Upregulation of Renal and Systemic Cyclooxygenase-2 in Patients with Active Lupus Nephritis

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Abstract. In lupus nephritis (LN), renal thromboxane A₂ (TXA₂) production is increased, and inhibition of TXA₂ activity improves renal function. In patients with LN, renal function depends very much on vasodilatory prostaglandins, and indeed inhibiting the prostaglandin-forming enzyme cyclooxygenase (COX) with aspirin or related compounds was detrimental on renal hemodynamics in these patients. There are no data so far on whether the excessive TXA₂ production in LN derives from upregulation of type I or type II isoforms of COX. It was found that TXB₂ synthesis and COX-2 gene expression were higher in peripheral blood mononuclear cells from patients with active LN compared to patients in the inactive form of the disease and to healthy subjects. Unlike COX-2, levels of COX-1 mRNA were comparable in lupus patients and control subjects and were not influenced by the disease activity. Immunoperoxidase studies on kidney biopsies showed COX-1 staining in glomerular arterioles and other renal vessels, with no evident difference between lupus biopsies and control specimens taken from either individuals who were free of renal disease or patients with non-lupus nephropathies. In contrast, COX-2 staining was definitely stronger in specimens from patients with active LN than control specimens. In active LN, COX-2-specific staining was localized mainly in the glomeruli, with a weaker signal on tubuli and in the interstitium. Double-staining studies with an antibody against the macrophage marker CD68 and an anti-COX-2 antibody definitely showed that COX-2 and CD68 often colocalized on the same cell, with only occasional glomerular COX-2-stained mesangial areas. Patients with non-lupus nephropathies had no increase in renal COX-2 expression. These results indicate that COX-2 upregulation is a specific finding of active LN and that monocytes infiltrating the glomeruli contribute to the exaggerated local synthesis of TXA₂. If this is correct, COX-2 may soon become a target for therapeutic intervention in this disease.

If nephritis develops in systemic lupus erythematosus (SLE), a disease of B cell activation and immune complex deposition in various organs (1,2), morbidity and mortality increase (3,4), much more so in cases of diffuse proliferative glomerular lesions. These lesions reduce the glomerular filtration properties of the kidney, and the severity of renal dysfunction mainly depends on the degree of mononuclear inflammatory cell infiltration in the glomeruli and renal interstitium (5,6).

Many studies have investigated the signaling molecule(s) that attract inflammatory cells into the kidney upon immune complex deposition (7,8) and the mediators actually involved in the subsequent cascade of events leading to tissue injury. There is evidence that abnormalities in the metabolism of arachidonic acid in the kidney take part in the dynamics of tissue injury and modulate the severity of the inflammatory reactions. Thromboxane A₂ (TXA₂) is one of the arachidonic acid metabolites widely implicated as a mediator of damage in renal diseases (9). In an experimental model of immune-mediated mesangial cell injury in the rat, glomerular synthesis of TXA₂ was increased, and pretreatment with a TXA₂ synthase inhibitor prevented the drop in GFR (10). In a murine model of lupus nephritis (LN), TXA₂ was produced within the kidney in much larger amounts than in control mice, and correlated with the amount of proteins in the urine, as well as with the severity of renal pathology (11,12). In the same model, long-term pharmacologic blocking of TXA₂ synthesis limited proteinuria and renal lesions and prolonged survival (12).

Similarly, in patients studied during the active phases of LN, renal TXA₂ synthesis was enhanced, as reflected by increased urinary excretion of its stable breakdown product TXB₂, whose levels in the urine correlated with the degree of renal lesions and with deteriorating renal function (13). In addition, both renal blood flow (RBF) and GFR were increased by pharmacologic blockade of TXA₂ receptors in patients with LN (14). However, in LN renal function depends on vasodilatory prostaglandins and, in fact, inhibiting the prostaglandin-forming enzyme cyclooxygenase (COX) with nonsteroidal anti-inflammatory compounds (13,15–17) was detrimental, as docu-
mented by a lowering of RBF and GFR in patients with LN given aspirin or ibuprofen.

There are two distinct isoforms of COX (18): COX-1 is constitutively expressed in inflammatory and other cells and is involved in a variety of physiologic functions (19,20), including cytoprotection of the gastric mucosa, regulation of RBF, and platelet aggregation; COX-2 is expressed only in the context of inflammation (21–25), particularly in mononuclear leukocytes, endothelial cells, and mesangial cells upon induction by cytokines (26–28).

