Cyclooxygenase-2 Overexpression Inhibits Platelet-derived Growth Factor-induced Mesangial Cell Proliferation through Induction of the Tumor Suppressor Gene p53 and the Cyclin-dependent Kinase Inhibitors p21waf-1/cip-1 and p27kip-1*

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Cyclooxygenase-2 (COX-2) is an inducible enzyme and serves as a source of paracrine prostaglandin E2 (PGE2) formation in many tissues. In glomerular immune injury, COX-2 formation is up-regulated in association with increased mesangial cell growth. To examine whether COX-2 exerts growth modulating effects on glomerular cells, we established two separate COX-2-overexpressing mesangial cell lines (COX-2+/+) and assessed their proliferative response to the potent mesangial cell growth-promoting factor, platelet-derived growth factor (PDGF). PDGF increased proliferation in mock-transfected cells. In contrast, PDGF did not induce proliferation in COX-2+ cells. Our results also showed that the tumor suppressor protein p53 and the cyclin-dependent kinase inhibitors p21cip-1 and p27kip-1 were up-regulated in COX-2+ cells de novo as well as under PDGF-stimulated conditions. To study whether COX-2 products are required for these effects, COX-2+ cells were treated with indomethacin (1 μg/ml) or NS-398 (3 μM). Unexpectedly, both COX inhibitors had no significant effect on cell proliferation, not on the protein levels of p53, p21cip-1, or p27kip-1. To evaluate the role of p21cip-1 and p27kip-1, COX-2 was overexpressed in mesangial cells derived from p21cip-1−/− (p21−/−/COX-2+) and p27kip-1−/−/COX-2+ (p27−/−/COX-2+) null mice. In contrast to the wild type COX-2+ cells, p21−/−/COX-2+ and p27−/−/COX-2+ cells proliferated in response to PDGF. These data suggest that COX-2 inhibits mesangial cell proliferation by a novel mechanism that is independent of prostaglandin synthesis, but involves p53, p21cip-1, and p27kip-1.

The rate-limiting enzymes in the formation of prostaglandins are the cyclooxygenases (1). Two cyclooxygenase isoforms, called cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), are currently known. The COX-1 isoenzyme is constitutively expressed in many tissues and is assumed to be responsible for the physiological functions of prostaglandins (PG) such as maintenance of the integrity of gastric mucosa and regulation of renal blood flow (2). In contrast, COX-2 is an immediate early response gene that is undetectable in most mammalian tissues, but is rapidly induced by proinflammatory cytokines, growth factors, and tumor promoters such as interleukin 1β (3), PDGF (4), and phorbol myristate acetate (5, 6). The subcellular localization of COX-1 and COX-2 is similar. Both isoenzymes are present in the endoplasmic reticulum as well as the outer and inner membranes of the nuclear envelope (7).

Classical nonsteroidal anti-inflammatory drugs such as indomethacin or aspirin inhibit both cyclooxygenases (8, 9). More recently, a new class of COX-2 specific inhibitors, such as NS-398 or SC-58635, has been characterized, which target cyclooxygenases more specifically and therefore may have specific treatment implications (10).

COX-2 gene expression is increased in proliferative diseases such as cancer and rheumatoid arthritis (11, 12). Moreover, COX-2 stimulates the proliferation of cancer cells in colorectal and gastric cancer via prostanooids in the complex growth regulation of resident glomerular cells. Many forms of glomerulonephritis are characterized by MC proliferation, and therefore understanding the mechanisms regulating this is critical in determining treatment strategies.

Proliferation is governed at the level of the cell cycle by specific cell cycle regulatory proteins. Proliferation requires that cyclins activate target cyclin-dependent kinases (CDK). In contrast, the CDK inhibitors p21cip-1 and p27kip-1 limit proliferation by inhibiting cyclin-CDK complexes. Studies have shown a role for specific CDK inhibitors in renal and non-renal diseases. To further delineate the role of the COX-2 isoform in governing specific cell cycle proteins, we stably overexpressed COX-2 in mesangial cells derived from wild type rats and p21cip-1−/− mice or p27kip-1−/− mice. Our studies demonstrated that COX-2 overexpression inhibits PDGF-induced proliferation of wild type MC. Moreover, COX-2 overexpression increases the expression of the tumor suppressor p53 and the CDK inhibitors p21cip-1 and p27kip-1 in a prostaglandin-independent manner. COX-2 overexpression also increased p53 in p21−/− and p27−/− MCs. These data suggest that COX-2 inhibits the growth of MC by a novel mechanism, independent
of prostaglandin synthesis, but involves p53 as well as the concerted action of p21cip-1 and p27kip-1.

