Cyclosporine triggers endoplasmic reticulum stress in endothelial cells: a role for endothelial phenotypic changes and death

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Bouvier N, Flinois JP, Gilleron J, Sauvage F, Legendre C, Beaune P, Thervet E, Anglicheau D, Pallet N. Cyclosporine triggers endoplasmic reticulum stress in endothelial cells: a role for endothelial phenotypic changes and death. Am J Physiol Renal Physiol 296:F160–F169, 2009. First published November 5, 2008; doi:10.1152/ajprenal.90567.2008.—Calcineurin inhibitors cyclosporine and tacrolimus are effective immunosuppressants, but both substances have the same intrinsic nephrotoxic potential that adversely affects allograft survival in renal transplant patients and causes end-stage renal disease in other solid organ or bone marrow transplant recipients. Endothelial cells are the first biological interface between drugs and the kidney, and calcineurin inhibitors may influence endothelial function and viability in a number of ways. Notably, endothelial cells have recently been shown to contribute to the accumulation of interstitial fibroblasts in nonrenal models, through endothelial-to-mesenchymal transition. Here we demonstrate that cyclosporine, but not tacrolimus or its metabolites, induces morphological and phenotypic endothelial changes suggestive of a partial endothelial-to-mesenchymal transition in human umbilical arterial endothelial cells. We identify for the first time a contingent of interstitial myofibroblasts that coexpress endothelial markers in rat kidneys treated with cyclosporine, suggesting that endothelial-to-mesenchymal transition could occur in vivo. Finally, our findings suggest that endoplasmic reticulum stress triggered by cyclosporine induces endothelial cells to undergo endothelial phenotypic changes suggestive of a partial endothelial-to-mesenchymal transition, whereas salubrin partially preserves the endothelial phenotype. Inversely, tacrolimus does not induce endothelial-to-mesenchymal transition or endoplasmic reticulum stress. In conclusion, this study demonstrates for the first time that cyclosporine, and not tacrolimus, induces endoplasmic reticulum stress in endothelial cells. Our findings also suggest that endoplasmic reticulum stress contributes to endothelial cell death and phenotypic changes similar to a partial endothelial-to-mesenchymal transition.

tacrolimus; endothelial-to-mesenchymal transition; epithelial-to-mesenchymal transition

The introduction of the calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus (TRL) in solid organ transplantation has led to a significant reduction in the incidence of acute allograft rejection and an improvement in short-term transplant survival (12). However, these early benefits have had little impact on long-term survival, and CNI use remains the chief cause of kidney failure (26–28). In renal transplantation, interstitial fibrosis (IF) and tubular atrophy (TA) are associated with a number of contributing factors such as immunological insults (acute rejection), hemodynamic changes (ischemia-reperfusion injury, hypertension), and toxicity (CNI nephrotoxicity). CNIs chronically destroy kidney parenchyma with the organization of suggestive lesions including TA, IF, and nodular arteriolar hyalinosis (5, 26). Although a number of therapeutic strategies have been proposed to avoid or reduce the use of CNI to halt or reverse IF/TA, CNI use remains the mainstay in solid organ transplantation (9, 17, 19, 20, 31).

With nephrotoxicity remaining a major contributing factor to late kidney damage, it is of the utmost importance to increase our understanding of the mechanisms involved in drug-induced nephrotoxicity to define new therapeutic strategies and identify early biomarkers. The pathogenesis of chronic nephrotoxicity in patients receiving CNI is still not well understood, and many potential mechanisms have been proposed, including vasoconstriction and ischemia, transforming growth factor-β (TGF-β) secretion, renin-angiotensin system activation, endothelin-1 release, nitric oxide dysregulation, tubular cell apoptosis, stimulation of inflammatory mediators, enhanced immunogenicity, and epithelial-mesenchymal transition in tubular epithelial cells (5, 6, 24, 36). Recently, we and others demonstrated that endoplasmic reticulum (ER) stress was an important contributor of CsA nephrotoxicity in mediating renal epithelial cell death and phenotypic changes suggestive of a mesenchymal transition (13, 32). Whether CNIs induce such effects in endothelial cells is unknown.

