Marian Danilewicz, Małgorzata Wągrowska-Danilewicz

Analysis of Renal Immunoexpression of Cyclooxygenase-1 and Cyclooxygenase-2 in Lupus and Nonlupus Membranous Glomerulopathy

Department of Nephropathology (Morphometry Division) Medical University of Łódź

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Recently a role of the upregulation of cyclooxygenase isoforms in renal injury and modulation the severity of the inflammatory reactions is suggested. Cyclooxygenase exists as two isoforms COX-1 and COX-2 which are poorly understood with regard to their roles in renal function. Thereby, the present study was undertaken to ascertain the immunoexpression of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in lupus (LMGN) and nonlupus (NLMGN) membranous glomerulopathy and to examine the possible relationship between this immunoexpresion and inflammatory infiltrates. Eleven renal biopsy specimens from patients with class V lupus glomerulopathy and 16 from patients with primary (nonlupus) membranous glomerulopathy were examined by percutaneous renal biopsy. As a control 10 biopsy specimens of the kidneys removed because of trauma were used. In each specimen staining intensity of COX-1 and COX-2 in glomeruli, tubuli, arterioles and interstitial cells were recorded semiquantitatively whereas CD68+ cells, CD3+ cells and CD20+ cells were assessed quantitatively using computer image analysis system.

Our study revealed that the mean scores of COX-1 immunoexpression did not differ significantly in all groups investigated whereas immunoexpression of COX-2 in LMGN was significantly stronger as compared with both NLMGN and controls. Moreover, in LMGN a significant positive relationship was noted between COX-2 immunoexpression and CD 68+ cells. In NLMGN and controls the correlations between COX-2 immunoexpression and CD 68+ cells were positive, but they have not reached statistical significance.

In conclusion, our findings point that glomerular inflammation in lupus and non-lupus membranous glomerulopathy have different signalling pathways and suggest that in lupus nephritis COX-2 and monocytes/macrophages but not COX-1 isoform are involved in the inflammatory process.

Introduction

That membranous glomerulopathy occurs as one variant of lupus nephritis is well recognised [5, 23] but reports on lupus membranous nephropathy (LMGN), or class V lupus glomerulonephritis according to World Health Organisation criteria, are few and often include heterogeneous populations, with patients presenting nihil to severe proliferative superimposed lesions [8, 20]. If nephritis develops in systemic lupus erythematosus morbidity and mortality increase [2, 25]. Certain pathologic features are known to occur more frequently in LMGN than in nonlupus membranous nephropathy (NLMGN). Especially predictive values of mesangial dense deposits, subendothelial dense deposits, tubular basement membrane deposits, increased density of the subepithelial deposits, increased glomerular basement membrane thickness, intense C1q deposition and glomerular hypercellularity were suggested [6, 12]. Moreover, the previous morphometric study revealed that relative interstitial volume was significantly greater in LMGN patients as compared with NLMGN [7]. Although a great number of cytokines and growth factors produced by the leukocyte subpopulations are

probably involved in renal lesions [26], the mechanisms of renal injury operating locally in the kidney are not well understood at the present time [15]. Recently a role of the upregulation of cyclooxygenase isoforms in renal injury and modulation the severity of the inflammatory reactions is suggested [9, 25]. Cyclooxygenase exists as two isoforms COX-1 and COX-2 which are poorly understood with regard to their roles in renal function [18]. Thereby, the present study was undertaken to ascertain immunoexpression of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) in lupus and nonlupus membranous glomerulopathy and to examine the possible relationship between this immunoexpression and inflammatory infiltrates.

Materials and Methods

Patients

Eleven renal biopsy specimens from patients with class V (according to WHO [3, 23]) lupus membranous glomerulopathy with subepithelial deposits resembling those seen in stage III of MGN according to the scheme proposed by Churg's group [3], and 16 from patients with primary (nonlupus) membranous glomerulopathy (stage III according to this scheme) were examined by percutaneous renal biopsy. All the LMGN patients fulfilled the preliminary criteria for the diagnosis of systemic lupus erythematosus [22] and all these patients were treated with corticosteroids, which were associated to cytotoxic drugs in 4 cases. Morphological diagnosis of LMGN and NLMGN was established independently by two experienced nephropatologists and based on light microscopy, immunofluorescence and electron microscopy. As a control 10 biopsy specimens of the kidneys removed because of trauma were used (the male to female ratio was 7:3, the mean age was 38.1). None of the persons from whom renal tissue originated were known to have had previous or actual renal disease. Before the quantitative examination was carried out, all control specimens were histologically examined by a nephropathologist and found to be normal renal tissue.

