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Malignancy

Heparin Induces Apoptosis in Lymphocytes from B-cell Chronic Lymphocytic Leukemia

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It has been shown that glycosaminoglycans play a role in the regulation of immune response. In particular, heparin exerts an antiproliferative and apoptotic action in different cellular systems. In this study we evaluate whether heparin can also induce a naturally occurring programmed cell death in lymphocytes from B-chronic lymphocytic leukemia (B-CLL), a neoplastic lineage where apoptosis is blocked by the expression of the proto-oncogene bcl-2.

Peripheral blood lymphocytes (PBL) from 7 cases of B-CLL patients in different stages were cultured with three different heparin sodium concentrations for 4 days. Apoptosis was evaluated by agarose gel electrophoresis and by cytofluorimetric analysis. Bcl-2 expression was tested by flow cytometric analysis and immunohistochemistry on cytospin preparations.

Agarose gel electrophoresis showed the characteristic DNA fragmentation pattern of apoptosis in all the cases of B-CLL stage III and IV after heparin incubation. DNA from normal and neoplastic lymphocytes cultured without heparin did not undergo spontaneous apoptosis. Cytofluorimetric analysis confirmed the agarose gel pattern and found a level of apoptosis over 50% after culture of neoplastic PBL with heparin. In these cases bcl-2 expression was found to be significantly reduced after heparin incubation when comparing to bcl-2 level before incubation.

Our data adds further evidence regarding the potential role of heparin in oncogene inhibition and in apoptosis induction. In particular, the induction of apoptosis in neoplastic lymphocytes by heparin may have a role in the complicated field of interactions between the immune system and the blood vessels by glycosaminoglycans.

Keywords: Heparin, apoptosis, B-CLL, bcl-2

INTRODUCTION

Heparin, a sulfated glycosaminoglycan isolated from mammalian tissues, binds with a plasma protein facilitating the inhibition of intrinsic and common coagulation pathways: this interaction produces a potent anticoagulant effect. Moreover, many recent studies have suggested that heparin has an antiproliferative actior directed

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to a variety of cultured cells and neutrophils. Prevention of the cellular progression through the G1 phase, reduction of mRNA transcription of c-fos and c-myc and interference with growth-factor are the main processes involved [1–3]. Recently, apoptosis induction in human neutrophils has been proposed as a possible anti-inflammatory mechanism of heparin [4].

There is growing interest in the potential impact of glycosaminoglycans on the regulation of the immune response. In fact, it has been shown that heparan sulfate, a molecule related to heparin, plays a role in lymphocyte activation and proliferation [5]; the same molecule has inhibitory or stimulatory effects on murine splenocytes [6]. It is noteworthy that lymphocytes possess a membrane glycoprotein namely CD44 which participates in several lymphocyte functions, including adhesion, lymph-node homing by virtue of its capacity to bind glycosaminoglycans. Moreover, growth factors binding heparin may have mitogen and survival effects in several cellular system as well as in lymphocytes.

Lymphocytes of B-cell chronic lymphocytic leukemia (B-CLL) highly express the protooncogene bcl-2 which confers a selective growth advantage blocking programmed cell death (apoptosis) [7]. This well defined scenario gave us the opportunity to further explore the effect of heparin on oncogene expression and apoptosis induction in normal and neoplastic lymphocytes.

MATERIALS AND METHODS

Patients

Four normal age-matched male volunteers and seven male patients with clinical, morphological and immunological features characteristic of B-CLL were studied (Tab. I). Age ranged from 53 to 73 years. None of the patients had received any treatment before entering in the study. Total lymphocyte count ranged from 13 to $29,3 \times 10^9$/L. According to the Rai staging system, stage 0 was diagnosed in three patients, two cases were in stage III and other two patients in stage IV.

Cell Cultures

Peripheral blood lymphocytes (PBL) were obtained from freshly citratated blood by centrifugation on Ficoll-Hypaque gradient. The cells were then washed three times with phosphate-buffered saline (PBS) and 5×10^6 cells were resuspended in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 IU penicillin, 100 mg/mL streptomycin, 50 mg/mL gentamycin. Cells were then incubated for 4 days at 37°C in a humidified atmosphere of 5% CO_2 in presence the of sodium heparin at three different concentrations (10 U/mL, 50 U/mL and 100 U/mL) or in medium alone.

Cell cultures were prepared in triplicate using three different sodium salt heparin preparations from porcine mucosa purchased from Sigma (S. Louis, Missouri), from Biologici Italia Laboratories (Milan, Italy) and from Calbiochem (S. Diego, California).

Apoptosis Assay

At the end of incubation, cells were washed, resuspended in 1 mL of RPMI and lysed by the addition in sequence of 200 µg of proteinase K (Boheringer Mannheim, Milano, Italy). DNA extraction was carried out through the salting-out method as follows: 400 µL of 5 M NaCl was added to the lysate which was shaken vigorously and centrifuged at 800 g for 15 min. The supernatant was collected and 5 mL of absolute ethanol was added. After washing with 70% ethanol, DNA was resuspended in TE (10 mM Tris-HCL, 0,2 mM Na-EDTA, pH 7,5) and treated with 0,5 mg/mL Rnase at 37°C for 30 min. DNA was finally run on 1.5% agarose gel containing

TABLE I Haematological parameters of B-CLL patients

Patients	Age (years)	Stage (Rai)	Leukocytes ($\times 10^9$/L)	Lymphocytes ($\times 10^9$/L)	Hemoglobin (g/dL)	Platelets ($\times 10^9$/L)	Phenotype
1	63	0	15,7	13	13,5	150	CD19/CD23/CD5
2	53	0	17,2	13,7	14,2	210	CD19/CD23/CD5
3	66	0	16	13,4	13,8	140	CD19/CD23/CD5
4	68	III	19,8	17	10,3	170	CD19/CD23/CD5
5	70	III	33	29,3	9,8	155	CD19/CD23/CD5
6	73	IV	24,6	21,8	9,8	85	CD19/CD23/CD5
7	61	IV	28,7	24,4	8,8	70	CD19/CD23/CD5

