Suppression of *N*-Methyl-*N*-nitrosourea/Testosterone-induced Rat Prostate Cancer Growth by Celecoxib: Effects on Cyclooxygenase-2, Cell Cycle Regulation, and Apoptosis Mechanism(s)[1]

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ABSTRACT

Purpose: **This study was aimed at examining the mechanisms underlying the chemopreventive effect of celecoxib against prostate cancer. We focused our attention on events at the cellular level to show the ability of celecoxib to inhibit prostate cancer growth, by inducing cell cycle arrest and apoptosis. Moreover, we attempted to demonstrate the expression of genes involved in the downstream events related to cyclooxygenase-2 (COX-2) regulation and apoptosis.**

Experimental Design: **To determine the level of COX-2 expression, we used paraffin-embedded tumor tissue sections and cancer cells (I-26) derived from *N*-methyl-*N*-nitroso-urea/testosterone-induced rat dorsolateral prostate, and we used immunofluorescence detection and Western blot analyses with anti-COX-2 monoclonal antibodies. We conducted clonogenic cell survival assays to demonstrate cell growth inhibition at very low doses of celecoxib. Flow cytometric analysis demonstrated the effects on the cell cycle. Reverse transcription-PCR and Western blot analyses were performed to show the effect of celecoxib on the downstream events of COX-2 and apoptosis-related targets.**

Results: **The summary of our findings indicates that (*a*) these cells from chemically induced rat prostate tumors express COX-2 at both the mRNA and the protein level; (*b*) celecoxib significantly reduces COX-2 expression in these cancer cells; and (*c*) celecoxib induces cell cycle arrest at the G_1-S phase transition point and modifies cell cycle regulatory proteins such as cyclin D1, retinoblastoma (Rb), and phosphorylated Rb, cyclin E, $p27^{KIP1}$, and $p21^{WAF1/CIP1}$. Furthermore, celecoxib inhibits DNA synthesis and induces apoptosis. Most importantly, celecoxib-induced apoptosis was associated with down-regulation of COX-2, nuclear factor κBp65, and with activation of peroxisome proliferator-activated receptor γ, apoptosis activating factor-1, and caspase-3.**

Conclusion: **Results from the present study clearly indicate that celecoxib exerts its anticancer effect partly through COX-2-independent mechanisms in addition to the known primary function of COX-2 inhibition.**

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related deaths in American men. African Americans have the highest rate of prostate cancer incidence (1, 2). A long-term goal of our research is to develop innovative strategies for prostate cancer prevention. A number of studies indicate a strong correlation between the levels of arachidonic acid metabolites and accumulation of prostaglandins in prostate carcinogenesis (3–7). COX-2,[3] one of the key rate-limiting enzymes involved in arachidonic acid metabolism, has been shown to be involved in prostate cancer, as well as in several other human cancers and inflammatory diseases (8–15). Epidemiological studies have revealed a decreased risk of colon cancer among people who have regularly taken COX-2 inhibitors, like aspirin or other NSAIDs (14, 16). Both human studies and preclinical model assays testing COX-2 inhibitors support the role of NSAIDs in the prevention of cancer of the colon and other organs (8, 11, 14, 17–23). In this context, it is noteworthy that a cohort study suggested that regular use of NSAIDs could protect against prostate cancer (24). Although selective COX-2 inhibitors are known to induce tumor growth inhibition through COX-2 down-regulation, not much is known about COX-2-mediated tumor growth inhibition in prostate cancer, mainly because there is little expression of COX-2 in the initial stages of this cancer, and

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[3] The abbreviations used are: COX-2, cyclooxygenase-2; DAPI, 4′,6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; NSAID, nonsteroidal anti-inflammatory drug; MNU, *N*-methyl-*N*-nitrosourea; PPAR, peroxisome proliferator-activated receptor; PS, phosphatidylserine externalization; Rb, retinoblastoma; pRb, phosphorylated Rb; RT-PCR, reverse transcription-PCR; PI, propidium iodide; Cdk, cyclin-dependent kinase; NF, nuclear factor.

also because of a lack of proper animal models. Because the MNU/testosterone-induced prostate tumor is a fully developed tumor model, we have chosen it for investigating the role of celecoxib in tumor growth inhibition and related mechanism(s). Expression of COX-2 at the mRNA and protein level in human prostate tumors has demonstrated a direct association between COX-2 levels and prostate cancer (25–32). Despite considerable enthusiasm about the use of celecoxib against cancer of the colon and of the breast, studies on its use against prostate cancer are few (33–37). Rats treated with MNU/testosterone develop a high incidence of prostate tumors (38), which is an advantage for testing the efficacy of celecoxib and elucidating its molecular mechanism(s). Earlier studies by Conden *et al.* (39) had suggested multiple pathways for MNU/testosterone-induced prostate carcinogenesis in the rat model. Even though studies by Pollard and Luckert and Pollard *et al.* (38, 40–43) with NSAIDs, such as peroxicum and indomethacine in MNU-treated rats, indicated effects on both intestinal and prostate tumors, effects of celecoxib on COX-2 regulation or apoptosis mechanisms have not been delineated. Interestingly, this rat model was used for several chemoprevention studies, such as (*a*) the evaluation of the activity of 9-*cis*-retinoic acid against prostate carcinogenesis in male (HsdCpb:Wu) rats (44); (*b*) very effective dose-related tumor growth-inhibition studies with dehydroepiandrosterone (DHEA) in MNU-induced rat prostate tumors (45); and (*c*) treatment with difluoromethyl ornithine or finasteride of MNU-induced rat prostate tumors showed chemopreventive efficacy by inhibiting prostate polyamine synthesis (46).

We investigated the effect of celecoxib on COX-2 expression, cell growth, DNA synthesis, cell cycle distribution, and apoptosis as well as on expression of cyclin D1, cyclin E, and cyclin kinase inhibitors, such as $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ to verify cancer growth inhibition and induction of apoptosis via cell cycle regulatory mechanisms in dorsolateral prostate cancer cells. Taken together, our observations strongly indicate a correlation in the down-regulation of COX-2 with targets such as NF-κBp65 that makes cells susceptible to the actions of apoptosis-inducing mechanisms that are believed to be simultaneously activated by celecoxib.