MATERIALS AND METHODS

COX-2 Expression Plasmid, Cell Culture, and Stable Transfection—An EcoRI/KpnI full-length PCR construct of rat COX-2 (17) was cloned into the mammalian expression vector pcDNA3.1-Zeo (Invitrogen). This plasmid was sequenced to confirm the identity and orientation of rat COX-2. Wild type rat MC cultures were established from glomeruli isolated from kidneys of male Sprague-Dawley rats (80–100 g of body weight) by differential sieving as previously described (16). For transfection, 2 × 10⁵ rat MC between passage 15–18 or mouse MC from p21−/− (18) or p27−/− (19) mice were seeded in RPMI 1640 media containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.66 units/ml bovine insulin (Invitrogen). A mixture of 5 µg of plasmid and 20 µg/ml Lipofectin (Invitrogen) in RPMI 1640 without penicillin and streptomycin and FCS were added to the 70–80% confluent MCs for 6 h at 37 °C 5% CO₂. Cells were maintained in normal 10% FCS RPMI 1640 growth media for 48 h before selection was started by adding 200 µg/ml zeocin (Invitrogen) for 2 weeks. Single cell clones of stable transfected MCs were established through limiting dilution and were cultured in 10% FCS RPMI 1640 supplemented with 100 µg/ml zeocin at 37 °C in 5% CO₂.

FACS Analysis—To characterize the transfected cell lines as mesangial cells, the cell surface antigen Thy1.1 was detected. Rat MCs were trypsinized, washed in 1× PBS, and incubated for half an hour with a 1:100 dilution of FITC-labeled mouse-anti rat Thy 1.1 (BD Biosciences). For detection of intracellular desmin, rat MCs were trypsinized and washed with 1× PBS. The cells were then resuspended in 0.25 ml cytofix/cytoperm solution (BD Biosciences) for 20 min at 4 °C. Cells were then washed twice in 1× wash/cytoperm solution (BD Biosciences) before a 1:50 dilution of mouse-anti human desmin in 1× wash/cytoperm solution containing 5% bovine serum albumin was added for half an hour at 4 °C. After washing, cells were incubated in a 1:100 dilution of FITC-labeled goat-anti mouse IgG (BD Bioscience) for 30 min at 4 °C in the dark. FACS analysis was performed with FACS.
Calibur (BD Bioscience). Data were analyzed with the FACScomp software.

Western Blot Analysis—Cells were washed with 1 × PBS and lysed in 1 × cell lysis buffer (150 mM Tris-HCl, pH 6.8, 6.6% SDS). Equal amounts of protein were treated with 0.25 volume reducing buffer (50% mercaptoethanol, 50% glycerol) as well as 0.20 volume gel loading buffer (42.5% glycerol, 0.05% bromphenol blue), and samples were boiled for 10 min. The solution was loaded onto a 12% polyacrylamide SDS gel and electrophoresed at a constant current of 20 mA for 4 h. A molecular mass marker (10.0–250 kDa, Amersham Biosciences, Inc.), was run in parallel. After completion of electrophoresis, proteins were electroblotted semidry (blotting buffer: 25 mM Tris, 200 mM glycine, 20% methanol) for 1 h at 1 mA/cm² onto a PVDF membrane (Hybond ECL, Amersham Biosciences, Inc.). The membrane was blocked in 5% nonfat dry milk in washing buffer (1 × PBS, 0.1% Tween 20) for 1 h at room temperature and then incubated for another hour with the primary antibody in the same buffer. The following primary antibodies were used: anti-human COX-2, anti-human CDK-2, and p27kip-1 were obtained from Transduction Laboratories; anti-human p53 and p21cip-1 were purchased from PharMingen. All primary antibodies were used in a dilution of 1:1000. After rinsing the membrane in washing buffer for 2 × 10 min, an anti-mouse-IgG antibody conjugated to alkaline phosphatase (Southern Biotechnology) was added at a concentration of 1:1000. Detection of the alkaline phosphatase activity was performed with CDP-Star (Tropix) in an assay buffer (10 mM Tris HCl, pH 9.6, 150 mM NaCl, 50 mM MgCl₂) according to the manufacturer’s recommendations. Chemiluminescence detection of the Blots as well as densitometric evaluation were performed with the FluorS imager system (Bio-Rad).
**PGE2-ELISA and Cell Proliferation Assay**—The extracellular PGE2 content was measured by a PGE2 ELISA obtained from Cayman Chemicals according to the manufacturer’s recommendations. Cells (5 × 10^4 cells/well) were plated on 96-well plates (Nunc) and maintained in RPMI 1640 medium supplemented with 10% FCS overnight. In one set of experiments, the media was changed to serum-free media for 48 h. DNA synthesis was measured by [3H]thymidine incorporation. In the control group 2 Ci/ml [3H]thymidine (90 Ci/mmol; Amersham Biosciences, Inc.) was added to the serum-free media for 4 and 24 h. [3H]thymidine incorporation of the appropriate control group was compared with cells that were additionally treated with 50 ng/ml PDGF for 4 and 24 h.

