

Nonsteroidal Anti-inflammatory Drugs Suppress Glioma via 15-Hydroxyprostaglandin Dehydrogenase

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Abstract

Studies have conjectured that nonsteroidal anti-inflammatory drugs (NSAID) inhibit growth of various malignancies by inhibiting cyclooxygenase-2 (COX-2) enzyme activity. Yet, several lines of evidence indicate that a COX-2-independent mechanism may also be involved in their antitumor effects. Here, we report that NSAIDs may inhibit the growth of glioblastoma multiforme (GBM) cells through COX-2-independent mechanisms, including up-regulation of both 15-hydroxyprostaglandin dehydrogenase (15-PGDH, the key prostaglandin catabolic enzyme) and the cell cycle inhibitor p21. Using Western blot and real-time PCR analysis in various GBM cell lines, we observed up-regulation of 15-PGDH and p21 after NSAIDs treatment. To elucidate the role of 15-PGDH in GBM, transfection assays were conducted using the T98G GBM cell line. Overexpression of 15-PGDH suppressed cell growth and was associated with increased expression of p21. In an attempt to investigate the roles of COX-2, 15-PGDH, and p21 in the inhibition of growth of GBM, small interfering RNA (siRNA) against each of these proteins was transfected into T98G cells. Inhibition of growth mediated by NSAIDs was partially reversed after knockdown of either 15-PGDH or p21, but not after COX-2 knockdown. Moreover, expression level of p21 was not affected in COX-2 siRNA transfected cells. Our studies provide evidence that the up-regulation of 15-PGDH induced by NSAIDs has the potential to inhibit growth of GBM, in part, by up-regulation of p21 possibly independent from COX-2 enzymatic function. [Cancer Res 2008;68(17):6978–86]

Introduction

Nonsteroidal antiinflammatory drugs (NSAID) have been used to treat inflammatory diseases, such as arthritis, because of their potent relief of pain and inflammation. Accumulating epidemiologic studies suggest that use of NSAIDs is associated with a reduced risk of colorectal cancer, as well as other malignancies (1–12). In general, this antitumor activity of NSAIDs is believed to be mediated via inhibition of cyclooxygenase-2 (COX-2) enzymatic activity in a manner similar to its antiinflammatory effect (13).

15-Hydroxyprostaglandin dehydrogenase (15-PGDH) catalyzes the oxidation of the 15(*S*)-hydroxyl group of prostaglandins

resulting in the production of 15-keto-prostaglandins and 15-keto-lipoxins, which have greatly reduced biological activities (14). Therefore, this protein has been considered a key enzyme involved in biological inactivation of prostaglandins. COX-2 promotes prostaglandin synthesis, whereas 15-PGDH behaves as an antagonist of COX-2. Recent reports suggest that 15-PGDH has activities similar to a tumor suppressor gene in cancers of the lung (15), gastrointestinal tract (16), and breast (17). The role of 15-PGDH in glioblastoma multiforme (GBM) and its ability to inhibit growth of GBM cells have not been elucidated yet.

GBM is one of the most common tumors of the central nervous system. Despite many efforts to overcome this aggressive disease with the use of surgery, radiation, and conventional chemotherapy, the median survival for patients with GBM is only 1 year (18). Whereas the molecular events involved in the development of GBM are not fully understood, studies have led to the recognition of the importance of COX-2. Its expression has been detected in brain tumors (19, 20), and high COX-2 expression in gliomas is associated with a poor prognosis (21). Recently, several phase I and phase II trials investigated the addition of NSAIDs to standard therapeutic modalities in GBM and found the combinations to be safe and active (22–24). The mechanisms involved in NSAID-induced growth inhibition in GBM are not fully elucidated. For example, growth inhibitory activity of the COX inhibitor indomethacin was observed in COX-deficient GBM cells (25), and COX-2 inhibition was found to be neither necessary nor sufficient for the growth inhibitory activity of the COX-2 inhibitor celecoxib in GBM cells (26). In this study, we investigated the growth inhibitory activity of NSAIDs on GBM cells and found it to be partially COX-2-independent. We identified up-regulation of 15-PGDH and p21 as important mechanisms involved in these COX-2-independent activities of NSAIDs. Furthermore, our studies found, for the first time, that 15-PGDH was able to inhibit growth of GBM cells.

Materials and Methods

Cells and compounds. T98G, U118, U343, and U373 cells (American Type Culture Collection) were maintained at 37°C in 5% CO₂ in DMEM (Invitrogen) with 10% fetal bovine serum (Invitrogen). Diclofenac sodium and meloxicam sodium (Sigma Chemical Co.) were dissolved in sterile water and DMSO (Sigma Chemical Co.), respectively. CAY10397 (Cayman Chemical Co.), a 15-PGDH specific inhibitor, was dissolved in DMSO. Final concentration of DMSO in the experiments was never above 0.5%, and control dishes having the same concentration of DMSO had no detectable effect.

