Cyclooxygenase-2 inhibits TNFα-mediated apoptosis in renal glomerular mesangial cells

Adiba Ishaque, Michael J. Dunn and Andrey Sorokin*

Department of Medicine and Cardiovascular Research Center, Medical College of Wisconsin, 8701 Watertown Plank Road Milwaukee WI, 53226. * Corresponding author: Tel.: 414-456-4438; Fax: 414-456-6515; E-mail: Sorokin@mcw.edu

Running title: Anti-apoptotic activity of COX-2 expression

Abbreviations

Cyclooxygenase-2 (COX-2)
Renal mesangial cells (RMC)
Wild type (WT)
Adenovirus (Ad)
Tumour necrosis factor α (TNFα)
Endothelin-1 (ET-1)
Interleukin 1β (IL-1β)
Prostaglandin E2 and prostaglandin I2 (PGE2 and PGI2)
Annexin V (AV)
Fluorescein isothiocyanate (FITC)
Propidium iodide (PI)
Viable (V), early membrane intact apoptotic (EA) and necrotic (N)
Hanks Buffered Saline Solution (HBBS)
Phosphate buffered saline (PBS)
Multiplicity of infection (MOI)
Green Fluorescent Protein (GFP)
Acridine Orange (AO)
Positive (+), negative (-)
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
ABSTRACT

Renal mesangial cell apoptosis is a crucial repair mechanism in glomerular nephritis (GN). These cells express receptors to TNFα, a cytokine with pro-apoptotic 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 (RMC) and to determine whether expression of the inducible form of cyclooxygenase, cyclooxygenase-2 (COX-2) inhibits this apoptosis. By 24h significant levels of apoptosis were induced by TNFα (100ng/mL) or etoposide control (100µM), as shown by phosphatidylserine externalization, caspase-3 activation, development of a sub-G0/G1 region and distinct chromatin condensation. 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 IL-1β also inhibited TNFα-mediated but not etoposide-mediated apoptosis, to an extent, similar to adenoviral COX-2 infection. Selective COX-2 inhibition by NS-398 restored TNFα-mediated apoptosis. PGE2 and PGI2 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.

Key words: cyclooxygenase-2, apoptosis, mesangial cells and flow cytometry
INTRODUCTION

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 (2,3,4). 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 over-expression 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 pro-inflammatory 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 (23,24,25). 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 if 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 PGI₂ on TNFα mediated apoptosis, after establishing that those PGs were the major PG metabolites generated by COX-2 over-expression. 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 (San Diego, CA), respectively. NS398 was obtained from Cayman Chemical (Ann Arbor, MI). RPMI 1640 medium and fetal bovine serum were from Life Technologies, Inc. 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 Labs (Hercules, CA). Protein was determined by a BCA assay from Pierce (Rockford, IL). All other reagents were obtained from Sigma Chemical (St. Louis, MO). Flow cytometry experiments were carried out using a Becton Dickinson 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 (Becton Dickinson).

**Cell culture**-Primary glomerular mesangial cells from male Sprague-Dawley rats were isolated and characterized as previously described (35,36). Rat mesangial cells (RMC) were maintained in RPMI 1640 medium containing 17% heat inactivated fetal bovine serum, 5 µg/ml each of insulin, transferrin and 5ng/ml sodium selenite, 100ug/ml penicillin, 100ug/ml streptomycin at 37ºC 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 24h in basal RPMI 1640 culture medium before the experiments.

**Adenovirus mediated COX-2 gene transfer**-The recombinant adenoviral vectors (Ad) AdCOX-2 and AdWildType (AdWT) expressing the constitutively active 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 [MOI] 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 (AdGFP) at an MOI 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (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 proteins 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, Piscataway, NJ).

