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Antitumor Effects of Celecoxib on K562 Leukemia Cells Are Mediated by Cell-Cycle Arrest, Caspase-3 Activation, and Downregulation of Cox-2 Expression and Are Synergistic With Hydroxyurea or Imatinib

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Celecoxib, a specific cyclooxygenase-2 (Cox-2) inhibitor, has been shown to possess antitumor activity in a variety of cancer cells. However, the antitumor activity of celecoxib in hematopoietic tumors, especially in chronic myeloid leukemia (CML), has not been well established. This study was designed to investigate the effect of celecoxib on growth and apoptosis in a human CML cell line (K562 cells) or in primary CML cells, and to examine the synergistic actions of celecoxib and hydroxyurea or imatinib on K562 cell proliferation and apoptosis. Celecoxib significantly inhibited the growth of both K562 and primary CML cells and induced apoptosis in a dose-dependent fashion. The IC_{50} of celecoxib was 46 μM for inhibition of K562 cell proliferation. The effect of celecoxib on growth inhibition was accompanied by the downregulation of cyclin D_1 and cyclin E and p-Rb expression, the upregulation of P_{16}^{INK4a} and $P27^{KIP}$ expression, and a G_1-S phase arrest of the cell cycle. The pro-apoptotic effect of celecoxib was determined to be mediated by caspase-3 activation. When K562 cells were pretreated with DEVD-fmk, a specific inhibitor of caspases, the apoptotic activity of celecoxib was, in part, abrogated. Importantly, we demonstrated for the first time that K562 cells were Cox-2-positive both at the mRNA and protein levels. We noted the following observations: (i) we detected Cox-2 mRNA in K562 cells by reverse transcription-PCR (RT-PCR) and protein expression by western blot analysis; (ii) Cox-2 expression in K562 cells was stimulated by IL-1β, a specific inducing agent of Cox-2 expression; (iii) primary CML cells from CML patient bone marrow also exhibited Cox-2 protein expression. Furthermore, Cox-2 expression was downregulated at higher doses of celecoxib (80–160 μM), suggesting a Cox-2-dependent mechanism was involved in the drug's effects of growth inhibition and induction of apoptosis. In addition, a synergistic effect was observed when cells were exposed to low-dose celecoxib (40 μM) and hydroxyurea (10 mM) or a combination of celecoxib (40 μM) and imatinib (0.2 μM). These findings provide the basis for uncovering the mechanism of celecoxib's antitumor effects and developing a new therapeutic strategy for treating CML. Am. J. Hematol. 81:242–255, 2006. © 2006 Wiley-Liss, Inc.

Key words: leukemia; cyclooxygenase-2; cyclooxygenase-2 inhibitor; apoptosis; cell cycle

INTRODUCTION

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by a 9;22 translocation resulting in the expression of a fusion oncoprotein, BCR/ABL. This oncoprotein not only exhibits constitutively active kinase activity that confers growth potential and apoptosis resistance for CML cells [1] but also becomes a drug target, based on molecular therapies including imatinib

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mesylate, a selective inhibitor of the BCR/ABL tyrosine kinase [2,3]. However, resistance to imatinib has occurred in a few CML patients [4,5]. In view of the difficulties of current CML therapy, new approaches remain a high priority. The cyclooxygenase (Cox) family of enzymes has been implicated in the processes of cell proliferation and angiogenesis for many tumors, including colon, non-small-cell lung, breast, gastric, esophagus, prostate, head/neck, and cholangiocarcinoma [6–9]. Cox-2 is an immediate early gene that is induced by a variety of stimuli, such as cytokines, hormones, mitogens, and growth factors [10–14]. Cox-2 and its metabolic products, such as PGE_2, may induce inflammation, mediate a number of apoptotic and cell proliferation signaling pathways, and play a role in carcinogenesis [15]. The proposed mechanisms by which nonsteroidal anti-inflammatory drugs (NSAIDs) prevent or block cancer growth include inhibition of cell proliferation and angiogenesis and induction of apoptosis [13–17]. Our previous studies suggest that indomethacin (IN) is able to inhibit cell proliferation and induce apoptosis in either a dose- or time-dependent manner, both in primary CML cells and in K562 cells. The mechanism partially involves the down-regulation of the BCL-2 gene or altered protein expression of the BCL-2/Bax ratio [18]. A further study indicated that apoptosis of CML cells induced by IN was mediated by the activation of either caspase-3 or caspase-8, with an elevation of intracellular free calcium [19]. Because traditional NSAIDs inhibit Cox-1 and cause unwanted side effects, such as gastrointestinal tract bleeding, we chose celecoxib, a specific Cox-2 inhibitor, and observed its effects on cell growth and apoptosis in K562 cells.

Although several mechanisms are proposed to explain the antitumor action of Cox-2 inhibitors in epithelial cancer cell lines, their effects on apoptotic signaling and cell proliferation have been gaining attention [20,21]. The central component of a cell's apoptotic machinery is a proteolytic system that involves a family of proteases called caspases. In the caspase family, caspase-3 is a key signal of apoptosis whose cleavage contributes to the morphological and functional changes associated with cell death [22]. Apoptosis, induced by DNA damaging agents, often involves the activation and cleavage of caspase-3. Another mechanism by which Cox-2 inhibitors may suppress carcinogenesis is by attenuating cell proliferation and inducing cell-cycle arrest [23]. These actions may occur because of the ability of Cox-2 inhibitors to deregulate the activity of factors involved in the regulation of cell-cycle progression, such as cyclin-dependent kinases or $P21^{waf1/cip1}/p27^{kip1}$ [9,24]. Additionally, Cox-2 inhibitors may play a role in affecting the dynamic interaction between apoptosis and cell growth, resulting in its antitumor activity.

Prior studies have demonstrated Cox-2-independent anti-proliferative effects of NSAID-like compounds on epithelial cancer cell lines. Another study found that the magnitude of the anti-proliferative effects of celecoxib were similar for both hematopoietic and epithelial cancer cell lines, despite the fact that expression of both Cox-2 mRNA and protein was negative in most hematopoietic cell lines (including K562 cells) [25]. However, some studies found that Cox-2 expression was positive in K562 cells. Giles et al. reported that Cox-2 protein levels in bone marrow cells were elevated in chronic-phase CML patients and increasing levels of Cox-2 were significantly associated with shorter survival time spans [26]. Because of the similar genetic backgrounds of both K562 and primary CML cells, and apparent discrepancies in reported results [25,26], it remains to be confirmed whether these cells actually express Cox-2 [26].

In this study, we examined the efficiency of celecoxib on K562 cell proliferation and apoptosis, and explored its anti-proliferation and apoptosis induction mechanisms. Furthermore, we demonstrated that both K562 and primary CML cells express both Cox-2 protein and mRNA. Thus, we concluded that CML cells are Cox-2-positive. Also, we assessed the effectiveness of celecoxib in combination with hydroxyurea or imatinib on its antitumor activity.

