Treatment With Interleukin-3 Plus Granulocyte-Macrophage Colony-Stimulating Factors Improves the Selectivity of Ara-C In Vitro Against Acute Myeloid Leukemia Blasts

By Kapil Bhalla, Charles Holladay, Zalmen Arlin, Steven Grant, Ana Maria Ibrado, and Michelle Jasiok

Hematopoietic growth factors (HGFs) interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) individually have been shown to increase the percentage of acute myeloid leukemia (AML) blasts in S phase and enhance the cytotoxic effects of Ara-C against these blasts in culture. We compared in vitro the effects of a combined treatment with GM-CSF (10 ng/mL) plus IL-3 (10 ng/mL) on the metabolism and cytotoxicity of Ara-C in normal bone marrow mononuclear cells (NBMMC) and AML blasts. NBMMC from six healthy volunteers and AML blasts from 10 patients were incubated for 20 hours with or without IL-3 plus GM-CSF, followed by a concurrent treatment with Ara-C for 4 additional hours. Exposure to the HGFs and Ara-C produced significantly higher intracellular Ara-CTP levels as well as higher Ara-CTP/dCTP pool ratios in AML blasts as compared with NBMMC. Treatment with HGFs resulted in [3H] Ara-C DNA incorporation that was significantly higher in AML blasts versus NBMMC. This selective improvement of Ara-C metabolism in AML blasts was associated with an enhanced Ara-C-mediated leukemia colony-forming unit (CFU) growth inhibition. In contrast, exposure to HGFs resulted in an improved colony growth of normal CFU granulocyte-monocyte and CFU-granulocyte, erythroid, monocyte, megakaryocyte. These in vitro studies indicate that a combined treatment with IL-3 plus GM-CSF may improve the selectivity of Ara-C against AML blasts.

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 MATERIALS AND METHODS

Drugs and Chemicals

Ara-C and [3H] Ara-C (26 Ci/mmol) were purchased from Sigma Chemicals (St Louis, MO) and Amersham Radiochemicals (Arlington Heights, IL), respectively. Human recombinant (Escherichia coli derived) IL-3 was a gift from Genetics Institute (Cambridge, MA). The specific biologic activity of recombinant IL-3 (rIL-3) is 4.6 x 10^6 U/mg of protein. Human recombinant GM-CSF (Schering-
ing 39300) was kindly provided by Dr Paul Trotta (Schering Corp, Nutly, NJ). Schering 39300 has a molecular weight of 14,477 and is E coli derived. The specific biologic activity of GM-CSF is 10^8 U/mg of protein. Drugs were stored at −20°C and reconstituted in McCoy's 5A medium immediately before use.

**Procurement of Normal BM Mononuclear Cells (NBMMC) and AML Blasts**

BM aspirates were obtained with informed consent from six healthy volunteers, and peripheral blood with high blasts count, or BM aspirates, were taken from 10 patients with AML at presentation or in relapse. Patients with relapsed AML had been previously treated with an intermittent high-dose Ara-C regimen during the consolidation phase of chemotherapy and were in their first relapse 3 to 15 months after receiving consolidation chemotherapy, except for the patient who had AML primarily refractory to standard-dose Ara-C. These studies were sanctioned by the Investigational Review Board of the Medical University of South Carolina. BMMC were isolated by the density gradient centrifugation method. Cells were washed twice with supplemented McCoy's 5A medium (GIBCO, Grand Island, NY), and adherent BM cells containing 90% or greater monocytes and macrophages were removed by incubation for 1 hour in tissue culture flasks. Nonadherent BMMC were depleted of T lymphocytes by rosetting with sheep red blood cells. In the case of AML, the samples consisted of greater than 90% myeloblasts or promyelocytes.

**Intracellular Ara-CTP Relative to dCTP Formation**

Normal BM MMC and leukemic blasts were isolated and suspended in McCoy's 5A medium containing 10% fetal calf serum (FCS) at a cell density of 10^6 cells/mL. Cell suspensions were incubated with or without the presence of rGM-CSF (10 ng/mL) and rIL-3 (10 ng/mL) in a 37°C, 5% CO₂ incubator for 20 hours. For the HGFs these dosages and exposure intervals were selected because they had previously been determined to have a maximal cytokinetic effect (percent increase in the S-phase cells), and in the case of Ara-C, this dose of IL-3 had produced a plateau in the intracellular dCTP pool expansion in the normal BMMC. The cells were further exposed for 4 hours to the HGFs alone, Ara-C (10 μmol/L) alone, or a combination of the HGFs and Ara-C. Subsequently, neutralized, perchloric acid-soluble extracts of the cell pellets were obtained and subjected to periodate oxidation to remove endogenous ribonucleotides. Ara-C-CTP and dCTP were quantitated by a previously described high performance liquid chromatography (HPLC) method using a Waters HPLC system (Waters, Milford, MA).