Light microscopy

Tissue specimens were embedded in paraffin, sections cut precisely at 4 μm, and stained by hematoxylin and eosin, periodic acid-Schiff (PAS)-alcian blue, trichrome light green (Masson), and by silver impregnation (Jones). Thickness of each section was controlled according to the method described by Weibel [27].

Immunofluorescence microscopy

Tissue was snap frozen, sectioned at 5 μm and fixed in 95% alcohol for 10 min. Sections incubated with FITC-antisera (DakoCytomation, Denmark) to human IgG, IgA, IgM and complement (C3 and C1q) were viewed on Olympus BX 41 microscope, using proper filters.

Electron microscopy

Tissue was fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide, embedded in epon and sectioned on a LKB ultratome. Sections were stained by lead citrate and uranyl acetate, and viewed in a JEM 100B electron microscope.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized, then (for COX-1, COX−2 and CD 68 only) treated in a microwave oven in a solution of citrate buffer, pH 6.0 (DakoCytomation) for 20 min and transferred to distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in distilled water for 5 min, and then sections were rinsed with Tris-buffered saline (TBS, DakoCytomation, Denmark) and incubated with: polyclonal rabbit anti-human antibodies anti-COX-1 (Cayman Chemical, USA,; dilution: 1:450), anti-COX-2 (Cayman Chemical, USA, dilution 1:450), monoclonal mouse anti-human CD20 B cell antibody (DakoCytomation, Denmark, dilution 1:100) monoclonal mouse anti-human CD3 T cell antibody (DakoCytomation, Denmark, dilution 1:50) and monoclonal mouse anti-human CD68 antibody (DakoCytomation, Denmark, dilution 1:100). Afterwards LSAB+/HRP Universal kit (DakoCytomation, Denmark) was used prepared according to the instructions of the manufacturer. Visualisation was performed by incubating the sections in a solution of 0.5 mg 3,3'-diaminobenzidine (DakoCytomation, Denmark), per ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10 min. After washing, the sections were counter-stained with hematoxylin and coverslipped. For each antibody and for each sample a negative control were processed. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results. In each specimen staining intensity of COX-1 and COX-2 in glomeruli, tubuli, arterioles and interstitial cells were recorded semiquantitatively by two independent observers in 7-10 adjacent high power fields and graded from 0 (staining not detectable), 1 (minimal immunostaining in some cells), 2 (weak immunostaining intensity in all cells) and 3 (strong staining in all cells). The mean grade was calculated by averaging grades

assigned by the two authors and approximating the arithmetical mean to the nearest unity.

Morphometry

Histological morphometry was performed by means of image analysis system consisting of a IBM - compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true-colour, real-time), produced by Indeo (Taiwan), and colour TV camera Panasonic (Japan) linked to a Carl Zeiss Jenaval microscope (Germany). This system was programmed (MultiScan 8.08 software, produced by Computer Scanning Systems, Poland) to calculate the number of objects.

The immunophenotype of leukocyte glomerular and interstitial infiltration was determined by counting all positive cells for each monoclonal antibody (semiautomatic function) in a sequence of ten consecutive computer images of 400 x high power fields - 0.0047 mm^2 each. The only adjustments of field were made to avoid large vessels. The results were expressed as a mean number of immunopositive cells per mm^2.

Results

Clinical and laboratory findings at the time of biopsy in cases with LMGN and NLMGN are summarized in Table 1. Most of our patients were young adults (the mean age was 29.9 in LMGN group and 35.5 in NLMGN group). In LMGN group female predominance was conspicuous. At the time of renal biopsy, a high percentage of patients in both groups showed nephrotic syndrome or heavy proteinuria. Clinical renal impairment (serum creatinine greater than 1.5 mg/dl) was noted in 3 LMGN patients and in 1 NLMGN patient. Elevated blood pressure was observed in 7 LMGN and 6 NLMGN cases. Hematuria accompanied proteinuria in 3 LMGN and 7 NLMGN patients.