MATERIALS AND METHODS

Materials

The human chronic myeloid leukemia cell line (K562) was provided by the Institute of Blood Physiology, Xiang Ya Medical College, and the human epithelial cancer cell line (A549) used as a Cox-2-positive control was obtained from the Laboratory of Respiratory Diseases at The Second Xiangya Hospital. Four samples of primary CML cells were collected from CML patients by regular bone marrow puncture; all patients gave written informed consent to the use of BM cells for research purposes. In each case, diagnosis was based on morphological and cytochemical staining and cytogenetic analyses. All cases were diagnosed in chronic phase and Ph chromosome were positive. Celecoxib was obtained from Pfizer; MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], trypan blue, propidium iodide, and acridine orange were obtained from Sigma Chemical Co. (St. Louis, MO); RNase A and proteinase K were purchased from the Hua-Mei Company (Shanghai, China); protein mo-

lecular weight standards were from Bio-Rad (Hercules, CA); Trizol solution was obtained from Gibco (Bethesda, MD); reverse-transcription reagents and DNA molecular weight standards were provided by Promega (Madison, WI); rabbit anti-human caspase-3 antibody was from StressGen Biotechnologies Inc. (San Diego, CA); mouse anti-human Cox-2 monoclonal antibody was provided by Cayman Chemical (Ann Arbor, MI); goat anti-human β-actin antibody, mouse anti-human cyclin D_1 antibody, rabbit anti-human cyclin E antibody, goat anti-human p-Rb (ser795) antibody, rabbit anti-human $P27^{KIP}$ (c-19) antibody, and HRP-labeled rabbit anti-goat IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); HRP (horseradish peroxidase)-labeled goat anti-rabbit IgG and goat anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA); mouse anti-human $P16^{INK4a}$ and mouse anti-human $P21^{wif1}$ monoclonal antibodies were provided by Neo-Markers (Fremont, CA) and Pharmingen (Franklin Lakes, NJ), respectively. IL-1β was provided from PeproTech EC Ltd (London, England); and DEVD-fmk was from Enzyme Systems Products (Livermore, CA). Annexin V-FITC apoptosis detection kit was provided by BD Biosciences Pharmingen (Franklin Lakes, NJ). Imatinib (Novartis Pharma, Basel, Switzerland) was kindly provided Dr. F.Y. Meng (Southern Medical University, Guangzhou, China).

Cell Culture

K562 cells were cultured in RPMI medium, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 3.0×10^5 cells were added to each well on a 24-well plate, in triplicate. Celecoxib was diluted with DMSO (Sigma) in a 2-fold dilution series (10, 20, 40, 80, and 160 μM). An equal volume of DMSO was added to control wells. The plates were incubated at 37°C in 5% CO_2 for 36 hr. For primary CML cells, BM mononuclear cells (BMMNCs) were isolated by means of Ficoll density gradient centrifugation. The treatment method was the same as described above. For A549 cells, RPMI culture medium was replaced with DMEM (Dulbecco's modified eagle's medium).

Cell Proliferation Assay

(1) MTT assay. Briefly, 200 μL (6×10^4 cells) of a K562 cell suspension were plated in each well of a 96-well plate. After 12 hr, the cells were treated with varying concentrations of celecoxib (10, 20, 40, 80, and 160 μM). An equal volume of DMSO was added to the control well, and the cells were cultured an additional 32 hr; then 20 μL MTT (5 mg/mL) in growth medium was added per well, and the plates incubated at 37°C for 4 hr. Plates were then centrifuged at 400 g for 10 min. Supernatants were removed from the wells, and the reduced MTT dye in each well was solubilized in 200 μL DMSO. Absorbance was measured on an ELX-800 microplate reader at 490 nm. Viability was calculated as follows: Cell Viability (%) = $(OD_{test}/OD_{control}) \times 100\%$.

(2) Trypan blue exclusion assay. K562 or primary CML cell viability levels were evaluated by the trypan blue exclusion method. Cells were treated as described above. After the cells had been harvested, routine trypan blue staining was performed and viable cells were counted using light microscopy. For each celecoxib concentration, the cell count was duplicated and the average value was obtained. Viability was calculates as follows: Cell Viability (%) = (Viable cell $number_{test}$/Viable cell $number_{control}$) × 100%.

(3) Colony formation-inhibiting test. K562 cells (1×10^3 cells/mL) were treated with different doses of celecoxib. After 36 hr, cells were seeded in triplicate into 35-mm dishes containing agarose and RPMI 1640. The plates were incubated at 37°C, in 5% CO_2, for 2 weeks. Colonies (cell numbers ≥ 50 = colony) were counted and photographed. The inhibition rate was calculated as follows: Colony inhibition rate (%) = (1 − Average colony number in treated group/Average colony number in blank control) × 100%.

(4) Cell-cycle analysis by flow cytometry. First, 1×10^6 cells/mL from each plate were fixed with 70% ethanol at 4°C. RNaseA (20 μg/mL) and PI (50 μg/mL) were then added, and cell suspensions were incubated for 30 min in the dark. Stained cells were analyzed on a FACScan flow cytometer (FACSCalibur, BD Biosciences, Franklin Lakes, NJ). The data were analyzed using ModFit software (Verity Software House, Topsham, ME).

Apoptosis Assays

K562 or primary CML cells were treated with varying concentrations (10–160 μM) of celecoxib and a "no drug treatment" was designated as the control. After 36 hr of culture, cells were harvested and apoptosis assays were performed as follows:

(1) Morphological identification of apoptotic cells. Cells were collected, washed, and stained with acridine orange/ethidium bromide (AO/EB), according to the method of Nicolatti et al. [27].

(2) DNA ladder agarose-gel electrophoresis assay. K562 or primary CML cells were harvested and lysed in buffer containing 20 mM EDTA, 100 mM Tris, pH 8.0, and 0.8% SDS. RNaseA (10 mg/mL) was added, and the cells were incubated for 2 hr at 37°C. Proteinase K (25 mg/mL) was added, and

cells were incubated overnight in a 50°C water bath. DNA samples were electrophoresed on a 1.5% agarose gel, and DNA bands were visualized using a GDS-8000 gel document device (GDS-8000 PCA, Bio-Rad).

(3) Analysis of PS exposure using annexin V. The assay was performed as described by the manufacturer (BD Biosiences Pharmingen Co.). Briefly, K562 cell were harvested, washed, stained with annexin V and propidium iodide, and analyzed with a FACS caliber cytometer (BD Biosciences).

(4) Caspase-3 activity assay and DEVD-fmk blocking test. Activation of caspase-3 cleavage was determined by western blot. Briefly, K562 cell lysates were prepared using RIPA buffer [5 mL of cold RIPA; 50 μL PMSF (10 mg/mL stock); 5 μL of aprotinin (10 mg/mL stock); 5 μL of leupeptin (10 mg/mL stock)]. Protein was quantitated using the Bradford method. Protein samples were separated on a 12% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated in blocking buffer and then incubated in a rabbit anti-human caspase-3 antibody solution (1:4000 dilution of antibody in blocking buffer) for 4 hr at room temperature. After being washed, HRP-conjugated goat anti-rabbit IgG (1:5000) was added. Protein signals were detected using the ECL system (Amersham Biosciences AB, Uppsala, Sweden). To confirm whether caspase-3 cleavage was activated after treatment of cells with celecoxib, a blocking test was carried out in which a 100-μM dose of DEVD-fmk, a specific inhibitor of caspase-3, was applied to K562 cells for 2 hr. Varying concentrations of celecoxib were then added onto the cells and incubated for 36 hr. An MTT assay, apoptotic cell identification, DNA fragment assay, and western analysis were then performed as described above.

Demonstration of Cox-2 Expression in K562 Cells or Primary CML Cells

(1) Stimulating effect of IL-1 on Cox-2 protein expression in K562 cells. IL-1β (5 ng/mL) was added to K562 cells (3×10^6 cells/mL) and incubated at 37°C, in 5% CO_2, for 12 hr. A549 cells were also treated with IL-1β (5 ng/mL). After protein extraction, Cox-2 detection was performed by western blot (primary antibody—mouse anti-human Cox-2 monoclonal IgG, 1:2000 dilution; secondary antibody—HRP-conjugated goat anti-mouse IgG, 1:4000 dilution). β-actin blots were designated as internal controls.

