Modulation of the Cellular Metabolism of Cytarabine and Fludarabine by Granulocyte-Colony-stimulating Factor during Therapy of Acute Myelogenous Leukemia

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ABSTRACT

Previous in vitro investigations demonstrated that human leukemia cells, when incubated with hematopoietic growth factors such as granulocyte-colony-stimulating factor (G-CSF), augment the accumulation of the triphosphate 1-β-D-arabinofuranosylcytosine (ara-C cytarabine). To test whether G-CSF infusion prior to ara-C infusion would biologically modulate the accumulation of ara-9-β-D-arabinofuranosylcytosine 5'-triphasphate (ara-CTP) and other ara nucleotides in the leukemia blasts during therapy, protocols were designed to infuse G-CSF prior to fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate) and ara-C to increase the accumulation of the active triphosphates [9-β-D-arabinofuranosyl-2-fluoroadenine 5'-triphosphate (F-ara-ATP) and ara-CTP] in acute myelogenous leukemia (AML) blasts during therapy. To complement these in vitro studies, ex vivo accumulation of ara-CTP was also investigated before and after G-CSF infusion. Patients (n = 5) treated on the fludarabine/ara-C/G-CSF regimen received a 30 mg/m² dose of fludarabine followed by a 2 g/m² dose of ara-C infused iv. for 4 h. Beginning at 24 h, and every day, patients received a 6-h infusion of 400 μg/m² G-CSF. At 48 h, the fludarabine and ara-C couplet was repeated. Comparison of F-ara-ATP pharmacokinetics in circulating AML cells of patients on the fludarabine/ara-C/G-CSF regimen demonstrated that the area under concentration time curve (AUC) of F-ara-ATP increased significantly (median, 1.4-fold; range, 0.9–1.5; P = 0.045) after G-CSF infusion. This was due to an increased rate of F-ara-ATP accumulation by AML cells. The AUC of ara-CTP, on the other hand, was not affected (median, 1.0-fold; range, 1.0–1.2; P = 0.571) after G-CSF infusion. Because fludarabine potentiates the accumulation of ara-CTP, the effect of G-CSF on ara-CTP metabolism may not be evident in the AML blasts of patients on the fludarabine/ara-C/G-CSF regimen. To determine the effect of G-CSF when ara-C was infused alone, four additional patients were treated on a pilot protocol in which ara-C (2 g/m²) was infused on days 1 and 3 and G-CSF on day 2. The AUC of ara-CTP accumulation in these patients decreased by a median of 48% after G-CSF infusion. Consistent with these in vivo investigations, ex vivo ara-CTP accumulation was decreased in the AML blasts after G-CSF infusion. Based on these data it could be concluded that (a) infusion of G-CSF before fludarabine augmented the rate of F-ara-ATP synthesis in circulating AML blasts during therapy, suggesting that G-CSF may benefit fludarabine therapy by biological modulation; (b) G-CSF did not increase ara-CTP accumulation, rather it may have caused it to decrease; and (c) these data imply that when G-CSF and ara-C are used in combination, administration of fludarabine prior to ara-C may maintain the ara-CTP AUC.

INTRODUCTION

ara-C, one of the most effective agents for therapy of AML (1), must be phosphorylated to its 5'-triphosphate (ara-CTP) to act as a cytotoxic agent (2, 3). Statistically significant correlations have been observed between clinical response to single-agent high-dose ara-C therapy given either on an intermittent schedule (4, 5) or by continuous infusion (6) and the pharmacokinetics of ara-CTP in circulating leukemia blasts. These results illustrate the importance of ara-CTP accumulation and retention to treatment outcome, and suggest the utility of therapeutic strategies that augment ara-CTP levels in leukemia blasts. Our previous studies demonstrated that infusion of fludarabine (F-ara-A monophosphate) prior to ara-C increases ara-CTP accumulation in circulating AML blasts (7), leukemic lymphocytes (8, 9), and leukemic lymphoblasts (10) of patients receiving this combination regimen. This biochemical modulation was due to the influence of F-ara-ATP on the rate of ara-CTP accumulation (7, 11). To further improve on this combination therapy, human recombinant G-CSF was selected to be included with the fludarabine and ara-C regimen (FLAG) based on clinical, biological, and pharmacologic rationales.

