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Original Article

G-CSF does not Stimulate Leukemic Stem Cells *in Vivo*.

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The effect of G-CSF on leukemic stem cells, which can support the long-term proliferation of leukemic cells *in vivo*, needs to be clarified to confirm the long-term safety of clinical use of G-CSF. When we examined the effect of G-CSF on leukemic stem cells in NOD/SCID mice using a transplantation assay, the engraftment of leukemic cells derived from 8 AML cases was neither supported by G-CSF alone, nor enhanced by G-CSF plus GM-CSF, IL-3 and SCF. These findings suggest that G-CSF was not able to stimulate leukemic stem cells into maintaining the long-term proliferation of leukemic cells *in vivo*.

Key words G-CSF, AML, leukemic stem cell, NOD/SCID mouse

INTRODUCTION

We have reported previously that granulocyte colony-stimulating factor (G-CSF) stimulated normal hematopoietic progenitor cells and also leukemic progenitor cells from patients with acute myelogenous leukemia (AML) into forming leukemic colonies *in vitro*[1-3]. However, the effect of G-CSF on more primitive leukemic progenitor cells (leukemic stem cells), which can support the long-term proliferation of leukemic cells *in vivo*, has not been sufficiently evaluated. Because G-CSF has been used frequently to treat AML patients, the effect of G-CSF on leukemic stem cells should be clarified to confirm the long-term safety of clinical use of G-CSF. Recently, it has been possible to characterize human leukemic stem cells using a transplantation assay in severe combined immunodeficient (SCID) or nonobase diabetic/severe combined immunodeficient (NOD/SCID) mice[4-6]. Human granulocyte/macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and stem cell factor (SCF) were reported to stimulate leukemic stem cells into maintaining the long-term proliferation of leukemic cells in immunodeficient mice. In the present study, therefore, we examined the effect of G-CSF on leukemic stem cells using this transplantation assay.

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MATERIALS AND METHODS

Leukemic blast cells.

Peripheral blood specimens from 8 different AML patients with high leukemic blood cell counts were sampled after obtaining informed consent at the time of diagnosis. The patients were classified according to French-American-British (FAB) subtypes as AML-M1 (n=2), M2 (n=4), M4 (n=1) and M5 (n=1). Mononuclear cells were obtained by density gradient centrifugation. All of the samples contained at least 95% leukemic cells by morphology.

Transplantation of leukemic blast cells into NOD/SCID mice.

NOD/SCID mice were bred and maintained in sterile microisolator cages at the Kyushu University Animal Center. This experiment was

reviewed by the Committee on the Ethics of Animal Experiments in the Graduate School of Medical Science, Kyushu University. It was carried out under the control of the Guidelines for Animal Experiments in the Graduate School of Medical Science, Kyushu University.

Twenty-four hours before transplantation, 6- to 8-week-old mice were irradiated at 350 cGy. Each sample, containing 2×10^6 leukemic cells in 0.2 ml alpha medium (Gibco, Grand Island, NY) with 5% fetal calf serum (FCS; Flow Laboratories, McLean, VA), was injected intravenously into the lateral tail vein of the mice. Mice were given intraperitoneal injections of 20 μl anti-asialo GM1 (Wako Pure Chemical Industries, Ltd.) once a week and were killed at 8 weeks post-transplantation. Bone marrow was removed from the femoral bones by flushing with alpha medium containing 5% FCS. The human cytokines administered to mice were diluted in sterile phosphate-buffered saline (PBS) with 0.1% bovine serum albumin and injected intraperitoneally three times a week.

Cytokines

Human recombinant G-CSF, GM-CSF, IL-3 and SCF were provided by Kirin Brewery Co., Ltd. (Tokyo, Japan).

Flow cytometry analysis

Cell suspensions from femoral bone marrow were incubated on ice with ammonium chloride for 20 minutes, to lyse red blood cells, and then washed in PBS with 5% human serum. Cells were stained with fluoresceinated anti-CD45, a human pan leukocyte antibody (Becton Dickinson) for 30 min on ice. Separate aliquots were stained with fluoresceinated mouse IgG1 (Becton Dickinson) as an isotype control. The stained cells were then analyzed using a FACScan (Becton Dickinson). Cells were defined as positive using a gate setting that excluded >99% of cells in the matched isotype control.