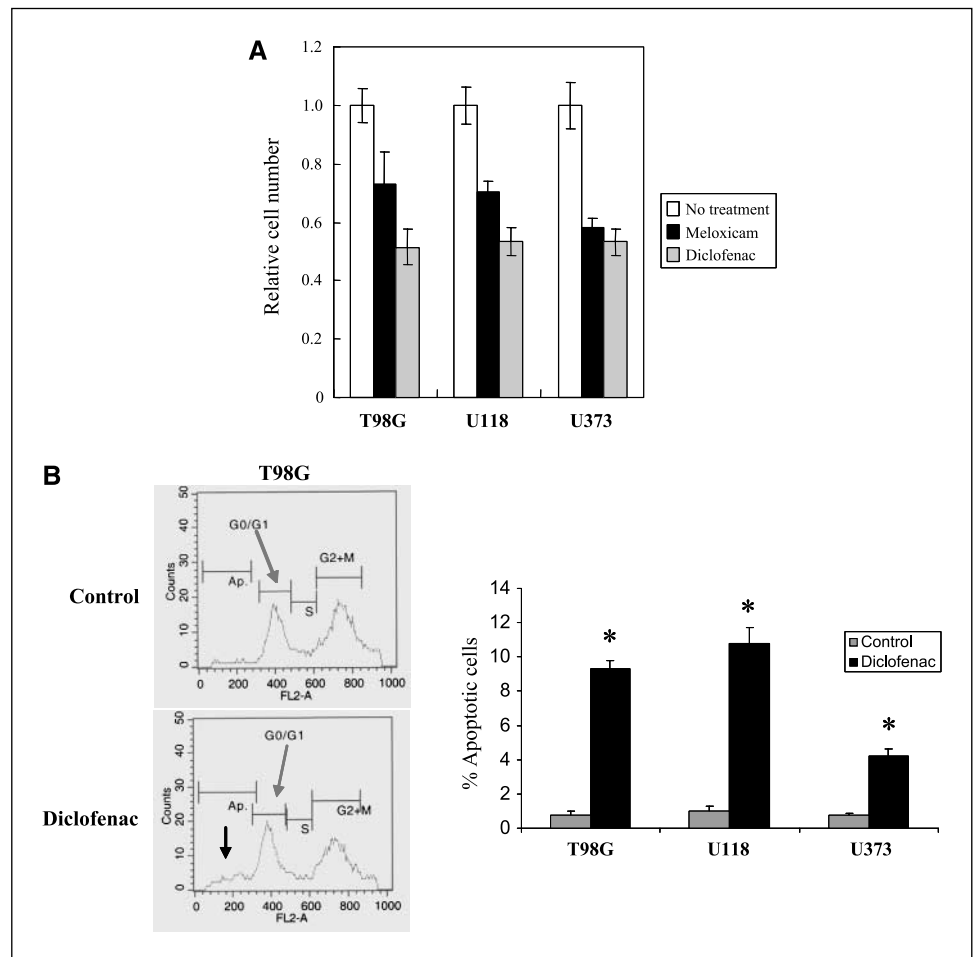
Western blotting. Total cell lysates were extracted using radioimmuno-precipitation assay buffer [1% Triton × 100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4)] containing a mixture of protease inhibitors (Roche Diagnostics GmbH). The mixture was run through a needle to shear the DNA and then centrifuged at 13,000 rpm for 20 min at 4°C. Protein concentrations were determined using the Bio-Rad protein

Note: N. Wakimoto and I. Wolf are coprimary authors.

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Figure 1. Growth inhibition of GBM cells mediated by NSAIDs. **A**, T98G, U118, and U373 GBM cells were plated either with or without diclofenac sodium (100 $\mu\text{mol/L}$) or meloxicam sodium (10 $\mu\text{mol/L}$). Cell numbers were counted 48 h after drug treatment. Results represent the mean \pm SD of three experiments done in triplicate dishes. **B**, cell cycle analysis. GBM cells were treated with diclofenac sodium (100 $\mu\text{mol/L}$, 24 h). Cell cycle was determined by staining with propidium iodide. *Left*, a representative experiment conducted on T98G cells. *Right*, mean \pm SD of three independent experiments; *, $P < 0.05$ for the number of sub- G_0 cells.



assay dye reagent (Bio-Rad Laboratories), according to the manufacturer's instruction. Western blotting was done, as previously described (17). Briefly, ~50 μg of total protein extracts were denatured in sample buffer by boiling for 5 min and then subjected to 4% to 15% SDS-PAGE followed by electrotransfer to polyvinylidene difluoride membrane. The blots were probed with chemiluminescence Western blotting detection reagents (Pierce). The following primary antibodies were used: anti-p21, anti-cyclin D1, anti-COX-2 from Santa Cruz Biotechnology, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Fitzgerald Industries. Anti-PGDH antibodies were described previously (16).

Real-time reverse transcription-PCR. Total cellular RNAs were isolated from cells using RNeasy minikit (Qiagen), and cDNAs were synthesized using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Expression levels were determined using Platinum Taq DNA polymerase (Invitrogen) and SYBERGreen I (Molecular Probes) with the following primers: PGDH forward 5'-AAGCAAATGGAGGTGAAGG-3', PGDH reverse 5'-CCAAC-TATGCCATGCTTTGA-3', COX-2 forward 5'-GCTCAGCCATACAGCAA-ATC-3', COX-2 reverse 5'-TGTGTTTGGAGTGGGTTTCA-3', p21 forward 5'-CACCTACCTGCTCTGCTG-3', p21 reverse 5'-AATCTGTCATG-CTGGTCTGC-3', Snail forward 5'-CTGCAGGACTCTAATCCAGAGTTT-3', Snail reverse 5'-GGACAGAGTCCCAGATGAGC-3', actin forward 5'-TACATGGCTGGGGTGTGAA-3', β -actin reverse 5'-AAGAGAGGCATCT-CACCT-3'.

All reactions were done in triplicate using an iCycler iQ system (Bio-Rad Laboratories). The thermal cycling conditions were as follows: 3 min at 95°C followed by 50 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and 84°C for 20 s. For each sample, the amount of the target gene was divided by the reference gene (β -actin).

Small interfering RNA. For design of small interfering RNA (siRNA) oligos targeting PGDH, a DNA sequence of type AA (N19), was selected to correspond with the nucleotides 444-462 located 3' to the first nucleotide of the start codon of the human 15-PGDH cDNA (BC018986). BLAST search against the human genome sequence showed that only the 15-PGDH gene was targeted. Both of the siRNAs against COX-2 and p21, and control siRNA were from Santa Cruz Biotechnology.

Transfections. T98G cells were plated onto six-well plates at 2×10^5 per well. After 24 h, cells were transfected with 8 μg of pcDNA3 plasmid using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The transfected cells were selected with 50 $\mu\text{g/mL}$ G418 (Omega Scientific). Two stable clones (P5, P6) expressing PGDH and four empty vector clones (E1-E4) of T98G GBM cells were expanded and used for the following studies. For the transfection of siRNA against COX-2 and 15-PGDH, as well as control siRNA, the siRNAs were transfected using OligofectAMINE (Invitrogen) according to the manufacturer's instructions. Briefly, OligofectAMINE was incubated with serum-free medium for 10 min. Subsequently, a mixture of siRNA was added. After incubation for 20 min at room temperature, the mixture was diluted with medium and added to each well. Thirty-six hours later, cells were harvested.