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The abbreviations used are: ara-C, cytarabine/1-β-D-arabinofuranosylcytosine; AGA, ara-C/G-CSF/ara-C; AML, acute myelogenous leukemia; ara-CTP, 1-β-D-arabinofuranosylcytosine 5'-triphosphate; AUC, area under concentration time curve; dCK, deoxycytidine kinase; F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-ATP, 9-β-D-arabinofuranosyl-2-fluoroadenine 5'-triphosphate; FLAG, fludarabine/ara-C/G-CSF; fludarabine, F-ara-A monophosphate; G-CSF, granulocyte-colony-stimulating factor.
Clinically it has been demonstrated that administration of G-CSF following completion of chemotherapy shortens the duration of neutropenia by approximately 1 week and reduces the frequency of documented infections in patients with previously treated acute leukemias (12). Because the fludarabine and ara-C combination therapy resulted in prolonged myelosuppression (13), addition of G-CSF would be beneficial with regard to faster recovery of neutrophils. Additionally, biological modulation by hematopoietic growth factors has shown that G-CSF, granulocyte-macrophage colony-stimulating factor, and interleukin 3 given alone or in combination before chemotherapy recruits AML blasts into the cell cycle, hence making them more vulnerable to chemotherapeutic agents such as ara-C and fludarabine (14, 15). Independent of these investigations, studies using cell lines derived from patients with AML suggested that G-CSF, but not granulocyte-macrophage colony-stimulating factor or interleukin 3, makes cells more sensitive to the cytotoxic effects of ara-C (16, 17). Finally, ara-C metabolism studies conducted in vitro demonstrated that incubation with growth factors alone and in combination results in potentiating intracellular accumulation of ara-CTP (15, 18, 19), its incorporation into DNA (20), and an increased incidence of apoptosis (21). Taken together, these rationales compelled us to add G-CSF to the fludarabine and ara-C combination regimen and conduct a Phase II study in patients with AML or myelodysplastic syndrome. The clinical analysis of this trial has recently been published (22).

The objective of the present investigation was to study the pharmacology of G-CSF in plasma and to analyze the influence of G-CSF on the pharmacokinetics of ara-CTP and F-ara-ATP in the circulating leukemia blasts of patients receiving this therapy. Because the biochemical modulation effect of fludarabine on ara-CTP accumulation may mask the possible biological modulation of ara-C metabolism by G-CSF, the pharmacokinetics of ara-CTP were studied in four additional patients when ara-C was infused without fludarabine. To complement these in vitro investigations, ex vivo studies were also conducted in circulating leukemia blasts obtained pre- and post-G-CSF infusion and incubated in vitro with ara-C and F-ara-A.

PATIENTS AND METHODS

Patients and Treatment Plans. Patients with newly diagnosed high-risk AML were treated with the FLAG protocol. Patient characteristics, along with the definition of high-risk AML and the clinical responses to these treatments have been published (22). The protocol was slightly modified for pharmacology and stipulated that the treatment (Fig. 1A) start with a 30-min infusion of fludarabine (30 mg/m²) followed 4 h later by a dose of 2.0 g/m² of ara-C infused over 4 h. Twenty-four hours after the start of therapy, patients received 400 μg/m² of G-CSF infused i.v. over 6 h. On day 3, a second and identical couplet of fludarabine and ara-C was infused. Subsequently, to complete one course of therapy, fludarabine and ara-C were administered daily for 4 days, whereas G-CSF, which started on day 2, was given each day until the recovery of neutrophil counts. For F-ara-ATP and ara-CTP pharmacokinetic investigations, five patients were studied who were receiving the FLAG protocol (Fig. 1A). One additional patient was investigated only for ex vivo accumulation.

