Immunopathology and Infectious Diseases

Potential Pathogenetic Implications of Cyclooxygenase-2 Overexpression in B Chronic Lymphoid Leukemia Cells

Paola Secchiero,* Elisa Barbarotto,*
Arianna Gonelli,* Mario Tiribelli,[†]
Carlotta Zerbinati,[‡] Claudio Celeghini,[‡]
Claudio Agostinelli,[§] Stefano A. Pileri,[§] and
Giorgio Zauli[‡]

From the Department of Morphology and Embryology, Human
Anatomy Section, University of Ferrara, Ferrara; the Department
of Medical and Morphological Researches,[†] Division of
Hematology and Bone Marrow Transplantation, University
Hospital, Udine; the Department of Normal Human Morphology,[‡]
University of Trieste, Trieste; and the Unit of Haematopathology,[§]
Institute of Haematology and Clinical Oncology "L. and A.
Seragnoli", University of Bologna, Bologna, Italy*

Evidence suggests that cyclooxygenase-2 (COX-2) increases tumorigenic potential by promoting resistance to apoptosis. Because B chronic lymphoid leukemia (B-CLL) cells exhibit a defective apoptotic response, we analyzed CD19$^+$ B lymphocytes purified from the peripheral blood of B-CLL patients. Microarray analysis showed a variable (up to 38-fold) increase in the steady-state mRNA levels of COX-2 in B-CLL lymphocytes compared with normal CD19$^+$ B lymphocytes. The up-regulation of COX-2 in B-CLL cells was confirmed by reverse transcriptase-polymerase chain reaction and Western blot analyses. Moreover, immunohistochemical analysis of B-CLL bone marrow infiltrates confirmed clear expression of COX-2 in leukemic cells. *Ex vivo* treatment with the COX-2 inhibitor NS-398 significantly decreased the survival of leukemic cells by increasing the rate of spontaneous apoptosis in 13 of 16 B-CLL samples examined, but it did not affect the survival of normal lymphocytes. Pretreatment with NS-398 significantly potentiated the cytotoxicity induced by chlorambucil in 8 of 16 B-CLL samples examined. Moreover, although recombinant tumor necrosis factor-related apoptosis inducing ligand (TRAIL)/Apo2L showed little cytotoxic effect in most B-CLL samples examined, pretreatment with NS-398 sensitized 8 of 16 B-CLL samples to TRAIL-induced apoptosis. Taken together, our data indicate that COX-2 overexpression likely represents an additional mechanism of resistance to apoptosis in B-CLL and that pharmacological suppression of COX-2 might enhance chemotherapy-mediated apoptosis. *(Am J Pathol 2005, 167:1599–1607)*

Chronic lymphocytic leukemia (CLL) is a highly heterogeneous disease with one-third of patients never requiring treatment, whereas in other patients, the disease progresses at a variable rate.[1,2] CLL represents a quintessential example of human malignancies that are caused primarily by defects in apoptosis.[3,4] Defects in apoptotic pathways contribute to chemoresistance, rendering tumor cells less sensitive to the cytotoxic actions of currently available anticancer drugs.

Cyclooxygenase (COX)-1 and -2, also known as prostaglandin H synthases (PTGSs), catalyze one of the rate-limiting steps in the prostanoids biosynthesis.[5,6] COX isoenzymes, which are encoded by two different genes, possess the same oxygenase and peroxidase activities and catalyze the formation of prostaglandin H$_2$ from arachidonic acid. Despite the remarkable structural and functional homologies, COX-1 and COX-2 have been shown to preferentially couple with different isoforms of prostaglandin synthases and subserve distinct functions even within the same cell.[5] In particular, it has been clearly established that the *COX-2* (or *PTGS2*) gene behaves like an immediate-early gene, being rapidly induced in response to mitogenic or inflammatory stimuli.[5] The premise that COX-2 is involved in growth and progression of several types of solid cancers is strongly supported by both epidemiological and animal studies.[7–10] Evidence suggests that the increase in tumori-

Table 1. Clinical Characteristics of the B-CLL Patients

Patient	Sex/Age	Months since diagnosis	WBC*	Stage†	Doubling time‡
1	F/74	171	29.7	1	12
2	M/65	20	25.6	0	20
3	M/73	83	42.8	4	20
4	F/60	59	47.6	0	29
5	F/84	16	28.8	0	30
6	M/71	27	18.6	1	33
7	M/75	107	42.8	1	36
8	M/53	8	12.1	0	45
9	M/61	36	46.9	1	46
10	M/61	34	46.3	1	46
11	M/73	64	35.8	0	47
12	M/63	40	25.9	1	49
13	F/65	50	31.5	0	60
14	M/63	64	24.6	0	96
15	M/69	181	47.4	0	125
16	M/58	158	27.7	2	130

*$\times 10^3$ cells/ml.
†Rai classification.
‡Months.

genic potential by COX-2 overexpression is associated with resistance to apoptosis.[11] Selective inhibitors of COX-2 have been shown to induce apoptosis in a variety of cancer cells, including those of colon, stomach, prostate, and breast.[6,12–15] Although the role of COX-2 in lymphoid carcinogenesis is poorly defined, high levels of prostaglandins have been found in patients with lymphoma, and elevated levels of COX-2 protein have been detected in lymphoma cell lines.[16,17]

Microarray technology has been previously used to profile gene expression in B-CLL and to subcharacterize patients with heterogeneous clinical outcome.[18–23] Because it has been clearly shown that B-CLL cells are defective in their apoptotic response, in searching for new potential therapeutic targets, we have analyzed by cDNA microarray the expression profile of a set of stress- and toxicity-associated genes, including *COX-2/PTGS2*, in B-CLL samples in comparison with normal B lymphocytes. Because *COX-2* was identified as one of the up-regulated genes, we have further characterized the expression of COX-2 protein in B-CLL and its role on cell viability. Moreover, to investigate the therapeutic potential of a strategy based on COX-2 inhibition, viability of B-CLL cells was analyzed in response to NS-398, a selective pharmacological inhibitor of COX-2 activity, used alone or in association with chemotherapeutic agents or recombinant tumor necrosis factor-related apoptosis inducing ligand (TRAIL).