Although the response of the tubular epithelium has been widely studied during exposure to CNI (5, 6, 21, 24, 33, 34, 36), comprehensive and comparative analysis of the arteriolar endothelial response to CsA and TRL is lacking (23, 29, 39, 41). Endothelial cells are one of the most important targets of CNI because they are in continuous contact with these drugs, and the arteriolar endothelium is both a target (e.g., through endothelial cell death during thrombotic microangiopathy) and a mediator (e.g., through secretion of vasoactive compounds, such as endothelin 1, leading to tissue ischemia) of CNI nephrotoxicity (29). A puzzling issue is the relation between the respective roles of CsA and TRL in endothelial injury and the resulting nephrotoxicity. Although it is usually accepted that CsA and TRL are responsible for similar structural lesions in treated patients, experimental data support different and sometimes conflicting toxicity profiles, depending on the biological process being analyzed (23).

Endothelial cells have been recently demonstrated to contribute to fibrosis through a process called the endothelial-to-
mesenchymal transition (EndMT), by which endothelial cells lose their endothelial phenotype and gain myofibroblastic properties (3, 10, 43, 44). The occurrence of EndMT in kidney has not been demonstrated to date, and, because IF is a hallmark of CNI nephrotoxicity, one may wonder whether CNI could induce such a process.

The aim of the present study is to comparatively analyze CsA- and TRL-induced endothelial phenotypic changes (EPCs) and death in the same range of concentrations. We report that CsA induced EPCs suggestive of a partial EndMT in human umbilical artery endothelial cells (HUAEcs), whereas TRL and its demethylated metabolites did not. Our results also support that such EPCs suggestive of a partial EndMT occur in vivo in rats treated with CsA. As an explanatory mechanism, our data suggest that ER stress contributes to CsA-induced EPCs and death. Finally, our results suggest that salubrinal (SAL), a molecule alleviating cells from the deleterious effects of ER stress, partially protects endothelial cells against CsA-induced EPCs and death.

MATERIALS AND METHODS

Reagents. All chemicals, including CsA and TRL, were obtained from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France). Cell culture medium and other cell culture products were supplied by Promocell (Heidelberg, Germany).

Cell culture. HUAECs were purchased from Promocell. Cells were not trypsinized more than three times and were tested for their expression and the lack of trypsinized more than three times and were tested for their expression and the lack of

In vivo study. Adult male Sprague-Dawley rats (Charles River laboratories, L’Arbresle, France) weighing 170–180 g were allowed free access to tap water. Two groups of five rats were treated intraperitoneally for 28 days with 15 mg/kg per day of CsA or placebo. Rats were then euthanized under thiopental anesthesia, and blood was collected; the kidneys were then rapidly retrieved, washed with heparinized saline, and either fixed in neutral-buffered formalin or snap frozen. The experimental protocol was approved by the animal care committee of the University Paris Descartes.

Immunofluorescence microscopy. For cellular immunofluorescence analysis, HUAECs were cultured on glass coverslips, fixed with 4% paraformaldehyde, rinsed with PBS, and blocked with 2% BSA/PBS. Cells were permeabilized with 0.1% Triton X-100 and incubated with a 1:200 dilution of mouse anti-vinculin antibody (Sigma-Aldrich Chimie) for 30 min in the dark at room temperature. Samples were then incubated with a 1:500 dilution of chicken anti-mouse Alexa Fluor 488 antibody (Invitrogen SARL, Cergy Pontoise, France). Actin stress fibers were stained in red with 1:1,000 tetramethylrhodamine isothiocyanate-labeled phalloidin (n° 77418, Sigma-Aldrich Chimie). Nuclei were counterstained and slides mounted in Vectashield Mounting Medium with 4,6-diamidino-2-diphenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Slides were mounted and viewed using a Zeiss Axioscopic2 imaging fluorescence microscope.

For immunofluorescence analysis of kidney cryosections, frozen tissues were cut into 5-μm-thick sections, which were fixed in 100% acetone at −20°C for 1 h. Sections were incubated with primary antibodies at room temperature for 2 h. The primary antibodies were mouse anti-CD31 (1:200, n° 550300, BD Pharmingen, Le Pont-de-Claix, France), rabbit anti-fibroblast-specific peptide 1 (FSP-1) (1:200, n° ab27975, Abcam) and rabbit anti-α SMA (Abcam n° ab27975). The sections were next washed with PBS Tween 20 0.1% and were subsequently stained with fluorescent secondary antibodies, rabbit anti-mouse Alexa Fluor 488 antibody and goat anti-mouse Alexa Fluor 546 antibody. The nuclei were counterstained with DAPI. Cell images for deconvolution were taken using a Nikon epifluorescence TE-2000E imaging microscope at ×10 and ×40 magnifications, and Z-stacks of ~20 incremental slices (0.4 μm step size) were captured. Auto-Quant software was used for image deconvolution.