To study the influence of G-CSF on ara-CTP metabolism when ara-C was infused without fludarabine, four additional patients were studied who were on an amended pilot protocol (AGA, Fig. 1B). The doses of ara-C and G-CSF were identical to those in the FLAG protocol. After 3 days of AGA treatment, patients were treated as with the FLAG regimen. The patients were selected for pharmacology studies on the basis of adequate numbers of circulating blasts (>5000/μl) to conduct the investigation and laboratory preparedness. They were informed about the investigational nature of this program in accord with institutional policies, and they signed an informed consent document to participate in the treatment and pharmacology studies.

Drugs. Berlex Laboratories, Inc. (Alameda, CA) provided fludarabine as a sterile, lyophilized powder free of antibacterial preservatives. Clinical ara-C was obtained commercially as Cytosar-U from the Upjohn Co. Recombinant human G-CSF (300 μg/vial) was obtained from Amgen, Inc. (Thousand Oaks, CA). The specific activity was 1 × 10⁶ units/mg protein as assessed by the cell mitogenesis assay. For in vitro investigations, F-ara-A was obtained by alkaline phosphatase treatment of fludarabine. F-ara-ATP was chemically synthesized (11). ara-C and ara-CTP were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Blood Samples for Clinical Pharmacology. To determine the pharmacokinetics of F-ara-ATP and ara-CTP in circulating blasts in patients treated with the FLAG protocol (Fig. 1A), 40-ml blood samples were obtained on days 1 and 3 before therapy and 10-ml samples were taken at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, and 9 h after the start of therapy. This schedule enabled the investigation of F-ara-ATP pharmacology for 9 h and ara-CTP for 5 h after the start of infusion with the respective drugs. On the AGA protocol (Fig. 1B), samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h after beginning ara-C infusion on days 1 and 3. All blood samples were collected in vacutainer tubes containing heparin. The tubes were immediately placed in an ice-water bath and transported promptly to the laboratory for processing. Control studies have demonstrated that under these conditions, leukemia cells are stable for at least 15 h with respect to size, membrane integrity, and cellular nucleotide content (23).
G-CSF Pharmacology. To determine G-CSF levels in patients on the FLAG regimen, blood samples obtained at 0, 1, 2, 3, 4, 5, and 7 h after the end of G-CSF infusion were centrifuged to remove cells. Plasma samples were diluted 100-fold before the assay was performed. G-CSF concentrations were determined by sandwich enzyme immunoassays using the Quantikine kit from R & D Systems, Inc. (Minneapolis, MN). The assay was performed as directed by the manufacturer (24) and G-CSF provided with the kit was used as a standard after reconstituting it in plasma obtained from healthy human donors. One hundred μl of standard (80 pg/ml to 5000 pg/ml) or diluted sample were added to each well, and 100 μl of diluent buffer were added to each before the assay was performed. The lower limit of detection was 100 pg/ml; the reaction was linear to 2500 pg/ml, which was the maximum concentration used. The results are expressed as the mean ± SD of values from patients 1–4.

Cellular Pharmacology. After removal of plasma, the cell pellet was resuspended in PBS (8.1 g NaCl, 0.22 g KCl, 1.14 g Na2HPO4, and 0.27 g KH2PO4/liter H2O, pH 7.4), and mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation procedures (23). After enumeration and cell size determinations with a Coulter counter (Coulter Electronics, Hialeah, FL), natural and arabinosyl nucleotides were extracted from blasts by HClO4, and ara-CTP and F-ara-ATP were separated from ribonucleoside triphosphates by high-pressure liquid chromatography on an anion exchange Partisol-10 SAX column (25). Ara-CTP and F-ara-ATP were quantified at 262 nm by electronic integration with reference to external standards. The intracellular concentrations of nucleotides were calculated and expressed as the quantity of nucleotides contained in the extract from a given number of cells of a determined mean volume. This calculation assumes that nucleotides are uniformly distributed in total cell water. The lower limit of sensitivity of this assay is 25 pmol triphosphate in an extract of 2 × 107 cells, which corresponds to a cellular concentration of about 5 μM (25).

