Cell Cycle-Dependent Priming Action of Granulocyte Colony-Stimulating Factor (G-CSF) Enhances in vitro Apoptosis Induction by Cytarabine and Etoposide in Leukemia Cell Lines

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We investigated the priming effect and mechanism of granulocyte colony-stimulating factor (G-CSF) in chemotherapy with low-dose Ara-C and VP-16 for acute myeloid leukemia. We analyzed cell proliferation, apoptosis, and cell cycle in vitro using leukemia cell lines 32Dcl3, U937, HL-60, and Ba/F3. Cell proliferation assays were performed using the Trypan Blue dye exclusion method. For detection of apoptosis, the Annexin V-binding capacity of treated cells was examined by flow cytometry. To evaluate the cell cycle, we used an FITC BrdU Flow kit and conducted analysis by flow cytometry. The combination of Ara-C and VP-16 significantly enhanced the observed effects compared with those of Ara-C or VP-16 alone. Concurrent administration of G-CSF further reduced the cell number and viability of 32Dcl3 and U937 cells, but not of HL-60 and Ba/F3 cells. Apoptotic cells were significantly increased in number by the addition of G-CSF to 32Dcl3 and U937 cells, while G-CSF had no significant effect on HL-60 and Ba/F3 cell lines. The addition of G-CSF significantly decreased the percentage of G0/G1-phase cells and significantly increased that of S-phase cells among 32Dcl3 and U937 cells. No significant effect was observed for HL-60 and Ba/F3 cells. An enhancement was confirmed for the combination of Ara-C, VP-16, and G-CSF for 32Dcl3 and U937 cells but not for HL-60 and Ba/F3 cells. It was thought that this difference was a result of different responses to G-CSF. G-CSF potentiates Ara-C- and VP-16-induced cytotoxicities through apoptosis induction by mobilizing resting G0-G1-phase cells into S phase.

Keywords: apoptosis, cell cycle, leukemia cell line, low-dose cytarabine (Ara-C) and etoposide (VP-16) and granulocyte colony-stimulating factor (G-CSF) therapy (AVG therapy)

INTRODUCTION

We recently reported the favorable clinical effects of combination chemotherapy with low-dose cytarabine (Ara-C) and etoposide (VP-16) (AV therapy) and low-dose Ara-C, VP-16, and granulocyte colony-stimulating factor (G-CSF) (AVG therapy) in elderly patients with acute myeloid leukemia (AML) who were ineligible for intensive chemotherapy. The latter regimen consisted of continuous drip infusion of low-dose Ara-C (20 mg/body) and VP-16 (50 mg/body) with concurrent administration of G-CSF (150 mg/m²). The aim of this study is to elucidate in vitro the mechanism of the priming effect of G-CSF in AVG chemotherapy for AML.

G-CSF has already been reported to potentiate in vitro the anti-leukemic effect of Ara-C or VP-16 alone. The mechanism of such action is assumed to involve recruitment of quiescent G0 leukemic cells into the cell cycle. We hypothesized that the higher cytotoxicity of AVG was due to the enhancement of apoptosis by G-CSF, and this priming effect of G-CSF depended on the cell cycle. We analyzed the effect of AVG on proliferation, apoptosis, and cell cycle in AML cell lines with or without G-CSF receptor expression.
MATERIALS AND METHODS

Materials

Ara-C was purchased from Sigma Chemical Co. (St. Louis, MO). VP-16 was provided by Bristol-Myers Japan (Tokyo, Japan). G-CSF was provided by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). RPMI-1640 medium, α-minimal essential medium, fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine were purchased from Gibco BRL Life Technologies Inc. (Grand Island, NY). Sodium pyruvate was purchased from Sigma Chemical Co. (St. Louis, MO).

Cell lines and cell culture

32Dcl3 [interleukin (IL)-3-dependent cells from Friend leukemia virus-infected mouse bone marrow], U937 (monocytic leukemia cells), and HL-60 (human promyelocytic cells) were obtained from RIKEN Bioresource Center Cell Bank (Ibaraki, Japan). Ba/F3 IL-3-dependent pro B cell line was kindly provided by Dr. Akihiro Kume, Jichi Medical School, Tochigi, Japan. HL-60 and U937 were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. 32Dcl3 and Ba/F3 were maintained in RPMI-1640 medium supplemented with 10% FCS, 10 U/mL recombinant mouse IL-3, 100 U/mL penicillin, and 100 μg/mL streptomycin. These cells were cultured in an incubator containing humidified air with 5% CO₂ at 37°C.