**Ara-C DNA Incorporation**

After the cells were isolated, washed, and suspended, as described above, they were incubated with or without the presence of GM-CSF (10 ng/mL) and IL-3 (10 ng/mL) at 37°C for 20 hours. For an additional 4 hours, the cells were concurrently exposed to 10 μmol/L [H]Ara-C. At the end of the incubation period, the cells were centrifuged at 400g for 8 minutes at 4°C. The cell pellet was washed twice with cold phosphate-buffered saline (PBS) to remove the radiolabeled drug. DNA was isolated and purified by pronase and RNase digestion, phenol/chloroform extraction, and ethanol precipitation as previously described. After centrifugation at 9,000g at 4°C for 30 minutes, the DNA was resuspended in Tris-HCl buffer, quantitated spectrophotometrically, and aliquots were removed and placed in scintillation vials to determine radioactivity. Values for each condition are expressed as femtmoles of Ara-C per microgram of DNA.

**Culture of Normal and Leukemic Human BM Progenitor Cells**

**Colony-forming unit granulocyte-monocyte (CFU-GM) assay.** After exposure to the designated concentration and schedule of GM-CSF plus IL-3 and Ara-C, cells were washed twice and suspended in a mixture consisting of McCoy's 5A medium, 20% FCS, and 0.3% Bacto agar containing 2 × 10⁶ cells/mL. Aliquots (0.5 mL) of the mixture were pipetted into 18-mm, 12-well plates (Costar, Cambridge, MA) containing a bottom layer consisting of McCoy's 5A medium, 0.5% agar, 20% FCS, and 10% 5637 CM as a source of colony-stimulating activity. The plates were incubated at 37°C, 5% CO₂ atmosphere, and colonies consisting of ≥ 50 cells were counted after 10 days.

**CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM) assay.** After incubation with HGFs and Ara-C as described above, 5 × 10⁵ cells were cultured in 1 mL of 1.2%, 1500 centipoise methylcellulose (Sigma), 30% FCS, 1% essentially fatty acid and globulin-free, crystallized and deionized bovine serum albumin (BSA; Sigma), 100 μmol/L 2-mercaptoethanol, 2 U of erythropoietin, and 10% 5637 CM. Plates were incubated for 14 days at 37°C in a humidified, 5% CO₂ atmosphere and erythroid and mixed (CFU-GEMM) colonies consisting of ≥ 50 cells were counted.

**Culture of Leukemic Blast Progenitors**

Leukemic blasts were separated as described above and incubated with the designated concentrations and schedule of the HGFs and Ara-C. Subsequently, the cells were washed and plated in 18-mm, 12-well plates at a concentration of 2 × 10⁶ cells/mL in 1 mL of McCoy 5A medium containing 0.8% methylcellulose, 20% FCS, and 10% 5637 CM as a source of colony-stimulating activity. Plates were incubated for 10 days in a 37°C, 5% CO₂, 5% O₂, fully humidified atmosphere. Colonies (> 20 cells) were scored and evaluated for morphology by Giemsa stain.

**Statistical Analysis**

Significant differences between values obtained in NBMMC and AML blasts treated identically were determined by using t-test, while significant differences between values obtained in a population of cells (NBMMC or AML blasts) treated with different experimental conditions were determined by paired t-test analyses.

