-6) and hematopoietic growth factors (EPO, leptin, and IGF-1) involved in hematopoiesis, was performed in a group of AN patients, before and during their weight gain phase. These were correlated to the metabolic characteristics of these patients aiming at understanding the link between hematopoiesis, systemic cytokine production and undernutrition.

2. Results

All subjects on admission suffered from undernutrition, indicated by low weight as a percent of ideal weight for height, low body fat percent and low resting energy expenditure (REE) as a percent of REE predicted (REEPP) for their age, weight, height and sex (Table 1) [17]. Along refeeding, all these parameters increased (Table 1). Serum albumin levels were within normal limits on admission and along refeeding. Hemoglobin and total red blood cell counts did not change during refeeding but white blood cell counts increased significantly (p < 0.003) (Table 2). No change was observed in PMN and lymphocyte differential counts during refeeding.

BFU-E and GM-CFC of the AN patients on admission were significantly lower than their age-matched controls (7.00 ± 5.15 vs 34.6 ± 22.3 and 4.38 ± 4.10 vs 15.0 ± 10.7, p < 0.0009 and p < 0.007, respectively) (Figs. 1 and 2). After 5% and 10% weight gain no differences were found between controls and AN patients in GM-CFC (10.75 ± 9.05 and 10.29 ± 4.89, respectively) (Fig. 1) and BFU-E (22.4 ± 11.3 and 18.71 ± 4.57, respectively) (Fig. 2). Erythropoietin serum levels were different from age-matched controls on admission (13.4 ± 2.0 vs 17.7 ± 7.5, p < 0.03) and increased significantly along refeeding (Fig. 3). Serum leptin levels were in the lower range of normal on admission and increased significantly after 5% weight gain and did not change at 10% weight gain (Fig. 3). Mean IGF-1 levels increased along refeeding but did not reach statistical significance (Fig. 3). Mean IL-1β were lower than normal on admission (0.33 ± 0.10 vs 1.38 ± 1.33 pg/mL p < 0.05) and did not change significantly along weight gain (Fig. 4, Table 3). TNF-α levels were not different from the controls on admission (211.42 ± 340.24 vs 21.18 ± 9.19 pg/mL) and tended to decrease along refeeding, but no statistical significant differences were found (Fig. 4, Table 3). IL-3, IL-6 and GM-CSF were not detected in the serum neither on admission nor during refeeding (Table 3).

When correlating hematopoietic parameters with nutritional characteristics and different cytokines’ levels, significant statistical correlations were found between both the changes in BFU-E and GM-CFC and the changes in REE parameters. After 10% weight gain, the change in BFU-E positively correlated with the increase in REE (p < 0.04), REEPP (p < 0.0003) and FFM

### Table 1

Changes in nutritional parameters along refeeding (mean ± 1 SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>On admission</th>
<th>5% Wt gain</th>
<th>10% Wt gain</th>
<th>p &lt;</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (kg)</td>
<td>38.96 ± 6.74</td>
<td>42.17 ± 6.28</td>
<td>44.65 ± 5.34</td>
<td>0.0001</td>
<td>38–67.5</td>
</tr>
<tr>
<td>Wt/ht (%)</td>
<td>77.01 ± 16.22</td>
<td>82.89 ± 10.16</td>
<td>88.58 ± 14.30</td>
<td>0.0001</td>
<td>90–110</td>
</tr>
<tr>
<td>Fat/ht (%)</td>
<td>15.94 ± 7.21</td>
<td>18.96 ± 6.63</td>
<td>23.04 ± 4.77</td>
<td>0.0001</td>
<td>16.0–33.8</td>
</tr>
<tr>
<td>REE (kJ/d)</td>
<td>3092.0 ± 617.34</td>
<td>4058.91 ± 442.79</td>
<td>4212.0 ± 695.97</td>
<td>0.01</td>
<td>3786–6470</td>
</tr>
<tr>
<td>REEPP (%)</td>
<td>–28 ± 8.07</td>
<td>–22.71 ± 6.05</td>
<td>–20.88 ± 10.70</td>
<td>0.037</td>
<td>90–110</td>
</tr>
<tr>
<td>RQ</td>
<td>0.81 ± 0.07</td>
<td>0.91 ± 0.03</td>
<td>0.91 ± 0.03</td>
<td>0.004</td>
<td>0.83–0.87</td>
</tr>
</tbody>
</table>

Comparison between time points by the Wilcoxon signed-rank test. Means denoted by different superscript letters (A, B, C) are significantly different. Wt, weight; Ht, height; Wt/ht %, weight as a percent of ideal weight for height; Fat %, fat as a percent of body weight; REE, resting energy expenditure; REEPP, REE as a percent of predicted; RQ, respiratory quotient.
The increase in GM-CFC correlated with the increase in REE \((p < 0.01)\), REEPP \((p < 0.08)\) and FFM \((p < 0.03)\). The increase in erythropoietin correlated with the increased weight and FFM only at 5% weight increase. The increase in leptin levels correlated with the increase in REE \((p < 0.025)\), weight \((p < 0.04)\) and body fat percent \((p < 0.03)\).

