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ORIGINAL ARTICLE

Ultrastructural alterations in endothelial mitochondria are associated with enhanced nitrotyrosine accumulation and progressive reduction of VEGF expression in sequential protocol renal allograft biopsies with calcineurin inhibitor toxicity

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Summary

Calcineurin inhibitors (cyclosporine and tacrolimus; CNIs) continue to be used as constituents of post-transplant immunosuppression in most centers. However, renal toxicity associated with the use of these drugs remains a problem adversely affecting the long-term graft survival. Fifteen adequate protocol renal allograft biopsies, with histological features of CNI toxicity among 140 protocol biopsies performed at 1-, 6-, and 12-month post-transplant, were included. Mitochondrial alterations in the tubular epithelial cells and endothelia of glomerular, peritubular capillaries and arterioles were graded semiquantitatively and further ultrastructural morphometric evaluation of numerical density and area of the mitochondria was performed. Immunohistochemical staining for nitrotyrosine (marker of peroxynitrite formation) and vascular endothelial growth factor (VEGF) was performed and expression graded semiquantitatively. Higher grades of alterations were seen in endothelial mitochondria as compared with tubular mitochondria in biopsies with calcineurin inhibitor toxicity (CNIT). Endothelial mitochondrial numerical density showed progressive decline over 1-, 6- and 12-month biopsies while area showed progressive increase in biopsies with CNIT as compared with controls. Upregulation of nitrotyrosine was seen even at 1-month post-transplant, persisted at 6 and 12 months, and was significantly greater than that in control biopsies. Intense VEGF expression was noted in early CNIT while progressive reduction was seen in 6- and 12-month protocol biopsies. This study shows a relatively high incidence of CNIT in protocol renal allograft biopsies, indicating that this might be an important mechanism of background damage to the allograft. Structural alterations in endothelial mitochondria are consistent findings in protocol biopsies with CNIT and this relatively specific mitochondrial damage may stem from the peroxynitrite-mediated damage associated with progressive loss of protective function of VEGF.

Introduction

Establishment of renal transplantation as the definitive treatment for end-stage renal failure is among the major medical advances in the last century. Although successful treatment of acute allograft rejections and the availability of newer and more potent immunosuppressive agents have led to a better short-term graft survival, the

long-term survival has not witnessed any major improvement [1,2].

Calcineurin inhibitors (cyclosporine and tacrolimus) continue to be used as important constituents of post-transplantation immunosuppression protocol in most renal transplantation programs. However, several studies have conclusively demonstrated the nephrotoxicity of these drugs [3–5]. Although the mechanisms involved in development of calcineurin inhibitor toxicity (CNIT) are complex, oxidative and nitrosative stress-mediated renal damage in the form of increased superoxide and peroxynitrite formation (manifesting as nitrotyrosine accumulation in tissues has been reported in experimental and cell culture-based studies as an important mechanism for development of CNIT [6–8]. In addition, the role of VEGF in development of CNIT has recently emerged. Studies have shown the various renoprotective functions of this multifunctional protein encompassing both the tubular as well as vascular compartments [9,10]. The most convincing evidence of these effects has been provided by demonstration of renal-limited thrombotic microangiopathy by selective inhibition of VEGF [11].

Ultrastructural changes in calcineurin inhibitor nephrotoxicity have not been adequately reported in the literature [12]. Although involvement of mitochondria has been recognized in development of CNIT, none of the studies on this topic have adequately evaluated structural alterations in this organelle. Mitochondria, particularly those located in vascular endothelium (endothelial mitochondria) are now recognized to be important regulators of vascular function and disease and have been implicated in pathogenesis of various disorders including atherosclerosis, diabetes mellitus and ischemia-reperfusion injury [13]. We noticed striking morphological alterations in the endothelial mitochondria during routine ultrastructural evaluation of biopsies with CNIT and decided to investigate these further.