0,1 mg/mL of ethidium bromide and viewed under UV light. Apoptosis was also evaluated using a cytofluorimetric method as previously described [8]. Briefly, the samples were fixed in ethanol 70% and subsequently treated with RNAse 0,5 mg/mL for 15 min at 37°C. Cell pellets were resuspended in 50 mg/mL propidium iodide (Sigma Chemical, St. Louis, MO) in PBS. Analysis was performed by a FACScan flow cytometer Ortho Cytoron Absolute using the Research software. Cell cultures were evaluated after 24, 48 and 96 hours of incubation.

Bcl-2 Analysis

Bcl-2 expression was evaluated by immunohistochemistry and by flow cytometric analysis.

Immunohistochemistry was performed on cytospin preparations using the antibody anti-bcl2 (DAKO), as previously described [9]. Briefly, cytospins were fixed with acetone-chloroform solution for 6 min or by microwave (2 min at 100 W) followed by incubation with the primary antibody for 15–20 min. As a revelation system, alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique was used.

To perform the two-color immunofluorescence analysis, the cells were fixed in 4% paraformaldehyde for 20 min and permeabilised in 0,1% Triton X-100 in Tris-buffered saline with 0,1% bovine serum albumin for 5 min. After washing in PBS with 3% FBS and 0,1% sodium azide, cells were incubated with anti-bcl-2 for 20 min on ice. Anti-bcl2-treated cells were treated with FITC-conjugated anti-mouse IgG1 antibody at a 1 : 1.000 dilution for 20 min. on ice and for 10 min. with 10% mouse serum to block unbinding sites of the second antibody. Next, PE-labelled CD 19 antibodies were added to anti-bcl2 treated cells for 20 min. on ice. The stained cells were analysed using a cytometer Ortho Cytoron Absolute. Two color staining patter was obtained for lymphocytes gated forward and 90° light scatters.

Bcl-2 evaluation was performed in viable cells separated from apoptotic cells by the measurement of forward and side light scatters on a flow cytometer, as previously shown [10, 11].

RESULTS

PBLs were exposed to different concentrations of heparin for various times to evaluate the dose response and the kinetic. After 96 hours, agarose gel electrophoresis of DNA showed a sharp characteristic fragmentation pattern of apoptosis in stage III and IV B-CLL cases. PBL from normal cases and from stage 0 CLL showed a high-molecular weight DNA pattern after incubation with heparin (Fig. 1). Flow cytofluorimetric analysis of ethanol-fixed PI-stained cells showed that treatment with 100 U/mL of heparin increased the level of apoptosis after 96 hours in all the advanced B-CLL stages (Fig. 2b) whereas no significant change was observed in stage 0 and normal cases, compared with cells incubated with medium alone (Figs. 2a, c). Table II summarises the percentage of apoptotic cells for each case examined.

Figure 3 shows the profile of apoptosis correlated to the time and the dose of heparin in all the

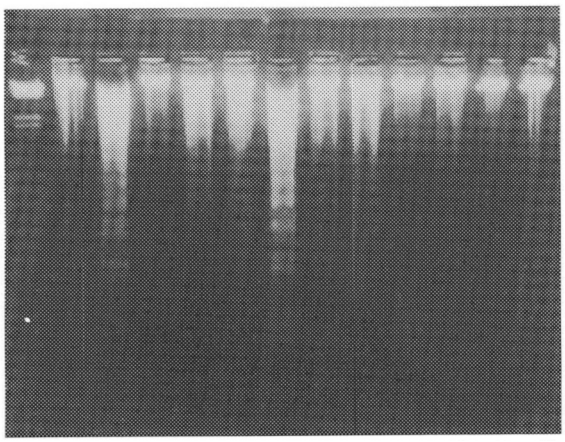


FIGURE 1 Agarose gel electrophoresis of total cellular DNA from CLL and from normal lymphocytes incubated with 100 U/mL sodium heparin for 4 days at 37°C in RPMI. Lanes 1–5: DNA from patients n.4 and n.5 without heparin; lanes 2,6 DNA from patients n.4 and n.5 after incubation with heparin; lanes 3,7: DNA from patients n.1 and n.2 without heparin; lanes 4,8: DNA from patients n.1 and n.2 after incubation with heparin. DNA from normal lymphocytes without heparin (lanes 9,11) and with heparin (lanes 10,12). M, Molecular weight marker II.

cases examined. *The degree of apoptosis is increased over a 24–48 hour time period in a dose and time dependent manner but the amount of apoptosis levels off at 96 hours.* Panel **a** and **c** show the profile of apoptosis in stage 0 and one of the four controls, respectively. Thus, heparin appears to induce apoptosis in lymphocytes from patients affected by B-CLL in advanced stages.

In lymphocytes from patients in stage III and IV, flow cytometric analysis indicated that the majority of viable PBL incubated with heparin displayed much less bcl-2 compared to the relatively strong staining with anti-bcl-2 antibody before incubation (Figs. 4 b, c), suggesting that bcl-2 downregulation is probably responsible of apoptosis. On the contrary, even if lymphocytes from patients in stage 0 did express a high level of bcl-2, no significant difference after incubation with heparin was found (Fig. 4 a). Figure 4 d shows the bcl-2 expression in a normal case incubated with heparin and in a B-CLL patient after incubation with medium alone.

Immunohistochemistry for bcl-2 showed a visible reduction of the expression after heparin incubation when comparing to bcl-2 level before incubation (Figs. 5 a, b).

