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Parte de este trabajo se encuentra en revisión para publicación con el título: **"Role of the STAT1 pathway in apoptosis induced by fludarabine and JAK kinase inhibitors in B-cell chronic lymphocytic leukemia"**.
L. Martinez-Lostao, J. Briones, I. Forné, M. Martínez-Gallo, B Ferrer, J. Sierra, J.L. Rodríguez-Sánchez, C. Juárez.

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Fludarabine-Induced Apoptosis in CD19+/CD5+ B-CLL Cells is a Direct and Nurse-Like-Cell Independent Effect

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B-cell chronic lymphocytic leukemia (B-CLL) is a hematological malignancy characterized by the accumulation of mature CD5+ B lymphocytes with a defective apoptosis. A subset of blood monocyte-derived adherent cells generated *in vitro* protects B-CLL cells from apoptosis playing a role as nurse-like cells (NLCs). Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-A) is an adenine nucleoside analog used to treat B-CLL. To gain insight into the mechanisms implicated in the antitumoral effect of fludarabine in B-CLL cells, we performed cross-cultures with B-CLL cells and NLCs treated and untreated with fludarabine. Our results showed that fludarabine blocked the development of NLCs and induced apoptosis in these cells when they were present in culture. Moreover, CD19+/CD5+ B-CLL cells treated with fludarabine underwent apoptosis and this event was not related with the presence of NLCs whether treated or not with fludarabine. In conclusion, apoptosis induced by fludarabine in CD19+/CD5+ B-CLL cells was due to a direct effect on these cells and not due to its effect in the NLCs.

Keywords: Nurse-like cells; B-CLL; Fludarabine; Apoptosis

INTRODUCTION

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the Western world. B-CLL is characterized by the accumulation of mature CD5+ B lymphocytes, that are long-lived cells arrested in the G0/early G1 phase of the cell cycle, supporting the hypothesis that the B-CLL is primarily related to defective apoptosis [1,2]. Genetic defects and external stimuli may both influence the cell's abnormal behavior [3]. Despite their longevity *in vivo*, B-CLL cells often undergo spontaneous apoptosis *in vitro* [4,5]. This implies that programmed cell death in cultured B-CLL cells results of the absence of essential survival signals and the resistance to apoptosis is not intrinsic to the B-CLL cells [6]. These external signals can arise from other cell types either producing growth factors [7] or by direct contact with B-CLL cells [8]. Therefore, regulatory signals in different loci, could play a key role in the prolonged survival of B-CLL, disease progression or resistance to therapy *in vivo* [9]. Nevertheless, neither the cells nor the factors that are involved in this support of B-CLL cells are clearly known.

Among the cells that have been implicated in the survival of B-CLL *in vitro*, a subset of blood-derived cells that spontaneously differentiate *in vitro* into round or fibroblast-like adherent cells has been described [10]. The effect of these adherent cells on B-CLL viability is mediated in part by the chemokine stromal-derived factor 1 [11]. These cells attract B-CLL cells and support their survival through a contact-dependent mechanism, so they have been termed nurselike cells (NLCs). It has been hypothesized that these cells probably represent a distinctive hematopoietic cell type of monocytic lineage *in vivo*, which differentiates from CD14+ cells and protect B-CLL cells from apoptosis [12].

Therefore, certain classes of cells play a fundamental role in the survival of B-CLL cells. Currently used therapeutic approaches or approaches that may be applied in the future would not only work because of

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their effect on B-CLL cells but also because of their effect on cells which play a role as "nurse" cells.

Fludarabine (9-β-D-arabinofuranosyl-2-fluoroadenine; F-ara-A) is an adenine nucleoside analog resistant to adenosine deaminase that has been extensively used to successfully treat various hematological malignancies such as B-CLL [13]. Although F-ara-A's major action is inhibition of DNA synthesis [14], clinical investigations have shown strong therapeutic activity in B-CLL and other indolent lymphocytic malignancies with a very low rate of proliferation. Other mechanisms of action have been shown for F-ara-A, such as effects on methylation [15], DNA repair [16], and nicotinamide adenine dinucleotide metabolism [17]. Although the incorporation of fludarabine into DNA could be the key event in causing cytotoxicity in proliferating leukemia cells, the precise mechanisms by which fludarabine kills B-CLL cells remain unclear because of the quiescent nature of this malignancy. Inhibition of STAT1 signaling [18] and inhibition of mRNA transcription [19] have been implicated in the effects of this drug on B-CLL.

