



Complexity of apoptotic pathways in leukemia treated with chemotherapy or cellular immunotherapy

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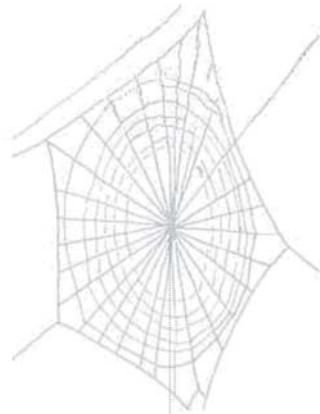
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CHAPTER

2

The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells



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ABSTRACT

Background and objective. Cytarabine (Ara-C) is commonly used for the treatment of acute leukemia. Incorporation of Ara-C into DNA is a key event in the mechanism of killing of proliferating leukemic cells. Previously, we demonstrated cytotoxicity of Ara-C to proliferating, but unresponsiveness to resting (G₀) malignant cells from patients with acute leukemia. In contrast, here we show unexpected apoptosis of G₀-B-CLL cells by Ara-C in a dose dependent manner. In this study we analyzed which cellular processes were involved in Ara-C-mediated killing of G₀-B-CLL cells.

Experimental design. Using primary B-CLL cells (>98% in G₀), we examined the mechanisms of Ara-C-mediated apoptosis in resting G₀ cells. CFSE-based cytotoxicity assays combined with cell cycle analysis, were used to perform long-term analysis of Ara-C-mediated killing of B-CLL cells. The effect of Ara-C on DNA and RNA synthesis effects were studied using various ³H-incorporation experiments.

Results. Ara-C-mediated cell death of B-CLL cells showed the characteristics of normal apoptosis, such as phosphatidyl serine exposure and caspase activation. The mechanism of killing of quiescent B-CLL cells by Ara-C was shown not to be dependent on DNA replication. In contrast, CD40L-activated B-CLL cells showed S-phase specific depletion of proliferating CLL cells. We demonstrated Ara-C to be converted into its active triphosphate by G₀-B-CLL cells, coinciding with 30% inhibition of RNA synthesis.

Conclusions. In conclusion, our data indicate that Ara-C can induce apoptosis in resting G₀-B-CLL cells using a mechanism independent of cell proliferation and DNA replication but associated with inhibition of RNA synthesis and downregulation of Mcl-1.

INTRODUCTION

Ara-C, an analogue of deoxycytidine (dC), has been the most important compound in the chemotherapy regimens of patients with acute myeloid leukemia for 40 years. The close structural similarity of Ara-C with dC allows it to be metabolized by the same metabolic pathways as used for the normal processing of dC.¹ Cytarabine is transported into cells in its nucleoside form (Ara-C), and is then converted by a series of cellular enzymes to the triphosphate Ara-CTP, the active metabolite of cytarabine. In proliferating cells, Ara-CTP competes with the natural nucleoside triphosphate dCTP for incorporation into replicating DNA. This incorporation causes termination of the daughter strand DNA synthesis at the sites of drug incorporation. Furthermore, Ara-C inhibits DNA repair by blocking topoisomerase I-mediated DNA religation.² Since the cytotoxicity of Ara-C is dependent on DNA synthesis, maximal effect of Ara-C on cell death is observed during the S-phase of the cell cycle. In line with this,

we previously demonstrated selective lysis of leukemic cells from patients with acute leukemia in active stage of the cell cycle, whereas G₀ cells were unresponsive to several cytostatic agents including Ara-C.^{3,4}

In contrast, here we show Ara-C-mediated cell death of resting cells derived from patients with chronic lymphocytic leukemia (CLL). CLL is an indolent malignancy, characterized by progressive expansion of a leukemic lymphoid clone, resulting in the accumulation of leukemia cells at the G₀ quiescent stage of the cell cycle in bone marrow, blood, and other extramedullary sites such as spleen, liver and lymph nodes. There is only a small pool of proliferating cells,⁵ as determined by the extremely low incorporation rate of ³H-thymidine by leukemic lymphocytes,⁶ or by flow cytometric analysis of newly synthesized DNA.⁷

Recently, nucleotide analogues like fludarabine (F-Ara-A) have been introduced as front-line therapy in the treatment of CLL.⁸ Since the incorporation of nucleotide analogues like fludarabine and cytarabine (Ara-C) into DNA has been considered to be a key event in causing cytotoxicity in proliferating leukemic cells, we hypothesized that nucleotide analogues induce cell death of B-CLL cells by a different mechanism. Some studies already revealed that mechanisms of killing resting cells include inhibition of cellular DNA repair, inhibition of RNA synthesis, activation of pro-apoptotic proteins (Apaf-1), and downregulation of survival proteins (Bcl-2).^{9,10}

In this study, we analyzed which cellular processes were involved in Ara-C-mediated killing of B-CLL cells. We first examined the relation between Ara-C-mediated apoptosis and the cell cycle status of the B-CLL cells using both quiescent and proliferating (CD40L-activated) B-CLL cells. Since we observed that different mechanisms were involved in Ara-C-mediated killing of G₀-B-CLL cells compared to proliferating B-CLL cells, we studied whether Ara-C could be converted into Ara-CTP in quiescent B-CLL cells, a reaction essential for the cytotoxicity of Ara-C in proliferating cells.^{2,11} Furthermore, the effect of Ara-C on RNA synthesis in G₀-B-CLL cells was investigated. Finally, we analyzed whether Ara-C-mediated killing of G₀-B-CLL cells was initiated via downregulation of survival proteins and dependent on caspase activation. In contrast to the mechanism of killing by Ara-C of proliferating leukemic cells, our results demonstrate that Ara-C-mediated killing of B-CLL cells is not dependent on cell proliferation and DNA incorporation, but probably requires Ara-CTP formation and may involve inhibition of RNA synthesis.