Yeast microsome extraction and TRL metabolites production. Saccharomyces cerevisiae yeast (both expressing the P450 3A5 cytochrome and empty) were cultured in SGM medium (Bacto casamino acids, yeast nitrogen base and glucose) at 28°C for 24–30 h. Yeast were next incubated with tryptophan at 28°C for 24 h. Two hundred milliliters of 2× yeast extract (2%, and 2% bactopeptide) together with ethanol was used for conversion under anaerobic conditions. Yeast microsomes were extracted with ultracentrifugation and solubilized in NaPO4:H2O 100 mM, MgCl2 10 mM, glycerol 20%, and pH 7.4 buffer.

TRL metabolites were produced as previously reported (16). Briefly, 10 μM of TRL dissolved in ethanol were incubated with 6 mg/ml yeast microsomes, 0.5 mM NADP+, 5 mM of glucose 6 phosphate, 1 U/ml of glucose 6-phosphate dehydrogenase, 5 mM MgCl2, 50 μM of EDTA, and 0.1 M of potassium phosphate buffer pH 7.4, in a total volume of 1 ml. The reaction was conducted at 28°C for 2 h. The solution was centrifuged at 35,000 revolution/min for 1 h at 4°C and filtered with a 0.2-μm sterile filter. Demethylated metabolites were detected in supernatants using high performance liquid chromatography coupled to mass spectrometry. Supernatants were then incubated for up to 72 h with HUAECs in culture medium.

Statistical analysis. All data were expressed as means ± SE of three different experiments, unless otherwise specified. Biological and histological data were compared using nonparametric tests. We used
the Mann-Whitney U-test for comparison between two groups. Statistical analyses were performed using Prism software. *P < 0.05 were considered significant.

RESULTS

CsA, but not TRL, induces HUAEC morphological changes and death. As a first step, we analyzed the morphological and viability changes in HUAECs cultured with CsA and TRL in the same range of concentrations. Ten micromolar CsA decreased HUAEC viability up to 40% in a concentration-dependent manner compared with the vehicle, whereas TRL did not induce mortality in a wide range of concentrations (10 nM to 10 μM) (Fig. 1A). CsA induces striking morphological alterations resulting in a fibroblastic shape. Indeed, phase contrast analysis shows that untreated HUAECs are small, round, and adhere to each other. When exposed to 10 μM CsA, HUAEC size increases (Fig. 1C), and they progressively exhibit an elongated shape with cytoplasmic extensions suggestive of filopodia and a high degree of cell detachment. These morphological changes are not observed in TRL-exposed cells (Fig. 1B). HUAECs exposed to CsA also develop actin stress fibers across the cytoplasm, whereas, in untreated cells, actin filaments are smooth and distributed in the periphery of the cytoplasm. Vinculin redistribution beneath filopodia, a feature suggestive of myofibroblasts (14, 38), is observed during CsA exposure (Fig. 1B), whereas this redistribution is not visualized in vehicle- or TRL-exposed cells.

Altogether, these data suggest that CsA, but not TRL, induces endothelial morphological changes suggestive of a myofibroblastic shape.

CsA, but not TRL, induces EPCs. We next analyzed whether CsA and TRL could alter endothelial marker expression. Endothelial cells exposed to CsA are subjected to endothelial phenotypic alterations with downregulation of the endothelial marker CD31 (PECAM-1) and vWF transcripts that become significant after 48 h of treatment (Fig. 2A). We confirmed CD31 downregulation during CsA exposure at the protein level (Fig. 2B). Unlike CsA, TRL does not modify CD31 and vWF expression (Fig. 2, C and D).

Myofibroblasts are fibroblasts that express α-SMA and thereby gain contractile and migratory properties. CsA, unlike TRL, induced a significant upregulation of α-SMA at both the mRNA and protein levels (Fig. 2, A, B, C, and D), suggesting that CsA induces α-SMA expression in endothelial cells.