To gain insight into the mechanisms implicated in the antitumoral effect of fludarabine in B-CLL cells, we decided to study the effect of this drug in both B-CLL cells and NLCs. The goal of the study was to know if the alterations induced by fludarabine in the NLCs had any effect on the survival of B-CLL cells, or if the direct action of fludarabine in B-CLL cells, was responsible for their programmed cell death.

MATERIALS AND METHODS

Patients and Cell Samples

Three B-CLL patients diagnosed according to standard criteria were included in the study. Patients were untreated in all cases. Table I summarizes clinical features of the three patients. In all cases, more than 90% of total lymphocytes were CD19 + /CD5 + B-cells as determined by flow cytometry analysis.

Reagents

Fludarabine phosphate was obtained from Schering (Madrid, Spain) and was used in cell cultures at a final concentration of 50 μM. Fludarabine dose was chosen on the basis of our previous experiments, showing that 50 μM dose was able to block the growth of NLCs (data not shown). This dose was also able to induce apoptosis of B-CLL cells in agreement with previous reports [14,20]. We did not observe differences in the susceptibility of B-CLL cells and NLC to the action of different fludarabine doses (data not shown).

Cells Samples

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples from B-CLL patients by density-gradient centrifugation over Ficoll-Hypaque (Nycomed, Oslo, Norway) and were suspended in RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% Fetal Bovine Serum (FBS: Bio Whittaker, Verviers, Belgium), penicillin 100 U/ml streptomycin 100 μg/ml (Bio Whittaker, Verviers, Belgium) and 2 mmol/l glutamine (Gibco BRL, Rockville MD, USA) at concentration of 2 to 4 $\times$ 10^6 cels/mL. PBMC were plated in tissue culture flasks (TPP, Trasadingen, Switzerland) and cultured at 37°C and 5% of CO_2 for 14 days in order to allow the growth of NLCs.

Immunophenotype of Nurse-Like Cells

Phenotype of NLCs was performed by immunohistochemical staining. After cell culture for 14 days suspension cells were removed from the flask and NLCs were washed 3 times in Phosphate-Buffered Saline (PBS) and twice in methanol. Adherent cells were fixed with methanol:acetone 1:1 for 10 min, washed once in PBS and incubated with primary monoclonal antibodies against CD13, CD14, CD15, CD19, CD33, CD34, CD45, CD123 and HLA-DR (Dako, Glostrup, Denmark) at room temperature for 30 min, washed 4 times in PBS and incubated at room temperature for 30 min with alkaline phosphatase conjugated secondary antibody (APAAP mouse monoclonal antibody from Dako, Glostrup, Denmark)). A color reaction was developed using the Fast Red Substrate System (Dako, Glostrup, Denmark) according the manufacturer's instructions. Specimens were examinated on a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY) and digital images were captured with a Spot Coge Digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

TABLE I Clinical features of the B-CLL patients included in the study

ID patients	Age (years)	Sex	Leucocyte count ($\times$ 109/1)	Lymphocyte count ($\times$ 109/1)	Binet's staging
Patient 1	70	Male	36.72	29.01	A
Patient 2	51	Female	69.07	63.10	A
Patient 3	75	Male	18.14	13.26	A

Analysis of the "Nurse" Effect of NLCs on B-CLL Cells

After 14 days in culture, we performed cultures of B-CLL cells with or without NLCs in order to examine the effect of NLCs on the survival of B-CLL cells (Fig. 1A). In order to asses the possible effect of manipulation on the survival of B-CLL cells, suspension cells from 1 tissue culture flask were separated from NLCs, both cell types were washed in PBS 3 times and cultured together again. Suspension cells from another culture flask were separated from NLCs, washed 3 times in PBS and cultured in a new culture flask without NLCs. A 3rd culture flask was maintained untreated during the experiment as control of spontaneous apoptosis.

Cross-Cultures

After 14 days in culture, we performed cross-cultures (Fig. 1B). Four culture flasks of each B-CLL were treated with 50 μM fludarabine for 24 h and 1 flask was maintained without treatment. Suspension cells were separated from NLCs, both washed in PBS and mixed again in such a way that fludarabine treated and untreated suspension cells were placed in presence of drug-treated or untreated NLCs in the 4 possible combinations. As control of apoptosis induced by fludarabine, 1 flask was treated with fludarabine and maintained during the experiment (Fig. 1B: F1).