(2) Effect of celecoxib on Cox-2 expression in K562 cells and Cox-2 expression in primary CML cells. K562 cells, treated with different concentrations of celecoxib, were collected. Cell lysates were prepared and Cox-2 protein levels were analyzed by western blot; β-actin protein was designated as internal controls. For the evaluation of Cox-2 expression in primary CML cells, heparin-treated bone marrow (BM) samples were obtained from 4 CML patients. BM mononuclear cells (BMMNCs) were isolated by Ficoll density gradient centrifugation. About 5×10^6 cells were washed in PBS and then lysed with RIPA buffer. Total protein (100 μg) was run on 10% SDS-polyacrylamide gel, and then mouse anti-human Cox-2 was transferred to PVDF membrane, probed with antibody (1:2000) and secondary antibody (1:4000), and finally visualized with an ECL system.

(3) Reverse-transcription-PCR assay of Cox-2 mRNA. RNA from cultured K562 or A549 cells was extracted with Trizol solution. Purified total RNA was suspended in DEPC-treated water and used for further experiments. Total RNA was used to generate first-strand complementary DNA by the following reaction: 2 μg of total RNA; 4 μL of $MgCl_2$ (25 mM stock); 2 μL of 10× buffer; 2 μL each of dNTP (10 mM stocks); 1 μL of oligo-dT (500 mg/mL stock), 0.5 μL of RNasin (40 U/μL stock), 1 μL AMV (20 U/μL stock), and ddH_2O to 20 μL and incubated for 45 min at 42°C. PCR was then performed using a PE-2400 PCR thermal cycler. Oligonucleotides for Cox-2 were 5′-ATCCTTGCTGTTCCCACCCA-3′ and 5′-CTTTGACACCCAAGGGAGTC-3′, and the expected product size was 402 bp. PCR amplification was initiated with a pre-denaturation step for 6 min at 94°C. The samples then were subjected to 35 cycles of denaturation (50 sec at 94°C), annealing (50 sec at 50°C), extension (60 sec at 72°C), and an additional extension (7 min at 72°C). The housekeeping gene for β-actin served as an internal control. Primer sequences for β-actin were 5′-TGACGGGGTCACCCACAC-3′ and 5′-CTAGAAGCATTTGCGGTGGA-3′, and the expected PCR fragment was 661 bp. The PCR products were then visualized by silver staining after electrophoresis on an 8% polyacrylamide gel.

Western Blots of Cell-Cycle-Regulating Proteins

K562 cells were treated with varying concentrations of celecoxib. Whole-cell lysates were prepared as described above. Eluates were separated on 10–15% SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membrane. Mouse anti-human cyclin D_1 antibody (1:800 dilution), rabbit anti-human cyclin E antibody (1:1000 dilution), rabbit anti-human $P27^{KIP}$ antibody (1:500), goat anti-human p-Rb antibody (1:500), mouse anti-human $P16^{INK4a}$ antibody (1:800 dilution), and mouse anti-human

$P21^{waf1}$ antibody (1:1000 dilution) were used to probe the membranes. After being washed, peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:4000–1:5000 dilution) was applied. The membranes were then visualized using the ECL system, exposed to film, and developed. As a control, β-actin blots were performed simultaneously.

Synergistic Effects of Celecoxib and Hydroxyurea or Celecoxib and Imatinib on CML Cells

K562 or primary CML cells were treated with low-dose celecoxib (40 μM) or hydroxyurea (10 mM) alone or in combination for 36 hr. Cell proliferation, apoptosis, Cox-2 protein, and mRNA expression assays were carried out as described above. In addition, the combined effects of celecoxib and imatinib on K562 cells were investigated. Briefly, K562 cells were treated with 40 μM celecoxib or imatinib (0.2 μM) respectively or in combination for 36 hr. MTT assay and apoptosis assessment (annexin V staining) were performed as described above.

Statistical Analysis

For analysis of cell proliferation and colony formation–inhibition, the values shown represent the means ± SD for at least 3 separate experiments repeated in triplicate. Significance was determined by one-way ANOVA using the SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL). Differences were considered significant at $P < 0.05$.

RESULTS

Celecoxib Has Anti-proliferation Effects in Both K562 and Primary CML Cells

We tested celecoxib to assess its growth-inhibiting action on K562 and primary CML cells. After K562 or primary CML cells were exposed to celecoxib (10, 20, 40, 80, and 160 μM) for 36 hr, cell growth was measured by trypan blue exclusion and MTT assay. Results suggested that celecoxib treatment induced a dose-dependent inhibition of cell growth both in K562 and primary CML cells (Fig. 1). MTT results are consistent with those using the trypan blue exclusion assay. The IC_{50} value for celecoxib in K562 cells was 46 μM. After a celecoxib dose of 20–160 μM, significant effects on growth inhibition of K562 or primary CML cells were observed ($P < 0.05$). The colony formation–inhibition test showed that after an increased celecoxib dose (10–160 μM), mean colony numbers were significantly reduced. Corresponding to celecoxib concentrations 10, 20, 40, 80, and 160 μM, the inhibitory rates of colony formation were 57.7%, 82.7%, 94.3%, 98.3%, and 100%, respectively (Table I). In addition, the size of the colony changed with varying celecoxib dose. These results demonstrated the inhibitory effect of celecoxib on proliferation of human CML cells.

Celecoxib-Induced Inhibition of Cell Growth Is Mediated in Part Through G_1-S Arrest

To elucidate the mechanism by which celecoxib inhibits K562 cell proliferation, flow-cytometry analysis was performed to determine the effects on cell-cycle progression in cells treated with varying doses of celecoxib. As shown in Table II, celecoxib treatment led to a significant inhibition of cell-cycle progression in a concentration-dependent fashion, causing arrest at the G_1-S checkpoint, with no effect on G_2-M transition. This result indicates that the celecoxib-induced inhibition of K562 cell growth is mediated, at least in part, through cell-cycle inhibition at the G_1-S checkpoint.

Celecoxib Downregulates Cyclin D_1, Cyclin E, and p-Rb Expression and Increases $P16^{INK4a}$ or $P27^{KIP}$ Expression

Because cyclin D_1, cyclin E, $P16^{INK4a}$, $P21^{waf1}$, $P27^{KIP}$, and phosphorylated Rb (p-Rb) play important roles in cell-cycle progression and regulation, further experiments were carried out to check whether celecoxib treatment might influence their expression. Figure 2 shows that celecoxib treatment of K652 cells induced a significant decrease in cyclin D_1 and cyclin E protein levels in a dose-dependent manner; $P16^{INK4a}$ and $P27^{KIP}$ were also significantly upregulated following an increased celecoxib dose; corresponding with a down-regulating expression of cyclin D_1, p-Rb expression was decreased following raised celecoxib concentration; and celecoxib did not affect $P21^{wif1}$ protein expression. These results further support that celecoxib induces G_1-S arrest through suppression of cyclin D_1 and/or cyclin E, or inhibits Rb phosphorylation, and induces expression of $P16^{INK4a}$ and $P27^{KIP}$ in K562 cells.