DISCUSSION

This study is based on the well-known influence of glycosaminoglycans on activation, recirculation and adhesion of lymphocytes [5]. Our data suggests that exposure of lymphocytes from B-CLL to heparin may trigger DNA fragmentation characteristic of apoptosis as defined by agarose gel electrophoresis of DNA and by propidium iodide cytofluorimetric analysis. The possibility that contaminants or preservatives could account for the apoptotic effect was ruled out by using three different preparations of sodium heparin containing only sterile water as eccipient (pH 7,4). We found a significant dose-effect relationship only after 24 and 48 hours of incubation whereas in the successive hours a ≪plateau≫ effect was observed.

Different mechanisms may be proposed to explain our observation. If we consider the previously described immunostimulatory properties of heparin and heparan sulfate [6] and equally the fact that lymphocytes from B-CLL downregulate bcl-2 after mitogenic stimulation [12], then we could surmise that the induction of apoptosis by heparin is related to an activating signal able to downregulate bcl-2 expression and to induce programmed cell death.

Alternatively, if one considers the evidence that heparin can reduce or interfere with the transcription of mRNA and of growth-factor, then we could maintain the existence of an inhibitory mechanism able to directly influence the RNA stability of bcl-2.

Interestingly, we found a different degree of apoptosis in lymphocytes from patients in stage 0 compared to those in advanced stages of disease. The limited experiments performed did

