original paper
Haematologica 1994; 79:127-131

APOPTOSIS INDUCTION WITH FLUDARABINE ON FRESHLY ISOLATED CHRONIC MYELOID LEUKEMIA CELLS

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ABSTRACT

Background. **Fludarabine (FLU) is a fluorinated purine analogue with antineoplastic activity in lymphoproliferative malignancies. Recently, some in vitro reports have showed the effective role of FLU on the activation of apoptosis.**

Materials and Methods. **The induction of programmed cell death (apoptosis) by fludarabine (FLU), an adenine nucleoside analogue, α-interferon (α-IFN), and FLU plus α-IFN was evaluated in vitro against freshly isolated, chronic-phase Ph^{1+} chronic myeloid leukemia (CML). Apoptosis was detected by electrophoresis gel of DNA oligonucleosomal fragments in 8 CML samples.**

Results. **Only FLU and FLU plus α-IFN significantly activated apoptosis in all the samples, suggesting selective activity on CML cells. On the other hand, α-IFN alone did not activate programmed cell death.**

Conclusions. **Our data show apoptotic activity for FLU on CML cells. Programmed cell death may be suppressed in cells carrying the bcr-abl transcript, and FLU might remove this resistance in the neoplastic cell cycle. This preliminary report justifies using FLU in pilot clinical trials for chronic phase Ph^{1+} CML patients.**

Key words: CML, fludarabine, apoptosis

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Chronic myeloid leukemia (CML) is a myeloproliferative disorder that can lead to death within 3-4 years of diagnosis.[1-3] In addition to its clinical importance, CML holds a unique place in oncological research as the first malignancy to be linked clearly to a particular cytogenetic abnormality: the reciprocal chromosomal 9;22 translocation.[4-6]

This fatal course of patients with CML on conventional therapy has prompted the investigation of new approaches and treatments for the chronic phase of the disease. So far, more promising clinical experience has been obtained with α-interferon (α-IFN) as a single agent because it has produced a clinical hematological response and has controlled disease progression in more than two-thirds of the patients treated.[7-15] Furthermore, α-IFN can also induce a partial or complete karyotypic conversion in 30-40% of treated CML patients.[7-15]

Fludarabine (FLU) is an adenine nucleoside analogue resistant to adenosine deaminase that shows promising therapeutic activity in the clinical treatment of chronic lymphoproliferative disorders.[16-23] Recent *in vitro* reports.[24,25] showed that FLU activates programmed cell death.

Here we report the *in vitro* induction of apoptosis by FLU, α-IFN, and a combination of the two on freshly isolated samples obtained

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Acknowledgements: this work was supported by Progetto Finalizzato ACRO C.N.R. N.92.02280.PF39, Rome (Italy) and by A.I.R.C., Milano (Italy)
Received November 30, 1993; accepted February 21, 1994.

from 8 patients with chronic-phase CML, in order to evaluate the synergistic and additive role of these two drugs.

Patients and methods

Isolation and purification of CML cells

Eight patients with diagnosis of chronic phase Ph^{1+} CML, who received no therapy for the previous month, were selected for this study. Mononuclear cells from peripheral blood samples were obtained after centrifugation of the cell suspension over a Ficoll/Hypaque gradient. The following mean percentages were observed in the mononuclear fraction of the samples: lymphocytes 42%, monocytes 18%, myelocytes 15%, metamyelocytes 12%, promyelocytes 7%, and blast cells 5%.

Drugs

FLU was purchased from Inveresk Clinical Research (Edinburgh, Scotland) and was used at a final concentration of 50 μg/mL. α-IFN was provided by Hoffmann-La Roche (Human Recombinant α 2a-IFN, Roferon-A) and was used at a final concentration of 100 U/mL; the same concentrations were used for both drugs when combined.

Apoptosis assay

Tumor cells were harvested, counted and added at a concentration of 5×10^5 into 25 cm^2 culture flasks; drugs were added and cultures were incubated for 3 days at 37°C. Cells were resuspended in 20 μL 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5% (w/v) sodium lauryl sarcosinate, and 0.5 mg mL^{-1} proteinase K, and incubated at 50° C for 1 hour. Ten μl 0.5 $mgmL^{-1}$ RNase A were added to each sample; incubation at 50°C was continued for another hour. Samples were heated to 70°C, and 10 μL 10 mM EDTA (pH 8.0) containing 1% (w/v) low-temperature gelling agarose, 0.25% (w/v) bromophenol blue, and 40% (w/v) sucrose were mixed with each sample before being loaded with a siliconized pipette tip into the dry wells of a 2% (w/v) agarose gel containing 0.1 μg mL^{-1} ethidium bromide. Electrophoresis was performed in 2 mM EDTA, 800 mM Tris-phosphate (pH 7.8) overnight. The gels were photographed under UV light.

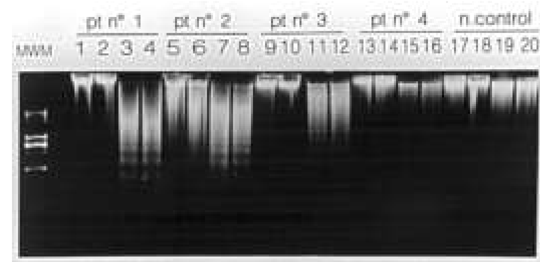

Figure 1. DNA fragmentation pattern of 4 CML patients (lanes 1 to 16) and 1 of normal control cells (lanes 17 to 20). Cells were incubated with: no addition (lanes 1, 5, 9, 13, 17); 100 U/mL α-IFN (lanes 2, 6, 10, 14, 18); 50 μg/ml FLU (lanes 3, 7, 11, 15, 19); α-IFN and FLU combined (lanes 4, 8, 12, 16, 20) as described under Patients and Methods. MWM= DNA molecular-weight marker VI, Boehringer Mannheim, Germany.