In the renal specimens cellular localisation of the immunoexpression of COX-1and COX-2 was similar in LMGN, NLMGN and controls. Focal staining of COX-1 was present on glomerular tuft, smooth muscle cells of vessels and on some epithelial tubular cells whereas COX-2 isoform showed focal staining on glomerular tuft, some epithelial cells of Bowman's capsule, focally on epithelial tubular cells and on some interstitial cells (Fig.1-4). The

TABLE 1
Clinical and laboratory findings at the time of biopsy in cases with LMGN and NLMGN

Number of cases	Micro-hematuria	Gross hematuria	Proteinuria <1g/24h	Proteinuria 1-2 g/24h	Proteinuria 2-3,5g/24h	Nephrotic syndrome	Renal function impairment[1)]	Hypertension (>90/160)
LMGN (n=11)	2	1	1	-	3	7	3	7
NLMGN (n=16)	4	3	-	3	4	9	1	6

[1)] Serum creatinine > 1.5 mg/dl

TABLE 2
Data of the immunoexpression of COX-1 and COX-2 as well as leukocyte infiltrates in LMGN, NLMGN groups and controls

Number of cases	COX-1 (mean score±SD)	COX-2 (mean score±SD)	Number of immunopositive cells per $1mm^2$		
			CD68+	CD3+	CD20+
Controls (n=10)	0.74±0.55	0.23±0.21	28.55±15.61	36.27±14.25	0.98±0.72
LMGM (n=11)	0.95±0.72	1.37±0.71	77.23±35.33	198.35±111.86	16.25±6.22
NLMGN (n=16)	0.81±0.69	0.56±0.54	46.22±22.18	85.78±39.11	1.77±1.01
P value	0.46 (NS) [1)] 0.76 (NS)[2)] 0.62 (NS) [3)]	<0.001[1)] 0.8 (NS)[2)] <0.004[3)]	<0.001[1)] <0.04 [2)] <0.01[3)]	<0.001[1)] <0.001[2)] <0.001[3)]	<0.001[1)] <0.05[2)] <0.001[3)]

[1)] between LMGN and controls, [2)]between NLMGN and controls,[3)] between NLMGN and LMGN, NS- not significant

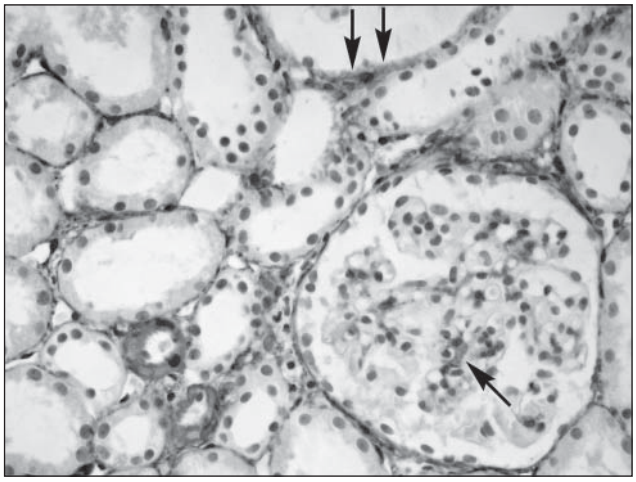

Fig. 1. LMGN. COX-1 immunoexpression on smooth muscle cells, focally on the glomerular tuft (arrow), and on some epithelial tubular cells (double arrows). Magn. 400x.

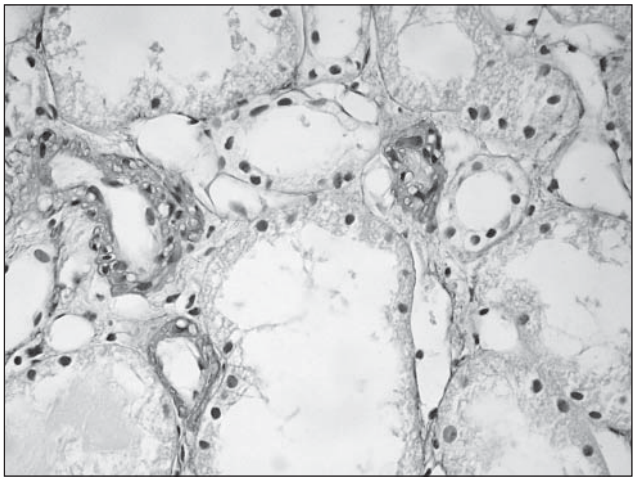

Fig. 2. NLMGN. COX-1 immunoexpression on smooth muscle cells. Magn. 400x.