Celecoxib Induces Apoptosis in Both K562 and Primary CML Cells

Consistent with its anti-proliferative effects, the effect on apoptosis induction by celecoxib was observed after a 36-hr exposure to celecoxib at varying doses in both K562 and primary CML cells. Morphological identification of apoptosis (AO/EB staining) is shown in Fig. 3A, in which 20 μM celecoxib was able

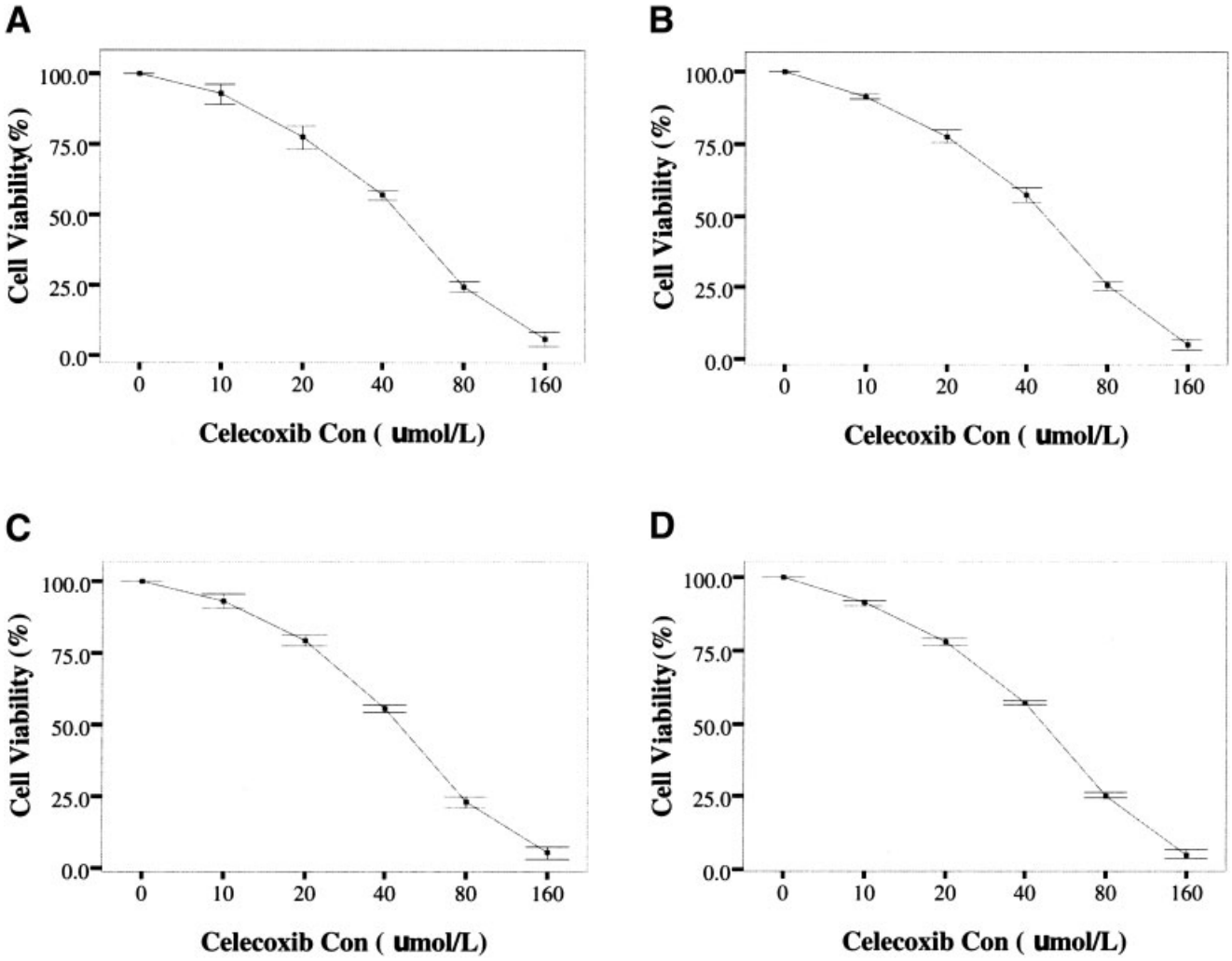

Fig. 1. Effects of celecoxib on the proliferation of K562 and primary CML cells. (A) Trypan blue exclusion test in K562 cells (n = 3); (B) MTT assay for K562 cells (n = 3); (C) trypan blue exclusion test in primary CML cells (n = 4); (D) MTT assay for primary CML cells (n = 4). Dots and lines are shown as the mean ± SD of cell activity value. Differences between every two means are significant (<0.001) at each chart.

TABLE I. Effects of Celecoxib on Colony Formation of K562 Cells ($\bar{X}$ ± SD, n = 3)

Celecoxib Con (μmol/L)	Colony number (colony count/plate)	Inhibitory rate of colony formation (%)
0	56.3 ± 7.0	0
10	24.0 ± 5.3	57.7 ± 4.0
20	10.0 ± 4.0	82.7 ± 5.0
40	3.3 ± 2.1	94.3 ± 3.4
80	1.0 ± 1.0	98.3 ± 1.8
160	0	100

TABLE II. Cell Cycle Analysis of Celecoxib-Treated K562 Cells ($\bar{X}$ ± SD, n = 3)

Celecoxib Con (μmol/L)	G0/G1 phase (%)	S phase (%)	G2 phase (%)
0	32.59 ± 1.23	56.58 ± 0.79	10.84 ± 1.28
10	32.39 ± 1.61	55.79 ± 0.46	11.82 ± 1.19
20	36.11 ± 0.97	54.13 ± 1.84	9.76 ± 1.29
40	40.91 ± 1.53	59.09 ± 1.53	0
80	60.73 ± 1.41	39.27 ± 1.41	0
160	65.94 ± 2.07	34.06 ± 2.07	0

to induce apoptosis in K562 cells. Annexin V assay showed K562 cells rapidly underwent apoptosis in the presence of celecoxib and the percentage of apoptotic cells was directly proportioned to the dose of celecoxib (Fig. 3C). Flow-cytometry results and DNA ladder gel patterns confirmed that celecoxib effectively induces apoptosis in K562 cells in a dose-dependent manner (Fig. 4). Results demonstrated that the threshold concentration was about 40 μM, similar to the IC_{50} concentration for its anti-proliferative effects.

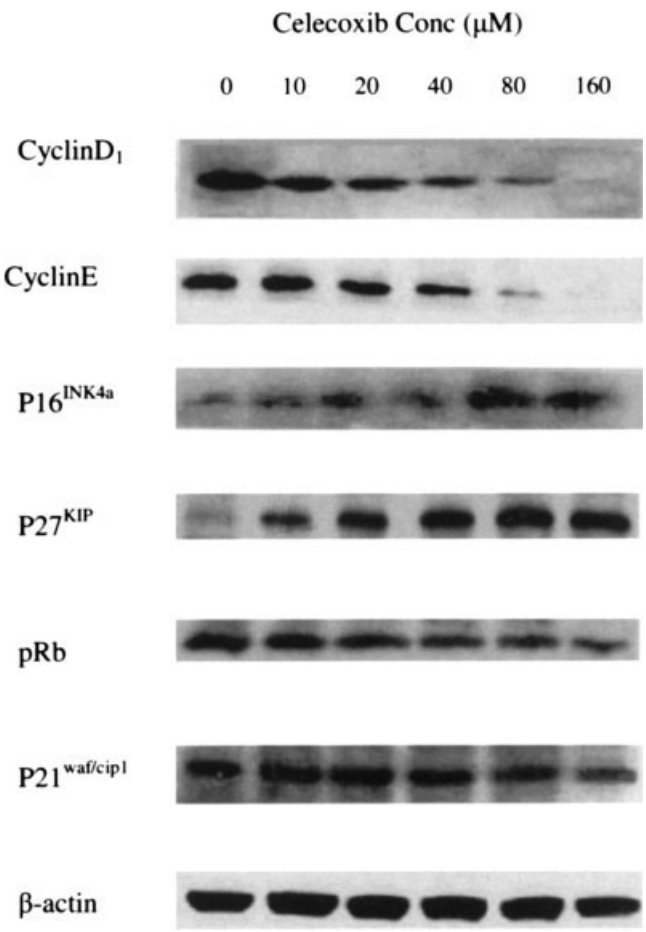

Fig. 2. Effects of celecoxib on cell-cycle-related protein expression. K562 cells were treated with varying doses of celecoxib or DMSO (negative control) for 36 hr, and the cell lysates were prepared for western blot analysis. Equal amounts of cellular protein was subjected to SDS-PAGE, followed by western blot analysis for cyclin D_1, cyclin E, $P16^{INK4a}$, $P21^{waf/cip1}$, $P27^{KIP}$, and p-Rb. β-Actin was used as an internal control.

