Cyclooxygenase-2 Inducing Mcl-1-dependent Survival Mechanism in Human Lung Adenocarcinoma CL1.0 Cells

IN Volvement of Phosphatidylinositol 3-Kinase/Akt Pathway

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Cyclooxygenase 2 (COX-2) has been reported to be commonly expressed in advanced stages of human lung adenocarcinoma. In this study, the COX-2 constitutive expression vector was transfected into a human lung adenocarcinoma cell line CL1.0 and several clones were obtained which stably expressed COX-2. These COX-2-overexpressed clones demonstrated remarkable resistance to apoptosis induced by Ultraviolet B (UVB) irradiation, vinblastine (BVL) cell lymphoma-2 (Bcl-2), or other anti-cancer drugs. To understand how COX-2 prevents apoptosis, the investigators examined the expression level of Bcl-2 family members. Mcl-1, but not other Bcl-2 members, was significantly up-regulated by COX-2 transfection or prostaglandin E2 (PGE2) treatment. Treatment of COX-2-overexpressed cells (cox-2/cl4) with two specific COX-2 inhibitors, NS-398 and celecoxib, caused an effective reduction of the increased level of Mcl-1. These data suggest that the expression level of Mcl-1 is tightly regulated by COX-2. Moreover, transfection of cox-2/cl4 cells with antisense Mcl-1 enhanced apoptosis induced by UVB irradiation, revealing that Mcl-1 plays a crucial role in cell survival activity mediated by COX-2. Furthermore, COX-2 transfection or PGE2 treatment evidently activated the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Inhibition of the PI3K pathway by LY294002 or wortmannin effectively attenuated the increased level of Mcl-1 induced by COX-2 or PGE2. Blocking the PI3K activity with a dominant-negative vector, DN-p85, also greatly diminished the level of Mcl-1 and enhanced UVB-elicited cell death in cells transfected by COX-2. In a similar way, LY294002 inhibited cell survival and Mcl-1 level in PGE2-treated CL1.0 cells. These findings suggest that COX-2 promotes cell survival by up-regulating the level of Mcl-1 by activating the PI3K/Akt-dependent pathway.

Cyclooxygenases (COX)

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1 The abbreviations used are: COX, cyclooxygenase; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; VBL, vinblastine; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl-

transferase-mediated dUTP nick end labeling; PPAR, peroxisome proliferator activator receptor; Bcl, B cell lymphoma; Mcl, myeloid cell leukemia.
Apoptotic cells were visualized by fluorescence microscopy.

**Experimental Procedures**

**Cell Culture and Transfection**—Human lung adenocarcinoma cell line CL1.0 was cultured as previously described (16). CL1.0 cells accurately expressing the COX-2 were established by transfection with a COX-2 constitutive expression vector, pSG5-Cox-2, employing the TransFast™ transfection reagent (Promega, Madison, WI). After 48 h of transfection, cells were trypsinized and replated in RPMI 1640 with 10% fetal calf serum and 300 μg/ml hygromycin B. Hygromycin B-resistant clones were selected and expanded. The level of COX-2 and COX-1 was determined by Western blotting.

**PGE₃ Assay**—5 × 10⁴ cells/well of different stable COX-2-overexpressed clones (cox-2/cl.2, cox-2/cl.4, and cox-2/cl.17) were plated in 6-well dishes and grown to a 60–70% confluence in growth medium by 24 h. Then the culture medium was collected with an enzyme immunosay to verify amounts of PGE₃ secreted by these stable clones. The production of PGE₃ was standardized to protein concentrations.

**Transient Transfection with Antisense Mcl-1**—COX-2-overexpressing CL1.0 cells (cox-2/cl.4) were plated 24 h before transfection at a density of 5 × 10⁴ cells onto a cover glass. Cells were transfected with 5 μg of antisense Mcl-1 plasmid (pcDNA3-mcl-1-AS) or control plasmid (pcDNA3) utilizing TransFast™ transfection reagent. Transfections were performed in triplicate. Twenty-four hours after transfection, transfected cells were changed to a serum-free medium for a further 12 h and were then treated with UVB irradiation (100 mJ/cm²) or none. After treatment, the extent of apoptosis was determined by TUNEL assays with the In Situ Death Detection Kit, Fluorescein (Roche Molecular Biochemicals) according to the manufacturer’s instructions. Apoptotic cells were visualized by fluorescence microscopy.