**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 A3281 (1 mg/mL) for 15 mins at 37°C (38). The total cell lysates were run on a solid phase extraction device for analysis of PGE₂, PGI₂ (detected as 6-keto-PGF₁α, a stable product of PGI₂), PGF₂α, PGJ₂, PGD₂ prior to measurement by liquid-chromatography mass spectrometry (LC-MS) (39). Internal standards, 1.0 ng each of [d⁴]-PGE₂, [d⁴]-PGF₂α, [d⁴]-6-keto-PGF₁α, [d⁴]-PGD₂ or [d⁴]-PGJ₂, 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 x 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 5ml of ethanol and 15 ml water. The columns were washed with 29ml of water and allowed to run dry. Then the PGs were eluted from the column with 6ml of ethyl acetate. A vacuum was applied to 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 1ml of ethyl acetate. The resulting two ethyl acetate portions were combined for each sample and dried under the stream of nitrogen gas until completely dry. The sample was then redissolved in 20µl of acetonitrile and transferred to an insert in the sample vial.

The derivatized extracts were subject to LC-MS 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 (MOI of 200) for 24h were incubated with TNFα (100ng/mL) or etoposide (100µM). 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 (10µg/ml) for ≈2 min. Typically 3 fields were randomly selected from each 60mm 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 (5x10^5 cells) infected with either AdWT or AdCOX-2 were incubated with TNFα (100ng/mL) or etoposide (100µM). At the end of the incubation time the
supernatants were collected. The remaining cell monolayer was washed in Hanks Buffered Saline Solution (HBBS) without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, and then incubated in a solution of trypsin–EDTA at 37\degree 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 x g, 4\degree C for 5mins and the resulting pellet was resuspended in PBS. The cell suspension was centrifuged again at 179 x g, 4\degree C and the pellet was resuspended in 70\% ice cold-ethanol. Cells fixed in ethanol were stored at -20\degree C for up to 3 days. The cells were precipitated from the ethanol by high-speed centrifugation at 358 x g, 4\degree C for 15mins. The pellet was resuspended in PBS and centrifuged again at 358 x g, 4\degree C for 15mins. The final pellet was resuspended in 1 mL of RNase A (prepared in PBS without Mg\textsuperscript{2+} or Ca\textsuperscript{2+} and heat-treated to inactivate DNase) to give a final concentration of 250\mu g/mL. The mixture was incubated at 37\degree C for 30min. At the end of the incubation PI (1mg/mL ) was added to give a final concentration of 50 ug/mL. The suspension was incubated at room temperature for 10 min in the dark. The cells were then analysed by flow cytometry.

Annexin-V FITC and propidium iodide double staining-RMCs (5x10\textsuperscript{5} cells) infected with either AdWT or AdCOX-2 were treated with TNF\alpha (100ng/mL) or etoposide (100\mu M) and stained with annexin-V (AV) labeled to FITC in combination with propidium iodide (PI), for FACS analysis of apoptosis. FITC-AV/PI staining was optimized for attached cells according to the instructions outlined by manufacturers of the AV assay kit (Oncogene Research Products).

Caspase-3 assay-FITC conjugated to a monoclonal 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 TNF\alpha (100ng/mL) or etoposide (100\mu M), following cell permeabilization and fixation according to the instructions outlined by manufacturers of the kit.

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 (100nM) or IL-1\beta (2ng/mL) and on cells treated with either PGE\textsubscript{2} (500nM) or PGI\textsubscript{2} (500nM) to investigate the effects of these various treatments on TNF\alpha (100ng/mL) or etoposide (100\mu M) mediated apoptosis. For PG addition, conditioned
media was removed and replaced with fresh basal media to ensure removal of any existing PGs. PGs were re-administered after 6 and 12h of co-incubation with the apoptotic inducers to replenish depleted PGs. Effect of NS398 (25µM) on ET-1 protection of apoptosis was also assessed by these assays. Control cells received DMSO at the same concentration and time of incubation as PGE₂ (500nM) and NS398 (25µM).

