Effects of the immunosuppressants, FK506, deoxyspergualin, and cyclosporine A on immature human hematopoiesis

By Atsushi Hirao, Yoshifumi Kawano, and Yoichi Takaue

Effects of the immunosuppressants, FK506, deoxyspergualin (DSG), and cyclosporine A (CsA) on the growth of human hematopoietic progenitor cells were tested in the presence of interleukin-3 (IL-3) with purified bone marrow and blood cells as targets in methylcellulose culture. FK506 had a significant stimulatory effect on the growth of colony-forming units/granulocyte-macrophage colony-forming units/erythroid colony-forming units (CFU-GM) and burst-forming units/erythroid (BFU-E) from peripheral blood and cord blood cells but not from bone marrow cells. Neither DSG nor CsA had an effect on any type of target cell. Liquid-suspension-limiting dilution assay with IL-3 showed that FK506 directly stimulated the growth of blood progenitors in a dose-dependent manner with single-hit kinetics. Liquid-suspension preincubation of blood cells with FK506 before culture in methylcellulose induced a significant increase in the amount of IL-3-supported growth of CFU-GM and BFU-E, whereas initial preincubation with IL-3 and subsequent culture with FK506 plus IL-3 exerted its stimulatory effect only on BFU-E. These data suggest that the stimulation of hematopoietic progenitor cells by FK506 occurs at a very early stage of maturation and diminishes with further myeloid development.

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Materials and methods

Cell preparation. Peripheral blood mononuclear cells were collected by apheresis from children who were in complete clinical remission from cancer. Procedural risks were explained and informed consent was obtained from the guardians, as previously reported.13 The mononuclear cells were separated by 40%/60% Percoll gradients (Pharmacia, Uppsala, Sweden),14 and purified as described previously.15 Briefly, the cells were washed three times with phosphate-buffered saline (PBS) solution, without Ca2+ or Mg2+, and were resuspended in Dulbecco's modified Eagle's medium (DMEM). They were incubated at 4°C with neuraminidase-treated sheep RBCs to deplete T cells. The T-depleted fraction was plated on plastic dishes and incubated at 37°C with 5% carbon dioxide in a humid atmosphere for 2 hours. Nonadherent cells were recovered and incubated with magnetic polystyrene microspherical particles coated with anti-CD2 and -CD19 antibodies (Dynabeads 450 pan-T and pan-B, Dynal Corp, Oslo, Norway) at 4°C for 40 minutes. Antibody-coated residual T and B cells were then removed by a cobalt samarium magnet (MPC1, Dynal Corp), according to the method of Lea et al.15 Finally, PME solution (5 mmol/L; Sigma, St Louis, MO) was added to the cell suspension to remove remaining monocytes. After 40 minutes of incubation at room temperature, purified progenitor cells were collected by 25%/100% Percoll gradient (700g for 15 minutes) and washed twice with PBS solution.

In some experiments, CD34-positive cells were positively purified and used. Briefly, the purified cells were incubated with anti-CD34 antibody (anti-HPCA-1; Beckton Dickinson, Mountain View, CA) at 4°C. After 30 minutes of incubation, the cell-bound and free microspheres were attached to the tube wall by the magnet. The free cells that did not bind to microspheres were removed by washing the tube three times with Hanks' medium containing 10% fetal bovine serum (FBS). The cells, microspheres, and free microspheres were pipetted for 30 seconds with 1 mL of Hanks' medium containing 125 U/mL chymopapain (Sigma) at 37°C. The tube was attached to the magnet, and the cells that were released from the microspheres were collected by 25%/100% Percoll gradient (700g for 15 minutes) and used.

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were collected. Cord blood cells and bone marrow cells obtained from healthy donors were also purified by the same procedures. The purity of these cells was confirmed by the presence of immunofluorescent CD34-positive cells. Consent forms were obtained from all donors.

**Cytokines and antibodies.** Human recombinant IL-3 (1 x 10^4 U/mg) was provided by Kirin Brewery Company (Tokyo, Japan). Antitumor necrosis factor (TNF) polyclonal antibody from rabbit was a gift from Ajinomoto Company, Inc. All antibodies were used at prescreened concentrations.

