Polymerized type I collagen reduces chronic cyclosporine nephrotoxicity

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Abstract

Background. Polymerized type I collagen (P-collagen) has been successfully used to reduce human hypertrophic scars due to its anti-fibrotic and anti-inflammatory properties. We therefore carried out a study to determine if P-collagen reduces functional and structural injury in chronic cyclosporine [cyclosporine A (CsA)] nephropathy.

Methods. Four groups of six male Wistar rats fed with a low sodium diet were treated with vehicle, P-collagen (0.8 mg/day, i.p.), CsA (15 mg/kg) or CsA + P-collagen for 15 days. Mean arterial pressure, renal blood flow and glomerular filtration rate were measured in all groups. Structural injury such as arteriolopathy, tubulo-interstitial fibrosis (TI-fibrosis) and positive apoptotic cells were quantified. The mRNA expression levels of transforming growth factor-β (TGF-β), kidney injury molecule (Kim-1), α-smooth muscle actin (α-SMA), glutathione peroxidase, catalase and Cu/Zn superoxide dismutase (SOD) as well as MnSOD were assessed. Antioxidant enzyme activity, renal lipoperoxidation and urinary excretion of oxygen peroxide (UH₂O₂V) were determined.

Results. Cyclosporine produced renal dysfunction and induced the development of arteriolopathy, TI-fibrosis and tubular apoptosis. These alterations were associated with increases in TGF-β, Kim-1 and α-SMA mRNA levels as well as with a significant increase of oxidative stress and a reduction of SOD activity. P-Collagen partially ameliorated CsA-induced renal dysfunction and structural injury and prevented both tubular apoptosis and increased oxidative stress. This renoprotective effect was found to be associated with a reduction of TGF-β, Kim-1 and α-SMA mRNA levels.

Conclusions. This study has therefore demonstrated that P-collagen appears to have anti-fibrotic and anti-apoptotic properties and highlights the possibility that the compound might be useful in a strategy to reduce chronic CsA nephrotoxicity.

Keywords: anti-fibrotic drug; oxidative stress; tubular apoptosis

Introduction

The introduction of the calcineurin inhibitor, cyclosporine A (CsA), into clinical transplantation procedures as an immunosuppressive agent to prevent allograft rejection has led to significant improvements over the last three decades, in both allograft half-life and patient survival [1]. Currently, 94% of kidney transplant recipients are treated with a calcineurin inhibitor-based immunosuppressive regimen [1].

The therapeutic benefits of calcineurin inhibitors have unfortunately been limited by side-effects, such as nephrotoxicity, which occur in almost all treated patients after long-term use [2,3]. Two forms of CsA renal toxicity have been described: acute nephropathy and chronic nephropathy. The acute form is characterized by renal vasoconstriction that is induced by an imbalance in vasoactive substance release, which in turn results in renal vasoconstriction and dysfunction. In contrast, the chronic form of nephrotoxicity is characterized not only by renal vasoconstriction but also by the development of structural damage, including arteriolopathy and tubulo-interstitial fibrosis (TI-fibrosis) that may lead to end-stage renal disease [3–5].

Several factors have been implicated in the development of structural injury during chronic CsA nephrotoxicity. Among them are (i) activation of the renin angiotensin aldosterone system, which not only induces renal vasoconstriction but also promotes fibrotic processes [6,7]; (ii) renal hypoxia, as a consequence of renal vasoconstriction induced by unbalanced release of vasoactive substances; this leads to reactive oxygen species generation, which causes
additional cellular injury and consequently cellular death by apoptosis [8,9]; and (iii) transforming growth factor-β (TGF-β) up-regulation, which promotes renal fibrosis by increasing the production and decreasing the degradation of extracellular matrix proteins [7,10–12].

Numerous drugs that switch off some of these pathways have been studied to reduce chronic CsA nephrotoxicity, such as agents that improve renal perfusion, anti-inflammatory drugs, anti-fibrotic agents, drugs that antagonize mineralocorticoid receptors and magnesium supplementation (for review see [13]). However, most of these treatments are unable to prevent both functional and structural injury; the most promising treatment has been mineralocorticoid receptor blockade, which completely prevents renal failure and reduces structural injury by about 50% [10,14,15].

