Cyclooxygenase-2 Inhibits Tumor Necrosis Factor α-mediated Apoptosis in Renal Glomerular Mesangial Cells*

Received for publication, October 15, 2002, and in revised form, December 12, 2002
Published, JBC Papers in Press, January 1, 2003, DOI 10.1074/jbc.M210559200

Adiba Ishaque, Michael J. Dunn, and Andrey Sorokin‡

From the Department of Medicine and Cardiovascular Research Center, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Cyclooxygenase-2 (COX-2) is an inducible form of cyclooxygenase involved in chronic inflammation (12, 13). Several studies have highlighted an up-regulation of COX-2 expression in proliferative GN (14, 15). However, the precise role of COX-2 has not been investigated. It is plausible that COX-2 is responsible for the progression of proliferative GN by an anti-apoptotic mechanism. A growing body of evidence that COX-2 has an anti-apoptotic role in the pathogenesis of epithelial cell carcinomas, in particular colorectal cancer, supports this hypothesis (16). An overexpression of COX-2 conferred a survival advantage in rat intestinal epithelial cells by inhibiting apoptosis (17). COX-2 selective inhibitors induced apoptotic cell death in HCA-7, HT-29 (18), and CaCo-2 colon cancer cell lines, which constitutively expressed COX-2 (19).

Within the inflamed glomerulus, TNFα is produced locally by mesangial and epithelial cells as well as by infiltrating monocytes/macrophages (20). TNFα alone may be a key component for the resolution phase of glomerular inflammation and may enhance death receptor initiated-apoptosis of mesangial cells by an autocrine and/or paracrine mechanism. TNFα can also stimulate the release of other proinflammatory cytokines including IL-1β (21, 22). Endothelins, particularly ET-1, are mitogenic to mesangial cells in vivo and may act in concert with other vasoconstrictor peptides or cytokines to promote glomerular inflammation (23–25). Several studies utilizing rat primary cultures of renal mesangial cells (RMC) have demonstrated enhanced COX-2 protein levels induced by either ET-1 (26, 27) or IL-1β (28, 29). TNFα alone had little effect on COX-2 but the combination of TNFα plus IL-1β dramatically increased

Renal mesangial cell apoptosis is a crucial repair mechanism in glomerular nephritis (GN). These cells express receptors to tumor necrosis factor α (TNFα), a cytokine with proapoptotic properties implicated in the resolution of GN. Progression to proliferative GN is accompanied by cyclooxygenase-mediated formation of prostaglandins and inefficient apoptosis of mesangial cells. The aims of this study were to quantify TNFα-mediated apoptosis in renal mesangial cells and to determine whether expression of the inducible form of cyclooxygenase, cyclooxygenase-2 (COX-2), inhibits this apoptosis. By 24 h significant levels of apoptosis were induced by TNFα (100 ng/ml) or etoposide control (100 μM), as shown by phosphatidylserine externalization, caspase-3 activation, development of a sub-G0/G1 region, and distinct etoposide-mediated apoptosis. Using adenoviral-mediated delivery of the COX-2 gene (AdCOX-2) apoptotic features were prevented from appearing in AdCOX-2 cells treated with TNFα, whereas etoposide-treated AdCOX-2 cells were not protected. Furthermore, COX-2 expression, induced by the vasoconstrictor peptide ET-1 or the cytokine interleukin-1β also inhibited TNFα-mediated but not etoposide-mediated apoptosis, to an extent, similar to adenosine COX-2 infection. Selective COX-2 inhibition by NS-398 restored TNFα-mediated apoptosis. Prostaglandin (PG) E2 and PGl2 were shown to be the major prostaglandin metabolites in AdCOX-2 cells. The addition of PGE2 and PGI2 protected against TNFα-mediated apoptosis. These results demonstrate COX-2 anti-apoptotic activity via a death receptor route and suggest that selective COX-2 inhibition may augment TNFα apoptosis in chronic inflammatory conditions.

Mesangial cells line the blood vessels of the renal glomerulus, provide structural support, and regulate glomerular ultrafiltration (1, 2). Importantly mesangial cells undergo a change in phenotype during glomerular inflammation in which they become proliferative and matrix-secreting myofibroblasts before they are eliminated by apoptosis (2–4). Apoptosis has been identified as the mechanism responsible for the deletion of excess myofibroblasts on completion of the inflammatory response in skin (5), liver (6), and renal glomerulus (7). In the glomeruli of patients experiencing acute glomerular inflammation, referred to as glomerular nephritis (GN), apoptotic bodies were detected as a compensatory response, instigated to counterbalance mesangial hypercellularity thereby permitting normal structure and function to return (8). Mesangial/myofibroblast apoptosis was also identified in the rat in vivo model of experimental GN, anti-Thy1.1 nephritis (7, 9, 10). Proliferative forms of GN are characterized by a dysregulation of mesangial cell apoptosis allowing a chronic secretion of proinflammatory stimuli and prostaglandins (PG), which leads to an excess deposition of extracellular matrix proteins, post-inflammatory scarring, and renal failure (7, 10). Proliferative GN remains a leading cause of end-stage renal failure (11). Consequently, to avoid progression of glomerular inflammatory disease it is important to define the mechanisms underlying the inhibition of mesangial cell apoptosis.

