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Original Article

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. 〔*J Clin Exp Hematopathol 50(2) : 99-105, 2010*〕

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)[1] 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.[2] 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/m^2).[2] 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.[3-5] The mechanism of such action is assumed to involve recruitment of quiescent G0 leukemic cells into the cell cycle.[4] 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.

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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% CO_2 at 37°C.

Cell proliferation assays

HL60, U937, 32Dcl3, and Ba/F3 cells (5×10^5 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) in 6-well flat-bottomed plates for 72 hr. 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 protocol. Since externalization of phosphatidylserine occurs in the early stages of apoptosis, Annexin V-FITC staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. 32Dcl3, HL-60, and Ba/F3 cells (5×10^5 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 washed twice with cold phosphate-buffered saline, and then Annexin V-FITC and propidium iodide (PI) were added for 15 min on ice. Stained cells were analyzed by flow cytometry using FACScan (Becton Dickinson, San Jose, CA) within 1 hr. Since Annexin V-FITC staining precedes the loss of membrane integrity that accompanies the later stage identified by PI, Annexin V-FITC-positive, PI-negative indicates early apoptosis, while viable cells are Annexin V-FITC-negative, PI-negative, and cells that are in late apoptosis or already dead cells are both Annexin V-FITC- and PI-positive.[6-11]

Cell-cycle analyses

To evaluate the cell cycle, we used an FITC BrdU Flow kit purchased from BD Pharmingen™ (San Jose, CA) according to the manufacturer's instructions. 32Dcl3, HL-60, and Ba/F3 cells (5×10^5 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 FACScan (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).[12,13] 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,

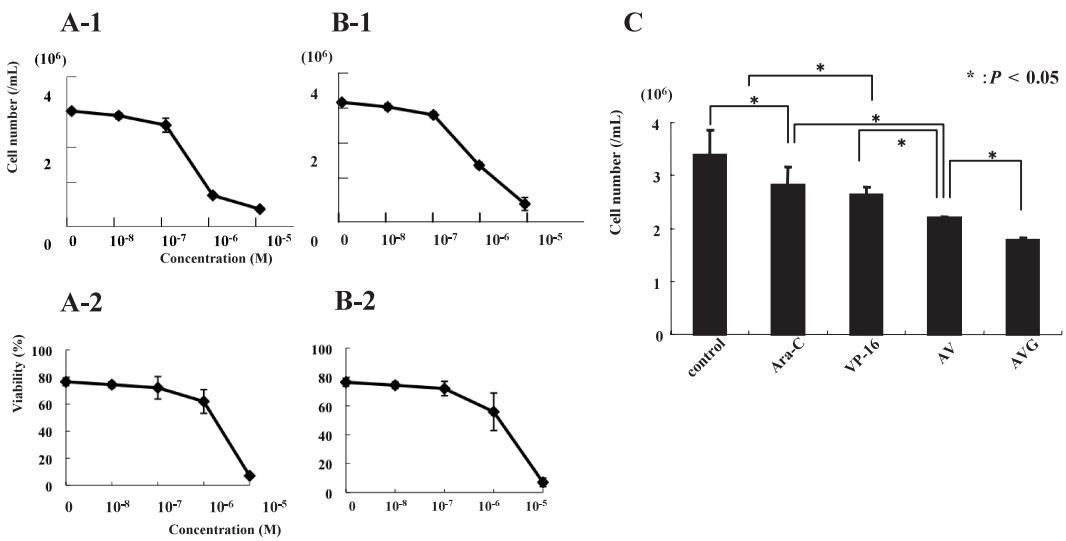

Fig. 1. Growth inhibition of 32Dcl3 cells by various indicated concentrations of Ara-C (*A*) or VP-16 (*1B*). The cytotoxicity was expressed as cell number (*A-1, B-1*) and viability (*A-2, B-2*). Growth inhibition of 32Dcl3 cells by Ara-C alone (10^{-7} M), VP-16 alone (10^{-7} M), Ara-C and VP-16 (AV), and AV plus G-CSF (50 ng/mL) (AVG) (*C*). The cytotoxicity was expressed as cell number. These data represent the mean ± SD of three independent experiments, each of which was performed in triplicate.
Ara-C, cytarabine ; VP-16, etoposide ; G-CSF, granulocyte colony-stimulating factor ; AV, Ara-C plus VP-16 ; AVG, AV plus G-CSF