MATERIALS AND METHODS

Cells and culture conditions

Peripheral blood was obtained from 4 CLL patients (3 female, 1 male) after informed consent. The materials we used were collected at times that patients had not received any form of therapy concerning their CLL, except one patient who was mildly treated with chlorambucil 2 years earlier. The percentage of CLL cells in peripheral blood samples was in all cases over 90% and these cells showed typical CLL characteristics including expression of CD19, CD5 and CD23. Mononuclear cells were separated by density centrifugation using Ficoll Isopaque, and either cryopreserved in liquid nitrogen or used directly for experiments. Isolated B-CLL cells were cultured at 37°C and 5% CO₂ for a maximum of 10 days in IMDM supplemented with 3 mM L-glutamine, 50 µg/mL streptomycin, 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium), and 10% pooled human serum. To induce proliferation, B-CLL cells were cultured for 7 days on irradiated (70 Gy) CD40L-transduced mouse fibroblasts ¹² (LTK-CD40L; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). Irradiated LTK-CD40L cells were cultured overnight at 37°C and 5% CO₂ in a 24 wells plate at a concentration of 2 x 10⁵ cells/well in IMDM supplemented with 10% fetal bovine serum (FBS, Cambrex), 3 mM L-glutamine, 50 µg/mL streptomycin and 50 U/mL penicillin. After one day, the medium was removed, and B-CLL cells were added at a concentration of 1x10⁶/mL/well, and cultured in the medium described for resting B-CLL cells, supplemented with 500 IU/mL IL-4 (Schering-Plough, Amsterdam, The Netherlands).

Reagents

Apoptosis was induced with the following agents: cytosine arabinoside (Ara-C), 9-β-D-arabinosyl-2-fluoroadenine (fludarabine) (both from Sigma-Aldrich, St. Louis, MO, USA), camptothecin (Alexis Corp., Lausanne, Switzerland), and as a negative control deoxycytidine (Sigma) was used.

Cytotoxicity assays

Cytotoxicity was measured using 24 or 40-hr ⁵¹Cr release assays as described previously ³ or using carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity assays as described by Jedema *et al.*, ¹³ with some alterations. Target cells were labeled with 5 µM CFSE (Molecular Probes Europe, Leiden, The Netherlands). For the cytotoxicity assay, 25,000 cells/well (100 µL) were plated in 96-well microtiter plates (all in triplicate). Ara-C (10⁻⁵ M) was added in a volume of 50 µL/well. After 24, 48, 72 and 120 hrs, FACS analysis was performed. To exclude dead cells from the analysis, 7-amino-actinomycin D (7-AAD) (2 µg/mL final concentration) or Propidium Iodide (PI) (1 µg/mL) (both Sigma-Aldrich) was added, and samples were mixed properly and directly analyzed on a flowcytometer (Becton Dickinson (BD), San Jose, CA, USA). To allow quantitative analysis of the viable cells, the wells were harvested and transferred to FACS tubes containing 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). This was done immediately prior to the analysis to avoid the formation of complexes between the cells and the beads. For each sample 5,000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (7-AAD/PI⁻) CFSE⁺ target cells. The percentage of specific lysis was calculated as follows:

$$[(\text{mean absolute number of viable CFSE}^+ \text{ target cells in control medium} - \text{absolute number of viable CFSE}^+ \text{ target cells}_{\text{experimental}}) / (\text{mean absolute number of viable CFSE}^+ \text{ target cells in control medium})] * 100.$$

To determine the role of caspases in the induction of Ara-C-mediated cell death, CFSE-labeled B-CLL cells were pre-incubated for 2 hrs with 100 µM of irreversible cell-permeable broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK), caspase-3- (z-DEVD-FMK), caspase-8 (z-LETD-FMK) or caspase-9 inhibitor (z-LEHD-FMK) (all Alexis) prior to apoptosis induction. After 48 hrs of exposure to Ara-C, inhibition of apoptosis was determined using the CFSE-based cytotoxicity assay. To specifically calculate the effect of the caspase-inhibitors on Ara-C-induced apoptosis, caspase-inhibitors were also added to the medium control, and a correction was performed for inhibition of spontaneous cell death by the caspase-inhibitors.

Annexin V / PI staining

Apoptosis was determined by Annexin V and PI staining. Annexin V specifically binds to phosphatidyl serine (PS), a phospholipid that becomes exposed on the surface of cells undergoing apoptosis. Dual staining with PI enables the identification of early apoptotic cells that have not yet lost their membrane integrity.¹⁴ Cells were collected and resuspended in binding buffer 10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) at a concentration of 10⁶ cells/mL. Cells were incubated at room temperature for 20 min with 0.1 µg/mL FITC-labeled Annexin V (Bender MedSystems, Vienna, Austria), washed, and resuspended in binding buffer. PI was added at a final concentration of 1 µg/mL, and the cells were directly analyzed on a flowcytometer.