Materials and Methods

Patients and Cell Purification

Peripheral blood (PB) samples were collected from 10 healthy human blood donors and 16 B-CLL patients after informed consent, in agreement with institutional guidelines. The diagnosis of B-CLL was made by PB morphology and immunophenotyping. Patients were staged according to the Rai system (Table 1). Two independent blood collections were obtained from each B-CLL patient within a clinical follow-up time of 6 to 18 months. During this period, patients were clinically stable, with a lymphocyte doubling time of >12 months, free from infectious complications, and with no evidence of disease progression. Moreover, none of the patients received cytoreductive chemotherapy both before entering this study and during the follow-up period.

PB mononuclear cells (PBMCs) were separated by gradient centrifugation with lymphocyte cell separation medium (Cedarlane Laboratories, Hornby, Ontario, Canada). T lymphocytes and monocytes were depleted from normal PBMCs and, in some cases, from B-CLL with immunomagnetic microbeads (MACS microbeads; Miltenyi Biotech, Auburn, CA), resulting in purity of >90% CD19+ B cells, as assessed by flow cytometry. After purification, CD19+ B lymphocytes were resuspended at a cell density of 1×10^6 cells/ml in RPMI supplemented with 10% fetal calf serum (Gibco-BRL, Grand Island, NY), 2 mmol/L L-glutamine, and 40 mg/ml gentamicin sulfate in the absence of exogenous cytokines.

Flow Cytometric Analysis of Cell Surface Antigens

Surface expression of CD19 and CD5 was evaluated by direct staining with fluorescein isothiocyanate (FITC)-CD19 (Becton Dickinson Biosciences, San Jose, CA) and PE-CD5 (Immunotech, Marseille, France) monoclonal antibodies (mAbs). Surface expression of TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 was evaluated by staining with primary mAbs (all from Alexis Biochemical, Lausen, Switzerland) followed by PE-conjugated anti-mouse secondary Ab (Immunotech). Nonspecific fluorescence was assessed using isotype-matched Abs. Flow cytometry analyses were performed by FACScan (Becton Dickinson).

cDNA Microarray and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from purified normal or B-CLL CD19+ cells by using the Qiagen RNeasy mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. Three micrograms of total RNA were transcribed into cDNA using GEArray AmpoLabeling-LPR kit (Superarray Bioscience Corporation, Frederick, MD). An *in vitro* linear polymerase reaction was then performed to generate biotinylated cRNA. Labeled cDNA was hybridized with a customized cDNA microarray containing a panel of genes associated to stress and toxicity response (GEArray HS-603; SuperArray Bioscience Corporation). Hybridization was revealed by alkaline phosphatase-conjugated streptavidin, using a chemiluminescent detection kit (Superarray Bioscience Corporation). Signal intensity was measured for each microarray, the minimal intensity was used for background subtraction, and the values were normalized to the median signal value for each array. Expression levels were compared between the

leukemic samples and normal CD19$^+$ B lymphocytes, and then data were filtered for the genes whose expression level increased by at least twofold, that is, filtering the ratio for values ≥2.0.

To validate the accuracy of the genes selected on the microarray, the same RNA samples used for microarray hybridization were analyzed by semiquantitative RT-PCR. In particular, COX-2 mRNA amplification was performed using the following primers: forward, 5′-TCCTGGCGCT-CAGCCATACAG-3′; reverse, 5′-GTAGCCATAGCTAGC-ATTGTA-3′.

Western Blotting Analysis

Western blot was performed on approximately 5 to 10 × 10^6 cells/sample. To obtain cell lysates, cell suspensions were mixed with a lysing buffer containing 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS and protease inhibitors (Protease inhibitor cocktail P8340; Sigma Chemical, St. Louis, MO). Protein determination was performed by Bradford assay (Bio-Rad, Richmond, CA). Equal amounts of proteins for each sample were migrated in 10% polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters. Blotted filters were blocked for 60 minutes in a 3% suspension of dried skimmed milk in phosphate-buffered saline and incubated overnight at 4°C with Abs anti-COX-2 (Cayman Chemicals, Ann Arbor, MI), caspase-3, poly (ADP-ribose) polymerase (PARP), and tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Filters were washed and further incubated for 1 hour at room temperature with 1:1000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma). Specific reactions were revealed with the ECL Western blotting detection reagent (Amersham Corp., Arlington Heights, IL).

