Aplastic Anemia: Analysis of Stromal Cell Function in Long-Term Marrow Cultures

By Leona A. Holmberg, Kristy Seidel, Wendy Leisenring, and Beverly Torok-Storb

Marrow samples from 89 patients with aplastic anemia (AA) were evaluated for their ability to grow stromal layers in standard long-term marrow cultures (LTMCs). Results were highly variable: 6.8% failed to grow any stromal cells (group I); 42.5% either failed to grow to confluency or appeared to have a decreased number of adipocytes and/or macrophages (group II); and 52.8% appeared as normal confluent cultures with fibroblasts, adipocytes, and macrophages (group III). Analyses of patient data suggested that group I patients had a longer disease duration and poorer survival ($P = .07$). Enzyme-linked immunosorbent assay analysis of cytokine production was performed on 20 of the normal-appearing AA LTMCs and 12 LTMCs established from normal donors. Significant differences between the AA and control groups were apparent for macrophage inflammatory protein-1α (MIP-1α), interleukin-1 receptor antagonist (IL-1ra), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and leukemia-inhibitory factor (LIF). The most dramatic differences observed were elevated levels of MIP-1α and GM-CSF and decreased levels of IL-1ra, particularly after IL-1α stimulation. In contrast, IL-1α stimulation of AA LTMCs produced levels of IL-6, LIF, and G-CSF comparable with those of controls. These data suggest that defects exist within the microenvironment of some AA marrows. Whether the majority of these defects are the cause or consequence of aplasia is not clear. However, we speculate that some of these abnormalities may contribute to the maintenance of the hypoplastic state and, in extreme cases, prevent engraftment of donor marrow.

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HISTORICALLY, aplastic anemia (AA) has been attributed to either defective stem cells or a defective microenvironment (ME). The defects responsible for AA have been reported to be introduced by etiologic agents, such as viruses, toxins, and autoimmunity. The relative ease with which AA can be cured by marrow transplantation and the observation that patients' stroma posttransplant is host in origin has led many to believe that the vast majority of AA cases are caused by damaged stem cells rather than by a compromised ME. However, in addition to the stromal components of the ME, which include fibroblasts, reticular adventitial cells, endothelial cells, and adipocytes, the marrow ME also contains accessory cells such as macrophages, which are transplantable. Because interactions among all of these cells are required to produce a competent ME, defects within the accessory cell population could cause marrow failure in the presence of competent stem cells. Given this premise, it is reasonable to speculate that bone marrow transplantation (BMT) can cure AA caused by certain defects in the ME.

It is our hypothesis that a defective ME can be responsible for maintaining the hypoplastic state seen in some patients with aplasia. To pursue this hypothesis, we have examined stromal cell function in AA as manifested in long-term marrow cultures (LTMCs) established from these patients. Our data indicate that there is considerable variability among stromal cell cultures established from AA patients, ranging from normal cellularity and function to a complete absence of stromal components. Furthermore, even among marrow samples that appear to establish competent stromal layers as determined by phase microscopy, the production of cytokines that modulate hematopoiesis can be significantly different from that produced in cultures from normal donors. The activities most dramatically affected were macrophage inflammatory protein-1α (MIP-1α), interleukin-1 receptor antagonist (IL-1ra), and granulocyte-macrophage colony-stimulating factor (GM-CSF).

MATERIALS AND METHODS

Patients and Controls

After informed consent was obtained (as defined by the Internal Review Board of the Fred Hutchinson Cancer Research Center [FHCRC]), BM was collected from AA patients and normal donors by aspiration from the posterior iliac crest. From 1987 to 1994, BM samples from 89 AA patients and 75 normal donors were evaluated for their ability to grow stromal layers in LTMC. An enzyme-linked immunosorbent assay (ELISA)-based analysis of cytokine production by patient stroma was performed on 20 AA cultures that appeared to have normal LTMC morphology and on 12 concurrent controls.

