Autologous Stem Cell Transplantation: Sequential Production of Hematopoietic Cytokines Underlying Granulocyte Recovery


ABSTRACT

We investigated the serum concentrations of a variety of cytokines (granulocyte-macrophage-colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin (IL) 1α, IL-3, IL-6, IL-8, erythropoietin, tumor necrosis factor α, γ-interferon) in 10 patients with advanced ovarian cancer undergoing autologous peripheral blood stem cell (PBSC) harvesting followed by treatment with high-dose cisplatin, etoposide, and carboplatin and PBSCT transplantation (chemotherapy was administered on days 1 through 3, PBSCT on day 6). Preliminary observations on cytokine serum levels were performed for 4 patients; on this basis, the kinetics of cytokines was then investigated in greater detail at closely sequential times in 6 further patients.

We observed a consistent pattern of sequential GM-CSF, G-CSF, and IL-8 release after chemotherapy/PBSCT in all 10 cases, including the 6 patients monitored in detail: (a) at days 5–10 a GM-CSF peak; (b) at days 12–14 a pronounced release of both G-CSF and IL-8, which always preceded granulocyte recovery by ~7 days. At days 17–23, a second GM-CSF peak was monitored in 5 of the 6 patients analyzed in detail, as well as in the other 4 cases. Particularly relevant are the observations that: (a) the peak of G-CSF serum concentration and neutrophil number in the recovery phase are strikingly and directly correlated, thus indicating a key role for G-CSF in granulocyte rescue; (b) the time courses of G-CSF and IL-8 levels are strictly parallel, thereby suggesting a coordinate stimulus for production of granulocytes, mediated by G-CSF, and their activation/migration capacity, mediated by IL-8.

Results were essentially negative for IL-3, tumor necrosis factor α, and γ-interferon concentrations (except in one case for each cytokine). An early peak of IL-1α was observed in all 3 analyzed patients, while an IL-6 peak was monitored at days 13–15 in all 4 patients analyzed in detail.

The present results indicate a sequential coordinate pattern of cytokine release after ablative therapy and PBSCT and shed light on the mechanisms mediating the recovery of granulocytes, and more generally of hematopoiesis, after stem cell transplantation. Furthermore, these studies may contribute to the design of improved protocols for cytokine administration following myelosuppressive anticaner therapy, as well as to the prediction of granulocytic response.

INTRODUCTION

Autologous hematopoietic SCT is widely used to facilitate BM recovery following marrow ablative therapy. In this regard, PB SCT has been indicated for treatment of hematological malignancies as well as for solid tumors (1–4).

Following marrow-ablative chemotherapy, the recovery of hematopoiesis is dependent on stem cell self-renewal and differentiation to lineage-committed progenitors, which undergo further differentiation and then maturation to morphologically recognizable precursors and terminal cells circulating in PB (5).

Both autologous BMT and PBSCT following intensive chemotherapy or chemoradiation are associated with absolute leukopenia before hematopoietic recovery (6–9). Several attempts have been made to alleviate the period of leukopenia by treatment with recombiant HGFs, e.g., G-CSF and GM-CSF; the results indicate a faster engraftment of the transplanted autologous stem cells and a reduction of the period and severity of neutropenia (10–12).

Little is known about the in vivo secretion of endogenous HGFs following high-dose chemotherapy and autologous SCT. Although the kinetics of GM-CSF and G-CSF levels has been investigated after chemotherapy and/or BMT (13–15), a systematic and detailed evaluation of HGFs released following SCT has not been performed thus far. Cairo et al. (16) have recently reported that after allogeneic or autologous BMT a rise of endogenous circulating G-CSF levels precedes and correlates with the myeloid engraftment.

In September 1989, we started a phase II study with high-dose combination chemotherapy and PBSCT in advanced ovarian cancer (17). One of the objectives of this study was to evaluate the kinetics of a variety of HGFs/cytokines (GM-CSF, G-CSF, IL-1α, IL-3, IL-6, IL-8, Ep, TNF-α, IFN-γ) in response to the myelosuppressive regimens. Results indicate a coordinate cascade of early GM-CSF production followed by peak G-CSF and IL-8 release after high-dose chemotherapy and PBSCT.