**RESULTS**

Table 1 shows the effect of IL-3 plus GM-CSF on the intracellular accumulation of Ara-CTP relative to dCTP in Ara-C–treated blasts from 10 patients with AML (French-American-British [FAB] subtype M₆;6; M₃;3; M₁;1). Mean intracellular dCTP levels in AML blasts (1.5 ± 0.1 pmol/10⁶ cells) decreased after treatment with Ara-C (0.9 ± 0.1 pmol/10⁶ cells) (P < .01), while exposure to the HGFs alone did not affect dCTP pools. Intracellular Ara-C-CTP accumulation or Ara-C/dCTP pool ratios were not significantly different between the FAB M₆ or M₃ subtypes, although each subgroup consisted of a small number of patients. After exposure to the HGFs in conjunction with Ara-C, mean Ara-CTP accumulation increased from 17.6 to 30.9 pmol/10⁶ cells in AML blasts (P < .01) while mean Ara-C/dCTP pool ratios increased from 27.0 to 54.1 (P < .02). In previous studies, we had found that treatment of AML blasts with high-dose Ara-C plus GM-CSF or IL-3 produced Ara-C/dCTP pool ratios that were either
were used for comparison with AML blasts with respect to would be considered as the true, normal counterparts of Ara-CTP and dCTP pools in NBMMC from AML blasts. The adherent cell-depleted NBMMC, which was used for comparison with AML blasts with respect to intracellular Ara-CTP level$ are expressed as pmol/106 cells, while Ara-CTP/dCTP pool ratios, in parenthesis, are without units.

*FAB classification for AML.
†Refers to the dose of Ara-C used in the treatment before relapse. H, high-dose Ara-C; N, no prior treatment; R, AML refractory to induction chemotherapy with conventional doses of Ara-C and daunorubicin.
‡Ara-CTP levels are expressed as pmol/106 cells, while Ara-CTP/dCTP pool ratios, in parenthesis, are without units.

Table 1. Effect of HGFs (IL-3 + GM-CSF) on the Metabolism of Ara-C in AML Blasts

<table>
<thead>
<tr>
<th>FAB*</th>
<th>Patient</th>
<th>Ara-C†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>M2</td>
<td>1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>4</td>
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<td></td>
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<td>3.3</td>
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<tr>
<td></td>
<td>6</td>
<td>0.8</td>
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<tr>
<td>Mean</td>
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<tr>
<td>SEM</td>
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<tr>
<td>M4</td>
<td>1</td>
<td>1.8</td>
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<tr>
<td></td>
<td>2</td>
<td>2.1</td>
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<tr>
<td></td>
<td>3</td>
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<td>M1</td>
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<td>Mean of all samples</td>
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<td>1.5</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>

Mean dCTP, pmol/10⁷ cells; Ara-CTP, fmol/pg of DNA. Values are expressed as pmol/10⁷ cells and represent the means for six samples ± SEM.

*Values significantly different from those in control cells (P < .01).

Table 2. Effect of HGFs (IL-3 + GM-CSF) on the Metabolism of Ara-C in Normal BM Cells

<table>
<thead>
<tr>
<th>Conditions</th>
<th>dCTP</th>
<th>Ara-CTP</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6  ± 0.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>HGFs</td>
<td>9.5  ± 0.7*</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Ara-C</td>
<td>2.3 ± 0.3*</td>
<td>24.8 ± 5.2</td>
<td>10.7</td>
</tr>
<tr>
<td>HGFs + Ara-C</td>
<td>4.4 ± 1.2</td>
<td>18.8 ± 3.1</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Values significantly different from those in control cells (P < .01).

lower (with GM-CSF) or similar (with IL-3) to those seen with treatment of AML blasts with Ara-C alone.7,8

Table 2 shows the effects of IL-3 plus GM-CSF on the Ara-CTP and dCTP pools in NBMMC from six BM samples. To perform these studies, it is difficult to purify sufficiently large numbers of normal BM myeloblasts that would be considered as the true, normal counterparts of AML blasts. The adherent cell-depleted NBMMC, which were used for comparison with AML blasts with respect to intracellular Ara-CTP/dCTP pool ratios and Ara-C DNA incorporation, is a heterogenous population of cells and only contains a small number of early myeloid progenitor cells. Mean intracellular dCTP pools were significantly higher in untreated NBMMC versus AML blasts (Table 1) (P < .01). Exposure to the HGFs resulted in an expansion of intracellular dCTP pools in NBMMC (P < .01), which was not seen in AML blasts (Table 1). In addition, treatment with HGFs caused a decline in Ara-CTP accumulation in NBMMC (24.8 ± 5.2 to 18.8 ± 3.1 pmol/10⁶ cells).

However, this decline did not achieve statistical significance (P > .05).