Bone Marrow Specimens and Immunohistochemical Analysis

Needle biopsies taken from bone marrow of three B-CLL patients at Rai stage 2 were used for immunohistochemical analysis. Sections were processed as previously described.[24] Briefly, antigen retrieval was performed by microwaving (three cycles lasting 5 minutes each at 900W) in 1 mmol of ethylenediamine tetraacetic acid (pH 8.0), and a three-layer alkaline phosphatase-anti alkaline phosphatase (APAAP) technique was applied for antigen detection. For COX-2 immunohistochemistry, the anti-COX-2 mAb (Cayman Chemicals; 1:20 dilution) was used. The specificity of the staining was ensured by using isotype-matched irrelevant antibody, as a substitute for primary antibody, and the immuno-alkaline phosphatase was adopted to avoid any disturbance from the endogenous peroxidase activity. Slides were analyzed blinded by two independent people.

Culture Treatments

NS-398, a relative specific pharmacological inhibitor of COX-2 isoenzyme (Biomol, Plymouth Meeting, PA) was

dissolved in ethanol, and stock solution was stored at −20°C for no more than 3 months. NS-398 was used at the final concentrations of 12.5 to 200 μmol/L. DuP697, an unrelated pharmacological inhibitor of COX-2 (Sigma Chemical) was dissolved in ethanol and used at the final concentration of 100 μmol/L. Chlorambucil and Fludarabine (F-ara-A) (Sigma) were added at the final concentration of 10 μmol/L. Recombinant histidine 6-tagged TRAIL$_{(114-281)}$, produced in bacteria and purified by chromatography, as described previously,[25] was added at the final concentration of 1 μg/ml.

PGE$_2$ levels were measured in the supernatants of cell cultures using the prostaglandin E$_2$ EIA kit-monoclonal (Cayman Chemicals), following manufacturer's instructions. Cell viability was assessed by trypan blue dye exclusion, and the degree of apoptosis was assessed by annexin V-FITC/propidium iodide double staining (Trevigen Inc., Gaithersburg, MD) followed by flow cytometric analysis and in parallel by analyzing cell lysates for PARP cleavage in Western blot.

Statistical Analysis

Box plots were used to show the median, minimum, maximum values and 25th to 75th percentiles of the RNA levels for each group of data. Correlation coefficients were calculated by Spearman's method. Results were evaluated by using analysis of variance with subsequent comparisons by Student's t-test and with the Mann-Whitney rank-sum test. Statistical significance was defined as $P < 0.05$.

Results

COX-2 Is Up-Regulated in B-CLL in Comparison with Normal B Cells

All B-CLL patients who entered this study (Table 1) were previously untreated, and we did not pre-select any subgroup of B-CLL patients to represent the heterogeneity of this disease course. RNA was extracted from eight freshly purified B-CLL PBMCs containing an excess of B leukemic cells (>85% of CD19$^+$/CD5$^+$ B cells) and from three normal B lymphocyte samples (>90% CD19$^+$) and analyzed by cDNA microarray for a set of stress and toxicity associated genes (GEArray HS-603; SuperArray Bioscience Corporation), which included *PTGS2/COX-2*. The concept of peripheral blood B cells being appropriate controls for B-CLL cells in microarray analysis has been recently validated by different authors.[18,22] Genes up-regulated in all B-CLL included *IL-1β*, *IL-8*, *BCL3*, and *NOS2A* in agreement with previous studies.[23,26–28] For the purpose of this study, however, it is particularly remarkable that *PTGS2/COX-2* was also consistently up-regulated, at variable levels (mean fold of increase compared CD19$^+$ B lymphocytes, 12.4; range, 2 to 33.3) in all of the B-CLL samples examined. Validation of the COX-2 microarray results was performed by RT-PCR on the same samples analyzed by microarray approach (Figure 1A).
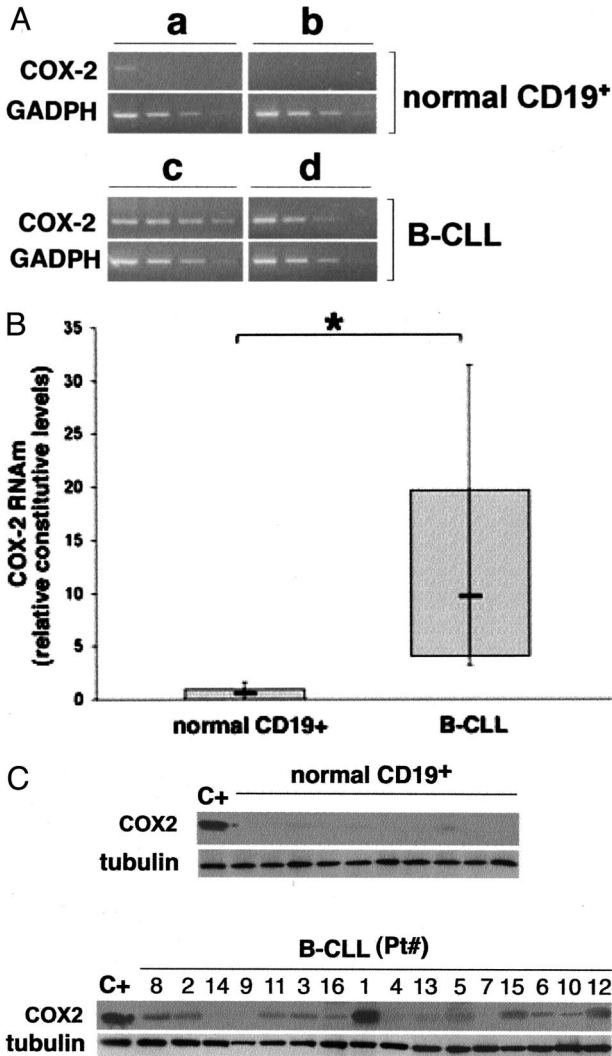
A
a   b   normal CD19⁺
c   d   B-CLL