Cell proliferation assays

HL60, U937, 32Dcl3, and Ba/F3 cells (5 × 10⁵ cells/mL) in conditioning medium were cultured with various concentrations of Ara-C (10⁻⁵-10⁻⁸ M) and/or VP-16 (10⁻⁵-10⁻⁸ M) in the presence or absence of G-CSF (50 ng/mL) for 72 hrs. Cell proliferation assay and viability examination were performed using the Trypan Blue dye exclusion method. All experiments were performed in triplicate.

Apoptosis assays

For detection of apoptosis, the Annexin V-binding capacity of treated cells was examined by flow cytometry using the ANNEXIN V-FITC APOPTOSIS DETECTION KIT I purchased from BD Pharmingen™ (San Jose, CA) according to the manufacturer’s instructions. 32Dcl3, HL-60, and Ba/F3 cells (5 × 10⁵ cells/mL) were treated with Ara-C (10⁻⁷ M) and VP-16 (10⁻⁷ M) in the presence or absence of G-CSF (50 ng/mL) for 72 hrs. Cultured cells were then labeled with BrdU for 45 min, washed, and fixed and permeabilized with BD Cytofix/Cytoperm Buffer. After repeated incubation on ice (30 min, 10 min, and 5 min), washes, and centrifugation, cells were treated with DNase to expose BrdU epitope for 1 hr at 37°C, washed, then stained with fluorescent anti-BrdU for 20 min at room temperature, washed again, and centrifuged. Staining buffer containing 7-amino-actinomycin D (7-AAD; 1 mL; BD Pharmingen™) was added to each tube to resuspend the cells, and the cells were analyzed by flow cytometry using FACSscan (Becton Dickinson, San Jose, CA). Acquired multiparameter data were analyzed using CellQuest software. With the combination of BrdU and 7-AAD, two-color flow cytometric analysis permits the enumeration and characterization of cells that are actively synthesizing DNA (BrdU incorporation) in terms of their cell cycle position (i.e., G0/1, S, or G2/M phases defined by 7-AAD staining intensities). As shown by the region gates applied to the 7-AAD versus BrdU dot plot (see Fig. 4A), flow cytometric analysis of cells stained with the reagents allowed the discrimination of cell subsets that were in the S phase (R1), G0/G1 phase (R2), G2/M phase (R3), and apoptotic (sub-G0/G1) (R4).

Statistical analysis

Statistical analyses of the cell proliferation assay, apoptosis assay, and cell cycle assay were performed using Student’s t-test, Scheffe’s t-test, or repeated-measure ANOVA. Statistical significance was declared when p < 0.05. All analyses were performed using Statview® program version 5.0 (SAS Institute Inc., Cary, NC).
RESULTS

Proliferation assay

We first examined the in vitro anti-leukemic effects of Ara-C and VP-16, and confirmed that both Ara-C and VP-16 dose-dependently inhibited proliferation and viability of each leukemic cell line (Fig. 1A, B). The combination of Ara-C and VP-16 (AV) significantly enhanced the observed effect compared with those of Ara-C or VP-16 alone (Fig. 1C). Concurrent administration of G-CSF further reduced the cell number for 32Dcl3 (Fig. 1C, 2A) and U937 cells (Fig. 2B) (p < 0.05), but not for HL-60 (Fig. 2C) and Ba/F3 cells (Fig. 2D).

Apoptosis assay

We next examined whether Ara-C- and VP-16-induced apoptosis in leukemic cell lines was affected by the concurrent use of G-CSF. As shown in Fig. 3, the percentage of apoptotic cells was significantly increased to 20.7 ± 0.58% from 13.0 ± 0.38% by the addition of G-CSF to 32Dcl3 cells (p < 0.05) (Fig. 3A) and increased to 63.5 ± 4.04% from 54.1 ± 3.29% by the addition of G-CSF to U937 cells (p < 0.05) (Fig. 3B), while G-CSF had no significant effect on the HL-60 (Fig. 3C) and Ba/F3 cell lines (Fig. 3D).

Cell-cycle analysis

We analyzed the cell cycle using BrdU and 7-AAD to confirm whether promotion of apoptosis by G-CSF was related to cell cycle progression in 32Dcl3 cells and U937 cells. As shown in Fig. 4, the addition of G-CSF significantly decreased the percentage of G0/G1-phase cells (to 36.6 ± 3.51% from 44.8 ± 1.01% ; 32Dcl3 cells : Fig. 4B-1) (to 26.6 ± 2.05% from 42.7 ± 2.98% ; U937 cells : Fig. 4C-1) (p < 0.05 for both) and significantly increased that of S-phase cells (to 30.3 ± 0.88% from 24.3 ± 0.52% ; 32Dcl3 cells : Fig. 4B-2) (to 42.1 ± 0.94% from 35.2 ± 1.14% ; U937 cells ; Fig. 4C-2) (p < 0.05 for both). No significant effect was observed for HL-60 and Ba/F3 cells.

