Paper

The blockade of the renin-angiotensin system reverses tacrolimus related cardiovascular toxicity at the histopathological level

Mehmet Agirbasli, Nurdan Papila-Topal,* Betul Ogutmen,† Hicran Deniz,§ Fulya Cakalagaoglu,# Serhan Tuglular,† Emel Akoglu†*

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* Department of Cardiology, Marmara University Medical School, Istanbul, Turkey.

† Division of Nephrology, Marmara University Medical School, Istanbul, Turkey.

§Department of Internal Medicine, Marmara University Medical School, Istanbul, Turkey.

#Department of Pathology, Marmara University Medical School, Istanbul, Turkey.

Correspondence to: Dr Mehmet Agirbasli, Marmara University Hastanesi, Kardiyoloji ABD, Tophanelioglu Cad, Altunizade Kadikoy, Istanbul 34662, Turkey.
Tel: +90 532 746 8840
Fax: +90 216 327 6035
E-mail: agirbasli@gmail.com

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Abstract

Introduction. In this study, we investigate the toxic effects of tacrolimus (FK506) on the cardiovascular system at the histopathological level in a rat model and whether these effects can be reversed by the blockade of the renin-angiotensin system (RAS) by either an angiotensin-converting enzyme inhibitor (ACE-inhibitors) or an angiotensin receptor antagonist (ARB).

Methods and results. Thirty-one Wistar rats were divided into four groups. FK506 group was treated with FK506 intraperitoneally (i.p.), FK506+ACE-inhibitors and FK506+ARB groups were treated with either quinapril or valsartan orally in addition to FK506. Control group was treated with saline i.p. Histological and immunohistochemical staining of cardiovascular tissue in the FK506 group showed increased vacuolar degeneration (11.2 *vs.* 5.8, p=0.008), arterial hyalinosis (10.7 *vs.* 6.3, p=0.036), transforming growth factor-beta (TGF-β) (12.2 *vs.* 4.8, p=0.001) and vascular endothelial growth factor expression (VEGF) (10.7 *vs.* 6.3, p=0.036), elastic van Gieson (11.5 *vs.* 5.5, p=0.004), and periodic acid Schiff stain scores (12.5 *vs.* 4.5, p<0.001) compared to the control group. Immunohistochemical scores showed that expression of TGF-β is up-regulated, and bone morphogenic protein (BMP-7) is down-regulated with FK506 toxicity. Adding RAS blockade with either an ACE-inhibitor or an ARB could reverse FK506 induced changes. Both FK506+ACE-inhibitors and FK506+ARB groups demonstrated decrease in arterial hyalinosis (22.1 *vs.* 14.4 (FK506+ACE-inhibitor) and 13.6 (FK506+ARB), p=0.09) and vacuolar degeneration (23.1 *vs.* 16.1 (FK506+ACE-inhibitor) and 12.4 (FK506+ARB), p=0.006) scores compared to the FK506 group.

Conclusion. Blockade of RAS could reverse the histopathological signs of FK506 induced cardiac toxicity in a rat model.

Introduction

Tacrolimus (FK506) is a potent immunosuppressive agent widely used in reducing the incidence and severity of allograft rejection after organ transplantation. FK506 belongs to the family of calcineurin inhibitors, a protein phosphatase with important regulatory effects such as blocking the expression of T cell activation genes.[1] The therapeutic potential of FK506 is limited by its toxic side-effects which include neurotoxicity, nephrotoxicity, diabetogenicity, and gastrointestinal disturbances.[2] Arrhythmia is the most commonly reported clinical cardiac side-effect after FK506 treatment.[3-5] Alterations in cardiac morphology and pathology have been reported in patients receiving FK506 treatment.[6]

Although purported mechanisms for FK506 related toxicity include renal and systemic vasoconstriction, increased release of endothelin-1, decreased production of nitric oxide, and increased expression of transforming growth factor (TGF-β),[7,8] the underlying mechanisms for FK506 related cardiac toxicity remain unclear.

In this study, we investigate the effects of FK506 on the cardiovascular system at the histopathological level and whether these effects can be reversed by the blockade of the renin-angiotensin system (RAS) by either an angiotensin-converting enzyme inhibitor (ACE-inhibitor) or an ARB.

Materials and methods

The study was performed at the animal laboratories of the Marmara University Medical School between July 2004 and June 2005. The study design was randomised and prospective.

Experimental animals and procedures

All animal procedures were performed in accordance with the Declaration of Helsinki, and the Guide for the Care and Use of Animals. The study protocol was approved by the local ethics committee.

Thirty-one male Wistar rats weighing 300–350 g were used in the study. Throughout the study, the animals were housed in four cages and maintained under controlled environmental conditions (a 12-hour light/dark cycle and a temperature of 21°C). The animals had free access to water and they were fed a low-sodium diet starting one week

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before the study. Animals were weighed daily throughout the study period. Study medications were administered under anaesthesia with ether and blood samples were taken by heart puncture at the end of the study period. FK506 levels were determined by a radioimmunoassay using Tacrolimus II ref 3C10 34-3091/R6 1MX System (Abbott Laboratories, USA).