Since CsA induces downregulation of CD31 expression and de novo expression of the myofibroblastic marker α-SMA, we conclude that CsA, and not TRL, induces in vitro EPCs, suggestive of a partial EndMT. These changes seem to be independent of TGF-β because we did not find any TGF-β

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**Fig. 1.** Cyclosporine (CsA), not tacrolimus (TRL), induces endothelial morphological changes and death. **A:** cell viability determined using the MTS assay after 72 h of exposure to various CsA and TRL concentrations, *P < 0.05, n = 3. B:** cellular morphology after 48 h of exposure to 10 μM CsA, 10 μM TRL, or vehicle (1/1,000 ethanol) determined with phase contrast microscopy (top), bar = 75 μm; immunofluorescence after phalloidin staining to analyze actin cytoskeleton (middle), bar = 25 μm. White arrows demonstrate stress fibers. **Bottom:** vinculin redistribution was detected using mouse antibody (green) together with phalloidin to detect actin cytoskeleton (red), bar = 25 μm. White arrows demonstrate vinculin redistribution beneath filopodia.
expression at either the mRNA or the protein levels during CsA treatment (Supplemental Fig. S1). Interestingly, we found that Snail (snail homologue 1, SNAI1) mRNA, a transcription factor known to upregulate mesenchymal markers and to repress the epithelial phenotype during the epithelial to mesenchymal transition (37), was transiently and significantly upregulated during CsA treatment (Supplemental Fig. S2). This finding raises the possibility that Snail could be involved in CsA-induced EPCs.

CYP3A5-mediated TRL metabolites induce neither HUAEC death nor phenotypic alterations. Since TRL itself has no effect on HUAECs in vitro, whereas it is known to be nephrotoxic in vivo, we hypothesized that TRL toxicity could be mediated by its demethylated metabolites produced by P450 3A5 cytochromes (CYP3A5). Since HUAECs do not express CYP3A5 (data not shown), the absence of TRL metabolites in culture medium could explain the absence of TRL cytotoxicity. Therefore, we incubated 10 μM TRL with CYP3A5-expressing yeast microsomes and an NADPH-generating system to obtain demethylated TRL metabolites (Fig. 3A). The quantity of demethylated TRL metabolites represented roughly 40% of the total TRL at the end of incubation with CYP3A5-expressing yeast. The incubation of these metabolites with HUAECs for 48 and 72 h did not alter either cell morphology or cell viability (Fig. 3, B and C). These results suggest that demethylated metabolites of TRL produced by CYP3A5-expressing yeasts are not responsible for the nephrotoxic effects of TRL in vivo.

CsA induces EPCs in vivo. To test whether partial EndMT might occur in vivo, we analyzed whether myofibroblasts (α-SMA-positive cells) coexpress the endothelial marker CD31 (α-SMA+/CD31+ double-labeled cells). Rat kidneys treated with 15 mg/kg CsA for 28 days express many interstitial fibroblasts in the vicinity of tubular sections (Fig. 4A, white arrows; Fig. 4B, white arrow heads), whereas untreated rat kidneys did not. Among these fibroblasts, ∼20% coexpressed α-SMA and CD31 (Fig. 4B.1, white arrows and see Fig. 4B.2, white arrows for higher magnification). To further prove the occurrence of endothelial cells coexpressing fibroblast markers in kidney interstitium, we analyzed
the expression of the fibroblastic marker FSP-1 in endothelial cells. During CsA treatment, a significant number of FSP-1-positive cells appeared in rat kidney interstitium (Supplemental Fig. S3A), whereas these cells were not observed during treatment with the vehicle. Interestingly, ~10% of the FSP-1-positive cells coexpressed CD-31 (FSP-1/CD31 double-labeled cells) (Supplemental Fig. S3B). Taken together, the observation that a significant proportion of endothelial cells express myofibroblastic markers in the kidney interstitium strongly suggests that partial EndMT might occur in vivo in rat kidneys during CsA treatment.