Type I collagen–polyvinylpyrrolidone or polymerized type I collagen (P-collagen) is a biodrug comprised of a γ-irradiated mixture of pepsinized porcine type I collagen and polyvinylpyrrolidone (Aspid, Mexico). Animal studies have shown that P-collagen may reduce the chronic inflammatory process and improve both skin wound repair and bone fractures in rats by stimulating the healing process and accelerating new bone formation [16]. In addition, this biopolymer was able to decrease the incidence and size of intra-abdominal adhesions in appendectomized rabbits [17]. Moreover, at the site of tracheoplasty, P-collagen reduces inflammatory lymphocytic infiltration and the degree of fibrosis and tracheal stenosis in dogs [18]. Studies performed in humans have shown that acute and chronic P-collagen administration is not genotoxic; it does not induce a localized hypersensitivity reaction, fibroproliferation, lymphoproliferation, human antiporcine collagen or anti-collagen–polyvinylpyrrolidone antibodies [19,20]. In addition, a protective effect of P-collagen has been reported in patients with rheumatoid arthritis [21–24], scleroderma [25], pressure ulcers [26] and hypertrophic scars [20]. Together, each of these studies has shown that P-collagen possesses immunomodulatory, fibrolytic, anti-fibrotic, haemostatic and tissue regeneration properties. Thus, due to the beneficial anti-fibrotic effects of P-collagen in animal models or in human diseases, we reasoned that P-collagen might represent a useful biomolecule to reduce renal fibrosis induced by chronic cyclosporine administration.

Materials and methods

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, USA 1996) and were approved by the Animal Care and Use Committee of our institution. Twenty-four male Wistar rats weighing 270–320 g were used for the study. The rats were divided into four groups: rats that received olive oil as vehicle 0.1 ml daily s.c. (V), rats treated with P-collagen intraperitoneally 0.2 mg daily (P-collagen, Aspid SA de CV, Mexico), rats administered with cyclosporine 15 mg/kg s.c. (CsA) and rats that received cyclosporine and P-collagen simultaneously (CsA + P-collagen) for 15 days. This P-collagen dose was chosen because it has been previously tested [19,22,25] and because we performed preliminary experiments using 0.1, 0.2 and 0.4 mg of P-collagen. The lower dose produced a minor renoprotective effect, whereas the higher dose produced similar renoprotection to the middle P-collagen dose (data not shown). Thus, we decided to use in this study 0.2 mg of P-collagen. Treatments were administered for 15 days. All animals were fed a low sodium rat diet (0.02%) and water ad libitum. In addition, rats were kept under a 12-h light–dark cycle. Rats receiving vehicle and P-collagen were pair-fed with the CsA and CsA + P-collagen groups, respectively.

Renal functional studies

At the end of the experiment, rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.), and supplemental doses were instilled as required. The rats were placed on a homeothermic table to maintain corporal temperature at 37°C. Trachea, both jugular veins, femoral arteries and the bladder were catheterized with polyethylene tubing PE-240, PE-50 and PE-90, respectively. During surgery, rats were maintained under euvoletic conditions by infusion with 10 ml/kg of body weight of isotonic rat plasma followed by an infusion of 5% low calorie commercial sugar (METCO, Mexico City, Mexico) at 1.6 ml/h as a marker of glomerular filtration rate (GFR). We have previously shown that this compound has sufficient sensitivity to measure GFR under normal and pathophysiological conditions to a similar extent as the standard measurement using polyfructosan [27,28]. Mean arterial pressure (MAP) was continuously monitored with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA, USA). Via a midline abdominal incision, the left renal artery was exposed, and an ultrasound transit-time flow probe (IRB, Transonic, Ithaca, NY) was placed in the left renal artery and filled with ultrasonic coupling gel (HR Lubricating Jelly, Carter-Wallace, New York City, NY, USA) to record renal blood flow (RBF). GFR was measured by the Davidson method [29].