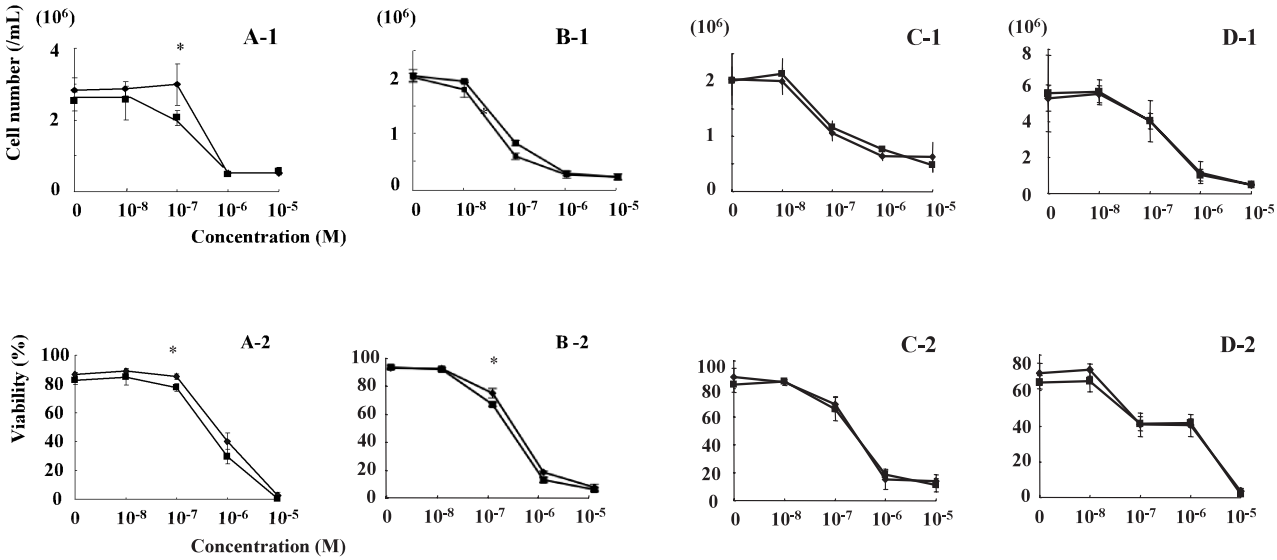

Fig. 2. 32Dcl3 (*A*), U937 (*B*), HL-60 (*C*), and Ba/F3 (*D*) cells were cultured for 72 hr with the indicated concentrations of both Ara-C and VP-16 in the presence (■) or absence (◆) of 50 ng/mL G-CSF (*A-1, B-1, C-1, D-1* : cell count and *A-2, B-2, C-2, D-2* : viability). The data represent the mean ± SD of three independent experiments, each of which was performed in triplicate. * : $P < 0.05$
G-CSF, granulocyte colony-stimulating factor ; Ara-C, cytarabine ; VP-16, etoposide

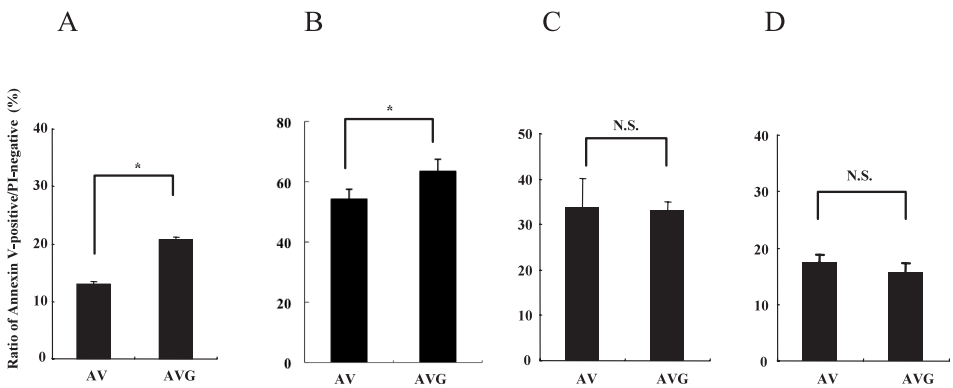

Fig. 3. Effect of the combination of Ara-C/VP-16 and G-CSF on the induction of apoptosis in 32Dcl3 (*A*), U937 (*B*), HL-60 (*C*), and Ba/F3 cells (*D*). The cells were treated for 72 hr with 10^{-7} M Ara-C and 10^{-7} M VP-16 (AV), or Ara-C and VP-16 plus 50 ng/mL G-CSF (AVG). Values are expressed as the mean ± SD of three independent experiments, each of which was performed in triplicate. * : $P < 0.05$
Ara-C, cytarabine ; VP-16, etoposide ; G-CSF, granulocyte colony-stimulating factor ; AV, Ara-C plus VP-16 ; AVG, AV plus G-CSF