Ex Vivo Studies. Blasts isolated before treatment or 1 day after G-CSF infusion were washed with PBS, suspended in an RPMI 1640 medium containing 10% FCS, and kept at 37°C in a humidified incubator containing 5% CO2. These were divided into two portions: one was incubated with 5 μM F-ara-A, whereas the other was treated with 10 μM ara-C for 4 h. Samples were taken every hour and processed for F-ara-ATP and ara-CTP analysis. The concentration of each drug was selected based on the plasma levels achieved in vivo as described before (7). Samples obtained from patients on the AGA protocol before and 24 h after G-CSF infusion were incubated with 10 μM ara-C. All samples were processed as described above to quantitate the accumulation of ara-CTP and F-ara-ATP.

Cell Cycle and Cell Type Distribution. Leukemia blasts obtained before the start of therapy and 24 h after G-CSF infusion were fixed in ethanol and analyzed for cell cycle distribution after staining DNA with propidium iodide. The percentage of each phase was determined with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To determine the percentage of leukemia cells (blasts plus promyelocytes) in the mononuclear cell preparations, 1–4 × 107 cells from samples obtained at 0 h and 8–10 h after the start of chemotherapy on days 1 and 3 were suspended in 100 μl medium with 20% FCS and spread on slides by cytopsin. Following staining with Giemsa dye, the differential morphology was analyzed using a total of 200 cells in each sample.

Calculations and Statistical Analysis. The levels of ara-CTP and F-ara-ATP obtained by high-pressure liquid chromatography analysis were normalized based on the concentrations of endogenous nucleotides during sampling times in each individual (8). The AUC for the accumulation of ara-CTP or F-ara-ATP in leukemia cells was estimated by gravimetric procedures. On the FLAG protocol, the AUC of F-ara-ATP represented the total AUC of accumulation and elimination, whereas the AUC of ara-CTP was the accumulation AUC until 4 h. On the AGA protocol, ara-CTP AUC was calculated by triangulation and represented the sum of the accumulation and elimination AUCs. Pharmacological data obtained during the first and second doses of ara-C or fludarabine were compared using the two-tailed, paired t test.

RESULTS

Plasma Pharmacology of G-CSF

The in vitro incubations with G-CSF, with or without other hematopoietic growth factors, that resulted in biological modulation or chemosensitization of AML cells were achieved at a G-CSF concentration of 500 units/ml (14). Hence, it could be postulated that plasma levels of 500 units/ml or greater would be sufficient for in vivo modulation by G-CSF. With this in mind, G-CSF pharmacokinetics was determined in the plasma of four patients following a 6-h infusion of 400 μg/m2 of G-CSF. The peak levels of G-CSF achieved at the end of the infusion in these patients were similar, ranging between 230 and 310 ng/ml (Fig. 2). The elimination of G-CSF, studied until 7 h after the end of infusion, was monophasic with a t1/2 of about 1.9 h. With these elimination kinetics, the plasma G-CSF levels were 20 ng/ml or 2000 units/ml 7 h after the end of the infusion. Extrapolating this rate of elimination, plasma G-CSF levels would be approximately 250 units/ml when patients received the next G-CSF infusion.

Effect of G-CSF on F-ara-ATP Pharmacokinetics

For illustrative purposes, the pharmacokinetics of F-ara-ATP in circulating leukemia blasts of two patients (patients 3 and 5) after the first two doses of fludarabine is presented in Fig. 3. F-ara-ATP accumulation was linear for 2 h; thereafter the rate diminished until a peak was reached 3 or 4 h after the start of the infusion. G-CSF augmented the rate of F-ara-ATP accumulation in these patients (Fig. 3), which resulted in an increase in the peak concentration of F-ara-ATP. The elimination of F-ara-ATP, studied for 5 to 6 h after peak levels, was linear when evaluated by semilogarithmic plots (not shown), and was not altered after G-CSF infusion. As summarized in Table 1, the increase in the peak F-ara-ATP in the blasts of four of five patients ranged between 1.3- and 2.0-fold (P = 0.045). In one patient (patient 4), G-CSF infusion did not potentiate F-ara-ATP accumulation. Comparison of the F-ara-ATP AUC of accumulation and elimination before and after G-CSF demonstrated that the AUC increased by a median of 1.4-fold in the cells of these patients.
Modulation of ara-CTP and F-ara-ATP Accumulation by G-CSF

