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ORIGINAL ARTICLE

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Molecular and clinical response to angiotensin II receptor antagonist in kidney transplant patients with chronic allograft nephropathy

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Abstract Chronic allograft nephropathy (CAN) represents an important cause of graft loss after kidney transplantation. TGF-β1 is a key factor in fibrogenesis, and the angiotensin II receptor antagonist losartan may decrease the intra-graft synthesis of TGF-β1. The aim of this study was to determine the clinical and molecular effect of losartan in kidney transplant patients (KTPs) with CAN. We studied nine KTPs, after the first year of transplantation, with proteinuria (more than 500 mg/24 h), stable renal function, and histological signs of CAN. Immunosuppression was cyclosporine, azathioprine, and corticoids. Kidney biopsy was performed in all patients at the beginning of the study and 12 weeks after treatment with 50 mg/day of losartan. Quantitation of intra-graft expression of TGF-β1 was performed in all biopsies, by real-time PCR. After losartan treatment there were no differences in patients' BP and blood creatinine level. The proteinuria significantly dropped to 414.2 ± 377 mg/24 h, $P = 0.001$. Intra-graft expression of TGF-β1 was decreased after treatment. In conclusion, losartan significantly decreases the intra-graft expression of TGF-β1 and proteinuria in KTPs with CAN.

Keywords Angiotensin II receptor antagonist · Angiotensin II · Chronic allograft nephropathy · TGF-β1 · Kidney transplantation · Losartan

Introduction

Chronic allograft nephropathy (CAN) remains an important cause of long-term graft loss, despite the use of new and more potent immunosuppressive drugs that prevents acute rejection. Even though immunological and non-immunological factors are involved in its pathogenesis, the exact molecular mechanism remains unclear [1].

The histopathology is non-specific and consists of fibrous intimal thickening of the arteries and arterioles, glomerulosclerosis, interstitial fibrosis and tubular atrophy [2].

Fibrogenesis and extracellular matrix accumulation leads to renal fibrosis and loss of renal function. This is the final common pathway by which the kidney responds to a variety of insults [3]. In chronic nephropathies, as well as in CAN, a myriad of mediators,

including proinflammatory cytokines, enzymes and growth factors, appears to play a critical role. There is evidence to support the notion that TGF-β1, is one of the main factors involved [3, 4]. A large number of stimuli induces their expression as cell damage or lyses, immune complex deposition, high glucose levels, advanced glycosylation end products, mesangial cell stretch and platelet-derived growth factor (PDFG), among others.

Recent studies have shown that angiotensin II (Ang II) is involved in the regulation of TGF-β1 and collagen production [4]. The intra-renal renin–angiotensin system (RAS) has been shown to be important in the progression of renal disease. Ang II, after binding the high-affinity type 1 receptor (AT1), stimulates mesangial and tubular cell growth and collagen production, and this effect appears to be mediated by TGF-β1 [4]. We, and others, had reported an increased intra-graft expression of TGF-β1 and angiotensinogen in kidney transplant patients with histological and clinical diagnosis of CAN [5, 6].

The goal of this study was to analyse the clinical and molecular responses to an Ang II receptor antagonist, losartan, in renal transplant patients with histological evidence of CAN.

Patients and methods

Nine kidney transplant patients (six deceased donors, three living donors) were prospectively recruited and comprised five men and four women with a median age of 40.0 ± 16.0 years. The mean post-transplantation follow-up period was 36.5 ± 11.2 months. A triple-drug immunosuppressive protocol was used that included cyclosporine (CsA), trough level 254.0 ± 33.0 ng/ml. The inclusion criteria were: stable long-term kidney transplant patient with more than 12 months since transplantation, serum creatinine < 1.5 mg/dl, proteinuria > 500 mg/24 h, BP $\leq 140/90$ mmHg, and histological evidence of CAN in the graft. All patients gave written informed consent, and the Ethical Committee of the Hospital Privado de Cordoba, Argentina, approved the protocol.