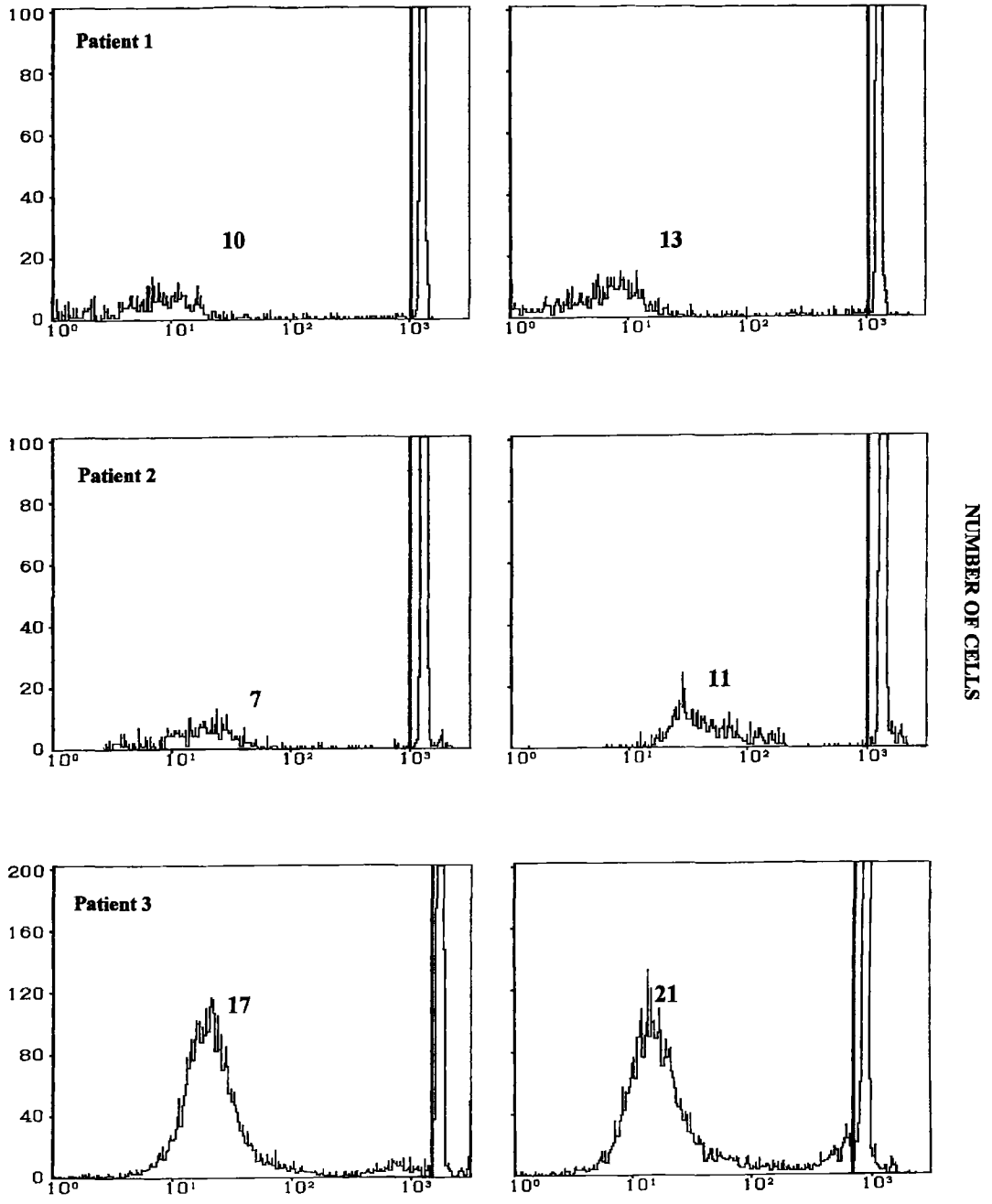
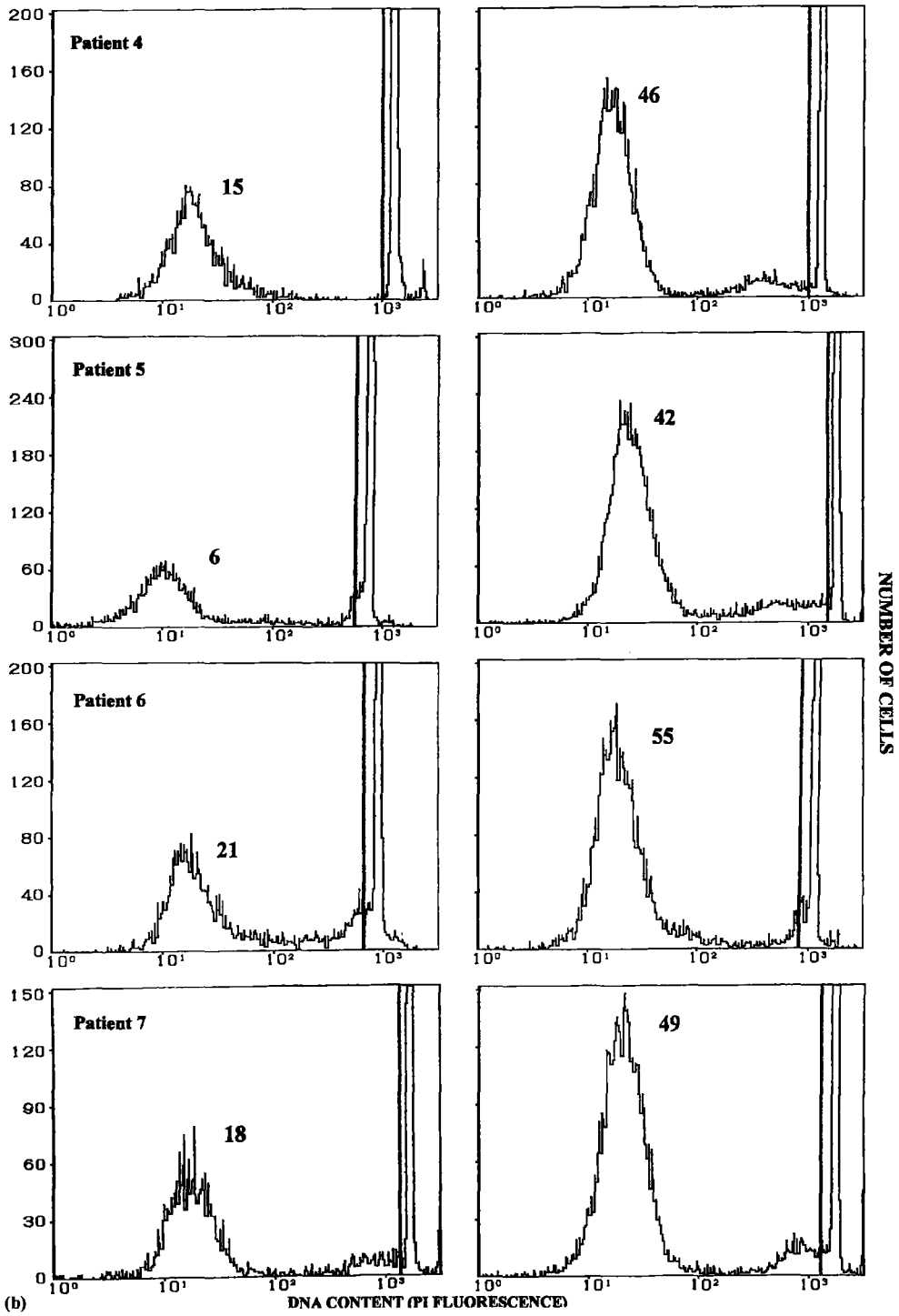
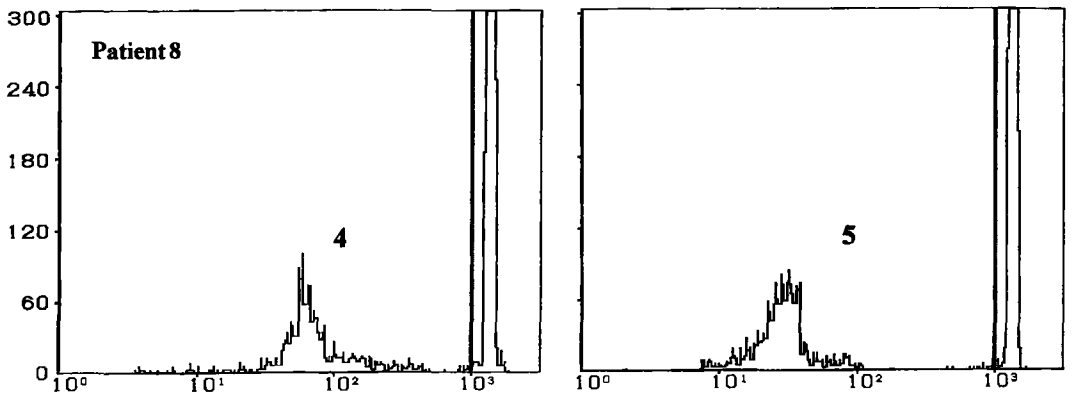


FIGURE 2 DNA fluorescence flow cytometric profiles of PI-stained PBL from two cases of CLL after 96 hours of incubation with medium alone (left panels) or 100 U heparin (right panels). Panel **a** refers to stage 0 B-CLL cases, panel **b** refers to advanced stages and panel **c** shows the normal control incubated with heparin. The numbers represent the percentage of apoptotic cells located in the hypodiploid region of the histogram.



(b)

FIGURE 2 (Continued).



(c) DNA CONTENT (PI FLUORESCENCE)

FIGURE 2 (Continued).

