



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

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Background and Objectives. 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 that Ara-C was cytotoxic to proliferating but not to resting (G_0) malignant cells from patients with acute leukemia. In contrast, here we show unexpected apoptosis of G_0 B-chronic lymphocytic leukemia (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 G_0 -B-CLL cells.

Design and Methods. Using primary B-CLL cells (>98% in G_0), we examined the mechanisms of Ara-C-mediated apoptosis in resting G_0 cells. CFSE-based cytotoxicity assays combined with cell cycle analysis were used to perform a long-term analysis of Ara-C-mediated killing of B-CLL cells. The effects of Ara-C on DNA and RNA synthesis 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 that Ara-C was converted into its active triphosphate by G_0 -B-CLL cells, coinciding with a 30% inhibition of RNA synthesis.

Interpretation and Conclusions. In conclusion, our data indicate that Ara-C can induce apoptosis in resting G_0 -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.

Key words: chronic lymphocytic leukemia, Ara-C, cell cycle, RNA incorporation, apoptosis.

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Ara-C, an analog 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 to dC allows the former 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 the action of a series of cellular enzymes into 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 re-ligation.² Since the cytotoxicity of Ara-C is dependent on DNA synthesis, the 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 an active stage of the cell cycle, whereas G_0 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 G_0 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 analogs such as fludarabine (F-Ara-A) have been introduced as front-line therapy in the treatment of CLL.⁸ Since the incorporation of

nucleotide analogs such as 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 analogs 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 was dependent on caspase activation.

Design and Methods

Cells and culture conditions

Peripheral blood was obtained from four CLL patients (3 females, 1 male) after informed consent. The material we used was collected at times that patients had not received any form of therapy concerning their CLL, except for one patient who had received mild treatment with chlorambucil 2 years previously. 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 in 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 fibroblasts¹² (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 in 5% CO₂ in a 24-well plate at a concentration of 2×10⁵ 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 1×10⁶ mL/well, and cultured in the medium described for resting B-CLL cells, supplemented with 500 IU/mL interleukin-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-hour ⁵¹Cr release assays as described previously³ or using carboxy-fluorescein 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 at a volume of 50 µL/well. After 24, 48, 72 and 120 hours, 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 from Sigma-Aldrich) was added, and samples were mixed properly and directly analyzed on a flow cytometer (Becton Dickinson (BD), San José, 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:

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

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 hours 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 from Alexis) prior to the induction of apoptosis. After 48 hours 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, 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 phosphate-buffered saline (PBS), fixed and permeabilized for 10 min at 4°C using a solution of 80 µg/mL L- α -lyssolecithin (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 monoclonal antibodies (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 reported by Veuger *et al.*¹¹ [³H]-labeled Ara-C (specific activity 24 Ci mmol⁻¹; Campro Scientific BV, Veenendaal, The Netherlands) was used as the 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-well plate (0.5×10⁶ cells/150 µL) in a humidified atmosphere at 37°C in 5% CO₂. To measure DNA or RNA synthesis, after 4 hours 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 hours. Prior to harvesting, from each ³H-thymidine-incubation four wells were pooled (total amount of cells, 2×10⁶/incubation). Using an automatic microharvester cells were harvested on 96-well 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).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis

Apoptosis was induced in B-CLL cells using 10⁻⁵ M Ara-C over a time range from 0 to 48 hours of exposure. At each time point, whole cell lysates of 6×10⁶ cells were obtained by freeze-thawing the cells in 100 µL NP40-lysis buffer (50 mM Tris-HCl, pH 7.6, 5 mM dithiothreitol, 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 hours 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, CA, USA). Bcl-2 antibody (1:50,000) was from Pharmingen (San Diego, CA, USA), and 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 three washing steps, membranes were incubated for 1 hour 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 minutes at 65 °C with buffer containing 0.5% SDS using anti- β -actin clone AC-15 monoclonal antibodies (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 hours of exposure is illustrated in Figure 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. 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 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 in the first 48 hours, and only after 5 days declined from 100 to 83%, due to spontaneous apoptosis. The CFSE intensity of the B-CLL cells was constant, showing that culturing in medium did not induce cell proliferation.