RESULTS

Characterization of COX-2 over-expression by adenovirus mediated infection—Previous work from this laboratory demonstrated a transient over-expression 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 renal mesangial cells (RMC). The transgenic AdGFP construct showed ≈100% transfection efficiency in RMC at 24h post-adenoviral infection, as visualized by fluorescence microscopy (Fig. 1B), compared to 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 24h 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 transgenic 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 over-expression 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 over-expression of COX-2 as determined by FITC-AV/PI double staining—Our first task was to develop an apoptosis model in RMC cultures. In order to conclusively demonstrate the elicitation of apoptotic cell death by a cytokine/receptor mediated route, the effect of TNFα was compared to 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 24h, and reached maximum effect by 40h 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). Since the 24h incubation time frame imposed the earliest, significant induction of apoptosis by TNFα, we used it to compare an inhibition of apoptosis by COX-2 over-expression 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. Since 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 N [AV (- and +)/PI (+)] cell fractions (Fig. 2) and simultaneously quantify this transition (Table 1). AdWT cells incubated with either TNFα (100ng/mL) or etoposide (100µM) produced distinct fractions of EA cells (28% and 30%, respectively) (Table 1 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 over-expressing 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 to AdWT treated cells (Table 1 and Fig. 2). A substantial fraction of AdCOX-2 cells were N (~18) in response to TNFα (Table 1 and Fig. 2). The transition in cell death for AdCOX-2 cells exposed to TNFα was therefore
from V directly to N. AdCOX-2 cells treated with etoposide, on the other hand, gave rise to a distinct EA cell fraction (~27%) (Table 1 and Fig. 2). It was noted, that AdCOX-2 infected cells displayed higher levels of N cell fractions in control cultures suggesting that the procedure of adenovirus infection was also inducing necrotic cell death as shown for AdWT infected control cells.

**Morphological analysis of apoptosis induction and its inhibition by an over-expression of COX-2** - Chromatin condensation is an early event of apoptosis that requires a supply of ATP (44) and may be independent of internucleosomal DNA fragmentation (45). It was measured by AO staining of AdWT and AdCOX-2 cells treated with either TNFα (100ng/mL) or etoposide (100µM) for 24h. Nuclei of control AdWT cells were composed of diffuse chromatin as depicted by a homogenous pattern of AO staining (Fig. 3A and Fig. 3G). In response to TNFα substantial chromatin margination and condensation became apparent, compared to AdWT control cultures (from ~8 to 18 %) (Fig. 3B and Fig. 3G). Etoposide also increased the proportion of AdWT cells with apoptotic nuclear phenotype (from ~8 to 28%) (Fig. 3C and Fig. 3G). 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 over-expressing COX-2 compared to 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 over-expression 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 capase-3. A marked increase in the fraction of active caspase-3-FITC positive AdWT cells treated with either TNFα (100ng/mL) (from 7 to 22%) or etoposide (100µM) (from 7 to 35%) was observed by 24h (Fig 4). Caspase-3 inactive cells were the predominant cell population (> 90%) in control AdWT and uninfected cells (Fig. 4). For cells over-expressing 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). Since 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 occurring specifically as a consequence of the adenovirus mediated infection procedure, as detected by this method.

**Suppression of the Sub-G1 population by an over-expression of COX-2**-Further evidence for an apparent inhibition of TNFα (100ng/mL) induced apoptosis by COX-2 over-expression was obtained by cell cycle analysis and the measurement of a sub-G1 region. FACS analysis of nuclear DNA showed a profound sub-G1 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-G1 region was also produced by TNFα in AdWT cells (Fig. 5). The sub-G1 region was considerably smaller in AdCOX-2 cells compared to AdWT cells following treatment with TNFα but was still visible in the presence of etoposide (Fig. 5).

**Profile of prostaglandin synthesis following over-expression of COX-2**-In order to assess the impact of an over-expression of COX-2 on prostaglandin production in RMC, AdWT and AdCOX-2 cells were stimulated with the calcium ionophore A23187 (1mM, 15min at 37°C) to release cellular arachidonic acid from membrane phospholipids, as described elsewhere (38). The PGs were then extracted and separated by LC-MS (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 over-expressed with COX-2 there was a selective enhancement of PG production. AdCOX-2 cells showed a two-fold increase in PGE2 and PGI2 (detected as 6-keto-PGF1α). There was no evidence of PGF2α, PGJ2, PGD2 and Thromboxane A2 (TxA2) production by AdWT or AdCOX-2 cells. Since COX-2 was the major active isoform due to 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 over-expression of COX-2, we attempted to mimic COX-2 activity by exogenous addition of either PGE₂ or PGI₂. PGE₂ (500nM) and PGI₂ (500nM) were each co-incubated with either TNFα (100ng/mL) or etoposide (100µM) 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 2). 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 to the anti-apoptotic effect of an over-expression 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 2 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 2).

