Paradoxical Effect of Cytosine Arabinoside on Mouse Leukemia Cell Line L1210 Cells

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Abstract. We investigated the effects of 1-β-D-arabinofuranosylcytosine (ara-C) on the growth of murine leukemic L1210 cells, which were cultured with high (2.0 × 10^3 ng/ml), middle (100 ng/ml) and low doses (5.0 ng/ml) of ara-C. In the analysis by flow cytometry, high dose ara-C arrested the cell cycle in the G0/G1-phase. Middle and low doses ara-C induced a block in the S-phase, that was not completely blocked by the low dose. Analysis of DNA fragmentation revealed that ara-C dose-dependently induced apoptosis, which was only slightly induced by the low dose. We measured activities of cellular thymidylate synthase (TS) and thymidine kinase (TK) after 24-h culture. Low and middle doses, but not high dose ara-C markedly enhanced TS activity to 2.9- in low and 5.3-fold in middle doses ara-C, and TK activity to 1.3- in low and 2.2-fold in middle doses, respectively, compared with those of the control. The cells accumulated in the S-phase by 48-h culture with low dose ara-C and markedly proliferated compared to that of the control in ara-C-free medium. These results indicate that non-high dose ara-C enhances DNA-synthesizing enzyme activities in L1210 cells, and withdrawal of the non-high dose ara-C results in paradoxical cell proliferation. Thus, daily intramuscular injections with an insufficient dose of ara-C may induce cells into S-phase, resulting in the proliferation of leukemic cells.

Cytosine arabinoside (ara-C) represents one of the potent agents in the treatment of acute myeloid leukemia. Ara-C induces inhibition of DNA synthesis by competing with binding of deoxycytidine 5’triphosphate (dCTP) to DNA polymerase and the incorporation of its intracellular metabolite ara-CTP into the DNA molecule as a chain terminator (1). The activity of ara-C in cancer cells is suspected to vary under different doses and/or delivery system, i.e. bolus or intermittent intravenous injections and continuous intravenous infusion, and after the treatment of the leukemic cells, the withdrawal rebound phenomenon occurs. Thymidylate synthase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21), key enzymes in the de novo and salvage pathways for the synthesis of pyrimidine nucleotides, respectively, have high activity in rapidly proliferating tissues (2-5).

In the present study, in an attempt to establish the appropriate approach for ara-C to lead the leukemic cells into remission, we investigated the withdrawal effects of various concentrations of ara-C on the growth of the murine leukemia cell line L1210. The cell cycle, cell alteration, appearance of apoptosis and activities of TS and TK of L1210 cells under the various concentrations of ara-C were examined.

Materials and Methods

Cell culture. The L1210 cell line (Health Science Research Resources Bank, Osaka, Japan) was cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Gibco-BRL, NY, USA) and 100 μg/ml kanamycin sulfate (Meiji Pharmaceutical, Tokyo, Japan) at 37°C under 5% CO2 in a humidified atmosphere. Live cells were adjusted to a density of 4 × 10^5 cells/ml every 4 days before all experiments. Cell viability was assessed using the trypan blue exclusion test, and was found to be greater than 99% at this stage. L1210 cells (4 × 10^5 cells/ml) were incubated for 48 h at 37°C in the absence (control group) or presence of ara-C (Nippon Shinyaku, Tokyo, Japan) in the various concentrations, i.e. group A: 5.0 ng/ml, group B: 100 ng/ml, group C: 2.0 × 10^3 ng/ml (Experiment I). Then, the 48-h-cultured cells in each group (Experiment I) were washed three times with PBS and adjusted to 4 × 10^5 cells/ml again, and thereafter cultured in the ara-C-free medium for 96 h, as described above (Experiment II).

Measurements of DNA-synthesizing enzyme activities. As previously reported (6), activities of TS and TK in L1210 cells were determined...
by the methods of Dunlap et al. (7) and Taylor et al. (2), respectively (Experiment I). Enzyme activity was normalized to the number of cells, and expressed as pmol/min/10^7 cells (TS) and fmol/min/10^7 cells (TK).

