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ORIGINAL ARTICLE

Expression of cyclooxygenase-1 and cyclooxygenase-2 in human renal allograft rejection – a prospective study

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Summary

Cyclooxygenases (COX) are known to be involved in inflammatory kidney diseases. However, there are no data available about the expression of COX-1 and only preliminary reports about the expression of COX-2 in biopsies of patients undergoing acute renal allograft rejection. We conducted this prospective study to analyze the expression, distribution, and cellular localization of COX-1 and -2 and thus to elucidate the role of COX in human kidney transplantation. One hundred forty-four biopsies were included from patients without rejection and unaltered morphology ($n = 60$), with acute interstitial rejection ($n = 7$), with acute vascular rejection ($n = 21$), with chronic allograft nephropathy ($n = 16$), without rejection but with various other lesions ($n = 40$). COX-1 and -2 expression was localized in each biopsy by immunohistochemistry. We found a highly significant up-regulation of COX-1 in vessels and in infiltrating interstitial cells of patients with acute allograft rejection compared with biopsies with well-preserved tissue. Also, COX-2 expression was significantly elevated in infiltrating interstitial cells of biopsies with acute rejection. This is the first prospective study demonstrating a significant induction of both COX-1 and -2 in human allograft biopsies with acute rejection after renal transplantation.

Introduction

Prostaglandins are well known as important mediators of inflammation involving cell-mediated immune responses such as those that occur in allograft rejection [1]. Cyclooxygenases (COX) are rate-limiting enzymes in the biosynthesis of prostaglandins. Two distinct COX isoforms exist: a previously called 'constitutive' COX-1 and an 'inducible' COX-2. Both isoenzymes catalyze the same reactions, share approximately 60% homology within a given species and exhibit remarkable structural homology [2]. Nevertheless, they are encoded by two different genes, are located on distinct chromosomes, and potentially have different functions even within the same cell type [3]. The role of renal COX-1 and COX-2 in physiology and disease states has been extensively reviewed [4,5].

Cyclooxygenases-1-dependent prostaglandin production is thought to occur in normal cell physiology, such as generation of pro-aggregatory thromboxane A2 (TxA2) by platelets, cytoprotective functions in the gastric mucosa and nephron-compartmentalized synthesis of prostanoids. In addition, COX-1 has been shown to be developmentally regulated in many different tissues including thymus [3,4]. In normal human and animal kidneys, COX-1 has been described at high levels in the collecting duct epithelium, and with lower expression in arteries and arterioles, descending thin loops of Henle, glomeruli, and renal medullary interstitial cells [6,7].

Unlike COX-1, which has limited inducibility and is constitutively present in most cells, COX-2 is inducible by proinflammatory and mitogenic stimuli and is therefore thought to be responsible for mediating inflammation and tumorigenic events associated with prostaglandins [3]. COX-2 is described to be the major COX isoform contributing to the regulated production of prostaglandins affecting the renal vascular tone and salt and water homeostasis. COX-2 has two proposed main functions in the renal cortex namely dilation of afferent arterioles and control of renin secretion [4,5].

Comparing animals and humans, renal localization of COX-2 seems to differ. In normal animal kidneys, published studies have documented a widely accepted pattern of COX-2 expression in the macula densa/thick ascending limb (cTAL) in the cortex and to a subset of interstitial cells in the medulla [8]. Furthermore, COX-2 was demonstrated in glomerular podocytes and small blood vessels [6]. In contrast with animal models, there is only a small number of studies investigating the expression of renal COX-2 in humans. Data on the cellular localization of COX-2 in human kidney are inconsistent. Initial studies of COX-2 localization in normal human kidney failed to detect COX-2 in the cTAL or macula densa and instead reported expression in glomerular podocytes and arteriolar smooth muscle cells [6,9–11] whereas other reports showed an age- or disease-dependent expression of COX-2 in the macula densa [7,12,13] and a functional role of COX-2 for stimulation of renin secretion in humans [14,15]. In addition, COX-2-expression was detected in endothelial cells of arteries, arterioles and glomeruli in the cortex and in vasa recta, and collecting ducts in the medulla in immunohistochemical studies of normal human kidney sections [7,16].

Up-regulation of COX-2 has been observed in renal biopsies from patients with renal arterial stenosis, diabetic nephropathy, lupus nephritis, hypertension, congestive heart failure, and children with Bartter syndrome [6,7,10,13,16–18]. The impact of blocking COX-2-derived prostaglandins for the development of acute renal failure has been shown in several reports [5,19,20].