3. Discussion

Our results indicate a significant reduction in the number of both GM-CFC and BFU-E in peripheral blood of patients with AN. Refeeding was accompanied by an increase in both, along with the changes in nutritional parameters. Some of the cytokines and hormones which are involved in hematopoiesis were decreased on admission and tended to increase along with weight gain. The nutritional parameters that best correlated with the increase in GM-CFC and BFU-E were nutritional parameters such as REE parameters and body composition (FFM and body fat percent).

GM-CFC and BFU-E in PB are a certain fraction of the bone marrow population regularly mobilized into the peripheral blood [18]. Decreased numbers of GM-CFC and BFU-E in peripheral blood may thus result from a decrease in their absolute numbers or decreased cell mobilization. Since these circulating early progenitors represent only a fraction of the total progenitors, especially the erythropoietic progenitor cells, their levels do not necessarily correlate with clinically overt anemia or neutropenia [19], as has been shown in our subjects. The decrease in absolute numbers may be due in part to a decrease in cell division or a relative lack of growth factors. Past studies indeed have indicated atrophy of the marrow pool in undernutrition with a proportional reduction in the efflux of cells [20]. Decreased cell division in response to undernutrition was previously reported in the GI tract. The changes included reduced brush border enzyme activity, reduced protein content as well as impaired nutrients’ absorption [21]. By analogy, our results may suggest, therefore, a hematopoietic cell loss in semi-starvation, indicated by a reduction in circulating progenitor cells, alleviated by an improvement in the nutritional status.

What are then the mediators of these changes? Few cytokines and hormones were suggested to inhibit human hematopoiesis. TNF-\(\alpha\), for example, was found to inhibit cell growth [22]. Previously we have shown increased levels of TNF-\(\alpha\) in cell-free PBMC supernatants in patients with AN that tended to decrease with weight gain [23]. In the current study we have found again high levels of TNF-\(\alpha\) before treatment that tended to decrease along weight gain. The decrease did not reach statistical significance, most probably due to a large standard deviation and the relative small number of patients. This may suggest an involvement of TNF-\(\alpha\) in the hematopoietic progenitor cell reduction in patients with AN. IL-1\(\beta\) on the other hand, was very low before treatment and increased along weight gain although not reaching statistical significance. This cytokine is known to play a central role in hematopoiesis and is required for IL-3 and GM-CSF induction [24].

![Fig. 1. Changes in GM-CFC of anorectic patients along weight gain (on admission and after 5% and 10% weight gain). *Significant statistical difference.](image1)

![Fig. 2. Changes in BFU-E of anorectic patients along weight gain (on admission and after 5% and 10% weight gain). *Significant statistical difference.](image2)
Leptin is an adipocyte-derived signalling protein which is involved in energy homeostasis [25]. Recently, leptin was also suggested to interact with hematopoietic cells and induce proliferation, differentiation and functional activation of hematopoietic cells [14]. As expected, leptin levels in our patients tended to increase with weight and body fat gain and thus may also have contributed to the increase in GM-CFC and BFU-E. Leptin has also been shown to have an inverse relationship to TNF-α which was also suggested in our study [25]. Cytokine levels in patients with AN were studied by several groups in the last few years [10]. Corcos et al. did not find any difference in serum levels of IL-1, IL-4, IL-6, IL-10 TNF-α or interferon-γ in anorectic patients as compared to controls, while IL-2 and TGF-β 2 were significantly decreased in anorectic patients [26]. In another study Raymond et al. found increased interferon-γ and a tendency to increased IL-6 serum levels in AN patients [27]. In supernatants of mononuclear cells cultures IL-1β was slightly increased in AN patients while TNF-α was not different from the controls [28]. Nagata et al. found an increased capacity to produce cytokines along with weight gain in patients with AN [29]. In a recent study by Nova et al., in-vitro cytokine production by blood mononuclear cells stimulated by PHA was assessed in 40 adolescent girls with AN [30]. The production of TNF-α and IL-6 was lower than in the controls while IL-1β was higher. Refeeding for 1 month did not reverse these changes. The differences from our results are most probably explained by the fact that our results reflect in-vivo production with no exogenous stimulation and may reflect as well compensatory mechanisms taking place during prolonged disease course [30].

Erythropoietin is the major growth factor of erythropoiesis, and its administration to anemic uremic patients was shown to promote erythropoiesis and thus improve the anemia [31]. In our study, erythropoietin levels tended to increase along with weight and BFU-E. Although both levels are within the normal range for this hormone, this may suggest that erythropoietin levels are affected by the nutritional status. IGF-1 was also suggested to stimulate BFU-E [32]. In our study, although IGF-1 increased considerably along with weight gain, it did not reach statistical significance. This hormone may also play a putative role in the observed increase in GM-CFC and BFU-E of our patients.