In a second set of experiments, cells were grown in serum-free media in which 3 µM NS-398 (Alexis Biochemicals) or 1 µg/ml indomethacin (Sigma) was added for 48 h. 2 µCi/ml [3H]thymidine was added for 4 and 24 h in serum-free medium in the presence of either 3 µM NS-398 or 1 µg/ml indomethacin. The media of the control cells was changed to serum-free media for 48 h, and subsequently 2 µCi/ml [3H]thymidine was added for 4 and 24 h. At the end of the incubation period, cells were washed twice with 1× PBS and then trypsinized. The cell suspensions were subsequently harvested onto a filterpaper (Whatman) using an automated cell harvester (Dynatech) before [3H]thymidine incorporation was measured in a β-scintillation counter (Packard).

**Non-radioactive Northern Blot Analysis**—Cells were washed twice with sterile 1× PBS and then directly solubilized in 5 ml of buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium lauroyl-sarcosinate, and 0.7% β-mercaptoethanol. RNA was extracted by repetitive phenol-chloroform extraction and precipitated with ice-cold isopropanol (20). The quantity and purity of the preparations were assessed by measurement of absorption at 260 and 280 nm. To separate total RNA, 20 µg was denatured in formamide-formaldehyde and loaded onto a 1.2% agarose gel containing 2.2 M formaldehyde. RNA was vacuum blotted to a nylon membrane (Hybond-N, Amersham Biosciences, Inc.) and UV-cross-linked. Prehybridization was performed at 50°C for 1 h in specific non-radioactive hybridization buffer (Roche Molecular Biochemicals). A digoxigenin-labeled p21cip-1 cDNA fragment was added and hybridized overnight at 50°C. The membranes were washed once in 250 mM sodium-phosphate buffer, pH 7.0, and 1% SDS for 15 min at 65°C and subsequently twice in 100 mM sodium-phosphate buffer, pH 7.0, 1% SDS 15 min at 65°C. Detection of the digoxigenated hybrids were performed with a chemiluminescence based northern hybridization detection kit (Roche Molecular Biochemicals). A digoxigenin-labeled p21cip-1 cDNA fragment was added and hybridized overnight at 50°C. The membranes were exposed on X-ray films (Hyperfilm ECL; Amersham Biosciences, Inc.) at room temperature without intensifying screen. Membranes were stripped for 1 h in 5× Tris-HCl, pH 8.0, 0.5% sodium pyrophosphate, 5× Denhardt’s (100× denhardt’s:2% Ficoll 400, 2% polyvinylpyrrolidone, 2% bovine serum albumin), and 0.2 mM EDTA at 65°C and reblood with a digoxigenin-labeled 2.0-kb human cDNA probe of 18 S rRNA to account for small RNA loading and transfer variabilities. Exposed films were scanned with a FluorS-imager system (Bio-Rad). The intensities of the hybridization signals were normalized to 18 S rRNA.

**FIG. 4.** A, COX-2 overexpression stimulates p53, p21cip-1. Western analysis of the tumor suppressor p53 and the CDK inhibitor p21cip-1 are shown. Four h of PDGF exposure maximally stimulates p53 protein expression in both COX-2+ cell lines (3.08 ± 0.41-fold and 8.24 ± 2.41-fold). Protein expression of p21cip-1 is also strongly enhanced in untreated and PDGF-treated COX-2+ #1 and #2 cells and varies between 3.6 ± 0.95-fold and 4.61 ± 0.71-fold. Both COX-2+ cell lines were compared with the appropriately treated mock-transfected control cell line. B, expression of p21cip-1 was stimulated on mRNA levels. The mRNA expression of p21cip-1 was stimulated under serum-free conditions in COX-2+ cells. PDGF stimulation for 1 and 4 h significantly reduced p21cip-1 mRNA expression in control cells, whereas the appropriate mRNA expression of COX-2+ cells were only slightly affected.
Statistical Analysis—All values are presented as means ± standard deviation (S.D.). All experiments were repeated a minimum of three times. Statistical significance between individual groups were tested using the nonparametric unpaired Mann-Whitney U test. A p value of < 0.05 was considered significant.