MATERIALS AND METHODS

Cell Culture and Treatments. The Searle Research and Development, Pharmacia, St. Louis, MO, kindly provided celecoxib. Solutions of 0.5- to 10-, 20-, and 40-μM concentrations of celecoxib were prepared in DMSO. The cell cultures used in the present study were established as described previously (39, 47) from rat prostatic adenocarcinomas induced by MNU plus testosterone in Wistar WU rats (48–50). Examination of H&E-stained sections of the original tumors indicated a dorsolateral prostatic origin. *In vitro*, the cells appear polyhedral in shape and grow in a "cobblestone" pattern, suggesting their epithelial nature, which was confirmed by positive immunohistochemical staining for cytokeratin 7. The cell cultures used in this study (I-26) are tumorigenic when injected s.c. into syngeneic hosts. They consistently form metastases in the lung and regional lymph nodes. The *in vitro* growth rate (doubling-time) of these cultures is within 12 to 24 h, and cells from passage 8 were used in all of these studies. The cells were propagated in F-12K medium containing 50 nM testosterone (Steraloids, Wilton, NH), 5% fetal bovine serum, 100 μg/ml penicillin and 100 mg/ml streptomycin. A uniform number of cells plated in T-25 or 35-mm cell-culture dishes were placed in a humidified incubator at 37°C with 5% CO_2 before treatment with celecoxib. Controls received 1% DMSO only. The experiments were repeated three times for each dose and time period after exposure to the groups, celecoxib, and control.

Cell Viability. Cell viability after celecoxib treatment was determined by the trypan blue (0.2%) exclusion assay. After 6, 12, 24, and 48 h of every experiment, adherent and floating cells were harvested by trypsinization and recovered by centrifugation. Trypan blue staining of cells enables easy identification of dead cells because these take up the dye and appear blue with uneven cell membranes. By contrast, living cells repel the dye and appear refractile and colorless.

Clonogenic Survival Assay. The soft-agar (Life Technologies, Inc.) clonogenic assay was performed to determine the efficacy of celecoxib at very low doses, ranging from 0.5 to 10 μM. Briefly, rat prostate cancer cells were grown on soft-agar plates (~50,000 cells/plate) made with F-12K medium containing 1.5% agar. Celecoxib solutions of 0.5-, 2.0-, 5.0-, or 10.0-μM concentrations were added to the culture medium containing the agar. For each dose, triplicate plates were used along with the control. The plates were incubated for 10 days at 37°C in 5% CO_2. Colonies identified by crystal violet staining that showed more than 25 cells/colony were precisely counted to determine the efficacy; the results are presented as a percentage of the control (with DMSO).

Flow Cytometric Analysis. Control cells and cells treated with celecoxib as described above, were harvested after 48 h by trypsinization and fixed in ice-cold 80% ethanol for up to 24 h. The cells were then washed twice with PBS, suspended in 1 ml of 0.25% Triton X-100 in PBS, kept on ice for 5 min, and centrifuged. The resulting pellet was suspended in 100 μl of PBS. After a washing with PBS, cells were resuspended in 5 μg/ml PI (Molecular Probes, Eugene, OR) with 0.1% RNase A (Sigma) in PBS. After incubation for 20 min at room temperature in the dark, the cells were analyzed by flow cytometry with the Coulter Epic Elite ESP. Cell cycle analysis was performed as described by Darzynkiewicz *et al.* (51) and Gong *et al.* (52).

BrdUrd Labeling. The effect of celecoxib on DNA synthesis was measured by assessing BrdUrd incorporation into DNA during the S phase as described earlier (53), with some modifications. Briefly, rat prostate cancer cells grown in 35-mm dishes were incubated with BrdUrd (30 μg/ml) along with different concentrations of celecoxib (10, 20, or 40 μM) in the cell culture medium; after 48 h, the experiments were terminated by immediately fixing the cells in 10% formalin. The PBS-washed cells were pretreated with 0.1% Triton X-100, and 2 N HCl at 37°C for 10 min, and were then treated with 0.1 M sodium borate for 5 min. The cells were incubated with mouse monoclonal antibody for BrdUrd (NCL-BrdUrd; Novacastra) for 1 h and, thereafter, with FITC-conjugated secondary antibody (goat antimouse IgG) for 30 min to detect the incorporation of BrdUrd in the S phase of the cells. Cells were viewed for BrdUrd incorporation with a fluorescence microscope (AX-70 Epi-fluorescence scope; Olympus) equipped with a computer-controlled digital camera (Spot) for imaging. Cells showing the

FITC signal for BrdUrd incorporation were quantified from three independent experiments.

Detection of Apoptosis. The rate of apoptosis induced by celecoxib in rat prostate cancer cells was assessed first with DAPI staining of the nuclear material. Briefly, cells grown in 35-mm dishes were treated with celecoxib for 48 h; after termination of the experiments, the floating and adherent cells were first fixed in 10% formalin for 15 min. After a washing with PBS, cells were treated with 0.1% Triton X-100, 4 M HCl, and sodium tetraborate; each treatment was performed for 15 min and was followed by a PBS wash. Cells were then stained with DAPI in 80% methanol for 30 min and again washed with PBS. The cells were viewed under a fluorescence microscope as described previously (54). In addition, annexin V staining of the membrane for PS in the apoptotic cells was performed in the control and celecoxib-treated cells. Cells with the characteristic morphological changes of apoptosis were quantified among 100 cells per field. Representative cells of more than three fields were selected for quantification.

Immunofluorescence Detection of COX-2. To determine whether rat prostate tumor, as well as the cancer cells, expresses COX-2, we used immunofluorescence detection techniques for both paraffin-embedded tumor tissue sections and the cancer cells derived from the tumor. First, deparaffinized tumor tissue sections were incubated with FITC-conjugated anti-COX-2 primary antibody for 6 h (1:1000) after a brief heat treatment, COX-2-positive cells in the nontumor and tumor regions were detected by comparison with the H&E-stained sections. Incubations of cells treated with 10, 20, or 40 μM celecoxib were performed after 48 h. PBS-washed cells, fixed in 10% formalin and pretreated with 0.1% Triton X-100 and 2 N HCl at 37°C for 10 min, were then treated with 0.1 M sodium borate for 5 min. COX-2-positive cells were localized using human monoclonal COX-2-specific antibody (Cayman, Ann Arbor, MI) covalently linked to FITC. The cells were viewed under a fluorescence microscope as described elsewhere (54).

Western Blot Analysis. Rat prostate cancer cells treated with celecoxib for 48 h were harvested by trypsinization. Cellular protein was isolated with protein extraction buffer containing 150 mM NaCl, 10 mM Tris (pH 7.2), 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, and 2% SDS, in addition to a mixture of protease inhibitors (Boehringer Mannheim, GmbH, Mannheim, Germany). Equal amounts of protein (50 μg/lane) were fractionated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The Western blot procedure was carried out as described earlier (55). The antibody used for Western blotting was from Santa Cruz Biotechnology, Santa Cruz, CA (cyclin D1, pRb, cyclin E, p27, and p21). The antibodies for COX-1 and COX-2 were from Cayman, Ann Arbor, MI. Reactive protein bands were developed using chemiluminescence detection reagents (ECL, Amersham). Densitometric analysis of the protein bands was performed with the software Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD).