The study consisted of 12 weeks' active treatment with losartan (50 mg/day). Allograft biopsy and laboratory and molecular studies were performed at baseline (pre-treatment) and at 12 weeks after treatment. Needle renal allograft biopsies were performed in all patients (in accordance with the protocol of the renal transplant programme of the Hospital Privado). Tissue biopsies were classified on the basis of Banff working classification criteria (1997) [7]. The histological diagnosis was based on the presence of interstitial fibrosis and tubular atrophy and additional vascular changes including arterial fibrous intimal thickening, and glomerular tuft shrinkage, sclerosis and thickening or wrinkling of the basement membrane. The glomerular changes, interstitial fibrosis, tubular atrophy and vascular changes were graded on a scale of 0 to 3, as proposed [7].

Total RNA was extracted from biopsies with the SV Total RNA Isolation System (Promega, Madison, Wis., USA). Briefly, guanidine thiocyanate (GTC) and β-mercaptoethanol were used to inactivate the ribonucleases found in the tissue. After centrifugation, the RNA was selectively precipitated out of solution with ethanol and bound to the silica surface of the glass fibres found in the spin basket. RNase-free DNase I (Promega) was applied directly to the silica membrane to digest contaminating genomic DNA. Finally, the total RNA was eluted from the membrane by the addition of nuclease-free water. RNA was quantified by ultraviolet spectrophotometry.

The real-time PCR was performed on an ABI Prism 7700 (Perkin-Elmer, Applied Biosystems, Foster City, Calif., USA) PCR and detection instrument. The detection and quantitation of TGF-β1 was performed with TaqMan pre-developed assay reagents for gene expression (Applied Biosystems). Each assay was performed with 2X mix of forward primer, reverse primer, and 6-FAM (6-carboxyfluorescein) dye-MGB labelled probe and TaqMan Universal Master mix. Quantitative RT-PCR was performed in duplicate and included a no-template control as a negative control. Two positive controls of known concentrations (high positive control and low positive control) were used for the quantitation. These positive controls (internal RNA quantitative standard) were created with a PCR Mimic construction kit, according to the manufacturer's specifications (Clontech, Palo Alto, Calif., USA) as previously published [5]. In brief, v-erb B DNA fragment was used for PCR amplification, using 65-mer and 40-mer oligonucleotides, respectively. These oligonucleotides contained 20 nucleotides of the T7 promoter sequence, 25 nucleotides homologous to the v-erb B DNA and 20 nucleotides that corresponded to each of the primers of TGF-β1. The desired primer sequences were thus incorporated during the PCR reaction. A dilution of the first PCR reaction was re-amplified with the TGF-β1-specific primers and a T7 promoter in the sequence 5′. We used 10 µl of DNA quantitative standard for the RNA quantitative standard construction. Using transcription with T7 polymerase and posterior digestion with DNase-RNase-free, we obtained RNA quantitative standard (RNA-QS). The RNA-QS was diluted in high-performance liquid chromatography (HPLC) water, RNase-free, and quantified for subsequent use.

The expression levels of BCR mRNA in each sample were used as endogenous control (VIC dye-MGB labelled probe). During the real-time RT-PCR reaction, the emission is proportional to the amount of amplified product, until it reaches the log-phase of the PCR. A

Table 1 Clinical parameters in kidney transplant patients with CAN, before and after losartan treatment (*NS* not significant)

Parameter	Baseline	After losartan treatment	*P*
Creatinine (mg/dl)	1.35 ± 0.2	1.40 ± 0.3	NS
Proteinuria (mg/24 h)	1,117 ± 470	414.2 ± 377	0.001
Systolic BP (mmHg)	129 ± 11	125 ± 18	NS
Diastolic BP (mmHg)	78 ± 7	73 ± 10	NS
Cyclosporine level (ng/ml)	254 ± 33	259 ± 30	NS

threshold value is set above the baseline, reflected by the average change in emission during the first PCR cycles. The Ct value (cycle number) is the point at which the emission passes above the baseline and thus mirrors the accumulation of PCR product in a specific well of the PCR reaction. The Ct value for every sample at the FAM layer (TGF-β1) was averaged among duplicates. That average was subtracted from the Ct value for every sample at the VIC layer (endogenous control), generating a ΔCt value. That ΔCt value was then related to one of the positive quantitative standards controls (high and low) at a time, by the $\Delta\Delta$Ct method, where $\Delta\Delta CT = \Delta Ct_{control} - \Delta CT_{sample}$. Finally, the results were expressed as transcripts/μg total RNA.

