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ScienceDirect
Leukemia Research 32 (2008) 755–760
Leukemia Research
www.elsevier.com/locate/leukres

Flavopiridol-induced iNOS downregulation during apoptosis of chronic lymphocytic leukemia cells is caspase-dependent

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Received 11 June 2007; received in revised form 24 September 2007; accepted 27 September 2007
Available online 5 November 2007

Abstract

We previously reported that flavopiridol-induced apoptosis of B cell chronic lymphocytic leukemia (CLL) patients' cells *ex vivo* is associated with downregulation of both the inducible nitric oxide (NO) synthase (iNOS) that produces the antiapoptotic molecule NO, and the CDK inhibitor p27kip1 that is thought to block the cell cycle of CLL cells. Here, we show that iNOS downregulation is caspase-dependent and thus can be considered as one of the effector mechanisms of apoptosis, but not a primary triggering event induced by flavopiridol. Furthermore, we also find that this flavone favors the entry into the S and G2 phases of the cell cycle of a subpopulation of the leukemic cells, confirming that flavopiridol might be useful for improving the efficacy of cell cycle-dependent cytostatic agents in the therapy of CLL.

Keywords: CLL; Flavopiridol; Apoptosis; iNOS; p27kip1; Cell cycle

1. Introduction

B cell chronic lymphocytic leukemia (CLL) is characterized by a low responsiveness of the patients to chemotherapy (Ref. [1] for review). This could be explained by the remarkable defective apoptosis of the CLL cells observed in the different forms of the disease, as well as by the fact that 95% of the leukemic cells are arrested in the G0/G1 phase of the cell cycle (Ref. [2] for review). In addition, no reliable features of prognostic significance are available to the clinicians, except the cytogenetic analysis, in order to decide appropriate therapeutic strategies [3].

Flavopiridol, a semi-synthetic plant-derived flavone (*N*-methylpiperidinyl, chlorophenyl flavone), is a potent inducer of apoptosis of CLL cells *ex vivo* [4] and is currently under clinical evaluation (Refs. [1,5–7] for reviews). The apoptotic effect of flavopiridol is p53-independent and involves in particular caspase activation, the mitochondrial pathway and inhibition of two crucial antiapoptotic proteins, Mcl-1 and XIAP, while Bcl-2 downregulation does not prove to be critical [4,6]. In addition, we recently reported that flavopiridol inhibits the expression of both the inducible NO synthase (iNOS) and p27kip1 [8], two enzymes which are constitutively overexpressed in CLL cells compared to normal B lymphocytes [9,10]. The former protein catalyzes the formation of nitric oxide (NO) which displays antiapoptotic properties in CLL cells [9,11] as well as in hairy cells derived from another type of B cell chronic leukemia [12]. Based on our data that caspase-3 activity is inhibited by a NO donor and enhanced by an iNOS inhibitor in CLL cells, we proposed that NO may act by S-nitrosylation of cysteine residues present in the catalytic site of caspases, thus resulting in inhibition of caspase activity [11]. As flavopiridol-promoted apoptosis of CLL cells was found to be partly reverted by a NO donor, we suggested that the inhibition of the NO pathway by flavopiridol could be a molecular switch contributing to restore apoptotic processes [8]. Whether iNOS downregulation is actually a primary mechanism by which flavopiridol is capable of triggering apoptosis, or rather subsequent to

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0145-2126/$ – see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2007.09.016

caspase-dependent pathways induced by the flavone remains yet to be evaluated.

The other enzyme which is overexpressed in CLL cells, p27kip1, is a CDK inhibitor thought to be responsible for the cell cycle arrest observed in at least 95% CLL cells [10]. Our finding that p27kip1 is downregulated *in vitro* by flavopiridol through a cleavage into a p23 form suggested the possibility that the flavone could favor G0/G1-arrested CLL cells to re-enter into the cell cycle in addition to stimulate apoptosis [8].

The aims of the present work were to investigate whether iNOS downregulation observed during flavopiridol-elicited apoptosis of CLL patients' cells *ex vivo* is a caspase-dependent event or not, and whether the flavone is capable of inducing cultured CLL cells to go through S and G2 phases of the cell cycle.