**DNA Fragmentation Assay**—The DNA fragmentation on agarose gel electrophoresis was detected as described previously (17). 

**Immunoblotting**—The cellular lysates were prepared as described previously (18). A 50–100 μg sample of each lysate was subjected to electrophoresis on 10% SDS-polyacrylamide gels. The samples were then electroblotted on a nitrocellulose paper. After blotting, blots were incubated with anti-Mcl-1, anti-Bcl-2, anti-Bcl-xL, anti-Bax (Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX-1, anti-COX-2 (Transduction Laboratories, Lexington, KY), anti-Akt, and anti-phospho-Akt (New England Bio- Lewis, Beverly, MA) antibodies in PBS containing 5% non-fat dry milk, 5% fetal calf serum, and 0.1% Tween 20 h at 4 °C. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence reagents (Amer sham Pharmacia Biotech) were employed to depict the protein bands on membranes.

**P38 Kinase Assay**—The P38 kinase activities were assayed as described previously (19). Briefly, 10⁶ cells received different treatments and were washed twice with ice cold phosphate-buffered saline and lysed with 1 mM lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% Nonidet P-40, 10% glycerol, 1 mg/ml bovine serum albumin, 20 mM Tris, pH 8.0, 2 mM orthovanadate). Cell extracts were incubated with 1 μg of anti-phosphotyrosine antibody overnight at 4 °C. The immunocomplex was precipitated with 50 μl of protein A-Sepharose for 1 h at 4 °C and washed three times with lysis buffer, twice with LiCl buffer (0.5 M LiCl, 100 mM Tris, pH 7.6), and twice with TNE buffer (10 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA). The immunocomplex was preincubated with 10 μl of 20 μg Hepes (pH 7.4), containing 2 mg/ml propidium iodide (Sigma) on ice for 10 min. Kinase reaction was performed by adding 40 μl of reaction buffer (10 μl of 15 mM MgCl₂, 1 mM Hepes, pH 7.4, 100 μM 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), 50 μM 4-methylumbelliferyl-β-D-galactopyranoside (X-gal; added just before use) for 4–24 h, and washed twice with PBS. The β-galactosidase-positive cells (blue living cells) in each well were counted.

**Results**

**COX-2 Expression Causes Resistance to Apoptosis in Human Lung Adenocarcinoma CL1.0 Cells**—The human COX-2 constitutive expression plasmid, pSG5-Cox-2, and the control vector were transfected into human adenocarcinoma CL1.0 cells, which have a relatively low level of COX-2 protein. This action was aimed at determining whether alteration of the COX-2 level would change the cellular sensitivity to apoptosis. After transfection, cells were cultured in a medium containing 300 μg/ml hygromycin B. Each colony that grew after hygromycin selection was picked and expanded. Western blot analysis revealed that three stable colonies (cox-2/cl.2, cox-2/cl.4, and cox-2/cl.17) were randomly selected and expressed a 4–6-fold increase of COX-2 protein compared with the control vector cells (Fig. 1A). The level of COX-1 protein in these transfectants and control cells remained unaltered (Fig. 1A). Expression of COX-2 protein in the human lung cancer CL1.0 cells. This implies that the exogenously overexpressed COX-2 displayed enzymatic activities in CL1.0 cells.
COX-2 Inhibits Apoptosis in Human Lung Adenocarcinoma CL1.0 Cells