Microscopic observation of apoptotic bodies in cultured cells. One spot of the 48-h-cultured cell suspension was applied to the end of a glass slide, spread into a thin film using the edge of the other slide, and prepared as a “smear” for the detection of apoptotic bodies after drying (Experiment I). The microscopic examination of each specimen, stained with the Wright staining, was performed at magnification x 400.

Flow cytometry (FCM) analysis for detection of cell cycle and apoptosis. After staining the cells with propidium iodide (PI; CycleTEST plus DNA Reagent Kit, Becton Dickinson & Co., NJ, USA), flow cytometry (FCM) analysis was performed using Facsan (Japan Becton Dickinson, Tokyo, Japan). The DNA fraction just before the G0/1 fraction in the FCM histogram was considered apoptotic.

Analysis of DNA fragmentation by agarose gel electrophoresis. DNA fragmentation was analyzed by agarose gel electrophoresis. Samples were extracted using the ApopLadderEX kit (Takara Biochemicals, Tokyo, Japan) (Experiment I). The extracted DNA was resuspended in the buffer containing 10 mM Tris and 1 mM Na2EDTA, pH 7.5, and the DNA concentration was determined by the absorbance at 260 nm. The DNA sample was electrophoresed on 1.5% agarose gel, which was subsequently stained in a 5 μg/ml solution of ethidium bromide before UV transillumination.

Statistical analysis. The resultant values were expressed as mean±SEM. Statistical significance of differences among groups was evaluated by the Student’s t-test and p<0.05 was considered significant.

Results

The behavior of the cells incubated for 48 h under the absence or presence of ara-C. Although the cell number of L1210 cultured without ara-C gradually increased to 1.7-fold that before culture (control group), the L1210 cells incubated with the various concentrations of ara-C for 48 h significantly decreased to 36.8 – 63.3% of that before culture in groups A to C (p<0.01) (Figure 1) (Experiment I).

Activities of thymidylate synthase (TS) and thymidine kinase (TK) of the cultured cells. Activities of TS and TK in the cultured cells in each group (Experiment I) were increased from baseline and peaked at 24 h after the onset of the cell culture, and returned to approximate basal levels at 48 h. The TS activities in the cells
incubated for 24 h with 5.0 ng and 100 ng/ml of ara-C (groups A and B) markedly increased to 2.9- and 5.3-fold that of the control, respectively (p<0.01) (Figure 2a). TK activities in the 24-h-incubated cells with 5.0 ng and 1.0 x 10^2 ng/ml of ara-C (groups A and B) markedly increased to 1.4- and 2.2-fold that of the control, respectively (p<0.01) (Figure 2b).

**Induction of apoptosis in the cultured cells.** Apoptotic bodies and enlarged cells were observed in the cells incubated with 100 ng and 2.0 x 10^3 ng/ml of ara-C for 48 h (groups B and C) (Experiment I). The internucleosomal fragmentation of the chromatin was found to increase in the 48-h-cultured cells proportional to the increase of the additional doses of ara-C, as estimated by electrophoretic analysis (data not shown).

**Cell cycle of the cells incubated with or without ara-C for 48 h.** The representative DNA histogram of the control cells after the 48-h culture (Experiment I) showed the same shape as that before culture. The concentrations of 5.0 ng and 100 ng/ml of ara-C (groups A and B) induced a block in the G2/M-phase and an accumulation of the cells in the S-phase with an increase of apoptotic fraction. Addition of 2.0 x 10^3 ng/ml of ara-C induced a block in the G1-phase with an increase of apoptotic fraction (group C) (data not shown).

**S-phase ratio of the cells incubated with or without ara-C.** The representative increase and decrease of the S-phase cell ratio of the cells cultured with ara-C is shown in Figure 3 (Experiment I). Although the S-phase ratio of the cells cultured with 2.0 x 10^3 ng/ml of ara-C (group C) kept the same low level throughout the experiment, the S-phase ratio was enhanced by the addition of 5.0 ng and 100 ng/ml of ara-C to the culture medium (groups A and B) compared to that of the control, i.e. 1.7- and 2.1-fold that of the control (Experiment I).