Analysis of Apoptosis by Annexin V Binding

Exposure of traslocated phosphatydilserine in apoptotic CD19 + /CD5 + B-CLL cells was quantified by surface annexin V binding staining as described previously [21]. Briefly, aliquots were removed from each flask at the time points indicated below, and incubated with phycoerythrin (PE)-conjugated anti-CD19 monoclonal antibody (Immunotech, Marseille, France), phycoerythrin-cyanin 5 (Pc5)-conjugated anti-CD5 monoclonal antibody (Immunotech, Marseille, France) and annexin V-fluorescein isothiocyanate (FITC) (Bender MedSystems,

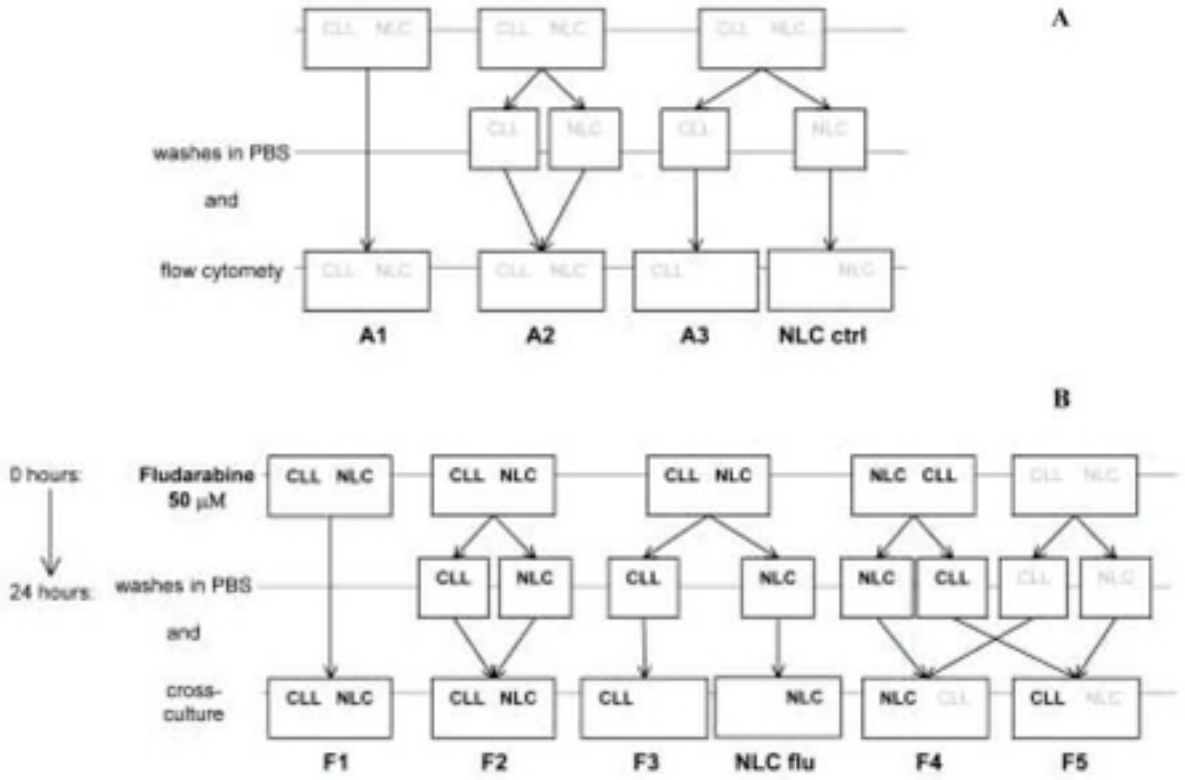

FIGURE 1 *Panel A*: Schematic representation of analysis of the "nurse" effect of NLCs on B-CLL cells. PMBC from three B-CLL patients were cultured for 14 days in culture flasks to allow the growth of NLCs. One of them was maintained as control of spontaneous apoptosis (A1). As control of manipulation, suspension cells (CLL) and NLCs (NLC) from another culture flask were separated, washed in PBS, mixed and cultured together again (A2). Suspension cells and NLCs from a 3rd culture flask were separated, both washed in PBS and then, suspension cells were cultured without NLCs (A3). *Panel B*: Schematic representation of cross-culture of fludarabine treated cells. PMBC from 3 B-CLL patients were cultured for 14 days in culture flasks to allow the growth of NLCs. Thereafter, 4 culture flasks of each B-CLL were treated with 50 μM fludarabine for 24 h (**bold letters**) and 1 culture flask were untreated. Then, suspension cells (CLL) and NLCs (NLC) were separated, washed in PBS and mixed as follows: F1: control of fludarabine-induced apoptosis (non-separated suspension cells and NLCs treated with fludarabine); F2: suspension cells and NLCs treated with fludarabine; F3: Suspension cells treated without NLCs; F4: untreated suspension cells and NLCs treated with fludarabine and, F4: suspension cells treated with fludarabine and untreated NLCs. Apoptosis of CD19 + /CD5 + B-CLL cells from each flask was analyzed by annexin V staining at 0 h (immediately before treatment with fludarabine), 24 h (immediately after the cross-culture), 48, 72 and 96 h after treatment with fludarabine.