Cyclooxygenase-2 (COX-2) is an inducible form of cyclooxygenase involved in chronic inflammation (12, 13). Several studies have highlighted an up-regulation of COX-2 expression in proliferative GN (14, 15). However, the precise role of COX-2 has not been investigated. It is plausible that COX-2 is responsible for the progression of proliferative GN by an anti-apoptotic mechanism. A growing body of evidence that COX-2 has an anti-apoptotic role in the pathogenesis of epithelial cell carcinomas, in particular colorectal cancer, supports this hypothesis (16). An overexpression of COX-2 conferred a survival advantage in rat intestinal epithelial cells by inhibiting apoptosis (17). COX-2 selective inhibitors induced apoptotic cell death in HCA-7, HT-29 (18), and CaCo-2 colon cancer cell lines, which constitutively expressed COX-2 (19).

Within the inflamed glomerulus, TNFα is produced locally by mesangial and epithelial cells as well as by infiltrating monocytes/macrophages (20). TNFα alone may be a key component for the resolution phase of glomerular inflammation and may enhance death receptor initiated-apoptosis of mesangial cells by an autocrine and/or paracrine mechanism. TNFα can also stimulate the release of other proinflammatory cytokines including IL-1β (21, 22). Endothelins, particularly ET-1, are mitogenic to mesangial cells in vivo and may act in concert with other vasoconstrictor peptides or cytokines to promote glomerular inflammation (23–25). Several studies utilizing rat primary cultures of renal mesangial cells (RMC) have demonstrated enhanced COX-2 protein levels induced by either ET-1 (26, 27) or IL-1β (28, 29). TNFα alone had little effect on COX-2 but the combination of TNFα plus IL-1β dramatically increased
COX-2 expression (28). The contribution of TNFα may depend on its mediation of either one of two conflicting pathways; cell survival via activation of NF-κB (30) or caspase-mediated apoptosis (30, 31). In previous studies of RMC apoptosis, sensitivity to TNFα could only be achieved in the presence of cycloheximide or actinomycin D, to prevent synthesis of survival factors (32) or by a specific inhibition of NF-κB (33, 34).

The overall objective of this study was to determine whether COX-2 expression inhibits apoptosis in RMC. Because mesangial cells express TNFα receptors and represent a potential route of apoptosis induction in the resolution of proliferative GN, we evaluated the extent of this apoptosis, and its inhibition by COX-2. We first established a suitable cell culture model in which TNFα-mediated apoptosis was measured in multiple apoptosis assays. We then used two different methods to induce COX-2 expression, i.e. induction by ET-1, or IL-1β, and forced expression using adenoviral-mediated gene transfer. We also investigated the effects of PGE₂ and PGF₂ on TNFα-mediated apoptosis, after establishing that those PGs were the major PG metabolites generated by COX-2 overexpression. Using these multiple approaches combined with several apoptosis assays, an anti-apoptotic effect of COX-2 on TNFα-mediated apoptosis was conclusively demonstrated in renal mesangial cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant IL-1β was obtained from R&D Systems (Minneapolis, MN). Recombinant ET-1 was obtained from Calbiochem (La Jolla, CA). Annexin V and the caspase-3 apoptosis assay detection kits were purchased from Oncogene Research Products (Boston, MA) and BD Pharmingen, respectively. NS398 was obtained from Cayman Chemical (Ann Arbor, MI). RPMI 1640 medium and fetal bovine serum were from Invitrogen. Polyclonal goat and polyclonal rabbit anti-COX-2 (N-20) and bcl-2 (N-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated donkey anti-goat and goat anti-rabbit immunoglobulins (IgGs) were from Bio-Rad. Protein was determined by a BCA assay from Pierce (Rockford, IL). All other reagents were obtained from Sigma. Flow cytometry experiments were carried out using a BD Biosciences FACS calibur (Mountain View, CA). In all experiments 10,000 live cells, based on light scatter properties, were gated and analyzed using Cell Quest Software (BD Biosciences).

Cell Culture—Primary glomerular mesangial cells from male Sprague-Dawley rats were isolated and characterized as previously described (35, 36). RMC were maintained in RPMI 1640 medium containing 17% heat-inactivated fetal bovine serum, 5 μg/ml each of insulin and transferrin, and 5 ng/ml sodium selenite, 100 μg/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified incubator (5% CO₂, 95% air). All experiments were performed with cells cultured in 60-mm dishes and used at 8–20 passages. After the cells were grown to ~60% confluence they were starved for 24 h in basal RPMI 1640 culture medium before the experiments.

Adenovirus-mediated COX-2 Gene Transfer—The recombinant adenoviral vectors (Ad) AdCOX-2 and Ad wild-type (AdWT) expressing the COX-2 and empty adenovirus vector, respectively, were constructed from the replication-deficient adenovirus type 5 (Ad5) as previously described (37). RMC were incubated with AdCOX-2 or AdWT (at a multiplicity of infection (m.o.i.) of 200) for 1 h at 37 °C with periodic shaking, followed by addition of complete medium. At 24 h after infection, cells were lysed for Western blot analysis. Efficiency of gene transfer in RMC was determined by adenoviral infection of green fluorescent protein at an m.o.i. of 200 and visualized by fluorescence microscopy at 24 h.

Western Blot Analysis—Cells were washed in ice-cold phosphate-buffered saline (PBS) and then harvested in lysis buffer as previously described (35). Cleared total cell lysates (20–40 μg) were resolved by criterion SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes (Micron Separation Inc.). Equal protein loading was confirmed by staining the membranes with Ponceau S, which stains all the pro-
teins on the membrane; an intensely stained band of a distinct molecular weight sufficiently different from the protein under investigation was selected from each membrane, to show that the amount of protein in each lane was identical. Membranes were probed with either COX-2 (1:1000 dilution) or bcl-2 (1:300 dilution) antibodies overnight at 4 °C. Primary antibodies were detected with goat anti-rabbit IgG-horseradish peroxidase conjugates for bcl-2 identification or anti-goat IgG-horseradish peroxidase antibodies for COX-2 detection (1:2000 dilution), followed by extensive washing of the membranes. The membranes were visualized by enhanced chemiluminescence (Amersham Biosciences).