Histological studies

At the end of the experiment, one kidney from each rat was removed and quickly frozen for molecular studies. The other kidney was perfused through the femoral artery and fixed in a 4% paraformaldehyde solution. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 3 μm and stained with routine periodic acid-Schiff and Masson trichromic. The arteriopathy percentage was evaluated by counting at least 100 afferent arterioles and determining the proportion with lesions. In Masson-stained slides, the degree of Tii-fibrosis was evaluated by morphometry, as previously reported by our laboratory [10]. For this purpose, 10 cortex fields (magnification ×200) were randomly selected in kidney sections from the different experimental groups. The images were recorded using a digital camera mounted on a Nikon microscope; the affected areas were delimited and quantified using eclipse net processing and analysis software (Nikon Instruments Inc., Whitman Road, Melville, NY). Tii-fibrosis consisted of extracellular matrix expansion and collagen deposition together with distortion and collapse of the tubules; fibrosis was evidenced by blue coloration in Masson stain. The proportion of fibrosis was calculated by dividing the area of interstitial fibrosis by the total area per field, excluding the glomerular and luminal tubular areas. The histological analysis was performed without knowledge of the experimental group in which each kidney belonged.

Terminal transferase dUTP nick end labelling assay

Apopotosis in the kidney sections was determined by the terminal transferase dUTP nick end labeling (TUNEL) assay using an ApopTag in situ apoptosis detection kit (S7101, Chemicon International, Temecula, CA). Sections were de-waxed and treated with proteinase K (400 mg/ml) for 5 min at room temperature and incubated with 3% hydrogen peroxide (H2O2) for 20 min at room temperature to inactivate endogenous peroxidase. Sections were rinsed with phosphate buffered saline (PBS) three times for 5 min; they were then incubated in Labeling Safe buffer (TaKaRa Biomedicals) with terminal deoxynucleotidyl transferase (TaKaRa Biomedicals) at 37°C for 60 min. Sections were washed in PBS and incubated with anti-fluorescein isothiocyanate horseradish peroxidase (HRP) conjugate at 37°C for 30 min. Apoptotic nuclei were visualized by the addition of diamobenidine and counterstained with methyl green. TUNEL-positive cells were counted in the cortical tubular cells at ×200 magnification in a minimum of 10 fields; images were then recorded and analysed blindly.

CsA blood levels

Blood CsA concentration was determined by monoclonal radioimmunoassay kit (TDx/TDXFlx; Abbott Laboratories, Abbott Park, IL, USA) exclusively in the groups that received CsA.
RNA isolation and real-time PCR

Total RNA was isolated from each kidney following the guanidine isothio- cyanate-cesium chloride method and checked for integrity by 1% agarose gel electrophoresis; RNA concentration was measured by absorbance at 260 nm (Beckman DU640). Reverse transcription was carried out using 2.5 μg of total RNA from each rat at 37°C for 60 min using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen). The mRNA levels of soluble superoxide dismutase (Cu/ZnSOD), mitochondrial superoxide dismutase (MnSOD), glutathione peroxidase (GPx), catalase (Cat), TGF-β, kidney injury molecule (Kim-1) and procaspase-3 were quantified by real-time PCR with the ABI Prism 7300 Sequence Detection System (TaqMan, Applied Biosystems, Foster City, CA). FAM or VIC dye-labelled probes were selected from the Applied Biosystems Assays-On-Demand ABI product line and were specifically used to detect and quantify cDNA sequences without detecting genomic DNA. Primers and probes for Cu/ZnSOD, MnSOD, GPx, Cat, TGF-β, Kim-1 and procaspase-3 were ordered as kits: Rn00566938_m1, Rn00999008_s1, Rn01577994_g1, Rn00569300_m1, Rn00572010_m1, and Rn0057703_m1 (Assays-on-Demand, ABI). As the endogenous control, we used eukaryotic 18S rRNA (predesigned assay reagent, ABI, external run) to correct for potential variations in RNA loading or the efficiency of the amplification reaction. When the probes were able to amplify genomic DNA, the RNA samples were treated with RNase-free DNase I. The relative quantification of Cu/ZnSOD, MnSOD, GPx, Cat, TGF-β, Kim-1 and procaspase-3 gene expression was performed using the comparative CT method.