RT-PCR. Total RNA extracted from cells treated with celecoxib was subjected to RT-PCR analysis using gene-specific primer sequences by adopting protocols described earlier (54) to determine the expression of COX-2, PPARγ, Apaf-1, and caspase 3. All of the templates were initially denatured for 2 min at 94°C, and the amplification of the amplicon were extended at a final temperature of 72°C for 7 min. PCR amplification with glyceraldehyde-3-phosphate dehydrogenase was used as the internal control.

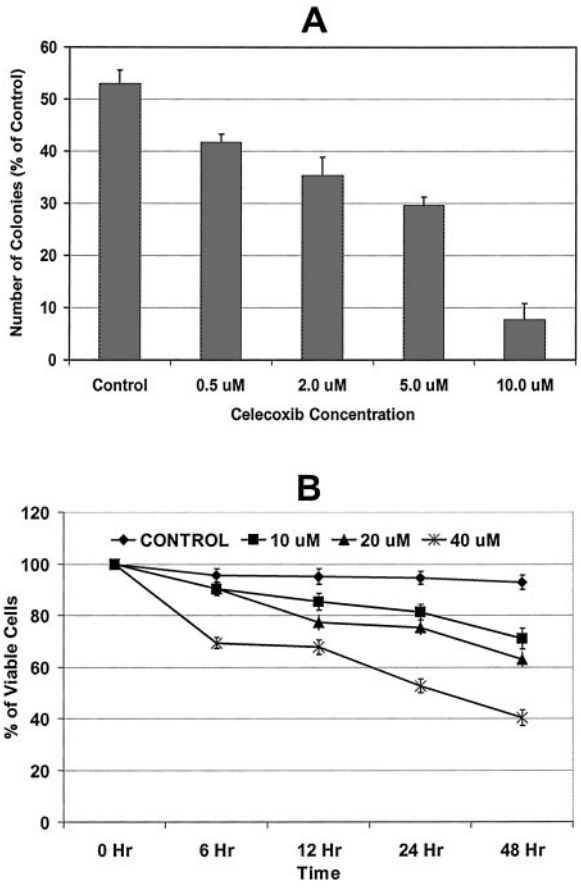

Fig. 1 Effect of celecoxib on cell viability. *A*, cell viability; clonogenic assay using soft agar to determine the efficacy of celecoxib at very low doses as described under "Materials and Methods." Colonies showing more than 25 cells/colony were counted and presented as percentage of control. *B*, viability of MNU/testosterone-induced rat dorsolateral prostate cancer cells exposed to celecoxib (10 μM, 20 μM, or 40 μM) at 6, 12, 24, and 48 h was detected using the trypan blue (0.2%) exclusion assay as described under "Materials and Methods." Data shown are the means and ± SE of three independent experiments.

RESULTS

Effect of Celecoxib on Cell Viability. Clonogenic cell survival assays were performed with very low doses of celecoxib as shown in Fig. 1*A*. There were remarkably fewer viable colonies with 10 μM compared with the effects with 2.0 and 5.0 μM. Viable cells detected by the trypan blue exclusion assay among the rat prostate cancer cells exposed to 10 μM, 20 μM, or 40 μM celecoxib after 6, 12, 24, and 48 h are presented in Fig. 1*B*. More than 50% of the cells were viable after 24 h at 40 μM, but more or less 50% of cell deaths occurred at the 48-h time point. Overall, at the tested levels, celecoxib induced a time- and dose-dependent growth inhibition in rat prostate cancer cells in a nontoxic manner. The IC_{50} was ~25–30 μM for this cell type,

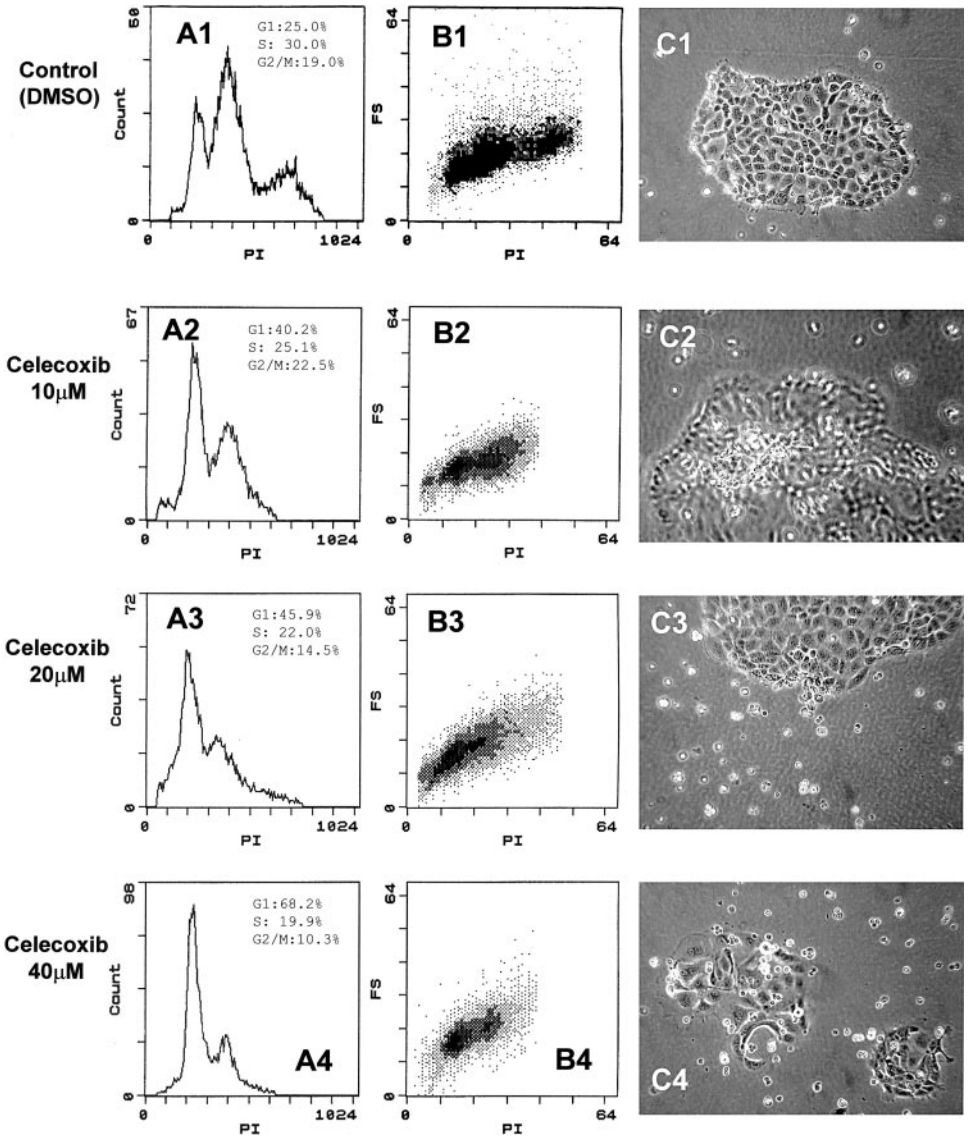

Fig. 2 Cell cycle analysis. Celecoxib induced cell cycle arrest after 48 h in rat prostate cancer cells in a dose-dependent manner (10 μM, 20 μM, and 40 μM). *In the left panel* (*A1–A4*), results from flow cytometric analysis, performed as described under "Materials and Methods"; *in the middle panel* (*B1–B4*), the same cells, gated for aggregates. PI-stained cells for DNA content in the G_1, S phase, and G_2-M fractions were quantitated using the "Multi Cycle" software package. *In the extreme right panel* (*C1–C4*), phase-contrast microscopic view of the cells exposed to celecoxib indicating the morphological changes, cell shrinkage, and appearance of apoptotic cells after 48 h.