VP-16 principally acts on the G2 and S phases of the cell cycle.[16] 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.[2]

A low-dose Ara-C regimen is currently regarded as the therapy of choice for advanced myelodysplastic syndrome and AML.[17] 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.[18,19] Yamada *et al*.[20] reported the CAG regimen, which consisted of low-dose Ara-C, aclarubicin, 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.[21] They reported that priming with G-CSF en-

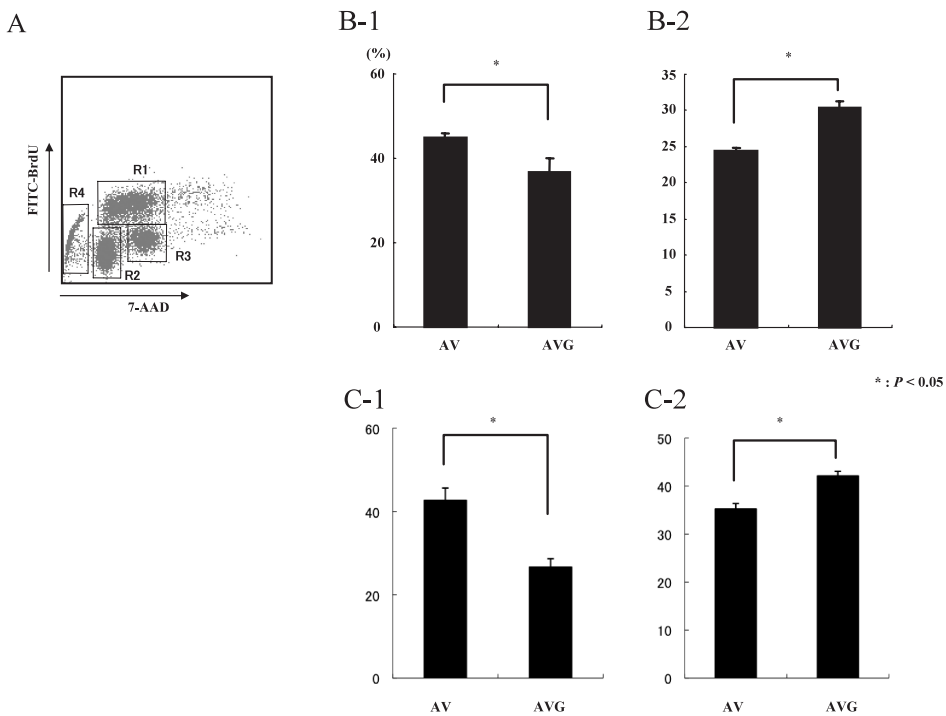

Fig. 4. Effect of the combination of Ara-C/VP-16 and G-CSF on the cell cycle for 32Dcl3 and U937 cells. The cells were treated for 48 hr with 10^{-7} M Ara-C and 10^{-7} M VP-16 in the presence or absence of 50 ng/mL G-CSF. The results of FACS dot plots (*A*) and the proportions of G0-G1- (32Dcl3 : *B-1*, U937 : *C-1*) and S-phase (32Dcl3 : *B-2*, U937 : *C-2*) cells are shown. Values are expressed as the mean ± SD of three independent experiments, each of which was performed in triplicate.
Ara-C, cytarabine ; VP-16, etoposide ; G-CSF, granulocyte colony-stimulating factor ; AV, Ara-C plus VP-16 ; AVG, AV plus G-CSF ; FACS, fluorescence-activated cell sorter ; G0, Gap 0, G1 ; Gap 1, S ; synthesis

hanced 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-3B[14] 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 STAT3β appears to be involved in G-CSF-mediated differentiation whereas STAT3α 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,[37-39] some cases of AML do not show the priming effect 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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REFERENCES

1 Tsurumi H, Kanemura N, Hara T, Kasahara S, Yamada T, *et al.* : Therapeutic strategy of untreated *de novo* acute myeloid leukemia in the elderly : the efficacy of continuous drip infusion with low dose cytarabine and etoposide. J Cancer Res Clin Oncol 133 : 547-553, 2007