Vienna, Austria) for 15 min in the dark. Samples were analyzed on Epics XL-MCL (Coulter, Miami, Florida, USA). Flow cytometry data were analyzed using XL2 software (Coulter, Miami, Florida, USA). Apoptosis of each sample of CD19 + /CD5 + B-CLL cells was analyzed at 0 h (immediately before the treatment with fludarabine), 24 h (immediately after the cross-culture), 48, 72 and 96 h after treatment with fludarabine.

Viability Analysis of NLCs

After 14 days in culture, NLCs were maintained untreated (Fig. 1A: NLC ctrl) or treated with 50 μM fludarabine for 24 h (Fig. 1B: NLC flu) from the 3 B-CLL patients. Then, NLCs were washed 3 times in PBS and the remaining NLCs were counted 10 days after the treatment in 6 random microscopy fields for each sample. In all cases, viability of untreated NLCs samples were more than 80% and less than 20% in fludarabine-treated NLCs

Statistical Analysis

Flow cytometry results are shown as mean $\pm$ SD of 3 experiments each. Statistical significance was evaluated using Student *t*-test. A P value < 0.05 was considered significant.

RESULTS

NLCs Are Detected in Long-Term Cultures of PBMC from Patients with B-CLL and Express Surface Markers of Blood Monocyte-Derived Cells

Large adherent cells with round or fibroblast-like morphology, appeared after several days in culture. When the surface markers of these cells were analyzed by immunohistochemical staining (Fig. 2), they were stained strongly with monoclonal antibodies against CD14, CD45 and HLA-DR (Fig. 2B,G,I) and stained