**Withdrawal effects of the various concentrations of ara-C on the cultured cells.** The behavior of the cells, which were cultured under the above conditions for 48 h, washed three times with PBS, adjusted to 4 x 10^5 cells/ml again and further cultured in the ara-C-free medium for 96 h, was very different according to the various concentrations of added ara-C (Figure 4) (Experiment II). The cells cultured with 2.0 x 10^3 ng/ml of ara-C for 48 h did not increase subsequently despite the withdrawal of ara-C (group C). Although the cells incubated with 100 ng/ml of ara-C did not increase for 48 h following the ara-C withdrawal, the cells began to increase afterwards (group B). However, the cells incubated with 5.0 ng/ml of ara-C significantly began to increase after the removal of ara-C from the culture medium compared with that of the control, i.e. 1.5-fold that of the control in the 48-h- and 96-h-cultured cells (p<0.01) (group A).

**Discussion**

TS is the enzyme responsible for de novo synthesis of deoxythymidine monophosphate (dTMP), catalyzing the methylation of deoxyuridine monophosphate (dUMP) with the
concomitant conversion of $N_6,N_10$-methylentetrahydrofolic acid to $7,8$-dihydrofolic acid. TK catalyzes the formation of dTMP by the phosphorylation of thymidine via a salvage pathway. The specific activities of TS and TK, among other enzymes in the biosynthesis of nucleotides, rise when DNA synthesis occurs (4, 8). The elevated activities are connected with the increased synthesis of TS (9) and TK (10) in the S-phase of the cell cycle. In the present study, middle and low dose ara-C treatment induced an increase in the activities of TS and TK. As a result, ara-C promoted DNA replication and accumulation of S-phase cells. However, while cells were exposed to ara-C, cell growth was restrained. Ara-C has cytotoxic and cytostatic effects (11). When cultured in ara-C-free medium after treatment with low dose ara-C for 48 h, the cells began to proliferate again.

The 48-h treatment with high dose ara-C did not increase the S-phase cell ratio and number of cells. However, the S-phase cell ratio of cells increased to approximately 70 – 80% after 48-h culture with non-high dose ara-C, though the cells did not proliferate. It is suspected that the $G_0/G_1$-phase cells accumulated with high dose ara-C gradually entered into the S-phase. The reduction in $G_0/G_1$-phase cells may suggest the entrance of resting L1210 cells into the cell cycle (12). These results suggest that non-high dose ara-C may enhance DNA replication, though high dose ara-C shows potent cytotoxic and cytostatic effects.

In previous studies, dCK has been examined as an enzyme of DNA synthesis, and ara-C has been reported to increase the activity of dCK (13, 14). On the other hand, in acute myeloid leukemia of rats, $[^3]$H thymidine incorporation of leukemia cells was increased after injection of ara-C (200 mg/kg) (12). The ara-C concentrations used in the present study are of clinical use. According to previous reports on serum ara-C concentrations (15, 16), the low dose, and both middle and high doses of ara-C in the present study are equivalent to 20 mg/kg/day and 200 mg/kg/day of ara-C as a continuous infusion in the clinical field, respectively. Low dose ara-C has been administered to patients with myelodysplastic syndromes (MDS) and to elderly leukemic patients. Thus, the DNA replication of leukemic cells may be increased by this therapy and, in leukemic patients, the interruption of ara-C treatment may induce the re-proliferation of leukemic cells. The current findings suggest that daily intramuscular injections of an insufficient dose of ara-C may lead the leukemic cells into the S-phase, resulting in the proliferation of leukemic cells. Treatment with high dose ara-C does not increase the S-phase cell ratio and number of cells in mouse leukemia L1210 cells. Cells cultured with high dose ara-C do not increase subsequently despite the withdrawal of ara-C. However, non-high dose ara-C enhances the DNA-synthesizing enzyme activities of cells, and withdrawal of the non-high dose ara-C can result in paradoxical increased cell proliferation.

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References