ER stress is involved in morphological and EPCs induced by CsA. To further decipher the toxicological mechanisms leading to in vitro EPCs and death during CsA exposure, we tested whether ER stress and the following unfolded protein response (UPR) were activated in HUAECs during CsA treatment. Indeed, ER stress is involved in CsA-induced nephrotoxicity and tubular incomplete epithelial-to-mesenchymal transition (32), a process that shares similarities with the EndMT we describe here. Moreover, ER stress is involved in various endothelial insults and contributes to vascular pathological process, including atherosclerosis (42, 45). In ER stress conditions, the aim of the UPR is to adapt to the changing environment and reestablish normal ER function by reducing mRNA translation and, therefore, the amount of new synthesized proteins in the ER, by increasing the proteasomal degradation of ER localized proteins, and by increasing the protein folding capacity of the ER. When adaptation fails and ER stress prolongs, cell triggers death programs, usually in the form of apoptosis. To analyze

Fig. 3. TRL metabolites do not induce either cell death or morphological alterations. A: TRL metabolite production followed by detection with HPLC-MS. On the first line, the grey spectrum represents the detection of nonmetabolized TRL (after incubation with empty yeasts). On the second line, the white spectrum at 3.77 ppm represents the theoretical profile of nonmetabolized TRL. On the third line, the grey spectrum at 3.61 ppm (arrow) represents demethylated metabolites of TRL (after incubation with CYP3A5-expressing yeasts). On the fourth line, the white spectrum at 3.61 ppm represents the theoretical profile of demethylated TRL. B: cellular morphology after 48 h of exposure after incubation with 10 μM TRL or TRL plus demethylated metabolites, determined with phase contrast microscopy; bar = 25 μm. C: cell viability determined using the MTS assay 72 h after incubation with 10 μM TRL or TRL plus demethylated metabolites.
whether the UPR is activated in endothelial cells during CsA and TRL treatments, we focused on four transcriptionally regulated markers. BiP/GRP78 and GRP94 are chaperones found to be upregulated during ER stress to increase the ER folding capacity. HERP is a component of the ER-associated degradation pathway, implicated in the degradation of accumulated proteins, thus lowering the protein charge in the ER lumen. Finally CCAAT/enhancer binding protein-homologous protein (CHOP) is a mediator of cell death program activated during ER stress. HUAECs exposed to CsA for various durations initiate the UPR with the upregulation of BiP/GRP78, GRP94, HERP, and CHOP (Fig. 5, A and B). Conversely, TRL, even at 10 \( \mu \)M, does not induce the UPR, suggesting that TRL does not induce ER stress (Fig. 5, C and D). Moreover, thapsigargin, a prototypical ER stress inducer, triggers EPCs including an elongated shape, a high degree of cell detachment, the development of actin stress fibers, and focal adhesions with vinculin redistribution at the level of filopodiae (Fig. 5E). CD31 expression was also strongly downregulated (Fig. 5F). However, thapsigargin did not induce \( \alpha \)-SMA synthesis. Similar morphological and phenotypic changes are also observed with tunicamycin, another ER stress inducer (Supplemental Fig. S4). Together, these results suggest that ER stress could contribute, at least partially, to the EPCs induced by CsA.

To clarify the role of ER stress in HUAEC phenotypic changes and death, we coincubated these cells with SAL, a molecule recently described to alleviate the cells from the deleterious effects of ER stress (4, 32). SAL reduces BiP expression in HUAECs during CsA treatment (Fig. 6A), suggesting that SAL reduces ER stress. CsA does not decrease cell viability when coincubated with SAL, suggesting that SAL protects against CsA-induced cytotoxicity (Fig. 6B). Finally, alleviating ER stress with SAL partially prevents CsA-induced EPCs. Indeed, SAL limits CsA-induced cell size increase, whereas it does not protect against the formation of filopodiae and actin stress fibers (Fig. 6C): SAL limits the downregulation of CD31 induced by CsA, at both the mRNA and protein levels (Fig. 6, D and E), whereas it did not do so for later \( \alpha \)-SMA expression (Fig. 6F). Taken together, these data suggest that ER stress contributes to the partial EndMT induced by CsA and that
SAL could partially reduce endothelial cell death and phenotypic changes.

DISCUSSION

The aim of our study was to decipher the toxicological response of arterial endothelial cells to CsA and TRL exposure. Our results bring important new insights to the mechanisms of CsA and TRL endothelial toxicity.

This work demonstrates that the endothelial responses to CsA and TRL are totally different, with CsA at near-therapeutic concentrations inducing cell death, ER stress, and EPCs suggestive of a partial EndMT and with TRL at concentrations 1,000 times higher than therapeutic concentrations altering neither cell viability nor endothelial phenotype.