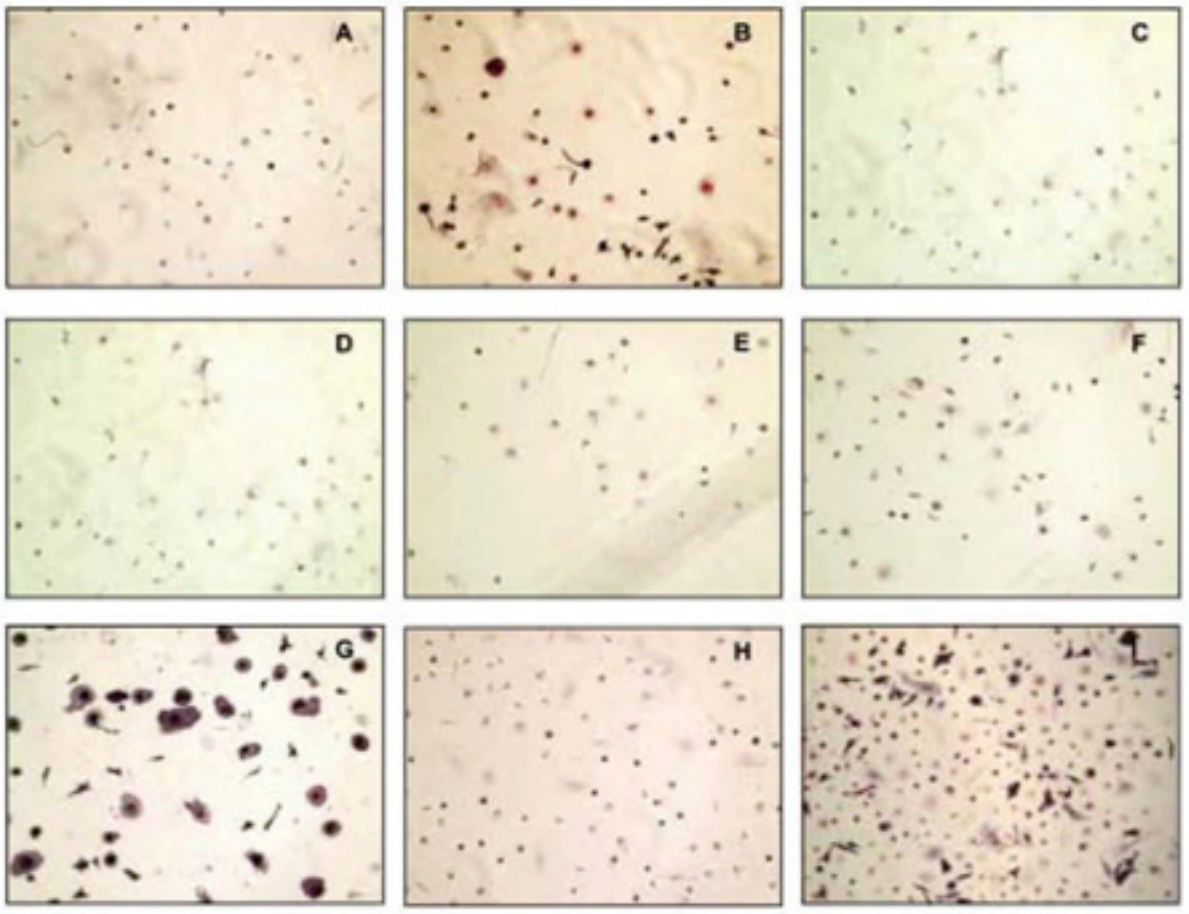

FIGURE 2 Immunophenotype of NLCs. PMBC from a representative B-CLL patient were incubated for 14 days and suspension cells were removed before immunohistochemical analysis. Remaining NLCs were incubated with mononuclear antibodies against surface markers; *Panel A*: CD13; *Panel B*: CD14; *Panel C*: CD15; *Panel D*: CD19; *Panel E*: CD33; *Panel F*: CD34; *Panel G*: CD45; *Panel H*: CD123; and *Panel I*: HLA-DR and then with alkaline phosphatase conjugated secondary antibody. A color reaction was developed resulting in a red stain for positive cells. Specimens were examined on a Zeiss Axioskop microscope (Carl Zeiss, Thornwood, NY, USA). Digital images were captured with a Spot Coge Digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) at 40 × magnification. NLCs from B-CLL patient were strongly positive for CD14 (*Panel B*), CD45 (*Panel G*) and HLA-DR (*Panel I*), and weakly positive for CD33 (*Panel E*) and CD34 (*Panel F*).