ET-1 inhibition of TNFα mediated apoptosis—Having determined the anti-apoptotic effect of an over-expression 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 (100nM) alone and more so by a combination of ET-1 (100nM) with TNFα (100ng/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 3). Fraction of TNFα treated RMC with caspase-3 activation was also reduced (from 21 to 8%) in TNFα cells pre-exposed to ET-
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 (25µM), a COX-2 selective inhibitor, was administered in combination with ET-1 for 24h prior to incubation with TNFα for an additional 24h. In this scenario the protective effect of ET-1 over TNFα induced apoptosis was eliminated, presumably due to an inhibition of COX-2 enzymatic activity by NS398 (Fig. 7B). NS398 alone induced neither significant fractions of EA (<10%, Table 3) cells nor cells with active caspase-3 (≤10%, Fig. 7B) as predicted by the lack 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 3 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 (2ng/mL) displayed increased endogenous COX-2 protein expression compared to TNFα (100ng/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, due to 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 4) 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 approximately 20 to 25% apoptotic cells in sub-confluent 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 sub-confluent rat mesangial cells, at a level of approximately 25% by 24h 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 over-expressing COX-2 were resistant to apoptosis induced by TNFα. PS exposure was reduced by approximately 50% in AdCOX-2 cells, as were nuclear apoptotic events, such as chromatin condensation and DNA fragmentation. Protection from TNFα-mediated apoptosis by an over-expression of COX-2 was due to the suppression of caspase-3 activation. On the other hand, AdCOX-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 over-expression in primary cell types such as RMC.

We were convinced of an anti-apoptotic activity of COX-2 expression since 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 over-estimating the level of apoptosis and incorrectly diagnosing necrosis as apoptotic cell death (43, 48). Consequently, the level of EA cell induction by TNFα at 24h was a significant parameter that we had consistently highlighted, and inhibited by an over-expression of COX-2. By contrast, the caspase-3 assay can exclusively detect the level of apoptotic cell death since 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, 100ng/mL), did not induce COX-2. However, a stimulatory effect on COX-2 expression by TNFα became synergistic with the pro-inflammatory 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), while 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. Since 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 over-expression of COX-2. By this analysis AdCOX-2 cells demonstrated a preferential synthesis of PGI2 and PGE2 with very little if any production of TxA2, PGF2α, PGJ2 and PGD2. Accordingly, both PGE2 and PGI2 inhibited the apoptotic parameters elicited by TNFα, suppressing caspase-3 activation and PS exposure, by approximately 80%. Therefore COX-2 may prevent TNFα apoptosis in RMC, at least in part, by generating anti-apoptotic products PGE2 and PGI2. 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 PGI2 and PGE2 participation in the progression of various inflammatory conditions and cancer progression (38,49).

Many studies correlate an over-expression of COX-2 and the prevention of apoptosis with an enhanced expression of bcl-2 (16,18,19). In this investigation bcl-2 was not evident in AdCOX-2 cells implying that an over-expression of COX-2 does not regulate bcl-2 activity in RMC, and bcl-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 dynein light chain (DLC) in PC12 cells (37), and P-glycoprotein (P-gp) expression in RMC, in response to an over-expression of COX-2 (40). In the former study DLC selectively prevented nitric oxide synthase activity and caspase-3 activation, in response to a trophic NGF withdrawal model of apoptosis, and the latter report correlated COX-2 activity with increased activity of P-gp. 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 over-expression 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 PGI2. 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 COX-2’s downstream components rather than TNFα or its receptor.