Only few studies describe the role of COX in renal transplantation. COX-2 has been detected to participate in the endothelial cell activation that follows ischemia-reperfusion injury in a rat model [21]. Two small studies observed the up-regulation of COX-2 in human renal allograft rejection [22,23]. A recent study demonstrated COX-2 induction during lung allograft rejection in inflammatory cells, especially in macrophages as well as in the airway epithelial cells, and fibroblasts [24]. An increased COX-2 expression has also been observed during the cardiac allograft rejection in rats [25] as well as during human cardiac post-transplant atherosclerosis [26]. Studying *in vivo* and *in vitro* models, it has been clearly demonstrated that immunosuppressants like glucocorticoids, cyclosporine A, and tacrolimus suppress renal COX-2 [27–32].

Recent data in a mouse model show that inhibition of COX-1/2 with nonselective and selective COX blockers is associated with an improvement in renal function and less parenchymal damages in animals with ischemia-reperfusion injury [33]. During cardiac allograft rejection, selective inhibition of COX-2 prolonged allograft survival and reduced myocardial damage and inflammation in a rat model [34].

To date, there exist no prospective studies analyzing the expression of both COX-1 and -2 in biopsies obtained from patients after renal transplantation. This study was performed to detect the expression, distribution, and cellular localization of COX-1 and -2 in different disease entities occurring after the renal transplantation like acute and chronic renal allograft rejection, acute renal failure, pyelonephritis, or atherosclerosis. Thus, we analyzed the expression of COX-1 and -2 prospectively in 144 biopsies, and correlated these data with clinical parameters.

Materials and methods

The study period was from July 2003 to December 2004. Protocol biopsies of kidney allografts were performed routinely 2 weeks and 3 months after transplantation, and additional biopsies were taken for diagnostic purposes during allograft dysfunction. A total of 144 prospectively collected, formalin-fixed, paraffin-embedded renal transplant biopsies were included in the analysis. C4d staining for antibody-mediated rejection identification was only performed in eight cases, where humoral rejection was considered and therefore could not be included in the analysis. Clinical data were routinely assessed in a database from all renal transplant recipients of our center. The following data were assessed for analysis: age of the patient, gender, timepoint of biopsy after transplantation and concentrations of serum creatinine, serum urea and serum albumin on the day of biopsy, and 14 days, 3, 6, 9 and 12 months after transplantation. The patients were treated with triple immunosuppressive therapy. Prednisolone was administered for at least 3 months after transplantation. Ninety-four patients were additionally treated by tacrolimus and mycophenolate mofetil, 35 by cyclosporine A and mycophenolate mofetil, six by tacrolimus and sirolimus, three by tacrolimus and azathioprine, five by cyclosporine A and FTY720 and one by cyclosporine A and everolimus.

The biopsies were graded according to the Banff 97 working classification [35] by a single pathologist.

Control native kidney sections were obtained from unaffected parts of tumor nephrectomies.

Human tissue was used following the guidelines of the Ethics Committee of the Medical Faculty of the University of Regensburg, Germany. Informed consent was obtained prior to renal transplantation.

Immunohistochemistry

Sections were deparaffinized and rehydrated. Endogenous peroxidases were blocked by hydrogen peroxide and antigen retrieval was performed by autoclave treatment for COX-1 antibody and by microwave treatment for COX-2 antibody in Antigen Unmasking Solution (Vector, Burlingame, CA, USA). Endogenous biotin was blocked using the Avidin/Biotin Blocking Kit (Vector). The polyclonal antihuman COX-1 antibody (C-20, sc-1752; Santa Cruz Biotechnology, Heidelberg, Germany) was used at 4 μg/ml and the polyclonal antihuman COX-2 antibody (M-19, sc-1747; Santa Cruz Biotechnology) was used at 10 μg/ml in 10% nonfat dry milk. After subsequent washing steps, the tissue was incubated with a biotinylated donkey antigoat IgG-B secondary antibody (1.3 μg/ml, sc-2042; Vector, Santa Cruz Biotechnology). For signal amplification, the ABC-Elite reagent (Vector) was used. 3.3′-diaminobenzidine with nickel enhancement, resulting in a black color product, served as chromogen. Slides were counterstained with hematoxyline, dehydrated, and coverslipped.

