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Basic fibroblast growth factor (bFGF) upregulates the expression of bcl-2 in B cell chronic lymphocytic leukemia cell lines resulting in delaying apoptosis

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Basic fibroblast growth factor (bFGF) is a pleiotropic cytokine which has recently been shown to delay fludarabine-induced apoptosis in B cell chronic lymphocytic leukemia (B-CLL) cells. To investigate the potential mechanism of bFGF-mediated delay of apoptosis, two EBV-transformed B prolymphocytic cell lines (JVM-2, JVM-13), one EBV-transformed B-CLL cell line (I83CLL), and one non-EBV-transformed B-CLL cell line (WSU-CLL) were used as a model for chronic lymphoid malignancies. Viability data of cells treated with fludarabine alone or in combination with bFGF demonstrated that the addition of bFGF to the cells resulted in prolonged survival. Quantitative assessment of apoptosis-associated DNA strand breaks by *in situ* TdT labeling showed a protective effect of bFGF on fludarabine-treated cells. The potential effect of bFGF on bcl-2 mRNA expression was analyzed by Northern blotting. Stimulation with bFGF led to a time-dependent accumulation of bcl-2 specific mRNA in all three cell lines. Maximal levels of bcl-2 mRNA expression were detected after 8 h in JVM-2, and after 18 h in JVM-13 and I83CLL. Intracellular bcl-2 protein was also found to be increased upon bFGF stimulation in both EBV- and non-EBV-transformed cells. In addition, exposure of cells from three patients with B-CLL to bFGF showed an upregulation of bcl-2 protein after 4–8 h. Our data demonstrate that bFGF upregulates the expression of bcl-2 in these cells, suggesting that this increase in bcl-2 expression may play a role in the delay of fludarabine-induced apoptosis.

Keywords: basic fibroblast growth factor; chronic lymphocytic leukemia; bcl-2; apoptosis

Introduction

Basic fibroblast growth factor (bFGF) is a pleiotropic cytokine which plays an important role in angiogenesis and mesodermal development,[1] and it has been postulated to play a role in malignancy.[2] In previous studies, increased levels of bFGF have been detected in urine from patients with a broad variety of neoplastic diseases including various leukemias.[3,4] Other investigators have demonstrated elevated levels of bFGF in cells derived from renal cell carcinoma[5] and malignant melanoma.[6] In addition, a correlation between increased levels of bFGF and impaired prognosis in these diseases has been established.[7]

Recently, we have been able to show, that intracellular protein levels of bFGF correlate with stage of disease in B cell chronic lymphocytic leukemia (B-CLL) cells and that these elevated levels are associated with enhanced survival of CLL lymphocytes upon treatment with fludarabine, a drug known to induce apoptosis in B-CLL cells.[8]

Apoptosis is an active process of programmed cell death which occurs during the senescence of normal cells. It is characterized by morphological changes, such as membrane blebbing, cell shrinkage, chromatin condensation and fragmentation.[9] The proto-oncogene bcl-2, an inner mitochondrial membrane protein, has been shown to play an important role in cell survival, preventing apoptosis in a variety of cell types *in vitro* and *in vivo*.[10] In human follicular lymphoma the gene is rearranged into the heavy chain Ig locus,[11,12] resulting in a permanent expression of bcl-2. High levels of both bcl-2 mRNA and protein have been documented in most B-CLL cells, whereas it has not been detected in normal $CD5^+$ B cells:[13] however, the mechanism by which bcl-2 expression is increased is unknown, since translocation of bcl-2 has been shown to occur infrequently in CLL.[14]

In order to investigate a potential mechanism by which bFGF might mediate a delay in apoptosis, two EBV-transformed B prolymphocytic cell lines (JVM-2, JVM-13),[15–17] one EBV-transformed B-CLL cell line (I83CLL),[18] and one EBV-negative B-CLL cell line (WSU-CLL)[19] were used as a model for chronic lymphoid malignancies of the B cell type. In addition, we examined lymphocytes from three patients with B cell chronic lymphocytic leukemia (B-CLL). Using the cell lines and the fresh CLL cells, we studied the ability of bFGF to improve the survival of fludarabine-treated cells and the potential effect of this cytokine on the expression of bcl-2 mRNA and protein levels, respectively.

Materials and methods

Reagents

Fludarabine des-phosphate (FAra, 9-β-D-arabinosyl-2-fluoroadenine) (Sigma, St Louis, MO, USA) was dissolved in 100% dimethylsulfoxide (DMSO) for a stock solution of 10 mM. It was further diluted in medium at final concentrations of 1–100 μM, respectively. Recombinant human basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN, USA) and diluted in medium.

Cells

The well characterized human B cell leukemia cell lines JVM-2[15,17] and JVM-13 were established from the peripheral blood of a patient with B-prolymphocytic leukemia by EBV transformation, respectively.[16] The human chronic B cell leukemia cell line I83CLL was derived from the peripheral blood of a patient with B cell chronic lymphocytic leukemia (B-CLL) by EBV transformation.[18] The human EBV-negative chronic B cell leukemia cell line WSU-CLL was established from a patient with advanced B-CLL.[19] All four cell lines were cultured in RPMI 1640, supplemented with 1% penicillin/streptomycin, 1 mM L-glutamine (Gibco, Grand Island, NY, USA), 10% heat-inactivated fetal bovine serum (FBS) (Gibco) at 37°C, 5% CO_2

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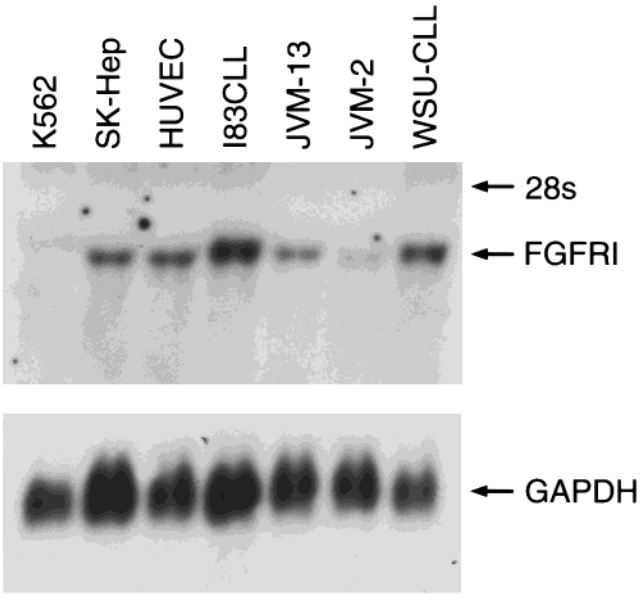