MATERIALS AND METHODS

Patients. Patients between 28 and 51 years of age, with stage III–IV untreated ovarian cancer (according to the International Federation of Gynecologists and Obstetricians) eligible for chemotherapy were included in the study. Patients with heart, lung, liver, or kidney impairment (creatinine clearance, <60 ml/min) and/or a <80 Karnofsky performance status were excluded. Patients entering this study had histological proof of stage III or IV ovarian carcinoma of grades 1–3 after diagnostic and staging laparotomy and a residual tumor of >0.5 cm after cytoreductive surgery. Written informed consent was provided by each patient, and the study has been approved by the Hospital Human Subjects Investigational Review Board (Catholic University, Rome, Italy). Patients were treated in private rooms and placed on a low bacterial and fungal diet.

Chemotherapy. The chemotherapy regimen and methods for harvesting PBSCT have been described elsewhere (17). Briefly, after surgery patients underwent two courses of induction chemotherapy consisting of cisplatin, 40 mg/m²/day i.v. from days 1 to 5, and cyclophosphamide, 1500 mg/m² i.v. given over 2 h on day 5. After 2–3 weeks from the start of each cycle, when the recovery from transient myelosuppression was observed (platelets >50 × 10⁹/liter, leukocytes >1 × 10⁹/liter), patients underwent PBSCT harvesting by repeated leukopherases.

All cases responsive to induction chemotherapy were eligible for high-dose chemotherapy on days 1–3. Patients were given cisplatin (100 mg/m² i.v.) on day 1, etoposide (600 mg/m² i.v.) on day 2 and carboplatin (1800 mg/m² i.v.) as continuous infusion over 24 h on day 3. Thawed autologous PBSCT were given in 3–4 infusions on day 6.
Table 1 Characteristics of ovarian cancer patients evaluated for serum cytokine concentration following PBSCT

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Histological type</th>
<th>Differentiation grade</th>
<th>Tumor stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>Serous</td>
<td>3</td>
<td>IIIC</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>Serous</td>
<td>3</td>
<td>IIIB</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>Serous</td>
<td>3</td>
<td>IIIB</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>Serous</td>
<td>3</td>
<td>IIIC</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>Endometrioid</td>
<td>3</td>
<td>IIIC</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>Serous</td>
<td>3</td>
<td>IIIC</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>Serous</td>
<td>1</td>
<td>IIIC</td>
</tr>
<tr>
<td>8</td>
<td>44</td>
<td>Serous</td>
<td>3</td>
<td>III</td>
</tr>
<tr>
<td>9</td>
<td>46</td>
<td>Serous</td>
<td>3</td>
<td>IIIC</td>
</tr>
<tr>
<td>10</td>
<td>47</td>
<td>Endometrioid</td>
<td>3</td>
<td>IIIC</td>
</tr>
</tbody>
</table>

Blood counts, including differentials and reticulocytes, were performed each day. Liver and renal functions were monitored three times a week.

Sera. Serum samples were obtained before high-dose chemotherapy and starting from PBSCT (day 6) at sequential times during the first month after chemotherapy. Serum was separated by centrifugation shortly after collection, aliquoted, and stored at -80°C until use. All blood samples used for cytokine evaluation were drawn from patients early in the morning (7-8 a.m.).

Cytokine Assays. Serum concentrations of cytokines/HGFs, including G-CSF, GM-CSF, IL-1α, IL-1β, IL-3, IL-6, IL-8, and TNF-α were evaluated by sensitive and specific immunoassays (British Biotechnology, Cowly, Oxford, United Kingdom). The detection threshold is 5 pg/ml for IL-1α, IL-1β, IL-6, and TNF-α, 10 pg/ml for IL-3, IL-8, and GM-CSF; and 20 pg/ml for G-CSF. Further controls, in addition to those performed by the supplier, were carried out to determine assay specificity (i.e., absence of cross-reactivity of each assay against a large panel of recombinant cytokines/proteins). Each serum cytokine value represents the mean value observed in three separate determinations. The intraassay variability for the various cytokine determinations was 5–10%. The interassay variability of serum cytokine immunoenzymatic analysis was 5–20%, using an identical immunoassay batch for each cytokine.