A chronic FK506 toxicity model was used in the study. The experimental animals were divided into four groups. Previous studies displayed that FK506 induced tubulointerstitial fibrosis and decrease in glomerular filtration rate were dependent on the sodium intake[9] and sodium depletion appeared to enhance FK506 related toxicity. Therefore, we decided to use low-salt diet to observe FK506 toxicity at the histological and immunohistochemical level.

Experimental rat models of FK506 therapy utilised a dose of 0.1-1 mg/kg/day.[10] We selected a similar dose and FK506 group (n=8) received tacrolimus (1 mg/kg/day) intraperitoneally (i.p.) for eight weeks. FK506+ACE-inhibitors (n=7) and FK506+ARB (n=8) groups received either quinapril (10 mg/kg/day) or valsartan (40 mg/ kg/day) administered via the progastric (og) route in addition to FK506 (1 mg/kg/day) i.p. for eight weeks. The control group (n=8) received 0.5 ml saline i.p.

Pathology

Cardiac tissue was removed immediately, cut from the right ventricle in 2 mm thick slices, fixed in 10% formalin and then embedded in paraffin. Pathology results were interpreted by a pathologist (blinded to the group) and semiquantitatively scored for the degree of histopathological changes as described previously.[11] The degree of fibrosis was evaluated semiquantitatively in trichrome-stained specimens. Arterial hyalinosis was assessed in periodic acid-Schiff (PAS) stained specimens. Vacuolar degeneration was scored semiquantitatively in haemotoxylin and eosin (HE) stained specimens. Elastic fiber and collagen formation were examined in elastic van Gieson (EVG) stained specimens.

Endocardium (thickness, cell type and number, stroma and thrombus), cardiac myocytes (size, localisation, vacuolar degeneration), interstitium (cell type and number, stromal composition and vessels) and arteriolar hyalinosis were examined in every specimen.[12] Histopathological findings were scored semiquantitatively as follows: (0) no damage, (1) mild, (2) moderate, (3) severe.[11,12]

Immunohistochemical evaluation was performed semiquantitatively for TGF-β (NCL-TGFB; Novocastra), VEGF (C-1; sc-7269; Santa Cruz Biotechnology), and bone morphogenic protein (BMP-7) (N-19; sc-6899; Santa Cruz Biotechnology). Light microscopic evaluation of the myocardial and interstitial tissue were performed under three different magnifications (Olympus BX50) and semiquantitative scores (0–3) were given based on the positively stained cell number.

Statistical analyses

Light microscopic and immunohistochemical findings were scored semiquantitatively by a blinded pathologist (Fulya Cakalagaoglu). Comparison between categorical groups and semiquantitative scores were performed using Kruskal-Wallis and Mann-Whitney U-tests. Bonferroni's method for multiple comparison was applied where appropriate. Continuous variables (FK506 levels) were compared by Student's *t*-test. All tests were two-tailed and a value of $p<0.05$ was accepted as significant.

Results

Mean FK506 levels were not significantly different among FK506, FK506+ACE-inhibitors and FK506+ARB groups (p=0.2). There were no significant differences in body weight between the control and FK506 groups at the beginning (236±21 *vs.* 251±42 g) and at the end of the experiment (264±37 *vs.* 290±31 g). Two rats died (one in the control group and one in the FK506+ACE-inhibitor group) throughout the experiment. Autopsy revealed lung abscess and infection in these two rats.

Kruskal-Wallis method was used to compare light microscopic and immunohistochemical findings in four groups. We then compared groups with each other by the Mann-Whitney test. Evidence for significant cardiac toxicity was noted after FK506 treatment. Cardiac myocytes in the FK506 group displayed significant vacuolar degeneration (mean rank 11.2 *vs.* 5.8, p=0.008) at the PAS and HE stains compared to the control group. Similarly, PAS stain showed that arterial hyalinosis also increased in the FK506 group compared to the control group (mean rank 10.7 *vs.* 6.3, p=0.036).

Histological and immunohistochemical staining in the FK506 group showed a significant increase in TGF-β immunoexpression (mean rank 12.2 *vs.* 4.8, p=0.001) and VEGF (mean rank 10.7 *vs.* 6.3, p=0.036), in addition to elastic van Gieson (mean rank 11.5 *vs.* 5.5, p=0.004) and PAS stain scores (mean rank 12.5 *vs.* 4.5, p<0.001) compared to the control group. These results indicate enhanced elastic fiber and collagen formation (elastic van Gieson stain) and arterial hyalinosis (PAS stain) as well as up-regulation of TGF-β and VEGF expression after FK506 toxicity.