High on the list of COX-2 inducers is interleukin-1β (IL-1β), which is generated in exuberant amounts in mononuclear leukocytes activated by immunologic/inflammatory events during the course of LN (29). It is not known whether the excessive TXA$_2$ formed in the kidney of lupus patients is of COX-1 or COX-2 origin, although this could be important with a view to opening up new ways of blocking one of the two pathways to reduce TXA$_2$ more selectively with little or no impact on vasodilatory prostaglandins.

To address this issue, we first evaluated the capacity of peripheral blood mononuclear cells (PBMC) from patients with active LN to generate TXA$_2$, compared with cells from patients studied in the remission phase of their nephritis when renal and systemic indexes of disease activity (i.e., renal function impairment, proteinuria, immunologic abnormalities) subsided. PBMC from patients with active LN were also studied for COX-1 and COX-2 isoform gene expression and compared with cells obtained during remission. Cellular localization of COX-1 and COX-2 in the kidney was studied in biopsy specimens from patients with active, severe nephritis requiring biopsy for diagnostic purposes.

**Materials and Methods**

**Experimental Design**

Eight adults with active LN (seven women, one man; 31 to 48 yr old) admitted to our hospital between June 1993 and September 1996, were enrolled in the study. All patients gave informed consent to participate in the study according to the locally approved procedures and to the Declaration of Helsinki. All patients fulfilled more than four of the revised criteria for the diagnosis of SLE proposed by the American Rheumatism Association (30). Patients were studied at admission before therapy. Renal biopsy was performed before therapy in all patients and classified according to the World Health Organization criteria (31) by light and electron microscopy and immunofluorescence examination. A group of seven patients with inactive LN receiving chronic steroid treatment was also studied. Clinical data of patients with active and inactive LN are given in Table 1. Both groups were compared with a group of eight age- and sex-matched healthy control subjects.

To assess whether LN was associated with excessive systemic TXA$_2$ production, PBMC were isolated from patients and control subjects, and *ex vivo* TXA$_2$ release was assessed. Because cytokines such as tumor necrosis factor-α and IL-1, which are highly expressed in monocytes of patients with LN (29), are potent inducers of COX-2 transcription (26,28), we also investigated whether the abnormal TXA$_2$ production in PBMC of patients with LN was associated with altered expression of COX-2. Total RNA was obtained from freshly isolated PBMC from patients and control subjects, and COX-2 expression was evaluated by Northern blot. Expression of the constitutive cytokine-insensitive COX isoform COX-1 was also evaluated.

In a separate experiment, PBMC from one healthy volunteer were incubated for 2 h without serum or with 10% fetal calf serum (FCS) to upregulate COX-2 gene expression (21,32). At the end of the

**Table 1.** Laboratory profiles of patients with lupus nephritis

<table>
<thead>
<tr>
<th>Category</th>
<th>Urinary Protein ($g/24$ h)</th>
<th>Serum Creatinine (mg/dl)</th>
<th>$C_3^c$ (mg/dl)</th>
<th>$C_4^c$ (mg/dl)</th>
<th>Anti-DNA (U/ml)</th>
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* Histologic classification proposed by the World Health Organization. For lupus nephritis patients in remission, it is referred to as the time of biopsy.

* 24-h urinary protein excretion.

* Serum activity of $C_3$ and $C_4$ fractions of complement (normal reference range: $C_3 = 83$ to $177$ mg/dl, $C_4 = 15$ to $45$ mg/dl).
incubation, total RNA was obtained and analyzed for COX-2 expression as a positive control (25,32).

To evaluate renal expression and localization of COX enzyme isoforms in LN, immunostaining studies with either anti-COX-1 or anti-COX-2 antibodies were performed on renal biopsy specimens from active LN patients. Renal infiltrating macrophages were identified by immunostaining with an antibody directed against a specific monoclonal/macrophage antigen, CD68. To establish whether renal COX-2 expression in LN patients was localized on infiltrating macrophages, the same biopsies were double-stained using anti-CD68 and anti-COX-2 antibodies.