Analysis of PG Production—RMC were infected with AdCOX-2 or AdWT as described above, but in this instance the conditioned medium was replaced with new basal medium devoid of any previously formed PGs. After the 24-h infection period, the supernatants were collected and the cell monolayers were washed twice with a HEPES-buffered medium (pH 7.4) and were incubated in this buffer containing the calcium ionophore A23187 (10 μM) for 15 min at 37 °C (38). The total cell lysates were run on a solid phase extraction device for analysis of PGE₂, PGL₃ (detected as 6-keto-PGF₁₅α), a stable product of PGI₃, PGI₂, PGI₃, and PGI₄ prior to measurement by liquid chromatography mass spectrometry (39). Internal standards, 1.0 ng each of d₄-PGE₂, d₄-PGF₁₅α, d₄-PGF₁₅α, d₆-keto-PGF₁₅α, d₄-PGD₂, or d₄-PGI₃, were added to the samples followed by ethanol, to give a final concentration of 15% in the samples. Glacial acetic acid (10 μl/ml) was then added. The samples were sonicated and vortexed three times before centrifugation at 300 × g, at room temperature for 5 min. The supernatants were loaded onto the solid phase extraction columns (C18Bond Elut SPE columns) that had been preconditioned with 5 ml of ethanol and 15 ml of water. The columns were washed with 29 ml of water and allowed to run dry. Then the PGs were eluted from the column with 6 ml of ethyl acetate. A vacuum was applied to a completely dry column. The top layer of ethyl acetate was removed from the water layer at the bottom of the reaction tubes. The compounds of interest were in the ethyl acetate layer. The water layer was extracted twice with 1 ml of ethyl acetate. The resulting two ethyl acetate portions were combined for each sample and dried under a stream of nitrogen gas until completely dry. The sample was then redissolved in 20 μl of acetonitrile and transferred to an insert in the sample vial.

The derivatized extracts were subject to liquid chromatography-mass spectrometry using LC-ESI-MS (Agilent 1100 LC/MSD, SL model) (39).
Concentrations of the different PGs were evaluated by comparing their ratios of peak areas to the standard curves. Results were expressed as picograms per milligram of protein per dish.

Morphological Analysis of Apoptosis—RMC infected with AdWT or AdCOX-2 (mo.i. of 200) for 24 h were incubated with TNFα (100 ng/ml) or etoposide (100 μM). After treatment, cells were morphologically assessed for apoptosis by acridine orange (AO) staining using inverted fluorescence microscopy. The cell monolayers were washed in PBS and incubated with AO in PBS (10 μg/ml) for ~2 min. Typically 3 fields were randomly selected from each 60-mm dish so that at least 80 RMC were counted. All cells produced a green fluorescence in response to a high affinity binding of AO to DNA. Apoptotic green cells were scored by their distinct morphology of cellular shrinkage and chromatin condensation. Attached apoptotic cells were scored as a percentage of the total number of cells counted for each dish.

Cell Cycle Analysis—RMCs (5 × 10⁵ cells) infected with either AdWT or AdCOX-2 were incubated with TNFα (100 ng/ml) or etoposide (100 μM). At the end of the incubation time the supernatants were collected. The remaining cell monolayer was washed in Hanks’ buffered saline solution without Ca²⁺ and Mg²⁺, and then incubated in a solution of trypsin-EDTA at 37°C. The reaction was terminated by addition of basal media. The trypsinized cells were then added to the previously collected culture supernatants. This cell suspension was centrifuged at 179 × g, 4°C, for 5 min, and the resulting pellet was resuspended in PBS. The cell suspension was centrifuged again at 179 × g, 4°C, and the pellet was resuspended in 70% ice-cold ethanol. Cells fixed in ethanol were stored at −20°C for up to 3 days. The cells were precipitated from the ethanol by high-speed centrifugation at 358 × g, 4°C, for 15 min. The pellet was resuspended in PBS and centrifuged again at 358 × g, 4°C, for 15 min. The final pellet was resuspended in 1 ml of RNase A (prepared in PBS without Mg²⁺ or Ca²⁺) and heat treated to inactivate DNase) to give a final concentration of 250 μg/ml. The mixture was incubated at 37°C for 30 min. At the end of the incubation propidium iodide (PI) (1 mg/ml) was added to give a final concentration of 50 μg/ml. The suspension was incubated at room temperature for 10 min in the dark. The cells were then analyzed by flow cytometry.

Annexin-V FITC and Propidium Iodide Double Staining—RMCs (5 × 10⁵ cells) infected with either AdWT or AdCOX-2 were treated with TNFα (100 ng/ml) or etoposide (100 μM) and stained with annexin-V (AV) labeled to FITC in combination with PI, for FACS analysis of apoptosis. FITC-AV/PI staining was optimized for attached cells according to the instructions outlined by the manufacturers of the AV assay kit (Oncogene Research Products).

Caspase-3 Assay—FITC conjugated to a monoconal rabbit antibody raised against the active fragment of caspase-3 was also used to determine apoptosis in AdWT or AdCOX-2 cells treated with either TNFα (100 ng/ml) or etoposide (100 μM), following cell permeabilization and fixation according to the instructions outlined by the manufacturer.