Tissues were dewaxed in xylene, and rehydrated in a graded series of ethanol. Antigen retrieval was performed in citrate buffer in a microwave (pH 7.3, 30 min, 250 W). [Incubation of the primary antibodies was for 24 min anti-CD4 (1F6) – antibody, mouse monoclonal IgG1 – Ventana, Strasbourg, France] and 32 min [anti-CD8-(1A5) – antibody, mouse monoclonal IgG1 – Ventana], respectively. This was followed by incubation with a biotinylated secondary antibody (antimouse IgG1, Ventana) for 8 min. 3′3′ diaminobenzidine (Ventana) with copper enhancement was used as detection system, resulting in a brown color product. Slides were counterstained with hematoxyline, dehydrated, and coverslipped. Because of the small size of each biopsy and the numeric limitation of biopsy slides from the prospectively collected biopsies, additional serial sections from allograft biopsies with defined disease entities and sections from human tonsils and human tumor nephrectomies were used.

Data analysis

The slides were studied under a light microscope. The staining of glomeruli, vessels, tubules, collecting ducts, and interstitium was analyzed by three observers in 10 high power fields (orig. ×400, covering an area of 296 μm × 222 μm) for each biopsy. Score 0 was attributed to basically no staining, score 1 to weak staining, score 2 to moderate staining, and score 3 to strong staining. Mean values were calculated and used for comparison of the different entities. For the comparison of means, the nonparametric Mann–Whitney *U*-test was used. $P < 0.05$ was considered to be statistically significant.

Results

The clinical information of the studied patient population according to the diagnosis based on the histomorphological evaluation of the biopsy is summarized in Table 1.

Expression of COX-1 and -2 in biopsies without rejection

Five pretransplant biopsies and 55 biopsies from 41 patients demonstrated no signs of rejection and well-preserved tissue without significant lesions (classified as Banff grade 1). Negative controls did not demonstrate positive staining (Fig. 1c and d). In glomeruli and vessels, there

Table 1. Demographic data.

Histomorphological diagnosis (n = number of biopsies)	Recipient age (years ± SD)	Sex (male/female)	Timepoint of renal biopsy after transplantation (days ± SD)
Banff 1 ($n = 60$)	51 ± 15	43/17	82 ± 103
Banff 4 I ($n = 7$)	54 ± 18	5/2	223 ± 422
Banff 4 II + III ($n = 21$)	57 ± 8	18/3	142 ± 317
Banff 5 ($n = 16$)	58 ± 10	11/5	1191 ± 1083
Banff 6			
Acute renal failure ($n = 21$)	50 ± 12	10/11	55 ± 83
Arteriosclerosis ($n = 10$)	62 ± 8	2/8	65 ± 79
Pyelonephritis ($n = 9$)	64 ± 6	7/2	351 ± 587