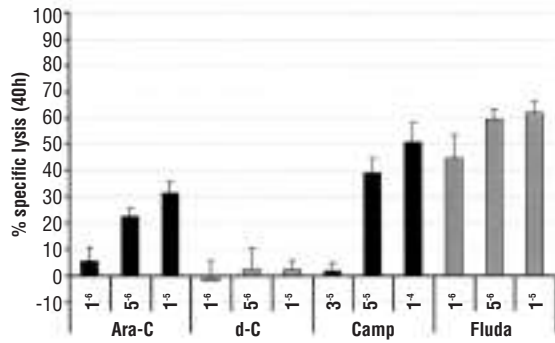


Figure 1. Susceptibility of B-CLL cells to various apoptotic agents. Lysis of B-CLL cells after exposure for 40 hours to different concentrations of Ara-C, deoxycytidine (dC), camptothecin (Camp) and fludarabine (Fluda) was determined using CFSE-based cytotoxicity assays. A representative result (in triplicate + SD) of three independent experiments is shown.

However, culturing in the presence of Ara-C caused a reduction of 50% of the cells after 48 hours, and almost 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 on B-CLL cells, we investigated whether Ara-C-mediated cell death of B-CLL cells was characterized by phosphatidyl serine exposure, an early characteristic of apoptosis. Annexin V binding and counterstaining with PI was used to distinguish between dead and early apoptotic cells. Figure 2B illustrates that B-CLL cells first became annexin V positive before staining with PI, showing that these cells died via an apoptotic mechanism.

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

To investigate the relation between Ara-C-induced apoptosis and 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 hours, and performed cell cycle analysis combining FITC-labeled anti-Ki-67 antibodies and PI DNA staining (Figure 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 the G₀ phase of the cell cycle. The two populations of B-CLL cells responded differently to Ara-C. Although after 48 hours of incubation already 50% of primary B-CLL cells were killed, no evidence of alteration in cell cycle status was observed (Figure 3Ai), indicating that these cells were killed by Ara-C in the G₀-phase of cell cycle. In contrast, in proliferating B-CLL cells slow lysis but specific deple-

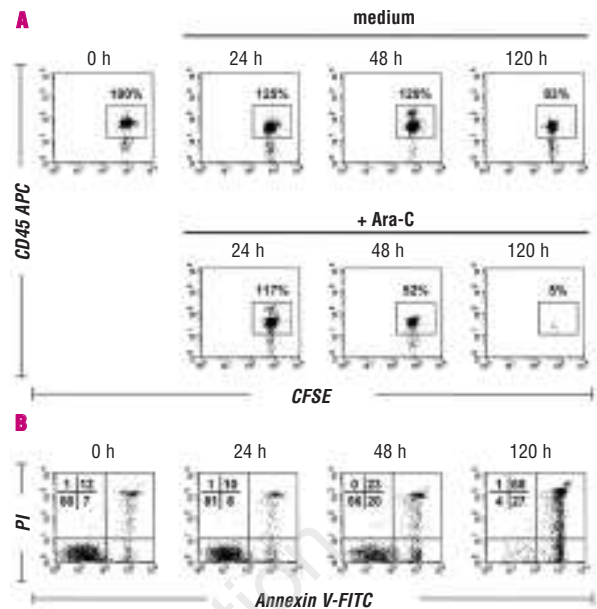


Figure 2. Long-term analysis of Ara-C-mediated apoptosis of B-CLL cells. (A) Kinetics of Ara-C-mediated and spontaneous lysis of B-CLL cells using the CFSE-based cytotoxicity assay. For each time point, the percentage of surviving (CFSE⁺, CD45⁺, 7-AAD⁻) cells, compared to the medium control at day 0,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