B

C
normal CD19⁺
B-CLL (Pt#)

Figure 1. COX-2 expression in B-CLL and in normal CD19⁺ B lymphocytes. **A:** Microarray results were validated by RT-PCR on serial scalar dilutions of the RNA templates. Amplification of the glyceraldehyde-3-phosphate dehydrogenase (GADPH) housekeeping gene was used to confirm comparability of the samples. Representative results obtained from two normal CD19⁺ B lymphocyte and two B-CLL samples are shown. **B:** COX-2 mRNA levels were measured in CD19⁺ B lymphocytes purified from PBMCs of 10 different healthy donors and in 16 B-CLL patients after normalization to the level of GADPH mRNA. Each sample was determined in duplicate. Horizontal bars are median; top and bottom edges of box are 75th and 25th percentiles; lines extending from box are 10th and 90th percentiles. *$P < 0.01$. **C:** COX-2 protein was analyzed by Western blot in lysates obtained from normal CD19⁺ cells purified from PBMCs of 10 different healthy donors, and from samples of 16 B-CLL patients (Pt.). Comparable loading of protein in each lane was confirmed by staining with the antibody to tubulin (C+, positive control).

Furthermore, quantitative analysis of the steady-state mRNA levels of COX-2 was extended to a total of 16 B-CLL samples and 10 normal B cell samples (Figure 1B). Overall, a significant ($P < 0.01$) increase of COX-2 mRNA levels was demonstrated in the B-CLL samples examined over normal B lymphocytes (Figure 1B). Because COX-2 is irreversibly inactivated following catalysis, it is assumed that COX activity is determined by the amount of the enzyme protein and is regulated exclusively at the levels of transcription and translation. Consistent with this hypothesis, the results of RT-PCR were
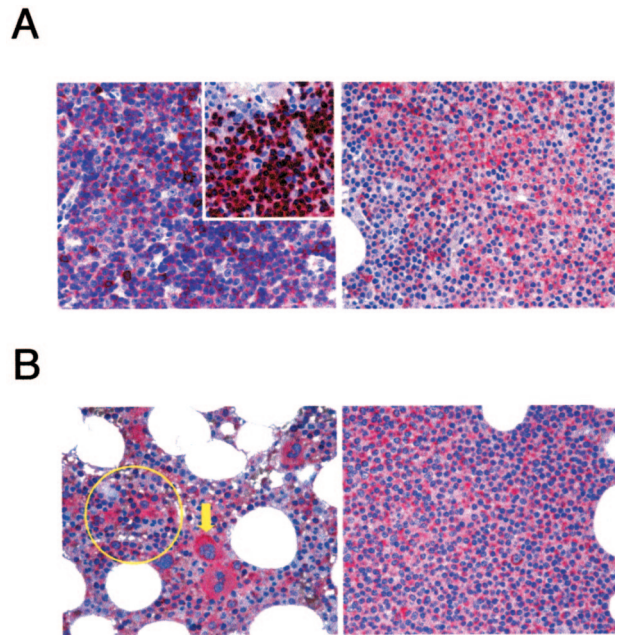

Figure 2. COX-2 expression in B-CLL bone marrow biopsies. Biopsies from bone marrow of B-CLL patients were analyzed by immunohistochemistry. **A:** Positivity for CD5 (**left panel**), CD79a (**inset**), and CD23 (**right panel**) of a nodular infiltrate in the bone marrow. **B:** COX-2 expression in normal erythroblasts (**circle**) and megakaryocytes (**arrowhead**) in the normal bone marrow (**left panel**) as well as in the nodular infiltrates (**right panel**). A representative sample is shown. Original magnification, ×400.

further confirmed by analyzing COX-2 protein at Western blot. In fact, as shown in Figure 1C, whereas COX-2 protein was virtually undetectable in normal B cells, it was detected, at variable levels, in the majority of B-CLL samples examined. The relative expression levels of COX-2 protein, determined by densitometry analysis, significantly correlated ($r = 0.81$, $P < 0.01$) with the steady-state mRNA levels.

In additional experiments, COX-2 expression was examined in biopsies obtained from B-CLL patients with leukemic bone marrow infiltrates. As shown in a representative sample (Figure 2A), the leukemic bone marrow infiltrate displayed a nodular pattern mainly consisting of small lymphocytes and pro-lymphocytes. These cells exhibited the characteristic B-CLL immunophenotype, ie, positivity for CD79a, CD23, and CD5. When the bone marrow biopsies were immunostained for COX-2 antigen, COX-2 expression was observed in normal erythroblasts and megakaryocytes (Figure 2B, left panel), as expected on the basis of previous studies of our and other groups of investigators.[29–31] More importantly, clear expression of COX-2 protein was detected also in the leukemic infiltrate (Figure 2B, right panel).