Statistical analysis was performed with the paired *t*-test. Data were reported as mean ± standard deviation. Pearson's correlation coefficient was calculated to examine the relation between proteinuria and intra-graft expression of TGF-β1 before and after losartan treatment. $P < 0.05$ was considered significant.

Results

Nine kidney transplant patients with more than 12 months of post-transplantation time were prospectively included in this study. As was previously mentioned, the patients presented proteinuria levels higher than 500 mg/24 h and histological evidence compatible with CAN. The glomerular changes, interstitial fibrosis, tubular atrophy and vascular changes were graded on a scale from 0 to 3. Among the 12 biopsies, three were graded as 2 and six as 1. After a complete 12 weeks of

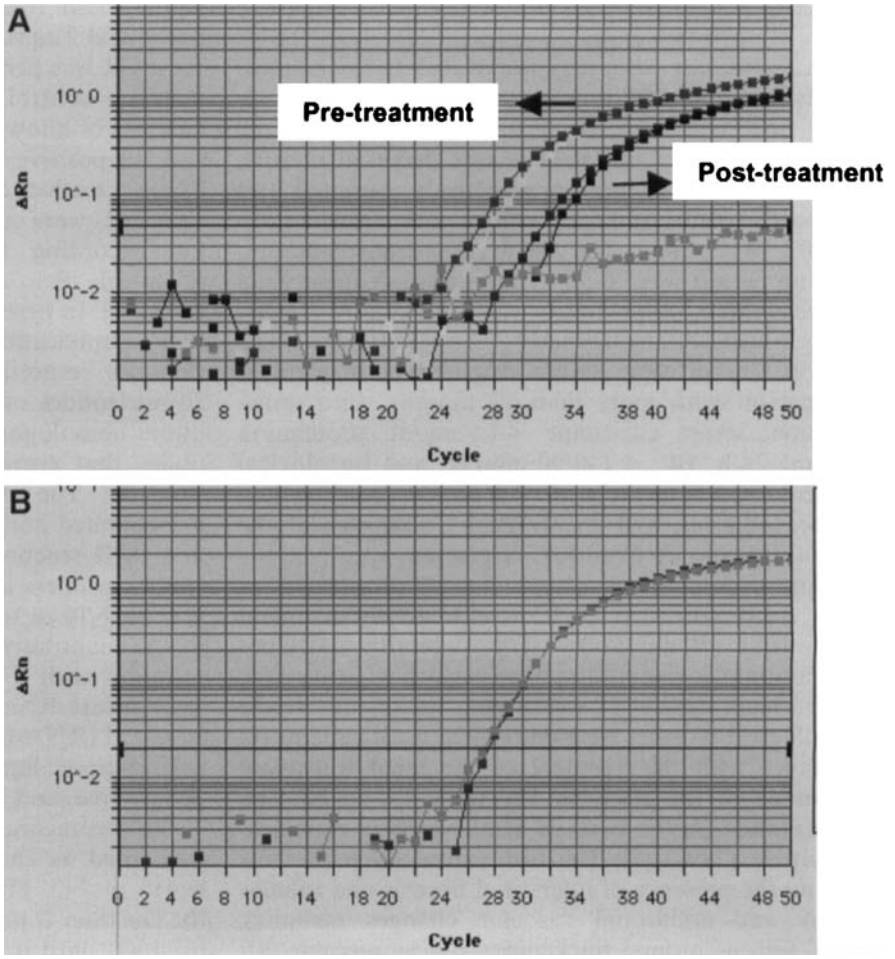

Fig. 1A,B Intra-graft expression of TGF-β1 in patient samples before and after losartan treatment. **A** Quantitation of TGF-β1 mRNA isolated from kidney biopsies by real-time RT-PCR. Samples were studied in duplicate. The Ct value (cycle number where the fluorescence crosses the threshold line) for every sample was averaged among duplicates. Pre-treatment samples crossed the threshold at earlier cycle numbers, indicating higher TGF-β1 expression, while post-losartan treatment samples crossed the threshold later in the cycling, indicating lower TGF-β1 expression. **B** RNA quality and loading was assessed with primers and probe for the gene BCR, used as an endogenous control. The RNA samples both before and after losartan treatment showed similar Ct values