Table 2 Cell infusion and PB recovery of granulocytes

Data represent mean values. The number of days is counted from the day of PBSCT.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Infused cells (CFU-GM* x 10^4/kg)</th>
<th>WBC &gt;1 x 10^9/liter (days)</th>
<th>PMN &gt;0.5 x 10^9/liter (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.0</td>
<td>+12</td>
<td>+13</td>
</tr>
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<td>2</td>
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</tr>
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<td>10.9</td>
<td>+10</td>
<td>+15</td>
</tr>
<tr>
<td>4</td>
<td>19.0</td>
<td>+12</td>
<td>+11</td>
</tr>
<tr>
<td>5</td>
<td>37.0</td>
<td>+11</td>
<td>+11</td>
</tr>
<tr>
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<td>+11</td>
<td>+14</td>
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<td>+13</td>
</tr>
<tr>
<td>10</td>
<td>26.3</td>
<td>+12</td>
<td>+12</td>
</tr>
</tbody>
</table>

* CFU-GM, colony-forming units, granulocyte-macrophage; PMN, polymorphonuclear leukocytes.

Table 3 Pretreatment and post chemotherapy/PBSCT peak serum levels of GM-CSF, G-CSF, and IL-8 in 10 ovarian cancer patients

Pretreatment value | Postchemotherapy/PBSCT peak value
<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td>GM-CSF (ng/ml)</td>
<td>G-CSF (ng/ml)</td>
</tr>
<tr>
<td>(days 7–9)</td>
<td>(days 12–15)</td>
</tr>
<tr>
<td>GM-CSF (ng/ml)</td>
<td>G-CSF (ng/ml)</td>
</tr>
<tr>
<td>(days 12–15)</td>
<td>(days 12–15)</td>
</tr>
</tbody>
</table>

* These 6 patients were analyzed in greater detail at closely sequential times (see Figs. 1–4 whereby patients 5–10 are shown from top to bottom and from left to right).

Fig. 1. Time course of PB neutrophil number and G-CSF serum level in 6 patients undergoing autologous PBSCT after high-dose chemotherapy (CTH) at the indicated times. These 6 cases (corresponding to patients 5–10 in Table 3, as indicated) were monitored in detail. Similar results were observed in the other 4 cases (patients 1–4 in Table 3).
IFN-γ serum concentration was evaluated using a commercial immunoenzymatic kit with a detection limit of 0.2 unit/ml (Medgenix, Brussels, Belgium). Ep serum concentration was assayed using a sensitive immunoenzymatic method with a detection threshold of 1 milliunit/ml (Amgen Diagnostics, Thousand Oaks, CA). The intra- and interassay coefficients of variation were 2.5–10% and 10–15%, respectively.

RESULTS

Clinical Data. Cytokine serum concentrations were evaluated in 10 patients included in the study. The main clinical and pathological features of these patients are reported in Table I; all met the criteria for inclusion in the present clinical trial. PBSC infusion data and kinetics of PB granulocytes recovery after high-dose chemotherapy and PBSC are detailed in Table 2. Hematopoietic recovery was prompt and sustained in all transplanted patients. Even if neutropenic fever occurred in most patients (average number of days with fever >38°C, 1.6; range, 0–3), in only 1 patient was an infectious episode clinically and microbiologically documented. All 10 patients are alive with normal blood counts with a median follow-up of 25 months (range, 10–30). Three and 7 patients had complete and partial responses, respectively.

In all patients, chemotherapy was given on days 1–3 and PBSC was performed on day 6.

Time Course of G-CSF, IL-8, and GM-CSF Serum Levels. G-CSF, IL-8 and GM-CSF serum concentrations were evaluated in all 10 patients before and after high-dose chemotherapy/PBSCT.

Preliminary observations in 4 patients (patients 1-4 in Tables 1 and 3) showed clear increases in serum GM-CSF, G-CSF, and IL-8 levels at sequential times after high-dose chemotherapy/PBSCT (Table 3).

On the basis of this finding, a detailed sequential analysis of these parameters was carried out in 6 further cases. In these patients we consistently observed a strikingly similar time course of G-CSF serum levels with a small peak at days 4–6 after initiation of chemotherapy followed by a pronounced increase at days 12–15 (Fig. 1).

The results monitored in the other four patients, although less detailed, are consistent with the time course described above: in all cases a peak of G-CSF was observed on days 12–15 (Table 3).