Cell cycle analysis

For cell cycle analysis, 10⁶ cells were washed with PBS, fixed and permeabilized for 10 min at 4°C using a solution of 80 µg/mL L-alpha-lysolecithin (Sigma-Aldrich) in 1% paraformaldehyde, washed with PBS containing 0.8 g/L human albumin, and stained for 30 min at 4°C with FITC-conjugated anti-Ki-67 MoAbs (clone MIB-1; Immunotech, Marseille, France) or isotype controls. After washing, cells were incubated with 20 µg/mL RNase (Sentra Systems, Minneapolis, USA) for 5 min at 37°C, and finally stained with 50 µg/mL PI. Cells were subjected to flow cytometric analysis of DNA content using a Becton Dickinson flow cytometer. The percentages of cell cycle distribution were calculated by CellQuest software.^{3,4}

Determination of dCK activity

dCK activity, expressed in amounts of substrate converted in time per mg of total protein (pmol min⁻¹ mg⁻¹), was measured in duplicate experiments using a dCK protocol as originally described by Cheng *et al.*¹⁵ with minor modifications described by Veugier *et al.*¹¹ [³H]-labeled Ara-C (specific activity 24 Ci mmol⁻¹; Campro Scientific BV, Veenendaal, The Netherlands) was used as substrate for dCK. Specific Ara-CTP formation was measured using more stringent washing of the DEAE-coated paper discs with a buffer consisting of 0.6 M HCl and 1.5 M NaCl.

³H-thymidine and ³H-uridine incorporation assay

B-CLL cells were incubated with either medium, or 10 µM of Ara-C or fludarabine in a round-bottom 96-wells plate (0.5x10⁶ cells/150 µL) in a humidified atmosphere at 37°C and 5% CO₂. To measure DNA or RNA synthesis, after 4 hrs of exposure ³H-thymidine or ³H-uridine (1 µCi/well; Amersham BioSciences, Freiburg, Germany) was added, respectively, and the cells were cultured for an additional 16 hrs. Prior to harvesting, from each ³H-thymidine-incubation 4 wells were pooled (total amount of cells is 2x10⁶/incubation). Using an automatic microharvester cells were harvested on 96-wells Unifilter type GF/C plates (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA), which specifically bind DNA and RNA. After drying, scintillation fluid (MicroScint-O; Perkin Elmer) was added and incorporation of radioactively labeled precursors was determined in a liquid scintillation counter and expressed in counts per minute (CPM).

SDS PAGE and Western Blot analysis

Apoptosis was induced in B-CLL cells using 10⁻⁵ M Ara-C using a time range from 0 to 48 hrs of exposure. At each time point, whole cell lysates of 6x10⁶ cells were obtained by freeze-thawing the cells in 100 µL NP40-lysisbuffer (50 mM Tris-HCl, pH 7.6, 5 mM dithiotreitol, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and Western Blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described.¹¹ Primary antibody incubations were performed for 2 hrs in 1% Ecl-blocking reagent. Cyclin D3 specific antibody (clone Ab-2) used for cell cycle analysis was purchased from Oncogene Research Products (San Diego, USA). Bcl-2 antibody (1:50,000) was from Pharmingen (San Diego, CA, USA), Bax (1:50,000) and Mcl-1 (1:1,000) specific antibodies were from SantaCruz Biotechnology (Santa Cruz, CA, USA). Caspase cleavage was detected using antibodies specific for caspase-8 (1:2,000; Pharmingen), caspase-9 (1:8,000; Calbiochem, San Diego, CA, USA), or cleaved caspase-3 (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA). After 3 washing steps, membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3,000; Promega, Madison, USA). β-actin expression was determined on the same blots after stripping for 30 min at 65°C with buffer containing 0.5% SDS using anti-β-actin clone AC-15 moAbs (1:100,000; Sigma-Aldrich).

RESULTS

Sensitivity of B-CLL cells to various apoptotic agents

We analyzed the sensitivity of B-CLL cells to Ara-C-, deoxycytidine-, camptothecin- and fludarabine-induced apoptosis using both ⁵¹Cr release and CFSE-based cytotoxicity assays. A representative result of a CFSE-based cytotoxicity assay showing lysis after 40 hrs of exposure is illustrated in Figure 2.1. B-CLL cells were sensitive not only to fludarabine, but also to other chemotherapeutic agents tested including the S-phase-specific agent Ara-C. Deoxycytidine (dC) used as a non-specific control, did not induce cell death.

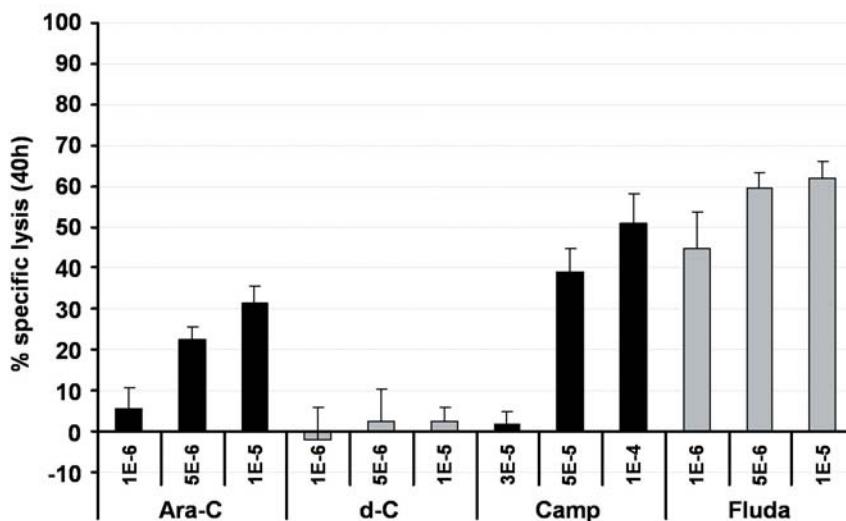


Figure 2.1. Susceptibility of B-CLL cells to various apoptotic agents.

Lysis of B-CLL cells after exposure for 40 hrs to different concentrations of Ara-C, deoxycytidine (dC), camptothecin (Camp) and fludarabine (Fluda) was determined using CFSE-based cytotoxicity assays. A representative result (in triplicate + SD) of 3 independent experiments is shown.