The effect of IL-3 and GM-CSF on Ara-C DNA incorporation is depicted in Fig 1. Treatment with HGFs decreased Ara-C DNA incorporation in NBMMC from six separate samples from 123.4 ± 12.4 to 98.5 ± 8.2 fmol/μg of DNA. However, it can not be ruled out that this reduction in Ara-C DNA incorporation in HGFs treated NBMMC may reflect the relatively lower proliferation status of the terminally differentiated, nondividing myeloid elements in the NBMMC population. In contrast, under identical conditions, Ara-C DNA incorporation increased in AML blasts from 155.6 ± 19.8 to 195.0 ± 24.4 fmol/μg of DNA. This increase did not reach statistical significance (P > .05). Nevertheless, after treatment with the HGFs, a significant difference in Ara-C DNA incorporation was observed between NBMMC and AML blasts (98.5 ± 8.2 vs 195.0 ± 24.4 fmol/μg of DNA) (P < .02). It is noteworthy that in our previous studies we had not found significantly higher Ara-C DNA incorporation in AML blasts versus NBMMC that were treated with either IL-3 or GM-CSF in conjunction with Ara-C in doses and schedule similar to the present study.7,8

Figure 2 highlights the differential effect of IL-3 and GM-CSF on Ara-C mediated inhibition of colony growth of normal CFU-GM and CFU-GEMM versus leukemia-CFU (L-CFU). Treatment with 100 μmol/L Ara-C alone for 4 hours inhibited L-CFU colony growth by 39.9% ± 6.5%, while under identical conditions CFU-GM and CFU-GEMM growth decreased by 44.3% ± 5.8% and 54.4% ± 9.2%, respectively. These differences in the inhibition of colony growth of normal and leukemic progenitor cells were not statistically significant (P > .05). Exposure to IL-3 plus GM-CSF in conjunction with 100 μmol/L Ara-C reduced its inhibitory effects on the colony growth of normal.
EFFECT OF IL-3 PLUS GM-CSF ON ARA-C ACTIVITY

Fig 1. NBMMC and AML blasts were incubated (E) with or (W) without IL-3 and GM-CSF for 20 hours followed by incubation with [3H]Ara-C (10 μmol/L) for an additional 4 hours. Subsequently, cells were pelleted and washed and their DNA was extracted as described in the text. The radioactive analogue incorporation was quantitated by liquid scintilligraphy. Values are expressed as femtomoles of analogue per micrograms of DNA and represent the means of six (NBMMC) or 10 samples (AML blasts) ± SEM.

Fig 2. NBMMC and AML blasts were incubated (E) with or (W) without IL-3 and GM-CSF for 20 hours followed by incubation with or without Ara-C (100 μmol/L) for an additional 4 hours. Subsequently, cells were washed and plated in soft agar (CFU-GM) or methylcellulose (CFU-GEMM and L-CFU) as described in the text. Plates were incubated under appropriate conditions and colonies were counted after 10 (CFU-GM and L-CFU) or 14 days (CFU-GEMM). The height of each bar corresponds to the percentage of colony formation for each condition relative to untreated control colony growth in 10% 5637 CM. Values represent the means for six separate NBMMC samples and 10 AML samples.

CFU-GM from 44.3% ± 5.8% to 20.7% ± 2.1% (P < .01) and normal CFU-GEMM from 54.4% ± 9.2% to 34.9% ± 4.4% (P < .05) (Fig 2). In contrast, IL-3 plus GM-CSF increased the inhibitory effects of Ara-C on L-CFU colony growth from 39.9% ± 6.5% to 57.1% ± 3.1% (P < .05). Therefore, coadministration of the two HGFs and Ara-C in the designated concentrations and schedule selectively enhanced the cytotoxic effects of high-dose Ara-C toward L-CFU but not against CFU-GM or CFU-GEMM in culture.

DISCUSSION

In previous studies we and others have shown the ability of IL-3 or GM-CSF to independently increase the percentage of cycling AML blasts in S phase and favorably affect the intracellular Ara-CTP accumulation and Ara-CTP/dCTP pool ratios in AML blasts versus NBMMC treated with Ara-C. Importantly, other studies have shown that IL-3 or GM-CSF increases the cytotoxic effects of Ara-C toward AML blasts. In the present study, we report for the first time that a combination of IL-3 plus GM-CSF significantly increases Ara-CTP/dCTP pool ratios in AML blasts and produces significantly higher Ara-C DNA incorporation in AML blasts versus NBMMC treated with high-dose Ara-C. Furthermore, these findings were associated with increased, in vitro, cytotoxic effects of high-dose Ara-C against L-CFU but not against normal CFU-GM or CFU-GEMM.