Cell cycle assays. After treatment with the indicated compounds, the cells were harvested, fixed in methanol, and stained with propidium iodide (Abcam). Flow cytometry was done at the Flow Cytometry Core facility of Sackler Faculty of Medicine, Tel-Aviv University.

Statistical analysis. The data are expressed as the means \pm SD. The study variables were compared between the study groups using Fisher's exact test for categorical variables and Student's *t* test for continuous variables. All calculations were two-tailed. Asterisks indicate significant

differences of between the variables. *P* values of <0.05 were considered statistically significant.

Results

Inhibition of glioma cell proliferation by NSAIDs. The effect of two different NSAIDs, diclofenac sodium and meloxicam sodium, on the proliferation of three GBM cell lines, T98G, U118, and U373, was studied by cell counts. The cells were treated with 100 $\mu\text{mol/L}$ of diclofenac or 10 $\mu\text{mol/L}$ of meloxicam, and the effect on proliferation was determined after 48 hours of culture. Both drugs suppressed the proliferation of the three GBM cell lines (Fig. 1A). Diclofenac had a more potent inhibitory effect than meloxicam on all of the glioma cells in these conditions.

Cell cycle analysis using propidium iodide at 24 hours after treatment with diclofenac (100 $\mu\text{mol/L}$) revealed a significant increase in the percentage of sub- G_0 apoptotic cells after treatment (Fig. 1B).

Up-regulation of 15-PGDH and p21 expression in glioma cells after treatment with NSAIDs. The effects of NSAIDs on the expression of 15-PGDH protein were tested using Western blotting. Three GBM cell lines were examined either with or without NSAIDs (Fig. 2A). Expression of 15-PGDH protein was increased by treatment with the NSAIDs in all GBM cell lines. p21 and cyclin

D1 protein expression was also examined in these cells (Fig. 2B). Cyclin D1 levels were significantly reduced by diclofenac in all cell lines, whereas meloxicam treatment reduced cyclin D1 levels in T98G and U118 cells, but not in U373 cells. The p21 levels were significantly up-regulated by both drugs in T98G cells and by meloxicam in U373 cells. As previously reported (27), U118 cells expressed high basal levels of p21 and therefore did not show p21 up-regulation after treatment. The level of mRNA expression of 15-PGDH and p21 after treatment was also determined by real-time reverse transcription-PCR (RT-PCR) in T98G cells (Fig. 2C and D). Levels of both transcripts were increased by the NSAIDs in parallel with the increase of their proteins. The level of cyclin D1 mRNA did not change (data not shown).

Inhibition of growth of GBM cells with forced expression of 15-PGDH. To elucidate the role of overexpression of 15-PGDH in glioma cells, T98G cells were stably transfected with a 15-PGDH expression vector. Two clones (P5 and P6) were established, which stably expressed 15-PGDH, and four clones containing empty vector were also isolated (Fig. 3A). Cell proliferation assays showed that the 15-PGDH clones (P5 and P6) grew 50% slower than the wild-type and empty-vector transfected clones (E1-E4) at 48 hours ($P < 0.05$; Fig. 3B). When NSAIDs were added, cell proliferation of the P5 and P6 clones (GBM overexpressing 15-PGDH) was