Celecoxib-Induced Apoptosis in K562 Cells Is Mediated by Caspase-3 Activation

To evaluate whether caspase-3 activation is involved in celecoxib-induced apoptosis in K562 cells, we assessed caspase-3 expression by western blot and performed a blocking test in which K562 cells were treated with a specific inhibitor of caspase-3 (DEVD-fmk) and different doses of celecoxib. Western blot results indicated that with increasing doses of celecoxib, caspase-3 expression was upregulated and activated; when K562 cells were pretreated with DEVD-fmk and celecoxib treatment was later initiated, apoptosis was significantly inhibited (Figs. 3B and 5A). These findings suggest that celecoxib-induced apoptosis in K562 cells involves caspase-3 expression and activation.

Both Cox-2 mRNA and Protein Are Expressed in K562 Cells or Primary CML Cells

To demonstrate whether K562 cells or primary CML cells are Cox-2 negative or positive and to elucidate the mechanisms of anti-proliferative activity and induction of apoptosis, we analyzed expression of Cox-2 mRNA by RT-PCR and Cox-2 protein expression by western blot. Because A549 is a strong Cox-2-positive cell line, stimulated by IL-1β [28], we chose these cells as a positive control. Our results indicated that both K562 and A549 cells express Cox-2 at both the mRNA and protein levels. More importantly, Cox-2 mRNA or protein expression in K562 cells may be activated by IL-1β (Fig. 5B). These results strongly indicate that K562 cells are Cox-2-positive for both mRNA and protein. In order to strengthen our conclusion, we measured Cox-2 protein expression by western blot from four primary CML cell samples and consistent results were obtained (Fig. 5C).

Celecoxib, at High Doses, Suppresses Cox-2 mRNA and Protein Expression in K562 Cells

To reveal whether the antitumor activity of celecoxib is related to the Cox-2 pathway, we investigated the expression of Cox-2 mRNA and protein in celecoxib-treated K562 cells. Results showed that, at higher celecoxib doses (80–160 μM), celecoxib significantly inhibited Cox-2 mRNA and protein expression (Fig. 5D). These findings suggest that the mechanism of the celecoxib antitumor effect is, in part, Cox-2-dependent.

Celecoxib Enhances the Antitumor Efficacy of Hydroxyurea and Imatinib

In another set of experiments, the effect of a combined treatment of celecoxib with hydroxyurea or celecoxib with imatinib on K562 cells was evaluated. K562 cells were exposed to celecoxib (40 μM) and hydroxyurea (10 mM) for 36 hr, and Cox-2 mRNA and protein levels were measured as described above. The results revealed that treatment with celecoxib or hydroxyurea individually resulted in minimal changes in K562 cell viability, apoptosis, and Cox-2 mRNA and protein expression. Combined treatment caused a significant growth inhibition, apoptosis, and down-regulation of Cox-2 mRNA and protein (Figs. 6A, C, D, and E). These findings indicate that celecoxib and hydroxyurea have a synergistic effect on apoptosis induction and inhibition of growth proliferation in K562 cells. The mechanisms involved seem to be related to downregulation of Cox-2 mRNA or protein expression. Interesting, when cells were exposed to the

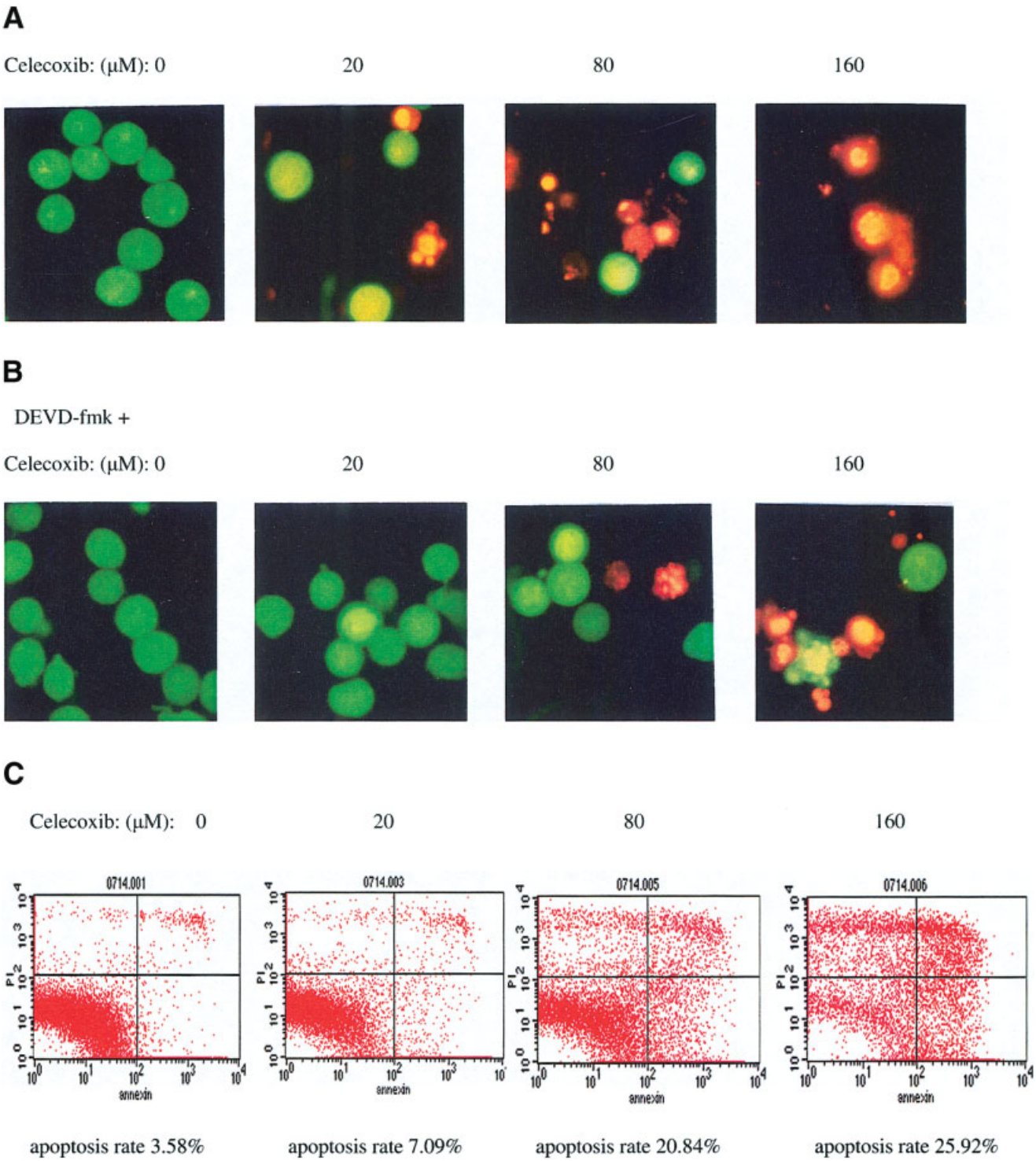

Fig. 3. Morphological identification and annexin V of apoptotic K562 cells after celecoxib treatment and DEVD-fmk blocking. (A) K562 cells were treated with celecoxib (0, 20, 80, and 160 μM) for 36 hr. Cells were then collected and stained with AO/EB and visualized by fluorescence microscopy. Viable cells exhibit a homogeneous green stain; apoptotic cells are orange-red and show evidence of cell shrinkage and nuclear condensation. (B) K562 cells were pretreated with DEVD-fmk (100 μM) for 2 hr. Cells then were incubated with varying doses of celecoxib (0, 20, 80, and 160 μM) for 36 hr and stained with AO/EB for morphological identification of apoptosis (original magnification 40x). (C) Annexin V-FITC assay: K562 cells was treated with celecoxib (0, 20, 80, and 160 μM) for 36 hr, stained with annexin V-FITC and propidium iodide, and analyzed by flow cytometry. Cells that were negative for annexin V and propidium iodide were counted as viable cells. Apoptotic cells were calculated as a percentage of those cells over the total cell population. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