RESULTS

Characterization of the COX-2 Overexpressing MC Lines—It is important to compare the nature of the genetically engineered cells with original rat MCs. Fig. 1, A–C shows the morphology of hematoxylin-stained rat MCs (A), mock-transfected control rat MC (B), and rat COX-2+ cells (C). The shape of all cell lines tested were similar, but COX-2+ cells (C) were smaller in size compared with mock-transfected control cells (B) and original rat MCs (A). Thy1.1 expression was measured by FACS analysis to confirm the mesangial cell origin of each cell line. Fig. 1, D–F depict Thy1.1 expression of rat MC (D), mock transfected control rat MC (E), and COX-2+ rat MC (F). All three cell lines expressed Thy1.1. Interestingly, COX-2+ cells had an approximate 10-fold increase in Thy1.1 compared with untreated MC and mock-transfected cells. Because we only utilized the Thy1.1 expression to characterize the cells, we did not further evaluate this phenomenon. Furthermore, Fig. 1, G–I demonstrates that all cell lines examined expressed significant amounts of desmin.

We selected two independent isolated clones of wild type COX-2+ rat MC lines, called COX-2+ #1 and COX-2+ #2. Fig. 2A shows that compared with mock-transfected control cells, COX-2 levels were increased 5-fold in stably transfected COX-2+ #1 and #2 cells. Furthermore, the production of PGE2 (21) was also significantly elevated in both COX-2+ cell lines (1.76 ± 0.09-fold for COX-2+ #1 and 2.91 ± 0.28-fold for COX-2+ #2) compared with control cells (Fig. 2B).

Proliferation after PDGF Stimulation—Studies have shown that PGE2 is anti-proliferative for MC (15, 16), whereas PDGF is proliferative (4). PDGF significantly increased DNA synthesis in mock-transfected control cells at 4 h (2.86 ± 0.46-fold) and 24 h (2.05 ± 0.07-fold) compared with mock-transfected control cells not exposed to PDGF.

To determine the role of COX-2 overexpression on basal levels of growth, proliferation was compared in COX-2+ overexpressing and mock-transfected cells when grown in serum-free media. COX-2+ #1 and #2 cell lines had a significantly reduced proliferative capacity at 4 h (0.45 ± 0.06 versus 0.65 ± 0.1) and 24 h (0.31 ± 0.08 versus 0.53 ± 0.08) compared with mock-transfected control cells. To test the hypothesis that COX-2 overexpression limits PDGF-induced proliferation, DNA synthesis was also measured at 4 and 24 h after exposure to PDGF, and the results are shown in Fig. 3, A and B. PDGF did not increase proliferation in COX-2+ #1 (0.63 ± 0.12) and COX-2+ #2 (0.68 ± 0.14) cells at 4 h. Although there was a mild increase in [3H]thymidine incorporation in both COX-2+ cell lines (0.53 ± 0.06 for COX-2+ #1; 0.66 ± 0.02 for COX-2+ #2) in response to PDGF stimulation at 24 h, [3H]thymidine incorporation was significantly reduced compared with PDGF stimulation of mock-transfected controls.

Cell proliferation was also assessed by cell count in serum-starved COX-2+ #1 and #2 cells, and mock-transfected control cells in the presence or absence of PDGF stimulation. As shown in Fig. 3C, cell number in PDGF-stimulated control cells was increased to 1.74 ± 0.04-fold. In contrast, there was no statistically significant increase in cell number in both COX-2+ cell lines (1.1 ± 0.12 and 1.14 ± 0.08) compared with control cells.

COX-2 Overexpression Increases the Expression of p53, p21cip-1, and p27kip-1 but Does Not Influence CDK-2 Protein