DISCUSSION

Ara-C is the major agent employed in the treatment of AML. The cytotoxic action of Ara-C is S-phase-specific, and thus depends on the growth characteristics of the target cells as well as the pharmacokinetics of the drug itself. It has been reported that the combination of Ara-C and G-CSF enhances myeloid differentiation in leukemic cells in vitro.14 Priming of myeloid leukemic cells with G-CSF in vivo has been reported to enhance the apoptosis induced by low-dose Ara-C.15 The anti-tumor effects of VP-16 are based on its ability to stimulate DNA cleavage by a DNA topoisomerase ; thus,
VP-16 principally acts on the G2 and S phases of the cell cycle. G-CSF has the ability to reintroduce resting-phase leukemic cells into the cell cycle. Therefore, we suggested and proved the utility of the AVG regimen in AML patients.

A low-dose Ara-C regimen is currently regarded as the therapy of choice for advanced myelodysplastic syndrome and AML. Combination therapies of low-dose Ara-C and other cytotoxic drugs (for instance AV therapy) have been found to be favorable. The combination of hematopoietic growth factors with cytotoxic agents has been investigated as an additional therapeutic option in AML patients. Yamada et al. reported the CAG regimen, which consisted of low-dose Ara-C, aclacinomycin, and G-CSF, as salvage chemotherapy for relapsed AML patients, and obtained a good complete response rate with low toxicity and improved quality of life. They reported that priming with G-CSF en-
enhanced the sensitivity of AML cells for Ara-C, presumably by recruiting leukemic cells into the cell cycle; however, this explanation has not yet been universally accepted. There have been some negative reports about the priming effect of G-CSF in past clinical studies.22-28

We first showed in the proliferation assay that AV therapy was more effective than Ara-C or VP-16 alone for 32Dcl3 cells (Fig. 1A, B). There was an additive effect (CI = 1.0), and this effect was further enhanced by the addition of G-CSF (Fig. 1C). In this study, an enhancement was confirmed for the combination of Ara-C, VP-16, and G-CSF in 32Dcl3 and U937 cells but not in HL-60 and Ba/F3 cells. In addition, there are some reports that state that priming with G-CSF enhanced Ara-C in myeloid leukemia cell lines such as WEHI-3B14 and OCI/AML1a.15 This was a different result although all cells have G-CSF receptors. We thought that this difference was a result of different responses to G-CSF by the cell lines. For instance, 32Dcl3 cells express G-CSF receptors, and show differentiation to neutrophils and proliferation subsequently in response to G-CSF.29 HL-60 cells also express G-CSF receptors, and differentiate in response to G-CSF, but do not proliferate thereafter.30 Recent studies on cytokine signaling have focused on whether the signals initiated by a receptor-ligand interaction are distinct between cell proliferation and differentiation.31-34 Several reports on G-CSF receptor and the gp130 signaling chain suggest that the intracellular domains of these receptors may be functionally sub-divided with a membrane-proximal region being critical for proliferation and a more distal domain providing differentiation-specific signals.31-35 In addition, it was stated that activation of STAT3 appears to be involved in G-CSF-mediated differentiation whereas STAT3a is linked to G-CSF-induced cell proliferation and transformation.36 We found that G-CSF mobilized G0/G1-phase cells into the S phase among 32Dcl3 and U937 cells (Fig. 4), but not HL-60 and Ba/F3 cells. We considered that this was the result of different responses to G-CSF by the cell lines. It was likely that G-CSF potentiated Ara-C-induced cytotoxicity through the mechanism of apoptosis by introducing resting G0-phase cells into the G1 phase, as well as the VP-16 additive effect in
AVG therapy.

We showed that the priming effect of G-CSF on AML cells in vitro depended on the mode of proliferation of G-CSF receptor-mediated action. Although most AML cells express G-CSF receptors, some cases of AML do not show the acute myeloid leukemia in clinical practice, which may be a result of AML heterogeneity. It would be useful to predict therapeutic outcomes by analyzing in vitro the priming effect of G-CSF in freshly isolated AML cells from patients before AVG chemotherapy.

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