Oxidative stress evaluation

Renal lipoperoxidation. Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid reactive substances. Briefly, after homogenization of the tissue, the reaction was performed in a 0.83% aqueous solution of thiobarbituric acid in 15% TCA and heated at 95°C for 45 min; the mixtures were then centrifuged at 3000 g for 15 min, and the absorbance of the supernatant was determined at 532 nm. Tissue protein levels were estimated with the Bradford method, and lipoperoxidation was expressed as nanomoles per milligramme of protein.

Urinary isoprostanes assay. The concentration of 8-epi-prostaglandin F2 alpha (8-epi-PGF _2α_ ) was determined in urine samples employing a urinary 8-iso-PGF _2α_ ELISA assay from Northwest (Vancouver, WA 98662) following the indications of the manufacturer. Briefly, 100 μl of each sample or standard was placed in wells that contained the adhered antibody anti 8-epi-PGF _2α_ and 100 μl of HRP-conjugated was added. Then 200 μl of TMB substrate was added to the wells and incubated again; the reaction was stopped by adding 50 μl H_2 SO_4 3 M, and the wells were read at 450 nm. The isoprostanes F _2α_ concentration was reported as nanograms per millilitre.

Urinary H_2O_2 assay. The amount of H_2O_2 in the urine samples was determined by using Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Carlsbad, CA). The manufacturer's instructions. Briefly, the assay was performed by employing a standard curve of 1–10 μM H_2O_2. A volume of 50 μl of each urine of the standard was placed in a microplate; 50 μl of the Amplex red reagent/HRP was then added, and the samples were incubated for 30 min at room temperature, protected from ambient light. The plate was then read at 560 nm. The H_2O_2 concentration in the samples is expressed as nanomoles per millilitre. The accuracy of this assay to detect H_2O_2 has been previously validated [30].

Antioxidant enzyme activity. Renal tissue was homogenized in a solution of 50 mM potassium phosphate, 0.1% Triton (pH 7.0). Crude homogenates were centrifuged at 15 300 rpm for 30 min at 4°C and used to assess antioxidant enzyme activities. Total protein concentration was measured by Lowry's method [31].

Cat activity assay. Renal Cat activity was determined according to the Aebi method [32]. This method is based on measuring the decomposition of H_2O_2 by Cat at an optical density of 240 nm at times 0 and 15 s (first-order kinetics) given the equation k = (1/ΔA) (2.3 × log A₁/A₁), where k is the first-order reaction rate constant and t is the time interval of decrease of H_2O_2. An enzyme activity unit is defined as the degradation of 1 μmol H_2O_2/s/mg tissue protein. Enzyme activity is expressed as k/mg protein as assayed at 25°C by a method based on the disappearance of H_2O_2 from a solution containing 30 mM H_2O_2 in 10 mM potassium phosphate buffer, pH 7.0 (output read at 240 nm).

GPx activity. GPx activity was indirectly detected by a method previously described by Lawrence and Burk [33]. GPx requires reduced glutathione, which is regenerated by glutathione reductase using oxidized glutathione, a process that consumes NADPH. The activity of GPx is defined as micromoles of NADPH oxidized per minute, taking into account that the millimolar absorption coefficient for NADPH at 340 nm is 6.22 l mmol⁻¹ cm⁻¹. Results are expressed as units per milligramme of protein.

Superoxide dismutase (SOD) activity. SOD activity was measured through the inhibition of nitroblue tetrazolium blue (NBT) reduction by O2⁻ generated through the xanthine-xanthine oxidase system, as previously reported [33]. One SOD activity unit is defined as the amount of enzyme promoting 50% inhibition of NBT reduction in 1 ml reaction solution per milligramme tissue protein. Results are expressed as units per milligramme of protein.

Statistical analysis

Results are presented as means ± SE. Significance of the differences among groups was tested by analysis of variance using Bonferroni's correction for multiple comparisons. All comparisons passed the normality test. Statistical significance was defined when the P-value was ≤ 0.05.

Results

Effect of P-Collagen on renal dysfunction induced by CsA

Figure 1 shows the main parameters of renal function in rats that received different treatments during 15 days. As expected, chronic cyclosporine administration produced a profound reduction in RBF, resulting in a significant decrease (>50%) of GFR (Figure 1A and B). Although P-collagen was unable to completely restore RBF and GFR, an improvement in renal dysfunction was observed. None of these findings were associated with changes in MAP (Figure 1C). Thus, the P-collagen effect was pressure-independent.