Patient selection

Protocol renal allograft biopsies were performed in a patient cohort of live related renal transplants (recruited at the time of transplantation) between 2006 and 2007 at our institute at 1-, 6- and 12-month post-transplant periods. Appropriate written consent was taken from patients for their inclusion in the study, which was duly approved by the Institute's human ethics committee. Clinical inclusion criteria included serum creatinine <1.8 mg/dl, normal graft ultrasound and diethylene triamine penta-acetic acid scans, no clotting abnormalities and tacrolimus and cyclosporine (CNI) levels within therapeutic range. Live unrelated/cadaver allografts and those performed in children <12 years were excluded. Also, protocol allograft biopsies with a coexistent rejection (cellular or humoral) and those containing insufficient tissue for immunohistochemical and ultrastructural evaluation were excluded. A total of 58 patients fulfilling the clinical inclusion criteria underwent the first protocol biopsy (1 month) while 45 and 37 patients reported for second and third biopsies at 6- and 12-month post-transplant respectively (total number of biopsies evaluated −140). Degree of pretransplant human leukocyte antigen (HLA) mismatch and history of induction were noted. Detailed laboratory data including renal functions, serum trough levels of drugs (cyclosporine or tacrolimus) were recorded. Estimated GFR by the modified diet for renal diseases formula was calculated for each patient at the time of biopsy. All the biopsies were processed for light microscopy, immunofluoroscence and ultrastructural examination. Seventeen biopsies had a light microscopic diagnosis of CNIT. Biopsies from patients who already had a diagnosis of CNIT in a previous protocol biopsy were excluded from further analysis, thus all the 17 cases were 'new' diagnosis. The study was designed with an 'intent to treat', implying that all the patients were treated by a suitable modification in their drug dose following a diagnosis of CNIT on protocol biopsy. Two biopsies which contained inadequate tissue for ultrastructural/immunohistochemical evaluation were excluded from analysis. Fifteen biopsies with CNIT contained adequate tissue for immunohistochemistry and ultrastructural examination and were finally included in this study. Age- and gender-matched biopsies from the same protocol cohort and performed in corresponding duration post-transplant did not show evidence of CNIT or rejection, were taken as controls ($n = 15$).

Light microscopic examination

Formalin-fixed and paraffin-embedded adequate renal biopsy (as per the Banff 2001 requirements) was sectioned at 3 μm and stained with hematoxylin and eosin, periodic acid Schiff and silver methenamine stains. Detailed light microscopic evaluation was performed to look for changes of calcineurin inhibitor toxicity. All the biopsies were independently evaluated in coded slides by three experienced renal pathologists and a case was included only when all the three concurred on the diagnosis of CNIT.

Ultrastructural examination

Biopsy core for ultrastructural examination was fixed in 3% glutaraldehyde solution, embedded in resin and ultrathin sections were double-stained with uranyl acetate and lead citrate. Ultrastructural examination was performed on Philips Morgagni 268 transmission electron

microscope with special emphasis on mitochondrial morphology. Mitochondria in the tubular epithelial cells and those in glomerular capillary, peritubular capillary and arteriolar endothelium were analysed and structural alterations were classified into four semiquantitative grades depending on the degree of cristal loss and membrane disintegration (adapted from Walker *et al.*) [14].

Grade 0: No evidence of cellular pathology/occasional mitochondrion with minimal loss of cristae

Grade 1: Discontinuous cristal membranes and or partial loss of cristae and matrix in few mitochondria.

Grade 2: Multiple disruptions of cristal membranes and substantial loss of cristae and matrix in approximately half of mitochondria.

Grade 3: Fragmented cristal membranes and effacement of central architecture in majority of mitochondria.

Ultrastructural morphometry

Further objective evaluation of morphological alterations in mitochondria was performed by ultrastructural morphometry. Numerical density and surface area of mitochondria in the renal proximal tubules, glomerular endothelium, arteriolar endothelium and peritubular capillary endothelium was measured. Mitochondrial numerical density was calculated on the captured images by enumerating the number of distinctly identifiable and non overlapping mitochondrial profiles in the unit area of the image. Mitochondrial surface area was measured by tracing the circumferential boundary of the mitochondrial profiles on digitally captured images using the area function on the Image Pro plus software (Media Cybernetics Inc., Bethesda, MD, USA), after appropriate calibrations for magnification. Mitochondria were analysed in at least three proximal tubular epithelial profiles, six peritubular capillary and glomerular endothelial profiles and arteriolar endothelial profile wherever it was included in the sections. A minimum of 200 tubular mitochondria and 50 endothelial mitochondria were analysed in each case.

Immunohistochemistry

Sections from paraffin-embedded renal allograft biopsy tissues were taken on poly-L-lysine-coated slides and immunohistochemistry for nitrotyrosine (NT) (Abcam Inc., Cambridge, MA, USA) and vascular endothelial growth factor (VEGF-1) (Abcam Inc) was performed. Nitrotyrosine antibody was diluted 1:30 with phosphate buffered saline (pH 7.4), heat-induced antigen retrieval was performed in ethylene diamine tetra acetic acid buffer (pH 8.0), while chromogen was diaminobenzidine (DAB). VEGF staining was performed after heat-induced antigen retrieval in citrate buffer at pH 8.0. The primary antibody was used in a dilution of 1:800 and chromogen was DAB. Expression of NT and VEGF was noted in tubules and vessels and graded semiquantitatively as follows:

Tubular expression of NT and VEGF

Absent/Positive in <5% of tubules:	Grade 1
Positive in 6–25% of tubules:	Grade 2
Positive in 26–50% of tubules:	Grade 3
Positive in >50% of tubules:	Grade 4

Vascular expression of NT and VEGF

Staining was assessed individually in the glomerular capillaries, peritubular capillaries and arterioles (endothelium and smooth muscle layer). As the degree of positivity was uniform in all three locations, all these were expressed as the mean vascular grade for each case.