although the results from the clonogenic assay clearly demonstrated cell growth inhibition at very low doses.

Effect of Celecoxib on Cell Cycle Distribution. To determine whether the observed cell growth inhibition was caused by cell cycle arrest, the distribution of cells in different phases of the cell cycle was assessed at the 48-h point after treatment with 10, 20, or 40 μM celecoxib. Flow cytometric analysis for DNA content revealed a dose-dependent G_1 arrest accompanied by a decrease of cells in the S and G_2-M phases (Fig. 2, *A1–A4*). Consistent with this observation, Fig. 2, *B1–B4*, depicts a dual parameter plot for cell count and PI uptake. Cells with high PI fluorescence have a disrupted surface membrane indicating a greater chance for the presence of apoptotic cells in the same cluster. Findings from the trypan blue exclusion assay and flow cytometric analysis were consistent with distinct morphological changes characteristic of apoptosis that occurred in celecoxib-treated cells but not in the control cells as shown in Fig. 2, *C1–C4*.

Celecoxib-induced Apoptosis. Apoptosis induced by celecoxib was confirmed with DAPI staining for chromatin material and annexin V for PS. Increases in the DAPI uptake and apoptotic cells with specific morphological and nuclear material changes characteristic of apoptotic cells were quantified and are presented in Fig. 3, *A1–A3*. PS is redistributed to the external surface of the villous plasma membrane. The level of PS expression increased at up to 48 h of treatment with 40 μM of celecoxib with the binding of annexin V. Similarly, the membrane localization of annexin-V apoptotic cells was quantified (Fig. 3, *B1–B3*). Results from DAPI and annexin-V localization demonstrated that more than 30% of apoptosis was induced by celecoxib at this time period.

Effect of Celecoxib on DNA Synthesis (BrdUrd Incorporation). A reduced peak for the S phase, observed by flow cytometric analysis, was consistent with less BrdUrd incorporation in cells exposed to different concentrations of celecoxib after 48 h. A gradual decline in the incorporation of BrdUrd at

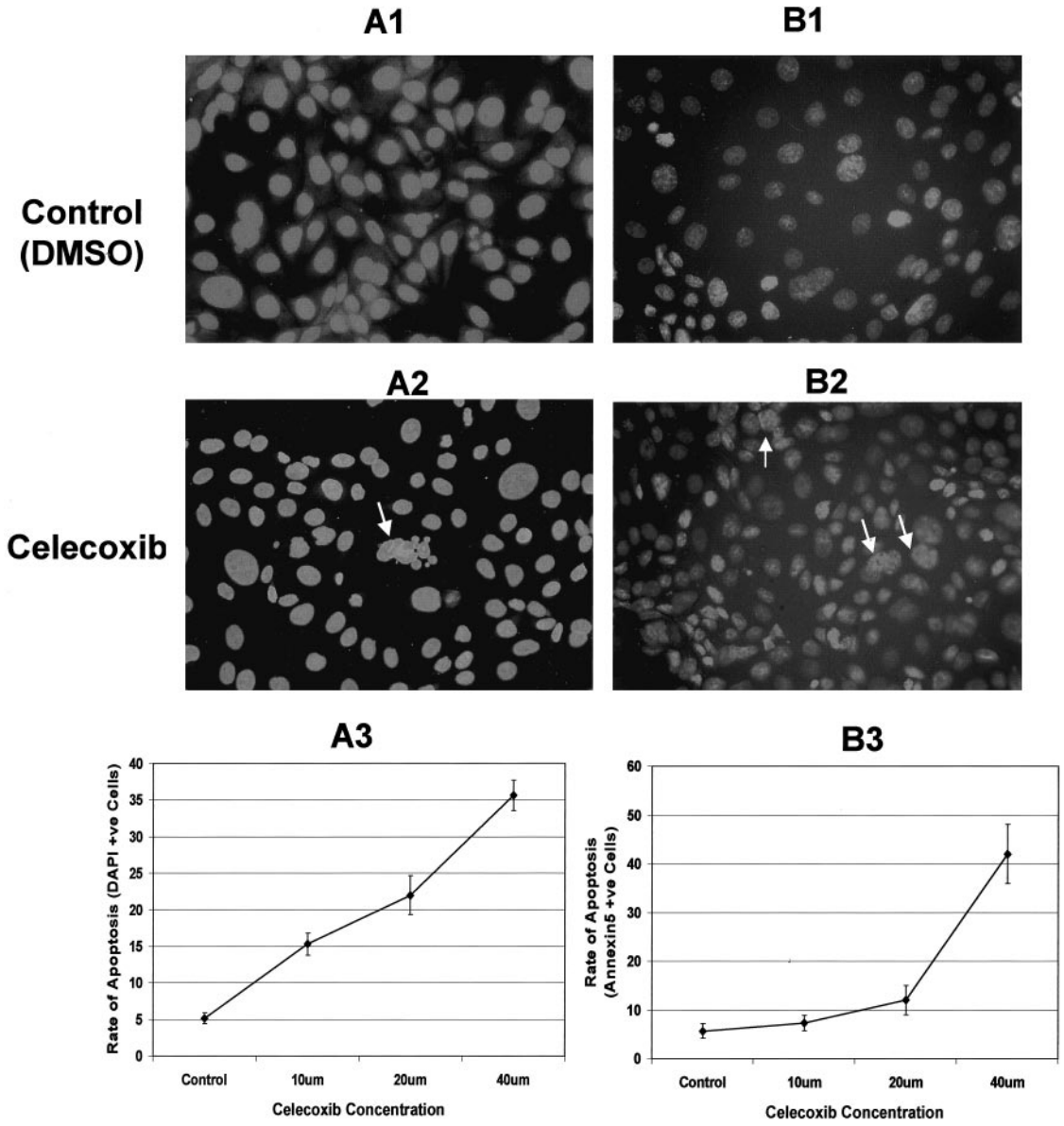

Fig. 3 Effect of celecoxib on apoptosis. Cells treated with celecoxib (40 μM) for 48 h display cytoplasmic shrinkage, membrane blebbing (*arrow*), and chromatin condensation detected by DAPI staining as described under "Material and Methods," These observations are presented in control (*A1*) and celecoxib-treated cells (*A2*). *A3*, quantitative data of apoptotic cells from three independent experiments. Annexin V-staining of the membrane for PS in the control (*B1*) and celecoxib-treated cells (*B2*), indicate the evidence for apoptosis. *B3*, quantitative data of the annexin V-stained cells, are presented from triplicate experiments.