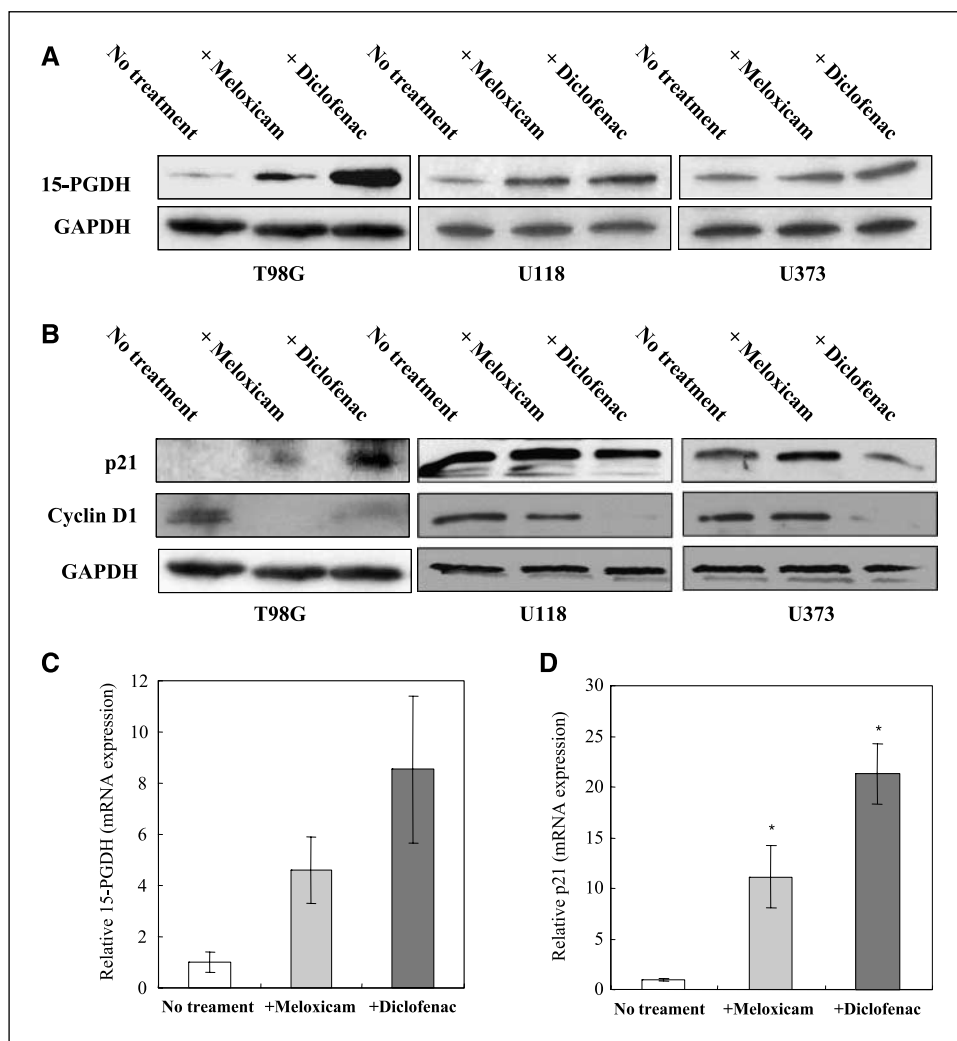
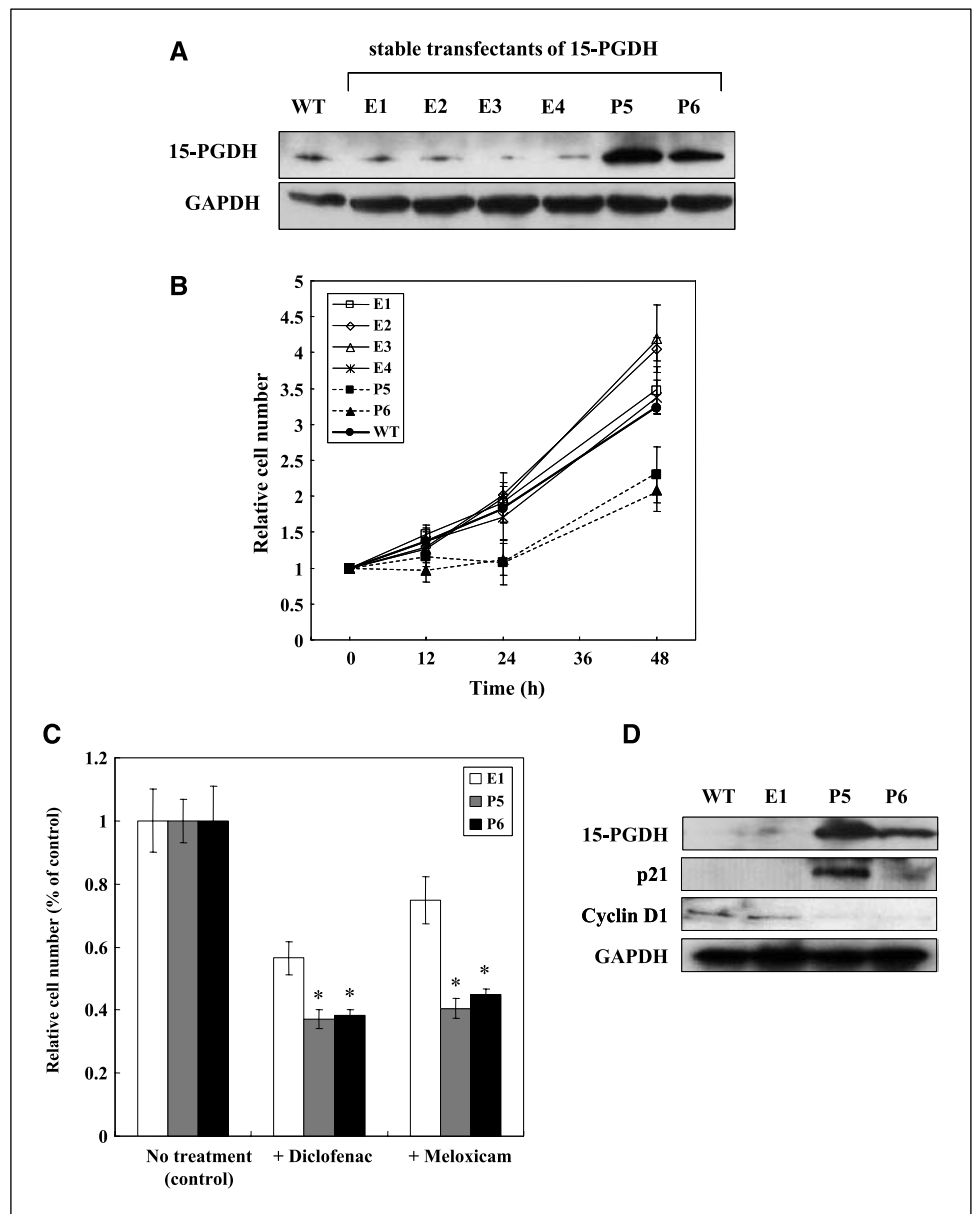


Figure 2. Up-regulation of 15-PGDH and p21 expression and down-regulation of cyclin D1 levels in NSAID-treated GBM cells. **A**, T98G, U118, and U373 GBM cells were cultured either with or without diclofenac sodium (100 $\mu\text{mol/L}$) or meloxicam sodium (10 $\mu\text{mol/L}$) for 48 h; cellular lysates were made, and 15-PGDH was determined by Western blotting. GAPDH was used as loading control. **B**, T98G, U118, and U373 cells were cultured either with or without diclofenac sodium (100 $\mu\text{mol/L}$) or meloxicam sodium (10 $\mu\text{mol/L}$) for 48 h; cellular lysates were made, and p21 and cyclin D1 levels were determined by Western blotting. GAPDH was used as a loading control. **C** and **D**, 15-PGDH and p21 mRNA levels were determined using real-time RT-PCR in T98G cells treated either with diclofenac sodium (100 $\mu\text{mol/L}$) or meloxicam sodium (10 $\mu\text{mol/L}$). Expression levels of the target mRNAs were divided by β -actin mRNA expression levels. Results are expressed as relative expression assuming untreated cells as 1.0. Columns, mean of triplicate plates; bars, SD; *, $P < 0.05$.

Figure 3. Growth suppression of T98G cells with forced expression of 15-PGDH. **A**, T98G cells (WT) were transfected with either an empty vector (E1–E4) or 15-PGDH expression vector having a *neo* selectable marker (P5, P6). After transfection, cells were selected with G418 and further cultured for 2 wk. Stable transfectants were isolated, and 15-PGDH levels were determined by Western blotting. GAPDH was used as loading control. **B**, stable transfectants of empty vector (E1–E4), 15-PGDH (P5, P6), or untransfected T98G GBM cells (WT) were plated in 24-well plates. Cell numbers were counted at indicated times. *Points*, mean of three experiments done in triplicate plates per experimental point; *bars*, SD. **C**, empty vector clone (E1) and two 15-PGDH transfected clones of T98G GBM (P5, P6) were cultured either with or without diclofenac sodium (100 $\mu\text{mol/L}$) or meloxicam sodium (10 $\mu\text{mol/L}$). Cell numbers were counted 48 h later. Relative cell numbers were expressed as a percentage of untreated control. *Columns*, mean of triplicate plates; *bars*, SD; *, $P < 0.05$. **D**, forced expression of 15-PGDH up-regulates p21 levels. T98G GBM cells (WT), empty-vector transfected clone (E1), or 15-PGDH transfected clones (P5, P6) were examined for expression of 15-PGDH, p21, and cyclin D1 levels by Western blotting. GAPDH was an internal loading control.



suppressed significantly compared with the GBM clone containing the empty vector (E1; Fig. 3C).