Positive in <10% of vessels:	Grade 1
Positive in 11–25% of vessels:	Grade 2
Positive in 26–50% of vessels:	Grade 3
Positive in >50% of vessels:	Grade 4

Calculation of mean vascular staining grade

Grade of glomerular capillary staining with NT and VEGF: vNTg & vVEGFg

Grade of peritubular capillary staining with NT and VEGF: vNTptc & vVEGFptc

Grade of arteriolar staining with NT and VEGF: vNTa & vVEGFa*

Mean vascular staining grade for NT = vNTg + vNTptc + vNTa/3*

Mean vascular staining grade for VEGF = vVEGFg + vVEGFptc + vVEGFa/3*

*Wherever arterioles were not included in sections, the respective value was derived after dividing the grade sum by 2.

Statistics

Statistical analysis was performed with the SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). All the values are depicted as mean ± standard deviation (mean ± SD). Non parametric tests (Mann–Whitney) were used for analysis of skewed data; Student's *t*-test was used for normally distributed data, Spearman's test was used for correlative analysis and test of significance between proportions for relevant data.

Results

Among the 140 protocol renal allograft biopsies, 17 (12.1%) showed light microscopic features of CNIT, and 15 biopsies which fulfilled clinical and pathological criteria for adequacy were finally included for analysis.

Mean age of the recipients was 27.2 ± 7.9 years while that of corresponding donors was 42.5 ± 12.3 years. Majority of recipients (80%) were males while most of the donors (77%) were females. The mean age of patients with CNIT was 31.5 ± 6.4 years while that of corresponding controls was 30.6 ± 7.2 years. The clinical parameters including the serum trough levels of cyclosporine/tacrolimus and mean HLA mismatch did not show a significant difference among cases and controls (Table 1). Three patients in each group received pretransplant daclizumab induction.

Among the 1-month protocol biopsies ($n = 58$), six showed histological evidence of CNIT (10.3%); all these patients were on tacrolimus. At 6 months ($n = 45$), five new cases showed CNIT (11.1%), four of these were on tacrolimus while one patient was on cyclosporine. At 12-month protocol biopsies ($n = 37$), four new cases of CNIT were seen (5.4%), two of these were on tacrolimus and the other two were on cyclosporine. Overall, among the 140 biopsies, 97 (69.3%) were from patients on tacrolimus while 43 (30.7%) were from patients on cyclosporine of which 12/97(12.3%) adequate biopsies in the former group and 3/43 (6.97%) from the latter group showed histological evidence of CNIT.

At 6 months, four out of the six patients with CNIT in the first biopsy were re-biopsied. None showed evidence of CNIT (one patient had an acute cellular rejection Banff Grade 1B and was appropriately managed). At 12-month protocol biopsy, four of the five new cases of CNIT diagnosed in 6-month biopsy were re-biopsied. Although there was persistence of striped pattern of fibrosis in two biopsies, the rest did not show any evidence of CNIT.

Light microscopic features

Specific histological features for diagnosis of CNIT in this study included a striped pattern of interstitial fibrosis and tubular atrophy, vacuolization of smooth muscle of arterioles and the arteriolar hyalinosis (particularly with peripheral/transmural nodules) (Table 2).

Ultrastructural examination

We noticed remarkable alterations in the mitochondrial morphology, particularly affecting the endothelial mitochondria in biopsies with CNIT. As the ultrastructural alterations in glomerular capillary, arteriolar and peritubular capillary endothelium were uniform, these were grouped together. The mitochondrial structural alterations in proximal tubules and endothelial cytoplasm were semiquantitatively graded. These included mitochondrial swelling, cristal swelling, disruption and partial or complete loss of outer membrane (Fig. 1).

Overall higher grades of mitochondrial alterations were seen in endothelial mitochondria (mean 2.2 ± 0.67) as compared with tubular mitochondria (mean 1.4 ± 0.82) in biopsies with CNIT. Mean endothelial mitochondrial grades were greater in cases as compared with controls at 1, 6 as well as 12 months and this difference was statistically significant in 12-month biopsies. On the other hand, the mean tubular mitochondrial grades did not

Table 1. Laboratory parameters.

Parameter	Patients with CNIT ($n = 15$)	Controls ($n = 15$)	*P* value
Urea (mg/dl)	44.2 ± 11.6	36.5 ± 10.7	0.69
Creatinine (mg/dl)	1.1 ± 0.5	1.0 ± 0.7	0.73
Estimated GFR	66. 2 ± 15.2	71.22 ± 18.4	0.42
Cyclosporine level (ng/ml)	946.3 ± 106.3	938.7 ± 88.7	0.83
Tacrolimus level (ng/ml)	8.3 ± 2.4	7.6 ± 3.1	0.69
Pretransplant HLA mismatches	2.1 ± 0.9	2.0 ± 0.7	0.73

Table 2. Histological features in biopsies with calcineurin inhibitor toxicity (CNIT) and controls.