20- and 40-μM concentrations after 48 h clearly revealed a difference between the control and the celecoxib-treated cells that showed only 8% BrdUrd-positive cells with 40 μM, indicating a dose-related, significantly decreased rate of DNA synthesis (Fig. 4, *A*–*C*).

Effect of Celecoxib on Rb Protein. The product of the *Rb* gene prevents S-phase entry during the cell cycle, and inactivation of this growth-suppressive function is presumed to result from Rb hyperphosphorylation during the late G_1 phase. To examine the effect of celecoxib on Rb, we used flow cytometric analysis to assess the expression of total Rb protein in rat prostate cancer cells exposed to 40 μM celecoxib for 48 h. We demonstrated an increase in the Rb/FITC incorporation in G_1-S phase-arrested prostate cancer cells (Fig. 5, *A* and *B*) that was consistent with an increase in the total Rb and pRb protein measured by Western blot analysis (Fig. 5*C*).

Effect of Celecoxib on Cyclin D and Cyclin E, and on cdk Inhibitors $p27^{KIP1}$ and $p21^{WAF1/CIP1}$. To examine the molecular mechanism that may be underlying changes in cell cycle profiles and apoptosis, we investigated the upstream events linked to Rb and pRb. We approached this study by measuring the effects of 10, 20, and 40 μM celecoxib on the expression of cyclin D1 and

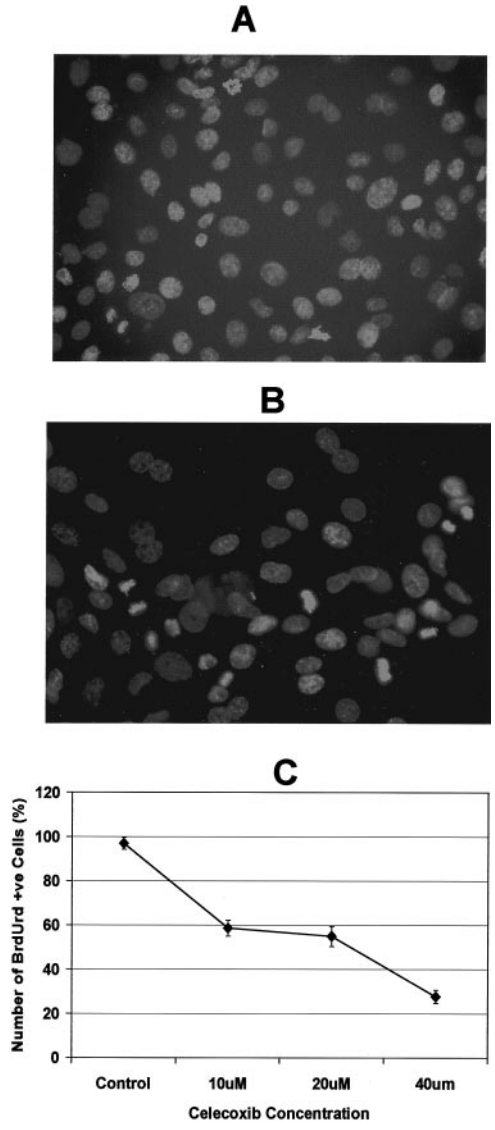

Fig. 4 Effect of celecoxib on DNA synthesis. BrdUrd incorporation of control (*A*) and 40 μM celecoxib-treated cells (*B*) after 48 h was detected with FITC-conjugated mouse monoclonal antibody for BrdUrd, as described under "Materials and Methods." FITC-positive (+*ve*) cells, visualized under a fluorescence microscope with ×40 magnification, were quantitated (*C*) from triplicate experiments and were presented as the percentage of BrdUrd-incorporated cells indicating the rate of DNA synthesis.

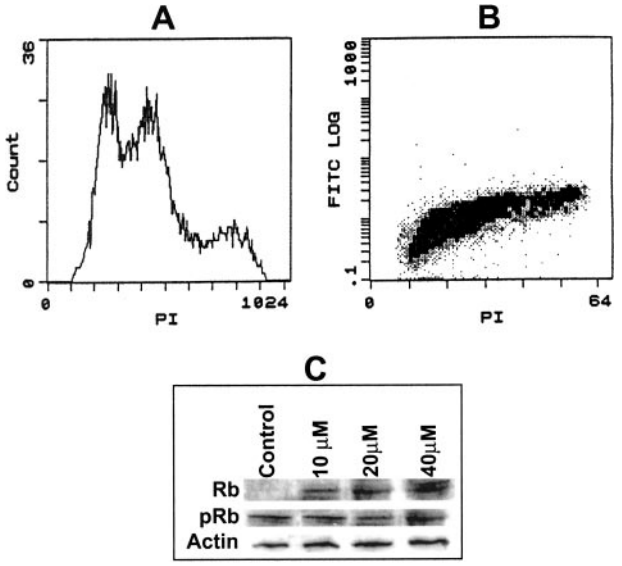

Fig. 5 Expression of Rb/pRb. *A*, cells treated with (40 μM) celecoxib for 48 h were harvested and stained for DNA content with PI; *B*, flow cytometric analysis shows the aggregates of cells labeled with FITC-conjugated antimouse Rb antibody; *C*, Western blot analysis for the total and pRb protein expression in the celecoxib-treated cells.

-E and the cdk inhibitors, $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ in rat prostate cancer cells. Western blots of these proteins indicated a 2-fold decrease in the level of cyclin D1 at 20 μM and a 3-fold decrease at 40 μM after 48 h (Fig. 6, *A* and *B*). In separate experiments, celecoxib had no effect on the expression of cyclin E in these cells, although a very minor change in the protein expression was observed with the 40-μM dose. By contrast, Western blot results showed an increase in the level of the cyclin kinase inhibitors $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ protein with different concentrations of celecoxib after 48 h. Although we were focusing on some of the downstream events related to the COX-2 inhibition and induction of apoptosis, we also observed that celecoxib down-regulated the levels of expression of the transcription factors NF-κB, and PPARγ very effectively within 48 h at the mRNA level (RT-PCR) in addition to affecting the activation of Apaf-1 and caspase 3 as shown in Fig. 6*C*.