Figure 1 Detection of bFGF receptor type 1 (bFGFR-1) mRNA in cell lines representative of chronic lymphoid malignancies of the B-cell type by Northern blot analysis. Ten micrograms per lane of total cellular RNA was size-fractionated, transferred to nylon membranes and hybridized with a DIG-labeled probe for FGFR-1, RNA of SK-Hep cells and human umbilical vein endothelial cells (HUVEC) were used as positive control. K562 cells were used as negative control. FGFR-1 mRNA is shown as a 4.8-kb band. The membrane was then rehybridized with a digoxigenin-labeled probe for GAPDH.

in air. The human promyelocytic cell line HL-60 (used as a positive control for bcl-2 expression) was grown in RPMI 1640, supplemented with 1% penicillin/streptomycin, 1 mM L-glutamine (Gibco), 10% FBS (Gibco) at 37°C, 5% CO_2 in air.

Patient samples

Peripheral blood was drawn from three patients with documented B cell chronic lymphocytic leukemia (B-CLL)[20] after informed consent. The isolation of the B-CLL cells was performed using Ficoll–Hypaque gradients (Pharmacia, Piscataway, NJ, USA). The cells were washed three times in PBS without magnesium chloride and resuspended in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 1 mM L-glutamine. 1.5×10^6 cells/ml were incubated with or without bFGF (100 ng/ml) for 4 or 8 h at 37°C, 5% CO_2 in air.

Viability

Cell viability was assessed by Trypan blue (Gibco) exclusion using a hemacytometer. At each time-point, samples were collected and each sample was counted in triplicate. The percentage of viable cells in treated samples (fludarabine alone or in combination with bFGF) was divided by the average viability control samples at each time-point, respectively.

RNA isolation

Total cellular RNA was prepared as described[21] with some modifications. Briefly, cells were lysed with 0.2 ml of RNAzol B (Tel-Test, Friendswood, TX, USA) per 10^6 cells. Chloroform (100 μl/ml of lysate) was added to the samples and after vortexing, the suspension was incubated on ice for 5 min followed by centrifugation at 12 000 *g* at 4°C for 15 min. The aqueous phase was then transferred into a fresh tube and an equal volume of isopropanol was added. Samples were kept on ice for 60 min and centrifuged at 12 000 *g* at 4°C for 15 min. The pellets were washed with 75% ethanol, dried, and resuspended in H_2O. RNA concentration was determined by spectrophotometer at 260 nm.

Nonradioactive Northern blot analysis

Ten micrograms of total cellular RNA/lane was size-fractionated by electrophoresis in a 1.2% agarose gel containing 0.7 M formaldehyde, transferred to nylon membranes (Hybond N; Amersham, Arlington Heights, IL, USA) by capillary suction overnight, and crosslinked by UV-light (Stratalinker; Stratagene, La Jolla, CA, USA). The cDNA probe for bcl-2, a 410-bp *Eco*RI fragment in pCR II Vector (TA cloning vector; Invitrogen, San Diego, CA, USA), digested with *Bam*HI was labeled with digoxygenin-UTP (DIG-UTP) (Boehringer Mannheim, Indianapolis, IN, USA) by *in vitro* transcription with respect to T7 RNA-polymerase. The cDNA probe for FGF receptor type 1 (FGFR-1), a 426-bp insert cloned into pCR-Script SK+, was digested with *Pvu*II and labeled with DIG-UTP by *in vitro* transcription with respect to T7 RNA-polymerase. The cDNA probe for glyceraldehyde-phosphate-dehydrogenase (GAPDH), a 1270-bp insert in pBS KS+ (ATCC), was linearized with *Eco*RI and labeled with DIG-UTP by *in vitro* transcription with respect to T7 RNA-polymerase. Hybridization was performed for 16 h at 66°C in 50% deionized formamide, 7% SDS, 5× salt saturated citrate (SSC), and 2% blocking reagent (Boehringer Mannheim), 50 mM sodium phosphate pH 7.0, and 0.1% *N*-laurylsarcosine. Washings were performed at high stringency (2× SSC, 0.1% SDS, 5 min at RT; 0.1× SSC, 0.1% SDS, 20 min at 68°C. Detection was accomplished by chemiluminescent reaction according to the Genius System (Boehringer Mannheim). Briefly, membranes were washed in buffer 1 (100 mM maleic acid, 150 mM sodium chloride, pH 7.5) for 1 min, followed by a 1 h incubation in buffer 1 containing 1% blocking reagent (buffer 2). Membranes were then incubated in buffer 2 containing a 1:10 000 dilution of anti-DIG Fab-fragments, conjugated with alkaline phosphatase (Boehringer Mannheim). Membranes were washed twice for 15 min in buffer 1 containing 0.3% Tween 20 (Sigma), followed by an equilibration step in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM sodium chloride) for 2 min. Disodium 3-(4-methoxyspirol[1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan]-4-yl)phenyl phosphate (CSPD; Tropix, Bedford, MA, USA) was used as substrate. The stock solution (11.8 mg/ml) was diluted 1:100 in buffer 3. Membranes were incubated for 30 min with diluted CSPD solution at 37°C. Substrate solution was removed and membranes were exposed to Cronex 4 film (Du Pont NEN, Boston, MA, USA) at RT for 2 min to 1 h.