RESULTS

The effects of human cytokines on leukemic stem cells from 8 AML patients were studied using the transplantation assay in NOD/SCID mice. As shown in Fig. 1, no long-term proliferation of leukemic cells from any of the patients was observed in the NOD/SCID mice without the injection of human cytokines. While the injection of 10 μg of human G-CSF did not support the long-term proliferation of leukemic cells, the simultaneous injection of three human cytokines (10 μg GM-CSF, 10 μg IL-3 and 10 μg SCF) was able to stimulate the long-term proliferation of leukemic cells from four of the 8 patients in the NOD/SCID mice ($p<0.05$, Friedman test). The level of engraftment of leukemic cells in NOD/SCID mice varied (5 -to 30%) among these four cases, which were, respectively, diagnosed as M5b, M4, M1 and M2, according to FAB subtype. The morphology of the engrafted leukemic cells was typical of the AML subtype. When we injected 10 μg human G-CSF in addition to the three human cytokines (10 μg GM-CSF, 10 μg IL-3 and 10 μg SCF) into the NOD/SCID mice, the level of engraftment of the leukemic cells was not enhanced in any of the AML cases. The morphologic differentiation of engrafted leukemic cells was not stimulated by the addition of G-CSF to the three cytokines.

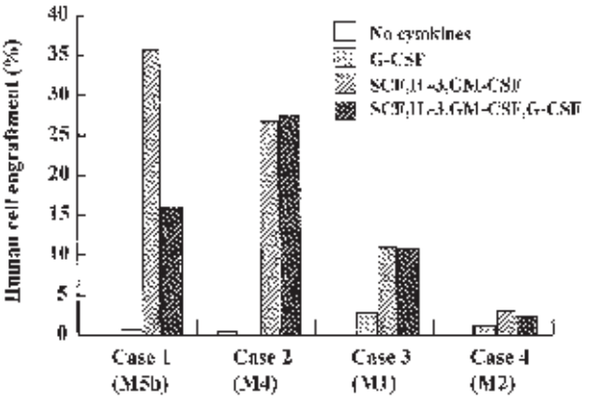

Fig. 1. Engraftment of AML cells in NOD/SCID mice.
The percentage of $CD45^+$ cells in bone marrow nuclear cells was determined at 8 weeks post-transplantation or when the mice began to exhibit clinical signs of the disease. When human cytokines were administered to mice, 10 μg G-CSF alone, or three cytokines (10 μg SCF, 10 μg IL-3 and 10 μg GM-CSF) either with or without 10 μg G-CSF were injected, intraperitoneally, three times a week.

DISCUSSION

Although AML is a clonal disorder resulting from leukemic transformation in a primitive hematopoietic progenitor cell, leukemic cells

from the malignant clone undergo slight differentiation, creating a hierarchy of cell populations that differ in their potential for self-renewal and proliferation. Previously, we reported that G-CSF could stimulate some populations of leukemic progenitor cells into forming leukemic colonies in semisolid culture medium[1-3]. Transient proliferation of leukemic cells is sometimes observed when G-CSF is administered to AML patients. This clinical experience also supports the in vitro effect of G-CSF on certain populations of leukemic progenitor cells to form leukemic colonies. However, the long-term proliferation of leukemic cells in AML patients is maintained by a more primitive population of leukemic progenitor cells (leukemic stem cells), that possess extensive proliferative capacity and the potential for self-renewal. Therefore, the effect of G-CSF on leukemic stem cells should be examined to evaluate the long-term influence of administration of G-CSF on AML patients.

Recently, it has been possible to characterize human leukemic stem cells using a transplantation assay in SCID or NOD/SCID mice[4-6]. Repeated, simultaneous injections of human GM-CSF, IL-3 and SCF were reported to be necessary to stimulate leukemic stem cells to achieve high levels of engraftment of leukemic cells in immunodeficient mice. In this study, we showed also that these three human cytokines could support the long-term proliferation of leukemic cells from 4 of 8 AML patients in NOD/SCID mice. Because the effect of G-CSF on leukemic stem cells has not been clarified, we injected human G-CSF alone or simultaneously with three human cytokines (GM-CSF, IL-3 and SCF) into NOD/SCID mice. The engraftment of leukemic cells from any of the cases was neither supported by G-CSF alone, nor enhanced by G-CSF plus the three cytokines. These findings suggest that G-CSF was not able to stimulate the leukemic stem cells into maintaining the long-term proliferation of leukemic cells *in vivo*.

Some clinical groups have conducted randomized studies to determine the efficacy and safety of G-CSF after intensive chemotherapy in AML patients[7,8]. They have reported that the use of G-CSF led to fewer documented infections, without increasing the risk of leukemic relapse. Our data using NOD/SCID mice seems to further confirm this clinical evidence.

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