No.	Light microscopic feature	Patients with CNIT (%)	Controls (%)
1	Glomerular mesangial matrix increase	4/15 (26.6)	2/15 (13.3)
2	Glomerular ischemic shrinkage	4/15 (26.6)	2/15 (13.3)
3	Nodular hyaline arteriolosclerosis	7/15 (46.6)	0/15 (0)*
4	Arteriolar smooth muscle vacuolization	12/15 (80)	2/15 (13.3)*
5	Striped tubular atrophy/interstitial fibrosis	8/15 (53.3)	0/15 (0)*
6	Juxtaglomerular apparatus hyperplasia	6/15 (40)	3/15 (20)

*$P = <0.05$.

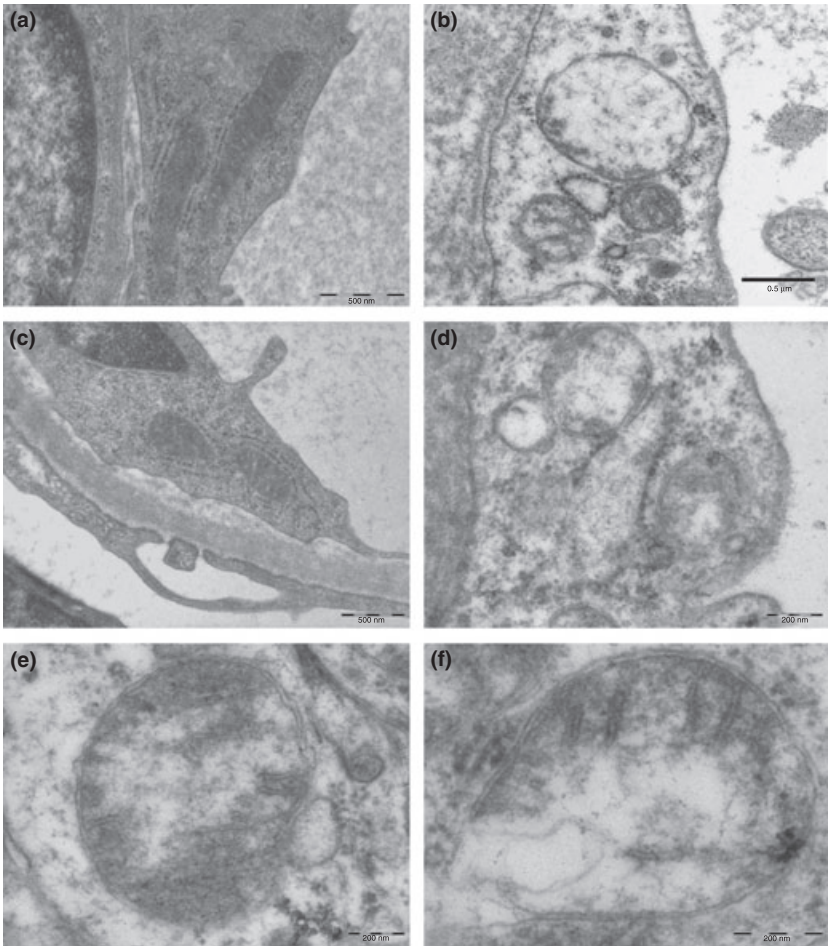

Figure 1 Ultrastructural micrographs showing the endothelial mitochondrial alterations. Peritubular capillary endothelium (a) from a control biopsy at 6-month post-transplant and glomerular capillary endothelium (c) from protocol biopsies at 12 months showing normal-appearing mitochondria with preserved cristal architecture (original ×10 000 and ×7100) while corresponding endothelial mitochondria from biopsies with calcineurin inhibitor toxicity (CNIT) (b and d) show marked mitochondrial swelling, cristal disintegration and loss with rarefaction of central mitochondrial matrix (original ×16 000). Higher magnification (×25 000) images show individual mitochondria showing central cristal disintegration and rarefaction (e) and membrane rupture (f).

show a significant difference amongst biopsies with CNIT and controls at any time point (Table 3). Grade 2/3 alterations in endothelial mitochondria were noted in 86.6% (13/15) of biopsies with CNIT while similar changes were seen in tubular mitochondria in only 46.6% (7/15) of the biopsies. In the control group, 13.3% (2/15) biopsies showed grade 2/3 changes in tubular mitochondria, while none of the biopsies showed similar changes in endothelial mitochondria (Figs 2 and 3). These findings indicate a more severe damage to endothelial mitochondria as compared with tubular mitochondria in biopsies with CNIT.

Morphometric analysis provided further objective substantiation of the structural mitochondrial alterations. The endothelial mitochondria showed a progressive reduction in the numerical density and were significantly more depleted in 12-month protocol biopsies as compared with the controls ($P = 0.02$). In addition, there was significantly more depletion of endothelial mitochondria in biopsies with CNIT at 12 months as compared with 1-month protocol biopsies (mean 0.17 ± 0.01; $P = 0.03$). The endothelial mitochondrial surface area showed an increase across the 1-, 6- and 12-month protocol biopsies indicating progressive mitochondrial swelling. The mean endothelial mitochondrial area was significantly more in biopsies from patients with CNIT as compared with controls at 12-month post-transplant.