The Selective COX-2 Inhibitor NS-398 Induces Cytotoxicity in Most B-CLL Samples but Not in Normal Purified CD19⁺ B Lymphocytes and PBMCs

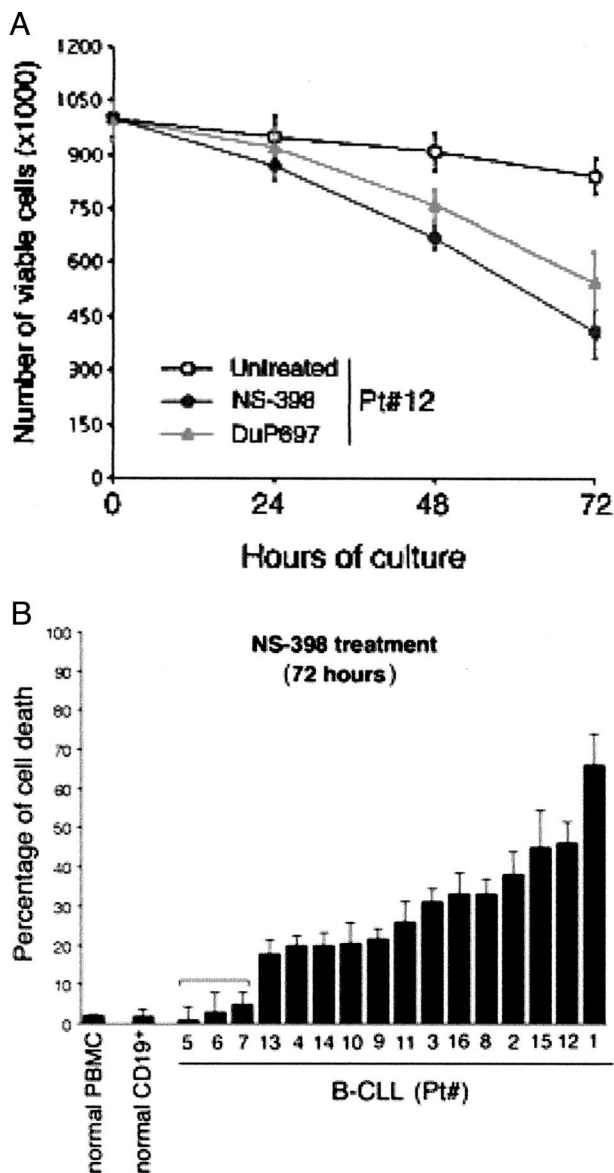In the next group of experiments, B-CLL cells were cultured *ex vivo* for 72 hours in the absence or presence of

A

B

Figure 3. Effect of NS-398 on *ex vivo* cultured B-CLL cells, normal CD19^{+} B lymphocytes and normal PBMCs. Primary normal CD19^{+} B lymphocytes, PBMCs, and leukemic cells isolated from B-CLL patients were cultured in the absence or presence of COX-2 inhibitors. **A:** After 24, 48, and 72 hours of treatment, cell viability was monitored by scoring the total number of viable cells. Shown are results from one representative B-CLL patient. Data are means ± SD of results from two independent experiments each performed in triplicate. **B:** Cytotoxicity measured after 72 hours of NS-398 treatment is reported for normal PBMCs (*n* = 8), for CD19^{+} B lymphocytes (*n* = 4), and for each B-CLL patient analyzed. Data are expressed as percentage of control untreated cultures and are means ± SD of three experiments, each performed in triplicate. In samples 5, 6, and 7, NS-398 treatment did not significantly affect viability.

the pharmacological inhibitor of COX-2, NS-398. This time frame was chosen taking into account that in the absence of exogenous cytokines, which are known to prolong the *in vitro* lifespan of B-CLL cells,[26–28] leukemic cells did not proliferate *ex vivo* and the number of viable cells remained relatively constant, never dropping below 80% of the total cell number seeded at time 0 (Figure 3A). The effect of NS-398 on cell viability was monitored every 24 hours, as exemplified in Figure 3A for one B-CLL

sample. In some B-CLL cultures (*n* = 3), a structurally unrelated COX-2 inhibitor (DuP697; 100 μmol/L) was also used, to rule out possible bystander effects of NS-398 on B-CLL cell viability. As shown in Figure 3A, also DuP697 induced a significant decrease in the number of viable cells, comparable with that observed with NS-398.

The susceptibility of each B-CLL sample to the *ex vivo* NS-398 treatment is reported for the end point (72 hours) in Figure 3B. In 13 of 16 B-CLL patients, NS-398 induced a significant (*P* < 0.01, cut-off >20%) decline in viability with respect to untreated control cultures (32 ± 16% of cytotoxicity, means ± SD of 13 cases), whereas no significant variations were observed in terms of viable cell number in only 3 of 16 B-CLL samples, as well as in normal purified CD19^{+} B lymphocytes (*n* = 4) and in total PBMCs (*n* = 8) (Figure 3B). There were no apparent correlations between sensitivity to NS-398 and clinical characteristics of the B-CLL patients. Moreover, analysis of the samples examined by cDNA microarray did not reveal any correlation between sensitivity to NS-398 and the steady-state mRNA levels of Bcl-2 (*r* = 0.48, *P* > 0.05).

Cell viability was next measured in either B-CLL or primary normal PBMCs incubated with various concentrations of NS-398 for 72 hours (Figure 4A). NS-398 induced a dose-dependent cytotoxic effect when added to B-CLL leukemic cells, whereas normal PBMCs were refractory to NS-398 cytotoxicity even at the highest drug concentrations (200 μmol/L) (Figure 4A). It should be noted that a 24-hour treatment with the concentration of NS-398 used in this and in the following set of experiments (100 μmol/L) was able to completely block the production and the release in culture of prostaglandins (PGE$_2$) in five different B-CLL samples examined (Figure 4B).