Effect of Celecoxib on COX-2 Expression. COX-2 expression was identified using FITC-immunofluorescence in the MNU/testosterone-induced rat prostate tumor, and the preexisting normal glandular regions indicated a significant level in the dorsolateral prostate tumor area (*T*) compared with the preexisting normal glandular regions (*N*), as indicated in Fig. 7, *A1–A2*. The H&E-stained sections were compared with sections used for COX-2 detection. The rat prostate cancer cells derived from these tumors showed a higher level of COX-2 expression (Fig. 7, *B1–B2*) than the cells exposed to celecoxib. Quantification of the COX-2-expressing cells as depicted in Fig. 7*B3*, showed a declining trend in the celecoxib-treated cells. In addition to the immunolocalization of COX-2 expression in the cells, we confirmed the presence of COX-2 protein by Western blot analysis (Fig. 7*C1*). A 2-fold decrease in the expression of COX-2 was observed with 20 μM celecoxib, and 40 μM caused complete inhibition after 48 h of treatment (Fig. 7*C2*). This clearly indicated the COX-2-inhibitory effect of celecoxib in carcinogen-induced rat prostate cancer cells. No significant changes were observed in the level of COX-1.

DISCUSSION

In recent years, there has been great interest in the question as to whether NSAIDs, including COX-2 inhibitors, affect carcinogenesis through COX-2 inhibition and/or via some other means. More than one molecular mechanism has been proposed by earlier investigators (17, 33, 34, 56–59) for the antiprolif-

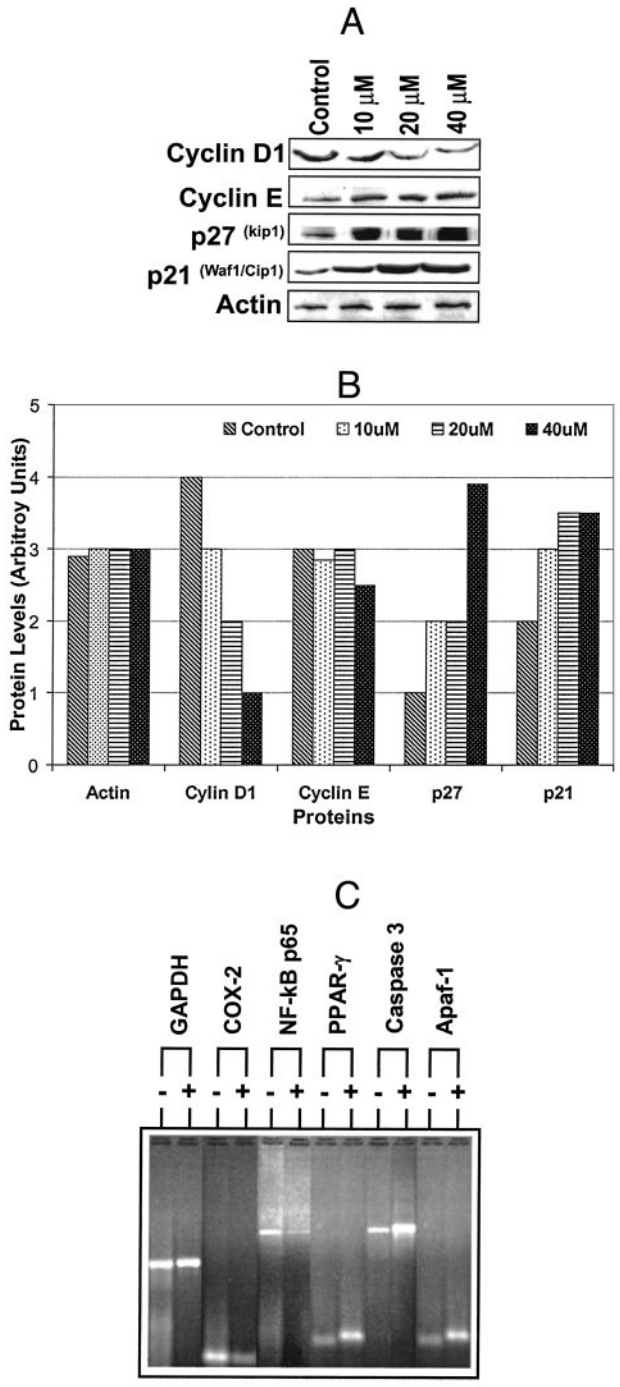

Fig. 6 Effect on cell cycle regulatory proteins. *A*, Western Blot analysis (as described under "Materials and Methods") showing the effect of celecoxib on cyclin D, cyclin E, and the expression of cdk inhibitors $p27^{KIP1}$ and $p21^{WAF1}$ in the control and 40 μM celecoxib-treated cells after 48 h; *B*, a comparative analysis of the level of expression of cyclin D1, cyclin E, $p27^{KIP1}$, and $p21^{WAF1}$, quantified by arbitrary units to indicate the extent of change in the level of expression compared with the control; *C*, total RNA extracted from cells treated with celecoxib was subjected to RT-PCR analysis, as described under "Materials and Methods," using sequence-specific primers for selected genes. (+), treated; (−), control.

erative effects of COX-2 inhibitors. Moreover, there appear to be multiple molecular targets through which celecoxib could exert its anticancer effects in addition to its primary function as a COX-2 inhibitor. Efficacy studies, both *in vitro* and *in vivo*, conducted with NSAIDs in both colon cancer and breast cancer models have demonstrated their anticancer effects; the few studies done on the chemoprevention of prostate cancer did not contribute much with regard to cellular and molecular mechanisms for prostate cancer prevention other than the modulation of Akt and *Bcl*II by celecoxib (59). To identify molecular targets other than COX-2 for prostate cancer prevention, we have investigated the functional mechanism of celecoxib focusing on COX-2-independent pathways involved in cell growth inhibition. We have demonstrated for the first time, a time- and dose-dependent effect of celecoxib on cell cycle arrest and growth inhibition of rat prostate cancer cells that were derived from chemically induced prostate cancer. Cell growth arrest at the G_1-S-phase transition point, associated with an inhibitory effect on the rate of DNA synthesis within 48 h at different doses of celecoxib, was evident from flow cytometric analysis and BrdUrd incorporation and Rb/pRb expression.

Additional attempts to detect and quantify cell death induced by celecoxib, via DAPI and annexin-V staining for apoptotic cells, revealed the presence of more than 30% apoptotic cells. Our observation was consistent with earlier studies, which indicated that colon cancer cell growth inhibition by celecoxib is associated with the induction of apoptosis (17, 60). In addition, as observed in the present study, cell cycle arrest associated with apoptosis is clearly one of the mechanisms by which celecoxib blocks cell cycle progression (59, 61), and it does so in a dose-dependent manner. Results from clonogenic survival assays further indicated that the lowest level of celecoxib that could be effective in preventing cancer cell growth in nude mice, in which the plasma concentrations of celecoxib were in the range of 3–4 μM (100 mg/kg), were similar to those found in humans after a single oral dose of 800 mg (3–5 μM). [Two × 400 mg is approved by the FDA for familial adenomatus polyposis patients as reported by Grosch *et al.* (60).] The results from the present *in vitro* study show that doses of 10 to 40 μM celecoxib over 48 h can still induce cellular and molecular changes that lead to apoptosis in a time-dependent manner.