Structural renal injury induced by CsA was reduced by P-Collagen

Fixed kidneys were used to assess arteriopathy and TIFibrosis. Figure 2A and B is a representative photomicrograph of renal slides stained with periodic acid-Schiff (PAS), showing arteriopathy in rats treated with CsA and CsA + P-collagen, respectively. Figure 2C depicts the quantification of the arteriopathy percentage in the four studied groups. As much as 43% of arterioles were injured in CsA-treated rats; whereas, only 23% were injured in the CsA + P-collagen group. In addition to the reduced percentage of arteriopathy in CsA + P-collagen treated rats, arteriolar thickness was lower in this group than in CsA-treated rats. This is also evident in Figure 2A and B, in which the capillary lumen is narrower in the CsA group than in the CsA + P-collagen group. Figure 2D and E is a representative photomicrograph of Masson-stained renal sections showing the tubulo-interstitial area affected by fibrosis. Extensive TIFibrosis was observed in CsA-treated group
P-collagen reduces chronic CsA nephrotoxicity

Because apoptosis has been suggested to play a role in renal injury induced by CsA [34–38], we determined whether P-collagen was able to modify tubular cell death by apoptosis [34]. The presence of apoptotic cells in the renal cortex of the studied groups was evaluated with the TUNEL technique. Representative photomicrographs of TUNEL staining in two rats treated with CsA for 15 days and two rats that simultaneously received CsA and P-collagen are shown in Figure 3A to D. About 20 digitalized images from renal cortex sections of CsA and CsA + P-collagen groups were obtained using Eclipse net software; the positive nuclei per square millimetre per kidney were then quantified. The results are graphically expressed in Figure 3E. Consistent with previous observations [15,34,39], after 15 days of CsA treatment, tubular cell apoptosis was evident at a level of 104 ± 15 positive nuclei per mm². P-Collagen prevented tubular cell death as compared with control group. Thus, the number of apoptotic cells was 15 ± 5 positive nuclei per mm². In addition to this finding, we observed that CsA-treated rats exhibited a 25% increase in renal cortex caspase 3 mRNA expression compared with the control group (Figure 3F). P-Collagen administration significantly reduced the expression of this pro-apoptotic protein. These results demonstrate that apoptosis induced by CsA is completely prevented by P-collagen.

The renoprotective effect of P-collagen also correlated with levels of Kim-1 mRNA, which we have demonstrated to be a sensitive marker of tubular injury [15,40,41]. Chronic CsA nephrotoxicity was associated with a marked increase (>150-fold) in Kim-1 mRNA levels (Figure 4A). The Kim-1 up-regulation was thus partially prevented by the use of P-collagen.

To determine if the reduction of fibrosis conferred by P-collagen was related to lower expression levels of TGF-β, mRNA levels of this profibrotic molecule were also measured. CsA-treated rats exhibited a 2-fold increase in TGF-β mRNA levels normalized with 18S expression, compared with the control group (Figure 4B). In contrast, administration of P-collagen did not result in up-regulation of TGF-β mRNA (P < 0.05).

Epithelial to mesenchymal transdifferentiation has recently been shown to play a role in the pathogenesis of renal fibrosis development [42–47]. We therefore evaluated renal α-smooth muscle actin (α-SMA) mRNA levels as a marker of this process. Renal fibrosis induced by CsA was associated with a significant 3-fold increase in α-SMA transcript levels (Figure 4C), suggesting that epithelial–mesenchymal transdifferentiation represents another mechanism contributing to this nephropathy. Interestingly, P-collagen prevented the α-SMA mRNA up-regulation detected in rats with chronic CsA nephrotoxicity, an effect that was associated with less severe TI-fibrosis.