Since Ara-C has been considered to be S-phase specific and B-CLL is characterized by the presence of mainly quiescent cells, we investigated the characteristics of Ara-C-mediated cell death of B-CLL cells in more detail.

Long-term analysis of Ara-C-treated B-CLL cells using a quantitative CFSE-based cytotoxicity assay was performed to study the kinetics of Ara-C-mediated killing of B-CLL cells (Figure 2.2A). When B-CLL cells were cultured for up to 5 days in the absence of Ara-C, the percentage of viable B-CLL cells did not diminish the first 48 hrs, and only after 5 days declined from 100 to 83%, due to spontaneous apoptosis. CFSE intensity of the B-CLL cells was constant, showing that culturing in medium

did not induce cell proliferation. Culturing in presence of Ara-C caused a reduction of 50% of the cells after 48 hrs, and almost a complete deletion of B-CLL cells after 5 days, demonstrating the susceptibility of B-CLL cells to the S-phase-specific agent Ara-C.

To exclude a direct non-specific cytolytic effect of Ara-C to B-CLL cells, we investigated whether Ara-C-mediated cell death of B-CLL cells was characterized by PS exposure, an early characteristic of apoptosis. Annexin V binding and counterstaining with Propidium Iodide (PI) was used to distinguish between dead and early apoptotic cells. Figure 2.2B shows that B-CLL cells first became annexin V positive before staining with PI, illustrating that these cells died via an apoptotic mechanism.

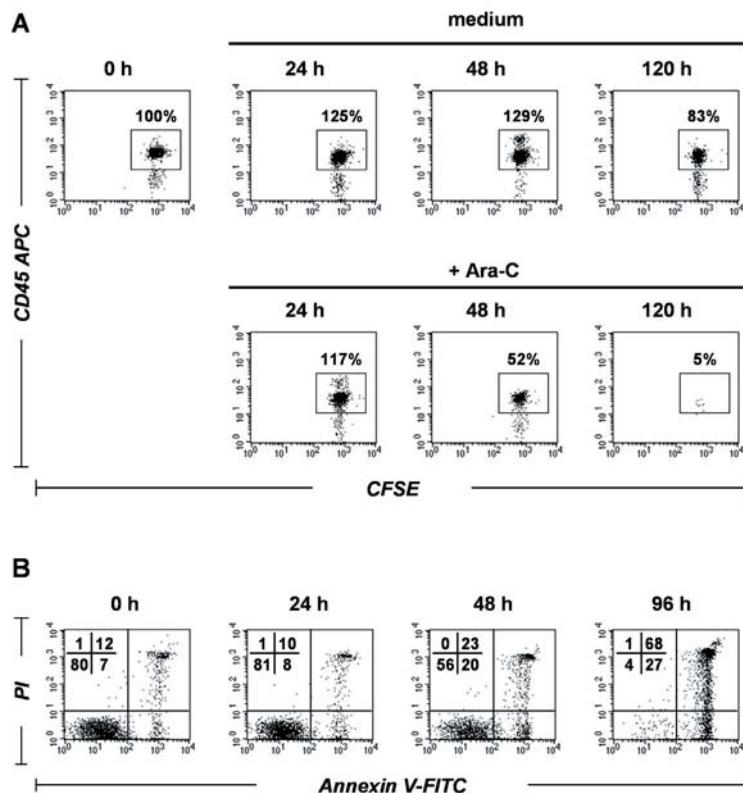


Figure 2.2. Long-term analysis of Ara-C-mediated apoptosis of B-CLL cells.

(A) Kinetics of Ara-C-mediated and spontaneous lysis of B-CLL cells using the CFSE-based cytotoxicity assay. For each time point, the percentage of surviving (CFSE⁺, CD45⁺, 7-AAD⁻) cells, compared to the medium control at day 0, is indicated. A representative result of 3 independent experiments is shown. (B) Phosphatidyl serine exposure by B-CLL cells treated for 0-96 hrs with 10^{-5} M Ara-C, as determined by FACS analysis using annexin V-FITC staining combined with PI. Dotplots are representative of 2 independent experiments.

Relation between Ara-C-induced apoptosis and cell cycle status of B-CLL cells

To investigate the relation between Ara-C-induced apoptosis and the cell cycle status of B-CLL cells, we exposed both resting (unmanipulated) and proliferating B-CLL cells (cultured for 7 days on CD40L)¹² to Ara-C for 0 to 120 hrs, and performed cell cycle analysis combining FITC-labeled anti-Ki-67 antibodies and Propidium Iodide (PI) DNA staining (Figure 2.3A). The percentages of cells in G₀/G₁, S or G₂/M phase were determined based on DNA content. Double staining with Ki-67-FITC was used to discriminate between cells in resting G₀ phase defined as cells lacking expression of the nuclear protein Ki-67,^{16;17} and cells in activated G₁ phase. At day 0 of the experiment, resting B-CLL cells were almost all (> 98%) in G₀-phase of the cell cycle, whereas proliferating B-CLL cells showed a cell cycle distribution of 15% of cells in S/G₂M, 69% in G₁, and only 16% in G₀ phase of cell cycle. Both populations of B-CLL cells responded in a different way to Ara-C. Although after 48 hrs of incubation already 50% of primary B-CLL cells were killed, no evidence of alteration in cell cycle status was observed (Figure 2.3Ai), indicating that these cells were killed by Ara-C in G₀-phase of cell cycle. In contrast, in proliferating B-CLL cells slow lysis but specific depletion of cells in S- and G₂/M phase of the cell cycle was observed during exposure to Ara-C.