FITC-AV/PI and active-caspase-3-FITC staining was also carried out on cells stimulated to express COX-2 by the addition of ET-1 (100 nm) or IL-1β (2 ng/ml) and on cells treated with either PGE₂, (500 nm) or PGJ₂ (500 nm) to investigate the effects of these various treatments on TNFα-(100 ng/ml) or etoposide-(100 μM) mediated apoptosis. For PG addition, conditioned media was removed and replaced with fresh basal media to ensure removal of any existing PGs. PGs were readministered after 6 and 12 h of co-incubation with the apoptotic inducers to replenish depleted PGs. Effect of NS398 (25 μM) on ET-1 protection of apoptosis was also assessed by these assays. Control cells received Me₂SO at the same concentration and time of incubation as PGE₂ (500 nm) and NS398 (25 μM).

RESULTS

Characterization of COX-2 Overexpression by Adenovirus-mediated Infection—Previous work from this laboratory demonstrated a transient overexpression of COX-2 in SV40-transformed human mesangial cells using an adenovirus-mediated transfer of COX-2 cDNA (37). In this study the AdCOX-2 construct was used to express COX-2 in rat primary RMC. The transgenic AdGFP construct showed ~100% transfection efficiency in RMC at 24 h postadenoviral infection, as visualized by fluorescence microscopy (Fig. 1B), compared with uninfected control cells (Fig. 1A). The ability of the virus to infect the cell is governed by specific cell receptors, and this infection process is not specific to the gene insert. Therefore AdGFP is a suitable control that can be used for assessing the level of expression by adenovirus-mediated gene delivery (40). Western blot analysis confirmed COX-2 protein expression, which was enhanced by a dose-dependent increase in AdCOX-2 after 24 h of infection (Fig. 1C). Certain regions of the kidney contain a higher constitutive expression of COX-2 than most tissues, however, mesangial cells are normally devoid of COX-2 (41). Uninfected RMC and RMC infected with the AdWT construct did not reveal any detectable COX-2 (Fig. 1C). In addition, we checked for anti-apoptotic bcl-2 protein in our system because...
an overexpression of COX-2 in colon cancer cells was accompanied by bcl-2 protein expression (18, 19). However, we could not detect bcl-2 in RMC transfected with AdWT or AdCOX-2 (Fig. 1D).

Inhibition of TNFα-mediated Apoptosis in RMC by an Overexpression of COX-2 as Determined by FITC-AV/PI Double Staining—Our first task was to develop an apoptosis model in RMC cultures. To conclusively demonstrate the elicitation of apoptotic cell death by a cytokine/receptor-mediated route, the effect of TNFα was compared with a cytotoxic insult using the DNA topoisomerase II inhibitor etoposide, which readily induces classic apoptotic changes such as phosphatidylserine (PS) externalization in different cell types. We found that TNFα at a dose of 100 ng/ml induced a time-dependent increase in apoptotic cells, which became significant by 24 h, and reached maximum effect by 40 h incubation, by which time, however, higher levels of necrotic cells were also appearing (results not shown). Lower doses of TNFα were ineffective at inducing cell death (results not shown). Because the 24-h incubation time frame imposed the earliest, significant induction of apoptosis by TNFα, we used it to compare an inhibition of apoptosis by COX-2 overexpression or following its up-regulation.

PS externalization is a characteristic hallmark of apoptotic cells, serving as a signal for their phagocytic recognition and removal in vivo (42). PS externalization was detected by FITC-AV binding in combination with PI to distinguish between viable (V), early membrane intact apoptotic (EA), and necrotic (N) cells. Because there is no phagocytic disposal mechanism in vitro apoptotic cells accumulate and continue to undergo degradation and membrane lysis. It was crucial, therefore, to include PI in the reaction, and highlight PS exposure on the surface of EA cells, and distinguish them from N cell populations, which may or may not have transited the process of apoptosis (43). By this analysis it was possible to both qualitatively determine viable, V (AV(−)/PI(−)), EA (AV(+)PI(−)), and necrotic (N) cell populations.

FITC-Caspase-3

**Fig. 4. Effect of COX-2 overexpression on caspase-3 activation.** AdWT- or AdCOX-2-infected cells were induced to apoptosis with either TNFα (100 ng/ml) or etoposide (100 μM) for 24 h and assayed for caspase-3 activation by flow cytometry. Histograms of cell count versus active caspase-3 labeled to FITC highlight the M1 and M2 regions, which represent cells with inactive and active caspase-3, respectively. Histograms are representative of three independent experiments.
and N (AV(− and +)PI(+)) cell fractions (Fig. 2) and simultaneously quantify this transition (Table I). AdWT cells incubated with either TNFα (100 ng/ml) or etoposide (100 μM) produced distinct fractions of EA cells (28 and 30%, respectively) (Table I and Fig. 2). However, some EA and N cell fractions were induced in control AdWT-infected cells implicating adenovirus-mediated cell death as a result of the infection procedure. Nevertheless the profile and extent of apoptosis induction by TNFα or etoposide in AdWT-infected cells was similar to uninfected cells treated with these inducers in which cell death progressed from V to EA to N cell populations. For cells overexpressing COX-2, the EA cell fraction induced in the AdWT cells by TNFα was significantly suppressed (Fig. 2). The annexin V assay highlighted a reduction in the maximum fraction of EA cells (from 28 to 6%) in AdCOX-2 cells treated with TNFα compared with AdWT control cultures (from 8 to 18%) (Fig. 3, A and G). Etoposide also increased the proportion of AdWT cells with apoptotic nuclear phenotype (from 8 to 28%) (Fig. 3, C and G). AdCOX-2 cells treated with TNFα were prevented from undergoing chromatin condensation (Fig. 3E), and appeared morphologically identical to AdCOX-2 control cells (Fig. 3D). TNFα-induced apoptosis was significantly reduced (from 18 to 6%) in cells overexpressing COX-2 compared with AdWT cells (Fig. 3G). By contrast etoposide-treated AdCOX-2 cells were not protected from apoptosis (Fig. 3F) and high levels of apoptotic cells were still observed (Fig. 3G).
Inhibition of Caspase-3 Activity by an Overexpression of COX-2