ACKNOWLEDGMENTS

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REFERENCES


FIGURE LEGENDS
FIGURE 1: **Characterization of RMC over-expressing COX-2 by adenovirus mediated infection.** Subconfluent serum starved cells untransfected (A) or infected (B) with the adenovirus (Ad) cDNA construct for Green Fluorescent Protein (GFP) at a multiplicity of infection (MOI) of 200 for 24h. Expression of COX-2 (C) 24h after infecting with increasing MOI’s of AdCOX-2, and bcl-2 expression after infection with AdCOX-2 at a MOI of 200 (D) was determined by western blot analysis with anti-COX-2 or anti-bcl-2 antibody, respectively. Cell lysates from AdCOX-2 cells were standardized for protein and extracts from LNcaP prostate cancer cells were used as positive (+) controls. Adenovirus Wild Type (AdWT) construct was used as a transfection control. AdWT cells did not show endogenous COX-2 or bcl-2 proteins (D). Ponceau S staining of the membrane bound protein confirmed equal protein loading. ECL exposure and fluorescence micrograph are representative of 3 independent experiments.

FIGURE 2: **Illustration of the transition in cell viability by FITC-AV/PI staining following COX-2 over-expression.** Cells infected with AdWT or AdCOX-2, were incubated with TNFα (100ng/mL) or etoposide (100µM) for 24h and subsequently stained with FITC-AV/PI to demonstrate fractions of V, EA and N cell populations. Cells that received no adenovirus are indicated as uninfected. Dot plot is representative of 5 independent experiments.

FIGURE 3: **Apoptotic morphology as detected by fluorescence microscopy (x40).** AdWT infected (A to C) or AdCOX-2 infected (D to F) cells were stained with AO and subsequently quantified for nuclear apoptotic morphology (G). Cells were treated with either TNFα (B and E) or etoposide (C and F) for 24h. Control cultures (A and D) were cultured in complete medium for 24h. Apoptotic cells were identified by ‘dots’ of chromatin condensation, which became pronounced in the shrunken apoptotic cells (indicated by arrows). Fluorescence micrograph is representative of 4 independent experiments. Data points (G) show the mean ± SE of the mean of triplicate readings in a representative experiment; significant difference form AdWT control cells (#p<0.01), and from AdWT treated cells (*p<0.001).
FIGURE 4: **Effect of COX-2 over-expression on caspase-3 activation.** AdWT or AdCOX-2 infected cells were induced to apoptosis with either TNFα (100ng/mL) or etoposide (100µM) for 24h and assayed for caspase-3 activation by flow cytometry. Histograms of cell count vs active-caspase-3 labeled to FITC highlight M1 and M2 regions, which represent cells with inactive and active caspase-3 respectively. Histograms are representative of 3 independent experiments.

FIGURE 5: **Cell cycle analysis.** Cells infected with AdWT or AdCOX-2, were analyzed for nuclear DNA content by PI staining, as described in the experimental procedures, after treatment with TNFα (100ng/mL) or etoposide (100µM) for 24h. Histograms are typical of 3 independent experiments.

FIGURE 6: **Effect of PGE2 and PGI2 on TNFα mediated apoptosis.** Cells were incubated for 24h in fresh basal media in the absence (control) or presence of PGE2 or PGI2, or each PG was co-incubated with either TNFα (100ng/mL) or etoposide (100µM). PGs were re-applied 6 and 12h during incubation to offset their metabolic degradation as described in experimental procedures. Cells were analyzed for active-caspase-3 FITC staining. Control cultures were also composed of an equivalent concentration of DMSO used to dissolve PGE2 (500nM). Histograms of cell count vs caspase-3 staining are representative of 3 independent experiments.

FIGURE 7: **Effect of ET-1 on TNFα mediated apoptosis and COX-2 expression.** Cells incubated with ET-1, TNFα (100ng/mL) or both for 24h 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 (100nM), NS398 (25µM), TNFα (100ng/mL) or etoposide (100µM) alone or in several combinations as indicated were stained with FITC-caspase-3 antibody and analysed by flow cytometry (B). Control cultures were also composed of an equivalent concentration of DMSO used to dissolve NS 398 (25 µM). Histograms and western blot results are representative of at least 3 independent experiments.
FIGURE 8: **Effect of IL-1β on TNFα mediated apoptosis and COX-2 expression.** Cells stimulated with IL-1β or TNFα or both cytokines were harvested and whole cell lysates were assessed for COX-2 protein by western blot analysis. Ponceau S staining of the membrane bound protein confirmed equal protein loading (A). FITC-capase-3 staining of cells incubated in the absence (control) or presence of IL-1β (2ng/mL) and/or TNFα (100ng/mL) was carried out after 24h incubation (B). Histograms and western blot results are representative of at least 3 independent experiments.
Table 1