The tubular mitochondria also showed progressive structural alterations, however these changes were less severe than those noticed in the endothelial mitochondria and the difference in tubular mitochondrial grade, numerical density and surface area in biopsies with CNIT did not achieve statistical significance as compared with the control biopsies at any time point (Table 4).

Immunohistochemistry

VEGF

In 1-month protocol biopsies, majority of cases with CNIT showed intense immunohistochemical expression of VEGF in both tubules as well as vessels (mean staining grades of 3.16 ± 0.4 and 3.0 ± 0.63 respectively). These changes were comparable in biopsies with CNIT and controls. However in 6- and 12-month protocol biopsies a progressive reduction in VEGF staining grade in both tubules and

Table 3. Mean ultrastructural mitochondrial grades in protocol biopsies with CNIT and control subjects.

	Duration of Protocol biopsy								
	One month			Six months			Twelve months		
	CNIT (n = 6)	Control (n = 6)	P	CNIT (n = 5)	Control (n = 5)	P	CNIT (n = 4)	Control (n = 4)	P
Tubular mitochondrial grade	1.0 ± 0.89	0.5 ± 0.54	0.39	1.4 ± 0.54	0.80 ± 0.83	0.31	2.0 ± 0.81	1.0 ± 0.81	1.0
Endothelial mitochondrial grade	1.83 ± 0.4	0.66 ± 0.51	0.15	2.2 ± 0.83	0.4 ± 0.54	0.06	2.75 ± 0.5	0.75 ± 0.5	0.01

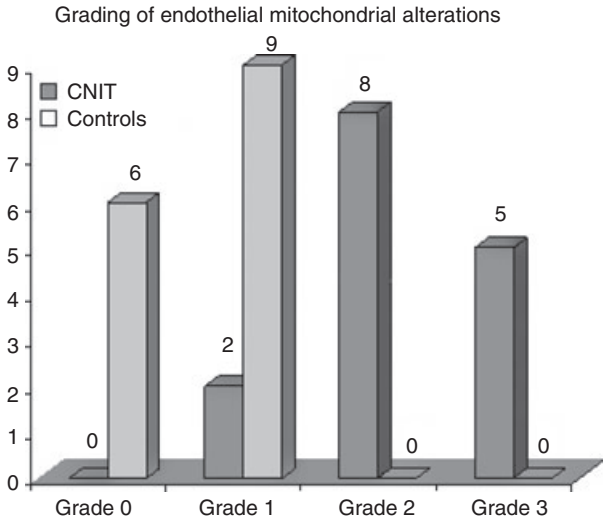

Figure 2 Bar diagram showing distribution of endothelial mitochondrial grades among the biopsies with calcineurin inhibitor toxicity (CNIT) and controls.

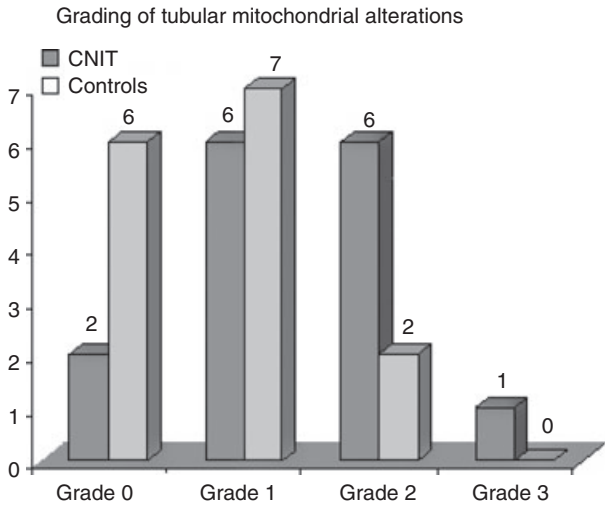

Figure 3 Bar diagram showing distribution of tubular mitochondrial grades among the biopsies with calcineurin inhibitor toxicity (CNIT) and controls.

vessels was noted, and in 12-month biopsies the mean grades of staining were significantly different among cases and controls in both tubules as well as vessels (P = 0.02). A persistent upregulation of VEGF expression was noted in the control biopsies (Table 5), (Fig. 4).

Nitrotyrosine

Enhanced nitrotyrosine expression was noted both in tubules and vessels in biopsies with CNIT as compared with the controls in 1-, 6- as well as 12-month protocol biopsies. The control biopsies, on the other hand, showed minimal expression of nitrotyrosine and the difference in mean staining grades amongst cases and controls was significant in 1-, 6- as well as 12-month protocol biopsies (Table 5).