Results

The results of our assay showed DNA isolated from CML cells presented the characteristic fragmentation pattern of apoptosis shown by electrophoresis (Figure 1, for 4 pts) in all 8 samples induced by FLU alone and by FLU plus α-IFN. No differences in apoptotic response were seen between FLU alone and the combination of α-IFN and FLU. On the contrary, α-IFN alone did not activate programmed cell death in any of the samples.

Light microscopy of CML cells incubated with FLU for up 72 hours showed characteristic apoptotic changes, including chromatin condensation, nuclear margination, and apoptotic body formation (see Figure 2). In addition, degenerate anucleated cells lacking chromatin appeared.

Discussion

The results of several clinical trials in CML patients with α-IFN[7-15] were encouraging, particularly because of the repeated observation of complete cytogenetic response. In an effort to

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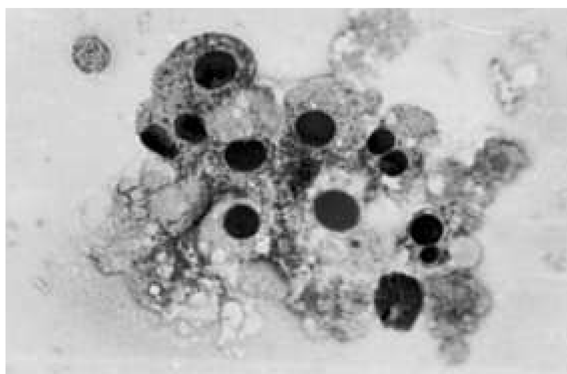

Figure 2. Morphologic features after 72 hours of CML cells treated with 50 μg/mL FLU. The specific morphologic changes include chromatin condensation, nuclear margination, and fragmentation of the nucleus into membrane-bound bodies.

improve on the incidence of complete and partial cytogenetic responses, a series of studies (in vivo and in vitro) are carrying out: α-IFN + homoharringtonine (HHT), α-IFN + antiviral agents (e.g., azidothymidine), α-IFN and low-dose ara-C.[26] In fact, HHT has showed encouraging results in most of the newly diagnosed CML patients.[27]

As far as the mechanisms of action are concerned, in vitro α-IFN suppresses normal and CML myeloid stem cell proliferation, probably by a direct growth inhibitory effect. This effect is non-selective and does not explain the clinical activity mediated by interferon in CML.[28] To be operative, α-IFN must bind to a specific receptor; however, no defects in receptor binding have been found in interferon-resistant CML.[29] Presently it is difficult, at least at the level of gene transcription, for one to detect overt defects in the interferon-signalling pathway.[30] On the other hand, FLU is cell-cycle specific and requires DNA synthesis for cytotoxicity. The active triphosphates of FLU interfere with the DNA polymerases and ribonucleotide reductase.[31,32] In addition, FLU blocks the action of DNA primase[33] and DNA ligase.[34] At high concentrations FLU has additional inhibitory effects on RNA and protein synthesis.[35]

A variety of cytotoxic cancer chemotherapy compounds, including cytosine arabinoside, methotrexate, adriamycin, nitrogen mustard, vincristine, 2-chlorodeoxyadenosine and FLU have been found to initiate apoptosis.

During the initial steps of apoptosis, the cell undergoes extensive membrane blebbing, nuclear condensation, cytoplasmic shrinking, and cleavage of nuclear DNA into fragments corresponding to 200 bp or multiples of this module. A few genes, among which bcl-2 and sgp-2,[36,37] have been isolated whose expression seems to be involved in the regulation of apoptosis, but the molecular details are unknown.

Our observations reveal that FLU, alone or in combination with α-IFN, directly activates or releases an apoptotic program; α-IFN, on the contrary, did not induce any programmed cell death (Figure 1).

These data demonstrate that FLU has apoptotic activity in CML cells and this justifies its use in pilot clinical studies on the treatment of chronic phase Ph^{1+} CML with or without α-IFN. The modality of α-IFN activity is not known; in fact, the rationale for interferon therapy was based on excessive myeloid proliferation in CML, on the suppression of growth, and on induction of differentiation of normal and CML myeloid progenitors *in vitro* by interferons. Furthermore, IFN has both direct and indirect cytotoxic, cytostatic, and immune-stimulatory effects. For this reason, one would find it interesting not only to study and verify the exact role of this cytokine in the apoptotic cycle, but also to evaluate its additive or synergistic action with various agents, such as FLU and 2-chlorodeoxyadenosine, which activate programmed cell death.

Some unresolved questions include: does α-IFN boost FLU activity? How does α-IFN act in this drug combination? There are probably two pathways with two end points: antiproliferative activity and the apoptotic program. Apoptosis may be suppressed or blocked in cells carrying the bcr-abl transcript, and FLU could selectively remove this blockage in the neoplastic CML cell-cycle.

Finally, we should point out that the most important objective for the next generation of studies will likely be examine synergistic combinations of α-IFN and other treatment modalities like FLU which, on the basis of our prelim-

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inary data, seems to be an interesting and active drug against CML cells. The future of chronic phase CML therapy must be concentrate on finding the optimal combined approaches able to achieve maximal and durable Ph^{1+} suppression, including combinations of biologic response modifiers and other anti-CML agents.

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