FITC-AV staining for uninfected, AdWT or AdCOX-2 infected cells treated with etoposide or TNFα

<table>
<thead>
<tr>
<th>Cell infection/Inducers(^1)</th>
<th>Cell fraction (%)(^2)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>EA</td>
<td>N</td>
</tr>
<tr>
<td>Uninfected:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>90 ± 1.8</td>
<td>4 ± 0.3</td>
<td>5 ± 1.2</td>
</tr>
<tr>
<td>TNFα</td>
<td>75 ± 1.5</td>
<td>20 ± 0.6</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>Etoposide</td>
<td>69 ± 1.8</td>
<td>22 ± 1.5</td>
<td>8 ± 3.3</td>
</tr>
<tr>
<td>AdWT:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75 ± 2</td>
<td>9 ± 1.7</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>54 ± 2</td>
<td>28 ± 0.6</td>
<td>14 ± 2</td>
</tr>
<tr>
<td>Etoposide</td>
<td>43 ± 1.2</td>
<td>30 ± 0.6</td>
<td>25 ± 2.1</td>
</tr>
<tr>
<td>AdCOX-2:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>78 ± 1</td>
<td>4 ± 1.4</td>
<td>16.0 ± 0.8</td>
</tr>
<tr>
<td>TNFα</td>
<td>76 ± 1.5</td>
<td>6 ± 0.9(^4)</td>
<td>17.7 ± 0.7</td>
</tr>
<tr>
<td>Etoposide</td>
<td>54 ± 2.3</td>
<td>27 ± 1.0</td>
<td>19.0 ± 1.2</td>
</tr>
</tbody>
</table>

\(^1\) Uninfected cells or cells infected with AdWT or AdCOX-2 for 24h were untreated (control) or treated with either TNFα (100ng/mL) or etoposide (100µM) for an additional 24h.

\(^2\) Cell viability was quantified in terms of the fraction of V [AV (-)/ PI (-)], EA [AV (+)/ PI (-)], or N [AV (+)/ PI (+)] cells, represented as a percentage of the total cells analysed by flow cytometry. Note staining procedure resulted in a loss ≤ (5%) of cells.

\(^3\) Values are mean ± SE (n ≥3)

\(^4\) Significantly different from treatment with TNFα alone in uninfected or AdWT infected cells (p<0.001)
Table 2

FITC-AV staining for cells incubated with PGE₂ or PGI₂ and treated with either etoposide or TNFα

<table>
<thead>
<tr>
<th>Cell treatment ¹</th>
<th>Cell fraction (%) ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
</tr>
<tr>
<td>Control</td>
<td>90 ± 3.5</td>
</tr>
<tr>
<td>TNFα</td>
<td>65 ± 2.9</td>
</tr>
<tr>
<td>Etoposide</td>
<td>71 ± 2.4</td>
</tr>
<tr>
<td>PGE₂</td>
<td>88 ± 1.2</td>
</tr>
<tr>
<td>PGE₂ + TNFα</td>
<td>84 ± 2.3</td>
</tr>
<tr>
<td>PGE₂ + etoposide</td>
<td>70 ± 0.6</td>
</tr>
<tr>
<td>PGI₂</td>
<td>87 ± 1.2</td>
</tr>
<tr>
<td>PGI₂ + TNFα</td>
<td>86 ± 2.3</td>
</tr>
<tr>
<td>PGI₂ + etoposide</td>
<td>69 ± 2.3</td>
</tr>
</tbody>
</table>

¹ Cells in the absence of PG were treated with either TNFα (100ng/mL) or etoposide (100µM) for 24h or were co-incubated with either PGE₂ (500nM) or PGI₂ (500nM) in the presence of each inducer. Control cells consisted of DMSO at the same concentration used to dissolve PGE₂.

² Data are mean ±SE of 2 independent experiments.