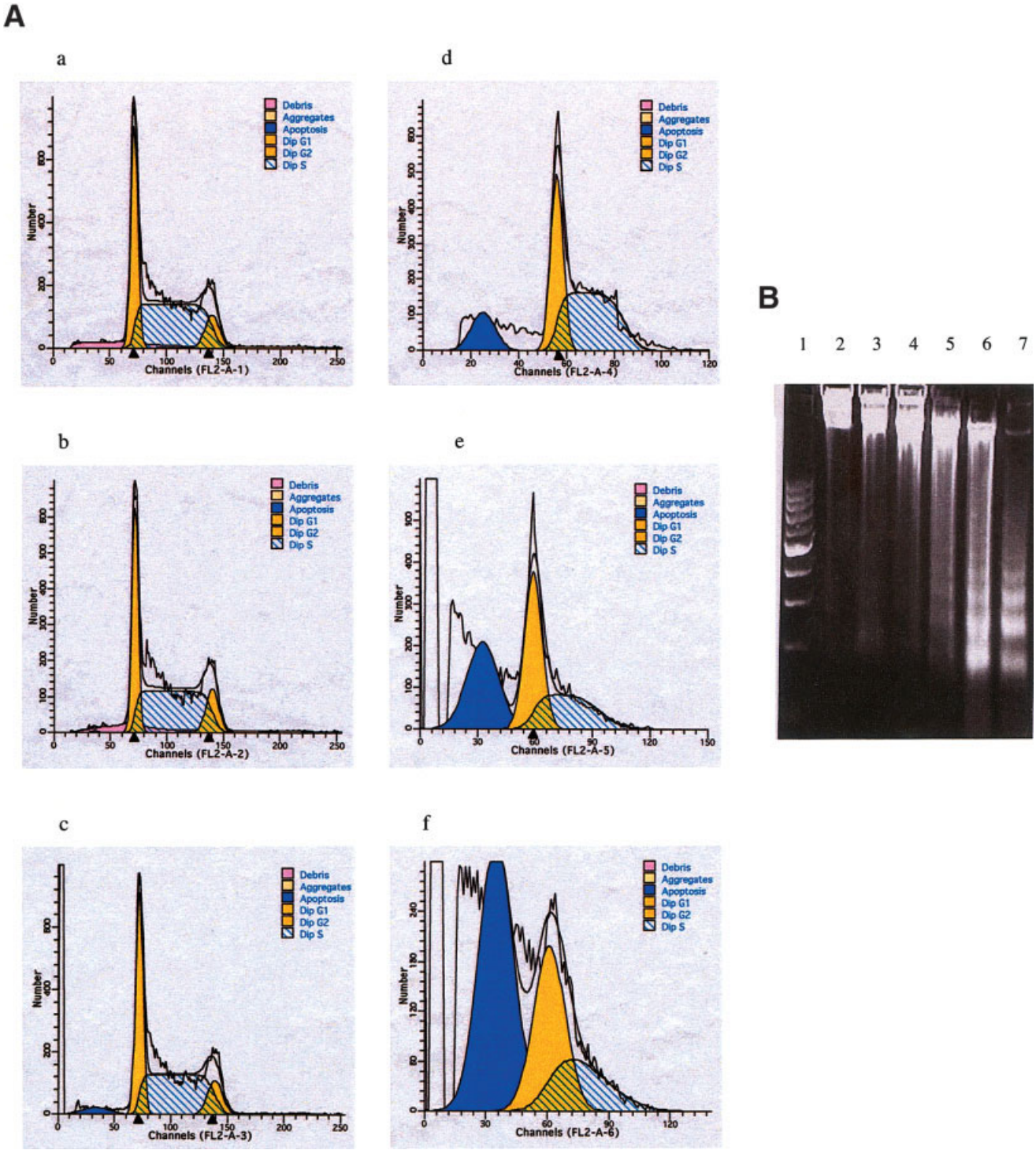

Fig. 4. Flow-cytometry assay and DNA ladder gel pattern in celecoxib-induced apoptosis in K562 cells. (A) Flow-cytometry results (a, b, c, d, e, and f represent celecoxib doses 0, 10, 20, 40, 80, and 160 μM, respectively) are shown. The percentage of apoptotic cells are indicated as the blue peak. K562 cells were treated with varying celecoxib doses for 36 hr. Cells were then harvested, and flow cytometry was performed. (B) DNA ladder gel pattern in celecoxib-induced apoptosis in K562 cells. Cells were treated as described for (A). Cells were then lysed, treated with RNase A or proteinase K, and subjected to 1.5% agarose gel electrophoresis. Lanes 2–7 represent celecoxib doses of 0, 10, 20, 40, 80, and 160 μM, respectively. Lane 1 shows the DNA molecular weight standard. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