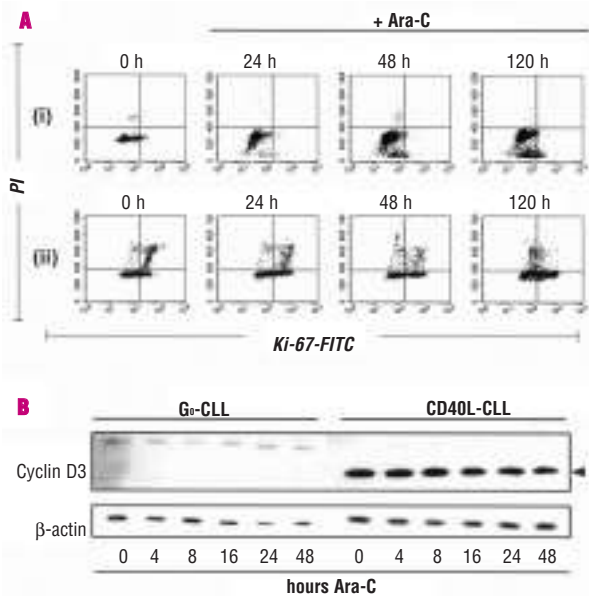


Figure 3. Cell cycle analysis of primary and proliferating B-CLL cells after treatment with Ara-C. (A) Cell cycle status of (i) primary B-CLL cells and (ii) CD40L-CLL cells after treatment with Ara-C for 0, 24, 48 or 120 hours determined by flow cytometric analysis using Ki-67-FITC and PI staining. Representative results of three 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 hours with Ara-C. Expression patterns were similar in two independent experiments.

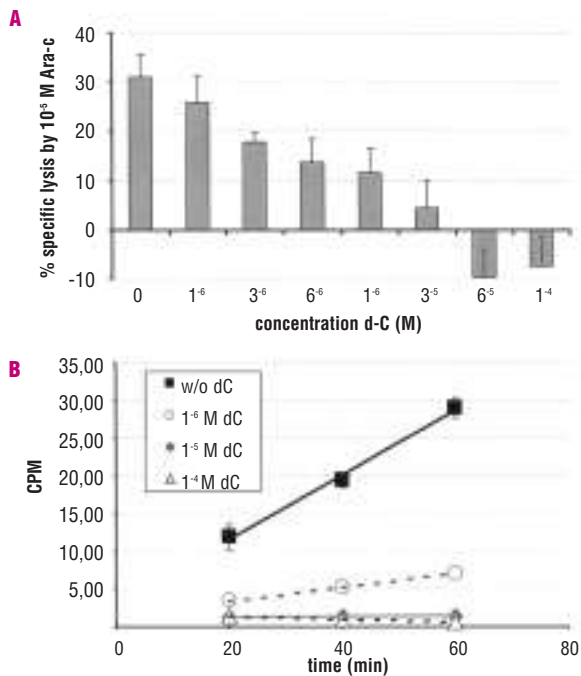


Figure 4. Mechanism of Ara-C-mediated killing of G₀-B-CLL cells. (A) Competition assay using increasing amounts of dC to block Ara-C-mediated cell death of G₀-B-CLL cells after exposure for 72 hours to 10⁻⁵ M Ara-C as determined by a CFSE-based cytotoxicity assay. A representative result (in triplicate) of three independent experiments is shown. (B) *In vitro* capability of G₀-B-CLL cell extracts to convert Ara-C into its active metabolite Ara-CTP, determined by dCK activity measurement using ³H-labeled Ara-C as a substrate. Total amounts of ³H-labeled Ara-CMP, -CDP and -CTP generated over 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 ³H-Ara-C. The graph shows mean values from duplicate measurements ± SD. Results are representative of two independent experiments.

tion of cells in the S- and G₂/M phases of the cell cycle was observed during exposure to Ara-C. To completely ensure that primary B-CLL cells did not leave the G₀-phase of the cell cycle before being eliminated by Ara-C, cyclin D3 protein expression, which is mainly found in the G₁ phase of the cell cycle, was analyzed (Figure 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 (G₀) B-CLL cells than to kill proliferating B-CLL cells.