As a control for immunostaining studies, biopsy specimens from two patients with no signs of glomerulopathy and five patients with non-lupus glomerulopathies (minimal change disease: n = 1; idiopathic diffuse proliferative glomerulopathy: n = 2; vasculitis: n = 1; IgA nephropathy: n = 1) were also studied.

**Isolation of PBMC**

Blood samples (50 ml) were drawn on 10% ethylenediaminetetraacetic acid, diluted 1:2 with 0.02 M phosphate-buffered saline (PBS), pH 7.3, and centrifuged at 150 × g for 20 min at 4°C. The supernatant was centrifuged at 1500 × g for 15 min at 18°C to eliminate platelets and used to reconstitute blood. Mononuclear leukocytes were then isolated on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) (33). After centrifugation at 400 × g for 40 min at 18 to 20°C, the mononuclear cell layer was collected, washed in PBS, and centrifuged at 500 × g for 7 min at 4°C; the cell pellet was resuspended in PBS. Cell viability, assessed by trypan blue exclusion, was approximately 90%. The average recovery was 68%; 95% of platelets were eliminated.

Aliquots of PBMC suspension (approximately 40 × 10⁶ cells) were used for Northern blot analysis. For evaluation of TXA₂ synthesis, PBMC were centrifuged at 500 × g for 7 min at 4°C and resuspended in Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution and layered on a Ficoll-Paque/sucrose gradient (1.5 vol of Ficoll-Paque, 1 vol for each sucrose: 20, 16, 12, 8, and 4%) to eliminate residual platelets (34) that may produce TXA₂.

After centrifugation at 200 × g for 15 min at 18 to 20°C, the lower phase was collected, centrifuged at 500 × g for 7 min at 4°C, and the cell pellet was resuspended at 2 × 10⁶ cells/ml in Hanks’ balanced salt solution supplemented with 1.87 mM CaCl₂ and 0.8 mM MgSO₄. After this procedure, more than 99% of platelets were eliminated.

Aliquots of PBMC (1 ml) were incubated at 37°C for 10 min and centrifuged at 500 × g for 7 min at 4°C, and the supernatant was stored at −20°C until assayed for TXB₂, the TXA₂ stable metabolite, by RIA.

**Radioimmunoassay**

TXB₂ in PBMC supernatant was measured by RIA, using a specific antibody as described previously (35). Briefly, the anti-TXB₂ antibody (final concentration in the assay, 1:300,000) was incubated for 18 h at 4°C with 2700 cpm of [³²P]TXB₂ and with increasing amounts of unlabeled TXB₂ (standard curve: 1 to 100 pg/ml) or with sample.

The bound antibody was separated from the free ligand by adding 0.1 ml of human prostaglandin free plasma and 0.1 ml of a charcoal suspension (Serva, Heidelberg, Germany; 0.1 g/ml) then by centrifugation for 10 min at 500 × g at 4°C. The smallest concentration that could be measured with 95% confidence was 2 pg/ml.

Intra- and interassay variability averaged 5 and 10%, respectively, over the range of TXB₂ concentrations of the standard curve. TXB₂ production by PBMC was expressed as pg/10⁶ cells.

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from PBMC samples by the guanidinium isothiocyanate/cesium chloride procedure, as described previously (36). Ten micrograms of total RNA was fractionated on 1.2% agarose gel and blotted onto nylon membranes (Gene Screen Plus, New England Nuclear, Boston, MA). COX-1 mRNA was detected using a 1.1-kb fragment of human COX-1 cDNA (Cayman Chemical Co., Ann Arbor, MI), and COX-2 mRNA was detected using a 1.1-kb fragment of murine COX-2 cDNA (Cayman Chemical Co.). The cDNA fragments were labeled with α-[³²P]dCTP by the random-primed method (37).

For COX-1 gene expression, membranes were hybridized for 20 h at 65°C with 1 × 10⁶ cpm labeled probe/ml of a solution containing 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 mg/ml salmon sperm DNA. The blots were washed twice with 0.1× SSC-0.1% SDS at 65°C for 45 min (1× SSC: 150 mM NaCl, 15 mM sodium citrate). For COX-2 gene expression, hybridization was performed at 60°C for 20 h with 1.5 × 10⁶ cpm labeled probe/ml, and blots were washed twice with 2× SSC-1% SDS at 60°C for 30 min.