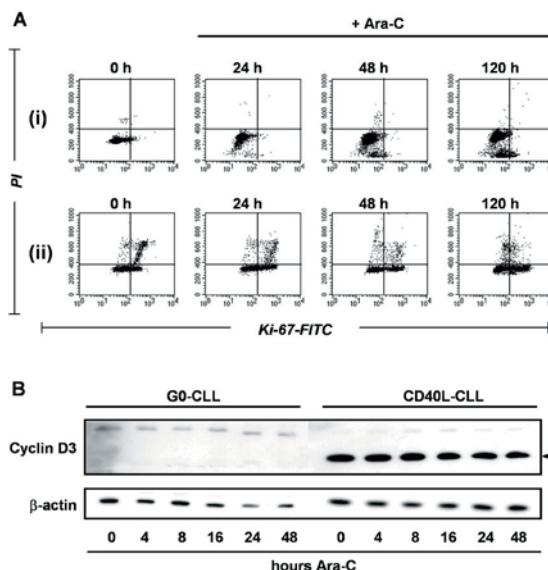


Figure 2.3. Cell cycle analysis of primary and proliferating B-CLL cells after treatment with Ara-C. (A) Cell cycle status of (i) primary B-CLL cells and (ii) CD40L-CLL cells after treatment with Ara-C for 0, 24, 48 or 120 hrs determined by flow cytometric analysis using Ki-67-FITC and PI staining. Representative results of 3 independent experiments are shown. (B) Cyclin D3 expression determined by Western Blot analysis of primary B-CLL cells and CD40L-activated B-CLL cells (cultured for 7 days on CD40L) treated for 0 to 48 hrs with Ara-C. Expression patterns were similar in 2 independent experiments.

To completely ensure that primary B-CLL cells did not leave G_0 -phase of the cell cycle before being eliminated by Ara-C, cyclin D3 protein expression, which is mainly found in G_1 phase of the cell cycle, was analyzed (Figure 2.3B). Cyclin D3 could not be detected in primary and Ara-C-treated B-CLL cells, but was present in CD40L-activated B-CLL cells exposed to Ara-C for the times indicated. These data confirm that Ara-C used a different mechanism to kill quiescent (G_0) B-CLL cells than to kill proliferating B-CLL cells.

Ara-C-induced apoptosis of G_0 -B-CLL cells is associated with inhibition of RNA synthesis

To study the mechanism of Ara-C-mediated apoptosis of G_0 -B-CLL cells, we examined whether Ara-C induced lysis could be blocked by dC, which is a structural analogue of Ara-C and the normal metabolite of DNA synthesis. As shown in Figure 2.4A, lysis of G_0 -B-CLL cells by 10^{-5} M Ara-C was inhibited by dC in a dose dependent manner, showing complete inhibition of lysis between 3×10^{-5} M and 6×10^{-5} M dC.

To investigate whether this inhibition of Ara-C-mediated lysis of G_0 -B-CLL cells by dC could be caused by competition for dC kinase (dCK), an enzyme involved in the phosphorylation of both Ara-C and dC, we investigated the capacity of G_0 -B-CLL cells to phosphorylate Ara-C, by measuring dCK activity using 3 H-labeled Ara-C as a substrate. dCK activity of G_0 -B-CLL cells was 81 (pmol $\text{min}^{-1}\text{mg}^{-1}$), illustrating that quiescent B-CLL cells show higher enzyme activity than expected based on their inactive state. The total amount of phosphorylated Ara-C (mono-, di- and triphosphate) produced in time is shown in Figure 2.4B (■). Specific conversion by G_0 -B-CLL cells of Ara-C into Ara-CTP, the active metabolite of Ara-C required for incorporation into DNA or RNA, was demonstrated as well (data not shown). Figure 2.4B further shows that increasing concentrations of dC caused complete inhibition of Ara-C conversion due to competition for dCK. Since both the phosphorylation of Ara-C in G_0 -B-CLL cells (Figure 2.4B) and Ara-C-induced cell death of G_0 -B-CLL cells (Figure 2.4A) could be completely blocked by increasing concentrations of dC, formation of Ara-CTP is likely to be important for the mechanism of Ara-C-mediated apoptosis of G_0 -B-CLL cells.

To determine whether Ara-C affected the amounts of RNA or DNA repair synthesis in G_0 -B-CLL cells, 3 H-uridine and 3 H-thymidine incorporation experiments were performed. G_0 -B-CLL cells were cultured for 20 hrs in either medium alone, or in the presence of Ara-C or fludarabine. After 4 hrs of pre-incubation, 3 H-labeled uridine or thymidine was added to all incubations, and incorporation into respectively RNA or DNA was measured after a 3 H-pulse of 16 hrs. Specific 3 H-thymidine incorporation (corrected for background) in 2×10^6 quiescent G_0 -B-CLL cells cultured in medium was only around 300 CPM (Figure 2.5A), compared to over 4000 CPM in 100,000

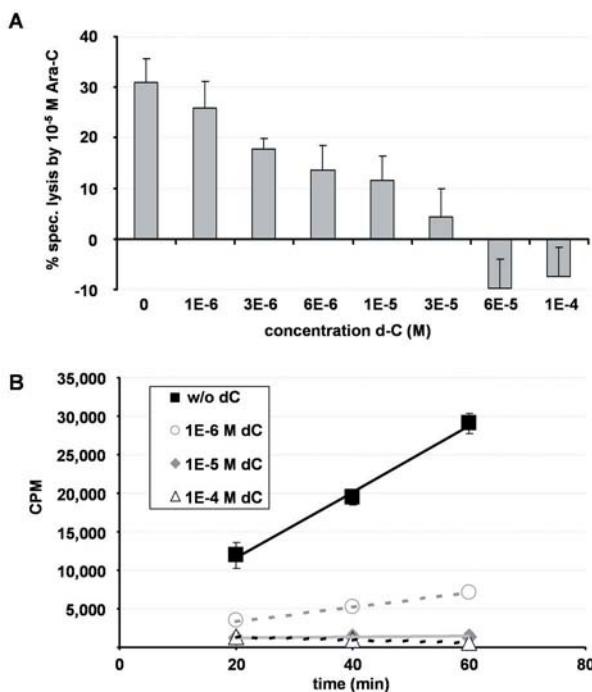


Figure 2.4. Mechanism of Ara-C-mediated killing of G_0 -B-CLL cells.