On nonparametric correlative analysis (Spearman's), a significant negative correlation was seen between the mean ultrastructural grade of endothelial mitochondrial alterations and VEGF expression in vessels (P = 0.04).

Discussion

Calcineurin inhibitor toxicity has emerged as an important factor affecting the renal allograft survival and function. The mechanisms of CNIT involve a complex interplay of several factors including oxidative damage mediated by reactive oxygen species (ROS), and nitrosative stress manifested as enhanced nitration of tyrosine residues of proteins leading to nitrotyrosine formation. Introduction of protocol biopsies has allowed insight into mechanisms of CNIT and helped in understanding the dynamics of 'background' damage to the allograft in the absence of clinical signs of renal dysfunction [15,16]. Incidence of CNIT in protocol allograft biopsies has been evaluated in a few studies with remarkable variation in the reported frequency, possibly because of lack of consensus on the defining histological features for diagnosis of CNIT. Kambham *et al.*, [17] in their study, reported an incidence of 41.5% in a 2-year follow-up study. Nankivell *et al.* [18] had earlier shown that

Table 4. Ultrastructural morphometry results.

	Duration of protocol biopsy								
	One month			Six months			Twelve months		
	CNIT (n = 6)	Control (n = 6)	P	CNIT (n = 5)	Control (n = 5)	P	CNIT (n = 4)	Control (n = 4)	P
Tubular mitochondrial density (no/μ^2)	1.13 ± 0.08	1.22 ± 0.08	0.42	1.04 ± 0.07	1.11 ± 0.03	0.20	0.90 ± 0.15	1.06 ± 0.10	0.10
Endothelial mitochondrial density (no/μ^2)	0.26 ± 0.03	0.31 ± 0.03	0.06	0.21 ± 0.01	0.31 ± 0.04	0.09	0.17 ± 0.01	0.28 ± 0.03	0.02
Tubular mitochondrial area (μ^2)	0.33 ± 0.11	0.31 ± 0.18	0.82	0.38 ± 0.19	0.32 ± 0.16	0.54	0.43 ± 0.17	0.34 ± 0.14	0.44
Endothelial mitochondrial area (μ^2)	0.38 ± 0.10	0.32 ± 0.09	0.30	0.46 ± 0.16	0.30 ± 0.16	0.15	0.58 ± 0.18	0.31 ± 0.10	0.04

Table 5. Immunohistochemical staining results for nitrotyrosine and vascular endothelial growth factor (VEGF).

	Duration of protocol biopsy								
	One month			Six months			Twelve months		
	CNIT (n = 6)	Control (n = 6)	P	CNIT (n = 5)	Control (n = 5)	P	CNIT (n = 4)	Control (n = 4)	P
VEGF staining grade tubules	3.16 ± 0.4	3.0 ± 0.63	0.69	2.2 ± 0.44	3.2 ± 0.44	0.03	1.5 ± 0.5	3.25 ± 0.9	0.02
VEGF staining grade vessels	3.5 ± 0.54	3.0 ± 0.63	0.24	2.2 ± 0.44	3.6 ± 0.54	0.01	1.5 ± 0.5	3.5 ± 0.57	0.02
NT staining grade tubules	3.16 ± 0.75	1.5 ± 0.54	0.00	3.2 ± 0.83	1.4 ± 0.54	0.01	3.2 ± 0.5	1.75 ± 0.5	0.02
NT staining grade vessels	3.16 ± 0.75	1.33 ± 0.51	0.00	3.2 ± 0.83	1.4 ± 0.54	0.01	3.5 ± 0.57	1.5 ± 0.57	0.02

histological features of CNIT were seen in virtually 100% of the biopsies in a follow-up study that spanned 7 years. As there are no reliable markers that can accurately predict the onset on CNIT, renal biopsy still remains the gold standard for diagnosis. Light microscopic features of CNIT in renal allograft biopsies have been the subject of various experimental and patient sample-based studies. A recent study on protocol allograft biopsies identified arteriolar peripheral hyaline nodules, striped pattern of interstitial fibrosis/tubular atrophy and tubular isometric vacuolization as relatively specific features of CNIT [17]. In addition, we also found smooth muscle cell vacuoles in arterioles to be a relatively specific feature for histological identification of CNI. Ultrastructural examination of renal allograft biopsies with CNIT has been the subject of very few studies in the available literature. Nacar *et al.* [12] reported ultrastructural features of allograft biopsies with CNIT and noted mitochondrial swelling in nine of the 26 biopsies examined. Though the authors did not mention the exact location (tubular or endothelial) of these mitochondria, presence of giant mitochondria with reduction in cristae was frequently noticed. This was in keeping with few earlier observations of mitochondrial morphological alterations in CNIT [19–21]. In this study, we also noted remarkable ultrastructural alterations in mitochondrial morphology during the routine evaluation of biopsies with CNIT and observed that these changes were more profound in endothelial mitochondria as as compared with tubular mitochondria. As energy requirements of endothelial cells are met predominantly from the anaerobic glycolytic metabolism of glucose [22–24], role of mitochondria in endothelial cells (which in other locations are the major energy providers to the cell), has been somewhat neglected. Endothelial mitochondria are now known to be important mediators involved in vascular homeostasis and disease [13]. In lieu of energy production, the endothelial mitochondrial respiratory chain produces ROS, which under physiologic conditions takes part in various intracellular signaling mechanisms. Normally, these potentially toxic ROS are dismutated to hydrogen peroxide by superoxide dismutase, however in face of a significant 'oxidative stress' the antioxidant mechanisms of the cellular machinery may be overwhelmed and then these ROS may assume a cytotoxic role. Oxidative stress and ROS are thought to play a pivotal role in genesis of CNI-induced renal toxicity [25–27]. Experimental evidence of this hypothesis is seen by amelioration of CNIT by administration of antioxidants and other agents such as beta blockers, mineralocorticoid