Analysis of the number of circulating neutrophils showed that the major peak of G-CSF constantly preceded neutrophils recovery by ~1 week (Fig. 1). Furthermore, a direct linear correlation (r = 0.92, P < 0.01) was found between the maximum level of G-CSF and the subsequent peak of neutrophils in the first 40 days after chemotherapy (Fig. 2), thus suggesting at least a partial cause-effect relationship between the two phenomena.
In all ten patients IL-8 serum levels showed a sharp rise, which strictly paralleled the second major peak of G-CSF (Fig. 3; Table 3), up to concentrations markedly more elevated (>500 pg/ml) than under physiological conditions (<10-20 pg/ml). IL-8 peak levels, however, did not directly correlate with maximum values of G-CSF concentration or neutrophil response (data not shown).

The time course of GM-CSF serum concentration consistently showed an early peak(s) at days 4–10 (Fig. 4; Table 3), with a late peak at days 17–23 in 5 of the 6 patients analyzed in detail (Fig. 4) as well as in the other 4 cases (data not shown). It is noteworthy that the first GM-CSF peak, although usually moderate (<100 pg/ml), always preceded the major G-CSF peak.

Kinetics of IL-3 and IL-6 Serum Levels. The serum concentrations of IL-3 and IL-6 were evaluated in 6 patients, with a more detailed analysis performed in 4 cases.

IL-3 serum levels remained undetectable in 5 of 6 patients. In one case (patient 8) we observed a small peak (~100 pg/ml) at days 5–7 followed by a late, more pronounced (~1000 pg/ml) peak at days 21–23 (Fig. 5); this patient also showed the highest G-CSF/neutrophils response.

Time course analysis of IL-6 serum concentrations showed clear elevations in the 4 patients analyzed in detail (data not shown). Two peaks were usually detected; a first one at days 13–15, in parallel with the major G-CSF release, followed by a second one at days 20–25 or later.

IL-1α and TNF-α Serum Levels. We analyzed the serum levels of IL-1α and TNF-α, because these cytokines stimulate HGF release by accessory cells, e.g., monocytes, fibroblasts, and endothelial cells (18).

IL-1α serum levels were monitored in detail in only 3 cases (data not shown). All these patients showed increased concentrations at days 3–15, thus suggesting a possible role for IL-1α in the induction of GM-CSF, G-CSF, and IL-8, as indicated above.

TNF-α levels were virtually undetectable in 9 of 10 patients (Fig. 6, data not shown). The only patient exhibiting elevated levels of TNF-α following chemotherapy/PBSCT suffered bacterial infections with pulmonary infiltrates. This finding implies that the increased...
Fig. 6. Time course of IFN-γ and TNF-α serum concentrations in 4 patients (patients 7–10 as in Fig. 1). In the other 6 cases the TNF-α levels were within the normal range (not shown). IFN-γ serum concentrations were not analyzed. CTH, high-dose chemotherapy.

production of HGFs following PBSCT cannot be ascribed to release of TNF-α.

IFN-γ and Ep Serum Concentrations. IFN-γ levels were analyzed in four patients: they were essentially normal with only small fluctuations (see Fig. 6). Since IFN-γ is produced only by activated T-cells, this finding suggests that these cells do not represent the main source of HGFs released during the early phases following PBSCT.

In the ten analyzed patients, Ep levels rose following the decline of hemoglobin after chemotherapy (Fig. 7; data not shown). However, the inverse relationship between hemoglobin decline and the rise in Ep serum levels is not significant (P > 0.05): this suggests that the increased Ep production observed following PBSCT may also be related to a factor(s) other than the decrease in hemoglobin levels.

DISCUSSION

Hematopoiesis is sustained by a pool of stem cells, which can self-renew and differentiate into progenitors (19–21). The progenitors are multipotent (CFU-granulocyte-erythrocyte-macrophage-monocyte) or committed to the erythroid series (burst-forming unit-erythrocyte, CFU-erythrocyte), the granulocytic-monocytic lineage (CFU-granulocyte-macrophage) and the megakaryocytic CFU series (19–21). The progenitors in turn differentiate into morphologically recognizable precursors that mature to terminal elements circulating in PB.