FIG. 2. COX-2-overexpressed or PGE$_2$-treated cells confer resistance upon apoptosis. A, COX-2 transfectant-resistant VBL-induced DNA fragmentation. Each cell clone was treated with 3 μM VBL for 16 h, and the cellular DNA was extracted as described under “Experimental Procedures.” The DNA laddering was analyzed with 1.8–2% agarose gel. B, morphological examination of cox-2/cl.4 and vector control cells after treatment with 100 mJ/cm$^2$ UVB. The apoptotic characteristics such as nuclear fragmentation and chromatin condensation were determined by staining with Hoechst 33258 fluorescent dye. COX-2 inhibitors attenuated the increased level of Mcl-1 in COX-2-overexpressed cells. Cox-2/cl.4 cells were plated at a density of 1–3 × 10$^6$ cells/100-mm dish. The cultures were treated with 10 μM celecoxib or 25 μM NS-398 for 16 h; then the treated cells were collected and cell lysates were prepared for immunoblotting. Lane 1, vector control cells; lane 2, cox-2/cl.4 cells; lane 3, cox-2/cl.4 cells treated with 10 μM celecoxib; lane 4, cox-2/cl.4 cells treated with 25 μM NS-398. C, transient transfection with COX-2 vector increased Mcl-1 protein level. CL1.0 cells were transiently transfected with 0, 2, or 4 μg of pSG5-Cox-2 or equal amounts of control vector pSG5 as described under “Experimental Procedures.” After transfection, each cell lysate was obtained and subjected to electrophoresis and immunoblotting with anti-Mcl-1, anti-Cox-2, and anti-Bcl-2 antibodies. D, PGE$_2$ elevated the level of Mcl-1 protein in CL1.0 cells. Human CL1.0 cells were serum-free for 24 h and then were treated with 4 μg/ml of PGE$_2$ for various periods of time as indicated. Cells were lysed, and the supernatants were subjected to immunoblotting with anti-Mcl-1, anti-Bax, and anti-Bcl-2 antibodies.cox-2/cl.4 cells had fewer apoptotic cells than the vector control cells when exposed to UVB irradiation (100 mJ/cm$^2$). Few apoptotic cells could be detected in cox-2/cl.4 or control cells without UVB exposure (data not displayed). Exposure of parental CL1.0 cells to 1–4 μg/ml PGE$_2$ consistently resulted in a dose-dependent decrease in apoptotic cells induced by UVB irradiation compared with cells in the absence of PGE$_2$, as determined by the TUNEL assay (Fig. 2C). These findings indicate that overexpression of COX-2 or its elevated product, PGE$_2$, renders cells more resistant to apoptosis stresses.

COX-2 Stimulation Up-regulates Mcl-1 but Not Other Bcl-2 Family Members—The expression level of the Bcl-2 family proteins was first examined to identify the possible downstream gene(s) regulated by COX-2 or PGE$_2$. Fig. 3A reveals that the level of Mcl-1 protein was significantly increased by 3–4 fold in cox-2/cl.4 and cl.4 stable clones compared with the vector control cells. In contrast, the levels of other Bcl-2 family members such as Bax remained unchanged after COX-2 stimulation.

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as Bcl-x<sub>L</sub> and Bcl-2 were only slightly affected either in COX-2 stable clones or control cells. We further examined whether the level of Mcl-1 protein in cox-2/cl.4 cells would be altered by treatment with COX-2 inhibitors. Fig. 3B shows, under the non-cytotoxic dose, that both celecoxib (10 μM) and NS-398 (25 μM) strongly diminished the endogenous level of Mcl-1 in the COX-2 stable clone. Under the same circumstance, both inhibitors could inhibit the level of PGE<sub>2</sub>, an indicator for COX-2 activity, in COX-2-overexpressed cells by 70–80% (data not shown).

To exclude the possibility that an artificial drug selection enhanced anti-apoptotic Mcl-1 protein in these COX-2-stable clones, investigators transfected CL1.0 cells with COX-2 expression vector or treated them with various concentrations of PGE<sub>2</sub> and then examined the expression of Mcl-1 protein. Western blot analysis revealed that CL1.0 cells displayed an apparent dose-dependent increase in Mcl-1 protein (~4–8-fold) when transfection occurred with the 2 or 4 μg of pSG5-Cox-2 vector but not with the control vector, pSG5 (Fig. 3C). A commonly used liposomal transfection method achieved a high transfection efficiency of >40% in the human adenocarcinoma CL1.0 cells (not displayed). CL1.0 cells were treated next with 4 μg/ml PGE<sub>2</sub> for various periods of time, and then the change of Mcl-1 protein level was detected by Western blotting. Fig. 3D clearly demonstrates that the level of Mcl-1 protein rapidly increased 1 h after PGE<sub>2</sub> treatment, peaked at 3–6 h, and then maintained a high level of Mcl-1 over 12 h. In contrast, the level of Bcl-2 protein was little affected during the PGE<sub>2</sub> treatment. These results suggest that COX-2 overexpression or PGE<sub>2</sub> elevated the Mcl-1 level in CL1.0 cells but not other Bcl-2 family members.