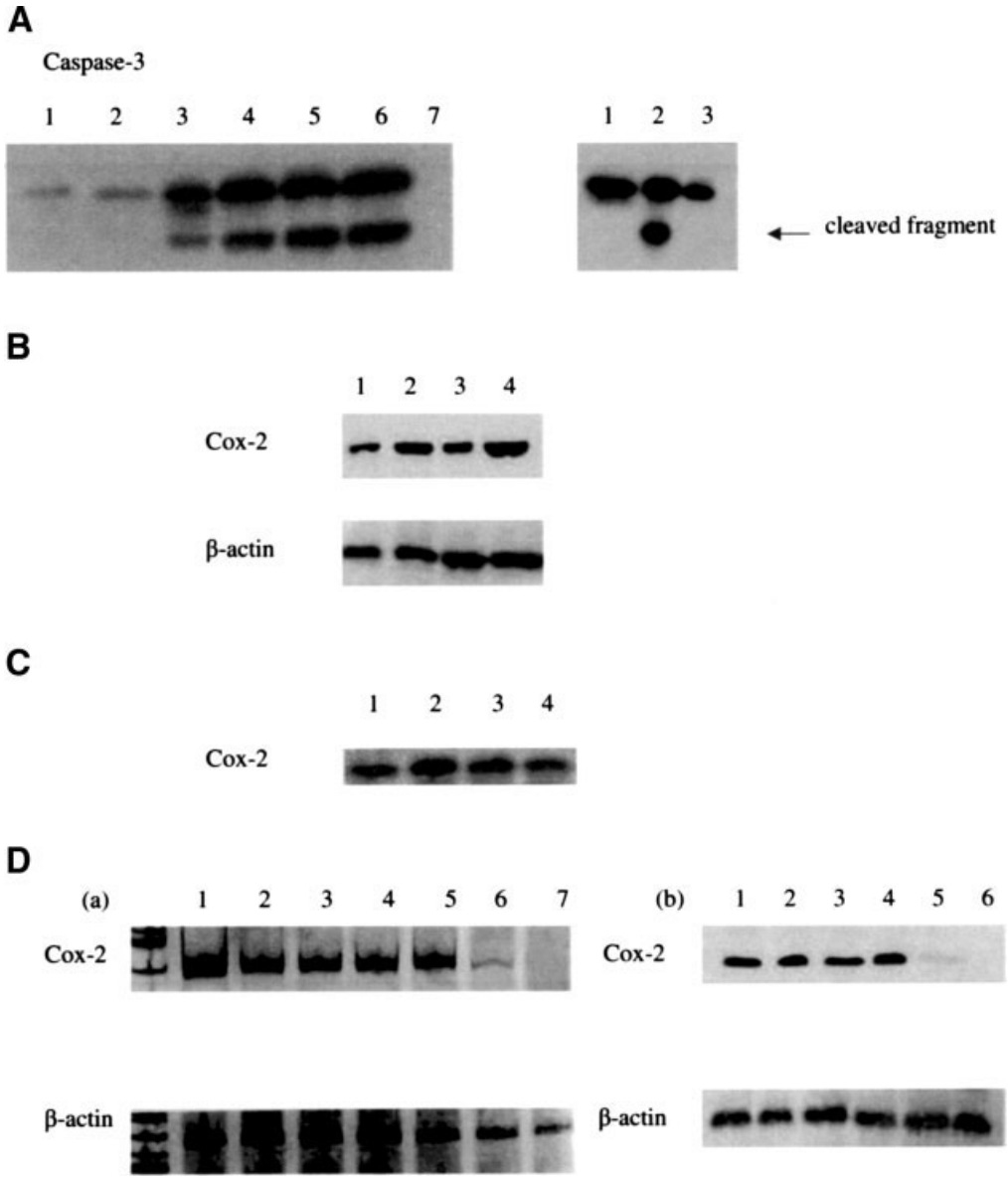

Fig. 5. (A) Caspase-3 expression and activation as assessed by western blot in celecoxib-induced apoptosis in K652 cells. The left panel shows that when celecoxib concentration increases, caspase-3 expression is upregulated and cleavage fragments appear (a 20-kDa fragment). Lanes 1–6 correspond to celecoxib doses of 0, 10, 20, 40, 80, and 160 μM. The right panel shows DEVD-fmk blocking: lane 1, blank control (only DMSO treatment); lane 2, K562 cells treated with 80 μM celecoxib alone; lane 3, K562 cells treated with 100 μM DEVD-fmk in combination with 80 μM celecoxib. (B) Expression of Cox-2 protein in K562 and A549 cells by western blot: lane 1, K562 cells; lane 2, K562 cells with 5 ng/mL IL-1β treatment; lane 3, A549 cells; lane 4, A549 cells with 5 ng/mL IL-1β treatment; β-actin: internal control. (C) Expression of Cox-2 protein in primary CML cells (Western blot result): lanes 1–4 correspond to CML cases 1–4. (D) Celecoxib inhibits Cox-2 protein (Western blot) and mRNA expression (RT-PCR) in a concentration-dependent manner. (a) Cox-2 mRNA: lane 1, A549 cells (positive control); lanes 2–7 correspond to celecoxib doses of 0, 10, 20, 40, 80, and 160 μM, respectively. β-actin mRNA amplification: internal control (b). Cox-2 protein: lanes 1–6 correspond to celecoxib doses of 0, 10, 20, 40, 80, and 160 μM, respectively.

combination of celecoxib and imatinib, K562 cell viability was inhibited more obviously than either agent alone (Fig. 6B); the combination of celecoxib and imatinib induced proliferation inhibition more rapidly than a single drug. Annexin V assay showed the average percentage of apoptotic cells were 4.91% (untreated cells), 14.28% (40 μM celecoxib), 12.81% (0.2 μM imatinib), and 18.59% (40 μM celecoxib + 0.2 μM imatinib) ($n = 3$), respectively. These results suggest that the synergistic effect of celecoxib and

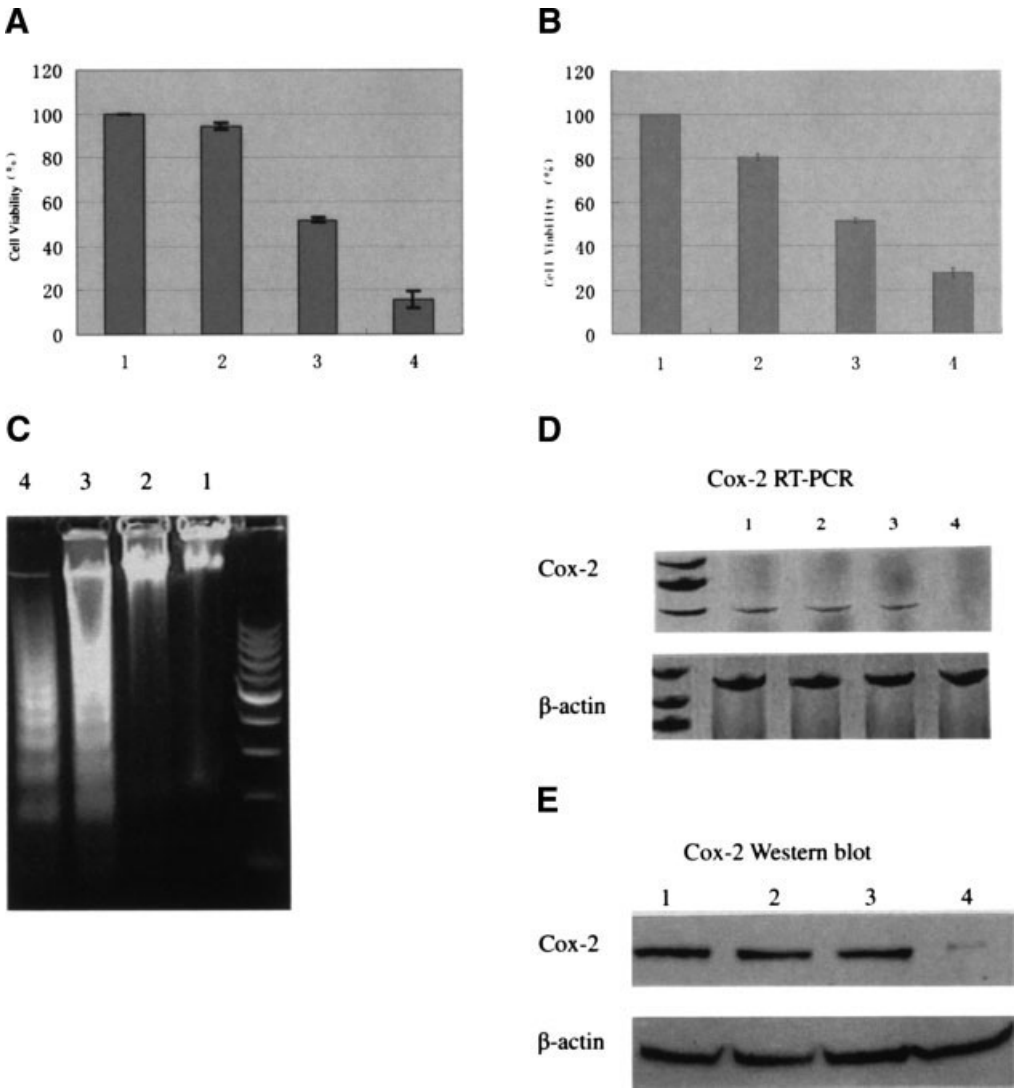

Fig. 6. Celecoxib interacts synergistically with hydroxyurea or imatinib to induce apoptosis, inhibit cell proliferation, and downregulate the expression of Cox-2 mRNA/protein in K562 cells. (A, B). Lane 1, K562 cells were treated with DMSO only (control, panel A or B); lane 2, 10 mM hydroxyurea (A) or 0.2 μM imatinib (B); lane 3, 40 μM celecoxib (A or B); lane 4, 40 μM celecoxib combined with 10 mM hydroxyurea (A) or 0.2 μM imatinib (B) for 36 hr. Cell growth inhibition was assessed by MTT assay, and results are representative of three separate experiments. (C) K562 cells were treated with DMSO alone (lane 1), hydroxyurea alone (lane 2), celecoxib alone (lane 3), or in combination (lane 4) for 36 hr. Cellular DNA was prepared and apoptosis was determined by DNA ladder gel electrophoresis. (D) Drug treatment of K562 cells was as described above. Cells lysates were prepared and Cox-2 mRNA expression was determined by RT-PCR (lane 1, control; lane 2, 40 μM celecoxib; lane 3, 10 mM hydroxyurea; lane 4, 40 μM celecoxib + 10 mM hydroxyurea). (E) Cox-2 protein levels were analyzed by western blot. For both Cox-2 mRNA and protein assays, β-actin was used as an internal control.

imatinib in terms of anti-proliferation was more evident than in apoptosis induction.