Caspase-3 activation is a specific biochemical event in apoptosis, responsible for cleaving cellular substrates that lead to characteristic apoptotic morphology (46). It was measured by FACS analysis of FITC conjugated to a monoclonal antibody raised against the active fragment of caspase-3. A marked increase in the fraction of active caspase-3-FITC positive AdWT cells treated with either TNFα (100 ng/ml) (from 7 to 22%) or etoposide (100 μM) (from 7 to 35%) was observed by 24 h (Fig. 4). Caspase-3 inactive cells were the predominant cell population (90%) in control AdWT and uninfected cells (Fig. 4). For cells overexpressing COX-2 the induction of the active caspase-3 FITC positive population remained high in response to etoposide (25%) (Fig. 4). Conversely, AdCOX-2 cells treated with TNFα were prevented from the induction of a separate population of cells, highlighting the increase in active caspase-3-FITC fluorescence. Instead the majority of AdCOX-2 cells displayed inactive caspase-3 (93%) with TNFα (Fig. 4). Because the level of caspase-3 activation in control AdWT or control AdCOX-2-infected cells was at a minimum identical to uninfected control cells (Fig. 4), cell death was probably not occur-

FITC-Caspase-3

**TABLE II**

FITC-AV staining for cells incubated with PGF2 or PGI2 and treated with either etoposide or TNFα

| Cell treatment | Cell fraction | V/E | A/N |
|---------------|--------------|-----|-----|
| Control       | 90 ± 3.5     | 2.5 ± 0.3 | 7.5 ± 1.3 |
| TNFα          | 65 ± 2.9     | 18.5 ± 2.9 | 14.0 ± 3.2 |
| Etoposide     | 71 ± 2.4     | 23 ± 4.0  | 5.8 ± 1.5  |
| PGE2          | 88 ± 1.2     | 5 ± 0.6   | 6.8 ± 0.88 |
| PGE2 + TNFα   | 84 ± 2.3     | 3.6 ± 1.6 | 11 ± 1.2  |
| PGE2 + etoposide | 70 ± 0.6 | 19 ± 0.8  | 8.0 ± 0.88 |
| PGI2          | 87 ± 1.2     | 4 ± 0.6   | 8.6 ± 1.7  |
| PGI2 + TNFα   | 88 ± 2.3     | 3.7 ± 1.2 | 10 ± 1.2  |
| PGI2 + etoposide | 69 ± 2.3 | 20 ± 0.88 | 11.0 ± 3.2 |

*Cells in the absence of PG were treated with either TNFα (100 ng/ml) or etoposide (100 μM) for 24 h or were co-incubated with either PGE2 (500 nM) or PGI2 (500 nM) in the presence of each inducer. Control cells consisted of MeSO at the same concentration used to dissolve PGE2.

*Data are mean ± S.E. of two independent experiments.

*Significantly different from TNFα alone (p < 0.0005).
ring specifically as a consequence of the adenovirus-mediated infection procedure, as detected by this method.

**Suppression of the Sub-G<sub>1</sub> Population by an Overexpression of COX-2**—Further evidence for an apparent inhibition of TNFα (100 ng/ml)-induced apoptosis by COX-2 overexpression was obtained by cell cycle analysis and the measurement of a

**FITC-Caspase-3**

---

**FIG. 7. Effect of ET-1 on TNFα-mediated apoptosis and COX-2 expression.** Cells incubated with ET-1, TNFα (100 ng/ml), or both for 24 h were harvested and the whole cell lysates were analyzed by immunoblotting with anti-COX-2 antibody. Ponceau S staining of the membrane-bound protein confirmed equal protein loading (A). Cells treated with ET-1 (100 nM), NS398 (25 μM), TNFα (100 ng/ml), or etoposide (100 μM) alone or in several combinations as indicated were stained with FITC-caspase-3 antibody and analyzed by flow cytometry (B). Control cultures were also composed of an equivalent concentration of Me<sub>2</sub>S0 used to dissolve NS 398 (25 μM). Histograms and Western blot results are representative of at least three independent experiments.
Anti-apoptotic Activity of COX-2 Expression

TABLE III

| Cell treatment | Cell fraction |
|---------------|---------------|
|               | V             | EA            | N              |
| Control       | 90 ± 1.2      | 5 ± 1.2       | 4.0 ± 1.2      |
| TNFα         | 74 ± 1.8      | 19 ± 1.2      | 6.7 ± 0.67     |
| NS 398        | 89 ± 2.3      | 7 ± 0.7       | 3.4 ± 0.2      |
| TNFα + NS 398 | 74 ± 2.3      | 19 ± 2.2      | 5.75 ± 0.98    |
| ET-1          | 85 ± 4.2      | 9 ± 1         | 3.5 ± 0.6      |
| ET-1 + TNFα   | 80 ± 3.8      | 8 ± 1.5†      | 9.75 ± 1.6     |
| ET-1 + NS 398 | 70 ± 1.76     | 21 ± 1.8      | 8.0 ± 1.52     |

|       | PGF2α        | PGI2          | 6-keto-PGF1α  |
|-------|--------------|---------------|---------------|
|       |              |               |               |
|       |              |               |               |

Sub-λ₀ region. FACS analysis of nuclear DNA showed a profound sub-λ₀ peak, produced by a leakage of DNA fragments from apoptotic cells following their fixation, in response to etoposide treatment in AdWT cells. A smaller but well defined sub-λ₀ region was also produced by TNFα in AdWT cells (Fig. 5). The sub-λ₀ region was considerably smaller in AdCOX-2 cells compared with AdWT cells following treatment with TNFα but was still visible in the presence of etoposide (Fig. 5).