Concurrently, we sought to elucidate whether the cytotoxicity of NS-398 toward B-CLL cells was caused by apoptosis. For this purpose, B-CLL cell lysates were examined for the levels of PARP, a downstream target of activated caspase-3 that is typically cleaved in the setting of caspase-mediated apoptosis. In NS-398-treated B-CLL cells, Western blot analysis revealed the increase of the 85-kd cleaved product of PARP (Figure 5A). Moreover, exposure to NS-398 resulted in a significant (*P* < 0.05) increase in the percentage of Annexin V-positive cells compared with untreated cultures (Figure 5B). Taken together, these findings suggest that COX-2 overexpression plays a role in counteracting the apoptotic pathway in B-CLL cells and demonstrate that B-CLL cells are more susceptible to NS-398-induced cell death compared with normal purified CD19^{+} B lymphocytes and PBMCs.

NS-398 Potentiates the Cytotoxicity of Chemotherapeutic Agents and TRAIL in a Subset of B-CLL Samples

We next investigated whether NS-398, besides showing a significant cytotoxic activity on B-CLL samples when used alone, was able to modulate the susceptibility of
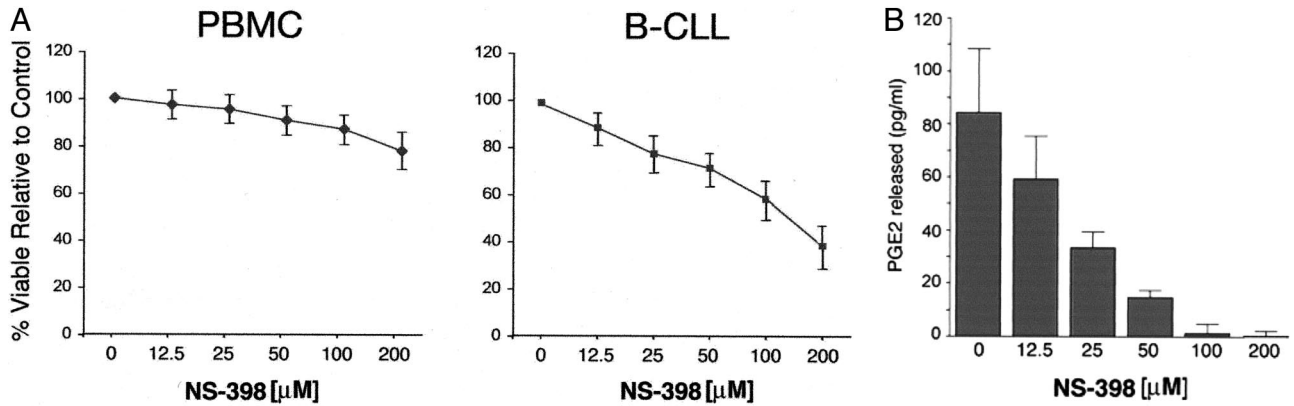
Figure 4. Dose-dependent cytotoxicity and inhibition of PGE$_2$ release by NS-398 in B-CLL samples. Normal PBMCs and leukemic cells isolated from B-CLL patients were cultured in the absence or presence of NS-398. **A:** Mean dose response curves for B-CLL cells isolated from patients ($n = 10$) and for normal PBMCs isolated from healthy blood donors ($n = 6$) cultured with increasing doses of NS-398 for 72 hours. Percentage of viable cells relative to control (vehicle alone) is expressed on the y axis. **B:** PGE$_2$ release was measured in the supernatant of B-CLL cells ($n = 5$) cultured with increasing doses of NS-398. Results are expressed as means $\pm$ SD.

B-CLL cells to chemotherapeutic agents. When B-CLL cells were exposed in culture to NS-398 for 24 hours before adding chlorambucil for additional 48 hours, a significant ($P < 0.05$) increase of cytotoxicity with respect to either chlorambucil or NS-398, used as single agents, was observed in 8 of the 16 patients examined (Figure 6). It is noteworthy that all B-CLL samples examined, except cells from patient 8, were susceptible to chlorambucil-mediated apoptosis. Interestingly, this sample was susceptible to NS-398-mediated apoptosis, suggesting that NS-398 might be pharmacologically active also in chlorambucil-resistant B-CLL cells. Similar findings were obtained when fludarabine (F-ara) was used instead of chlorambucil (data not shown).

In parallel, the ability of NS-398 to sensitize to cell death was also examined in cultures treated with recombinant TRAIL. Despite the fact that all B-CLL samples examined showed significant surface levels of the death receptor TRAIL-R2 (Figure 7A), recombinant TRAIL alone induced a modest cytotoxic response when added alone to B-CLL cells (Figure 7B). These findings were not unexpected on the basis of previous data of different groups of investigators including ourselves.[32,33] Nevertheless, when B-CLL cells were pretreated for 24 hours with NS-398 before adding TRAIL for additional 48 hours, a significant ($P < 0.05$) increase of cytotoxicity with respect to both recombinant TRAIL and NS-398, used as single agents, was observed in 8 of 16 B-CLL samples (Figure 7B).

