Hematopoietic Growth Factors Released by Marrow Stromal Cells From Patients With Aplastic Anemia

By Seiji Kojima, Takaharu Matsuyama, and Yoshihisa Kodera

We studied the production of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-6 (IL-6) by stromal cells from 33 patients with aplastic anemia (AA). Complete, confluent stromal layers were produced by 29 of the 33 samples using the long-term bone marrow culture (LTBMC) system. The concentration of G-CSF, GM-CSF, and IL-6 in culture media with or without interleukin-1 (IL-1) stimulation was determined by an enzyme-linked immunosorbent assay (ELISA). The spontaneous production of G-CSF, GM-CSF, and IL-6 did not differ significantly between normal controls and the patients with AA. The ability of stromal cells to release the three hematopoietic growth factors in response to IL-1 was either normal or elevated in all but one patient. We also studied the change in production of G-CSF, GM-CSF, and IL-6 by stromal cells before and after antilymphocyte globulin (ALG) therapy in 16 patients with AA. There was no correlation between the change in production of these cytokines and the response to ALG. In contrast to previous studies that showed a defect in the production of hematopoietic growth factors by stromal cells from patients with AA, the results indicated a normal or elevated production of G-CSF, GM-CSF, and IL-6 by marrow stromal cells in patients with AA.

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MATERIALS AND METHODS

Patients and controls. Informed consent was obtained from 33 consecutive patients with AA admitted for treatment. Clinical and hematologic data on these patients are presented in Table 1. The etiology of the aplasia was known in two patients (cases 19 and 33) in whom aplasia had developed following non-A, non-B hepatitis. Antilymphocyte globulin (ALG) was administered in combination with a bolus of methylprednisolone and danazol. The hematologic response was evaluated 3 months after the initiation of treatment. Those who showed an increase in at least two of the following parameters were considered responders: granulocytes greater than \(1.0 \times 10^{9}/\text{L}\), platelets greater than \(50 \times 10^{9}/\text{L}\), and reticulocytes greater than \(60 \times 10^{9}/\text{L}\). Samples of normal bone marrow were obtained from 12 donors of allogeneic bone marrow transplants, who ranged in age from 2 to 17 years. Informed consent had been obtained from these subjects to use a small aliquot of marrow for this experiment.

Stromal cell culture. Bone marrow specimens were obtained from the posterior iliac crests of the 33 patients and 12 normal controls. Mononuclear cells (MNCs) separated by centrifugation on Ficoll-Hypaque were used to establish LTBMC according to the method described by Gartner and Kaplan, with a slight modification. MNCs were plated at \(10^{7}\) cells/8 mL of a-medium (Flow, Irvine, Scotland) supplemented with 12.5% horse serum (Flow), 12.5% fetal calf serum (Flow), and \(10^{-5}\) mol/L hydrocortisone. The cultures were incubated at 37°C in 5% CO\(_2\)/95% air. They were refed once by exchanging half the supernatant for an equal volume of fresh medium.

Preparation of medium conditioned by stromal cells. After 4 weeks of culture (ie, the time required by marrow cells to form a confluent adherent layer), the adherent cells were detached by exposure to cold trypsin (Flow). The cells were resuspended in 24-well plates (Corning Glass Works, Corning, NY) in a dose of \(10^{6}\) cells/1 mL per well. After 5 days of culture, the confluent monolayers grown in 24-well plates were refed with fresh LTBMC medium (1 mL/well) with or without recombinant IL-1B (Otsuka Pharmaceutical, Tokyo, Japan) at 10 U/mL or 100 U/mL. The supernatants were removed after 48 hours of incubation and stored at \(-80^\circ\text{C}\) until assayed for G-CSF, GM-CSF, and IL-6.

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Assays for G-CSF, GM-CSF, and IL-6. The concentration of G-CSF in each culture supernatant was determined by a commercial enzyme-linked immunosorbent assay (ELISA; Oncogene Science, Manhasset, NY). The lowest level of G-CSF detected by this system was 50 pg/mL. GM-CSF and IL-6 concentrations were also quantified with an ELISA system (Genzyme, Boston, MA), according to the manufacturer’s instructions. The limits of sensitivity of the assay for GM-CSF and IL-6 were 1 pg/mL and 0.16 pg/mL, respectively.