Institute of Cell Technology (Tokushima, Japan). Anti-IL-6 antibody polyclonal antibodies against IL-

**In vitro experiments.** Human recombinant IL-3, Human recombinant erythropoietin, and 2,000 U/mL of recombinant IL-3, and the test drugs (FK506, DSG, or CsA) at various concentrations. Triplicate cultures were plated in 0.8 mL volumes in 12-well tissue culture plates (Coming, NY), and placed in an ESPEC N2-02-CO2 BNP-110 incubator (Tabai ESPEC Co, Osaka, Japan), which maintained a humidified atmosphere containing 5% carbon dioxide, 5% oxygen, and 90% nitrogen. Plates were incubated for 13 to 15 days. The mean number of colonies from three wells was calculated and statistical analysis was performed by using the Student’s t-test based on the number of colonies.

**Limiting dilution assay of progenitors.** The limiting dilution assay (LDA) was performed as previously described. Briefly, 2,000 U/mL of IL-3 was added to the purified peripheral blood cell suspension. Cells were mixed well by pipetting and seeded into 60-well microplates at four to five different dilutions (50 to 500 cells/well), completely aspirating the fluid around the 10-μL wells. The plates were incubated under the same conditions used for the methylcellulose method. After 48 hours, the same medium was added gently to prevent transfer of cells across the wells, leaving enough medium inside the plate to prevent evaporation of the contents of the microwells. Seven to 10 days later, using an inverted microscope, colony growth was scored as either positive or negative, based on a threshold number of cells (>40 cells) in the largest cluster in each well. The results were expressed as the log of the percentage of negative wells (y-axis) versus the number of cells plated per well (x-axis). Linear regression analysis was applied to the LDA data to calculate the best slope. Confidence intervals for the slope were calculated by using a variation of the Student’s t-test. The linearity of the data was tested using X^2 analysis.

**Preincubation experiments.** The purified peripheral blood cells were incubated in DMEM containing 10% FBS with or without 0.1 ng/mL FK506 or IL-3 2,000 U/mL for 48 hours. After the cells were washed three times with PBS, hematopoietic progenitor assay in methylcellulose was performed in the presence of IL-3 with or without FK506.

**RESULTS**

**Effects of immunosuppressants on progenitor growth.** The overall cell yields of the combined purification procedures were 5.4% in peripheral blood, 4.5% in cord blood, and 4.0% in bone marrow. The amount of CD34-positive cells in each purified sample was, respectively, 14%, 12%, and 11%. The actual number of colony-forming units/granulocyte-macrophage (CFU-GM) grown from 10^4 cells with IL-3 alone was 74 ± 11 (mean ± SD) for peripheral blood, 69 ± 5 for cord blood, and 45 ± 4 for marrow cells. The number of burst-forming units/erythroid (BFU-E) was 132 ± 23, 103 ± 12, and 92 ± 7, respectively. The addition of FK506 alone (0.1 ng/mL) to methylcellulose cultures of hematopoietic cells did not support the growth of blood progenitors. However, when combined with IL-3, this concentration of FK506 significantly increased the number of CFU-GM and BFU-E grown from peripheral blood and cord blood cells. The addition of diluted ethanol to the culture with IL-3 did not show any effect on colony growth. The results are shown in Fig 1. In 12 experiments with peripheral blood cells as a target, the degree of the increments at a concentration of 0.1 ng/mL FK506 varied between 1.08- and 2.69-fold (mean ± SD, 1.71 ± 0.53) for CFU-GM, and 0.85- and 1.64-fold (1.30
Currently available potent immunosuppressants include CsA, FK506, and DSG. CsA, a fungus-derived cyclic peptide, has contributed significantly to the remarkable progress over the past decade in human organ transplantations. The selective capacity of CsA to inhibit activation of CDC-positive lymphocytes and subsequent cytokine production by these cells has also led to an interest in its potential in the treatment of various autoimmune disorders in which T cells and their products play major pathogenic roles. FK506, a macrolide antibiotic obtained from Streptomyces tsukubaensis, has a powerful and selective anti-T cell effect that is similar to that of CsA. Like CsA, FK506 acts specifically by inhibiting the transcription of a limited set of early T-cell activation genes, including those encoding IL-2, IL-3, IL-4, and IFN-γ. However, these effects can be achieved at lower doses (10- to 100-fold) with FK506 than with CsA, giving FK506 a therapeutic advantage over other immunosuppressants. In organ transplantations, a superior therapeutic ratio resulting in improved clinical results has been reported with FK506. DSG, extracted from culture medium of Bacillus laterosporus, was initially used as an antitumor agent before its immunosuppressive actions were discovered.