Ara-C-induced apoptosis of G₀-B-CLL cells is associated with inhibition of RNA synthesis

To study the mechanism of Ara-C-mediated apoptosis of G₀-B-CLL cells, we examined whether Ara-C induced lysis could be blocked by dC, which is a structural analog of Ara-C and the normal metabolite of DNA synthesis. As shown in Figure 4A, lysis of G₀-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-medi-

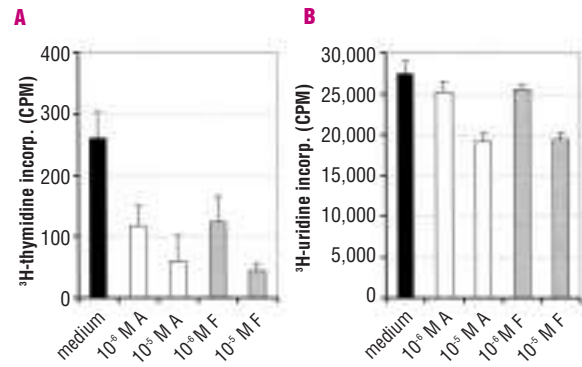


Figure 5. Inhibition of DNA and RNA synthesis by Ara-C or fludarabine. G₀-B-CLL cells (0.5 × 10⁶/150 μL) were cultured for 20 hours in the absence or presence of 10⁻⁶ or 10⁻⁵ M Ara-C (A) or fludarabine (F). ³H-uridine or ³H-thymidine incorporation was measured in the last 16 hours of incubation. To determine ³H-thymidine incorporation, prior to harvesting on Unifilter plates, four wells were pooled (total amount of cells, 2.0 × 10⁶ per incubation). The data presented are mean values (+ SD) of triplicate ³H-uridine (A) and ³H-thymidine (B) incorporations (in cpm). Inhibition of ³H-uridine or ³H-thymidine incorporation by Ara-C or fludarabine was similar in three independent experiments.

ated lysis of G₀-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 G₀-B-CLL cells to phosphorylate Ara-C, by measuring dCK activity using ³H-labeled Ara-C as a substrate. dCK activity of G₀-B-CLL cells was 81 (pmol per min.mg), 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 4B. Specific conversion by G₀-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 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 G₀-B-CLL cells (Figure 4B) and Ara-C-induced cell death of G₀-B-CLL cells (Figure 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 G₀-B-CLL cells.

To determine whether Ara-C affected the amounts of RNA or DNA repair synthesis in G₀-B-CLL cells, ³H uridine and ³H thymidine incorporation experiments were performed. G₀-B-CLL cells were cultured for 20 hours in either medium alone, or in the presence of Ara-C or fludarabine. After 4 hours of pre-incubation, ³H-labeled uridine or thymidine was added to all incubations and incorporation into, respectively, RNA or DNA was measured after a ³H-pulse of 16 hours. Specific ³H-thymidine incorporation (corrected for background) in 2 × 10⁶ quiescent G₀-B-CLL cells cultured in medium was only around 300 cpm (Figure 5A), compared to over 4000 cpm in 100,000 proliferating CD40L-B-CLL cells

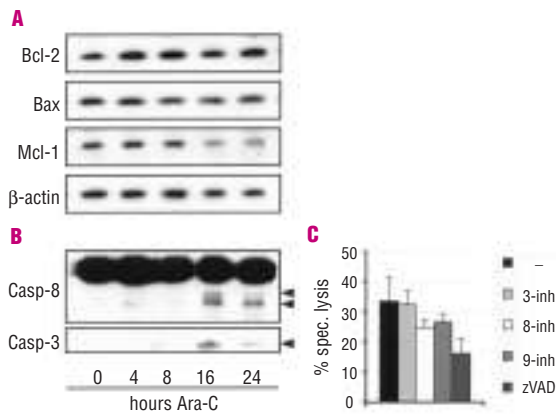


Figure 6. Expression of apoptotic proteins and involvement of caspases in Ara-C-mediated apoptosis of G₀-B-CLL cells. Cell lysates of G₀-B-CLL cells were prepared after apoptosis induction with 10⁻⁵ M Ara-C in a time course from 0 to 24 hours of exposure. Expression of various pro- and anti-apoptotic proteins (A) and caspase activation patterns (B) were determined using western blot analysis, and the data shown are representative of three 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 hours exposure in the 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).

(*data not shown*). These low levels of specific ³H-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 ³H-uridine incorporation of approximately 30,000 cpm in 0.5×10⁶ G₀-B-CLL cells (Figure 5B), which was only 5-10 times lower than in proliferating B-CLL cells (*data not shown*). ³H-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 hours of exposure.