Statistics. The results were analyzed using the Student’s t test and Fisher’s exact test, with P < .05 accepted as statistically significant.

RESULTS

Stroma formation. Within 3 to 4 weeks, all normal bone marrow samples generated a complete confluent layer of adherent cells composed of fibroblast-like cells, adipocytes, macrophages, and endothelial cells. Because complete, confluent stromal layers did not grow in four of the 33 samples from patients with AA within 4 weeks of culture, the following experiments were confined to samples from the remaining 29 patients. The four samples that did not develop complete adherent layers came from patients who suffered from very severe AA (cases 30 to 33) characterized by the total absence of peripheral neutrophils and reticulocytes. After transfer of stromal cells to 24-well plates, the confluent layers were produced within 5 days of culture, which were composed of mainly fibroblast-like cells scattered with adipocytes, macrophages, and endothelial cells.

Production of G-CSF, GM-CSF, and IL-6 by stromal cells. Figure 1 shows the spontaneous and IL-1–induced production of G-CSF by stromal cells in 29 patients with AA. In 12 of the 29 patients, the spontaneous production of G-CSF was below the detectable level. Detectable levels of spontaneous G-CSF production, ranging from 60 to 210 pg/mL (median, 100 pg/mL) were observed in 17 of the 29 patients.

Table 1. Clinical and Hematologic Data on Patients With AA

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Abbreviations: M, male; F, female; S, severe; NS, nonsevere; AS, anabolic steroid; HDMP, high-dose methylprednisolone; CsA, cyclosporine; ALG, antilymphocyte globulin; Hb, hemoglobin; RBC, red blood cell; NA, not available.

*At time of study.
Fig 1. Spontaneous and IL-1β–induced release of G-CSF by stromal cells from 29 patients with AA. The dots represent the individual value of G-CSF in each patient. The mean value is indicated by a horizontal bar. The mean ± SD for 12 normal controls is indicated by a vertical rule between bars.

and did not differ from the levels observed in normal controls. The production of G-CSF was increased by stimulation with 10 U/mL IL-1β both in patients with AA and in normal controls. The mean G-CSF production induced by IL-1β did not differ significantly between the patients with AA and normal controls (mean ± SD, 2,117 ± 2,162 pg/mL and 1,310 ± 1,103 pg/mL, respectively). However, the distribution of G-CSF levels was more scattered in the patients with AA than in the normal controls. In 8 of the 29 samples, the production of G-CSF exceeded the 95% confidence limit of the normal range when samples were stimulated by 10 U/mL IL-1β.

In case 22, the production of G-CSF did not increase in response to IL-1β. This patient also failed to produce GM-CSF and IL-6 following exposure to IL-1β.

The spontaneous and IL-1β–induced production of GM-CSF is shown in Fig 2. Detectable levels of spontaneous GM-CSF production ranging from 1.2 to 8.6 pg/mL (mean ± SD, 2.0 ± 3.5 pg/mL) were observed in 13 of the 29 samples tested. The difference in spontaneous GM-CSF production observed between the normal controls versus patients with AA was not statistically significant. Case 14 showed a marked increase in spontaneous GM-CSF production (8.6 pg/mL) and also produced an exceptionally high level of GM-CSF (250 pg/mL) when stimulated by 10 U/mL IL-1β. IL-1β stimulated increased GM-CSF production in patients with AA, as well as in normal controls. The difference in GM-CSF levels induced by concentrations of 10 U/mL and 100 U/mL IL-1β was not statistically significant (mean ± SD, 16.8 ± 48.7 pg/mL and 19.0 ± 37.1 pg/mL, respectively). The levels of GM-CSF induced by IL-1β were distributed more widely in the patients with AA than in the normal controls. In 10 of the 29 patients, the levels of GM-CSF exceeded the 95% confidence limit of the normal range when stimulated by 10 U/mL IL-1β.