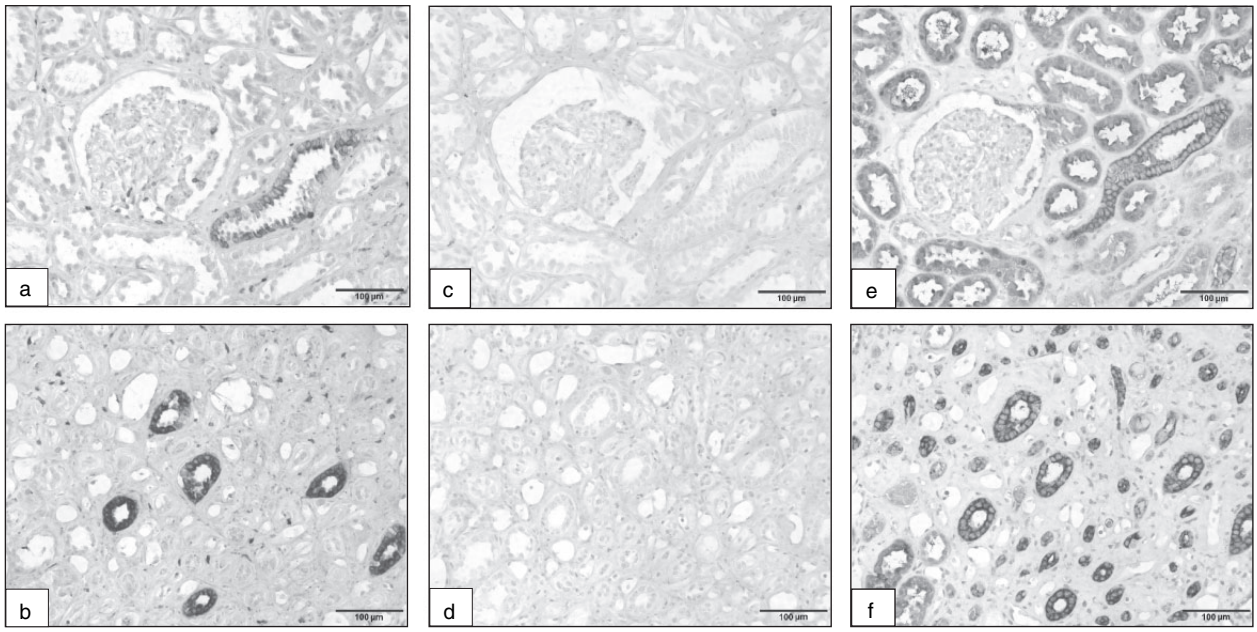

Figure 1 Cyclooxygenases-1 and -2 in a biopsy without signs of rejection. There is absent or only little COX-1 staining in glomeruli and vessels (a, orig. ×200). The most prominent staining of COX-1 is detected in collecting ducts, whereas in the other tubules, only distinct COX-1 staining is found (b, orig. ×200). No color product can be detected in the negative controls (c, d, orig. ×200). In some glomeruli, the cells of Bowman's capsule were positive for COX-2 staining. In the macula densa, no COX-2-staining was observed (e, orig. ×200). COX-2-staining in the epithelium of tubules and collecting ducts (f, orig. ×200).

was absent or only little COX-1 staining (Fig. 1a). The most prominent staining of COX-1 was detected in collecting ducts, whereas in other renal tubules, only limited COX-1 staining was found (Fig. 1b). In some glomeruli, the cells of Bowman's capsule were positive for COX-2 staining. In the macula densa, no COX-2 staining was observed (Fig. 1e). COX-2-positive cells were barely observed in arteries and arterioles, whereas a prominent staining was found in the epithelium of some proximal tubules and collecting ducts (Fig. 1f).

Expression of COX-1 and -2 in biopsies with acute allograft rejection

Seven biopsies from seven different patients were classified as Banff grade 4 type I, demonstrating signs of acute interstitial allograft rejection. Acute vascular rejection was present in 21 biopsies from 15 patients (12 biopsies classified as Banff grade 4 type IIA, six as Banff grade 4 type IIB, three as Banff grade 4 type III). We found a significantly higher expression of COX-1 in interstitial infiltrates ($P = 0.006$) in the patients with acute interstitial rejection and a significantly higher expression of COX-1 in interstitial infiltrates ($P = 0.001$, Fig. 2a) and in subendothelial cells of vessels ($P = 0.003$, Figs 2b and 6a) in the biopsies with acute vascular rejection. Elevation of COX-2 expression did only reach significance in the group of tubulointerstitial rejection regarding the interstitial infiltrates ($P = 0.038$, Figs 3 and 6b).

Expression of COX-1 and -2 in biopsies with chronic allograft nephropathy

Sixteen biopsies from 14 patients demonstrated signs of chronic allograft nephropathy (e.g. vasculopathy, interstitial fibrosis, tubular atrophy, seven classified as Banff grade 5 type I, nine as Banff grade 5 type II). Significant up-regulations were found only concerning COX-1 expression in subendothelial cells of vessels ($P = 0.002$, Fig. 4a) and in interstitial infiltrating cells ($P = 0.001$, Figs 4b and 6a).

Expression of COX-1 and -2 in biopsies with acute renal failure

Histological signs of acute renal failure (Banff grade 6) were seen in 21 biopsies from 17 patients. Compared with biopsies without rejection, COX-1 expression in biopsies with acute renal failure was significantly higher in vessels ($P = 0.02$), in tubular epithelial cells ($P = 0.005$) and in interstitium ($P = 0.001$, Figs 5a and 6a). The pattern of COX-2 staining in biopsies with acute renal failure did

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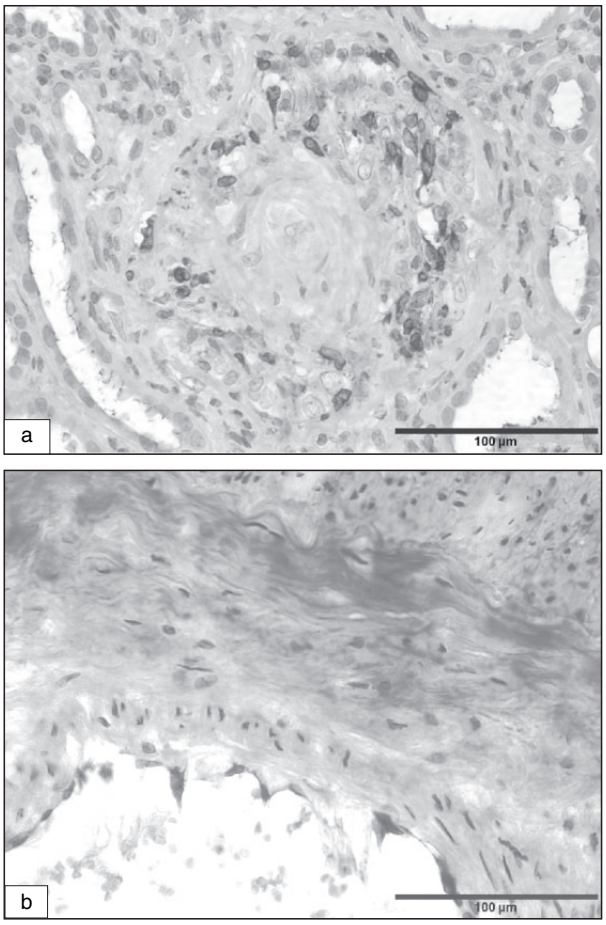