The ER stress response and the induction of EPCs are observed at the same concentration of CsA (10 μM). At 1 μg/ml after drug intake have been recommended, corresponding to 1.2 to 1.7 μM in renal transplant patients (7). Our working CsA concentration is therefore close to the whole blood concentrations observed in transplant patients. However, achieving a correspondence between CsA concentrations in patients’ blood or kidney tissue and CsA concentrations in culture medium is virtually impossible. CsA is a highly lipophilic drug that accumulates in cell membranes, thus producing higher concentrations within phospholipid membranes (18). Moreover, the concentration of CsA in the medium does not correspond to the concentration at the intracellular target site, because tubular cells express transporters such as P-glycoprotein that actively reject CsA from inside the cell (2).

The differential endothelial responses to CsA and TRL in vitro suggest that, rather than calcineurin (CN) inhibition, immunophilin [cyclophilin A (CyPA) and FK-BP12] enzymatic activity inhibition is probably involved in the cytotoxic effects we observed, at least in vitro. This hypothesis is supported by experimental data demonstrating the role of immunophilins in CNI nephrotoxicity. Mice overexpressing CyPA are protected against CsA nephrotoxicity, whereas mice expressing mutated CyPA spontaneously develop renal lesions similar to those observed in CsA nephrotoxicity and are more sensitive to CsA-induced nephrotoxicity (15). We also recently demonstrated that CyPA knockout with RNA interference induced ER stress and tubular cell death (32). FK-binding proteins share similar enzymatic activities with cyclophilins, but with a reduced array of substrate array.

To explain the differential effects of CsA and TRL in endothe-
Fig. 6. Salubrinal (SAL) partially protects against endothelial cell death and phenotypic changes induced by CsA. A: representative Western blot of BiP expression 24 h after incubation with vehicle, 10 μM CsA, or 10 μM CsA plus 25 μM SAL. B: cell viability determined using the MTS assay 72 h after incubation with increasing concentrations of CsA with or without 25 μM SAL. *P < 0.05. C: tubular cell surface measurement 72 h after incubation with 10 μM CsA with or without 25 μM SAL. *P < 0.05 compared with CsA + SAL. D: real-time PCR analysis of CD31 mRNA expression reported to vehicle-treated cells after 48 h of exposure to 10 μM CsA with or without 25 μM SAL. *P < 0.05 compared with CsA + SAL. E: Left: representative Western blot analysis of CD31 expression in whole cell lysates 48 h after incubation with 10 μM CsA with or without 25 μM SAL. Right: densitometric analysis of the CD31/actin ratio. *P < 0.05 compared with CsA + SAL. F: representative Western blot analysis of α-SMA expression in whole cell lysates 48 h after incubation with 10 μM CsA with or without 25 μM SAL.
cial cells in vitro, one can hypothesize that FK-BP12 isomerase activity inhibition by TRL is supplanted by CyPA isomerase activity in a redundant manner. Indeed, FK-BP12 inhibition does not induce nephrotoxicity, and a TRL analog, L-615,818, which inhibits FK-BP12 isomerase activity without inhibiting CN activity, does not induce in vivo nephrotoxicity (8). However, in vivo studies suggest that CN inhibition, rather than immunophilin enzymatic inhibition, mediates CN nephrotoxicity. Kidneys from mice knocked out for the calcineurin A α-subunit develop structural lesions resembling those induced by CsA, whereas deletion of the β-subunit does not induce any lesion (11). Together, these data lead us to propose that CsA nephrotoxicity could be mediated by both CyPA isomerase activity inhibition and CN inhibition, whereas TRL nephrotoxicity could be mediated by CN inhibition alone.

The fact that TRL is not cytotoxic in vitro, even at very high concentrations, although it induces chronic nephrotoxicity lesions similar to CsA in vivo, is very intriguing. One cannot exclude the possibility that our in vitro model is limited to correctly studying chronic toxic drug effects. Indeed, the treatment durations we used (days) are very short when compared those used for in vivo experiments (months) and for treating patients (years). When cells are cultured for long times, they lose their phenotypic characteristics, and it is virtually impossible to attest their origin after 1 wk of culture. However, this criticism applies to all in vitro models. CNi nephrotoxicity encompasses a single toxic affect on one kind of cell and results from the combination of multiple tissue insults such as ischemia, TGF-β and endothelin-1 secretion, oxidative stress, and so on. Despite the limitations inherent to the nature of the model, in vivo studies are necessary to better understand the biological pathways involved in CNi nephrotoxicity at a molecular level that cannot be achieved with laboratory animals.