Saturation of Ara-C phosphorylation in AML blasts occurs at plasma Ara-C concentrations ≥ 10 μmol/L that are reached by high-dose Ara-C infusion rates. Because stringent regulation of Ara-C phosphorylation and DNA incorporation has been attributed to intracellular dCTP levels, Ara-CTP to dCTP pool ratios may be an important biochemical correlate of high-dose Ara-C-mediated cytotoxicity. In this report, after treatment with the HGFs plus Ara-C, significantly higher mean Ara-CTP/dCTP pool ratios in AML blasts were associated with increased Ara-C DNA incorporation and growth inhibitory effects on L-CFU colony growth (P < .01). Ideally, it would be desirable to compare the generation of Ara-CTP/dCTP pool ratios and Ara-C DNA incorporation in AML blasts with their true normal counterparts, ie, normal BM blast CFU-Cs of equivalent degree of maturation. However, with currently available methods it is difficult to procure and purify a sufficiently large population of these normal BM elements to perform the biochemical studies detailed in this report.

It is noteworthy that IL-3 plus GM-CSF-mediated increase in the mean Ara-C DNA incorporation in AML blasts did not achieve statistical significance despite a significant increase in the mean Ara-CTP/dCTP pool ratio observed in these cells. It has been previously noted that the exposure to high concentrations of Ara-C causes high intracellular Ara-CTP levels that result in a significant deterioration in the efficiency of Ara-C incorporation into DNA (defined as the ratio of Ara-C incorporation to Ara-CTP pools), most likely due to the inhibitory effects of high levels of Ara-CTP on DNA polymerase. This finding may explain the discordance between the extent and the statistical significance of the increment in Ara-CTP/dCTP pool ratios and Ara-C DNA incorporation produced by HGFs in AML blasts.
Although GM-CSF and IL-3 receptors do not have endogenous tyrosine or serine threonine kinase activity, the receptor activation induces rapid tyrosine phosphorylation of 90- and 70-Kd proteins. Both GM-CSF and IL-3 have been shown to induce rapid, dose-dependent phosphorylation of a 74-Kd serine-threonine protein kinase, Raf-1, that results in an enhanced Raf-1 kinase activity. Also, activation of protein kinase C may be involved in IL-3 signal transduction, which controls the transcription of the growth regulatory oncogene c-myc. In this context it is noteworthy that a combined treatment with IL-3 and high-dose Ara-C was shown to decrease c-myc RNA in KG1 cells to a significantly lower level than that observed on treatment with high-dose Ara-C alone. Recently, in human leukemic cell line U-937, high-dose Ara-C was shown to cause an endonuclease mediated oligonucleosomal DNA fragmentation known to occur with programmed cell death or apoptosis, and this was correlated with Ara-C-mediated inhibition of clonogenic survival of the leukemic cells. The same study also showed that these molecular events associated with the cell lethality of Ara-C were related to the induction of early response genes, c-fos and c-jun, which encode for the AP1 transcription factor. Of note in this regard is the observation that an AP1 binding site is present in the 5' regulatory region of c-myc that is implicated in the downregulation of c-myc. Taken together, these studies raise the possibility that a combined treatment with GM-CSF plus IL-3 in conjunction with high-dose Ara-C may activate kinase cascades and transcription factors that may result in altered expression of growth regulatory genes or early response genes associated with apoptosis. Furthermore, these reports along with the results of the present study suggest that the selectively higher Ara-CTP/CTP pool ratios and Ara-C DNA incorporation produced by treatment of AML blasts with a combination of IL-3 plus GM-CSF and high-dose Ara-C may be exerting antileukemic selectivity through novel molecular mechanisms that are growth regulatory for AML blasts.

Clinical trials of concurrent treatment with GM-CSF and Ara-C in AML are currently underway. The present report provides a rationale to explore a combination of IL-3 plus GM-CSF to improve the antileukemic selectivity of Ara-C in vivo and ex vivo to purge AML blasts from remission BM samples for autotransplantation in AML.

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