(A) Competition assay using increasing amounts of dC to block Ara-C-mediated cell death of G_0 -CLL cells after exposure for 72 hrs to 10^{-5} M Ara-C as determined by a CFSE-based cytotoxicity assay. A representative result (in triplicate) of 3 independent experiments is shown. (B) *In vitro* capability of G_0 -B-CLL cell extracts to convert Ara-C into its active metabolite Ara-CTP, determined by dCK activity measurement using 3 H-labeled Ara-C as a substrate. Total amounts of 3 H-labeled Ara-CMP, -CDP and -CTP generated in time are depicted in the figure and indicated in CPM. Increasing amounts of dC were added to the cell extracts to compete for dCK activity and inhibit phosphorylation of 3 H-Ara-C. The graph shows mean values from duplicate measurements \pm SD. Results are representative of 2 independent experiments.

proliferating CD40L-B-CLL cells (data not shown). These low levels of specific 3 H-thymidine incorporation were blocked by both Ara-C and F-Ara-A, showing 40% inhibition at 10^{-6} M, and 60% inhibition at 10^{-5} M.

In contrast, RNA synthesis was much more pronounced than DNA (repair) synthesis, showing basal 3 H-uridine incorporation of approximately 30,000 CPM in 0.5×10^6 G_0 -B-CLL cells (Figure 2.5B), which was only 5-10 times lower than in proliferating B-CLL cells (data not shown). 3 H-uridine incorporation was hardly blocked by 10^{-6} M Ara-C or fludarabine (8%), but 10^{-5} M resulted in 30% inhibition of RNA synthesis after 20 hrs of exposure.

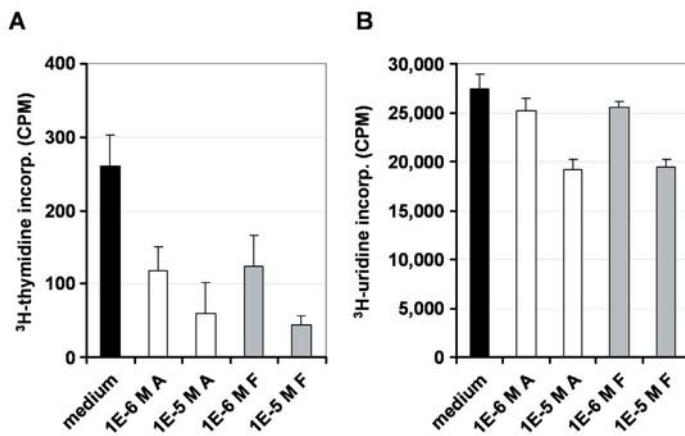


Figure 2.5. Inhibition of RNA synthesis by Ara-C or fludarabine.

G_0 -B-CLL cells ($0.5 \times 10^6/150 \mu\text{L}$) were cultured for 20 hrs in absence or presence of 10^{-6} or 10^{-5} M Ara-C (A) or fludarabine (F). The last 16 hrs of incubation, ${}^3\text{H}$ -uridine or ${}^3\text{H}$ -thymidine incorporation was measured. To determine ${}^3\text{H}$ -thymidine incorporation, prior to harvesting on Unifilter plates 4 wells were pooled (total amount of cells is 2.0×10^6 per incubation). The data presented are mean values (+ SD) of triplicate ${}^3\text{H}$ -uridine (A) and ${}^3\text{H}$ -thymidine (B) incorporations (in CPM). Inhibition of ${}^3\text{H}$ -uridine or ${}^3\text{H}$ -thymidine incorporation by Ara-C or fludarabine was similar in three independent experiments.

Effect of Ara-C on Bcl-2, Bax and Mcl-1 expression and caspase-activation in B-CLL

One of the initiating mechanisms for drug-induced apoptosis in B-CLL cells has been reported to be alteration of the Bcl-2/Bax ratio,⁹ either by downregulation of Bcl-2 or by upregulation of Bax. Moreover, downregulation of other survival proteins such as myeloid cell leukemia sequence 1 (Mcl-1) might contribute to the mechanism of Ara-C-mediated cell death of G_0 -CLL cells. Since exposure of G_0 -CLL cells to Ara-C resulted in decreased RNA synthesis in these cells, we analyzed whether this inhibition of RNA synthesis resulted in alterations in Bcl-2, Bax or Mcl-1 protein levels. Therefore, G_0 -B-CLL cells were exposed to Ara-C in a time range from 0 to 24 hrs, a time interval in which no cell lysis was observed (Figure 2.2). Bcl-2 and Bax expression levels were not significantly altered upon Ara-C treatment, whereas Mcl-1 expression was decreased after 16 hrs of exposure to Ara-C (Figure 2.6A), suggesting that downregulation of Mcl-1 may play an important role in Ara-C-induced apoptosis of G_0 -B-CLL cells.