Discussion

Gene expression profiling has been used in previous studies to compare the molecular differences and similarities between leukemic B-CLL cells and healthy cells as well as to compare the molecular signatures of subsets of malignant cells based on their biology or clinical outcome.[18–23] B-CLL samples have been shown to possess a gene expression profile related to resting and memory B cells, not in keeping with the hypothesis that CLL cells are derived from CD5$^+$ B cells.[18] These studies
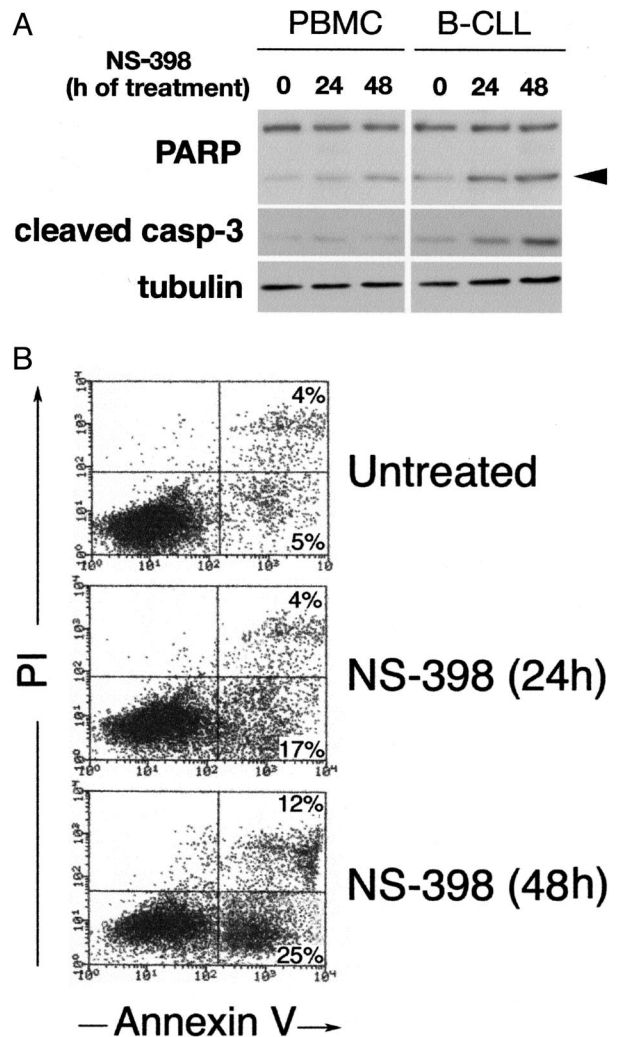

Figure 5. NS-398 induces apoptotic cell death in B-CLL cells. Normal PBMCs and leukemic cells isolated from B-CLL patients were cultured in the absence or presence of NS-398. **A:** Protein lysates from B-CLL cultures treated as indicated were analyzed in Western blot for PARP and caspase-3 cleavage. **Arrowhead** indicates the 85-kd cleaved product of PARP. Representative results obtained from PBMCs and B-CLL cells are shown. **B:** Apoptosis was monitored in flow cytometry by annexin V-FITC binding and propidium iodide staining. Representative results obtained from B-CLL cultures are shown.
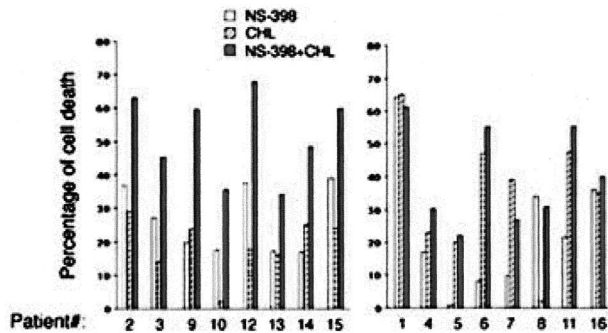
Figure 6. Cytotoxic effect of NS-398 in combination with chlorambucil on B-CLL cells. B-CLL samples were pre-incubated with NS-398 for 24 hours before exposure to chlorambucil (CHL) for an additional 48 hours. The cytotoxic effect of the treatment with the single agents (NS-398 and CHL alone) and with the combination is reported for each patient analyzed. Data are expressed as percentage of control untreated cultures and are means of the results from two independent experiments, each performed in duplicate. SDs were comprised within 10%. The patients in which NS-398+CHL treatment induced greater apoptosis levels versus the treatment with the single agents are grouped in the left graphic.

have increased our understanding of CLL pathogenesis, classification, and risk stratification.

By comparing B-CLL cells with healthy peripheral blood B cells, we have identified COX-2 as a potential therapeutic target for the treatment of B-CLL. In fact, the steady-state mRNA levels of COX-2 were variably up-
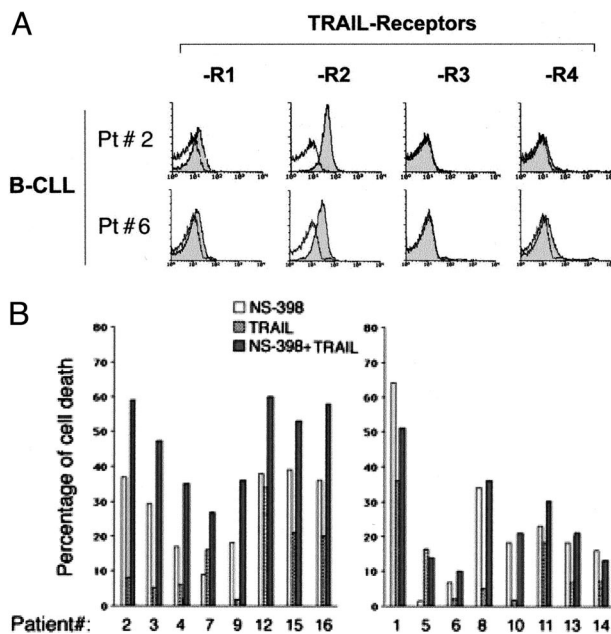