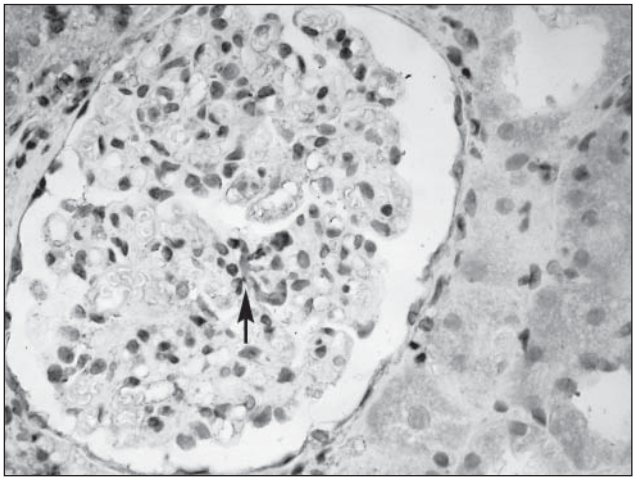

Fig. 3. LMGN. Prominent COX-2 immunoexpression on epithelial cells of Bowman's capsule and focally on glomerular tuft (arrow). Magn. 400x.

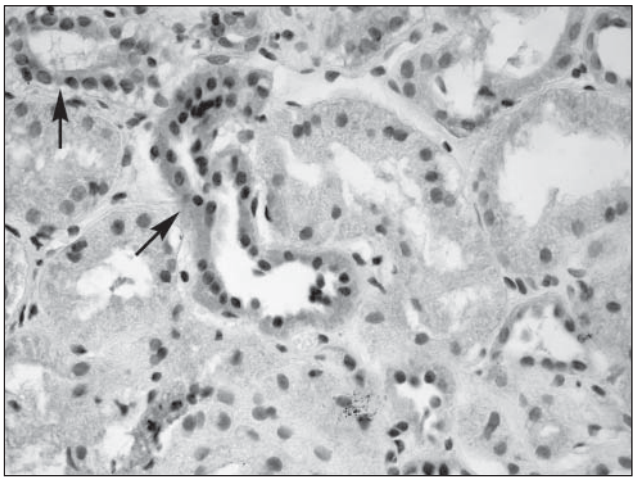

Fig. 4. NLMGN. Focal COX-2 immunoexpression on epithelial tubular cells (arrows). Magn. 400x.

semiquantitative data of the immunoexpression of COX-1 and COX-2 as well as morphometric data of leukocyte infiltrates in LMGN, NLMGN and controls appear from Table 2. The mean scores of COX-1 immunoexpression did not differ significantly in all groups investigated whereas immunoexpression of COX-2 in LMGN was significantly stronger as compared with both NLMGN and controls. Similarly leukocyte infiltrates in LMGN were significantly more numerous in comparison with both NLMGN and controls. The correlations between the immunoexpression of COX-1and COX-2 and CD 68, CD 3 as well as CD 20 positive cells are shown in Table 3. In LMGN a significant positive relationship was noted between COX-2 immunoexpression and CD 68+ cells. In NLMGN and controls the correlations between COX-2 immunoexpression and CD 68+ cells were positive, but they have not reached statistical significance. All other correlations were weak and not significant.

Discussion

The cyclooxygenase-1 isoform is constitutive in many organs and catalyses the prostaglandin synthesis in many physiologic function. The cyclooxygenase-2 isoform seems to be constitutive in some tissues as well, but may be also induced by bacterial endotoxins, cytokines and growth factors, and catalyzes synthesis pro-inflammatory prostaglandins [18].

TABLE 3
The correlations between COX-1 and COX-2 immunoexpression as well as leukocyte infiltrates in LMGN, NLMGN and controls

Correlation between:	LMNGN (n=11)	NLMGN (n=16)	Controls (n=10)
COX-1 and CD68+ cells	r=0.42, p=0.19(NS)	r=0.29, p=0.27(NS)	r=0.32, p=0.36(NS)
COX-1 and CD3+ cells	r=0.21, p=0.53(NS)	r=0.18, p=0.51(NS)	r=0.09, p=0.8(NS)
COX-1 and CD20+ cells	r=0.32, p=0.33(NS)	r=0.26, p=0.33(NS)	r=0.11, p=0.7(NS)
COX-2 and CD68+ cells	r=0.64, p<0.04	r=0.49, p=0.06(NS)	r=0.55, p=0.09(NS)
COX-2 and CD3+ cells	r=0.44 p=0.17(NS)	r=0.29, p=0.27(NS)	r=0.17, p=0.63 (NS)
COX-2 and CD20+ cells	r=0.19, p=0.57(NS)	r=0.37, p=0.15(NS)	r=0.22, p=0.54(NS)