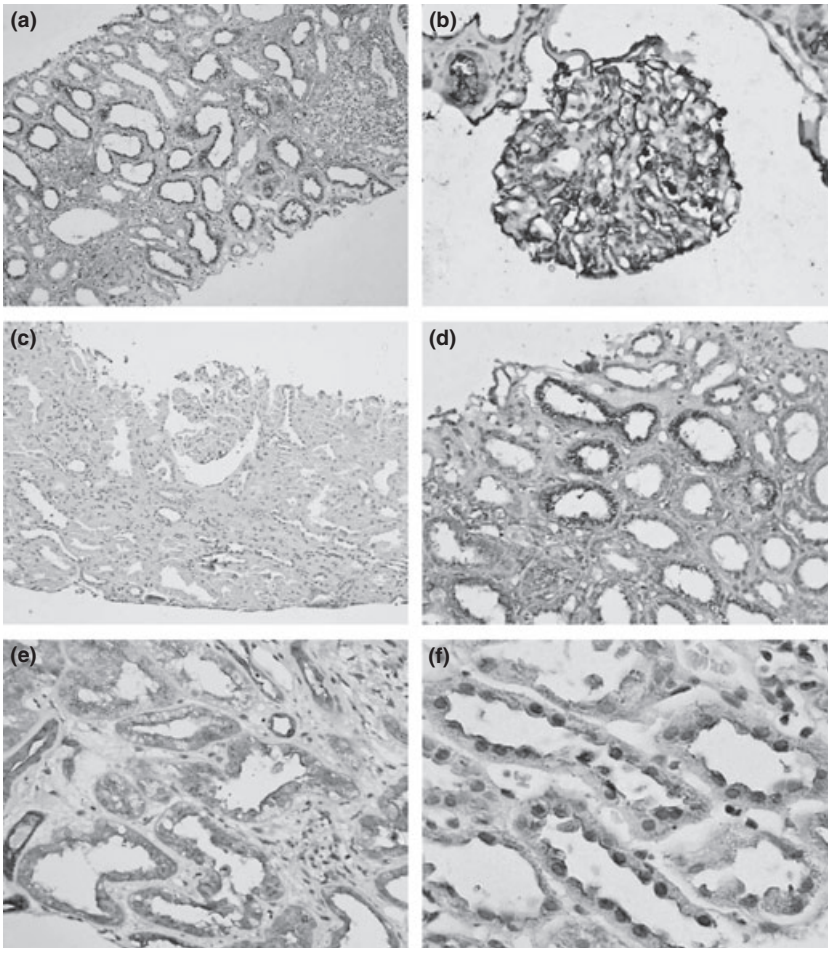

Figure 4 Representative photomicrographs of immunostaining for vascular endothelial growth factor (VEGF) (a–d) and Nitrotyrosine (e, f): Intense VEGF expression in tubules [a; immunoperoxidase (IHC) ×40] and glomerular capillaries (b: IHC ×100) in a 1-month protocol biopsy with calcineurin inhibitor toxicity (CNIT). Marked reduction of VEGF staining in a biopsy with CNIT at 12-month protocol biopsy (c; IHC ×40) (note the striped pattern of intertubular fibrosis), while a corresponding control biopsy shows marked VEGF up regulation (d; IHC ×100). Photomicrographs from sections stained for nitrotyrosine show marked up regulation in 1-month (e; IHC ×200) and 12-month protocol biopsy (f; IHC ×200) (note the apical pattern of staining in f).

receptor blockage among others [28–31]. Another important target of mitochondrial ROS is the mitochondrial electron transport chain itself. The reaction of superoxide radicals with nitric oxide in the endothelium leads to formation of peroxynitrite, which can irreversibly damage molecules including the proteins of the mitochondrial electron transport chain. Reaction of peroxynitrite with the tyrosine residues in proteins yields nitrotyrosine, which can be detected in the tissues by immunohistochemical techniques [32]. CNIs are known to induce oxidative stress and mitochondrial dysfunction in renal tubular epithelial cells [33] and peroxynitrite formation has been demonstrated in vascular endothelial cells exposed to cyclosporine *in vitro* [7]. In this study, enhanced peroxynitrite production as evidenced by persistent increase in nitrotyrosine staining was seen both in tubules and renal vessels in protocol biopsies with CNIT. On the other hand, low expression of nitrotyrosine, perhaps indicating physiological levels of production, was noted in control biopsies. These findings underscore the role of peroxynitrite radicals in development of CNIT.