Mcl-1 Protein Involved in the Anti-apoptotic Effect of Cox-2—The COX-2-overexpressed cells (cox-2/cl.4) were transfected with a constitutive antisense Mcl-1 expression vector to further investigate the role of Mcl-1 in COX-2-mediated anti-apoptotic activity. However, following several rounds of stable clone selection, none of the clones that survived could express antisense Mcl-1. This probably occurred because of the severely cytotoxic effect of the constitutive antisense Mcl-1 expression. A transient transfection death assay was therefore conducted to investigate this phenomenon. Cox-2/cl.4 cells were initially transfected with empty vectors or antisense Mcl-1 vectors. Twenty-four hours after transfection, transfected cells were changed to a serum-free medium for a further 12 h and were then treated with UVB irradiation (100 mJ/cm<sup>2</sup>) or none. After treatment, the extent of apoptosis was defined as the number of cells positively stained with TUNEL and identified under a fluorescence microscope. Fig. 4A reveals that cox-2/cl.4 cells displayed a resistant phenotype to UVB-induced apoptosis when transfected with a control vector or none. Transfection of cox-2/cl.4 cells with antisense Mcl-1 enhanced apoptotic cell death induced by UVB irradiation. Under the same circumstances, the antisense Mcl-1 vector effectively attenuated the endogenous level of Mcl-1 in cox-2/cl.4 cells by transfection (Fig. 4B). The above results strongly suggest that the mcl-1 gene is critically involved in the COX-2-mediated anti-apoptotic effect in human adenocarcinoma CL1.0 cells.

Function of PI3K/Akt Pathway in COX-2-mediated Anti-apoptotic Effect and Mcl-1 Up-regulation—Because the PI3K/Akt pathway plays a central role in integrating a diverse survival signal triggered by numerous growth factors (20–22), COX-2 was tested to ascertain whether it could activate this pathway. To evaluate this, the endogenous Akt activity in cox-2/cl.4 and cox-2/cl.4 cells was examined by determining the serine-phosphorylated status of Akt, employing an anti-phospho-Akt antibody. The Akt activity correlates well with the phosphorylation of Akt molecules on serine 473 (23). Interestingly, these COX-2-overexpressed cells displayed a significantly increased Akt phosphorylation over the vector control cells (Fig. 5A). Furthermore, treatment with a PI3K inhibitor, LY294002 (10 μM), greatly reduced the phosphorylation of Akt in these COX-2-expressed cells (Fig. 5A). This indicates that PI3K functions upstream of Akt in response to the COX-2-elicted survival signal. The authentic PI3K activity of CL1.0 cells after treatment with PGE<sub>2</sub> was subsequently determined. Immunoprecipitates with the anti-phosphotyrosine antibody revealed a substantial increase in PI3K activity 15–60 min after exposure to 4 μg/ml PGE<sub>2</sub>, and 10 μM LY294002 could completely inhibit this increase (Fig. 5B). The above results suggest that the PI3K/Akt pathway is indeed activated by COX-2 or its product, PGE<sub>2</sub>.

The next aim of the investigation was to ascertain whether PI3K activity inhibition might affect the anti-apoptotic activity and Mcl-1 up-regulation induced by COX-2 or PGE<sub>2</sub>. To address this issue, a COX-2 vector and a dominant-negative form of the regulatory subunit of PI3K, DN-p85, were concomitantly transfected into CL1.0 cells. Western blot analysis revealed that DN-p85 completely attenuated COX-2-mediated Mcl-1 up-regulation (Fig. 6A). Utilizing an anti-hemagglutinin antibody (data not shown) or detecting the serine phosphorylation of Akt (Fig. 6B) confirmed the successful expression of the DN-p85 in transfected cells. To determine the effect of DN-p85 on COX-2-mediated cell survival, a transient transfection death assay was conducted employing the β-galactosidase expression plasmid (pCMV-β-gal) as a survival marker. The transfection results revealed that the DN-p85 transfection, but not the control pDNA3, abolished the COX-2-induced cell survival activity when exposed to UVB irradiation, as determined by the decrease of blue surviving cells expressing β-galactosidase activity (Fig. 6C).
Akt antibody or anti-Akt antibody. B without 10 μM LY294002 (LY) for 6 h, and then each cellular lysate was prepared to perform electrophoresis and immunoblotting as described under “Experimental Procedures” employing a specific anti-phospho-Akt antibody or anti-Akt antibody. B, activation of PI3K activity by PGE2, in COX-1 cells. Cells were treated as indicated, and lysates with equal amounts of protein were subjected to immunoprecipitations with anti-phosphotyrosine antibody. The immunocomplex was employed for PI3K activity assays as described under “Experimental Procedures.”