Increased expression of p21 protein in GBM cells over-expressing 15-PGDH. As shown in Fig. 2A–C, p21 expression was up-regulated together with 15-PGDH when T98G cells were treated with NSAIDs. Therefore, we studied whether 15-PGDH over-expressing cells (clones P5 and P6) had increased expression of p21. By Western blotting, strong expression of the p21 protein was observed in these cells, whereas the empty vector containing E1 clone had negligible levels. Cyclin D1 protein was reduced in 15-PGDH over-expressing cells (Fig. 3D).

Down-regulation of COX-2 does not alter either 15-PGDH or p21 expression and does not affect proliferation in GBM cells. COX-2 is one of the key enzymes that regulates prostaglandin production and can modulate cancer cell growth. To examine the contribution of COX-2 to glioma cell growth, we knocked-down specifically COX-2. A siRNA against COX-2 and a control siRNA

were transfected into T98G cells, resulting in a marked decrease of COX-2 protein (Fig. 4A), but no significant alteration occurred in either levels of 15-PGDH, p21, or cyclin D1 proteins or p21 mRNA expression (Fig. 4A and B, respectively). The cells transfected with an siRNA against COX-2 did not show a significant difference in proliferation compared with the control siRNA transfected cells (Fig. 4C, left). When either diclofenac or meloxicam was added to the culture medium, the cells transfected with COX-2 siRNA did not change their growth rate compared with the control siRNA transfected cells (Fig. 4C, middle and right).

Down-regulation or inhibition of 15-PGDH restores growth inhibition of glioma cells by NSAIDs. An siRNA against 15-PGDH and a control siRNA were transfected into T98G cells. Analyses of 15-PGDH mRNA levels revealed that siRNA against 15-PGDH effectively reduced expression of the gene (Fig. 5A, left). p21 mRNA levels were also down-regulated in the cells transfected with the 15-PGDH siRNA (Fig. 5A, middle). Western blotting showed an

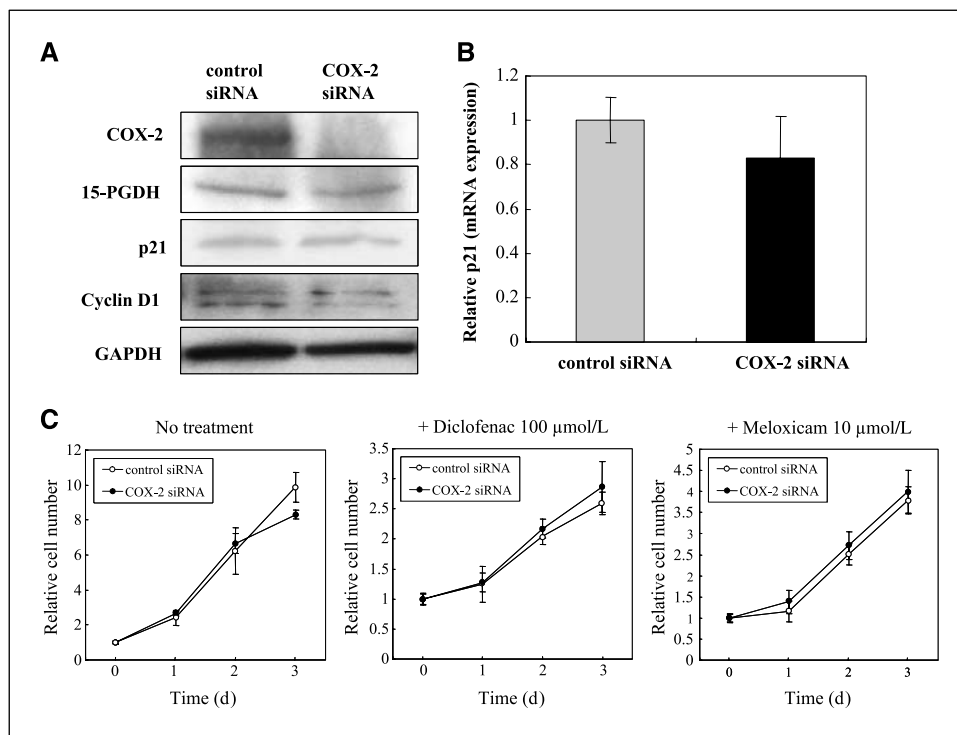


Figure 4. Effect of COX-2 siRNA on growth of T98G cells. *A*, T98G cells were transfected with either COX-2 siRNA or control siRNA. Forty-eight hours later, cells were harvested, lysed, and subjected to Western blotting with antibodies to COX-2, 15-PGDH, p21, cyclin D1, and GAPDH (loading control). *B*, p21 mRNA levels were determined using real-time RT-PCR in T98G cells transfected with either COX-2 siRNA or control siRNA. Expression of p21 mRNA was divided by β -actin mRNA levels and shown as the relative expression with the control value set as 1.0. *Columns*, mean of three experiments done in triplicate plates; *bars*, SD. *C*, T98G GBM cells transfected with either COX-2 siRNA or control siRNA were cultured with either no NSAID (*left*), diclofenac sodium (100 μ mol/L; *middle*), or meloxicam sodium (10 μ mol/L; *right*). Cell numbers were counted on days 1, 2, and 3. *Columns*, mean of three experiments done in triplicate plates; *bars*, SD.

increased expression of cyclin D1 protein in these 15-PGDH silenced cells (Fig. 5A, right). Cell proliferation assays showed that the cells transfected with siRNA against 15-PGDH had no change in proliferation compared with the cells transfected with control siRNA (Fig. 5B, left top). In contrast, when either diclofenac or meloxicam was added to the cultures, the cells transfected with 15-PGDH siRNA grew significantly faster than the cells with control siRNA (Fig. 5B, middle and right top). Moreover, in the 15-PGDH-deficient cells, 15-PGDH mRNA levels were suppressed and almost unchanged and p21 mRNA levels increased only mildly after the addition of NSAIDs (Fig. 5B, bottom).