Figure 2 Cyclooxygenases-1 in a biopsy of a patient with acute allograft rejection. Increased expression of COX-1 in interstitial infiltrates (a, orig. ×200) and in subendothelial cells of vessels (b, orig. ×400).

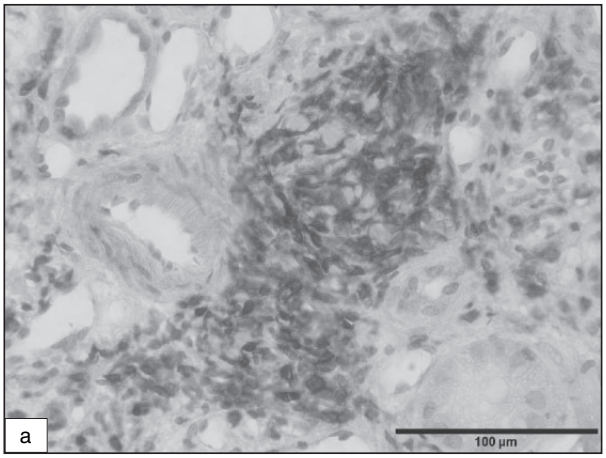

Figure 3 Cyclooxygenases-2 in a biopsy of a patient with acute allograft rejection. Positive COX-2 staining is detected in interstitial infiltrates (orig. ×200).

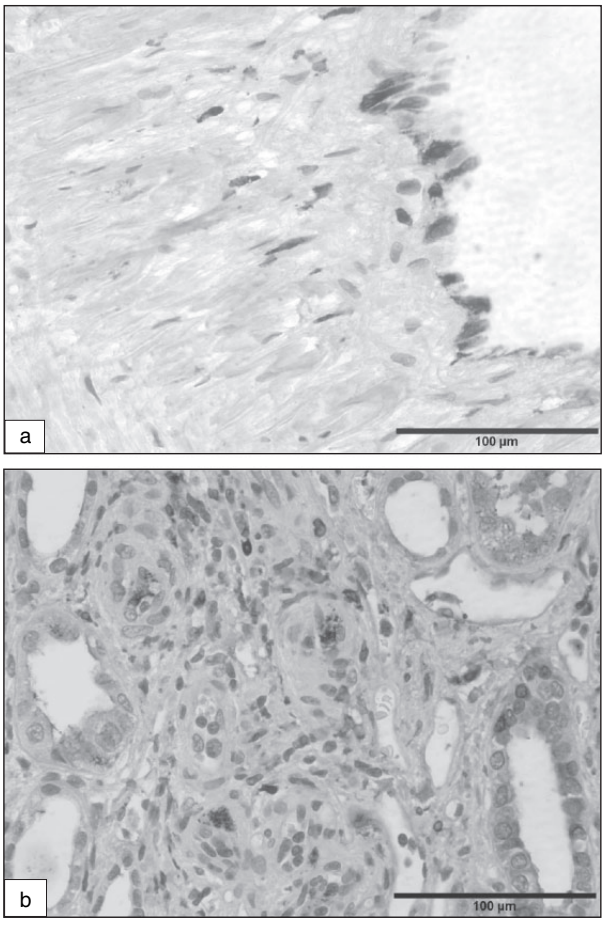

Figure 4 Cyclooxygenases-1 in a biopsy of a patient with chronic allograft nephropathy. Up-regulation is found in vessels (a, orig. ×400) and in interstitial infiltrating cells (b, orig. ×400).

not differ from that of biopsies without rejection (Fig. 6b).

Expression of COX-1 and -2 in biopsies with arteriosclerosis

This group included 10 biopsies from seven patients (Banff grade 6). COX-1 expression was up-regulated in arteries, arterioles ($P = 0.002$), and interstitium ($P = 0.042$, Fig. 5b) compared with biopsies without rejection, whereas no change in COX-2 expression could be detected (Fig. 6a and b).