Vascular endothelial growth factor is a multifunctional protein involved in vasculogenesis, angiogenesis and also promotes endothelial cell proliferation. It exerts its effects through two well-characterized tyrosine kinase receptors VEGFR1 and VEGFR2 present on the endothelial cells. In addition to its role in renal angiogenesis including maintenance of glomerular and endothelial integrity, VEGF also has tubular cytoprotective effects and is responsible for induction of cell resistance against injurious stimuli as varied as hypoxia, exposure to ROS, rupture of endothelial vascular endothelial cadherin junctions or exposure to fibroblast growth factor via an autocrine secretion pathway [34]. The finding of increased tubular expression of VEGF induced by CNIs favors the possibility that VEGF is endowed with tubuloprotective effects and may act as a survival factor for renal tubular epithelium *in vivo* [10].

We noted an initial VEGF upregulation (in both tubules and vasculature) in protocol biopsies with CNIT performed at 1 month post-transplant; this was comparable to the expression in the control group and probably represents a protective mechanism (Table 5). However, in the

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6- and 12-month protocol biopsies with CNIT, a decline in intrarenal expression of VEGF (both in tubules as well as vasculature) was noted. This was in contrast to the control biopsies which continued to show elevated expression of VEGF as evaluated by the mean immunostaining grades. The difference in mean VEGF immunostaining grades between CNIT and control group was statistically significant in 6- and 12-month biopsies in both tubules and vasculature. These findings may indicate the loss of VEGF support associated with establishment of CNIT.

Although tacrolimus and cyclosporine have similar toxicity profiles and mechanisms of action, little is known about the relative contribution of these drugs in development of CNIT. Solez *et al.* [35] reported CNIT in 24% of biopsies with tacrolimus and 17% in patients taking cyclosporine in a 2-year follow-up study. In this study, although a greater number of patients with CNIT were on tacrolimus as compared with those on cyclosporine i.e. 11/97 (11.34%) vs. 4/43 (9.3%) respectively, however this difference was not statistically significant. Interestingly, 10/11 (90.9%) of the biopsies with CNIT in the first 6-month post-transplant were from patients on tacrolimus indicating that it may be responsible for most of the acute toxicity associated with administration of calcineurin inhibitors. Although we did not find any significant difference in VEGF or nitrotyrosine expression or ultrastructural features among patients with CNIT on cyclosporine and tacrolimus (data not shown), a recent study has shown that tacrolimus but not cyclosporine induces the release of VEGF in MC3T3-E1 (osteoblast-like) cells by SAPK/JK pathway and modulation of p70S6 kinase activity [36]. This important finding clearly needs to be investigated further as it has important implications in the setting of renal transplantation.

The ultrastructural alterations in endothelial mitochondria along with the progressive loss of VEGF support and persistently elevated nitrotyrosine expression indicate an intricate relationship between these processes in the development of CNIT. These findings suggest that the oxidative stress-mediated mitochondrial injury coupled with loss of VEGF support may be important events in initiation and persistence of CNIT in renal allografts. Finding of these alterations in protocol biopsies (without clinical evidence of renal dysfunction) further emphasizes the impact of these events in long-term graft outcome and also highlights the importance of performing protocol renal allograft biopsies.

Conclusion

In conclusion, this study shows a relatively high incidence of CNIT in protocol renal allograft biopsies, asserting the fact that this might be an important mechanism of background damage to the allograft and may adversely affect the long-term graft survival. Our findings indicate that endothelial mitochondria could be important intracellular targets of CNIT in renal allografts and the structural alterations seen in them are associated with enhanced nitrotyrosine expression and progressively decreasing expression of intrarenal VEGF, suggesting a close pathologic link between these in the pathogenesis of CNIT in renal allografts. Although we have not investigated the functional aspects of mitochondrial dysfunction, we have provided evidence of structural alterations which are more profound in endothelial location indicating specific functional pathways operating at this location which merit further investigative studies.

Authorship

AS: performed research/study, compiled and analysed data and wrote manuscript. SJ: performed research, performed surgeries and biopsies, collected data. RG: collected and analysed data, wrote manuscript. KGB: collected and analysed data, performed literature review. SG: performed research, performed surgeries and biopsies. SKA: responsible for clinical care of patient, performed research, reviewed manuscript. AKD: conceptualized, planned the study, compiled and analysed data, wrote and reviewed manuscript.

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