DISCUSSION

In this paper, we have provided comprehensive evidence to demonstrate the antitumor activities of celecoxib by inhibiting cell growth and inducing apoptosis in K562 cells. We showed that this growth inhibition was dose-dependent. The IC_{50} of celecoxib was found to be 46 μM, consistent with results of Waskewich et al. [25]. Moreover, our findings confirm that the anti-proliferative effect is mediated through inhibition of cell-cycle progression. In eukaryotes, the cell cycle is tightly regulated at G_1-S and G_2-M checkpoints by several protein kinases, composed of a

cdk subunit and a corresponding regulatory cyclin subunit, and cdk inhibitors [29,30]. G_1-S progression is mediated by cyclin D_1 and cyclin E in complex with the cdks, which phosphorylate Rb and result in cell-cycle transition. $P21^{waf/cip1}$, $P27^{KIP}$, and $P16^{INK4a}$ are cdk inhibitors and prevent the transition of cells from G_1 to S phase [31,32]. In hematological malignancies, especially in CML, $P16^{INK4a}$ mRNA expression has been found to be either abnormally high or exhibit numerous deletions [33,34]. Our results suggest that celecoxib significantly decreases cyclin D_1, cyclin E, and phosphorylated Rb protein levels and increases $P16^{INK4a}$ and $P27^{KIP}$ protein expression in a dose-dependent fashion, without affecting $P21^{waf/cip1}$ protein levels. Whether an upregulated $P16^{INK4a}$ implies an enhanced tumor-suppressing effect remains to be elucidated. Flow-cytometric analysis showed that celecoxib causes G_1-S arrest with no obvious effect on G_2-M transition. These results provide a possible link between cyclin D_1/cyclin E/p-Rb/$P27^{KIP}$/$P16^{INK4a}$ and celecoxib induction of growth inhibition in K562 cells. Perhaps the lack of an effect on $P21^{waf/cip1}$ levels reflect a deregulated P53 variation in CML cells since $P21^{waf/cip1}$ is induced in part by P53 [35].

In the present study, we demonstrated that celecoxib induction of apoptosis in K562 cells is dependent on caspase-3 activation. Five different types of assays were performed to confirm the apoptotic activity of celecoxib: morphological, DNA ladder gel pattern, flow-cytometry analysis, annexin V assay, and caspase-3 activity. In addition, DEVD-fmk inhibited celecoxib-induced cell death. These results indicate that celecoxib-induced apoptosis, at least in part, is mediated through activation of a caspase pathway. However, our results argue that while caspase-3 activity is inhibited, the effect of apoptosis induction for celecoxib is not abolished. This suggests that potential mechanisms of celecoxib-induced K562 cells apoptosis may be involved in caspase-independent pathway. Additionally, we have demonstrated that the percentage of apoptotic cells with annexin V assay is lower than what has been observed by cell-cycle assay. The discrepancy may be related to the sensitivity of methods for detecting apoptosis and partially depends on various phase distribution of apoptotic cells.

Antitumor activity of Cox inhibitors has been reported previously in Cox-2-negative epithelial cell lines and xenografts. Williams et al. confirmed very similar growth inhibition patterns for celecoxib in Cox-2 (+/+), (+/−), and (−/−) mouse embryo fibroblasts [36]. Recently, Waskewich et al. reported that celecoxib exhibited a potent anti-proliferative activity in Cox-2-negative hematopoietic cell lines, including K562 cells, and therefore determined that the antitumor effect of celecoxib was independent of the Cox-2 pathway [25]. In this paper, we have provided strong evidence to support the fact that K562 cells are Cox-2-positive. First, Cox-2 mRNA was detected by RT-PCR in K562 cells. Second, Cox-2 protein was observed by western blot. Compared with Waskewich et al., we substituted a monoclonal antibody against Cox-2 for their polyclonal antibody, possibly improving the sensitivity of Cox-2 protein detection. Third, Cox-2 expression in K562 cells for both mRNA and protein was stimulated by IL-1β, a specific activator of Cox-2 expression. Fourth, more convincing data were derived from the studies using primary CML cells, in which both Cox-2 mRNA and protein were detected, consistent with Giles et al. [26]. Based on our results, we believe K562 cells are Cox-2-positive. More importantly, it has been shown that the levels of both Cox-2 mRNA and protein in K562 cells could be downregulated by higher doses of celecoxib (80–160 μM). Recently, Shishodia et al. reported that celecoxib suppresses NF-KB activation and inhibits cyclin D_1, Cox-2, and matrix metalloproteinase-9 expression in human non-small-cell cancers, suggesting that the antitumor activity of celecoxib involves Cox-2 inhibition [37]. Our results indicate that the effect of celecoxib on anti-proliferation and induction of apoptosis in K562 cells are, in part, related to Cox-2 inhibition.

Because unregulated cell proliferation and resistance to apoptosis contribute to malignant growth, simultaneously counteracting multiple processes provides a more effective approach toward inhibiting tumor growth or inducing tumor cell death [38–40]. Furthermore, studies in prostate cancer cell lines demonstrated that celecoxib induced rapid apoptosis at a concentration range of 25–100 μM [41]. Such data on the steady-state concentration of celecoxib are not obtained in vivo. To develop efficient synergistic or additive combinations of chemotherapeutic and/or Cox-2 inhibitors to inhibit K562 cell growth, induce apoptosis and the potential clinical application dose of celecoxib, we designed an experiment to test the application of celecoxib together with hydroxyurea. In addition, we tested the combined effects of celecoxib and imatinib. Our results suggested that treatment with a subtoxic concentration of celecoxib (40 μM) combined with hydroxyurea (10 mM) significantly suppressed cell proliferation and induced apoptosis. We also investigated the effect of celecoxib and hydroxyurea on Cox-2 mRNA and protein expression in order to understand how the combination of these agents would affect K562 cells. As observed, the concentration of celecoxib required to block expression of Cox-2 protein or mRNA was 80 μM. However, the combination of low-dose

celecoxib (40 μM) and hydroxyurea (10 mM) synergistically suppressed Cox-2 mRNA and protein expression. Apparently a combination of low-dose celecoxib and hydroxyurea enhanced the inhibition of both Cox-2 mRNA and protein expression, as compared to low-dose celecoxib or hydroxyurea individually. Importantly, we found an evidently synergistic effect of celecoxib (40 μM) and imatinib (0.2 μM) combination in terms of apoptosis induction was weaker than the combination of celecoxib and hydroxyurea. Systematic evaluation of the in vitro mechanism should be followed by appropriate preclinical assays using animal models before considering appropriate dosages of celecoxib and hydroxyurea or imatinib for use in clinical trials.

In summary, the present studies demonstrate for the first time the presence of Cox-2 mRNA and protein in CML cells, and that a combination of celecoxib and hydroxyurea or imatinib synergistically suppresses K562 cell growth as well as induces apoptosis. Celecoxib appears to play an antitumorigenic role by both growth inhibition and apoptosis induction. The mechanisms of antitumor activity may be associated, at least in part, with Cox-2 pathway inhibition.

The use of low levels of celecoxib in combination with hydroxyurea or imatinib appears to be a promising approach for CML therapy and deserves further investigation.

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