After washing, membranes were exposed to x-ray film for autoradiography. Membranes were subsequently probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (38), taken as internal standard for equal loading of samples on the membranes.

The autoradiographs were analyzed by densitometry to quantify the amounts of radioactively labeled probe for COX-2 and GAPDH. The ratios of intensity (arbitrary units of bands probed with COX-2 cDNA to bands probed with GAPDH cDNA) were calculated for RNA from patients and healthy subjects, and results were expressed as the patient-to-healthy subject ratio. COX-2 level for healthy subjects was normalized to one. At least one control was added on each membrane. Qualitative analysis was done for COX-1 because of the very low level of expression.

**Immunohistochemistry**

COX isoenzyme expression was analyzed on 3-μm frozen sections from renal biopsies processed for light microscopy by immunohistochemistry, using an avidin-biotin horseradish peroxidase complex technique (ABC method, ABC Elite, Vector Laboratories, Burlingame, CA). Either mouse monoclonal antibody against ovine COX-1, which recognizes human antigen (39) (Cayman Chemical Co.), or rabbit polyclonal antibody directed against human COX-2 (Cayman Chemical Co.) was used. Briefly, the sections were air-dried, fixed in acetone for 10 min, rehydrated, and incubated for 1 h with 0.3% H₂O₂ in methanol to quench endogenous peroxidase. Aspecificities were blocked by a 30-min incubation with nonimmune sera (5% horse serum for anti-COX-1, 5% goat serum for anti-COX-2). All of the above steps were carried out at room temperature. Slides were then incubated overnight at 4°C in a moist chamber with the primary antibody (anti-COX-1 1:500, anti-COX-2 1:200) in PBS/1% bovine serum albumin (BSA) (Miles, Bayer, Milan, Italy)/0.2% saponine (Sigma Chemical Co., St. Louis, MO)/1% normal human serum for COX-1, and PBS/1% BSA/0.2% saponine for COX-2, followed by the secondary antibody (biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG, respectively, for COX-1 and COX-2), ABC solution, and finally developed with diaminobenzidine (Merck, Darmstadt, Germany).

An additional section from each biopsy was labeled with EBM11 primary antibody (mouse monoclonal anti-human macrophage; Dako, Glostrup, Denmark) directed against the CD68 antigen present on human lung macrophages and blood monocytes (40,41), using a
streptavidin-alkaline phosphatase fast red technique (SAP red). The sections were air-dried, fixed in aceton for 10 min at room temperature, and rehydrated in distilled water for 10 min. Specificities were blocked by a 30-min incubation with 1% BSA/0.01 M PBS, pH 7.4. Slides were then incubated overnight at 4°C with the primary antibody EBM11 at a dilution of 1:100 in PBS/1% BSA, followed by the secondary antibody (biotinylated sheep anti-mouse IgG, Boehringer Mannheim, Mannheim, Germany, 1:100 in PBS) for 30 min at room temperature, streptavidin-alkaline phosphatase (Boehringer Mannheim) 1:500, and finally developed with fast red chromogen (Boehringer Mannheim) containing levamisole, an inhibitor of endogenous alkaline phosphatase activity. Negative controls were obtained by omitting the primary antibodies on a second section present on all of the slides.

All biopsies were also double-stained first with anti-COX-2 antibodies, following the same procedure as for single labeling with the exception that treatment with 0.3% H2O2 in methanol was performed before incubation with anti-COX-2 antibody because the fast red product is soluble in methanol. Negative controls consisted of the above protocol without either antibody.

All of the sections were counterstained with Harris-hematoxylin (Biopica, Milan, Italy). The slides were observed under the light microscope. Renal COX-2 staining was semiquantitatively analyzed by light microscopy by a pathologist blind to the protocol. At least eight to 10 glomeruli were evaluated for each patient. 0 was attributed to no staining, 1+ to weak staining, 2+ to moderate staining, and 3+ to strong staining.

The specificity of anti-COX-1 and anti-COX-2 antibodies was verified by immunofluorescence. Platelets were isolated from blood of a healthy volunteer and resuspended in Tyrode’s solution (42) at a final concentration of 200 × 10^9/ml. Samples of 500 ml were cytocentrifuged (Shandon, Cheshire, England) on glass slides, which were stained with either a 1:25 dilution of anti-COX-1 (followed by a 1:50 dilution of the secondary antibody, FITC-conjugated goat anti-mouse IgG; Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-COX-2 (followed by a 1:200 dilution of the secondary antibody, Cy3-conjugated goat anti-rabbit IgG; Jackson ImmunoResearch Laboratories).