Data on the cellular distribution of COX-1 and COX-2 in human kidney are inconsistent [14, 24]. We found in LMGN, NLMGN and controls focal staining of COX-1 on glomerular tuft, smooth muscle cells of vessels and on some epithelial tubular cells whereas COX-2 isoform showed focal staining on glomerular tuft, some epithelial cells of Bowman's capsule, focally on epithelial tubular cells and on some interstitial cells. Similar distribution of COX isoforms in human kidney was observed by Tomasoni et al. [25]. Although some authors suggested that COX-2 isoform is expressed only in the context of inflammation [4, 10, 21, 28] our results confirmed findings of Therland et al. [24] who found that both COX-1 and COX-2 cyclooxygenases are expressed constitutively in a human kidney.

As membranous glomerulonephritis in lupus erythematosus seems to be more aggressive nephropathy than non-lupus form, in our paper we compared immunoexpression of COX isoforms in renal biopsy specimens in these cases. To our knowledge no data have documented immunoexpression of COX-1 and COX-2 in LMGN and NLMGN. In the present study no significant differences were detected between COX-1 immunoexpression in controls, LMGN and NLMGN. This is in agreement with results of Tomasoni et al. who did not find evident difference between renal immunoexpression of COX-1 isoform in active lupus nephritis and control specimens taken from either individuals who were free of renal disease or from patients with non-lupus nephropathies [25]. These observations may point out that the mechanism of inflammation occurring in LMGN and NLMGN does not include COX-1 signalling pathway. On the other hand, when the COX-2 immunoexpression scores were compared, significant differences were apparent between LMGN and NLMGN as well as normal controls. Although the data of the immunoexpression of COX-2 in class V of lupus nephritis were not available it is worthy of note, that in class IV increased immunoexpression of COX-2 was noted by others [25]. Therefore, nephritis in lupus erythematosus seems to have different immunopathologic characteristics than non-lupus form. Upregulation of COX-2 has also been shown in the macula densa of human diabetic nephropathy [13] whereas in IgA nephritis COX-2 was strongly expressed in infiltrating interstitial cells [9]. In contrast, in our study the COX-2 signal in infiltrating interstitial cells was focal and very weak, although in LMGN interstitial infiltrates were significantly more numerous as compared with NLMGN and controls.

COX-2 is inducible in response to hypoxia, tissue injury, cytokines and mititogens, and participates in the process of inflammation [17]. Many experimental and clinical studies suggested that upregulation of COX-2 depends on interleukin-1β [1, 11, 19] or tumour necrosis factor-α [1]. Especially the role of inerleukin-1β is stressed, which is generated in mononuclear leukocytes activated by inflammatory events during the course of lupus nephritis [16]. It is also proposed that high level of IL-1 in the kidney in lupus nephritis may be one of the reasons for local COX-2 overexpression [25]. In view of the above, a major finding of the present study seems to be significant positive correlation between glomerular as well as interstitial monocytes/macrophages and immunoexpression of COX-2 in LMNGN. Although to our knowledge this is the first correlative analysis on the immunoexpressions of COX and monocytes/macrophages in lupus nephritis, the relationship between these immunoexpressions was found by others using double-staining method. Remuzzi group [25] using double-staining revealed that CD68 immunoexpression and COX-2 immunoexpression often colocalized on the same cells, what is in concordance with our results.

In conclusion, our findings point that glomerular inflammation in lupus and non-lupus membranous glomerulopathy have different signalling pathways and suggest that in lupus nephritis COX-2 and monocytes/macrophages but not COX-1 isoform are involved in inflammatory process.

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Address for correspondence and reprint requests to:
Professor Marian Danilewicz M.D. Ph.D.
ul. Zamenhofa 5 m. 4. 90-431 Łódź
Phone +48 42 6757633, fax +48 42 6790191
Phone GSM +48 601 283697
E-mail: hobo@csk.umed.lodz.pl