The limitation of the current study, apart from the small study population, is the fact that some of our patients did not reach normal nutritional status on discharge and contact was lost thereafter. Their residual impaired nutritional status is indicated by weight/height % < 90% and energy expenditure that did not normalize (REEPP = -20.88 ± 10.70%) after 10% weight gain. Finally, the fact that the levels of different cytokines and hormones changed during refeeding precluded our being able to identify which of them could be responsible for these changes.

In conclusion, we suggest that undernutrition in AN patients affects hematopoietic kinetics of both the erythropoietic and granulopoietic lineages. These changes may be attributed to decreased bone marrow stem cell
production, possibly due to changes in the concentrations of cytokines and hormones involved in hematopoietic growth.

4. Materials and methods

4.1. Patients

Seven adolescent girls, previously diagnosed as suffering from AN were studied before initiating therapy and after 5% and 10% weight gain. All patients fulfilled the DSM-IV criteria for AN [33]. Patients’ age ranged from 15 to 24 yr old, and their history of dieting ranged from 6 to 24 months; patients lost between 10% and 30% of their previous weight. All of the postmenarcheal patients had secondary amenorrhea, which lasted for 4–18 months. Subjects were first admitted to the hospital and followed up later at the out-patient clinic. None of the patients had any infection upon entering the study. No medications were used throughout the study and none of the subjects suffered from viral infection during the study course. Seven age- and sex-matched healthy controls were used for the study. The study was approved by the Human Subject Review Committee of the Kaplan Hospital, and an informed consent was obtained from each patient before the study.

4.2. Collection and preparation of peripheral blood (PB) cells

PB specimens were heparinized with 10 U/mL sodium heparin. PB cells were layered on Ficoll-Hypaque gradients (<1.077 g/mL) (Pharmacia Fine Chemical, Uppsala, Sweden) and centrifuged to recover the low density, GM-CFC and BFU-E enriched mononuclear cell fraction. Mononuclear fractions were separated by Ficoll-Hypaque gradient sedimentation [34,35]. Separated peripheral mononuclear cells (PMN) were washed with PBS, pH 7.2.

4.3. GM-CFC and BFU-E assays

Circulating GM-CFC and BFU-E were cultured in colony assays of hematopoietic cells using methyl cellulose media. Peripheral blood mononuclear cells (PBMC) were plated at a concentration of 5 × 10⁶ cells/mL in Metho-cult GF-H4433 methyl cellulose medium (Stemcells Technologies Inc., Vancouver). Cultures were incubated in a humidified atmosphere with 5% carbon dioxide at 37 °C and colonies containing at least 50 cells were counted in duplicate cultures after 10 days. The means of duplicate cultures never varied by more than 20% of each individual value.

4.4. Cytokine and growth factors assays

Serum levels of the different cytokines were measured by solid phase ELISA. IL-1β, IL-3 and GM-CSF (R&D Systems Inc., Minneapolis, USA) and tumor necrosis factor-α (TNF-α) and IL-6 (DPC, LA, USA). The minimal detectable dose of IL-1β is <0.1 pg/mL, IL-3 <14 pg/mL, GM-CSF <0.4 pg/mL and TNF-α <4 pg/mL. Growth factors were measured as follows: leptin by radioimmuno assay (RIA) (Linco, Missouri, USA), IGF-1 with radioisotopic assay (Nichols Institute Diagnostics, San Juan, California, USA); and EPO with radioimmuno assay (INCSKAR, Skillwater, Minnesota, USA). The minimal detectable dose of leptin is <0.5 ng/mL, IGF-1 <20 nmol/L and EPO <1.5 μU/mL.

4.5. Resting energy expenditure (REE) and body composition

REE was measured by Deltatrac indirect calorimeter (Datex, Instrumentarium OY, Helsinki, Finland) using the ventilated hood technique. After achieving a steady state, REE was measured for 45–60 min. Oxygen consumption, carbon dioxide production and respiratory quotient (RQ) were measured at 1-min intervals after being corrected for temperature, barometric pressure and humidity. Before each test the calorimeter was
calibrated with a reference gas mixture (95% oxygen, 5% carbon dioxide) and ethanol burning was performed in several occasions to assess the precision of the measurements. Four-site skinfold thickness measurements by Harpenden skinfold calipers were used to calculate body fat percentage, fat mass (FM) and free fat mass (FFM) [36].

4.6. Statistical analysis

Non-parametric tests were used for all comparisons due to non-normal distribution of cytokine concentrations. The AN patients were compared to control patients in each time point for every parameter using the Mann–Whitney test. Comparison between repeated measurement in the AN group was carried out using the Friedman rank test with days serving as blocks. Differences over-time were tested by the Wilcoxon paired-difference test. Pearson correlation coefficient was calculated between different cytokines and hormone levels and nutritional parameters. The SPSS for Window version 11.0 was used for statistical analysis. $p < 0.05$ was considered statistically significant.

References