Actinomycin D experiments

Cells were stimulated with bFGF (10–100 ng/ml) for 8 h before being exposed to actinomycin D (AcD; Sigma) (5 mg/ml). Cells were harvested at intervals of 30, 60, 120 and 180 min poststimulation. A second group enclosed unstimulated cells

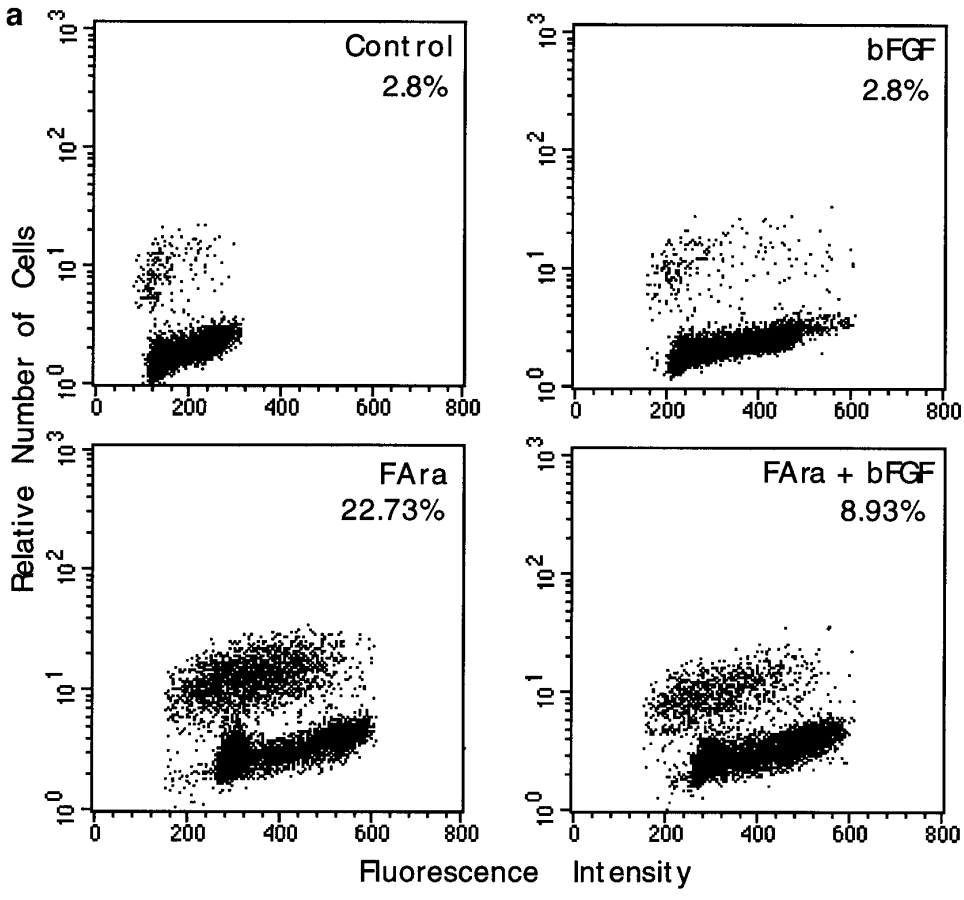

Figure 2 Detection of apoptosis-associated DNA strand-breaks by *in situ* terminal deoxynucleotidyl transferase assay (TdT assay). Cells were treated as follows: medium alone; medium plus bFGF (100 ng/ml); medium plus fludarabine (100 μm); medium plus fludarabine (100 μm) plus bFGF (100 ng/ml). The dots represent the distribution of individual cells with respect to their b-dUTP incorporation and DNA content. The degree of b-dUTP complexes correlated with DNA strand-breaks per cell. A representative experiment with I83CLL cells after 24 h (a) and 48 h (b) is demonstrated.

exposed to AcD in the same time intervals. Cytoplasmatic RNA was extracted and studied by Northern blot analysis for bcl-2 mRNA expression. Autoradiograms were quantitated by computer densitometry. In all experiments, data were normalized to the ethidium bromide staining of the 28S ribosomal RNA (rRNA).

In situ TdT assay

The *in situ* terminal deoxynucleotidyl transferase (TdT) assay was performed as previously described.[22] Briefly, 2×10^6 cells were collected from liquid culture, centrifuged and fixed in 0.3% buffered formaldehyde (pH 7.5). After washing with PBS, cells were resuspended in 70% ethanol (–20°C) and stored at –20°C. Following rehydration with PBS, the cells were resuspended in 50 μl cacodylate buffer containing 0.2 m potassium cacodylate, 2.5 mm Tris-HCl (pH 6.6), 2.5 mm cobalt chloride ($CoCl_2$), 0.25 mg/ml bovine serum albumin, 7.5 units of terminal deoxynucleotidyl transferase (TdT) and 0.5 nm biotinylated deoxyuracil-triphosphphate (b-dUTP) (all reagents from Boehringer Mannheim). After an incubation period in this solution for 30 min at 37°C, cells were washed with PBS and resuspended in 100 μl of a solution containing 4× SSC, 25 μg/ml avidin-fluorescein-isothiocyanate (avidin-FITC; Boehringer Mannheim), 0.1% Triton X-100 and 5% w/v non-fat dry milk, and incubated for 30 min in the dark at room temperature. Cells were washed with PBS containing 0.1% Triton X-100 and resuspended in 0.5 ml PBS containing 5 μg/ml propidium iodide (Sigma) and 1 mg/ml RNAse A (Sigma). Green fluorescence detecting FITC levels and red fluorescence measuring propidium iodide content of individual cells were quantified on a FACStar (Becton Dickinson, Franklin Lakes, NJ, USA), data from 2×10^4 cells was collected and used for analysis.

Western blot analysis

Pellets of 5×10^6 cells were resuspended in a lysis buffer containing 20 mm Tris-HCl, 1 mm EGTA, 50 μm $NaVO_4$, 50 mm NaF, 0.01 U/ml aprotinin, 10 μg/ml leupeptin, 1 mm elastinal, 10 μg/ml pepstatin, and 1 mm phenyl methyl sulfonyl fluoride (PMSF) (all Sigma). The lysates were then sonicated using a ultrasonic homogenizer (4710 series; Cole Parmer Instruments, Chicago, IL, USA), centrifuged at 7500 *g* in a microfuge (Sorvall Instruments, Wilmington, DE, USA), and the protein content of the lysates was determined (BioRad Protein Assay Kit I; Melville, NY, USA) at 595 nm with a BSA standard. Sample buffer containing 10% glycerol, 0.4% SDS, 0.3% bromphenol blue, 0.2% pyronin Y, in 1× stacking buffer (Tris base 0.5 m, 0.8% SDS), 20% 2-mercaptoethanol, was