Expression of COX-1 and -2 in biopsies with pyelonephritis

Nine biopsies from five patients demonstrated signs of pyelonephritis (Banff grade 6). The interstitial infiltrates contained a significant number of COX-1-positive cells

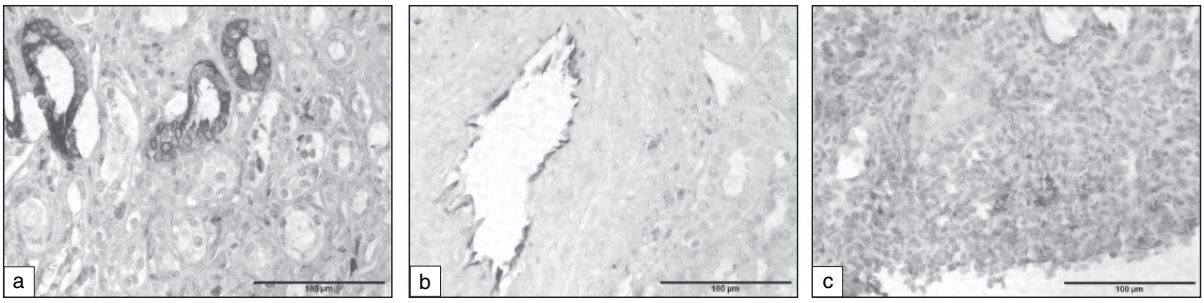

Figure 5 Cyclooxygenases-1 in biopsies of patients with *acute renal failure* (a, orig. ×400, COX-1 expression is significantly up-regulated in vessels, in tubular epithelial cells and in interstitial cells), *arteriosclerosis* (b, orig. ×400, COX-1 expression is up-regulated in arteries, arterioles and interstitial cells), and *pyelonephritis* (c, orig. ×400, COX-1 expression is up-regulated in interstitial infiltrates).