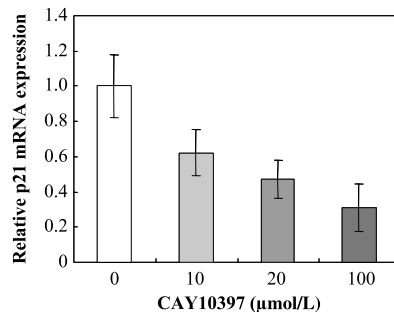
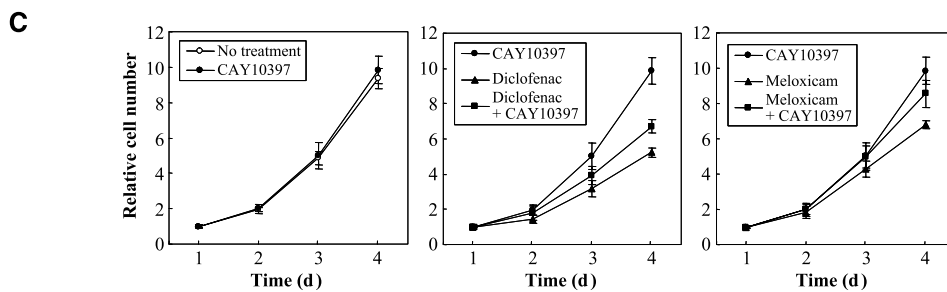
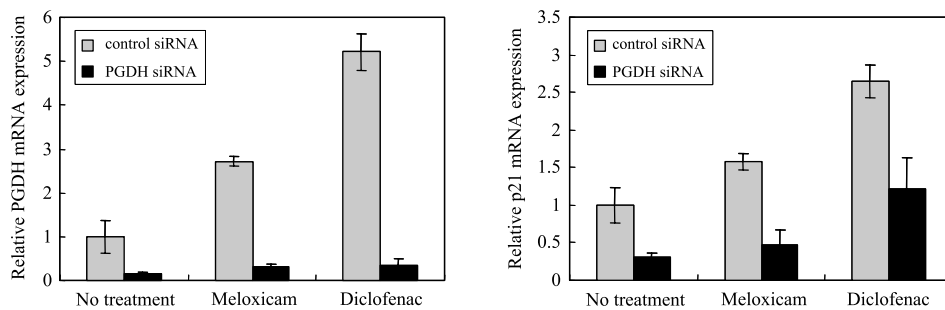
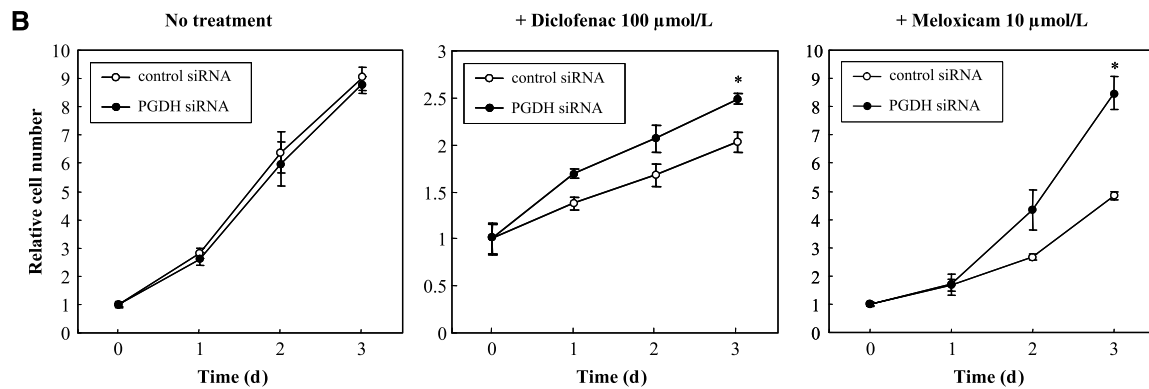
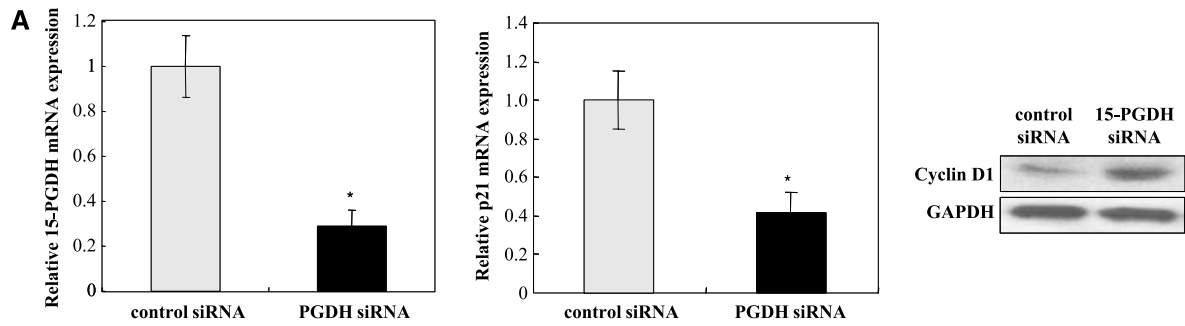
To validate the role of 15-PGDH in the growth inhibitory effects of NSAIDs, 15-PGDH activity was inhibited by CAY10397, a specific inhibitor of 15-PGDH. Cell proliferation assay showed that the cells treated with 20 μ mol/L of CAY10397 had no change in proliferation compared with untreated cells (Fig. 5C, left top). In contrast, when either diclofenac or meloxicam was added to the cultures, the cells with CAY10397 grew significantly ($P < 0.05$) faster than those cultured with either diclofenac or meloxicam alone (Fig. 5C, middle and right top). In parallel, CAY10397 reduced expression of p21 mRNA in a dose-dependent manner (Fig. 5C, bottom). Taken

together, these data suggest that 15-PGDH expression and activity is necessary for the growth inhibitory effects of NSAIDs in these cells.