Flow cytometric analysis of cell cycle distribution revealed that, in rat prostate cancer cells, celecoxib at 10, 20, and 40 μM caused an increase in the number of cells at the G_1-S-phase transition point with a trend toward accumulation of more cells in the G_1 phase. Although there was only a small apoptotic peak at the very lowest concentration (10 μM), the results suggest a possibly impaired progression through the S phase. At 40 μM of celecoxib, the cell cycle distribution was affected in two ways: (*a*) virtually all of the cells accumulated in the G_1-S phase, although it was not possible to rule out a few cells in the G2-M phase; and (*b*) at higher concentrations of celecoxib, the peak for apoptotic cells appeared small when compared with untreated control cells. Results from our present study on an inhibitory effect on DNA synthesis, determined by BrdUrd incorporation and induction of apoptosis by celecoxib, support the results of Leahy *et al.* (61). However, the observed time- and dose-dependent growth inhibition of carcinogen-induced dorsolateral prostate cancer cells by celecoxib seems to be caused by its effect on cell cycle perturbation.

In mammalian cells the Rb protein acts as a critical switch,

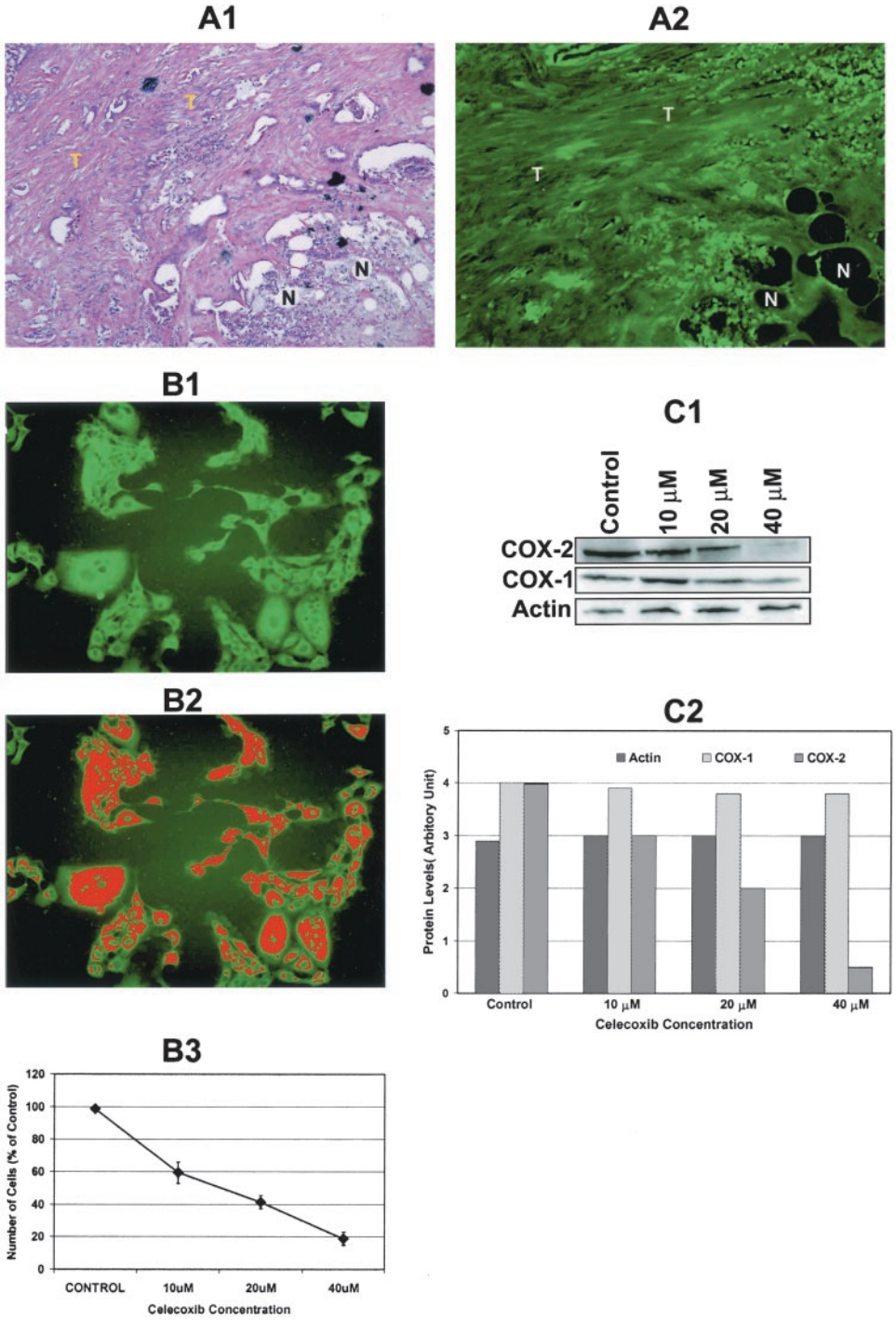

Fig. 7 COX-2 expression. *A1*, H&E staining of paraffin-embedded sections from the MNU/testosterone-induced rat dorsolateral prostate tumor, indicating normal and tumor regions of the prostate; *A2*, levels of COX-2 expression detected by immunofluorescence in the tumor sections; *B1*, level of COX-2 expression in rat prostate cancer cells; *B2*, COX-2 expression after treatment with celecoxib in rat prostate cancer cells; *B3*, quantification of the COX-2-expressing cells; *C1*, Western Blot analysis for COX-1 and COX-2 proteins in celecoxib-treated rat prostate cancer cells; *C2*, quantification of COX-2 protein by arbitrary units to indicate the extent of change in the level of expression compared with the control.

regulating entry into the DNA synthetic phase of the cell cycle. Results from the present study suggest a reversal of the stimulation on the Rb, associated with a reduced expression of cyclin D in celecoxib-treated cells. This was demonstrated by the dose-dependent down-regulation of cyclin D proteins that is consistent with the higher expression of total Rb in G_1-arrested cells. The inhibitory effect of celecoxib on cell cycle progression was directly related to an increase in the proportion of cells with total Rb labeled with FITC. At 30 μM celecoxib, the percentage of Rb-positive cells increased to 50%. This suggests that the suppression of cell cycle progression through the G_1-S transition phase that is induced by celecoxib seems to be mediated by the maintenance of Rb in its normal functional state. Rb is the master switch regulating cell cycle progression, and its status of phosphorylation parallels cell transit through G_1 into the S phase (62, 63). One of the pathways that regulates Rb