PBMC were also isolated from a healthy volunteer and resuspended in RPMI (Life Technologies, Grand Island, NY) at a density of 1 × 10^6 cells/ml and incubated in sterile conditions for 20 h in a 60-mm culture dish containing glass coverslips to allow adhesion. In some wells, 10% FCS was added to upregulate COX-2 protein expression (21,32). At the end of the incubation, the coverslips were washed, fixed in 2% paraformaldehyde, and incubated with either anti-COX-1 or anti-COX-2 antibodies, followed by the secondary antibodies. The slides were observed with an Olympus BH-2 epifluorescence microscope.

Statistical Analyses

Data are presented as mean ± SEM. The nonparametric Kruskal-Wallis test was used to test for differences between means (43). Statistical significance was defined as P < 0.05.

Results

TXB2 Release by PBMC

Ex vivo TXB2 production by PBMC from healthy subjects was 25.95 ± 3.84 pg/10^6 cells (Figure 1). In patients with active LN studied before steroid treatment, PBMC TXB2 release was almost 10 times higher (208.26 ± 51.86 pg/10^6 cells; P < 0.01 versus healthy subjects) (Figure 1). By contrast, PBMC TXB2 production from lupus patients studied in the inactive phase of the disease was comparable to healthy subjects (28.97 ± 8.1 pg/10^6 cells; P < 0.01 versus active lupus) (Figure 1).

PBMC COX-1 and COX-2 Gene Expression

To assess whether the abnormal production of TXA2 from PBMC was associated with altered expression of either of the COX isoforms, COX-1 and COX-2 gene expression was evaluated.

A faint band for COX-1 mRNA of approximately 2.8 kb (21) was present in PBMC from healthy subjects and LN patients and did not differ in the groups, indicating comparable expression of the transcripts (Figure 2A).

Hybridization with a specific COX-2 cDNA probe showed a transcript of approximately 4.1 kb in all samples (21). PBMC from healthy subjects gave a low signal for COX-2 mRNA (Figure 2A). Stimulation of control PBMC with 10% FCS increased the COX-2 signal approximately fourfold (data not shown).

In active LN, PBMC COX-2 mRNA was significantly, although variably, higher than in healthy subjects (Figure 2, A and B; 2.65 ± 0.33 arbitrary units, P < 0.01 versus healthy subjects). Consistent with TXA2 data, COX-2 expression in patients with inactive LN was comparable to healthy subjects (Figure 2, A and B; 0.69 ± 0.12 arbitrary units, P < 0.01 versus active lupus).

A second transcript for COX-2 mRNA of approximately 2.8 kb, whose signal was weaker than the 4.1-kb transcript, was detected in PBMC from active LN patients and from healthy subjects after stimulation with 10% FCS. The smaller band probably reflects a different site of polyadenylation of mRNA, as reported previously for murine COX-2 (21).
Immunohistochemistry

In preliminary experiments, the anti-COX-1 antibody brightly stained normal human platelets (which mainly express COX-1 isoform) (44), whereas the anti-COX-2 antibody gave a weak signal (data not shown). Unstimulated human PBMC were weakly stained by both antibodies. In addition, PBMC incubation with 10% FCS, which selectively induces COX-2 expression in monocytes (21), resulted in bright staining with anti-COX-2 antibody, whereas staining with anti-COX-1 was comparable to unstimulated cells. Taken together, the above results confirm the high specificity of anti-COX-1 and anti-COX-2 antibodies for the corresponding enzyme isoforms.

To evaluate renal expression and cellular localization of COX-1 and COX-2 isoforms, immunoperoxidase staining was performed on renal biopsy specimens. In control biopsies from patients either without glomerulopathy (n = 2) or with non-lupus glomerulopathies (n = 5), the COX-1 isoform showed focal staining on the glomerular tuft (Figure 3A) (45); COX-1 immunoreactivity was present in smooth muscle cells and endothelial cells of vessels (Figure 3C) (45). Weak staining was also seen on cortical tubuli (data not shown). No difference in COX-1 expression and distribution was found between LN patients and control subjects (Figure 3, B and D).