2 Kanemura N, Tsurumi H, Kasahara S, Hara T, Yamada T, *et al.* : Continuous drip infusion of low dose cytarabine and etoposide with granulocyte colony-stimulating factor for elderly patients with acute myeloid leukaemia ineligible for intensive chemotherapy. Hematol Oncol 26 : 33-38, 2008

3 Miyauchi J, Kelleher CA, Wang C, Minkin S, McCulloch EA : Growth factors influence the sensitivity of leukemic stem cells to cytosine arabinoside in culture. Blood 73 : 1272-1278, 1989

4 Tafuri A, Andreeff M : Kinetic rationale for cytokine-induced recruitment of myeloblastic leukemia followed by cycle-specific chemotherapy *in vitro*. Leukemia 4 : 826-834, 1990

5 te Boekhorst PA, Lowenberg B, Vlastuin M, Sonneveld P : Enhanced chemosensitivity of clonogenic blasts from patients with acute myeloid leukemia by G-CSF, IL-3 or GM-CSF stimulation. Leukemia 7 : 1191-1198, 1993

6 Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingsperger CP, *et al.* : Human neutrophils lose their surface FcγRIII and acquire Annexin V binding sites during apoptosis *in vitro*. Blood 85 : 532-540, 1995

7 Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, *et al.* : Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood 84 : 1415-1420, 1994

8 Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, *et al.* : Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus : inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182 : 1545-1556, 1995

9 Raynal P, Pollard HB : Annexins : the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim Biophys Acta 1197 : 63-93, 1994

10 van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP : Annexin V-affinity assay : a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 31 : 1-9, 1998

11 Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C : A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184 : 39-51, 1995

12 Dean PN, Dolbeare F, Gratzner H, Rice GC, Gray JW : Cell-cycle analysis using a monoclonal antibody to BrdUrd. Cell Tissue Kinet 17 : 427-436, 1984

13 Lacombe F, Belloc F, Bernard P, Boisseau MR : Evaluation of four methods of DNA distribution data analysis based on bromodeoxyuridine/DNA bivariate data. Cytometry 9 : 245-253, 1988

14 Katagiri T, Miyazawa K, Nishimaki J, Yaguchi M, Kawanishi Y, *et al.* : Combination of granulocyte colony-stimulating factor and low-dose cytosine arabinoside further enhances myeloid differentiation in leukemia cells *in vitro*. Leuk Lymphoma 39 : 173-184, 2000

15 Bai A, Kojima H, Hori M, Nara N, Komeno T, *et al.* : Priming with G-CSF effectively enhances low-dose Ara-C-induced *in vivo* apoptosis in myeloid leukemia cells. Exp Hematol 27 : 259-265, 1999

16 Rowe T, Kupfer G, Ross W : Inhibition of epipodophyllotoxin cytotoxicity by interference with topoisomerase-mediated DNA cleavage. Biochem Pharmacol 34 : 2483-2487, 1985

17 Visani G, Malagola M, Piccaluga PP, Isidori A : Low dose Ara-C for myelodysplastic syndromes : is it still a current therapy ? Leuk Lymphoma 45 : 1531-1538, 2004

18 Baer MR, Bernstein SH, Brunetto VL, Heinonen K, Mrozek K, *et al.* : Biological effects of recombinant human granulocyte colony-stimulating factor in patients with untreated acute myeloid leukemia. Blood 87 : 1484-1494, 1996

19 Rossi HA, O'Donnell J, Sarcinelli F, Stewart FM, Quesenberry PJ, *et al.* : Granulocyte-macrophage colony-stimulating factor (GM-CSF) priming with successive concomitant low-dose Ara-C for elderly patients with secondary/ refractory acute myeloid leukemia or advanced myelodysplastic syndrome. Leukemia 16 : 310-315, 2002

20 Yamada K, Furusawa S, Saito K, Waga K, Koike T, *et al.* : Concurrent use of granulocyte colony-stimulating factor with low-dose cytosine arabinoside and aclarubicin for previously treated acute myelogenous leukemia : a pilot study. Leukemia 9 : 10-14, 1995