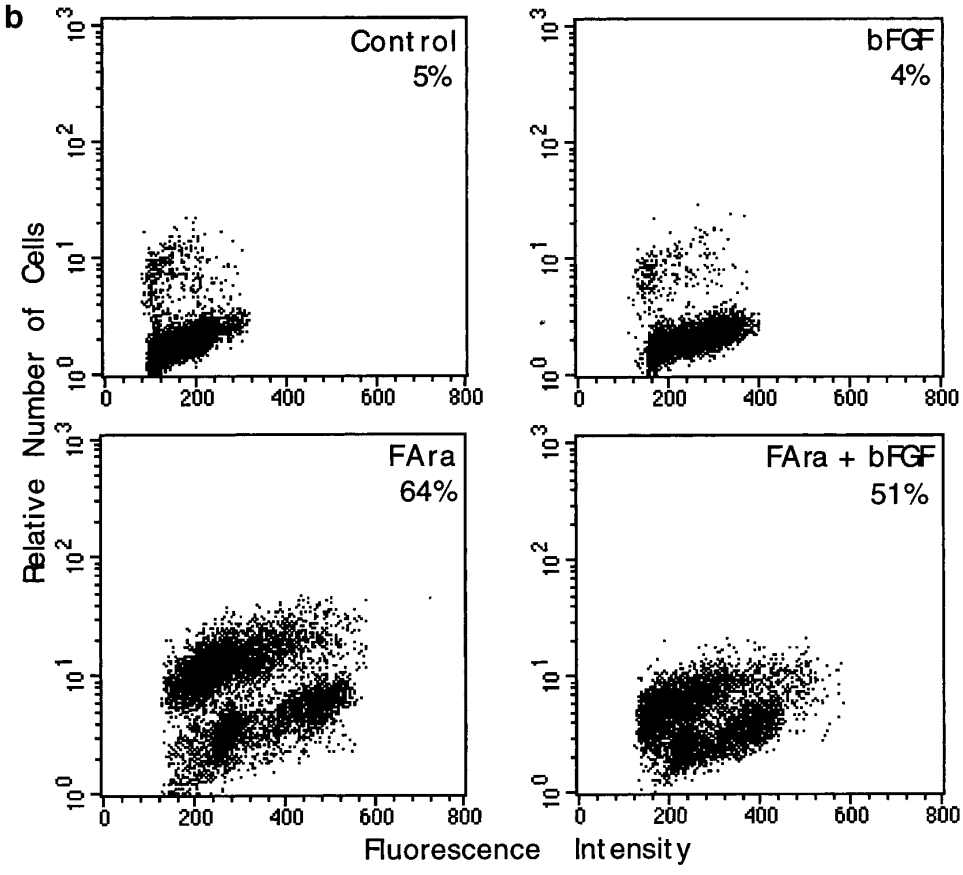

Figure 2(b) *Continued.*

added to the cell lysates which subsequently were heat-denaturated at 95°C for 3 min. Ten micrograms per lane of protein was loaded on a SDS-polyacrylamide gel containing 12.5% polyacrylamide, and size-fractionated by electrophoresis. Proteins were electroblotted on to Immobilon-P PVDF transfer membrane (Millipore, Bedford, MA, USA) and immunostained with a mouse anti-human monoclonal bcl-2 antibody (1:5000; Dako, Carpinteria, CA, USA). Bound antibody was detected using the ECL chemiluminescence detection system (Amersham). Protein bands were quantified by computer densitometry.

Statistical analysis

Student's unpaired, *t*-test was used to estimate statistical significance between the different groups whenever applicable.

Results

FGF-receptor expression

The cell lines JVM-2, JVM-13, I83CLL and WSU-CLL were initially examined for the presence of FGF-receptors. As analyzed by Northern blotting, all four cell lines were found to express mRNA for the FGF-receptor type 1 (FGFR-1) at ~4.8 kb (Figure 1).

Delay of fludarabine-induced cell death

In order to investigate the ability of bFGF to enhance the survival of the cells when exposed to an apoptotic stimulus, JVM-2, JVM-13, I83CLL and WSU-CLL cells were treated with fludarabine alone or in combination with bFGF. Viability, as determined by Trypan blue exclusion, was performed for each cell line, respectively, over a time period of 4 days. Experiments were performed in triplicate. Exposure of JVM-13 to 100 μm fludarabine alone resulted in 80% of cells being viable on day 1, 50% on day 2 and 44% by day 3 of culture. After 4 days of culture in the presence of fludarabine alone, only 33% of the cells were still viable (Table 1). The addition of bFGF at 100 ng/ml to JVM-13 resulted in a significant delay in the decline of viability ($P = 0.0087$) by day 4 of culture (Table 1). In JVM-2 cells treated with fludarabine alone, 46% and 33% of cells were viable on days 2 and 3, respectively; in contrast, in JVM-2 cells exposed to fludarabine and bFGF, 74% ($P = 0.0166$) and 52% ($P = 0.0036$) were found to be viable on days 2 and 3 of culture. On day 4 of culture, fludarabine-treated JVM-2 cells were found to be only 18% viable, whereas cells exposed to fludarabine and bFGF showed a viability of 38% ($P = 0.0213$) (Table 1). Similarly, protection from fludarabine-induced loss of viability was noted in I83CLL cells (Table 1).

WSU-CLL cells appeared to be more sensitive to fludarabine. Exposure of the cells to 1 μm of the drug resulted in 91% viable cells on day 1, 44% on day 2, 51% on day 3, and 37% on day 4. A protective effect of bFGF on the survival of the

Table 1 Basic FGF delays fludarabine-induced cell death in cell lines derived from chronic lymphoid malignancies

Cell line	*Day*	*Cell viability[a] (%)*			*P value[d]*
		Treatment control	*F-Ara*	*F-Ara + bFGF*	
JVM-2[b]	1	96 ± 2[e]	71 ± 6	85 ± 1	0.0019**
	2	92 ± 0	46 ± 10	74 ± 14	0.0166**
	3	96 ± 4	33 ± 6	52 ± 5	0.0036**
	4	94 ± 1	18 ± 4	38 ± 2	0.0213**
JVM-13[b]	1	89 ± 4	80 ± 2	74 ± 7	0.2077
	2	90 ± 2	50 ± 4	42 ± 10	0.2479
	3	89 ± 1	44 ± 9	53 ± 5	0.2050
	4	81 ± 1	33 ± 9	62 ± 6	0.0087**
I83CLL[b]	1	95 ± 3	47 ± 3	68 ± 0	0.0041**
	2	93 ± 2	18 ± 6	25 ± 4	0.2032
	3	97 ± 3	9 ± 6	29 ± 4	0.0345**
	4	94 ± 0	11 ± 0	20 ± 3	0.0435**
WSU-CLL[c]	1	99 ± 1	91 ± 3	89 ± 1	0.3348
	2	99 ± 0	44 ± 12	66 ± 2	0.0351**
	3	99 ± 1	51 ± 1	70 ± 2	0.0068**
	4	98 ± 2	37 ± 3	33 ± 10	0.5432