Fig. 4—continued. C, COX-2 overexpression promotes p27kip-1. Western analysis of the CDK inhibitor p27kip-1. Four h of PDGF stimulation maximally reduced p27kip-1 protein expression in control cells. In both COX-2+ cell lines, p27kip-1 protein stability does not significantly change following PDGF stimulation. Thus, p27kip-1 protein levels were increased in both COX-2+ cell lines when compared with the corresponding mock-transfected control cell line. D, COX-2 overexpression does not influence protein expression of CDK-2. Western analysis of CDK-2 is shown. CDK-2 protein expression in mock-transfected control cells and in both COX-2+ cell lines was not influenced under basal conditions and by 4 h of PDGF stimulation.
Expression—Because [3H] thymidine is incorporated in DNA during the S-phase of the cell cycle, earlier events in the G1-phase of the cell cycle might be responsible for the anti-proliferative effects of COX-2 in MC. We next elucidated if the changes in proliferation induced by COX-2 were due to specific cell cycle regulatory proteins. Fig. 4A shows a representative immunoblot for p53 and p21cip-1. Under serum-free conditions, protein expression for p53 was significantly increased in COX-2/H11001#1 cells (1.97 ± 0.43-fold) and COX-2/H11001#2 cells (2.37 ± 0.18-fold) compared with control cells. The addition of 50 ng/ml PDGF for 1 h augmented the increase in p53 protein expression in COX-2/H11001#1 (2.23 ± 0.85-fold) and COX-2/H11001#2 (2.88 ± 0.74-fold) cell lines compared with PDGF-stimulated control cells. The maximal effect on p53 protein expression was detected when COX-2/H11001#1 and #2 cells were stimulated with PDGF for 4 h (3.08 ± 0.41 and 8.24 ± 2.4-fold) compared with PDGF-treated control cells.

Our results also showed that the protein expression for p21cip-1 was markedly increased in serum-free conditions in COX-2/H11001#1 (3.97 ± 1.52-fold) and #2 cell line (4.19 ± 1.43-fold) compared with untreated control cells. Compared with control cells, p21cip-1 levels remained significantly elevated following

Table I

Summary of the COX inhibitor effects on the protein expression of p53 and the two CDK inhibitors with or without PDGF in COX-2+ cells. Indomethacin and the specific COX-2 inhibitor NS-398 diminished protein expression of all proteins studied in both COX-2+ cell lines when compared to the corresponding cell line without any COX inhibitor. The protein expression of COX inhibitor treated COX-2+ cells, however, remained significantly increased (p < 0.05 versus controls). All data were normalized to mock-transfected control cells after 4h of PDGF treatment and without COX inhibitors or to mock-transfected control cells without any PDGF and without any COX-inhibitors respectively.
PDGF stimulation in COX-2+ #1 cells at 1 h (2.79 ± 0.71-fold) and 4 h (4.21 ± 2.1-fold) and in COX-2+ #2 cells at 1 h (4.61 ± 0.71-fold) and 4 h (3.6 ± 0.95-fold).

Since p53 transactivates transcription of the p21<sup>cip-1</sup> gene, p21<sup>cip-1</sup> mRNA expression was measured (Fig. 4B). Under serum-free conditions the p21<sup>cip-1</sup> mRNA expression was increased in COX-2 #1 and #2 cells. The addition of PDGF for 1 and 4 h markedly decreased the mRNA expression of p21<sup>cip-1</sup> in mock-transfected control cells. In contrast, p21<sup>cip-1</sup> mRNA levels were only mildly affected in COX-2 #1 and #2 cells. Thus, compared with control cells, the p21<sup>cip-1</sup>-1 mRNA content of both COX-2+ cell lines were markedly increased after 1 h of PDGF stimulation (3.37 ± 0.74-fold, for #1) and 4.19 ± 0.42-fold, for #2) and 4 h of PDGF stimulation (2.19 ± 0.39-fold, for #1 and 2.65 ± 0.35-fold, for #2).

Growth arrest is also influenced by the CDK inhibitor p27<sup>kip-1</sup>, which is p53-independent. Fig. 4C shows that PDGF significantly reduced p27<sup>kip-1</sup> protein expression in control cells. In contrast, PDGF stimulation was associated with increased p27<sup>kip-1</sup> protein content in COX-2+ #1 and #2 cell lines (2.83 ± 0.97 and 2.2 ± 0.28-fold after 4 h of PDGF).

Cyclin/CDK-2 complexes are essential mediators of the cell cycle progression and are pivotal in G<sub>1</sub>-S-phase transition (22). Fig. 4D shows that CDK-2 levels did not change in both COX-2+ cell lines compared with mock-transfected control cell line. This result contrasts to the increased protein expression of the tumor suppressor p53 and the CDK inhibitors p21<sup>cip-1</sup> and p27<sup>kip-1</sup> under serum-free conditions and in the presence of PDGF.

**Inhibition of COX-2 Does Not Influence Proliferation**—To test whether prostaglandins were responsible for the growth inhibitory effects and the levels of p53, p21<sup>cip-1</sup>, and p27<sup>kip-1</sup>, prostaglandin formation was inhibited either with indomethacin (1 μg/ml), a nonspecific cyclooxygenase inhibitor, and with NS-398 (3 μM), a COX-2-specific cyclooxygenase inhibitor for 48 h. As demonstrated in Fig. 5, neither indomethacin nor NS-398 prevented cell cycle arrest due to the enhanced COX-2 expression. Moreover, neither indomethacin nor NS398 significantly reduced p53, p21<sup>cip-1</sup> and p27<sup>kip-1</sup> protein expression in COX-2+ #1 and #2 cell lines (Table I).