21 Saito K, Nakamura Y, Aoyagi M, Waga K, Yamamoto K, *et al.* : Low-dose cytarabine and aclarubicin in combination with granulocyte colony-stimulating factor (CAG regimen) for previously treated patients with relapsed or primary resistant acute myelogenous leukemia (AML) and previously untreated elderly patients with AML, secondary AML, and refractory anemia with excess

blasts in transformation. Int J Hematol 71 : 238-244, 2000
22 Estey E, Thall P, Andreeff M, Beran M, Kantarjian H, *et al.* : Use of granulocyte colony-stimulating factor before, during, and after fludarabine plus cytarabine induction therapy of newly diagnosed acute myelogenous leukemia or myelodysplastic syndromes : comparison with fludarabine plus cytarabine without granulocyte colony-stimulating factor. J Clin Oncol 12 : 671-678, 1994
23 Estey EH, Thall PF, Pierce S, Cortes J, Beran M, *et al.* : Randomized phase II study of fludarabine+cytosine arabinoside+idarubicin+/-all-trans retinoic acid+/-granulocyte colony-stimulating factor in poor prognosis newly diagnosed acute myeloid leukemia and myelodysplastic syndrome. Blood 93 : 2478-2484, 1999
24 Godwin JE, Kopecky KJ, Head DR, Willman CL, Leith CP, *et al.* : A double-blind placebo-controlled trial of granulocyte colony-stimulating factor in elderly patients with previously untreated acute myeloid leukemia : a Southwest oncology group study (9031). Blood 91 : 3607-3615, 1998
25 Goldstone AH, Burnett AK, Wheatley K, Smith AG, Hutchinson RM, *et al.* : Attempts to improve treatment outcomes in acute myeloid leukemia (AML) in older patients : the results of the United Kingdom Medical Research Council AML11 trial. Blood 98 : 1302-1311, 2001
26 Harousseau JL, Witz B, Lioure B, Hunault-Berger M, Desablens B, *et al.* : Granulocyte colony-stimulating factor after intensive consolidation chemotherapy in acute myeloid leukemia : results of a randomized trial of the Groupe Ouest-Est Leucémies Aigues Myeloblastiques. J Clin Oncol 18 : 780-787, 2000
27 Ohno R, Naoe T, Kanamaru A, Yoshida M, Hiraoka A, *et al.* : A double-blind controlled study of granulocyte colony-stimulating factor started two days before induction chemotherapy in refractory acute myeloid leukemia. Kohseisho Leukemia Study Group. Blood 83 : 2086-2092, 1994
28 Usuki K, Urabe A, Masaoka T, Ohno R, Mizoguchi H, *et al.* : Efficacy of granulocyte colony-stimulating factor in the treatment of acute myelogenous leukaemia : a multicentre randomized study. Br J Haematol 116 : 103-112, 2002
29 Ward AC, Smith L, de Koning JP, van Aesch Y, Touw IP : Multiple signals mediate proliferation, differentiation, and survival from the granulocyte colony-stimulating factor receptor in myeloid 32D cells. J Biol Chem 274 : 14956-14962, 1999
30 Bashey A, Healy L, Marshall CJ : Proliferative but not nonproliferative responses to granulocyte colony-stimulating factor are associated with rapid activation of the p21ras/MAP kinase signalling pathway. Blood 83 : 949-957, 1994
31 Dong F, van Buitenen C, Pouwels K, Hoefsloot LH, Lowenberg B, *et al.* : Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. Mol Cell Biol 13 : 7774-7781, 1993
32 Fukunaga R, Ishizaka-Ikeda E, Nagata S : Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74 : 1079-1087, 1993
33 Koay DC, Sartorelli AC : Functional differentiation signals mediated by distinct regions of the cytoplasmic domain of the granulocyte colony-stimulating factor receptor. Blood 93 : 3774-3784, 1999
34 Yamanaka Y, Nakajima K, Fukada T, Hibi M, Hirano T : Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. EMBO J 15 : 1557-1565, 1996
35 Yoshikawa A, Murakami H, Nagata S : Distinct signal transduction through the tyrosine-containing domains of the granulocyte colony-stimulating factor receptor. EMBO J 14 : 5288-5296, 1995
36 Chakraborty A, Tweardy DJ : Stat3 and G-CSF-induced myeloid differentiation. Leuk Lymphoma 30 : 433-442, 1998
37 Kondo S, Okamura S, Asano Y, Harada M, Niho Y : Human granulocyte colony-stimulating factor receptors in acute myelogenous leukemia. Eur J Haematol 46 : 223-230, 1991
38 Park LS, Waldron PE, Friend D, Sassenfeld HM, Price V, *et al.* : Interleukin-3, GM-CSF, and G-CSF receptor expression on cell lines and primary leukemia cells : receptor heterogeneity and relationship to growth factor responsiveness. Blood 74 : 56-65, 1989
39 Estey EH : Use of colony-stimulating factors in the treatment of acute myeloid leukemia. Blood 83 : 2015-2019, 1994