Establishment of LTMCs

Buffy coat BM cells were exposed to hemolytic buffer for 2 minutes at 37°C, washed, and then plated at 7.5 × 10⁶ cells/ml in Iscove's medium (GIBCO, Grand Island, NY) supplemented with 100 U/ml of penicillin G sodium, 100 µg/ml of streptomycin, 0.4 mg/ml of L-glutamine, 1 mmol/L pyruvate sodium, 10⁶ mol/L hydrocortisone sodium succinate (Sigma, St Louis, MO), 10⁴ mol/L β-mercaptoethanol (Sigma), and 12.5% each of lot-selected batches of heat-inactivated horse serum and fetal calf serum. Cultures were maintained at 37°C in an atmosphere of 5% CO₂ and air. Cultures reached confluency by 3 weeks for normal donors and by 4 to 7 weeks for AA patients.

Incubation of LTMCs in Serum-Deprived Medium, With or Without IL-1α

A total of 20 normal-appearing AA LTMCs plus 12 control LTMCs were evaluated for cytokine production. Once the LTMCs

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reached confluency, they were washed extensively and cultured for 4 days in serum-deprived medium, with or without recombinant human IL-1α (1 ng/ml; Genetics Institute, Cambridge, MA). Serum-deprived medium consists of 0.4 mg/mL of L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.11 mg/mL sodium pyruvate, 50% Iscove’s medium, 49% RPMI 1640 (GIBCO), 10 mmol/L HEPES buffer (GIBCO), and 1% Neutridoma-Hu (Boehringer Mannheim, Indianapolis, IN). Supernatants from the LTMCs were collected, clarified by centrifugation, and stored at −20°C. The supernatants were later evaluated in ELISAs for granulocyte colony-stimulating factor (G-CSF; minimum sensitivity, 7.2 pg/mL), GM-CSF (minimum sensitivity, 1.5 pg/mL), IL-6 (minimum sensitivity, 0.35 pg/mL), leukemia-inhibitory factor (LIF; minimum sensitivity, 2 pg/mL), MIP-1α (minimum sensitivity 2 pg/mL), and IL-1α (minimum sensitivity, 8.7 pg/mL; R&D, Minneapolis, MN). The data are presented as the amount of cytokine detected per milliliter in the LTMC supernatant minus the amount of cytokine detected per milliliter in LTMC media incubated without cells. All experimental samples were evaluated in duplicate wells per assay.

Statistical Methods

Baseline cytokine production refers to the concentration in supernatants from LTMCs without exogenous IL-1α added. We defined an individual’s response to IL-1α stimulation as the difference between the concentration in supernatants from LTMCs with IL-1α added and baseline. For each of the six cytokines, median baseline levels and responses for AA patients were compared with those of a group of 12 normal donors using Wilcoxon tests. We also assessed whether the two groups differed with regard to the variability of baseline production or response using nonparametric squared-ranks tests for unequal variances.

RESULTS

During the course of this study, marrow samples were obtained from 75 normal donors and from 89 AA patients at the time they were admitted for BMT or immunosuppression/growth factor therapy at the FHCRC. Marrow samples were evaluated for their ability to grow stromal layers in LTMCs. All samples obtained from normal donors grew confluent stromal layers containing macrophages, adipocytes, and fibroblasts. In contrast, patient samples were highly variable. Based on this variability, patients were divided into three groups (Table 1). Group I includes six patients whose marrow failed to grow any stromal fibroblastoid cells. Five of these six patients received transplants and failed to establish a normal allograft, and four of these five died before a second BMT could occur. The one surviving individual received a second BMT and remains a chimera with poor marrow function. The last patient failed to respond to immunosuppression/growth factor therapy and died from septic complications before a BM donor could be found.

Group II includes 37 patients whose marrow failed to establish a confluent fibroblastoid layer and/or had deficient numbers of adipocytes or macrophages. Twenty-nine of these patients received a marrow transplant, 20 of whom are long-term survivors. Eight patients in group II received immunosuppressive therapy only, 4 of whom are still alive.

Group III contained 46 patients whose marrow grew normal-appearing LTMCs as determined by inverted-phase microscopy. Thirty-four of the patients received transplants and 26 are long-term survivors. Twelve patients received immunosuppressive therapy only and 10 of these remain alive and well.