Figure 3 shows the production of IL-6. In normal controls, the spontaneous production of IL-6 was 4.1 ± 3.4 ng/mL. IL-1β increased the production of IL-6 to 33.0 ± 11.5 ng/mL (10 U/mL) and 41.0 ± 15.3 ng/mL (100 U/mL). In patients with AA, a wide range in the spontaneous production of IL-6 was observed: 1.6 to 100 ng/mL. In four of the 29 patients, spontaneous IL-6 production was

Fig 2. Spontaneous and IL-1β–induced release of GM-CSF by stromal cells from 29 patients with AA. The dots represent the individual value of GM-CSF in each patient. The mean values are indicated by horizontal bars. The mean ± SD values for 12 normal controls are indicated by the vertical rules between bars.
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Fig 3. Spontaneous and IL-1β-induced release of IL-6 by stromal cells from 29 patients with AA. The dots represent the individual value of IL-6 in each patient. The mean values are indicated by horizontal bars. The mean ± SD values for 12 normal controls are indicated by the vertical rules between bars.

outside the normal range. Case 14 showed a marked increase in spontaneous IL-6 production (100 ng/mL). Supernatants from the IL-1β-stimulated stromal layers contained significantly higher levels of IL-6 than those from unstimulated layers from patients with AA (P < .05). The levels of IL-6 were widely distributed, ranging from 1.0 to 170 ng/mL and 1.0 to 140 ng/mL when stimulated by 10 U/mL and 100 U/mL IL-1β, respectively. Although the median values did not differ significantly between the normal subjects versus the patients with AA (29.5 ng/mL and 33.0 ng/mL, respectively), five of the 20 patients had levels of IL-6 production that exceeded the 95% confidence limit of the normal range when stimulated by 10 U/mL IL-1β.

Changes in G-CSF, GM-CSF, and IL-6 production following ALG therapy. Repeated studies were performed in the 16 patients with AA. Of the 16 patients, the seven whose peripheral blood counts recovered following ALG therapy, allowing them to become transfusion-independent, were considered responders. The spontaneous production of the three hematopoietic growth factors was not changed significantly before versus after ALG therapy (data not shown). Figure 4 shows the changes in production of G-CSF, GM-CSF, and IL-6 by stromal cells before and after ALG therapy when stimulated by 10 U/mL IL-1β. Eleven of 16 patients showed an increase in G-CSF production posttreatment. Three patients showed a decrease, and two showed no change. The release of G-CSF increased in four of the seven responders and in seven of the nine nonresponders. There was no correlation between the change in G-CSF production and the likelihood of a hematologic response. The GM-CSF production increased in three of the seven responders and in two of the nine nonresponders. Nine of the 16 patients showed a decrease in IL-6 production after ALG therapy. Four patients showed an increase, and three showed no change. IL-6 production decreased in three of the seven responders and in six of the nine nonresponders. Changes in IL-6 production did not correlate with the therapeutic outcome.

DISCUSSION

The pathogenesis of AA remains unknown. Although several studies suggest that microenvironmental defects may play a role in the bone marrow failure, the mechanism is not clear. To evaluate the functional aspect of the stromal microenvironment, we studied 33 patients with AA, using LTBMC. Several investigators have used the LTBMC system to study marrow function in patients with AA.8-12 According to Fugiwara et al,11 only 12 (34%) of 35 patients with AA produced confluent stromal layers with LTBMC. In contrast, we observed the development of a complete, confluent stromal layer in 29 (88%) of 33 patients with AA. Other studies have also found that the majority of patients with AA are able to form confluent stromal layers within 3 to 5 weeks of culture.8,10,12