To determine whether the effect of G-CSF on the F-ara-ATP accumulation rate would occur during ex vivo incubation, leukemia blasts obtained from each patient before therapy and 24 h after G-CSF infusion were washed and incubated in vitro with 5 μM F-ara-A. Under these conditions, F-ara-ATP accumulated at linear rates during the 4-h incubation (not shown). Although there was interpatient heterogeneity in the absolute values, F-ara-ATP accumulation increased in cells from four patients studied after G-CSF infusion (Table 1, P = 0.049). The median increase (4.4-fold) was greater than that observed during therapy (Table 1).

Effect of G-CSF on ara-CTP Pharmacokinetics

FLAG Protocol. The observation that the plasma levels of G-CSF were in the range of those which potentiated ara-CTP accumulation in vitro and which augmented F-ara-A metabolism in vitro (Table 1) suggested that G-CSF would also modulate accumulation of ara-CTP. To investigate this possibility, ara-CTP concentrations in blasts were quantitated hourly until 5 h after the start of ara-C infusion. ara-CTP pharmacokinetic profiles in the circulating blasts of two representative patients (patients 3 and 5) are illustrated in Fig. 4. Consistent with previous findings (7), the peak concentrations of ara-CTP were generally achieved at 3 or 4 h after the start of ara-C infusion. In contrast with its actions on F-ara-ATP accumulation, G-CSF infusion did not significantly increase ara-CTP accumulation (Fig. 4). The ara-CTP peak and AUC values from all patients are summarized in Table 2. The peak concentrations of ara-CTP varied among patients, but were similar within a patient before and after G-CSF infusion. This is indicated by the median ratio of peak values after and before G-CSF, which was unity (range, 0.8–1.2; P = 0.571). Consistent with the peak values of ara-CTP, the ratio of AUC of ara-CTP remained constant after and before G-CSF infusion (median, 1.0, range, 1.0–1.2; Table 2).

To evaluate whether G-CSF infusion affected ara-CTP accumulation in vivo, blasts obtained before treatment and 1 day after G-CSF infusion were incubated in vitro with 10 μM ara-C. As illustrated in Fig. 5, the rate of ara-CTP accumulation began to plateau by 4 h when cells (patients 1 and 6) were obtained before treatment. After G-CSF infusion, however, the rate of ara-CTP accumulation was reduced compared with that observed before treatment. This resulted in lower ara-CTP peaks after G-CSF infusion (median, 1.6-fold; range, 1.2–3.6-fold, P = 0.032; Table 2).

AGA Protocol. To eliminate the interaction of fludarabine on ara-CTP metabolism during therapy or ex vivo, the effect of G-CSF on the pharmacokinetics of ara-CTP was studied in four patients on the AGA protocol. Leukemia blasts were isolated after the start of ara-C infusion, 1 day before, and 1 day after G-CSF administration. Pharmacokinetic profiles of two representative patients (patients 7 and 9) are shown in Fig. 6. The ara-CTP accumulation rate was generally linear during each ara-C infusion. After G-CSF infusion, the rate of ara-CTP accumulation was reduced compared with that observed before G-CSF infusion. This resulted in lower ara-CTP peaks after G-CSF infusion (P = 0.034, Table 3). In contrast, the rate of ara-CTP elimination from the leukemia blasts was not significantly different before and after G-CSF infusion (not shown), suggesting that the effect was on the accumulation of ara-CTP. These patterns were consistent in all four patients; ara-CTP AUC was decreased by a median of 48% in circulating blasts.