Control biopsies from patients without glomerulopathy (n = 2) showed weak focal staining for COX-2 enzyme in a few glomerular cells in the tuft (45) and in the epithelium of Bowman’s capsule. Some staining was also found on a few cortical tubuli (Figure 4A). Some COX-2 staining was also found on endothelial and smooth muscle cells of vessels (data not shown) (45). Glomerular COX-2 staining was much stronger in renal biopsies from patients with active LN. Increased COX-2 staining in glomeruli was associated with endocapillary proliferative lesions. The staining was present mainly on infiltrating cells, but also on some resident glomerular cells whose morphology suggested they were mesangial cells (Figure 4B and Figure 5C; Table 2). Some staining was also seen on cortical tubuli, which was focally greater than in control biopsies (not shown). The COX-2 signal in areas of interstitial inflammation was weak or absent (not shown). An antibody against the macrophage marker CD68 gave specific red staining in the glomeruli of LN patients (Figure 5A), whereas a low focal signal was found on inflammatory cells infiltrating the interstitium (6).

Analysis of serial sections indicated colocalization of anti-CD68- and anti-COX-2-positive areas (Figure 5, B and C). To confirm this, we double-stained LN biopsies with anti-CD68 and anti-COX-2 antibodies. In most patients, several glomerular cells stained dark brown for COX-2 and red for CD68 (Figure 5E). In some patients, focal COX-2 staining was also seen on resident anti-CD68-negative cells.

In renal biopsy specimens from patients with noninflammatory minimal change disease or inflammatory renal diseases (idiopathic diffuse proliferative glomerulopathy, vasculitis, IgA nephropathy), COX-2 staining was similar to control patients with no renal disease, in all structures examined (Table 2). No CD68 staining was found in patients with minimal change disease (Figure 5D); however, some staining was present in the interstitium and focally in glomeruli in patients with inflammatory renal disease. No staining was observed when the primary antibodies were omitted, in either single- (not shown) or double-staining experiments (Figure 5F).
Discussion

The first finding of the present study was that PBMC from patients with active LN synthesized 5 to 10 times more TXA$_2$ than PBMC from patients with inactive disease or PBMC from healthy subjects. We found no differences in PBMC TXA$_2$ synthesis in the latter two groups. These results may explain the high urinary levels of 2,3-dinor-TXB$_2$ and 11-dehydro-TXB$_2$, markers of extrarenal TXA$_2$ synthesis (46,47), in patients with LN and high disease activity (48,49).

The rationale for looking at a possible upregulation of COX-2 enzyme induced by IL-1$\beta$ (26–28) or tumor necrosis factor-$\alpha$ (26) in this setting rests on many recent experimental and clinical findings. In a rat model of adjuvant-induced arthritis, Anderson et al. (50) found upregulation of COX-2 but a normal signal for COX-1 in the arthritic paw concomitant with the development of edema. They also reported that selective inhibition of COX-2 rapidly reversed paw edema, reduced joint inflammation, and returned prostaglandin levels to normal. In another model of carrageenan-induced inflammation in rats, Masferrer et al. (51) found that COX-2 mRNA and protein were both induced in the pouch tissue. Carrageenan induced the expression of COX-2 in cells of the inflamed pouch lining; most of the immunoreactive COX-2 signal was on fibroblasts and macrophages. In the same model, dexamethasone inhibited COX-2 expression and eicosanoid production and reduced inflammation (51).

Consistent with experimental findings, there are now reports of remarkably high levels of COX-2 mRNA and enhanced protein in freshly explanted synovial tissues from patients with rheumatoid arthritis (24). In these specimens, de novo synthesis of the COX-2 peptide was induced by IL-1$\beta$ in vitro and was suppressed by dexamethasone (24). High levels of IL-1$\beta$ in synovial fluid and peripheral blood of patients with rheumatoid arthritis (52,53) could be instrumental to upregulate COX-2 in inflammatory cells, thus enhancing tissue damage.

Whether the same applies to LN has not been established, but indirect evidence indicates that in vitro short-term cultures of unstimulated PBMC from patients with SLE and active nephritis form more IL-1 than control cultures (54). On the other hand, IL-1 mRNA levels were very high in mononuclear cells freshly isolated from the peripheral blood of patients with SLE and active nephritis, again consistent with the possibility that more IL-1 is formed in vivo (55).