[a]Viability of the cells treated with fludarabine alone (100 μM[b] or 1 μMol[c]) or in combination with bFGF (100 ng/ml).
[d]The unpaired *t*-test was used to determine the *P* values.
**Indicates significantly different (level $P = 0.05$).
[e]Numbers represent the mean and standard deviation of a representative experiment out of three evaluated in triplicate.
F-Ara, fludarabine; bFGF, basic fibroblast growth factor.

cells treated with both fludarabine and bFGF, could be observed on day 2 and 3 of culture, where 66% ($P = 0.0351$) and 70% ($P = 0.0068$) of the cells were viable, respectively (Table 1).

Delay of fludarabine-induced apoptosis by bFGF

In order to demonstrate that the decrease in viability in these cells was due to fludarabine-induced apoptosis, I83CLL cells were treated with fludarabine alone 100 μm or with fludarabine and bFGF (100 ng/ml) and examined for apoptosis-associated DNA strand breaks using the *in situ* terminal deoxynucleotidyl transferase assay (TdT assay). The intensity of labeling of apoptotic cells with biotinylated dUTP correlated with the number of DNA strand breaks per cell. Treatment of I83CLL cells with fludarabine (100 μm) alone for 24 h resulted in 22.73% cells to be apoptotic; however, in the presence of bFGF, only 8.93% of the cells exhibited apoptosis (Figure 2a). After 48 h of incubation with fludarabine 64% of the cells were apoptotic, whereas the fraction of apoptotic cells treated with fludarabine plus bFGF was reduced to 51% (Figure 2b).

Regulation of bcl-2 mRNA after exposure to bFGF

To evaluate whether the protective effect of bFGF was due to an upregulation of bcl-2, cells were stimulated with bFGF (10 ng/ml) and the expression of bcl-2 mRNA was examined by Northern blot analysis. Both JVM-2 and I83CLL were found to express low amounts of steady-state bcl-2 mRNA, whereas no bcl-2 transcripts could be detected in unstimulated JVM-13 cells. Kinetic experiments with bFGF during a time period from 2 to 48 h led to a significant accumulation of bcl-2 transcripts in all three cell lines (Figure 3). The upregulation of bcl-2 mRNA occurred in a time-dependent manner: in JVM-2 an increase of bcl-2 specific transcripts could be detected after 2 h with a maximal mRNA accumulation after 8 h, followed by a decrease to basal expression after 24 h. During the whole period of stimulation, all three transcripts (~8.5 kb, ~6.5 kb and ~4.0 kb) were expressed concomitantly. In JVM-13 bcl-2 mRNA could be induced within a time period of 4 h with a maximal expression after 24 h. A decrease of expression towards basal levels could be observed within the next 24 h. This cell line also expressed three bcl-2 specific transcripts (Figure 3). In contrast to JVM-2 and JVM-13, the B-CLL cell line I83CLL was found to express a single mRNA for bcl-2 at ~8.5 kb. A significant accumulation of this transcript could be observed within 2 h of exposure to bFGF with a maximal expression after 24 h. Transcripts returned to basal levels after a time period of 48 h (Figure 3). To further determine whether the increase of bcl-2 mRNA expression is based on post-transcriptional mRNA stabilization by bFGF, we examined the half-life ($t_{1/2}$) of bcl-2 transcripts in each cell line. Unstimulated cells or cells pretreated with bFGF (100 ng/ml) were exposed to AcD (5 μg/ml) for various time intervals. No difference in $t_{1/2}$ of bcl-2 mRNA in unstimulated cells compared to cells stimulated with bFGF could be observed (data not shown).

Regulation of bcl-2 protein upon treatment with bFGF

In order to investigate whether the bFGF-induced regulation of bcl-2 mRNA was also reflected on the protein level, we examined cell lysates of the cell lines JVM-2, JVM-13, I83CLL and WSU-CLL for the presence and regulation of bcl-2 protein by Western blot analysis. At baseline JVM-2, I83CLL and WSU-CLL cells were found to express strong bcl-2 protein