2. Materials and methods

2.1. Patients, cells and cell culture

Peripheral blood samples from patients with CLL of the B cell phenotype (stage A according to Binet's classification) were obtained from the Hematology Department of Hôtel-Dieu hospital (Paris, France) after written informed consent, in agreement with the revised Helsinki protocol. Diagnosis was established according to standard clinical criteria and those of the International Workshop on CLL), including lymphocyte morphology and co-expression of CD5, CD20 and CD23. A total of 11 patients were studied: 6 men and 5 women with a mean age of 72 ± 14 years (range 58–84) with a time since diagnosis ranging from 0 to 10 years. The patients were randomly chosen because CD38 and ZAP-70 expression, cytogenetics and mutational V_H status were only available for a fraction of them, thus hampering a risk-group analysis. The leukemic B cells were purified as previously described, providing >95% cells of the CLL phenotype [9]. All cell cultures were performed with 2×10^6 cells/ml at 37 °C in an humidified atmosphere containing 5% CO_2, in RPMI-1640 medium supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES and 10% FCS (PAA Laboratories, Pasching, Austria). The Eskol cell line was also used in some experiments. This cell line, obtained through the courtesy of Dr. E.F. Srour, was derived from a patient with hairy cell leukemia [13], another chronic B cell malignancy that also shares with CLL apoptosis deficiency and a high constitutive iNOS expression [12]. In addition, Eskol cells display sensitivity to flavopiridol and several polyphenols regarding apoptosis induction and iNOS downregulation [14–16]. The Eskol cell line was routinely maintained in culture at densities between 10^5 and 10^6 cells/ml in the same medium as above but without HEPES. Experiments were carried out with exponentially growing cells at the seeding density of 2.5×10^5/ml.

2.2. Reagents

Flavopiridol is a gift from Aventis Pharmaceuticals (Bridgewater, NJ, USA). A 10 mM stock solution was prepared in DMSO, aliquoted and kept at −20 °C. *Trans*-resveratrol was purchased from Sigma (St Louis, MO, USA) and stock solutions (5 mM) were prepared in ethanol/water (1/1). Hyperforin was purified and kindly provided by Dr. J.D. Fourneron [17] and 1 mg/ml stock solutions were made in ethanol. The combretastatins JO1 and JO3 were synthesized and kindly provided by Dr. J.P. Finet [18]. The broad specificity caspase inhibitor z-VAD-fmk was obtained from Biomol (Plymouth Meeting, PA, USA). The other reagents and chemicals were from Sigma.

2.3. Western blot analysis

The expression of the iNOS, p27kip1, PARP-1 [Poly (ADP-ribose) Polymerase-1] was analysed by Western blotting, as detailed previously [8]. Briefly, cells were lysed with RIPA buffer for 30 min at 4 °C, and equal amounts of the cell lysates (30–50 μg of proteins in SDS-reducing buffer) were electrophoresed by SDS-PAGE (either 8 or 12% according to the proteins studied, or a gradient gel for simultaneous analysis). Separated proteins were electrotransferred on PVDF membranes which were further incubated with the antibodies recognizing the molecules of interest. The polyclonal rabbit antibodies against iNOS (N-20) and p27kip1 (N-20), recognizing both the p27 and its cleavage product p23, were from Santa Cruz Biotechnology, CA, USA. The anti-PARP-1 mAb (C2-10) specific for its native form (116 kDa) as well as for its cleaved fragment (85 kDa) was from Alexis Biochemicals (Coger, Paris, France). The immunoblotted proteins were revealed with HRP-goat anti-rabbit or anti-mouse antibodies (DakoCymation, Glostrup, Denmark) and a system of chemiluminescence (Western lightning chemiluminescence reagent plus; Perkin-Elmer, Boston, MA, USA). Finally, the membranes were also hybridized with a mouse anti-β-actin mAb (clone C4; ICN, Costa Mesa, CA, USA) for protein content monitoring.

2.4. Cell cycle analysis

To quantify cells in the different phases of the cell cycle, the method of incorporation of propidium iodide into DNA was used. Aliquots of cells (10^6) were washed in PBS and fixed in 70% ethanol at 4 °C for at least 2 h. After repeated washings with PBS containing 0.5% Tween 20, cell pellets were resuspended in the same buffer containing 50 μg/ml propidium iodide and 1 mg/ml of heated RNAse. Propidium iodide incorporation was then analysed by flow cytometry after excitation at 488 nm with at least 10,000 cells, and the percentage of diploid cells in each phase of the cell cycle was evaluated using the "MultiCycle" software.