The effect of G-CSF infusion on the ex vivo accumulation of ara-CTP was analyzed in blasts obtained before therapy and 1 day after the start of the first G-CSF infusion (Fig. 7). Consistent with the in vivo findings, ara-CTP accumulation was lowered in the blasts of all four patients (Fig. 7 and Table 3, P = 0.048); ara-CTP peak levels were decreased to a median of...
**Table 1** Effect of G-CSF infusion on F-ara-ATP accumulation during FLAG therapy and *ex vivo* F-ara-A incubations

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* Concentration at 3 h.
* Concentration at 2 h.
* nd, not determined.

Fig. 4 Effect of G-CSF on ara-CTP pharmacokinetics in circulating leukemia blasts during FLAG therapy of patient 3 (A) and patient 5 (B). Box on the abscissa in each panel, time of ara-C infusion. ara-CTP accumulation was quantitated before G-CSF infusion, *i.e.*, day 1 of therapy (○), and after G-CSF infusion, *i.e.*, day 3 of therapy (●).

74% after G-CSF. These data demonstrated that G-CSF had no positive effect on *in vivo* or *ex vivo* ara-C metabolism. Because G-CSF did not alter elimination of ara-CTP, the negative influence was most likely directed toward ara-CPT synthesis.

**Effect of G-CSF on Cell Cycle and Cell Type Distribution**

Mononuclear cell populations isolated before and after G-CSF infusion were evaluated for possible changes in cell cycle distribution and differences in morphology that might be related to the growth factor. The percentage of cells in S phase, which was between 2 and 4% at the start of therapy, did not change 1 day after G-CSF infusion (data not shown). Cytospin preparations from five patients (three on FLAG and two on AGA) were analyzed and scored for cell differential morphologies. Before therapy, the median blast percentage in the isolated mononuclear cell fraction was 83%; 24 h after G-CSF infusion this value was 70%. This was associated with a slight trend toward more mature cells (segments, bands, and metamyelocytes); 8% in the pre-G-CSF sample and 14% after G-CSF infusion.

**DISCUSSION**

Present studies conducted in blasts isolated during therapy and after *ex vivo* treatment demonstrated that G-CSF infusion augmented F-ara-ATP accumulation in four of five patients studied *in vivo* and in four of six patients studied *ex vivo* (Table 1). This action has clear implications for therapeutic applications involving combinations of G-CSF and fludarabine. Previous studies have demonstrated a direct relationship between loss of clonogenicity and the intracellular accumulation of F-ara-ATP (26) and its incorporation into DNA (27). Fludarabine administered at 100–125 mg/m²/day resulted in a greater accumulation of F-ara-ATP in blasts than lower doses during Phase I studies (28). Because of severe central nervous system toxicity associated with these doses (29, 30), the currently used doses range between 25 and 30 mg/m²/day for single-agent and combination therapies (13, 22, 31–33). Patients with AML and myelodysplastic syndrome with normal renal function when treated with the FLAG protocol which includes 25–30 mg/m²/day of fludarabine did not experience central nervous system-related toxicity (34). Because only this much fludarabine (25–30 mg/m²/day) can be given, combining G-CSF with fludarabine appears to be a means of biological modulation which increases the F-ara-ATP concentration in leukemia cells beyond that clinically possible with fludarabine alone. Furthermore, coadministration of fludarabine with G-CSF could benefit patients for whom fludarabine dose reduction is indicated, such as individuals with impaired renal function or when used in combination with other agents (35).

In contrast to the *in vivo* and *ex vivo* modulation of F-ara-ATP levels by G-CSF, ara-CTP accumulation was only increased during *ex vivo* incubations; no change in ara-CTP levels was observed during therapy in circulating blasts after G-CSF infusion. The following possibility may explain this discrepancy between the *ex vivo* and *in vivo* modulatory effect of G-CSF on ara-C metabolism in patients on the FLAG protocol. G-CSF may augment ara-CTP accumulation, but this would not be evident during *in vivo* ara-CTP accumulation because during therapy ara-C was not infused alone, but was coupled with fludarabine. Because fludarabine potentiates ara-CTP accumulation by biochemical modulation (7, 8), the rate of ara-CTP accumulation may be maximized by fludarabine and the influence of G-CSF would not be evident. Alternatively, there may be a negative mechanism, such as feedback inhibition of dCK by ara-CPT, that does not permit ara-CPT to exceed a
Table 2  Effect of G-CSF infusion on ara-CTP accumulation during FLAG therapy and ex vivo ara-C incubations

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* Four-h value.