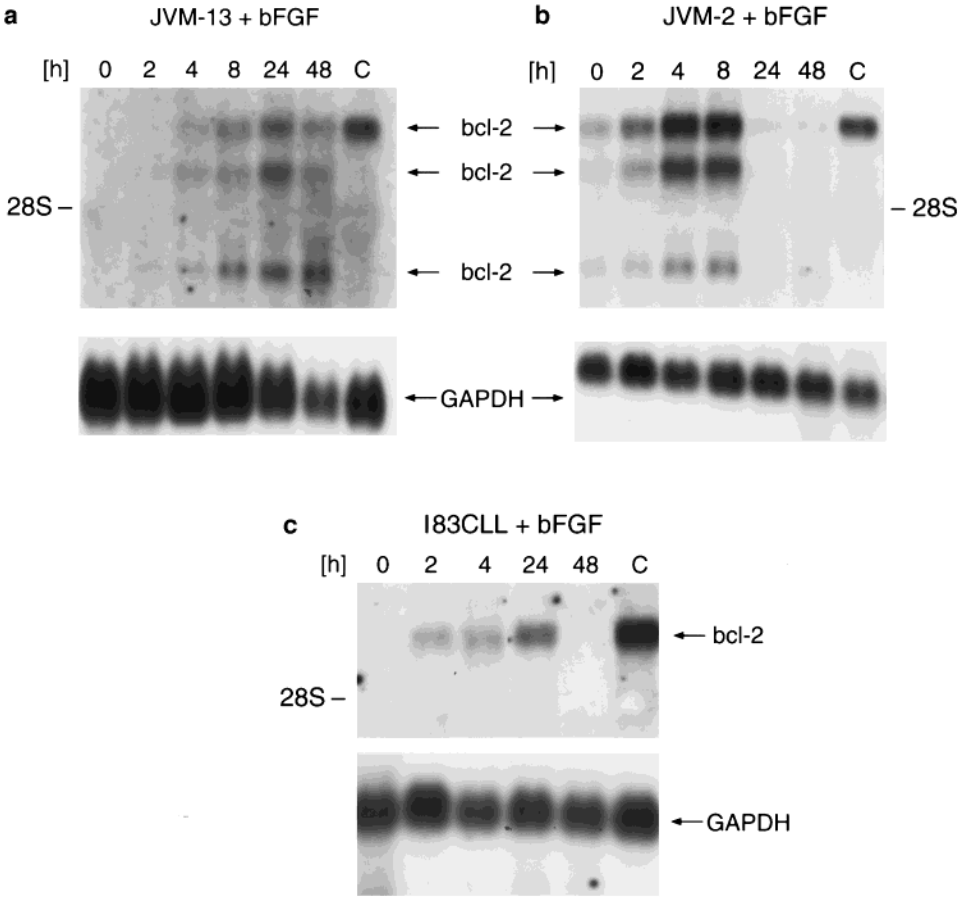

Figure 3 Northern blot analysis of bcl-2 mRNA expression in the human EBV-transformed B prolymphocytic cell lines JVM-2 (a) and JVM-13 (b), and the human EBV-transformed B-CLL cell line I83CLL (c). Total cellular RNA (10 μg/lane) of unstimulated cells (0) and cells cultured in the presence of recombinant bFGF (10 ng/ml) for a time period from 2 h to 48 h was hybridized using a digoxigenin-labeled bcl-2 probe. RNA of the human promyelocytic cell line HL-60 was used as control (c). bcl-2-specific transcripts are shown at 8.5 kb, 6.5 kb, and 4.5 kb (JVM-2 and JVM-13) and at 8.5 kb (I83CLL) as indicated by the arrows. The membranes were rehybridized with a digoxigenin-labeled GAPDH probe, respectively.

bands; in JVM-13 cells bands of detected bcl-2 protein appeared much weaker. Exposure of bFGF resulted in a marked increase of bcl-2 expression compared to unstimulated cells. Figure 4 shows a representative experiment of the expression of bcl-2 protein in JVM-2, JVM-13 and I83CLL cells after 8 h and 24 h of stimulation. Densitometric analysis of protein bands indicated that bFGF induced a 1.2-fold increase of bcl-2 protein expression in JVM-2 cells after 8 h and 24 h. The addition of bFGF to JVM-13 cells resulted in a 3- to 4.6-fold increase of bcl-2 protein after 8 h and 24 h, respectively. In I83CLL cells a 1.7-fold upregulation of bcl-2 protein could be observed after 24 h upon treatment with bFGF.

Lymphocytes from three patients (P1, P2, P3) with B-CLL were isolated and incubated for 4 or 8 h with or without bFGF (100 ng/ml). Cell lysates were examined for the regulation of bcl-2 protein using Western blot analysis. The data demon-

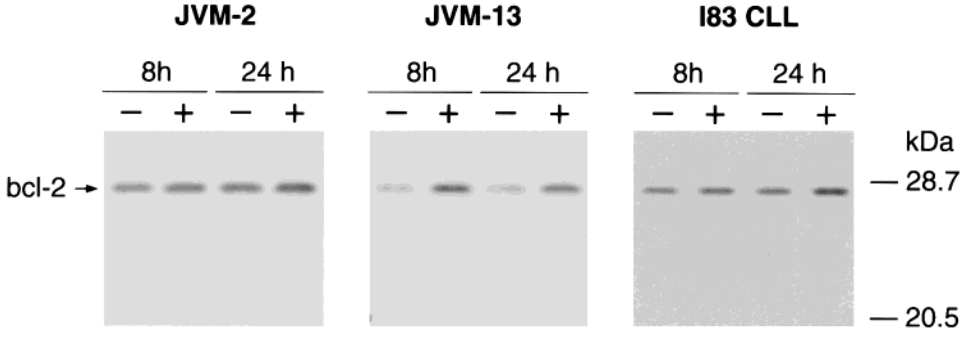

Figure 4 Regulation of bcl-2 protein expression in JVM-13, JVM-2 and I83CLL cells upon treatment with bFGF. Cells (1×10^6/ml) were cultured alone (–) or in the presence of bFGF (+) for 8 h and 24 h. Ten micrograms of protein/lane from cell lysates were examined by Western blot analysis using an anti-bcl-2 monoclonal antibody (Dako).

strated at 1.5- to 2.3-fold increase of bcl-2 protein in cells incubated with bFGF as compared to cells incubated without bFGF in the patients (Figure 5).

Discussion

In this study, we demonstrate that basic fibroblast growth factor (bFGF) (1) upregulates the expression of bcl-2 mRNA and bcl-2 protein in three cell lines, JVM-2[15] and JVM-13[16] (prolymphocytic, PLL) and I83CLL (chronic lymphocytic, CLL),[18] representative of chronic lymphoid malignancies and (2) that the enhanced bcl-2 expressed in these cells correlates with bFGF-mediated protection from fludarabine-induced apoptotic cell death.

Fludarabine is a drug that has been shown to induce apoptotic cell death in B-CLL cells.[23] We demonstrate here that the addition of exogenous bFGF to fludarabine-treated cells increased the survival of the cells in all three cell lines examined. The hypothesis that bFGF delays fludarabine-induced apoptosis in CLL cells was confirmed by the TdT assay. The addition of bFGF to fludarabine-treated cells resulted in a reduction of apoptotic cells compared to those treated with fludarabine alone. In this regard, Fuks *et al*[24] have demonstrated that exogenous bFGF increases the survival of bovine endothelial cells after radiation-induced apoptosis *in vitro*.[24] Furthermore, bFGF given *in vivo* to mice, prior to lung irradiation, resulted in a lower incidence of pneumonitis and lower content of apoptotic cells in tissue samples.[24] An apoptosis delaying effect upon serum starvation has also been shown in cell lines overexpressing bFGF following stable transfection with the gene for bFGF.[40]

The bcl-2 protein has been described as a repressor of apoptosis in many different cell types.[10] Furthermore, it is reported that fludarabine can reduce the expression of bcl-2 mRNA in fresh B-CLL cells *in vitro*.[25] Our data demonstrate that the protective effect of bFGF against fludarabine-induced apoptosis may be mediated by upregulating the expression of bcl-2. In accordance with previously published data,[13,26] we demonstrated that JVM-2 and I83CLL express low levels of bcl-2 mRNA at steady state. We demonstrate here, for the first time, that bFGF significantly upregulates the expression of bcl-2 mRNA in JVM-2 and I83CLL and that it induces bcl-2 gene expression in JVM-13. The accumulation of bcl-2 specific transcripts was time dependent with different kinetics among the three cell lines.