**COX-2 Overexpression in p21<sup>−/−</sup> and p27<sup>−/−</sup> MCs Restores PDGF-induced Proliferation**—To examine the role for p21<sup>−/−</sup> and p27<sup>−/−</sup> in COX-2-induced growth inhibition in MCs, p21<sup>−/−</sup> and p27<sup>−/−</sup> null (-/-) MCs were stably transfected with COX-2, and the results are shown in Fig. 6. There was an increase in p53 levels in p21<sup>−/−</sup> and p27<sup>−/−</sup> MCs. When grown in serum-free media, p27<sup>kip-1</sup> protein expression increased in p21<sup>−/−</sup> cells, and p21<sup>−/−</sup> increased in p27<sup>−/−</sup> cells.

Fig. 7A compares [<sup>3</sup>H]thymidine incorporation in mock-transfected p21<sup>−/−</sup> MC with p21<sup>−/−</sup> MC overexpressing COX-2+ (named p21<sup>−/−</sup>-COX-2+) and mock-transfected p27<sup>−/−</sup> MC with p27<sup>−/−</sup> MC overexpressing COX-2 (named p27<sup>−/−</sup>-COX-2). COX-2 overexpression significantly decreased DNA synthesis in p21<sup>−/−</sup>-cells at 4 h (0.12 ± 0.005) and 24 h (0.27 ± 0.04). COX-2 overexpression also reduced [<sup>3</sup>H]thymidine incorporation in p27<sup>−/−</sup>-cells at 4 h (0.16 ± 0.03) and 24 h (0.34 ± 0.04).

Finally, proliferation (as assessed by [<sup>3</sup>H]thymidine incorporation) in PDGF-stimulated (4 and 24 h) wild type control, wild type COX-2+ #1, p27<sup>−/−</sup>-COX-2+, and p21<sup>−/−</sup>-COX-2+ cell lines were compared with the corresponding nonstimulated cell line (Fig. 7B). PDGF-induced DNA synthesis in wild type control cells (3 ± 0.52-fold at 4 h and 2.05 ± 0.07-fold at 24 h). This effect was less pronounced in wild type COX-2+ #1 cells (1.28 ± 0.15-fold at 4 h and 1.4 ± 0.23-fold at 24 h). In contrast to wild type COX-2+ #1 cells, PDGF-stimulated DNA synthesis in p27<sup>−/−</sup>-COX-2+ cells (2.66 ± 0.32-fold at 4 h and 2.02 ± 0.11-fold at 24 h) and in p21<sup>−/−</sup>-COX-2+ cells (2.28 ± 0.25-fold at 4 h and 2.23 ± 0.07-fold at 24 h).

**DISCUSSION**

In specific renal diseases such as glomerulonephritis, the formation of the cyclooxygenase product PGE2 is enhanced (23–26). This prostaglandin exerts anti-proliferative effects on MC (15, 16) and could therefore counteract the growth-promoting events following glomerular injury.

To further characterize the role of COX-2 on mesangial cell growth, cell lines were generated that stably overexpress COX-2. Two independently isolated single clones overexpressing COX-2, named COX-2+ #1 and #2, were used in all the studies described. Both cell lines expressed large amounts of COX-2 protein and synthesize more PGE2 than control cells. COX-2 overexpression did not alter cell morphology nor expression for the cytoskeletal filament desmin (15). The COX-2+ cell line expressed about 10-fold more cell surface Thy1.1 than untransfected and mock-transfected MCs. Because the Thy1.1 expression served to identify our modified cells as true MCs we did not further examine this observation.