P-collagen prevents oxidative stress induced by CsA

To determine if P-collagen renoprotection is also correlated with a reduction in oxidative stress, renal lipoperoxidation and urinary excretion of H$_2$O$_2$ were measured in all studied groups. In fact, a statistically significant increase in renal lipoperoxidation levels in CsA-treated rats was detected (Figure 5A), suggesting that greater oxidative stress may contribute to the renal injury observed in this model. Interestingly, P-collagen completely prevented the increase in lipoperoxidation levels. Accordingly, the mean value of renal lipoperoxidation was 1.45 ± 0.24 in CsA-treated rats, compared with 0.24 ± 0.08 nmol/mg of protein in the V group. In contrast, the value in the CsA + P-collagen group was 0.45 ± 0.07 nmol/mg. We observed similar findings when oxidative stress was quantified by urinary H$_2$O$_2$ excretion.
(UH$_2$O$_2$V), as is shown in Figure 5B. Thus, UH$_2$O$_2$V levels in CsA-treated rats were 3-fold greater than in the V group. This increase was not seen in rats that received CsA and P-collagen simultaneously. Moreover, these findings were confirmed when the urinary isoprostanes F2α were assessed. As Figure 5C shows, CsA-treated rats had a sig-
significant increase in the urinary amount of 8-epi-PGF\(_2\alpha\) by >2-fold, effect that was not seen when the animals were simultaneously treated with CsA and P-collagen. Together, these results suggest that P-collagen prevents the oxidative stress induced by CsA.

In order to determine if the reduction in renal lipoperoxidation correlates with an improvement in the antioxidant system, the mRNA levels and activity of Cat, GPx, Cu/ZnSOD and MnSOD were evaluated (Figure 6A–F). Mild changes in mRNA levels and activity of antioxidant enzymes were observed. Cat mRNA levels were reduced by half in CsA-treated rats, an effect that was prevented by P-collagen (Figure 6A). Cu/ZnSOD was not modified, since the levels of Cu/ZnSOD/18S ratio in V, P-collagen, CsA and CsA-P-collagen were 1.0 ± 0.1, 0.8 ± 0.1, 1.1 ± 0.4 and 1.1 ± 0.2, respectively. In contrast, inducible MnSOD mRNA levels were significantly increased in the group treated with CsA and P-collagen compared with the rest of the groups, as Figure 6B shows. Accordingly, the reduction of SOD activity in CsA-treated rats was restored in CsA + P-collagen group. Since chronic CsA administration induced a 30% reduction in SOD activity, this reduction was not apparent in the CsA + P-collagen group. The activity of Cat and GPx was not modified by either CsA or P-collagen.

Discussion

In the present study, we have demonstrated that the administration of P-collagen partially prevented renal dysfunction and reduced arteriolopathy and TGF-\(\beta\) and \(\alpha\)-SMA mRNA levels. These effects were evidenced by the reduction of Kim-1 mRNA levels, an indicator of tubular injury.

CsA is an immunosuppressant drug that is widely used in solid organ transplantation. However, its use is limited due to its nephrotoxic effect. It is well known that CsA produces renal dysfunction due to an imbalance in the re-
lease of vasoactive factors and by the development of arteriolopathy. In this study, we observed that P-collagen significantly improved the hypoperfusion and hypofiltration induced by CsA. This effect appears to be mediated, at least in part, by a reduction in the degree of arteriolopathy in rats that received CsA and P-collagen together.

P-Collagen is a novel type I collagen that has been γ-irradiated and incorporated into a polyvinylpyrrolidone vehicle. Recent studies have shown that P-collagen down-regulates the expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, PDGF, ICAM-1 and VCAM-1 as well as molecules involved in the development of fibrosis, such as TIMP-1 and TGF-β [20,24,25], suggesting that P-collagen possesses anti-inflammatory and anti-fibrotic properties. In this study, we therefore determined if P-collagen might be able to reduce Ti-fibrosis and arteriolopathy that are characteristic of chronic CsA nephrotoxicity. In fact, both structural alterations were significantly reduced by P-collagen administration. This renoprotective effect was associated with prevention of TGF-β up-regulation, suggesting that P-collagen might have a renal anti-fibrotic property.