Down-regulation of p21 also restores growth inhibition of glioma cells by NSAIDs. An siRNA against p21 was also transfected into T98G cells. Analyses of p21 mRNA levels revealed that siRNA against p21 effectively reduced expression of the gene (Fig. 6A) and increased expression of cyclin D1 (Fig. 6B). Cell proliferation assays showed that the cells transfected with siRNA against p21 had no change in proliferation compared with the cells transfected with control siRNA (Fig. 6C, left). In contrast, when either diclofenac or meloxicam was added to the cultures, the cells transfected with p21 siRNA grew significantly ($P < 0.05$) faster compared with the cells with control siRNA (Fig. 6C, middle and right).

Snail expression in NSAID-treated glioma cells. A recent report showed that *Snail*, a transcription factor, repressed expression of 15-PGDH in colon cancer cells (28). To investigate whether *Snail* expression plays a role in the regulation of 15-PGDH in NSAID-treated glioma cells, mRNA levels of *Snail* was measured by real-time RT-PCR. No difference in *Snail* expression was noted between NSAID-treated and untreated cells (data not shown).

Figure 5. Effect of knockdown or inhibition of 15-PGDH on the growth of T98G cells. *A*, T98G GBM cells were transfected with siRNA against 15-PGDH (PGDH siRNA) or control siRNA and mRNA levels of 15-PGDH (*left*) and p21 (*middle*) were determined using real-time RT-PCR. Expression levels of the target mRNAs were divided by those of β -actin mRNA and displayed as relative expression setting control siRNA exposed cells at 1.0. *Columns*, mean of three experiments done in triplicate measurements; *bars*, SD; *, $P < 0.05$. *Right*, 48 h after transfection, cells were harvested, lysed, and subjected to Western blotting with antibodies to cyclin D1 and GAPDH (loading control). *B*, T98G GBM cells were transfected with either PGDH siRNA or control siRNA and cultured with no NSAID (*top left*), diclofenac sodium (100 μ mol/L; *top middle*), or meloxicam sodium (10 μ mol/L; *top right*). Cell numbers were counted on days 1, 2, and 3. 15-PGDH (*bottom left*) and p21 (*bottom right*) mRNA levels were determined using real-time RT-PCR. Expression levels of the target mRNAs were divided by β -actin mRNA expression levels and are expressed as relative levels assuming untreated cells as 1.0. Results represent the mean of three experiments done in triplicate plates; *bars*, SD; *, $P < 0.05$. *C*, T98G cells were cultured with no NSAID (*top left*), diclofenac sodium (100 μ mol/L; *top middle*), or meloxicam sodium (10 μ mol/L; *top right*) either with or without CAY10397 (20 μ mol/L). Cell numbers were counted on days 1, 2, and 3. $P < 0.05$ for cells treated with NSAIDs and CAY10397 compared with NSAIDs alone. *Bottom*, mRNA levels of p21 were determined using real-time RT-PCR in T98G GBM cells treated with various concentrations of CAY10397. Expression levels of p21 mRNAs were divided by those of β -actin mRNA and displayed as relative expression setting control siRNA-exposed cells at 1.0. Results represent the mean \pm SD of three experiments done in triplicate plates; *bars*, SD.