The growth factor, PDGF-BB is a potent MC mitogen (4), and has also been shown to play a critical role in the pathogenesis of glomerulonephritis (27, 28). Cell growth in wild type COX-2+ cells was significantly inhibited compared with mock-transfected wild type control cells. Our results show that COX-2 effects likely appear before the S-phase of the cell cycle, since [<sup>3</sup>H]thymidine incorporation into newly synthesized DNA was reduced. Events during the G<sub>1</sub>-phase may explain these differences. For example, the tumor suppressor p53 is able to inhibit cell growth by transactivation of the CDK inhibitor.
p21cip-1 gene transcription, which acts exclusively in the G1-phase of the cell cycle and can induce apoptosis in the G1-phase. Both mechanisms could account for the reduced [3H]thymidine incorporation. This mechanism may be operative because the reintroduction of p53 in p53-deficient mouse embryonic fibroblasts inhibits COX-2 gene expression (29). Thus, a regulation loop between p53 and COX-2 might exist. In our system, the cyclooxygenase inhibitors the proliferation of both independently isolated COX-2+ cells unexpectedly remained unaltered compared with untreated COX-2+ #1 and #2 cells. On the other hand, the protein expression of p53, p21cip-1 and p27kip-1, other molecules involved in the anti-proliferative effect seen in this study. To examine the role of cyclooxygenase cells were either treated with indomethacin or NS-398 (9, 10). Our results showed that when [3H]thymidine incorporation was measured in the presence of the cyclooxygenase inhibitors the proliferation of both independently isolated COX-2+ cells unexpectedly remained unaltered compared with untreated COX-2+ #1 and #2 cells. On the other hand, the protein expression of p53, p21cip-1 and p27kip-1, was partially reduced in the presence of the cyclooxygenase inhibitors in both COX-2+ cell lines; however, it remained significantly increased compared with control cells. Interestingly, Trifan et al. (37) transiently overexpressed a COX-2-GFP chimeric protein in NIH 3T3 and COS-7 cells and showed cell

In contrast, CDK-2 protein expression remained unaffected following COX-2 overexpression. Thus, it seems that COX-2 influences cell cycle indirectly through CDK inhibitors of the cip/kip family rather than directly through influencing protein expression of CDK-2.

To confirm the potential interrelation between p21cip-1 and p27kip-1 in the blockade of PDGF-induced proliferation of COX-2, p21cip-1 (18), or p27kip-1 (19) null MCs, which overexpress COX-2 were established. Exposure of both COX-2+ knockout cell lines to PDGF resulted in a significant induction of proliferation. This suggests that both CDK inhibitors have to be expressed together to inhibit PDGF-induced proliferation. Moreover, the behavior of the COX-2+ CDK inhibitor knockout cell lines strongly argues against an artificial effect due to the overexpression of the COX-2 protein.

Since prostaglandins are known to reduce MC proliferation (15, 16) it was expected that COX products are responsible for the anti-proliferative effect seen in this study. To examine the role of cyclooxygenase cells were either treated with indomethacin or NS-398 (9, 10). Our results showed that when [3H]thymidine incorporation was measured in the presence of the cyclooxygenase inhibitors the proliferation of both independently isolated COX-2+ cells unexpectedly remained unaltered compared with untreated COX-2+ #1 and #2 cells. On the other hand, the protein expression of p53, p21cip-1 and p27kip-1, was partially reduced in the presence of the cyclooxygenase inhibitors in both COX-2+ cell lines; however, it remained significantly increased compared with control cells. Interestingly, Trifan et al. (37) transiently overexpressed a COX-2-GFP chimeric protein in NIH 3T3 and COS-7 cells and showed cell
cycle arrest, independent of cyclooxygenase inhibition. Moreover, COX-2 mutants devoid of cyclooxygenase activity exhibit the same effect as wild type COX-2.

These results of Trifan et al. and now by our group suggest that COX-2 exerts its anti-proliferative effect independently of cyclooxygenase activity. Furthermore, because p53, p21cip-1, and p27kip-1 were partially attenuated by cyclooxygenase inhibitors, other factors such as the retinoblastoma protein and their related factors and/or the INK4 family of CDK inhibitors, might contribute to this complex COX-2 mediated inhibition of proliferation. Therefore the concerted action of multiple factors are necessary to induce the COX-2-dependent cell cycle arrest obviously partially independent of cyclooxygenase products. Finally, these data suggest the contribution of a novel COX-2 mechanism that does not require the formation of prostaglandins.