* nd, not determined.

Fig. 5  Effect of G-CSF on ara-CTP accumulation ex vivo in leukemia blasts of patient 1 (A) and patient 6 (B). ○, sample taken before the start of therapy; ●, sample taken 1 day after G-CSF infusion. Blasts isolated from these samples were incubated with 10 µM ara-C, and ara-CTP accumulation was quantitated every hour up to 4 h by high-pressure liquid chromatography as described in "Patients and Methods."

One possibility is inherent in the design of the treatment protocol (AGA); the interference of first ara-C dose infused 1 day prior to G-CSF administration may have adversely affected metabolism of the second ara-C infusion. One way to avoid the likelihood of this interference is to compare ara-CTP accumulation in two cohorts of AML patients: one receiving ara-C alone and the other infused with G-CSF followed by ara-C. However, the accumulation of ara-CTP in circulating blasts of patients show a wide range of interpatient heterogeneity (4–6). Therefore, it would be difficult to discern the effect of G-CSF on ara-C metabolism among two groups. With that in mind, it was necessary to measure ara-CTP accumulation within the same individual. Our previous studies have shown that AUC of ara-CTP remained similar during two consecutive doses of ara-C infusions (38, 39). Fludarabine, however, when administered between these two ara-C doses, increased the AUC of the second ara-CTP by 2-fold, demonstrating that a modulatory effect could be studied in individuals who served as their own controls (7, 8). Hence the decreased AUC of ara-CTP during the
second ara-C infusion in patients on AGA protocol appears to be the effect of G-CSF administration, which was in contrast to the
in vitro modulatory effect of G-CSF on ara-C.

There are other examples which demonstrate that the mechanisms by which growth factors affect the cells in vitro may not be directly applicable in vivo. For instance, in vitro GM-CSF incubations were reported to augment ara-CTP accumulation in blasts (18, 36), but failed to provide a similar response during therapy of CML in the blastic phase (40). In a report investigating a murine leukemia model, G-CSF promoted the growth of leukemia cells in vitro, yet when mice were given injections of the leukemia cells and then G-CSF, survival of the animals was improved (41).

The dose and duration of the exposure of AML blasts to the growth factor is important. Although there are relatively few investigations involving G-CSF, in vitro incubations with 500 units/ml for 24 to 48 h was sufficient to recruit cells into cell cycle and to increase the ara-C sensitization (14). The pharmacokinetic profile of G-CSF in plasma demonstrated a continuous exposure of circulating leukemia blasts with similar or higher levels of G-CSF during therapy (Fig. 2). Additionally, because G-CSF was given daily, it is likely that these levels were maintained throughout therapy. Hence, the discrepancy between in vitro and in vivo modulation of ara-CTP by G-CSF cannot be attributed to differences in G-CSF exposure.