Other cytokines such as interleukin-4 (IL-4), inteferon-alpha (IFN-α), and interleukin-10 (IL-10) have also been shown to regulate the expression of bcl-2 in CLL cells. In this regard, IL-4 has been reported to inhibit spontaneous and hydrocortisone-induced apoptotic cell death in fresh B-CLL cells by either increasing bcl-2 protein levels,[27] or by inhibiting the loss of the expression of bcl-2 protein.[28] IFN-α has been controversially demonstrated to prevent B-CLL cells from undergoing apoptosis: Jewell *et al*[29] found that IFN-α upregulates bcl-2 expression in fresh CLL cells and protects them from apoptosis induced by corticosteroids or γ-irradiation; however, other investigators have demonstrated that IFN-α prevents spontaneous apoptosis in B-CLL cells *in vitro* via a bcl-2 independent mechanism.[30]

Bcl-2 dependent mechanisms also play a role in the induction of apoptosis, where IL-10 has been shown to enhance spontaneous apoptosis in B-CLL cells by decreasing bcl-2 protein levels.[31]

Delay of apoptosis seems to play an important role in the clonal expansion, tumor progression, and resistance to cytotoxic therapy in a number of malignant disorders.[32,33] In this regard we have also recently extended these observations, where we have shown that DNA topoisomerase I + II inhibitor-induced apoptosis in K562 chronic myeloid leukemia cells (CML) is delayed in response to bFGF.[34]

In this study, we demonstrate that basic FGF confers a survival advantage to CLL cell lines by delaying their response to an apoptotic stimulus: this likely results in part from an upregulation of bcl-2. Increased expression of bcl-2 in CLL has been found frequently; however, the mechanism by which its expression is enhanced is unknown, since specific gene rearrangements appear to be very rare.[14,35] Other genetic alterations have been reported to be involved in the pathogenesis of CLL. This includes mutations in the tumor suppressor gene p53[36] and deletions or alterations of the retinoblastoma gene (RB).[37,38] Whether bFGF acts alone or works in concert with other cytokines in conjunction with these specific genetic alterations involved in the pathogenesis and evolution of CLL[27,29,39] to promote the growth advantage of these malignant cells, remains to be determined and is under investigation.

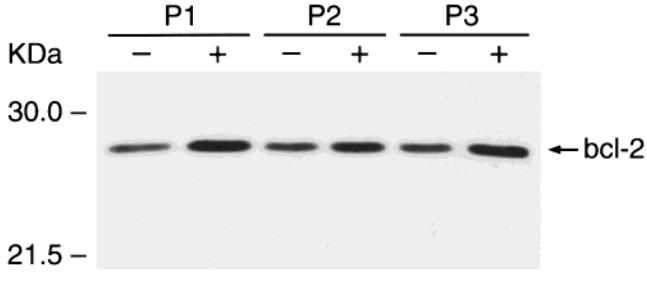

Figure 5 Expression of bcl-2 protein in fresh cells from three patients with B-CLL (P1, P2, P3). Unstimulated cells (–) and cells stimulated with bFGF (100 ng/ml) (+) were cultured for 4 h (P1, P2) or 8 h (P3). Fifty micrograms of protein/lane from cell lysates were loaded on a 12.5% SDS-PAGE gel and electrophoresed. Bcl-2-specific protein was detected using an anti-bcl-2 monoclonal antibody.

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References

1 Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. Structural characterization and biological functions of fibroblast growth factor. *Endocr Rev* 1987; **8**: 95–114.
2 Folkman J. The role of angiogenesis in tumor growth. *Semin Cancer Biol* 1992; **3**: 65–71.
3 Nguyen M, Watanabe H, Budson AE, Richie JP, Hayes DF, Folkman J. Elevated levels of an angiogenic peptide, basic fibroblast growth factor, in the urine of patients with a wide spectrum of cancers (see comments). *J Natl Cancer Inst* 1994; **86**: 356–361.
4 Perez-Atayde AR, Sallan SE, Tredow U, Connors S, Folkman J. Spectrum of tumor angiogenesis in bone marrow of children with acute lymphoblastic leukemia. *Lab Invest* 1995; **72**: 141A (Abstr.).
5 Eguchi J, Nomata K, Kanda S, Igawa T, Taide M, Koga S, Matsuya F, Kanetake H, Saito Y. Gene expression and immunohistochem-