Profile of Prostaglandin Synthesis following Overexpression of COX-2—To assess the impact of an overexpression of COX-2 on prostaglandin production in RMC, AdWT and AdCOX-2 cells were stimulated with the calcium ionophore A23187 (1 mM, 15 min at 37 °C) to release cellular arachidonic acid from membrane phospholipids, as described elsewhere (38). The PGs were then extracted and separated by liquid chromatography-mass spectrometry (39). By this analysis it was possible to determine the conversion of arachidonic acid to a variety of COX-2-mediated PG metabolites. AdWT cells contained low levels of PGE₂ and a substantial amount of PGI₂. When the cells were overexpressed with COX-2 there was a selective enhancement of PG production. AdCOX-2 cells showed a 2-fold increase in PGE₂ and PGI₂ (detected as 6-keto-PGF₁α). There was no evidence of PGF₂α, PGJ₂, PGD₂, and thromboxane A₂ as the major active isoform because of adenovirus transfection, PGE₂ and PGI₂ were deemed to be the principal PG metabolites induced by COX-2 in RMC.

Anti-apoptotic Effect of PGE₂ and PGI₂—Having established that PGE₂ and PGI₂ were generated by an overexpression of COX-2, we attempted to mimic COX-2 activity by exogenous addition of either PGE₂ or PGI₂ (500 nM) to PGE₂ (500 nM) cells co-incubated with either TNFα (100 ng/ml) or etoposide (100 μM) in uninfected RMC. Their effect on TNFα or etoposide-mediated apoptosis was monitored by FACS analysis using the previously established assays in this study. The fraction of active caspase-3-FITC cells was reduced from 16 to 1% by PGE₂ (Fig. 6) and from 16 to 3% by exogenous PGI₂ (Fig. 6). Furthermore, the EA cell fraction was significantly reduced in TNFα-treated cells, co-incubated with either PGE₂ or PGI₂ (Table II). In each instance the fraction of EA cells was reduced from ~18.5 to 3.6%. The extent of apoptosis inhibition by PGE₂ and PGI₂ was comparable with the anti-apoptotic effect of an overexpression of COX-2, suggesting that the cytoprotective effect of COX-2 on TNFα apoptosis was mediated by PGE₂ and PGI₂. Moreover, the inhibitory effect of PGE₂ and PGI₂ could not be elicited in the presence of etoposide as shown by a significant induction of EA cells (~20%) and cells with active caspase-3 (>25%) (Table II and Fig. 6, respectively). PGE₂ or PGI₂ alone had no effect on apoptosis, as shown by the low levels of caspase-3 activation (<5%, see Fig. 6) and EA cell induction (<10%, see Table II).

ET-1 Inhibition of TNFα-mediated Apoptosis—Having determined the anti-apoptotic effect of an overexpression of COX-2 and the cytoprotection afforded by the PGs generated by this mechanism we then wanted to examine the influence of other mediators that are responsible for proliferative GN. ET-1 may be important in the progression of proliferative GN (23, 24). Earlier investigations demonstrated a rapid induction of COX-2 gene expression in response to ET-1 in RMC (25, 26). Here COX-2 expression was enhanced in RMC by ET-1 (100 nM) alone and more so by a combination of ET-1 (100 nM) with TNFα (100 ng/ml) (Fig. 7A). In contrast, COX-2 was barely detected in cells incubated with TNFα alone (Fig. 7A).

This synergistic effect of ET-1 and TNFα on COX-2 expression was then tested on the inhibition of TNFα-mediated apoptosis. ET-1 in combination with TNFα reduced the EA cell fraction induced by TNFα alone (from 19 to 8%) (Table III). A fraction of TNFα-treated RMC with caspase-3 activation was also reduced (from 21 to 8%) in TNFα cells pre-exposed to ET-1 (Fig. 7B). ET-1 did not prevent apoptosis induced by etoposide as evidenced by the substantial number of cells with active caspase-3 (26%) (Fig. 7B). NS398 (25 μM), a COX-2 selective inhibitor, was administered in combination with ET-1 for 24 h prior to incubation with TNFα for an additional 24 h. In this scenario the protective effect of ET-1 over TNFα-induced apoptosis was eliminated, presumably because of an inhibition of COX-2 enzymatic activity by NS398 (Fig. 7B). NS398 alone induced neither significant fractions of EA (<10%, Table III) nor cells with active caspase-3 (<10%, Fig. 7B) as predicted by the lack of up-regulation of endogenous COX-2 in RMC, and the reported specificity of NS398 for this isoform. ET-1 alone failed to induce EA cell fractions or caspase-3 active populations (Table III and Fig. 7B).

Inhibition of TNFα-mediated Apoptosis by IL-1β—IL-1β in combination with TNFα was reported to elicit an additive induction of COX-2 in RMC, with IL-1β being the more potent inducer of COX-2 (28). With this observation in mind, RMC were treated with a combination of cytokines, and the effects on apoptotic cell death were evaluated. RMC stimulated with IL-1β (2 ng/ml) displayed increased endogenous COX-2 protein expression compared with TNFα (100 ng/ml)-treated cells (Fig. 8A). The combination of TNFα and IL-1β further enhanced COX-2 protein levels (Fig. 8A) and mitigated apoptosis resulting from TNFα alone, because of their synergism over COX-2 expression. As a result EA cell fractions and cells with active caspase-3 were reduced to almost that of control levels from 19 to 6% (Table IV) and 19 to 9% (Fig. 8B), respectively, in cells co-incubated with TNFα and IL-1β. This response further supports our hypothesis that COX-2 has an anti-apoptotic role during cytokine-mediated proliferative GN.