³ Significantly different from TNFα alone (p<0.005)
Table 3

FITC-AV staining for cells pre-incubated with ET-1, TNFα, and COX-2 inhibitor NS 398

<table>
<thead>
<tr>
<th>Cell treatment †</th>
<th>Cell fraction (%) ²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
</tr>
<tr>
<td>Control</td>
<td>90 ± 1.2</td>
</tr>
<tr>
<td>TNFα</td>
<td>74 ± 1.8</td>
</tr>
<tr>
<td>NS 398</td>
<td>89 ± 2.3</td>
</tr>
<tr>
<td>TNF + NS 398</td>
<td>74 ± 2.3</td>
</tr>
<tr>
<td>ET-1</td>
<td>85 ± 4.2</td>
</tr>
<tr>
<td>ET-1 + TNFα</td>
<td>80 ± 3.8</td>
</tr>
<tr>
<td>ET-1 + NS 398 + TNFα</td>
<td>70 ± 1.76</td>
</tr>
</tbody>
</table>

† ET-1 (100nM) or NS 398 (25µM) treatments were for 24h prior to incubation with TNFα (100ng/mL) for an additional 24h. Control cells were also composed of DMSO at the same concentration as NS 398 (25µM).

² Values are mean ±SE of 3 independent experiments.

³ Significantly different from TNFα alone (p<0.005)
**Table 4**

FITC-AV staining for cells treated with IL-1β, TNFα, or TNFα plus IL-1β

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Cell fraction (%)&lt;sup&gt;2&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
</tr>
<tr>
<td>Control</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>IL-1β</td>
<td>85 ± 3.5</td>
</tr>
<tr>
<td>TNFα</td>
<td>76 ± 1.2</td>
</tr>
<tr>
<td>IL-1β + TNFα</td>
<td>86 ± 0.6</td>
</tr>
</tbody>
</table>

<sup>1</sup> Cells were incubated with IL-1β (2ng/mL) or TNFα (100ng/mL) or IL-1β plus TNFα for 24h.

<sup>2</sup> Values are mean ±SE of 3 independent experiments.

<sup>3</sup> Significantly different from TNFα alone (p<0.01)
Figure 1
Uninfected control  
Uninfected + TNFα  
Uninfected + etoposide  

AdWT control  
AdWT + TNFα  
AdWT + etoposide  

AdCOX-2 control  
AdCOX-2 + TNFα  
AdCOX-2 + etoposide  

Propidium iodide  
FITC-Annexin V

Figure 2
Figure 3
Figure 4

FITC-Caspase-3
Figure 5

AdWT control
AdCOX-2 control

AdWT + TNFα
AdCOX-2 + TNFα

AdWT + etoposide
AdCOX-2 + etoposide

Cell count

Propidium Iodide
FITC-Caspase-3

Figure 6
A

<table>
<thead>
<tr>
<th>control</th>
<th>TNFα</th>
<th>ET-1</th>
<th>ET-1 + TNFα</th>
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<tbody>
<tr>
<td>COX-2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ponceau S</td>
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</table>

B

<table>
<thead>
<tr>
<th>control</th>
<th>ET-1</th>
<th>NS 398</th>
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<tbody>
<tr>
<td>TNFα</td>
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<td></td>
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<tr>
<td>ET-1 + TNFα</td>
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<td></td>
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<tr>
<td>TNFα + NS 398</td>
<td></td>
<td></td>
</tr>
<tr>
<td>etoposide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET-1 + etoposide</td>
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<td></td>
</tr>
<tr>
<td>ET-1 + NS 398 + TNFα</td>
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</table>

Cell count

FITC-Caspase-3

Figure 7
<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>TNFα</th>
<th>IL-1β</th>
<th>IL-1β + TNFα</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>93%</td>
<td>7%</td>
<td>81%</td>
<td>19%</td>
</tr>
<tr>
<td>M2</td>
<td>92%</td>
<td>8%</td>
<td>90%</td>
<td>9%</td>
</tr>
</tbody>
</table>

**A**

![Image of COX-2 and Ponceau S](image)

**B**

![Graphs of FITC-Caspase-3](image)

*Figure 8*
Cyclooxygenase-2 inhibits TNFα-mediated apoptosis in renal glomerular mesangial cells
Adiba Ishaque, Michael J. Dunn and Andrey Sorokin
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