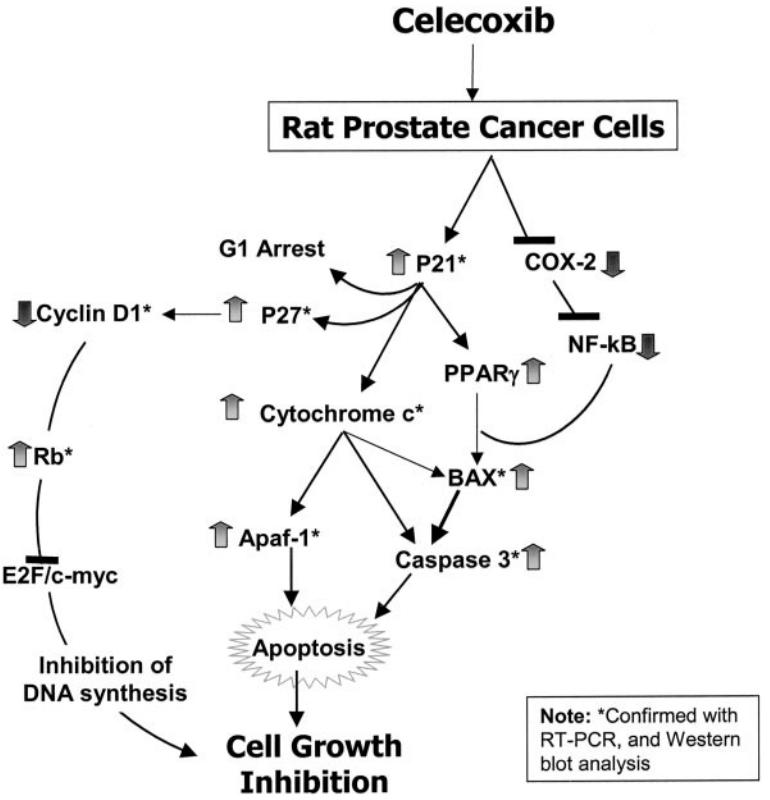

Fig. 8 Predicted mechanism(s) illustrating the cascade of events caused by celecoxib in rat prostate cancer growth prevention. At the cellular level, celecoxib is shown to inhibit DNA synthesis and to induce cell cycle arrest and apoptosis, in addition to its primary function as COX-2 inhibitor. The primary effect of celecoxib on COX-2 inhibition is associated with downstream events such as modulation of NF-kBp65; its possible role in blocking androgen receptor signaling clearly supports the COX-2 hypothesis. More importantly, this illustration also clearly explains the potential role of celecoxib on non-COX targets via modulation of cell cycle regulatory genes, such as proapoptotic effects on cyclins D and E, Rb, along with Bax, cytochrome *c*, Apaf-1, and caspase activation. Overall, a possible synergism in the COX-2 and non-COX-2 pathways at the point of cytochrome-*c* activation-mediated apoptosis is well implicated in this illustration. Recent results from our ongoing studies with celecoxib in rat prostate cancer on the differential expression of genes using microarray analysis[4] strongly support the events predicted in the present mechanistic pathways. ∗, gene or protein expression confirmed with RT-PCR and Western blot analysis; *solid downward arrow*, inhibitory effects; *solid upward arrow*, activation.

phosphorylation and controls the cell cycle from G_1 to S phase progression is through cyclin D and the cdk4/6 complex. The activated cyclin D/CDK4 complex can phosphorylate Rb and prevent its binding to E2F, resulting in the entry from G_1 into S phase (64). Several earlier studies indicated the potential role of cyclins D and E in growth-arrested prostate cancer cells and pointed to the distinct class of genes involved in prostate cancer cells (65–72). However, the role of COX-2 targets and cyclins in the progression of prostate cancer requires further investigation.

To better understand the inhibitory effect of celecoxib on prostate cancer growth via cell cycle regulatory mechanisms, we studied the expression of the direct inhibitors $p27^{KIP1}$ and $p21^{WAF1/CIP1}$ on cdk activity. The results of this study demonstrate that celecoxib increased the expression of $p27^{KIP1}$ protein by comparison with control. Interestingly, we observed an increase in the rate of apoptosis only in cells with a higher activation of $p27^{KIP1}$ and $p21^{WAF1/CIP1}$, which suggests that the up-regulation of $p27^{KIP1}$ may contribute to the induction of cell cycle arrest and apoptosis (73, 74).

Prostate cancer cells used in this study consistently express elevated levels of COX-2 both in the tumor and in the cells derived from the tumor, detected by immunofluorescence and Western blot analysis when compared with celecoxib-treated cells. These results suggest that the inhibitory role of celecoxib in prostate cancer chemoprevention could be mediated via COX-2-independent mechanisms in addition to COX-2-related signaling pathways. A close link between the cell cycle regulatory mechanisms and COX-2 signaling pathways in the presence of celecoxib in carcinogen-induced prostate cancer needs to be further investigated. Nevertheless, overall data suggest a synergism in the mechanisms of action of celecoxib, at least in prostate cancer cells, through the modulation of COX-2 activity and cell cycle regulatory proteins at nontoxic doses. A scheme of predicted mechanism(s) illustrating the cascade of events exerted by celecoxib in rat prostate cancer growth prevention is presented in Fig. 8. At the cellular level, the COX-2 inhibitor celecoxib is shown to induce cell cycle arrest and apoptosis and to inhibit COX-2 expression as a function of dose. This illustration also explains the significance of non-COX targets such as modulation of cell cycle regulatory genes and Bax, Apaf-1, and caspase activation; modulation of NF-κB and PPARγ further supports the non-COX-2 hypothesis to induce antineoplastic effects *in vitro*. A synergism in the mechanism(s) of COX-2 and non-COX-2 pathways at the point of cytochrome-*c* activation-mediated apoptosis is also implicated in this illustration. Recent results on the differential expression of genes by microarray analysis, from our ongoing studies with celecoxib in rat prostate cancer,[4] strongly support the events predicted in the mechanistic pathways shown here. To complement these results from *in vitro* studies, we are currently testing the anticancer effects of celecoxib at different stages of prostate cancer growth in the transgenic adenocarcinoma of the mouse prostate model. These findings are expected to demonstrate the chemopreventive effects of celecoxib in prostate cancer at clinically achievable doses, in addition to revealing the level of

[4] B. A. Narayanan, N. K. Narayanan, and B. S. Reddy, unpublished observations.

COX-2 expression in prostate tumors and other cell types associated with the tumor milieu at different stages of the disease.

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