To reveal whether caspases were involved in the induction of Ara-C-mediated cell death of G_0 -B-CLL cells, either indirectly occurring as a consequence of cellular stress caused by inhibition of RNA synthesis or by downregulation of the survival protein Mcl-1, or directly induced by Ara-CTP via activation of the apoptosome as described for F-Ara-ATP,¹⁸ caspase activation patterns were assessed in the same cell lysates

by Western Blot analysis (Figure 2.6B). Modest caspase-3 and -8 cleavage was observed after 16 hrs exposure to Ara-C, whereas caspase-9 cleavage could not be detected after apoptosis induction with Ara-C in a time interval from 0 to 24 hrs of exposure (data not shown).

To investigate whether this modest caspase activation was required for the execution of Ara-C-mediated apoptosis of G₀-B-CLL cells, we examined the effect of various caspase-inhibitors on Ara-C-induced cell death. G₀-B-CLL cells were exposed to Ara-C in presence or absence of caspase-3, -8, -9 or the pan-caspase-inhibitor z-VAD-FMK, and Ara-C-induced apoptosis was determined after 48 hrs exposure using quantitative CFSE assays (Figure 2.6C). In the presence of 10⁻⁴ M z-VAD-FMK, 50% inhibition of Ara-C-induced lysis was obtained ($p<0.01$), whereas no significant effect (0-25% inhibition) was observed with caspase-3, -8 or -9 inhibitor. These data suggest that caspases were at least partially involved in Ara-C-induced disassembly of the B-CLL cells, although we could not pinpoint which specific caspase plays a key role in Ara-C-mediated apoptosis of G₀-B-CLL cells.

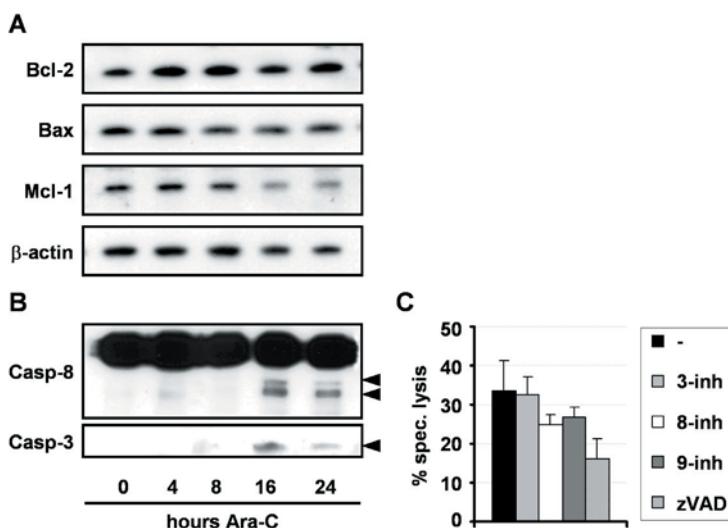


Figure 2.6. Expression of apoptotic proteins and involvement of caspases in Ara-C-mediated apoptosis of G₀-B-CLL cells.

Cell lysates of G₀-B-CLL cells were prepared after apoptosis induction with 10⁻⁵ M Ara-C in a time course from 0 to 24 hrs of exposure. Expression of various pro- and anti-apoptotic proteins (A) and caspase activation patterns (B) were determined using Western Blot analysis, and the data shown are representative for 3 independent experiments. Active subunits of caspase-8 (p40/42) and caspase-3 (p17/19) are indicated with arrows; caspase-9 cleavage could not be detected (data not shown). (C) Effect of various caspase-inhibitors (10⁻⁴ M) on Ara-C-induced apoptosis. Mean percentages of Ara-C-induced lysis ($n=4$) after 48 hrs exposure in presence or absence of specific caspase-inhibitors (caspase-3, -8, -9 inhibitor and zVAD-FMK) were determined using quantitative CFSE assays. Only inhibition by zVAD was significant ($p<0.01$).

DISCUSSION

Ara-C is the most important agent in the treatment of patients with acute leukemia. Incorporation of Ara-C into DNA is considered a key event in the mechanism of killing of proliferating leukemic cells.² We previously demonstrated specific depletion of leukemic cells in S- and G₂/M phase of the cell cycle by Ara-C, whereas resting (G₀) acute leukemic cells did not respond.^{3,4} We now observed unexpected lysis of B-CLL cells, which are mainly (>98%) in G₀ phase of the cell cycle, by Ara-C, being independent of cell proliferation and DNA replication. In this study we analyzed which cellular processes were involved in Ara-C-mediated killing of G₀-B-CLL cells. We showed that, in response to Ara-C treatment, G₀-B-CLL cells died via an apoptotic mechanism characterized by PS exposure and caspase activation, which was not initiated via downregulation of Bcl-2. Furthermore, G₀-B-CLL cells were able to convert Ara-C into Ara-CTP, and increasing concentrations of dC completely inhibited Ara-C-mediated apoptosis of G₀-B-CLL cells, indicating that phosphorylation of Ara-C is important for its cytotoxicity. Since unexpectedly high RNA synthesis was present in G₀-B-CLL cells, which was blocked by Ara-C for 30%, we hypothesize that inhibition of RNA synthesis is an important mechanism of killing G₀-B-CLL cells.