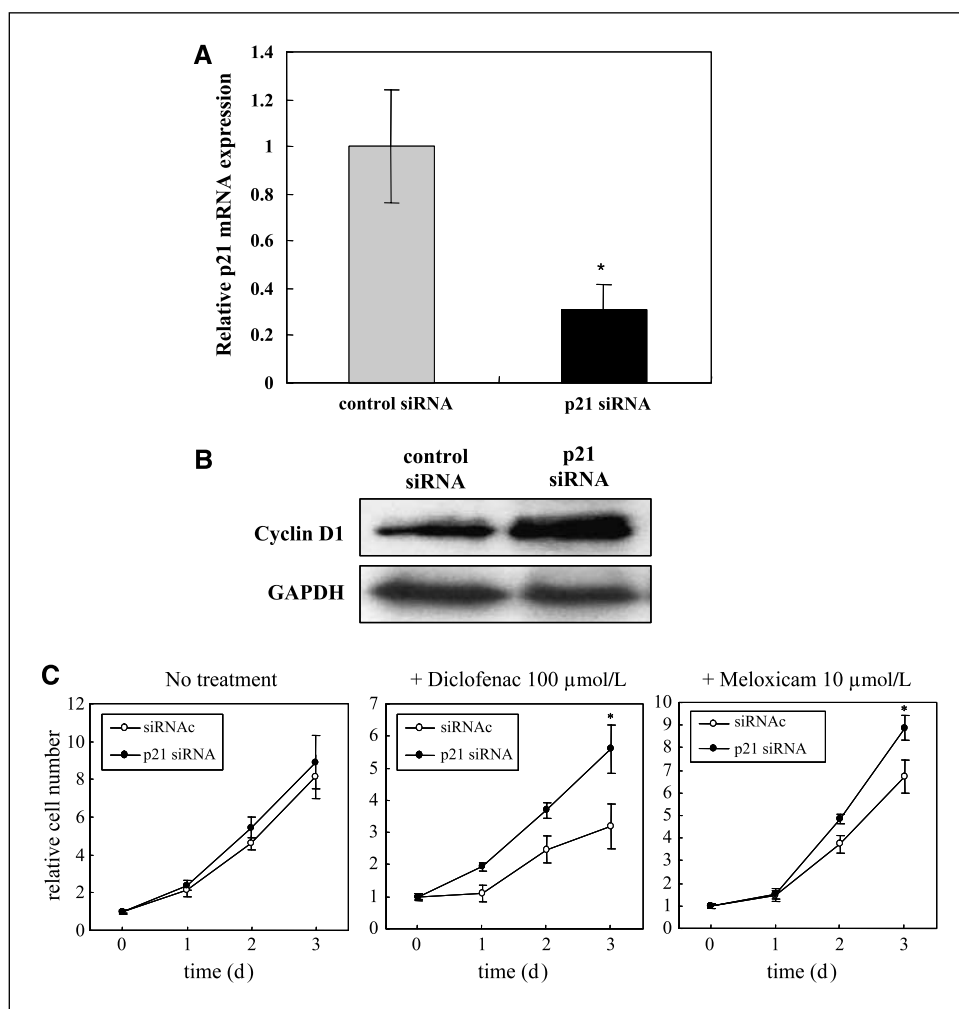


Figure 6. Effect of p21 siRNA on the growth of T98G cells. **A**, mRNA levels of p21 were determined using real-time RT-PCR in T98G GBM cells transfected with p21 siRNA or control siRNA. Expression levels of the target mRNAs were divided by those of β -actin mRNA and displayed as relative expression, setting control siRNA exposed cells at 1.0. Columns, mean of three experiments done in triplicate measurements; bars, SD; *, $P < 0.05$. **B**, 48 h after transfection cells were lysed and cyclin D1 levels were determined by Western blotting. **C**, T98G transfectants were cultured with no NSAID (left), diclofenac sodium (100 μ mol/L; middle), or meloxicam sodium (10 μ mol/L; right). Cell numbers were counted on days 1, 2, and 3. Points, mean of three experiments done in triplicate plates; bars, SD; *, $P < 0.05$.

Discussion

Beginning in the 1980s, studies reported that treatment with NSAIDs were associated with a reduced risk for colon cancer (29, 30). Since then, many more studies have confirmed that NSAIDs can reduce the risk of various types of cancers (9–14). Traditional NSAIDs are nonselective inhibitors of COX-1 and COX-2. Studies suggest that reduction of carcinogenesis by NSAIDs results from inhibition of the activity of COX-2. The premise that COX-2 inhibition is essential for this antitumor effect is based on the observation that prostaglandin E2 and other COX-2 generated downstream mediators promote tumor cell proliferation (31, 32), survival (33–36), and angiogenesis (37, 38) in an autocrine and/or paracrine manner. Recently, however, several lines of evidence indicate that a COX-2-independent mechanism may be involved in the antitumor activity of COX-2 inhibitors. For example, several COX-2 inhibitors have similar abilities to inhibit COX-2 activity but differ considerably in their potency to induce apoptosis of cancer cells (39). Also, metabolites of sulindac (an NSAID) induce apoptosis of prostate cancer cells despite their inability to inhibit COX-2 activity (40). Moreover, overexpression of COX-2 in immortalized human umbilical vein endothelial HEK-293, COS-7, and NIH3T3 cells led to their increased cell death and/or cell cycle arrest (41, 42), whereas COX-2 overexpression in gastrointestinal cells and PC-12 pheochromocytoma cells inhibited their apoptosis

(43–45). Also, an NSAID caused cell cycle arrest of ovarian cancer cells independently of COX-2 inhibition (46). Likewise, celecoxib inhibited growth of prostate cancer cells by the inhibition of 3-phosphoinositide-dependent protein kinase-1/Akt signaling pathway (47). Therefore, tissue specificity may explain these differences in responses and mechanisms of action of NSAIDs. COX-2 may not always be the major target of NSAIDs and may not necessarily be responsible for the antitumor activity in some types of cancer cells.

In this study, we observed, for the first time, up-regulation of 15-PGDH after treatment of GBM cells with NSAIDs. In addition, 15-PGDH overexpressing T98G cells had a further reduction of growth in the presence of NSAIDs compared with the cells transfected with an empty vector (Fig. 3C). When 15-PGDH was silenced by siRNA or inhibited by a specific inhibitor, growth of T98G cells was restored with exposure to NSAIDs (Fig. 5). These results suggest that 15-PGDH may interact with another target molecule of NSAIDs. In contrast, knockdown of COX-2 expression did not show any alteration in growth of T98G cells either in the presence or absence of an NSAID (Fig. 4C).

Exposure to NSAIDs was accompanied by an up-regulation of the cyclin-dependent kinase (cdk) inhibitor, p21 in T98G and U373 cells, which express low basal levels of p21 (27). In T98G cells, both mRNA and protein levels were increased after

treatment. Also, levels of p21 protein markedly increased with forced expression of 15-PGDH (Fig. 3D). Consistently, cyclin D1 protein decreased in NSAID-exposed cells and 15-PGDH-over-expressing cells (Figs. 2B and 3D), which may be explained by degradation of cyclin D1-cdk4 complex. In contrast, knockdown of COX-2 gene expression had no effect on p21 levels (Fig. 4A). This observation is consistent with a previous report in which the growth inhibition of the COX-2 inhibitors and their ability to induce p21 were not affected by COX-2 siRNA in lung cancer cell lines (48). Treatment with aspirin and indomethacin has been reported to induce apoptosis in T98G cells (49). Similarly, we noted increased levels of apoptosis after diclofenac treatment in all three GBM cells that were tested.

We observed tumor suppressor-like activities for 15-PGDH in glioma cells. Others have reported that 15-PGDH behaves as a tumor suppressor gene in lung (17), colon (18), and breast cancers (19). Our study showed that ectopic overexpression of 15-PGDH inhibited growth of the T98G glioma cells, and inhibition of expression of this enzyme blunted the antiproliferative activity of the NSAIDs.

Recently, Mann and colleagues reported that expression of 15-PGDH was repressed by epidermal growth factor (EGF) and Snail in colon cancer cells (28). The study suggested that signaling through the EGF receptor induced Snail which bound to the 15-PGDH promoter and repressed transcription of 15-PGDH. The report raised a question whether the alterations associated with

15-PGDH by NSAIDs might also involve *Snail* expression. We found no change in *Snail* expression in glioma cells either with or without treatment with NSAIDs.

In conclusion, this study identified 15-PGDH as a pivotal regulator of glioma cell growth. Forced expression of 15-PGDH in glioma cells slowed their growth and, blocking its expression, prevented the growth inhibition mediated by NSAID. The observed regulation of growth may be mediated in part by regulating levels of p21. The results suggest that 15-PGDH can behave as a tumor suppressor in glioma cells and should be considered as a target for therapeutic manipulation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Manuscript is dedicated to the loving memory of Matt Schreck.

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