Kim-1 is a cell-surface protein that is a member of the immunoglobulin superfamily; the protein is normally expressed only at a low level in the normal kidney, but it is highly up-regulated in injured kidney epithelial cells [40,48–50]. It has been recently demonstrated that after a renal insult, tubular cells that over-express Kim-1 acquire a phagocytic phenotype and are responsible for internalizing adjacent apoptotic bodies and necrotic cells, helping to remove irreversibly damaged tubular cells [51]. As we previously demonstrated [15], renal Kim-1 is up-regulated during chronic CsA nephrotoxicity, indicating that epithelial tubular cells are injured by CsA. The renoprotection conferred by P-collagen was found to be associated with a significant reduction of Kim-1 mRNA levels, indicating that this protein helps to prevent epithelial cell injury.

The imbalance in the release of vasoactive substances induced by CsA leads to a constriction of afferent and efferent arterioles that contributes to cause chronic hypoxia of the

Fig. 6. Renal mRNA levels (A–C) and activity (D–E) of Cat, MnSOD and GPx. White bars, vehicle rats (V); grey bars, P-clg group; black bars, CsA-treated rats; dark grey bars, rats that received simultaneously CsA + P-clg. Error bars represent SE. *P < 0.05 vs all studied groups.
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tubulo-interstitium. Recent studies suggest that hypoxia of tubular cells leads to apoptosis or epithelial–mesenchymal transdifferentiation, which in turn exacerbates the fibrosis of the kidney. This results in a loss of peritubular capillaries and further chronic hypoxia, setting in motion a vicious cycle that eventually leads to end-stage renal disease (for review see [42]). In this study, we demonstrated for the first time that chronic CsA nephrotoxicity is associated with an elevation of α-SMA, a protein that participates in the differentiation of the mesenchymal cells to myofibroblasts; several groups have demonstrated that increased levels of this protein are implicated in the development of renal fibrosis [42–47]. Accordingly, P-collagen administration restored α-SMA mRNA levels to normal values, an effect that was associated with a reduction of TGF-β fibrosis.

Thus, these results suggest that the pathophysiology of chronic CsA nephropathy includes epithelial–mesenchymal transdifferentiation.

As previously discussed, renal injury induced by CsA is associated with tubular cell death by apoptosis [34–38,52], and several experimental studies have demonstrated that interference with the apoptotic programme translates into a protective effect during chronic CsA nephrotoxicity [15,39,41,53]. Thus, the pathways associated with apoptosis may be critical in the cell tubular injury observed during CsA toxicity. In fact, CsA-treated rats exhibited a significant elevation of tubular cell death by apoptosis in subcortical sections. Interestingly, the renoprotection conferred by P-collagen was also found to be associated with the prevention of tubular apoptosis. This effect was associated with a significant reduction in mRNA levels of procaspase-3, an important effector enzyme in the apoptosis cascade.

Renal CsA toxicity has been associated with an increase in the production of free radicals, together with a lowered efficiency in the antioxidant system [54–57]. In the present study, we confirmed that CsA induced a significant increase in oxidative stress stage, an effect that was evidenced by the significant increase in urinary H₂O₂ and 8-epi-PGF₂α excretion, as well as in renal liperoxidation, measured using the MDA technique. In addition, the increased oxidative stress in CsA-treated rats was associated with a reduction of SOD activity. In contrast, the administration of P-collagen reestablished the urinary H₂O₂ and 8-epi-PGF₂α excretion, as well as renal MDA levels, but had little effect on antioxidant activity. Thus, it is unlikely that this partial change in SOD activity could explain how P-collagen prevents oxidative stress. It is therefore possible that P-collagen itself may act as an antioxidant molecule. Further studies are necessary to resolve this question. Furthermore, renal hypoperfusion is well known to trigger free radical generation with subsequent cell death by apoptosis [42]. It is therefore also possible that the improvement in renal perfusion and lower levels of arteriolopathy observed in rats that simultaneously received CsA and P-collagen might contribute to reduce oxidative stress and apoptosis in these animals.

In summary, our data demonstrated that P-collagen improves the renal dysfunction and significantly reduces renal structural injury induced by CsA. The mechanism of renoprotection includes the reduction of tubular cell death by apoptosis and a reduction of renal oxidative stress. Thus, P-collagen appears to possess anti-fibrotic, anti-apoptotic and antioxidant properties that are useful to reduce the chronic structural damage induced by CsA in the kidney.

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