With such a background, we reasoned that enhanced TXA$_2$
synthesis in patients with active LN was possibly the result of upregulation of COX-2 in inflammatory cells. COX-2 gene expression was indeed increased in circulating PBMC from patients with active LN, but not in those with inactive disease. The specificity of such phenomenon is documented by the finding that, unlike COX-2, levels of COX-1 mRNA were comparable in lupus patients and control subjects and were not influenced by the disease activity.

Excessive TXA₂ in the systemic circulation of experimental animals and humans with SLE (12,48,49) is paralleled by excessive TXA₂ formed in the kidney, as documented by several studies that measured urinary excretion of the TXA₂ metabolite, TXB₂ (12,13,48). Direct evidence of enhanced renal synthesis of TXA₂ was also found in MLR-lpr and in NZBxNZW mice (11) by looking at renal cortical and medullary homogenates.

An issue that has never been addressed so far in animal and human lupus concerns the enzymatic and cellular source of TXA₂ in the kidney. Using specific antibodies, we differentially stained renal specimens from human biopsies with immunoperoxidase, and documented COX-1-specific staining in glomerular arterioles and other renal vessels, with no difference between lupus biopsies and control specimens either from individuals with no renal diseases or from patients with non-lupus nephropathies. By contrast, COX-2 staining was definitely stronger in kidney specimens from patients with an active form of LN compared with control subjects. In active LN, COX-2-specific staining was mainly localized in the glomeruli, with a much weaker signal on tubuli and in interstitium, with a focal distribution. COX-2-positive glomerular cells had rounded or elongated morphology, suggesting a macrophage/macrophage lineage.

Double-staining studies to validate this interpretation definitely showed COX-2 and CD68, often on the same cell; glomerular COX-2 only occasionally stained mesangial areas. By contrast, interstitial infiltrates, mainly of lymphocyte origin (6), never stained for COX-2. Patients who had non-lupus nephropathies had no apparent increase in renal COX-2 expression, so the intensity of the signal in this group was comparable to that in subjects with no renal diseases.

These results were taken to indicate that renal COX-2 upregulation is a specific finding of active LN and that monocytes abundantly infiltrating the glomeruli of these patients, which were already recognized as instrumental to the development of glomerulonephritis (4–6), do substantially contribute to the exaggerated local synthesis of TXA₂.

In vitro monocytes (21,25) and mesangial cells (28) express COX-2 and synthesize TXA₂ in response to IL-1 and other cytokines. In patients with LN, IL-1 synthesis in the kidney is higher than normal (56,57), which could be one of the reasons for local COX-2 overexpression.

In vitro COX-2 mRNA and protein upregulation in monocytes by cytokines and growth factors is rapid but transient with maximum effect between 4 and 12 h after the challenge, followed by a progressive decline in the COX-2 transcript down to baseline levels within 24 h (18,21). This pattern of activation looks almost incompatible with the possibility that the enzyme in monocytes infiltrating the kidney was upregulated as a consequence of systemic events, and in fact suggests that mediators of renal origin are implicated, among which IL-1 appears one of the most likely candidates at the moment.

The functional consequence of renal COX-2 expression upregulation can be prevented by pharmacologic manipulation of the TXA₂ pathway, although most attempts to improve the course of experimental and human renal disease by this approach have been unsuccessful. Thus, selective TXA₂ receptor
blockade appears to some extent to slow the progression of LN in MRL-lpr mice (58), and a TXA2 synthase inhibitor reduces proteinuria and glomerular lesions in NZBxNZW mice (12); however, other studies in lupus mice (11,59) found no benefit on proteinuria and mortality by inhibiting renal TXA2. In 24 patients with LN, 22 of them class IV, a selective TXA2 receptor antagonist transiently increased GFR and RBF as measured by the clearance of inulin and para-aminohippurate (14). In another short-term study, a TXA2 synthase inhibitor appeared to help only a subgroup of patients (48). Most currently available TXA2 inhibitors, at doses fully suppressing platelet TXB2, only partially reduced urinary TXB2 excretion, either in healthy control subjects (60–62) or patients with various nephropathies (63) (F. Casiraghi, unpublished data). This suggests some lack of tissue selectivity of these molecules that might account for their limited therapeutic value. On the other hand, there is now solid evidence (13–15) that in human LN, glomerular filtration is critically dependent on intact renal COX-1, as unselective inhibitors reduced GFR by an effect attributed to reduced production of prostaglandin E2 (PGE2) and prostacyclin (PGI2) in the kidney (16,17,64). Thus, urinary excretion of PGE2 and PGI2 metabolites was substantially