TABLE II Percentage of apoptosis detected by DNA fluorescence flow cytometric profiles of PI-stained PBL after 96 hours of incubation with heparin compared to PBL cultured in medium alone

Patients	Heparin −	Heparin +
1	10%	13%
2	7%	11%
3	17%	21%
4	15%	46%
5	6%	42%
6	21%	55%
7	18%	49%
8	4%	5%
9	2%	5%
10	4%	6%
11	3%	6%

Patients 1 to 7 refer to Table I. Patients 8 to 11 refer to normal controls.

not enable us to draw any definite conclusion but our data suggest that glycosaminoglycans may interact with lymphocyte functions and influence apoptosis *via* the inhibition of bcl-2 expression. The presence of different expressions or different isoforms of CD44, the receptor for glycosaminoglycans present on the surface of normal and neoplastic lymphocytes, could play a role [13].

Another intriguing mechanism possibly playing a role in the induction of apoptosis by heparin could involved the basic fibroblast growth factor (bFGF), a pleiotropic cytokine with diverse roles on a variety of cell types. In CLL cells, bFGF has been shown to delay apoptosis probably by upregulating the expression of bcl-2 protein [14, 15]. Moreover, recent studies have found a direct correlation between the intracellulare level of bFGF and advanced stages of CLL.

Heparin strongly binds bFGF, in some cases with inhibitor effect [16] in other with potentiat-

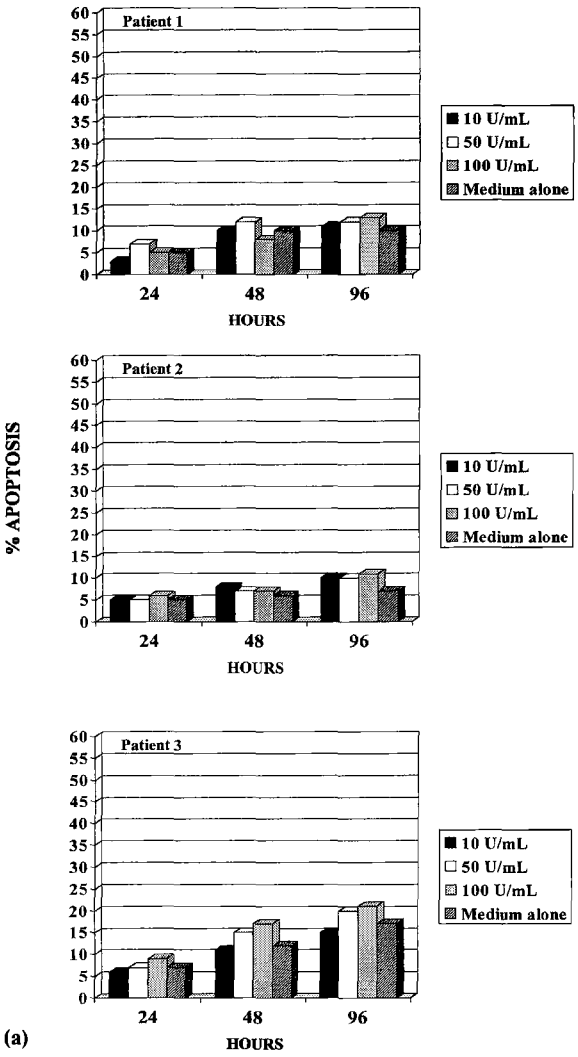


FIGURE 3 The dose response of heparin-induced DNA fragmentation in CLL cells. PBL were incubated in various concentrations of heparin or in medium alone for three different times. Panel **a** refers to stage 0 B-CLL cases, panel **b** refers to cases in stage III and IV and panel **c** refers to normal controls.

ing activity [17]. There is evidence that the binding of heparin to bFGF primarily serves to juxtapose components of the FGF signal to transduction pathway [18].

On this basis, independently by the mechanism, heparin could trigger apoptosis in B-CLL *via* the interaction with bFGF, or blocking its ability to prolong survival by a down-regula-