As shown in Table 1, the three groups do not differ with regard to severity of disease, patient age, or patient sex. However, there is a suggestion that the duration of disease differs among groups (P = .07; one-way analysis of variance [ANOVA]). Survival was evaluated separately for patients who underwent transplantation and those who did not. After controlling for the type of transplant (HLA-, MM, and URD), the risk of death was found not to be significantly higher in group II when compared with group III (P = .72); however, group I patients may fare worse than group III patients (P = .07). Among nontransplant patients receiving immunosuppression, there was no significant difference between groups II and III, whereas group I had only one such case making statistical analysis impossible.

LTMC-Derived Cytokines

Twenty AA LTMCs that appeared normal by morphology were studied in greater detail to determine their ability to secrete cytokines. Control LTMCs from 12 normal donors were studied concurrently. Replicate LTMCs were exposed to serum-deprived medium or serum-deprived medium to
Cytokines in AA LTMCs

LTMCs from matched unrelated donors. AA LTMCs had significantly higher, and more variable, baseline levels of MIP-1α than did control LTMCs (P < .01, medians; P < .01, variability). After exposure to IL-1α, MIP-1α levels were significantly more variable for AA LTMCs than for controls (P < .01), although we could not show a difference in median response. All 12 of the cultures established from normal donors showed an increase in MIP-1α production after stimulation with IL-1α, but 7 of 20 AA LTMCs decreased production sharply (Fig 1). Another 10 of the AA cultures showed dramatically greater increases than seen among the normal LTMCs.

IL-1ra. LTMCs from normal donors expressed high levels of IL-1ra in serum-deprived medium. Baseline levels of IL-1ra in AA LTMCs were significantly more variable than for controls (P < .01). However, in contrast to MIP-1α, there were both unusually high and low baseline values among the aplastic patients, resulting in no significant differences between the medians of the two groups. After IL-1α stimulation, AA LTMCs had significantly lower responses than did normal donor cultures (P < .01). As shown in Fig 1, 8 of 20 AA LTMCs produced lower levels of IL-1ra after exposure to IL-1α, whereas only 1 of 12 controls did so. Six of these AA LTMCs were from individuals who also displayed decreases in the amount of MIP-1α after IL-1α stimulation.

GM-CSF. LTMCs from normal donors had extremely low baseline levels of GM-CSF. Exposure to IL-1α increased or did not change the expression of GM-CSF in all control LTMCs (Fig 1). AA LTMCs yielded significantly higher and more variable baseline values (P < .01, medians; P < .01, variability). Responses to IL-1α stimulation were more variable for AA cultures (P < .01). Ten AA LTMCs with relatively high baseline levels of GM-CSF decreased production of GM-CSF after IL-1α exposure. Interestingly, 7 of these also expressed decreased MIP-1α after exposure to IL-1α.

We also noted that, of the 3 AA patients whose LTMCs had the highest levels of GM-CSF after IL-1α stimulation, 2 (340 pg/mL and 550 pg/mL) developed graft failure at day 90 and day 104, respectively, after their BMT. The other individual (340 pg/mL) is still aplastic 60 days after initiating treatment with a course of antithymocyte globulin (ATG), methylprednisolone, and oxymetholone.

IL-6. No significant differences between the groups of AA and normal donor LTMCs were found for IL-6.

LIF. The AA group had more variable baseline levels of LIF (P < .01). Median response to IL-1α exposure was significantly lower for AA LTMCs than for controls (P = .02), although none of the AA LTMCs showed responses dramatically outside the normal range.

G-CSF. The AA group had significantly higher baseline levels of G-CSF than did the control group (P < .01). Variability was also greater for AA LTMCs (P < .01). Median response to IL-1α stimulation was significantly less than for the control group (P = .01). However, we did not see dramatic decreases in G-CSF levels among certain aplastics as we did in MIP-1α, IL-1ra, and GM-CSF levels.