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 Mcl-1 might contribute to the mechanism of Ara-C-mediated cell death of G₀-CLL cells. Since exposure of G₀-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, G₀-B-CLL cells were exposed to Ara-C for a time range from 0 to 24 hours, an interval in which no cell lysis was observed (Figure 2). Levels of Bcl-2 and Bax expression were not significantly altered upon Ara-C treatment, whereas Mcl-1 expression was decreased after 16 hours of exposure to Ara-C (Figure 6A), suggesting that downregulation of Mcl-1 may play

an important role in Ara-C-induced apoptosis of G₀-B-CLL cells.

To reveal whether caspases were involved in the induction of Ara-C-mediated cell death of G₀-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 6B). Modest caspase-3 and -8 cleavage was observed after 16 hours exposure to Ara-C, whereas caspase-9 cleavage could not be detected after apoptosis induction with Ara-C over a period from 0 to 24 hours 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 the 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 hours exposure using quantitative CFSE assays (Figure 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.

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 phases 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 the 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 phosphatidyl serine 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 this drug's cytotoxicity. Since unexpectedly high RNA synthesis was present in G₀-B-CLL cells which was blocked by Ara-C by 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 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 is inhibition of DNA repair synthesis, which may also play a role in quiescent cells. 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 that in primary healthy donor cells and acute myeloid leukemia 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 hours) in 2×10⁶ G₀-B-CLL-cells, whereas Seto *et al.* showed ³H-thymidine incorporations of 2,000 cpm in 3×10⁶ normal resting lymphocytes within 2 hours of incubation. Huang *et al.*¹⁰ found slightly higher levels of DNA repair synthesis in CLL cells measuring incorporation of ³H-deoxycytidine during 4 hours (2,500 cpm in 5×10⁶ cells). Moreover, they showed that fludarabine inhibited DNA repair synthesis in B-CLL cells (75%), but that this inhibition did not contribute to the F-Ara-ATP-induced cytotoxicity observed in these cells. Similarly, we showed that 10⁻⁶ M Ara-C resulted in 40% inhibition of DNA synthesis after 16 hours (Figure 5A), but not in lysis after 48 hours of exposure (Figure 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 it is in the killing of proliferating cells.

A potential mechanism of killing of G₀-B-CLL cells by Ara-C is inhibition of RNA synthesis, which has also been described for fludarabine.²¹ We demonstrated that a remarkable amount of RNA was synthesized within 20 hours 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 hours resulted in 30% less incorporation of ³H-uridine compared to control G₀-B-CLL cells. Addition of 10⁻⁶ M Ara-C resulted in hardly any inhibition of RNA synthesis (8%, Figure 5B), which correlated well with the lack of cytotoxicity at this concentration after 48 hours (Figure 1). Our RNA inhibition data were in line with the results of the Huang's group¹⁰ showing a decrease of 25-50% in RNA synthesis in B-CLL cells treated for 24 hours with 3×10⁻⁶-10⁻⁵ 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 was incorporated into both RNA and DNA.²³ Therefore, Ara-C probably inhibits

RNA synthesis via inhibition of RNA polymerase, which has also been 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 speculate 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 co-operate with cytochrome C and apoptosis protein-activating factor-1 (Apaf-1) to form an apoptosome, and trigger apoptosis directly 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 have been expected when using caspase-9 inhibitor (Figure 6C). Moreover, this pathway is only described for purine analogs such as F-Ara-A and 2CdA, and not for pyrimidine analogs such 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 that in 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 coincided with downregulation of the survival protein Mcl-1, which may partly explain Ara-C-mediated apoptosis of G₀-B-CLL cells.

JFdV contributed to the design of the study, performed most of the experiments, analyzed and interpreted the data, and drafted the manuscript. JHFE, RW and RMYB contributed to the conception of the study, and the interpretation of the data. Furthermore they critically revised the manuscript and their intellectual input was indispensable. The authors thank Willy Honders for her technical assistance concerning the dCK activity experiments and declare that they have no potential conflict of interest. This work supported by a grant of the Dutch Cancer Society: NKB 99-2122.

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