Table 2 Intra-graft TGF-β1 expression at baseline and after losartan treatment. Values are expressed as TGF-β transcripts/μg total RNA

Patient	Baseline	After treatment
1	3.00×10^3	2.70×10^3
2	4.96×10^4	1.07×10^4
3	3.83×10^4	10.80×10^3
4	6.44×10^3	5.21×10^3
5	2.80×10^4	1.11×10^4
6	1.41×10^4	1.21×10^4
7	5.83×10^4	1.31×10^4
8	6.20×10^4	1.07×10^4
9	7.00×10^3	6.00×10^3
mean	2.96×10^4	9.17×10^3
std	2.20×10^4	3.50×10^3
p	0.02	

losartan treatment, proteinuria dropped significantly to 414 ± 377 mg/24 h, $P=0.001$, without any difference in blood pressure, CsA and blood creatinine levels (Table 1). At the molecular-marker level there was a decrease of intra-graft expression of TGF-β1 mRNA, with a reduction from the baseline measurement. TGF-β1 mRNA in each sample was down-regulated (from $2.96\times10^4\pm2.20\times10^4$ transcripts/μg total RNA baseline to $9.1710^3\pm3.50\times10^3$ transcripts/μg total RNA after losartan treatment ($P=0.02$) (Fig. 1). Individual values of TGF-β1 mRNA for each patient included in the study, at baseline and after 12 weeks of losartan treatment, are shown in the Table 2.

Allograft biopsies after treatment did not show changes in comparison with the baseline biopsies. We found correlation between proteinuria and intra-graft expression of TGF-β1 before and after losartan treatment ($r^2=0.79$, $P=0.01$ and $r^2=0.87$, $P=0.01$, respectively).

Discussion

Over the past several decades there have been significant advances in the ability to control acute rejection through more effective immunosuppressive regimens, but these improvements have had little impact on the long-term renal allograft survival rates [1].

CAN is a major problem, which limits long-term graft survival, but underlying molecular and immunological mechanisms remain poorly understood [1, 2].

In this study we demonstrated that losartan significantly decreased proteinuria as well as the intra-graft expression of TGF-β1 in patients with CAN. Proteinuria is considered a factor that may, itself, contribute to renal disease progression. A postulated hypothesis is that proteins exert a toxic effect on tubular cells with the release of lysozymes into the interstitium, inducing fibrosis. Therapeutic approaches directed at reducing proteinuria have been developed [8]. Inhibition therapy has been demonstrated to have a reno-protective effect. In renal transplant patients, proteinuria has been shown to decrease renal graft and patient survival times [9].

We found a down-regulation of intra-graft expression of TGF-β1 after losartan therapy in kidney transplant patients treated with CyA. Production of TGF-β1 may be modulated either by the intra-renal renin–angiotensin system [3] or by a direct effect of CyA [10]. In addition, CyA also causes over-expression of Ang II, inducing, by this mechanism, the synthesis of TGF-β1 [10]. Our patients maintained CyA trough levels of 254.0 ± 33.0 ng/ml during the entire treatment period with losartan.

Campistol et al., in previous studies, demonstrated a drop of TGF-β1 plasma level in patients with CAN after losartan treatment [11]. In this study we have seen a decrease in the intra-graft expression of TGF-β. The local RAS is increased in different progressive nephropathies, including CAN. Ang II, through the AT1 receptor, up-regulates TGF-β production. Losartan, by blocking the AT1 receptor, might, perhaps, interrupt the intra-renal pathway of Ang II and TGF-β production, limiting the perpetuation of renal tissue damage and fibrotic tissue formation.

In the study group, we did not observe differences in the histological findings of the allograft biopsies performed before and after treatment. These results might indicate that the tissue damage is a non-reversible process, indicating the importance of the prevention of CAN in the earlier stages of transplantation.

In conclusion, losartan efficiently reduced proteinuria in the studied group of kidney transplant patients with CAN, with an adequate tolerance and via a mechanism apparently different from its antihypertensive effect. By reducing proteinuria and TGF-β, Ang II receptor blockers might reduce, or even prevent, the development of CAN. This effect needs to be confirmed by prospective studies with larger numbers of patients. Multicentre and prospective studies directed to confirm these clinical observations are required.

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