DISCUSSION

Mesangial cells are myofibroblasts essential for maintaining immunological functions of renal glomeruli (1–4, 7). The mechanisms responsible for the impairment of myofibroblast/mesangial cell apoptosis, in severe proliferative GN remain poorly characterized. To address this issue our primary objective was to develop a suitable cell culture model of apoptosis in RMC. We were able to induce ~20 to 25% apoptotic cells in subconfluent RMC, in response to TNFα, highlighted by a number of apoptotic indices, without prior cellular or molecular manipulations as described in other published investigations (32–34). Our findings are in agreement with one other study whereby...
TNFα-mediated apoptosis was also restricted to subconfluent rat mesangial cells, at a level of ~25% by 24 h incubation, in the absence of any manipulations (47).

After establishing this primary cell culture model we were able to investigate the relationship between COX-2 expression and TNFα-mediated apoptosis in RMC. We used recombinant adenovirus-mediated gene transfer, which is an indispensable tool for driving gene expression in primary cell types, and by this mechanistic approach we were able to show that cells overexpressing COX-2 were resistant to apoptosis induced by TNFα. PS exposure was reduced by ~50% in AdCOX-2 cells, as were nuclear apoptotic events, such as chromatin condensation and DNA fragmentation. Protection from TNFα-mediated apoptosis by an overexpression of COX-2 was because of the
suppression of caspase-3 activation. On the other hand, Ad-COX-2 cells could not be rescued from etoposide-mediated apoptosis, suggesting that COX-2 anti-apoptotic activity was confined to the pathways mediated by TNFα. Although there is definitive evidence of COX-2 suppression of apoptosis in cancerous or transformed cells (16–19) this study is one of few to demonstrate the cytoprotection of COX-2 overexpression in primary cell types such as RMC.

We were convinced of an anti-apoptotic activity of COX-2 expression because we had utilized more than a single viability assay. There are several points of controversy for relying exclusively on the interpretation and specificity of a single assay. For instance the AV assay using FITC-AV/PI staining is a sensitive FC method for detecting PS exposure, which is an early and transient event in apoptosis that may be difficult to distinguish from the necrotic cell fraction. Studies utilizing the AV assay without including PI in the analysis run the risk of overestimating the level of apoptosis and incorrectly diagnosing necrosis as apoptotic cell death (34, 48). Consequently, the level of EA cell induction by TNFα at 24 h was a significant parameter that we had consistently highlighted, and inhibited by an overexpression of COX-2. By contrast, the caspase-3 assay can exclusively detect the level of apoptotic cell death because it measures a specific event of apoptosis not present in necrosis. As a result few caspase-3-positive cells were identified in AdWT and AdCOX-2 control cells, and as expected, the absolute quantity of apoptosis measured by the two methods was different. Nevertheless, all of our assays were consistent in showing the same ultimate conclusion: COX-2 inhibits TNFα-mediated apoptosis in RMC.

TNFα was investigated in this study because it plays an important role in the physiology of RMC. Elimination of mesangial/myofibroblast cells by immune surveillance may depend on TNFα-initiated apoptosis. Consequently, many studies have emphasized the importance of TNFα in the resolution of proliferative GN (32–34) and yet few have demonstrated the caspase-mediated pathway of TNFα, without implementing a prior deletion of the NF-κB survival pathway (32–34). We hypothesized that the observed cytotoxic effect of TNFα was a result of the absence of COX-2 expression in our cells. TNFα alone, at all the doses tested (25, 50, and 100 ng/ml), did not induce COX-2. However, a stimulatory effect on COX-2 expression by TNFα became synergistic with the proinflammatory cytokine IL-1β and the vasoconstrictor peptide ET-1. This observation is consistent with previous work showing that IL-1β potently induced COX-2 in RMC (28, 29), and in a host of other cell types relevant to the inflammatory process, e.g. in human gingival fibroblasts (49) and osteoblasts (50). Furthermore, TNFα plus IL-1β was shown to have an additive effect on COX-2 expression in RMC (29), and a synergistic effect in human gingival fibroblasts (49) with IL-1β as the more potent inducer of COX-2 in each instance (29, 49). Similarly, ET-1 by itself was shown to rapidly induce COX-2 in RMC (26, 27), whereas its anti-apoptotic effect was demonstrated in serum-deprived rat fibroblasts (51) and endothelial cells (52).

We found that the respective combinations of TNFα with ET-1 or IL-1β ameliorated TNFα-mediated apoptosis by >50%, as quantified by a reduction in PS exposure and caspase-3 activation. Because we also show that the cytoprotection induced by ET-1 was reversed by NS398, highlighting specific inhibition of COX-2 anti-apoptotic catalytic activity, we suggest that renal inflammation may be propagated by at least two pathways of COX-2 induction. TNFα may act as a bimodal ligand, at least in mesangial cells, by promoting cell survival in a synergistic action with other mediators, and cell death simultaneously, which may be circumvented by up-regulated COX-2 expression. Hence our results implicate a novel role for ET-1 and IL-1β as potent survival factors for renal mesangial cells against TNFα-mediated apoptosis.