### DISCUSSION

Table 1. Effect of FK506 on CD34-Positive Cells From Peripheral Blood

<table>
<thead>
<tr>
<th>Concentration of FK506 (ng/mL)</th>
<th>0</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFU-GM</td>
<td>88 ± 13</td>
<td>97 ± 23</td>
<td>126 ± 44*</td>
<td>188 ± 31*</td>
<td>231 ± 24*</td>
<td>314 ± 26</td>
<td>558 ± 29</td>
</tr>
<tr>
<td>BFU-E</td>
<td>51 ± 7</td>
<td>53 ± 6</td>
<td>70 ± 12*</td>
<td>93 ± 12*</td>
<td>58 ± 4</td>
<td>54 ± 3</td>
<td>50 ± 6</td>
</tr>
</tbody>
</table>

CFU-GM and BFU-E were produced from 10⁶ CD34-positive cells with IL-3. FK506 had a stimulatory effect on the growth of CFU-GM and BFU-E (n = 5; *P < .01).
potency of these drugs has been tested primarily in immunocompetent cells, their hematopoietic effects must also be studied, because patients receiving immunosuppressive therapy are at an increased risk of infection.

The inhibitory effect of CsA on the growth of CFU-GM has been controversial. Some of this confusion may be caused by the use of different target cells containing heterogeneous cell populations, or the use of hematopoietic progenitor cells at various stages of development. Immunosuppressants may also affect contaminating accessory cells, which directly or indirectly interfere with the growth of progenitors. Furthermore, it is obvious that the impact of drugs on immature hematopoiesis cannot be evaluated in target cell populations predominantly composed of mature progenitors. A highly purified immature progenitor cell population is essential for this research.

In this study, target cells purified from the peripheral blood of patients in the recovery phase of chemotherapy and from the cord blood and bone marrow cells of healthy donors were used to compare the effects of various immunosuppressive drugs. Peripheral blood in the recovery phase of chemotherapy contains an increased number of hematopoietic progenitor cells, including transplantable stem cells. Hematopoietic progenitor cells purified from peripheral blood and bone marrow respond differently to various cytokines. Nakahata et al identified hematopoietic colony-forming cells that had an extensive ability to generate progenitors for secondary colonies in human cord blood, but they were unable to identify blast cell colonies in cultured marrow cells. This suggests that the frequency of multipotential progenitor cells in human cord blood is higher than that in bone marrow.

In our experiments using combined purification procedures, the percentages of CD34-positive cells in the three sources of progenitors were quite similar. However, the plating efficiencies of cells derived from peripheral blood and cord blood were higher than that of bone marrow cells. Serke et al reported that the CD34-positive cells from peripheral blood differ substantially from marrow-derived CD34-positive cells in their response to growth factors.

This is the first study to report the enhancing effect of FK506 on immature human hematopoiesis. Although the purified blood cells used in this study were devoid of most accessory cells and the effect was confirmed in cultures with CD34-positive cells, a residual, small number of lymphocytes and monocytes can excrete a trace amount of stimulatory or inhibitory cytokines following stimulation by FK506. However, the stimulatory effect of FK506 was not eliminated by the addition of antibodies against TNF, IL-1α, IL-1β, and IL-6, thereby precluding the possibility that this effect results from the stimulation of accessory cells to produce these cytokines. In addition, LDA showed the direct effect of FK506 on hematopoietic progenitor cells. Kino et al reported that FK506 inhibited the growth of CFU-GM colonies at concentrations exceeding 320 nmol/L in murine marrow cells. We showed that the significant enhancing effect of FK506 becomes prominent with the use of blood-derived progenitors, and that preincubation with 0.1 ng/mL of FK506 induced a further increase in the number of IL-3-supported CFU-GM and BFU-E. IL-3 has been reported to support the proliferation of multipotential hematopoietic progenitors. However, progenitor assays performed with FK506 plus IL-3 in methylcellulose after the cells were preincubated with IL-3 showed that the enhancing effect of FK506 only occurred in the growth of BFU-E, which are more mature. In contrast, CsA or DSG essentially had no effect on colony formation in the culture method used. However, DSG is oxidized rapidly in culture containing FBS and confirmation of these data requires further evaluation under serum-depleted culture conditions.

These data suggest that FK506 stimulates very immature hematopoietic progenitor cells to yield more CFU-GM and BFU-E, but the stimulatory effect will diminish as the myeloid maturation process continues. Because current attempts to determine distinct maturation steps in erythroid progenitors are limited, it is not yet clear whether FK506 has a preferential effect on mature progenitors in erythroid development. FK506 preferentially stimulates blood progenitor cells. There is a possibility that FK506 has an advantage over other im-
munosuppressants for use in the clinical management of compromised patients with hematologic disorders, such as those undergoing high-dose chemotherapy treatment and transplantation with allogenic blood stem cells or cord blood cells.

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REFERENCES


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