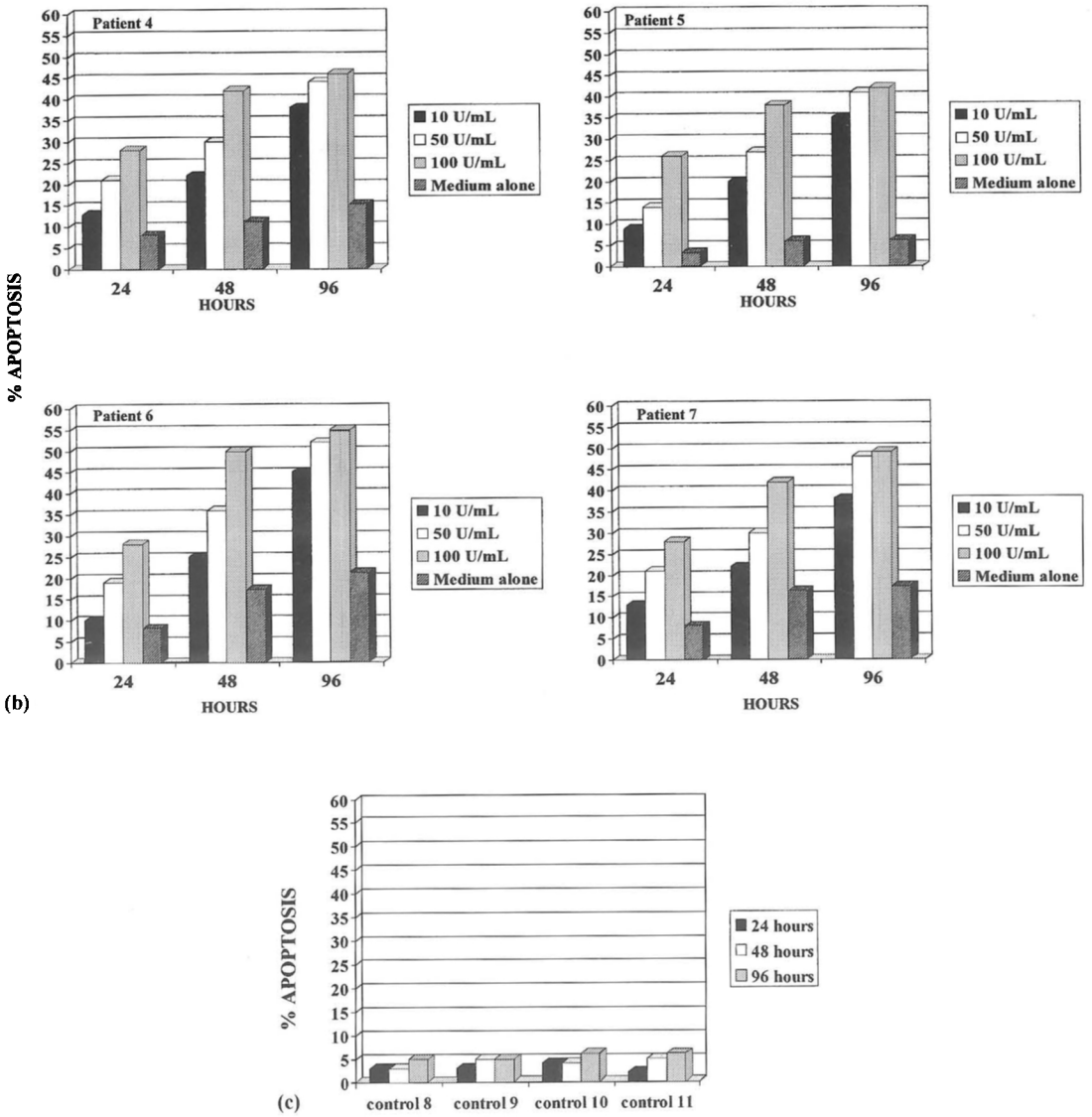


FIGURE 3 (Continued).

tion of bcl-2 or potentiating the effect of bFGF. This hypothesis could well explain why heparin did not act in stage 0 CLL, where the intracellular level of bFGF if lower than advanced stages.

In conclusion, although it is possible that heparin interferes with lymphocyte functions by more than one mechanism, we suggest that suppression of bcl-2 expression by heparin at least partially explain its antiproliferative activity.