Another important finding of our study is the fact that CsA induces partial EPCs, suggestive of EndMT both in vitro and in vivo. Growing data support the existence of EndMT in various fibrogenic processes. Although the understanding of the implications of epithelial-to-mesenchymal transition in kidney fibrosis and allograft nephropathy has been increasing for many years, EndMT is a very recent concept that has never been described in kidneys. EndMT has been described during hearth fibrosis, in the stroma surrounding tumoral cells, and during pulmonary hypertension (3, 10, 43, 44). Similar to the epithelial-to-mesenchymal transition, EndMT is characterized by the loss of the endothelial phenotype together with the gain of myofibroblastic markers such as FSP-1 and α-SMA. Very recently, two studies focusing on EndMT in nonembryological models, namely heart fibrosis (44) and carcinoma-associated fibroblasts (43), strongly supported that EndMT could be involved in myofibroblast formation in vivo and plays a pathogenic role during adult tissue injury. In their landmark study focusing on carcinoma-associated fibroblasts, ongoing EndMT was defined by the simultaneous expression of CD31 with α-SMA or FSP-1 (43). A complete prototype switch would be characterized by a CD31+/α-SMA+ or CD31-/FSP-1+ labeling, rendering these cells indistinguishable from resident myofibroblasts or epithelial-to-mesenchymal transition-derived myofibroblasts. Demonstrating a complete phenotypic switch requires a CreLox system model in which a LacZ protein coding gene is controlled by the Tie2 promoter. In this model, the detection of the LacZ protein in a CD31+/α-SMA+ or CD31-/FSP-1+ cell attests the endothelial origin of EndMT-derived myofibroblasts. Demonstrating the existence of partial EndMT in the kidney is an important step toward a better comprehension of CNI nephrotoxicity, but the precise role of EndMT in IF remains to be elucidated.

Our study also demonstrates for the first time that CsA, and not TRL, induces endothelial ER stress and that ER stress could, at least in part, mediate CsA-induced endothelial cell death and phenotypic changes. Cellular redox disturbances, aberrant Ca2+ regulation, glucose deprivation, viral infections, altered glycosylation, inhibition of folding enzymes, and overloading of cholesterol can interfere with the ER protein folding machinery and subsequently lead to the accumulation of unfolded or misfolded proteins in the ER lumen, a situation called ER stress (25, 42, 45). Importantly, many injuries that mediate chronic allograft nephropathy, such as chronic ischemia, ischemia-reperfusion, viral infections, and CsA nephrotoxicity are known inducers of ER stress. Thus the importance of the phenomenon in chronic structural damage and its contribution to the damage is probably underestimated. ER stress triggers an evolutionarily conserved response termed the UPR. The aim of this response is to adapt to the changing environment and reestablish normal ER function, to reduce the amount of new proteins translocated to the ER by reducing mRNA translation, to increase the proteasomal degradation of ER-localized proteins, and to increase the protein folding capacity of the ER. When adaptation fails and ER stress is prolonged, the cell triggers suicide programs, usually in the form of apoptosis. However, ER stress does not only mediate cell death; growing evidence implicates ER stress as a contributor to cell phenotypic and functional changes (1, 35, 40). SAL does not protect against the formation of filopodia. This suggests that ER stress partially participates to EPCs and that, because SAL partially limits ER stress, the whole phenotypic changes induced by CsA may not be blocked by SAL. Since compounds that target ER stress exist, such as SAL or 4-phenyl butyrate (4, 30), as do potential biomarkers (such as BiP, detected in renal biopsies), it is important to better understand the role of ER stress during kidney injury and to clarify its precise role in chronic kidney diseases.

In conclusion, we demonstrate that CsA, and not TRL, induces endothelial cell death and phenotypic changes suggestive of partial EndMT and that these features may also occur in vivo. This process is partially mediated by ER stress and reversed with SAL, which relieves the cell from ER stress.

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