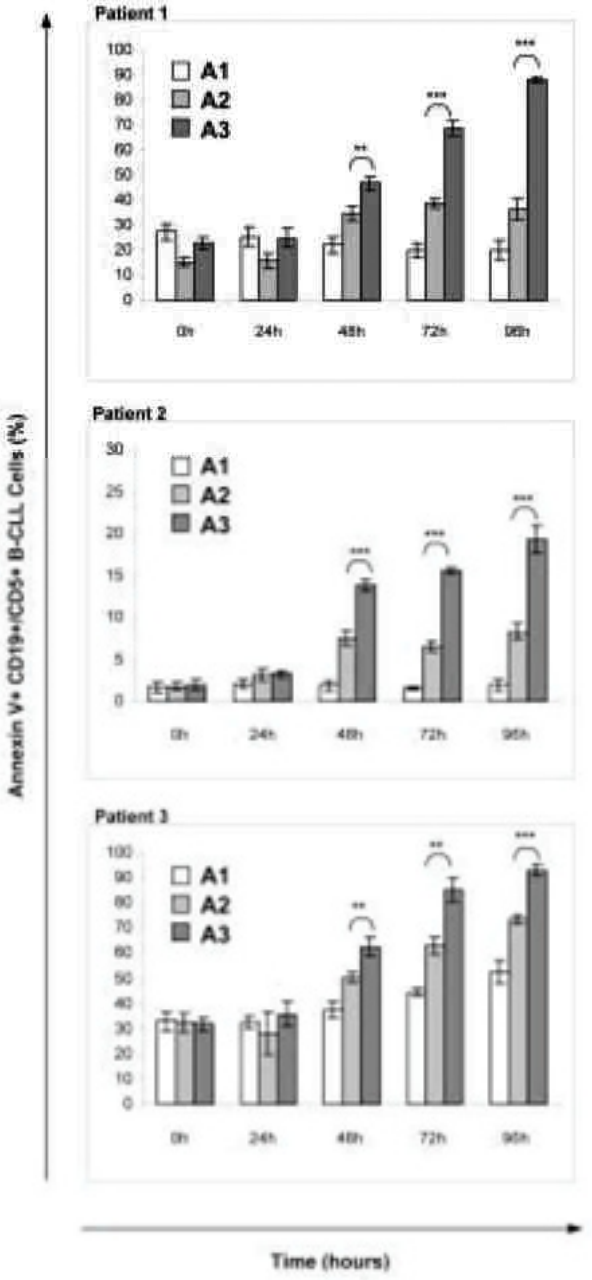

FIGURE 3 NLCs protect B-CLL cells from spontaneous apoptosis. Suspension cells were cultured in presence or absence of NLCs. PMBC from three B-CLL patients (Patient 1, Patient 2 and Patient 3) were cultured for 14 days in culture flasks to allow the growth of NLCs. One of them was maintained as control of spontaneous apoptosis (A1). In order to asses the effect of manipulation on cells, suspension cells (CLL) and NLCs (NLC) from another culture flask were separated, washed in PBS and mixed cultured together again (A2). Suspension cells and NLCs from a 3rd culture flask were separated, both washed in PBS and then, suspension cells were cultured without NLCs (A3). Apoptosis of CD19 + /CD5 + B-CLL cells from each flask was analyzed by annexin V staining at 0 h, 24 h (immediately after the cross-culture), 48, 72 and 96 h. ** Significant increase in percentage of apoptosis of CD19 + / CD5 + B-CLL cells cultured without NLCs (A3) compared to control (A2) value at $P < 0.01$. *** Significant increase in percentage of apoptosis of CD19 + /CD5 + B-CLL cells (A3) cultured without NLCs compared to control (A2) value at $P < 0.001$.

weakly with monoclonal antibodies against CD33 and CD34 (Fig. 2E,F). Cells were negative with antibodies anti-CD13, anti-CD15, anti-CD19, and anti-CD123. These data indicates that immunophenotype of these adherent cells in B-CLL are blood monocyte-derived cells and it is consistent with previously reported data for NLCs [12].

NLCs Protect CD19 + /CD5 + B-CLL Cells from *in vitro* Spontaneous Apoptosis

In order to examine the "nurse" effect of NLCs on the survival of B-CLL cells, suspension cells from 3 B-CLL patients were cultured in presence or absence of NLCs. In all cases, apoptosis of CD19 + /CD5 + B-CLL cells cultured without NLCs were increased progressively during the experiment (Fig. 3). Because of undergoing apoptosis of CD19 + /CD5 + B-CLL cells in part could be due to manipulation of the cells during the washes in PBS, comparison was performed between apoptosis values of B-CLL cells separated, washed, and again cultured with NLCs (Fig. 3: A2), and apoptosis values of B-CLL cells separated, washed and cultured again in absence of NLCs (Fig. 3: A3). In the 3 B-CLL samples, apoptosis of CD19 + /CD5 + B-CLL cells cultured without NLCs were significant greater than their respective controls of manipulation at 48, 72 and 96 h. These data show, in agreement with previously reported data, that NLCs protect CD19 + /CD5 + B-CLL cells from *in vitro* spontaneous apoptosis.

Fludarabine Eliminates Precursors of NLCs in B-CLL Cultures and Induces Death of NLCs

When cultures of PBMC immediately isolated from B-CLL were performed in presence of fludarabine, no NLCs were found even after several weeks in culture. Therefore, precursors cells that can generate NLCs were eliminated by fludarabine at 50 μM dose (data not shown). Moreover, when the growth of NLCs was allowed, and these cells were treated with 50 μM fludarabine for 24 h, the number of the remaining NLCs after 10 days of the treatment, was significant lower than untreated NLCs (Table II). Figure 4 shows a representative picture of NLCs untreated (Fig. 4A) or treated with fludarabine (Fig. 4B), in which, death NLCs were shown by trypan blue staining.

NLCs Do Not Protect CD19 + /CD5 + B-CLL Cells of the Apoptosis Induced by Fludarabine

In order to understand the role played by NLCs in the apoptosis induced by fludarabine in B-CLL cells, we performed cross-cultures incubating tumoral cells untreated and treated with fludarabine with untreated and treated NLCs in the 4 possible combinations (Fig. 1B). When CD19 + /CD5 + B-cells were treated with fludarabine the percentage of apoptotic cells observed was gradually higher during the experiment (Fig. 5: F1). Apoptosis in treated CD19 + /CD5 + B-cells was similar

TABLE II NLC count 10 days after the treatment with fludarabine

	Number of NLCs		
ID patients	Untreated NLCs mean ± SD†	Treated NLCs* mean ± SD†	*P* values
Patient 1	153.33 ± 21.20	46.00 ± 11.27	0.0042
Patient 2	189.67 ± 49.32	24.67 ± 5.50	0.0272
Patient 3	163.33 ± 14.47	39.33 ± 2.51	0.0036

* NLCs treated with 50 μM fludarabine for 24 h.
† Mean ± SD of total number of NLCs of 3 different counts of 6 random microscopy fields.