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REFERENCES

1. Herschman, H. R. (1996) Biochim. Biophys. Acta 1299, 125–140
2. Smith, W. L., Gavito, R. M., and DeWitt, D. L. (1996) J. Biol. Chem. 271, 33157–33160
3. Rzymkiewicz, D., Leingang, K., Baird, N., and Morrison, A. R. (1994) Am. J. Physiol. 266, F39–F45
4. Abboud, H. E. (1992) Kidney Int. 41, 581–583
5. Hla, T., and Neilson, K. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388
6. Rzymkiewicz, D., DuMaine, J., and Morrison, A. R. (1995) Kidney Int. 47, 1354–1363
7. Spencer, A. G., Woods, J. W., Arakawa, T., Singer, L. L., and Smith, W. L. (1998) J. Biol. Chem. 273, 9886–9893
8. Smith, W. L., and DeWitt, D. L. (1996) in Advances in Immunology (Dixon, F. J., ed) Vol. 62, pp. 167–215, Academic Press, Orlando, FL
9. Laneville, O., Breuer, D. K., DeWitt, D. L., Hla, T., Funk, C. D., and Smith, W. L. (1994) J. Pharmacol. Exp. Ther. 271, 927–934
10. Hla, T., Rutili, M., Appleby, S., and Barriocanal, J. G. (1993) Ann. N. Y. Acad. Sci. 696, 197–204
11. Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., and Vane, J. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7563–7568
12. Dubois, R. N., Abrams, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Nan De Putte, L. B., and Lipsky, P. E. (1998) FASEB J. 12, 1063–1073
13. Hla, T., Bishop-Bailey, D., Liu, C. H., Schaefer, H., and Trifan, O. C. (1999) Int. J. Biochem. Cell Biol. 31, 551–557
14. Kawamori, T., Tao, C. V., Seibert, K., and Reddy B. S. (1998) Cancer Res. 58, 409–412
15. Mene, P., Abboud, H. E., and Dunn M. J. (1990) Kidney Int. 38, 232–239
16. Stahl, R. A. K., Thaiss, F., Haberstroh, U., Kah J., Shaw, A., and Schoeppe, W. (1999) Am. J. Physiol. 259, F419–F424
17. Kennedy, B. P., Chan, C. C., Culp, S. A., and Cromlish, W. A. (1993) Biochem. Biophys. Res. Commun. 197, 494–500
18. Kim, Y. G., Alper, C. E., Brugarellos, J., Johnson, R. J., Couser, W. G., and Shankland, S. J. (1999) Kidney Int. 55, 2349–2361
19. Kimura, K., Pippin, J. W., Fere, M. L., Roberts, J. M., and Shankland, S. J. (1999) J. Clin. Invest. 103, 597–604
20. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
21. Brock, T. G., McNish, R. W., and Peters-Golden, M. (1999) J. Biol. Chem. 274, 11660–11666
22. Nigg, E. A. (1995) Bioessays 17, 471–480
23. Lianos, E. A., Andres, G. A., and Dunn M. J. (1983) J. Clin. Invest. 72, 1439–1446
24. Stahl, R. A. K., Adler, S., Baker, P. J., Chen, Y. P., Protzl, P. M., and Couser, W. G. (1987) Kidney Int. 31, 1126–1131
25. Stork, J. E., and Dunn M. J. (1985) J. Pharmacol. Exp. Ther. 233, 672–678
26. Stahl, R. A. K., Kufeikia, S., Paraviczini, M., and Schollmeyer, F. (1986) Nephron. 42, 252–257
27. Okuda, S., Languin, L. R., Rooslayi, E., and Border, W. A. (1990) J. Clin. Invest. 86, 455–462
28. Border, W. A. Okuda, S., Languin, L. R., and Rooslayi, E. (1990) Kidney Int. 37, 689–695
29. Subbarajiah, K., Alterki, N., Chung, W. J., Mestre, J. R., Sampa, A., and Dannenberg, A. (1999) J. Biol. Chem. 274, 10911–10915
30. Goroese, M., Cirilli, C., Wang, X., Seth, P., Capogrossi, M. C., and Holbrook, N. J. (1997) Oncogene 14, 929–935
31. McGinty, A., Chang, Y. W., Serokin, A., Bekeneyer, D., and Dunn, M. J. (2000) J. Biol. Chem. 275, 12105–12109
32. Chang, Y. W., Jakobi, R., McGinty, A., Foschi, M., Dunn, M. J., and Serokin, A. (2000) Mol. Cell. Biol. 20, 8571–8579
33. Shankland, S. J., Pippin, J., Flanagan, M., Coats, S. R., Nangaku, M., Gordon, K. L., Roberts, J. M., Couser, W. G., and Johnson R. J. (1997) Kidney Int. 52, 1088–1099
34. Wolf, G., Schroeder, R., Thaiss, F., Ziyadeh, F. N., Helmchen, U., and Stahl, R. A. K. (1998) Kidney Int. 53, 869–879
35. Wolf, G., Schroeder, R., Ziyadeh, F. N., Thaiss, F., Zahnier, G., and Stahl, R. A. K. (1997) Am. J. Physiol. 273, F348–F356
36. Wolf, G., and Stahl, R. A. K. (1996) Kidney Int. 50, 2112–2119
37. Trifan, O. C., Smith, R. M., Thompson B. D., and Hla, T. (1999) J. Biol. Chem. 274, 34141–34147