For Ara-C, only mechanisms of killing of proliferating cells have been described, such as inhibition of cellular DNA replication by inhibition of DNA polymerase or incorporation of Ara-CTP into DNA, and inhibition of DNA repair.² The absence of both ki-67 and cyclin D3 expression in Ara-C-treated B-CLL cells (Figure 2.3) revealed that Ara-C used a different mechanism to eliminate G₀-B-CLL cells. One of the mechanisms, demonstrated to be important in Ara-C in contrast to fludarabine-mediated cell death of proliferating cells,¹⁹ that also may play a role in quiescent cells, is inhibition of DNA repair synthesis. In this study, we showed that despite the quiescent stage of the cell cycle, B-CLL cells possessed unexpectedly high dCK activity (81 pmol min⁻¹mg⁻¹), comparable to primary healthy donor cells and AML cells (84 and 114 pmol min⁻¹mg⁻¹, respectively),¹¹ and that Ara-CTP could be formed. In 1986, Carson's group already showed that quiescent lymphocytes break and rejoin DNA at a slow and balanced rate.²⁰ Exposure to DNA repair blocking agents such as Ara-C will cause an accumulation in DNA strand breaks eventually leading to apoptosis. In our experiments, however, basal DNA repair synthesis was hardly detectable (260-400 CPM in 16 hrs) in 2 x 10⁶ G₀-B-CLL-cells, whereas Seto *et al.* showed ³H-thymidine incorporations of 2,000 CPM in 3 x 10⁶ normal resting lymphocytes within 2 hrs of incubation. Huang *et al.*¹⁰ found slightly higher levels of DNA repair synthesis in CLL cells measuring incorporation of ³H-deoxycytidine during 4 hrs (2,500 CPM in 5 x 10⁶ cells). Moreover, they showed that fludarabine inhibited DNA repair synthesis in B-CLL cells (75%), but that this inhibition did not contribute to F-Ara-ATP-induced cytotoxicity observed in these cells. Similarly, we showed that

10⁻⁶ M Ara-C did result in 40% inhibition of DNA synthesis after 16 hrs (Figure 2.5A), but not in lysis after 48 hrs of exposure (Figure 2.1). Overall, we suppose that inhibition of DNA repair is less important in the mechanism of Ara-C-mediated killing of G₀-B-CLL cells than of proliferating cells.

A potential mechanism of killing of G₀-B-CLL cells by Ara-C is inhibition of RNA synthesis, which is also described for fludarabine.²¹ We demonstrated that a remarkable amount of RNA was synthesized within 20 hrs in G₀-B-CLL cells (10-20% of RNA synthesis of proliferating B-CLL cells) and addition of 10⁻⁵ M Ara-C or fludarabine for 20 hrs resulted in 30% less incorporation of ³H-uridine compared to control G₀-B-CLL cells. Addition of 10⁻⁶ M Ara-C hardly resulted in inhibition of RNA synthesis (8%, Figure 2.5B), which correlated well with lack of cytotoxicity at this concentration after 48 hrs (Figure 2.1). Our RNA inhibition data were in line with the results of the group of Huang *et al.*¹⁰ showing a decrease in RNA synthesis of 25-50% in B-CLL cells treated for 24 hrs with 3 x 10⁻⁶-10⁻⁵ M fludarabine. Although we showed that Ara-C treatment resulted in 30% inhibition of RNA synthesis, we did not clarify whether this was due to incorporation of Ara-CTP into RNA or via inhibition of RNA polymerase. Some studies in proliferating cells revealed that Ara-CTP was not incorporated into RNA,^{22,23} whereas F-Ara-ATP incorporated into both RNA and DNA.²³ Therefore, Ara-C probably inhibits RNA synthesis via inhibition of RNA polymerase, which was also shown by some other groups in a non-human setting.^{24,25}

Inhibition of gene transcription by Ara-C may reduce expression of proteins that are important for survival of B-CLL cells such as Bcl-2 and Mcl-1, and in this way lead to apoptosis of G₀-B-CLL cells. Therefore, we investigated levels of Bcl-2 and Mcl-1 protein expression, and observed significant downregulation of Mcl-1 but not of Bcl-2 upon exposure to Ara-C, as was found by several groups after chemotherapy treatment.^{9,26}

We demonstrated that caspases play a role in Ara-C-mediated apoptosis of G₀-B-CLL cells, showing that the general caspase-inhibitor z-VAD-FMK reduced Ara-C-mediated cell death of G₀-B-CLL cells by 50%. In contrast to our findings, other groups argued that caspase activation was only a secondary event of fludarabine-induced apoptosis in G₀-B-CLL cells, showing that caspase inhibition only prevented specific manifestations of apoptosis, such as PARP cleavage and DNA fragmentation, but did not prevent cytotoxicity.²⁷⁻²⁹ We speculated that caspase-activation might be the consequence of the 30% inhibition of RNA synthesis by Ara-C, which will result in major cellular stress in G₀-B-CLL cells. Moreover, caspases might be activated in response to downregulation of the survival protein Mcl-1. An alternative pathway for fludarabine to induce caspase activation and apoptosis, proposed by Genini *et al.*,¹⁸ is activation of the apoptosome pathway. They demonstrated that,

similar to dATP, F-Ara-ATP can cooperate with cytochrome C and apoptosis protein-activating factor-1 (Apaf-1) to form an apoptosome, and directly trigger apoptosis via caspase-9 activation. However, if a similar mechanism plays an important role in Ara-CTP-induced apoptosis as well, a larger inhibition of apoptosis would be expected using caspase-9 inhibitor (Figure 2.6C). Moreover, this pathway is only described for purine analogues such as F-Ara-A and 2CdA, and not for pyrimidine analogues as Ara-CTP.

In conclusion, here we provide evidence for Ara-C-mediated apoptosis of G₀-B-CLL cells, using a different mechanism of killing than proliferating B-CLL cells being independent of cell proliferation and DNA replication. A potential mechanism of killing of resting G₀-B-CLL cells by Ara-C is inhibition of RNA synthesis, either by direct RNA incorporation or by inhibition of RNA polymerase. This decreased RNA synthesis was coincided with downregulation of the survival protein Mcl-1, which may partly explain Ara-C-mediated apoptosis of G₀-B-CLL cells.

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