ical localization of basic fibroblast growth factor in renal cell carcinoma. *Biochem Biophys Res Commun* 1992; **183**: 937–944.
6 Reed JA, McNutt NS, Albino AP. Differential expression of basic fibroblast growth factor (bFGF) in melanocytic lesions demonstrated by *in situ* hybridization. Implications for tumor progression. *Am J Pathol* 1994; **144**: 329–336.
7 Nanus DM, Schmitz-Drager BJ, Motzer RJ, Lee AC, Vlamis V, Cordon-Cardo C, Albino AP, Reuter VE. Expression of basic fibroblast growth factor in primary human renal tumors: correlation with poor survival. *J Natl Cancer Inst* 1993; **85**: 1597–1599.
8 Menzel T, Rahman Z, Calleja E, White K, Wilson EL, Wieder R, Gabrilove JL. Elevated intracellular level of basic fibroblast growth factor correlates with stage of chronic lymphocytic leukemia and is associated with resistance to fludarabine. *Blood* 1996; **87**: 1058–1063.
9 Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol* 1980; **68**: 251–306.
10 Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; **348**: 334–336.
11 Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chomosome 14 and near a transcriptional unit on 18. *Cell* 1985; **41**: 899–906.
12 Cleary ML, Sklar J. Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near transcriptionally active locus on chromosome 18. *Proc Natl Acad Sci USA* 1995; **82**: 7439–7443.
13 Schena M, Larsson LG, Gottardi D, Gaidano G, Carlsson M, Nilsson K, Caligaris-Cappio F. Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. *Blood* 1992; **79**: 2981–2989.
14 Adachi M, Tefferi A, Greipp PR, Kipps TJ, Tsujimoto Y. Preferential linkage of bcl-2 to immunoglobulin light chain gene in chronic lymphocytic leukemia. *J Exp Med* 1990; **171**: 559–564.
15 Melo JV, Brito-Babapulle V, Foroni L, Robinson DS, Luzzatto L, Catovsky D. Two new cell lines from B-prolymphocytic leukaemia: characterization by morphology, immunological markers, karyotype and Ig gene rearrangement. *Int J Cancer* 1986; **38**: 531–538.
16 Melo JV, Foroni L, Brito-Babapulle V, Luzzatto L, Catovsky D. The establishment of cell lines from chronic B cell leukaemias: evidence of leukaemic origin by karyotypic abnormalities and Ig gene rearrangement. *Clin Exp Immunol* 1988; **73**: 23–28.
17 Marti GE, Zenger V, Brown M, Marti DM, Melo JV, Crescenzi M, Dadey B, Han T, Bertin P, Caporaso NE, Noguchi N. Antigenic expression of B-cell chronic lymphocytic leukemic cell lines. *Leuk Lymphoma* 1992; **7**: 497–504.
18 Wendel-Hansen V, Sällström J, De Campos-Lima PO, Kjellström G, Sandlund A, Siegbahn A, Carlsson M, Nilsson K, Rosén A. Epstein–Barr virus (EBV) can immortalize B-CLL cells activated by cytokines. *Leukemia* 1994; **8**: 476–484.
19 Mohammad RM, Mohamed AN, Hamdan MY, Vo T, Chen B, Katato K, Abubakr YA, Dugan MC, Al-Katib A. Establishment of a human B-CLL xenograft model: utility as a preclinical therapeutic. *Leukemia* 1996; **10**: 130–137.
20 Rai KR, Han T. Prognostic factors and clinical staging in chronic lymphocytic leukemia. *Hematol Oncol Clin North Am* 1990; **4**: 447–456.
21 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**: 156–159.
22 Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z. The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* 1993; **53**: 3186–3192.
23 Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittelman WN, Plunkett W. Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2′-deoxyadenosine and 9-beta-d-arabinosyl-2-fluoroadenine. *Blood* 1993; **81**: 143–50.
24 Fuks Z, Persaud RS, Alfieri A, McLoughlin M, Ehleiter D, Schwartz JL, Seddon AP, Cordon-Cardo C, Haimovitz-Friedman A. Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death *in vitro* and *in vivo*. *Cancer Res* 1994; **54**: 2582–2590.
25 Gottardi D, Alfarano A, De Leo AM, Stacchini A, Bergui L, Caligaris-Cappio F. Defective apoptosis due to Bcl-2 overexpression may explain why B-CLL cells accumulate in G0. *Curr Top Microbiol Immunol* 1995; **194**: 307–312.
26 Hu ZB, Gignac SM, Quentmeier H, Pettit GR, Drexler HG. Effects of dolastatins on human B-lymphocytic leukemia cell lines. *Leuk Res* 1993; **17**: 333–339.
27 Dancescu M, Rubio-Trujillo M, Biron G, Bron D, Delespesse G, Sarfati M. Interleukin 4 protects chronic lymphocytic leukemic B cells from death by apoptosis and upregulates Bcl-2 expression. *J Exp Med* 1992; **176**: 1319–1326.
28 Panayiotidis P, Ganeshaguru K, Jabbar SA, Hoffbrand AV. Interleukin-4 inhibits apoptotic cell death and loss of the bcl-2 protein in B-chronic lymphocytic leukaemia cells *in vitro*. *Br J Haematol* 1993; **85**: 439–445.
29 Jewell AP, Worman CP, Lydyard PM, Yong KL, Giles FJ, Goldstone AH. Interferon-alpha up-regulates bcl-2 expression and protects B-CLL cells from apoptosis *in vitro* and *in vivo*. *Br J Haematol* 1994; **88**: 268–274.
30 Chaouchi N, Wallon C, Taieb J, Auffredou MT, Tertian G, Lemoine FM, Delfraissy JF, Vazquez A. Interferon-alpha-mediated prevention of *in vitro* apoptosis of chronic lymphocytic leukemia B cells: role of bcl-2 and c-myc. *Clin Immunol Immunopathol* 1994; **73**: 197–204.
31 Fluckiger AC, Durand I, Banchereau J. Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells. *J Exp Med* 1994, **179**: 91–99.
32 Miyashita T, Reed JC. bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res* 1992; **52**: 5407–5411.
33 McGahon A, Bissonnette R, Schmitt M, Cotter KM, Green DR, Cotter TG. BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. *Blood* 1994; **83**: 1179–1187.
34 Calleja E, Menzel T, Koenig A, Wrazel L, Maslak P, Gabrilove J. Basic fibroblast growth factor delays DNA topoisomerase inhibitor induced apoptosis in K562 chronic myeloid leukemia cells. *Blood* 1995; **86**: 522a (Abstr.).
35 Hanada M, Delia D, Aiello A, Stadtmauer E, Reed JC. bcl-2 gene hypomethylation and high-level expression in B-cell chronic lymphocytic leukemia. *Blood* 1993; **82**: 1820–1828.
36 el Rouby S, Thomas A, Costin D, Rosenberg CR, Potmesil M, Silber R, Newcomb EW. p53 gene mutation in B-cell chronic lymphocytic leukemia is associated with drug resistance and is independent of MDR1/MDR3 gene expression. *Blood* 1993; **82**: 3452–3459.
37 Liu Y, Grander D, Soderhall S, Juliusson G, Gahrton G, Einhorn S. Retinoblastoma gene deletions in B-cell chronic lymphocytic leukemia. *Genes Chromosom Cancer* 1992; **4**: 250–256.
38 Kornblau SM, Chen N, del Giglio A, O'Brien S, Deisseroth AB. Retinoblastoma protein expression is frequently altered in lymphocytic leukemia. *Cancer Res* 1994; **54**: 242–246.
39 Hoffbrand AV, Panayiotidis P, Reittie J, Ganeshaguru K. Autocrine and paracrine growth loops in chronic lymphocytic leukemia *Semin Hematol* 1993; **30**: 306–317.

Reference added in proof

40 Wieder R, Wang H, Shirke S, Menzel T, Feirt N, Jakubowski AA, Gabrilove JL. Low level expression of basic FGF upregulates bcl-2 and delays apoptosis, but high intracellular levels are required to induce transformation in NIH 3T3 cells. *Growth Factors* (submitted).