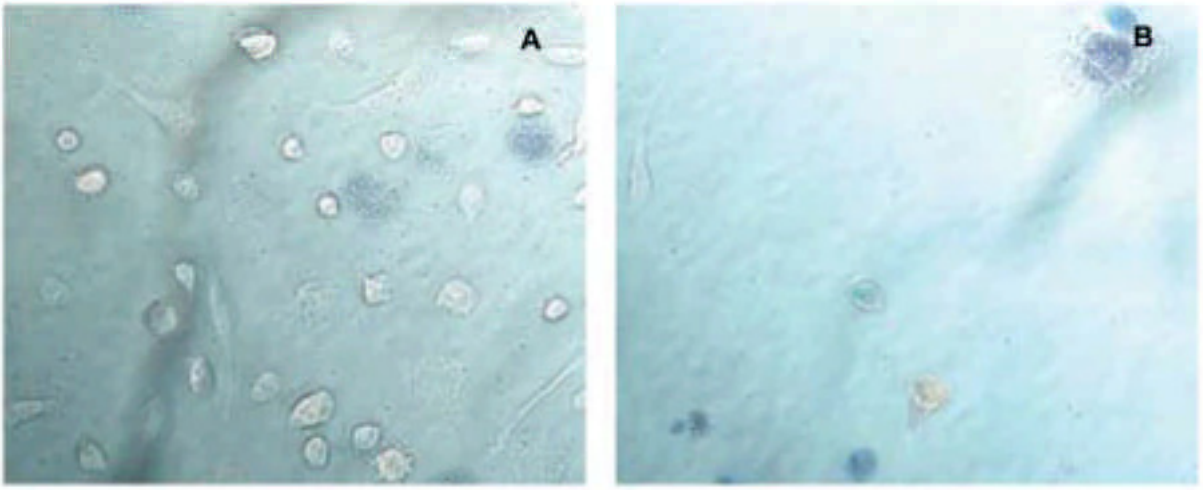

FIGURE 4 Fludarabine induces the death of NLCs. Representative experiment with NLCs from patient 1. *Panel A*: Differentiated untreated NLC; *Panel B*: Differentiated NLCs treated with fludarabine for 24 h. NLCs were examined by trypan blue exclusion after 10 days of the treatment. Digital images were captured with a Spot Coge Digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) at 40 × magnification The total number of NLCs treated with fludarabine was significant lower than untreated NLCs, and almost all NLCs were stained by blue trypan in these treated NLCs.

whether they were co-cultured with NLCs treated with fludarabine (Fig. 5: F2), without NLCs (Fig. 5: F3) or with untreated NLCs (Fig. 5: F5), reaching high percentages of apoptosis at 96 h after treatment with the drug.

DISCUSSION

The accumulation of tumoral cells in B-CLL is due to a deficient programmed cell death, as opposed to excessive cell proliferation [22,23]. Although the molecular events responsible for the development of B-CLL are not yet known, current data support the hypothesis that transformed CD5 + B cells are not autonomous in their ability to evade apoptotic signals. Culture of B-CLL cells *in vitro* is associated with the rapid down-regulation of antiapoptotic signal and apoptosis [24]. These data suggest that *ex vivo* conditions lack essential survival signals received by B-CLL cells *in vivo* [6,23]. Soluble factors [7] and cell to cell interactions [7,10] have been implicated in the antiapoptotic effects mediated by other cells that could play a role as "nurse cells". In agreement with data previously reported [12], when we performed long-term cultures of PBMC from B-CLL patients, a subset of adherent cells with large and fibroblast like morphology was observed. These adherent cells expressed surface markers of monocytic lineage and protected to B-CLL cell from spontaneous apoptosis playing a role as "nurselike" cells (NLCs).

Fludarabine is currently used alone or in combination with other drugs for the treatment of B-CLL [25,26]. The experimental approaches designed to analyze the action of this drug have been focused in the action of the drug in B-CLL cells, without paying special attention to its effect in other cells, such as NLCs, that could play an important role in the survival of B-CLL cells [27].