Figure 7. Cytotoxic effect of NS-398 in combination with TRAIL on B-CLL cells. **A:** Surface expression of TRAIL receptors (TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4) was analyzed by flow cytometry in B-CLL PBMCs. Shadowed histograms represent cells stained with mAbs for the indicated antigens, whereas unshadowed histograms represent the background fluorescence obtained from the staining of the same cultures with isotype-matched control mAbs. Representative phenotypes are shown. **B:** B-CLL samples were pre-incubated with NS-398 for 24 hours before exposure to TRAIL for additional 48 hours. The effect of the treatment with the single agents (NS-398 and TRAIL alone) and with the combination is reported for each patient analyzed. Data are expressed as percentage of control untreated cultures and are means of the results from two independent experiments, each performed in duplicate. SDs were comprised within 10%. The patients in which NS-398+TRAIL treatment induced greater apoptosis levels versus the treatment with the single agents are grouped in the left graphic.

regulated in B-CLL samples in comparison with normal B cells. In this respect, it is noteworthy that several mechanisms have been proposed by which COX-2 overexpression inhibits apoptosis, including increased Bcl-2 expression,[15] and up-regulation of the anti-apoptotic kinase Akt.[34] Consistently, we were able to document a variable up-regulation of the mRNA steady-state levels of Bcl-2 (from 2 to 6.5 fold) in a subset of B-CLL samples in our microarray analysis (data not shown). Moreover, other authors have shown that the levels of Akt activity are pathologically elevated in B-CLL cells in comparison with normal lymphocytes.[35,36]

The relevance of COX-2 up-regulation in protecting B-CLL cells from apoptosis was underscored by the fact that pharmacological inhibition of COX-2 activity by NS-398 induced a significant increase of cytotoxicity in most B-CLL samples examined and showed an additive or synergistic cytotoxic effect when added in combination with either chemotherapeutic agents or TRAIL. In a previous paper, Bellosillo et al[37] suggested a COX-2-independent cytotoxic effect of aspirin in a small cohort of B-CLL patients studied; and in five of seven patients examined, NS-398 (used at the same concentrations used in this study) failed to induce significant apoptosis. Although we do not have a ready explanation for these discrepancies, Bellosillo et al did not specify the percentage of leukemic cells in the PBMC samples examined. In this respect, it is important to underline that in our study only patients with a number of leukemic cells higher than 85% were enrolled. Because the mean apoptosis induced by NS-398 in B-CLL samples was 32 ± 16% over the untreated cultures, a low purity of starting cell population could easily mask the effect of NS-398.

Our current findings that COX-2 is overexpressed in primary leukemic B-CLL cells expand previous findings, which have documented that COX-2 is overexpressed in solid tumors and represent a potential target for therapy.[38-40] As with other malignancies, the accumulation of genetic abnormalities is required for malignant transformation of human lymphocytes. Whether COX-2 might contribute to malignant progression of B-CLL is uncertain. COX-2 is a bifunctional enzyme with both oxidation and peroxidation activities. There is recent evidence that COX-2 peroxidation can lead to the production of mutagens, such as malondialdehyde.[41] Although we cannot exclude that the increased COX-2 expression in B-CLL may represent an epiphenomenon representative of activation mechanisms, it is also possible that COX-2 overexpression plays a role in the pathogenesis of B-CLL, like in solid tumors.[38] Because *COX-2* is an immediate-early response gene, induced by a variety of cytokines, the observed up-regulation of COX-2 in B-CLL cells might be secondary to the release of cytokines in an autocrine fashion. Consistent with this hypothesis, our microarray analysis revealed a marked elevation of IL-8 and even more pronounced of IL-1β mRNA, in keeping with previous findings of other authors.[42,43]

In addition to genetic evidence, numerous pharmacological studies suggest that COX-2 is a bona fide therapeutic target in solid tumors.[38-40] Treatment with selective inhibitors of COX-2 reduced the formation of tongue,

esophageal, intestinal, breast, skin, lung, and bladder tumors in experimental animals.[38–40] Whether pharmacological inhibitors of COX-2 suppress carcinogenesis exclusively by inhibiting COX-2 is not certain. For example, high concentrations of selective COX-2 inhibitors suppress the growth of cells in culture that do not express COX-2, and new celecoxib derivatives without COX-2 inhibitory activity have been recently proposed for the treatment of B-CLL.[44,45] It is therefore possible that both COX-2-dependent and COX-2-independent anticarcinogenic effects account for the cytotoxicity of NS-398 also in B-CLL, as previously demonstrated in colon cancer.[46]

Although our study suggests that COX-2 inhibitors might be also effective in B-CLL, it seems unrealistic to expect that selective COX-2 inhibitors will be useful as monotherapy in treating B-CLL. In all likelihood, selective COX-2 inhibitors will need to be given in conjunction with either standard anticancer therapy or with innovative therapy, in the effort to re-establish a normal apoptotic process as a therapeutic approach in B-CLL.[47] Taken together, our results suggest that COX-2 warrants investigation as a molecular target for the prevention and treatment of B-CLL.

Acknowledgments

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