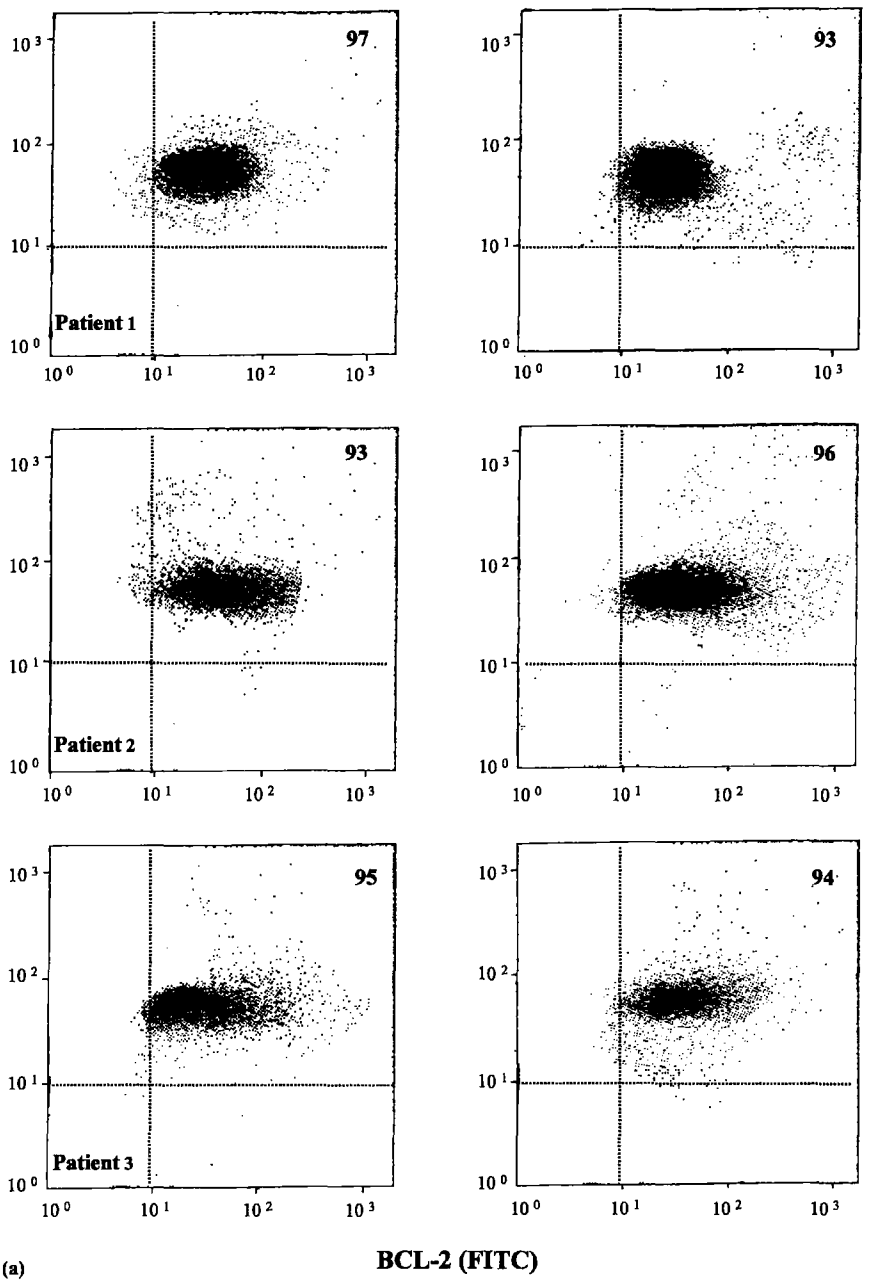
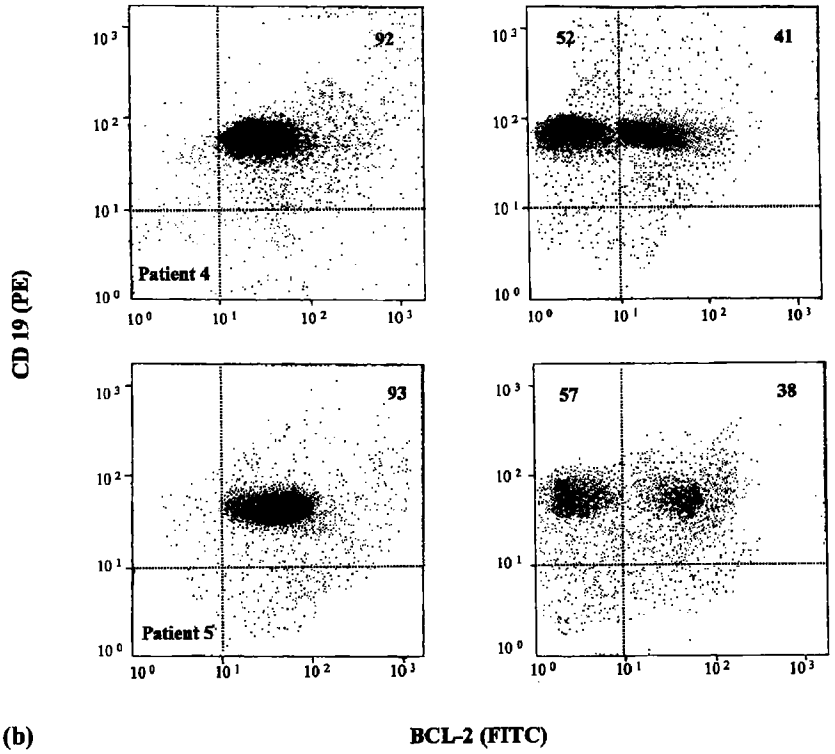
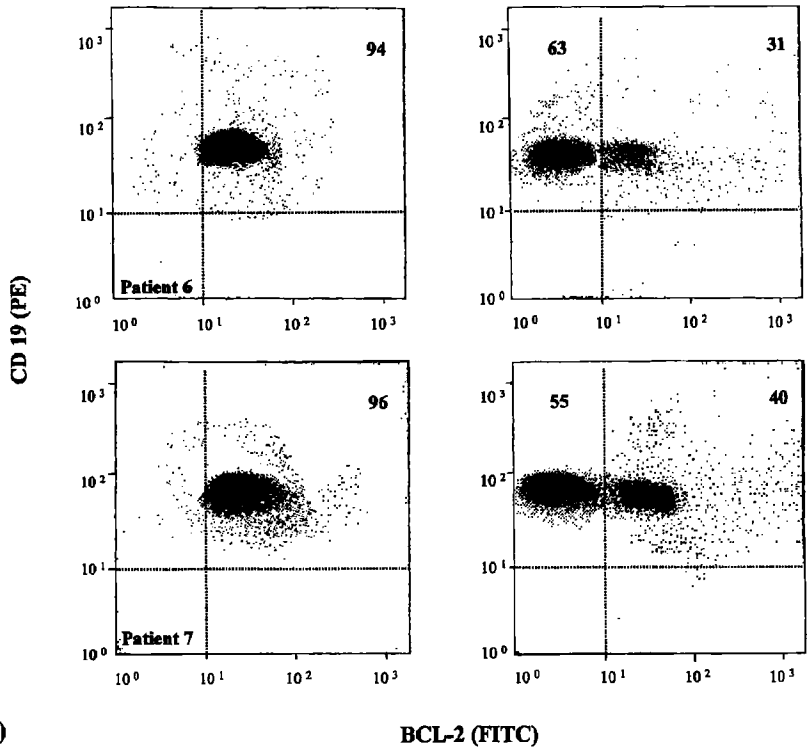


FIGURE 4 Two-color immunofluorescence profiles of bcl-2 expression evaluated by a flow-cytometric analysis using anti-bcl-2 antibody. Right panels show the percentage of bcl-2 positivity after 96 hours of incubation with heparin whereas left panels refer to bcl-2 level before incubation. Panel **a** refers to stage 0 B-CLL, panel **b** and **c** refers to advanced B-CLL cases, panel **d** shows a normal case before and after incubation with heparin and an advanced B-CLL stage before and after incubation in medium alone. Numbers refer to the percentage of cells.