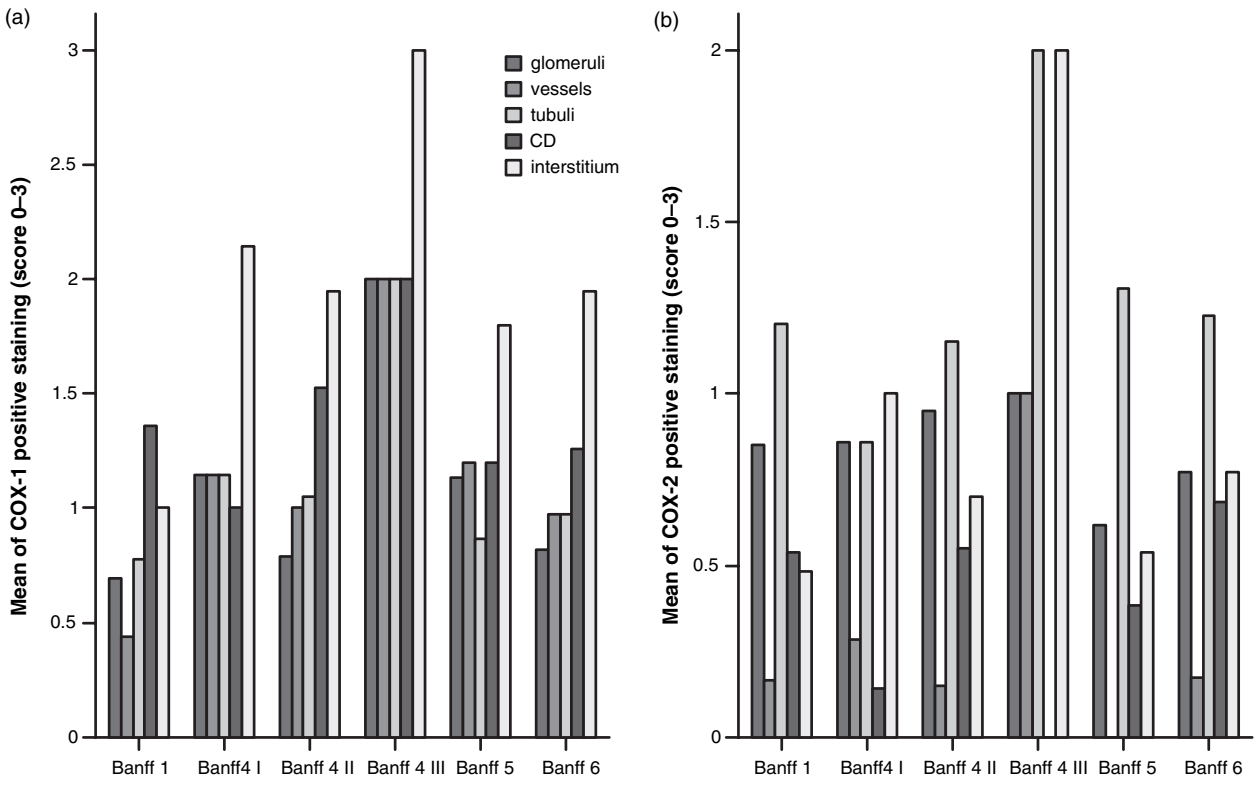

Figure 6 Mean of COX-1 (a) and COX-2 (b) positive staining in the different renal substructures [glomeruli, vessels, tubuli (except collecting ducts), CD (= collecting ducts), interstitium] according to Banff criteria. Score 0 was attributed to basically no staining, score 1 to weak staining, score 2 to moderate staining, and score 3 to strong staining.

($P = 0.001$, Fig. 5c) compared with biopsies with well-preserved tissue (Fig. 6a). The COX-2 expression pattern remained unchanged (Fig. 6b).

Discussion

Although several studies investigated the expression of COX after renal transplantation in animal models, only few data exist in humans.

In our study, an up-regulation of COX-1 could be seen in both intrinsic and infiltrating renal cells. In accordance with other previous animal and human studies [7], we detected a constitutive expression of COX-1 with a distinct and limited cellular localization in our large sample of 60 biopsies without signs of rejection and well-preserved renal tissue. The most prominent staining of COX-1 was found in collecting ducts whereas in other renal tubules, in glomeruli, arteries, arterioles,

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and capillaries, there was absent or only discrete COX-1 staining.

However, in comparison with biopsies with unaltered morphology, COX-1 was highly induced in acute allograft rejection. In the biopsies of patients with the diagnosis of acute tubulointerstitial rejection (Banff 4 I), we detected a significantly higher expression of COX-1 in infiltrating interstitial cells. In the biopsies with acute vascular rejection (Banff 4 II and III), COX-1 expression was significantly up-regulated in arteries and arterioles and in infiltrating interstitial cells. In previous studies, an increased formation of TxA2, which is COX-1 dependent, has already been associated with acute rejection episodes in rats and in patients [36,37]. TxA2 has been shown to potentiate the function of naïve and primed alloreactive T-cell population, and to stimulate the rejection of skin and renal allografts in rats while the administration of TxA2 synthase inhibitors was reported to delay kidney and skin MHC-incompatible rejection [38,39]. Therefore, this new finding of our study might be of great interest for human renal transplantation. On the other hand, Lewis rats, injected intrathymically with class II MHC allopeptides, which usually promote acceptance, reject their allografts when treated with TxA2 antagonists during the induction of tolerance [40].

Although published studies have documented a similar pattern of COX-2 expression (macula densa/cortical thick ascending limb of Henle and medullary interstitial cells) in kidneys of mouse, rat, rabbit, and dog [6,8,40], there are contradictory reports about the expression and localization of COX-2 in immunohistochemical studies of normal human kidney. As in our biopsies without signs of rejection, several studies did not detect COX-2 in the macula densa of normal adult human kidneys [6,10–11,17,22], whereas other reports showed a disease- and age-related expression of COX-2 in the macula densa [7,12,13]. We found COX-2-positive cells barely in vessels and only a moderate staining in cells of the Bowman's capsula. A more prominent staining was detected in collecting ducts, in epithelial cells of some proximal tubules and in infiltrating cells in our study sample with an unaltered morphology.

A recent retrospective study analyzed COX-2 expression in biopsies obtained from patients with acute vascular renal rejection in combination with interstitial cellular rejection and tubulitis [22]. They found that COX-2 expression was strongly up-regulated in proximal tubular cells with additional staining in the distal tubular epithelial cells [22]. In our study, elevation of COX-2 expression reached significance in the group of acute tubulointerstitial rejection (Banff 4 I) only in interstitial infiltrates. However, we confirmed the observations of these authors that few of the arteries showed distinct staining of endothelial cells, whereas most of the arteries were devoid of COX-2 immunoreactivity even if they showed morphologic signs of acute vasculitis.

Our finding of positive staining of COX-2 in infiltrating interstitial cells is new and in accordance with the recent observations of Rangel *et al.* [23] but not surprising, as prostaglandins have been previously described to be important in the pathogenesis of inflammation involving cell-mediated immune responses such as those that occur in allograft rejection [25,41]. Many cells are reported to synthesize COX-2, including macrophages, and monocytes [42]. The expression of COX-2 was also detected in patients with active lupus nephritis in infiltrating cells in the glomerulus, while little staining was observed in intrinsic renal cells of glomeruli, tubuli, and the interstitium [18]. There is evidence that COX-2 is transcriptionally up-regulated in T cells and that it behaves as an early inducible gene involved in the T-cell activation process [43].