Discussion

It has been reported that the LTMCs established from AA patients exhibit a more rapid decline in the production of nonadherent cells or committed hematopoietic progenitors than do normal LTMCs. Because this original observation was compatible with either a defect in the stromal cells or a defect in the hematopoietic progenitors, investigators have
Table 2. Concentrations of Cytokines in LTMCs Without IL-1α Stimulation

<table>
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<tr>
<th>Cytokine</th>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>Lower</th>
<th>Upper</th>
<th>Median Test</th>
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Table 3. Changes in Concentrations of Cytokines in LTMCs With Addition of IL-1α

<table>
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<th>Median Change</th>
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<th>Upper</th>
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<td>.37</td>
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<td>&gt;.5</td>
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patients in this group, data analysis suggests that the poor survival may be independent of transplant type. Therefore, we can hypothesize that graft failure in these few patients may be associated with the failure of compromised stroma to support donor stem cells.

In the current report, data are also provided on supernatants collected from 20 AA LTMCs that grew to confluency and appeared normal by morphology. Results showed that there was no statistical difference in the amount of detectable IL-6 in AA LTMCs before or after stimulation with IL-1α compared with normal LTMCs. However, statistical differences were detectable for G-CSF and LIF, but their differences were not as striking as those detected for GM-CSF, MIP-1α, and IL-1α. All of these cytokines can be produced by fibroblasts and monocytes, and both cell types are major components of the ME. However, because the responses to IL-1α addition were not uniform for each of these cytokines, our data suggest that the production or regulation of these cytokines may involve different components of the ME.

Among AA LTMCs, GM-CSF was secreted at higher levels than in normal LTMCs. This finding may reflect an appropriate response to the hypoplastic state. Regenerating marrow could be expected to produce increased levels of GM-CSF. The failure of most patients with high baseline levels to further augment GM-CSF production after exposure to IL-1α could mean that production had already reached maximum levels. In 2 patients, a large additional increment of GM-CSF was detected after IL-1α exposure, and both patients had problems with long-term engraftment after BMT. Hypothetically, the ability to produce such high amounts of GM-CSF could reflect abnormal feedback mechanisms within the ME that, if not overcome by the transplant, could adversely affect engraftment.

Another striking difference between AA and normal LTMCs was the increased levels of MIP-1α. In 14 of 20 AA LTMCs, baseline MIP-1α levels were greater than those observed among normal donors and, after exposure to IL-1α, 7 of these AA LTMCs further increased MIP-1α production. The remaining 7 actually decreased levels of MIP-1α in response to IL-1α; however, it is difficult to hypothesize which of these is the more appropriate response. In control cultures, 12 of 12 LTMCs increased levels of MIP-1α in response to IL-1α, yet these increased levels were significantly less than the majority detected in AA LTMCs.

Finally, the 20 AA LTMCs studied also had variable baseline levels of IL-1α that became significantly lower than control LTMCs after exposure to IL-1α. Eight patients actually decreased IL-1α levels after exposure to IL-1α, whereas IL-1α levels increased in 11 of 12 normal LTMCs. Taken together, these data suggest that cytokine regulation may be defective even in the AA LTMCs that appear normal. However, it is unclear as to what extent observations made in LTMCs may reflect the in vivo ME. In regard to this issue, recent data obtained from serum samples of 25 AA patients showed that 22 of 25 had levels of IL-1α lower than control sera (X ± SE was 73 ± 63 pg/mL v 174 ± 40 pg/mL). These observations support the validity of the in vitro model and make it reasonable to speculate that a number of individuals with AA may have problems modulating the effect of IL-1α through the generation of IL-1ra. A lower than normal amount of IL-1ra or the inability to increase its production could contribute to an unregulated inflammatory response, resulting in the overproduction of activities capable of suppressing hematopoiesis. Although such a mechanism might not be the primary cause of AA, one could easily hypothesize that the development of such a dysregulated system, after an initial insult to the marrow, would prevent appropriate regeneration.

The reasons for marrow failure in patients with AA are most likely multifactorial. Although many patients can be cured with a BMT, there will be patients who do not have a donor and cannot receive a transplant. These patients may benefit from different forms of therapy that could involve modifying the cytokine regulation of their ME. In such cases, treatment with recombinant IL-1ra or soluble IL-1 receptor may be of benefit. However, those few patients who have no detectable stroma and fail to engraft may require a more novel approach that allows for engraftment of donor stroma. Unfortunately, at the present time, there are no proven ways to transplant normal stroma.

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Aplastic anemia: analysis of stromal cell function in long-term marrow cultures

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