Cytokinetic changes have been observed in AML after growth factors (14, 15, 18, 42) or after high-dose ara-C therapy (43-45). The lack of S-phase recruitment 1 day after G-CSF infusion in the present study may reflect the short observation interval. Additionally, growth factor-mediated or ara-C-induced kinetic changes in blasts were generally observed in the bone marrow aspirates and not peripheral blood. Although a relationship has been observed in S-phase-specific changes in bone marrow and peripheral blood leukemia cells after ara-C infusion (43), the kinetic responses were not consistent in all patients (43-45) and where observed were maximum between 24 and 30 h (43, 45). The objective for cell cycle analyses in the Ficoll-Hypaque buoyant cells (present study) was to determine the percentage of S phase in pre- and postsamples used for ex vivo incubations. There is a prevailing perception that S-phase cells are capable of greater accumulation of ara-CTP. A rationale for this might be that because phosphorylation of ara-C by dCK is the rate-limiting step in ara-CTP accumulation (46, 47) and dCK activity has been shown to be doubled in extracts of S-phase cells (48-50), it may be expected that recruitment of blasts into S phase by growth factors (14, 15, 18, 42) would increase ara-CTP accumulation. However, in whole cells, such a cycle-phase dependency of ara-CTP accumulation has not been observed (51-54), probably because dCK is not a markedly cell cycle-specific enzyme (48-50, 54) and its activity is regulated in intact cells by dNTP pools (46, 47) which also fluctuate with the cell cycle (51, 52).

Another possibility to explain modulation of nucleotide metabolism arises from the observation that growth factors such as GM-CSF increase the number of nucleoside transporters on leukemia blasts during therapy (42, 55). It is possible that increased expression of nucleoside transporters, particularly those which function in a concentrative manner, could result in a higher concentration of nucleoside analogue in the cell for greater conversion to the active triphosphate. In the present trial, this would be an unlikely explanation for ara-CTP metabolism because at the ara-C infusion rate used (0.5 g/m²/h), the plasma level of ara-C was at least 10 μM before any growth factor infusions (7). At this concentration, the rate of ara-C phosphorylation in blasts is saturated (39, 56); hence, a further increase in the intracellular ara-C concentration would not augment ara-CTP accumulation. In fact, an increase in the concentration of ara-C intracellularly may result in a negative effect because
recent studies have shown that concentrations of ara-C greater than 10 \( \mu M \) result in a substrate inhibition of the dCK (57). This is consistent with the data which indicate that human leukemia cell lines or AML cells incubated with greater concentrations of ara-C have less ara-CTP accumulation compared with that formed by 10 \( \mu M \) ara-C (11, 53). If G-CSF increases the transport-dependent cellular concentration of ara-C, this hypothesis offers an explanation as to why the AGA protocol failed to augment ara-CTP accumulation. With the FLAG protocol, however, it is likely that ara-CTP accumulation was stabilized by the positive biochemical modulation effect of fludarabine which activates dCK (7, 8, 11), and the G-CSF-mediated negative effect was not evident.

In contrast, based on this hypothesis the effect of G-CSF on fludarabine would be positive. A G-CSF-stimulated increase in concentrative nucleoside transporters would also augment the entry of F-ara-A into blasts. At the present infusion rate, the peak plasma concentration of F-ara-A is about 3 \( \mu M \) (58). Unlike ara-C, the phosphorylation of F-ara-A by dCK is not saturated because the affinity of dCK for F-ara-A \( [K_m = 300-600 \mu M] \) is much less than that for ara-C \( [K_m < 10 \mu M] \) (59, 61). The correlation between higher plasma F-ara-A and greater cellular F-ara-ATP has been described during therapy when fludarabine was infused at 50 and 100 mg/m\(^2\) (28). Taken together, these results lead us to postulate that G-CSF may affect the nucleoside transporters, and that this action may be positive or negative depending on the plasma concentration of the nucleoside analog and the affinity of dCK for each nucleoside.

These studies demonstrate that in contrast to results observed during \textit{in vitro} incubation with G-CSF, the biological modulation of ara-C metabolism by G-CSF was not evident during therapy. Rather, both the \textit{in vivo} and \textit{ex vivo} data demonstrate that G-CSF infusion may result in lowered ara-CTP accumulation. When ara-C is administered as a couplet with fludarabine, the AUC of ara-CTP is maintained after G-CSF infusion. These data further emphasize the biochemical modulation of ara-CTP by fludarabine during therapy (7, 8). The positive results obtained regarding augmentation of fludarabine metabolism by G-CSF-mediated biological modulation warrant further investigations.

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Modulation of the cellular metabolism of cytarabine and fludarabine by granulocyte-colony-stimulating factor during therapy of acute myelogenous leukemia.


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