A recent study demonstrated COX-2 induction during lung allograft rejection in inflammatory cells, especially in macrophages [24] at an early stage of pulmonary allograft rejection. As experimental data suggest that COX-2 is also up-regulated in infiltrating macrophages of rejecting heterotopic rat cardiac allografts [25], renal COX-2 expression on infiltrating cells, its regulation, and the release of COX-2-derived prostanoids might be of particular interest for future research in renal transplantation.

In the present study, we found in addition a significant up-regulation of COX-1 expression in arterioles and arteries and in interstitial infiltrating cells in 16 biopsies with signs of chronic allograft nephropathy. COX-2 expression did not change compared with biopsies with well-preserved tissue. Experimental data have demonstrated that a tissue inflammatory response occurs following the renal ischemia-reperfusion injury, which is implicated as one of the potential contributors for the development of chronic allograft nephropathy, the main cause of graft loss after the first year of transplantation [33]. COX-2 has been reported to participate in the endothelial cell activation after ischemia-reperfusion injury, and thus may have an impact on its functional outcome [21]. It is well conceivable that even if COX-2 is involved in early vascular damage also in human renal transplantation, we might easily have missed this finding in biopsies carried out days or weeks after renal transplantation. Currently, except living donor transplantation, there are no effective therapeutical approaches to limit ischemia-reperfusion injury; thus, a better knowledge about its pathophysiology, i.e. a putative role of COX-1 and -2 is crucial. The benefit of nonselective blockade of COX-1 and -2 in a murine model of ischemia-reperfusion injury has already been described [2].

Our patients were treated with triple immunosuppressive therapy consisting of a calcineurin inhibitor (tacrolimus or cyclosporine A), mycophenolate mofetil, and prednisolone. The interference of COX-2 with calcineurin inhibitors and glucocorticoids is well documented: in rat mesangial cells, COX-2-expression was suppressed by cyclosporine A treatment whereas COX-1 expression was not affected by this treatment [28]. Another recent study reported a decrease of COX-2 in cyclosporine A-treated mouse medullary thick-ascending limb-cultured cells [32]. These data were furthermore confirmed by *in vivo* data in rats reporting that both cyclosporine A and tacrolimus markedly lowered COX-2 expression while COX-1 expression remained unaltered [30]. In rats, a down-regulation of COX-2 expression was also observed by endogenous glucocorticoids [44]. The up-regulation of COX-2 mRNA was also inhibited by cyclosporine A in human peripheral blood lymphocytes [45]. Combined treatment of tacrolimus and dexamethasone down-regulated synovial COX-2 expression in humans, whereas neither tacrolimus nor dexamethasone alone influenced COX-2 expression [31]. The pathophysiology of cyclosporine A-induced acute renal vasoconstriction with nephrotoxicity and blood pressure increase involves among other mechanisms a decrease of the vasodilating prostaglandins E_2 (PGE2) and 6-keto-prostaglandin $F_{1\alpha}$ [46]. In addition, PGE2 has been reported to increase the efficacy of immunosuppressive protocols in organ transplantation models [47]. Recently, the limited efficacy of mycophenate mofetil has been attributed to its down-regulation of PGE2 production in humans [48].

In our biopsies with acute allograft rejection, there was no increase of COX-2 expression in tubuli, vessels, or glomeruli. A down-regulation by the immunosuppressive treatments might possibly explain this phenomenon.

Whether COX are pro-inflammatory in the setting of acute rejection or might have protective properties is not clear yet. Data have shown that PGE2 modulates the T-helper cell type 1 response, impairing the expression of TNF-α, IL-12, and IFN-γ [49,50]. Furthermore, PGE2 recently has also been reported to suppress chemokine production in human macrophages through the EP4 receptor [51]. Even if COX-2 is considered a pro-inflammatory enzyme and a chief target for the treatment of inflammatory diseases, it has been described to be anti-inflammatory during a later, mononuclear cell-dominated phase of pleurisy by generating anti-inflammatory PGD_2 metabolites [52].

In summary, this is the first prospective study investigating COX-1 and -2 expression in human renal transplant biopsies. In our large sample of 144 biopsies, we clearly demonstrate a highly significant induction of renal COX-1 in vessels and of both COX-1 and -2 in interstitial infiltrating cells during acute renal allograft rejection. The pathophysiological role of COX in these cells has to be elucidated in further studies.

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