Our data showed that fludarabine was able to block the development of NLCs in culture of PBMC from B-CLL patients. Moreover, when the growth of the NLCs was allowed, treatment with fludarabine for 24 h also induced the death of these differentiated NLCs. Because of the effect of fludarabine in these NLCs could influence the viability of B-CLL cells, we performed cross-cultures to know the role that NLCs play in the ability of fludarabine to induce apoptosis in B-CLL cells. Our results showed

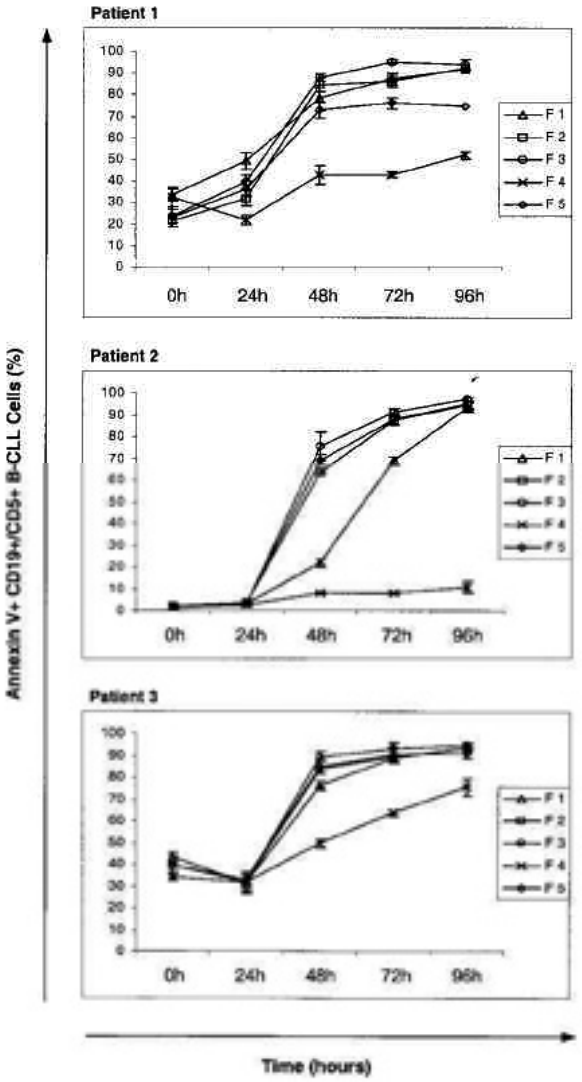

FIGURE 5 NLCs do not protect CD5 + B cells from apoptosis induced by fludarabine. Cross-cultures with PMBC from three B-CLL patients (Patient 1, Patient 2 and Patient 3) were performed to analyze the role played for NLCs in the apoptosis induced by fludarabine in CD19 + /CD5 + B-CLL cells. Aliquots was removed from each culture flask: F1: control of fludarabine-induced apoptosis (non-separated suspension cells and NLCs treated with fludarabine); F2: suspension cells and NLCs treated with fludarabine; F3: treated suspension cells without NLCs; F4: untreated suspension cells and NLCs treated with fludarabine; F5: suspension cells treated with fludarabine and untreated NLCs. Analysis of the apoptosis of CD19 + /CD5 + B-CLL cells was performed at 0 h (immediately before treatment with fludarabine), 24 h (immediately after the cross-culture), 48, 72 and 96 h. When suspension cells were treated with fludarabine, the percentages of annexin V positive CD19 + /CD5 + B-CLL cells were progressively higher and were similar whether they were co-cultured with treated NLCs (F2), without NLCs (F3) or with untreated NLCs (F5).

that in spite of fludarabine was able to induce the death of NLCs and CD19 + /CD5 + B-CLL cells, apoptosis induced by fludarabine on CD19 + /CD5 + B-CLL cells was due to a direct effect on these cells and not due to its effect on NLCs; that is, fludarabine treated CD19 + / CD5 + B-CLL cells underwent apoptosis at a similar rate no matter whether they have been cultured with NLCs treated or untreated with fludarabine (Fig. 5).

Whereas our study indicates that fludarabine was able to induce apoptosis in B-CLL cells *in vitro* mainly by a direct action, these data do not exclude that fludarabine could affect different cell types (stromal cells, macrophagic cells, etc) that play an important role in the survival of B-CLL cells *in vivo*.

In conclusion, NLCs protects B-CLL cells from *in vitro* apoptosis and fludarabine exerts its effect independently from NLCs on B-CLL cells.

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