HGFs control the survival, proliferation, and differentiation of stem and/or progenitor cells; in addition, they affect a variety of functional...
Our results indicate a sequential coordinate release of cytokines after chemotherapy (days 1–3) and PBSCT (day 6): (a) at days 4–10 a small GM-CSF peak(s) was consistently monitored, apparently concomitant with and/or followed by a moderate increase of IL-1α; (b) at days 12–14 a pronounced release of both G-CSF and IL-8 was constantly observed; (c) at later times (days 17–23) a small peak of GM-CSF was usually observed.

A crucial observation is represented by the massive G-CSF release, which constantly occurs at days 12–14 after chemotherapy and precedes neutrophils recovery by ~1 week. Furthermore, a striking correlation is observed between the peak values of G-CSF concentration and granulocyte number in the recovery period. It is also noteworthy that only 1 patient of 10 showed a clinically documented infection, correlated with a rise of TNF-α level (see below). These observations clearly suggest that G-CSF plays a key role in granulocyte rescue after chemotherapy and PBSCT. In line with our studies, endogenous G-CSF production correlates with myeloid engraftment after allogeneic or autologous BMT (16).

Interestingly, the time course of IL–8 strictly parallels that of G-CSF. In this regard, G-CSF stimulates neutrophil production by acting on lineage-specific progenitors (23), whereas IL–8 is a chemotactic stimulator and activator of neutrophils (32). The simultaneous induction of the two cytokines, preceding neutrophil recovery, may coordinate stimulate both neutrophil formation, mediated by G-CSF, and their activation/migration capacity, mediated by IL–8. It is of interest that neutrophil migration is defective during recombinant human GM-CSF or G-CSF infusion after autologous BMT (33).

The G-CSF/IL-8 peak is also consistently preceded by a discrete release of GM-CSF. It is hence apparent that the multilineage stimulus of GM-CSF precedes the unilineage proliferative action of G-CSF, thus indicating a sequential coordinate production of cytokines reflecting their hierarchical order. The late GM-CSF peak at days 17–23, observed in 9 of 10 patients (Fig. 4, results not shown), is of uncertain significance. Hypothetically, it may represent a stimulus for compensatory amplification of the progenitor pool following the initial, massive wave of differentiation.

The simultaneous release of G-CSF and IL–8 suggests that they are induced by the same mechanism(s). A candidate mediator is IL-1α, in view of its well-known capacity to elicit the release of both G-CSF and IL–8 by monocytes/macrophages, endothelial cells, and fibroblasts (34–38). This hypothesis is in line with our preliminary observations in 3 patients, who showed a peak of IL–1α production preceding and overlapping the large G-CSF and IL–8 release. However, the peak level of serum IL–1α was relatively low and its significance must be determined in a large number of cases.

In 4 patients analyzed in detail the IL–6 serum level showed variable fluctuations; however, a consistent peak was monitored at days 13–15, i.e., in parallel with the major G-CSF/IL–8 peak.

An increase in serum IL–3 was observed in only 1 of 6 patients after PBSCT. This finding suggests that either (a) IL–3 is not involved in the recovery of hematopoiesis following PBSCT or (b) IL–3 is released in and confined to the BM microenvironment. It has been hypothesized that HGFs exert their main effects within a local regulatory network: in mice, heparan sulfate binds HGFs suggesting a storage function within the BM stromal extracellular matrix (39).

TNF–α levels are usually normal after PBSCT. We observed a significant rise of serum TNF–α in only 1 of 10 patients, clearly correlated with a bacterial infection. This observation is in line with a previous study showing that increased serum levels of TNF–α precede...
major complications of BMT (i.e., interstitial pneumonia or graft-versus-host disease) (40).

In summary, this study shows a sequential coordinate release of HGFs following PBSC. These results shed light on the mechanism(s) involved in the recovery of hematopoiesis following SCT and may contribute to the design of improved protocols for cytokine administration following myelosuppressive anticancer therapy, as well as to the prediction of granulocytic response. However, since our results are based on the analysis of ten patients treated with a specific myeloablative therapy, they will be followed in the future by studies on a larger group of patients treated with various myeloablative protocols; thus far, two additional ovarian cancer patients treated with a modified antibiotic regimen have shown a cytokine (particularly IL-8/G-CSF) plasma profile strictly comparable to that described here (data not shown).

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REFERENCES


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