**Figure 5.** Staining with an antibody against CD68 macrophage marker gives specific red signal in the glomeruli of LN patients (A and B). Staining on serial sections confirms localization of COX-2 on macrophages (B and C). Magnification: ×265 in A; ×355 in B and C. (D) CD68 staining in one patient with minimal change disease. Magnification, ×265. (E) LN biopsy specimen double-stained with anti-CD68/SAP red (red) followed by anti-COX-2/ABC (brown). Glomerular infiltrating cells show staining with both red and brown chromogens. No signal is seen when the primary antibodies were left on (F) (Harris-hematoxylin counterstaining). Magnification, ×1330.
Table 2. COX-2 immunoperoxidase signal

<table>
<thead>
<tr>
<th>Case Biopsy No.</th>
<th>Diagnosis</th>
<th>COX-2 Signal Glomeruli</th>
<th>Tubules</th>
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<tr>
<td>2315</td>
<td>No renal lesions</td>
<td>0 to 2 f</td>
<td>Traces</td>
</tr>
<tr>
<td>2512</td>
<td>No renal lesions</td>
<td>Traces/2 f</td>
<td>0/traces</td>
</tr>
<tr>
<td>2375</td>
<td>Minimal change nephropathy</td>
<td>0/2 f</td>
<td>0/traces</td>
</tr>
<tr>
<td>2427</td>
<td>Extracapillary proliferative GN</td>
<td>0/1 f</td>
<td>0/traces</td>
</tr>
<tr>
<td>2434</td>
<td>Proliferative GN</td>
<td>0/1 f</td>
<td>Traces</td>
</tr>
<tr>
<td>2273</td>
<td>Vasculitis</td>
<td>0/1 + f</td>
<td>0</td>
</tr>
<tr>
<td>2405</td>
<td>IgA nephropathy</td>
<td>0/2 f</td>
<td>0/1 + f</td>
</tr>
<tr>
<td>2376</td>
<td>Lupus nephritis (class IV)</td>
<td>1+ to 3 f Traces/1+</td>
<td></td>
</tr>
<tr>
<td>2311</td>
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<td>1+ to 3 f Traces</td>
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<tr>
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<td></td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>1723</td>
<td>Lupus nephritis (class IV)</td>
<td>3+ 1+</td>
<td></td>
</tr>
</tbody>
</table>

* Immunostaining for COX-2 was graded as: 0, no staining; 1+, weak; 2+, moderate; 3+, strong. COX, cyclooxygenase; f, focal; GN, glomerulonephritis.
  * Values are range of the staining observed in at least 8 to 10 glomeruli for each patient.

impaired (>80%) after 1 wk of ibuprofen in these patients (15). In the above study, serum creatinine increased by 40% after ibuprofen treatment, and creatinine and para-aminophenylurate clearances were reduced by 28 and 35%, respectively. In another study in LN, ibuprofen suppressed renal TXA2 and PGL2 synthesis by 67 and 77%, respectively, and reduced RBF and GFR, confirming that in this setting renal vasodilatory prostaglandins are essential to maintain glomerular hemodynamics (13).

Our finding of a selective upregulation of COX-2 isoenzyme in circulating monocytes and kidney macrophages indicates a novel pathway of inflammatory injury in human LN. If this interpretation is correct, COX-2 may become a target for therapeutic intervention as soon as drugs selective enough to retain maximum anti-inflammatory activity with low toxicity become available for routine use. In the past few years, molecules with a favorable profile have been identified, and clinical development is under way (65–67). The ones with the best credentials for human use will have the advantage of selectively blocking COX-2-dependent TXA2 formation in infiltrating monocytes, sparing PGE2, PGL2 and other COX-1-dependent eicosanoids, which in numerous renal conditions appear essential to maintain hemodynamics and regulate water and sodium transport vis-à-vis the enhanced activity of other autacoids with vasoconstrictor properties (9,13).

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