The marrow stromal layers established with AA cultures exhibited a more rapid decline in the number of nonadherent and committed progenitor cells than did normal controls,8-10,12 implying a defect in the stem cell level or in the capacity of marrow stromal cells to support hematopoiesis. To distinguish them, investigators have used the two-step LTBMC system, in which the survival of the recharged normal granulocyte-macrophage colony-forming unit (CFU-GM) depends on the supportive ability of a stromal layer. Hotta et al8 demonstrated that marrow stromal layers from three of nine AA patients failed to maintain CFU-GM in vitro. Using the same technique, Marsh et al12 failed to show functional defects in the marrow microenvironment of AA patients. In their study, there was no significant difference between AA patients and normal controls in the ability of the stromal cells to support normal hematopoiesis. Most of the patients studied by Hotta et al had severe AA of short duration; however, most of the patients studied by Marsh et al had long-standing AA that was not severe. The discrepancy in these results may be due to differences in the populations studied.
Stromal cell–dependent hematopoiesis has been postulated to result from either a direct contact between stromal and stem cells,\textsuperscript{19} from the trophic effects of the stromal cell–derived extracellular matrix,\textsuperscript{20} or from the secretion of hematopoietic growth factors by stromal cells,\textsuperscript{13–17} such as G-CSF and GM-CSF, and IL-6. G-CSF and GM-CSF play critical roles in hematopoiesis. IL-6 has been found to act synergistically with IL-3 in supporting the formation of multilineage blast-cell colonies.\textsuperscript{21}

We studied the production of G-CSF, GM-CSF, and IL-6 by stromal cells from 29 AA patients. Our study demonstrated that the stromal cells' ability to release these three cytokines in response to IL-1 was either normal or elevated in all but one patient, a 2-year-old girl. The spontaneous production of these cytokines did not differ significantly between normal controls and AA patients. Juneja and Gardner showed that fibroblasts from AA patients have poor myeloid colony-stimulating activity (CSA) defined by their capacity to stimulate the formation of CFU-GM.\textsuperscript{22} In contrast to those investigators, we used the LTBMC system to assess the functional capacity of the stromal layer as an integral unit of many cell types, rather than evaluating the properties of such isolated cellular components as the fibroblast. Migliaccio et al also investigated the production of burst-promoting activity (BPA) and CSA from stromal layers derived from LTBMC in nine patients with AA.\textsuperscript{23} In contrast to our findings, the addition of IL-1 to AA stromal cultures failed to increase the BPA and CSA in the conditioned media. As in the study of Juneja and Gardner, Migliaccio et al used a bioassay to quantify levels of growth factors. The ELISA system we used can identify only a specific growth factor and neglects the influence of other cytokines. It is known that some populations of stromal cells produce not only stimulators, but also inhibitors of hematopoiesis, such as TNF-\(\alpha\) and transforming growth factor-\(\beta\) (TGF-\(\beta\)).\textsuperscript{24–26} The spontaneous and stimulated production of TNF-\(\alpha\) by peripheral blood MNCs was increased in patients with AA.\textsuperscript{27} The presence of such inhibitors to hematopoiesis in a bioassay system would interfere with the quantification of hematopoietic growth factors. The discrepancy between our study and others may be due to differences in patient characteristics or to the methods of quantifying the growth factors evaluated.

The role of ALG has been established in treating AA patients who lack an HLA-identical marrow donor.\textsuperscript{28} While its mechanism of action is still unknown, ALG is believed to restore the depressed hematopoiesis through its immunosuppressive effects. In addition, ALG has been shown to act as a mitogen for peripheral blood MNCs, resulting in the release of hematopoietic growth factors.\textsuperscript{29,30} ALG is not specific for lymphocytes and binds to a variety of cells, including marrow stromal cells.\textsuperscript{31} Therefore, we studied the changes in growth factors produced by stromal cells before and after ALG therapy in 16 patients with AA. We were not able to correlate the changes in cytokine production with the response to ALG therapy.

Although we did not demonstrate a defect in the production of growth factors by the stromal layers from patients with AA, marrow stromal cells exhibit other functions to maintain hematopoiesis. Additional research is required to clarify the role of marrow stromal cells in the pathogenesis of AA.

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Hematopoietic growth factors released by marrow stromal cells from patients with aplastic anemia

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