Another line of investigation is to analyze both the levels and types of PGs generated by COX-2 metabolism, which can change significantly during an inflammatory reaction. Several groups correlated a single measurement of PGE2 production with COX-2 expression in RMC (28, 29, 53, 54). Here, we sought to determine the profile of PG release from endogenous arachidonic acid derived from an overexpression of COX-2. By this analysis AdCOX-2 cells demonstrated a preferential synthesis of PGI2 and PGE2 with very little if any production of thromboxane A2, PGF2α, PGI2, and PGD2. Accordingly, both PGE2 and PGI1 inhibited the apoptotic parameters elicited by TNFα, suppressing caspase-3 activation and PS exposure, by ~80%. Therefore COX-2 may prevent TNFα apoptosis in RMC, at least in part, by generating anti-apoptotic products PGE2 and PGI1. Generally, PGE2 production has been correlated with the inhibition of apoptosis as shown in cancerous or transformed cells such as human colon cancer cells (19) and cholangiocarcinoma cells (55). The results from the present study support a growing recognition of both PGI1 and PGE2 participation in the progression of various inflammatory conditions and cancer progression (38, 49).

Many studies correlate an overexpression of COX-2 and the prevention of apoptosis with an enhanced expression of bel-2 (16, 18, 19). In this investigation bel-2 was not evident in AdCOX-2 cells implying that an overexpression of COX-2 does not regulate bel-2 activity in RMC, and bel-2 induction is dependent on the cell type and extent of expression or configuration of the proto-oncogene. Recent work from this laboratory identified an up-regulation of anti-apoptotic dynin light chain in PC12 cells (37), and P-glycoprotein expression in RMC, in response to an overexpression of COX-2 (40). In the former study dynin light chain selectively prevented nitric-oxide synthase activity and caspase-3 activation, in response to a trophic nerve growth factor withdrawal model of apoptosis, and the latter report correlated COX-2 activity with increased activity of P-glycoprotein. Studies are underway to determine whether these mechanisms can be applicable to a COX-2 suppression of TNFα-mediated apoptosis in RMC, enabling further novel observations of COX-2 anti-apoptotic activity in primary cell types.

In summary, the presented data suggest that COX-2 overexpression or induction may prevent apoptosis in renal mesangial cells. The observations implicate COX-2 expression and catalytic activity in proliferative GN by inhibiting TNFα-dependent apoptosis perhaps via the generation of PGE2 or PGI1. These results could be useful in elucidating the molecular mechanisms underlying the regulation of COX-2 and may open up specific strategies for the treatment of renal inflammatory diseases that specifically target COX-2 or the downstream components of COX-2 rather than TNFα or its receptor.
Acknowledgments—We gratefully acknowledge Dr. Kasem Nithipatikom and Marilyn Isbell for help and advice with liquid chromatography-mass spectrometry analysis and Dr. James Zanghi for editorial assistance.

REFERENCES

1. Davies, M. (1994) Kidney Int. 45, 320–327
2. Pfeilschifter, J. (1994) Physiol. Rev. 74, 271–276
3. Striker, G. E., Mannik, M., and Tung, M. Y. (1979) J. Exp. Med. 149, 127–136
4. Johnson, R. J., Ploeger, J., Yoshimura, H., Ida, W. G., Cousser, W. G., and Alpers, C. E. (1992) J. Am. Soc. Nephrol. 2, S190–S197
5. Desmouliere, A., Redard, M., Darby, I., and Gabbiani, G. (1995) Am. J. Pathol. 146, 56–66
6. Iredal, J. P., Benyon, R. C., Benyon, J., Pickering, M., McCullen, M., Northrop, S., Pawley, C., Hovell, and Arthur, M. J. (1996) J. Clin. Invest. 102, 538–549
7. Baker, A. J., Mooney, A., Hughes, J., Lombardi, D., Johnson, R. J., and Savill, J. (1994) J. Clin. Invest. 94, 2105–2115
8. Harrison, D. J. (1986) Histopathology 12, 679–683
9. Bagchus, W. M., Heedemancker, P. J., Roosing, J., and Bakker, W. W. (1986) Lab. Invest. 55, 680–687
10. Shimizu, A., Masuda, Y., Kitamura, H., Ichizaki, M., Sugisaki, Y., and Yamanaka, N. (1996) 74, 941–951
11. Kluth, D. C., and Rees, A. J. (1999) Nephrology 12, 66–75
12. Smith, W. L., DeWitt, D. L., and Garavito, R. M. (2000) Annu. Rev. Biochem. 69, 145–182
13. Hla, T., Ristimaki, A., Appleby, S., and Barriocanal, J. G. (1993) Ann. N. Y. Acad. Sci. 696, 197–204
14. Dubois, R. N., Abrahamson, S. B., Cruft, L., Gogta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky, P. E. (1996) FASEB J. 12, 1063–1073
15. Hirose, S., Yamamoto, T., Feng, L., Yaita, E., Kawasaki, K., Goto, S., Fujinaka, H., Wilson, C. B., Arakawa, M., and Kihara, I. (1998) J. Am. Soc. Nephrol. 9, 408–416
16. Cao, Y., and Prescott, S. M. (2002) J. Cell. Physiol. 190, 279–286
17. Tsuji, M., Kawano, S. S., Tsuji, S., Pawley, C., Hovell, and Arthur, M. J. (1996) J. Clin. Invest. 102, 538–549
Cyclooxygenase-2 Inhibits Tumor Necrosis Factor α-mediated Apoptosis in Renal Glomerular Mesangial Cells
Adiba Ishaque, Michael J. Dunn and Andrey Sorokin

J. Biol. Chem. 2003, 278:10629-10640.
doi: 10.1074/jbc.M210559200 originally published online January 1, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210559200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 53 references, 17 of which can be accessed free at http://www.jbc.org/content/278/12/10629.full.html#ref-list-1