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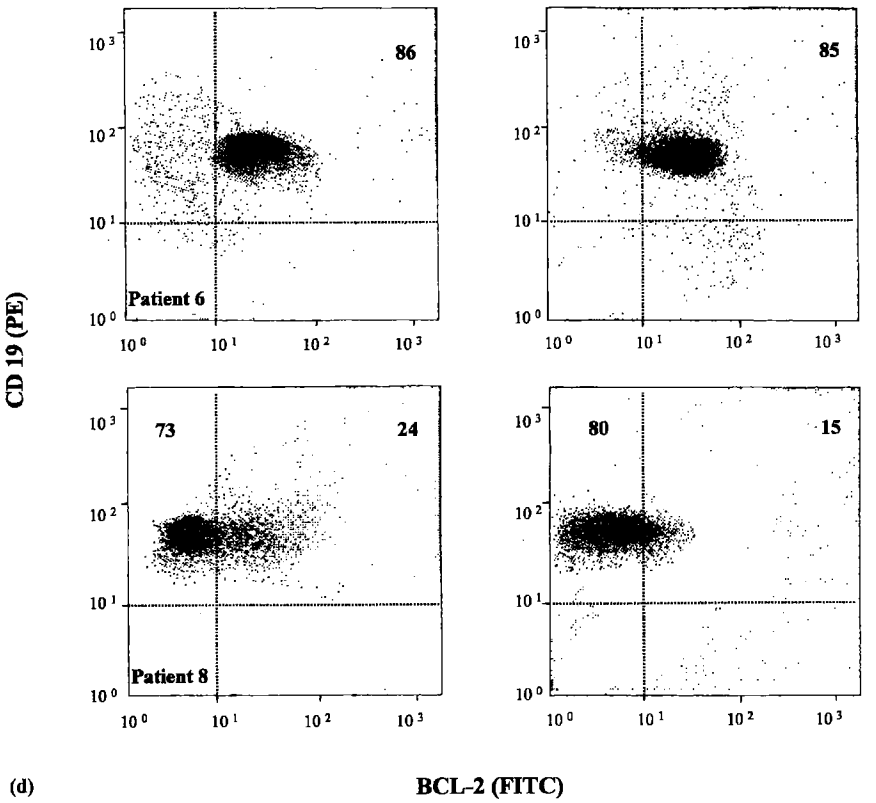

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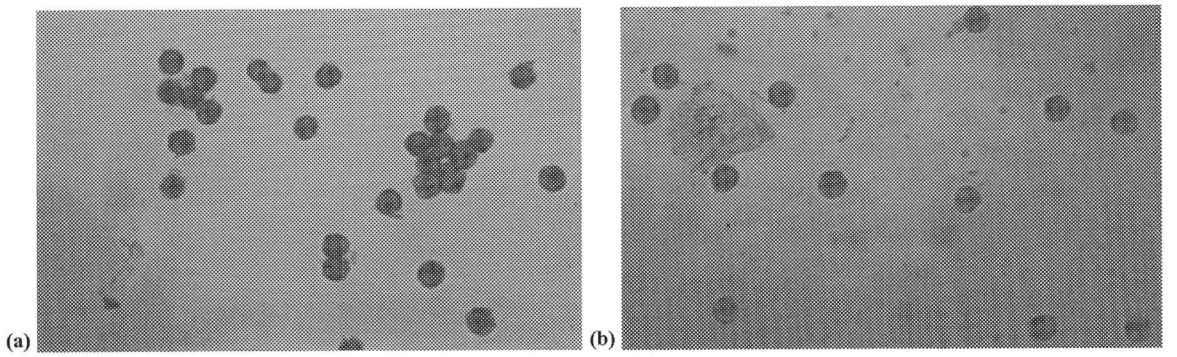

FIGURE 5 Immunohistochemistry for bcl-2 in lymphocytes from patient n.6 before incubation **(a)** and after incubation **(b)** for 96 hours with 100 U/mL sodium heparin. (See Color Plate I).

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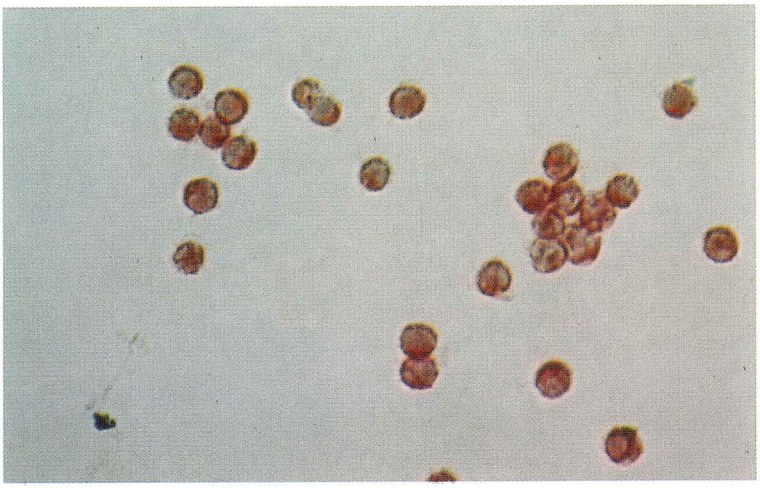
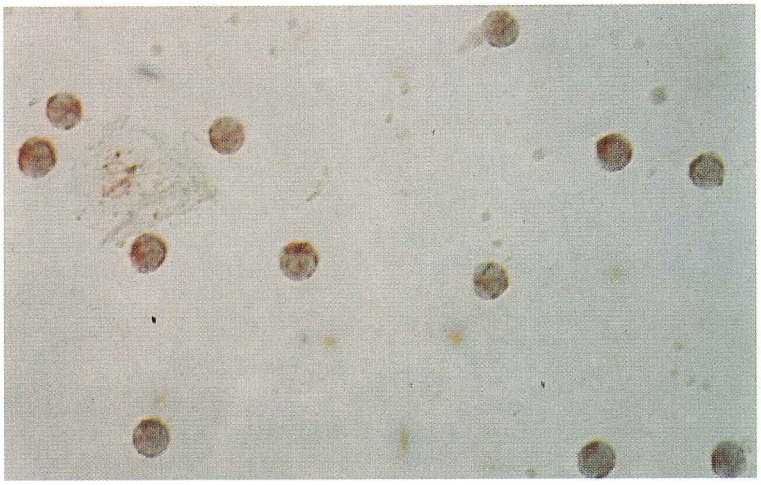


(a)



(b)

Color Plate I (See page 462, Figure 5) Immunohistochemistry for bcl-2 in lymphocytes from patient n.6 before incubation **(a)** and after incubation **(b)** for 96 hours with 100 U/mL sodium heparin.