A similar observation was made in COX-1 cells treated with PGE2. Fig. 7A reveals that PGE2-induced Mcl-1 up-regulation was almost completely attenuated in the presence of LY294002 or wortmannin. Also, LY294002 treatment greatly sensitized PGE2-treated cells to UVB irradiation-elicited apoptosis as determined by a TUNEL assay (Fig. 7B). The above data clearly reveal that COX-2-mediated cell survival activity requires activation of the PI3K/Akt-dependent pathway and its subsequent downstream gene, mcl-1.

DISCUSSION

Accumulating evidence has suggested that cancer cells expressing higher levels of COX-2 may obtain a survival advantage that eventually facilitates the tumor development and progression. Although these studies have established a direct relationship between COX-2 expression and cell survival in different cell systems, the precise mechanism by which COX-2 prevents cell death has seldom been investigated and remains elusive. This study demonstrated that COX-2 overexpression or PGE2 treatment induced an increase in a novel anti-apoptotic protein Mcl-1 through the PI3K/Akt-dependent pathway in human adenocarcinoma cells. An antisense Mcl-1 transfection assay ensured a crucial role for Mcl-1 in the COX-2-mediated anti-apoptotic effect in lung adenocarcinoma COX-1.0 cells. Other researchers distinctly observed that either forced expression of COX-2 in intestinal cells (13) or PGE2 exposure (14) to colon cancer cells caused up-regulation of the Bcl-2 protein. The level of Bcl-2 protein, however, was unchanged in our cell system, suggesting induction of certain members of the Bcl-2 family by COX-2, depending upon the cell context.

The mcl-1 gene, one of the Bcl-2 family members, was originally identified as an early gene induced during differentiation of ML-1 myeloid leukemia cells (24). Overexpression of Mcl-1 delays apoptosis induced by a broad array of agents such as c-Myc overexpression, growth factor withdrawal and other cytotoxic agents (17, 25, 26). These findings correspond to our current data, suggesting that certain types of cancer cells require Mcl-1 to survive. Many cytokines and growth factors have already been reported as able to induce Mcl-1 expression (27), but this is first time it has been demonstrated that the COX-2-derived prostanoids can do so. COX-2 has been reported to inhibit nerve growth factor withdrawal apoptosis in differentiated PC12 cells (28). A different study found that an apoptosis-related gene, dynemin light chain (DLC), was up-regulated in PC12 cells by COX-2 expression or PGE2 treatment (29). DLC expression prevented apoptosis of PC12 cells by reducing neuron nitric-oxide synthase activity. The mcl-1 and DLC genes are the only two downstream effectors responsible for COX-2-mediated anti-apoptotic signal identified thus far.

This investigation also revealed that the PI3K/Akt signaling pathway could be activated and involved in Mcl-1 up-regulation by COX-2 expression or PGE2. Emerging evidence has demonstrated that the PI3K/Akt signaling pathway promotes growth-
Although COX-2 expression is thought to be crucial for the development of certain human cancers, the downstream signal that mediates the neoplastic effects is poorly understood. The current investigation has revealed that either overexpression of COX-2 or exposure to PGE_2 can increase the apoptosis threshold in human lung adenocarcinoma cells by up-regulating the mcl-1 gene. It also found the PI3K/Akt signaling pathway to be involved in regulating McI-1 expression. This work verifies a new downstream agent for COX-2. That verification will help researchers to understand better the precise mechanism of the COX-2-mediated carcinogenic process.

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