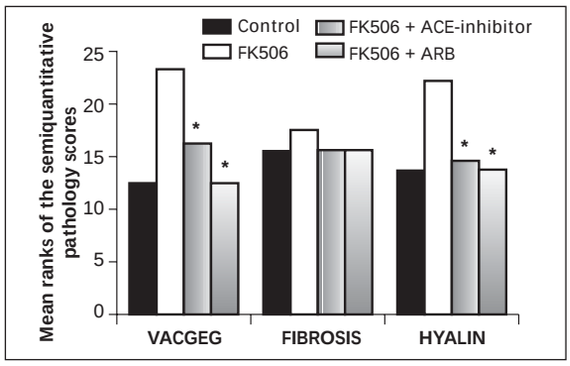

Figure 1
Light microscopy displayed partial reversal of the tacrolimus toxicity with renin angiotensin system blockade. Y Axis indicates mean ranks of the semiquantitative pathology scores by Kruskal-Wallis test. FK506: tacrolimus treated group, FK506+ARB received Valsartan in addition to tacrolimus, FK506+ACE-ihibitors received Quinapril in addition to tacrolimus; VACDEG: vacuolar degeneration; HYALIN: hyalin arteriolopathy; *= p<0.05

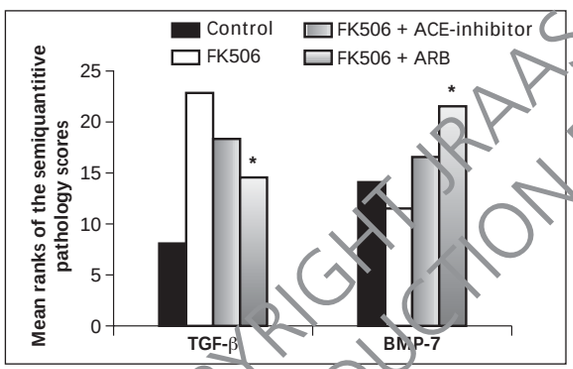

Figure 2
Immunohistochemical data showing TGF-β is significantly up-regulated, and BMP-7 is down-regulated with tacrolimus toxicity. Adding RAS blockade with either an ACE-inhibitor or AT1 antagonist reversed tacrolimus induced alterations in TGF-β, and BMP-7 expression.Y Axis indicates mean ranks of the semiquantitative pathology scores by Kruskal-Wallis test. FK506: tacrolimus treated group, FK506+ARB: received Valsartan in addition to tacrolimus. FK506+ACE-ihibitors: received Quinapril in addition to tacrolimus;
TGF-β = transforming growth factor; BMP-7: bone morphogenic protein. *= p<0.05

The differences in vacuolar degeneration, elastic van Gieson and PAS scores, and TGF-β expression remained significant after Bonferroni correction.

Cardiac toxicity after FK506 could be attenuated with RAS blockade. Compared to the FK506 group, both the FK506+ACE-inhibitor and FK506+ARB groups demonstrated a significant decrease in hyalinisation and vacuolar degeneration (p=0.01, p=0.01 for FK506+ACE-inhibitor and p=0.001, p=0.007 for FK506+ARB, respectively) (figure 1). The differences

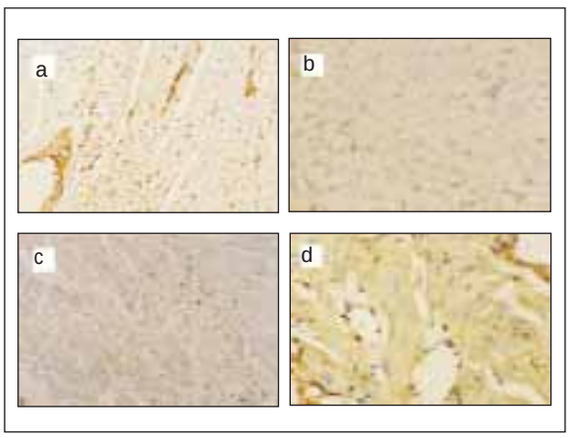

Figure 3
Immunohistochemical examination of the TGF-β and BMP-7 in FK506 and FK506 + ARB groups. a: TGF-β expression in FK506 group IHC; 100X; b: TGF-β expression in FK506 + ARB group IHC; 40X; c: BMP-7 expression in FK506 group IHC; 40 X; d: BMP-7 expression in FK506 + ARB group IHC; 40 X.

maintained significance after Bonferroni correction. The degree of fibrosis tended to decrease with RAS blockade without reaching statistical significance.

Immunohistochemical scores displayed that expression of TGF-β is up-regulated, and BMP-7 (bone morphogenic protein) is down-regulated with tacrolimus toxicity. Adding RAS blockade with either an ACE-inhibitor or ARB could reverse FK506 induced histopathological changes (figures 2 and 3).

Compared to the FK506 group, histological staining showed a significant decrease in TGF-β (mean rank 10.9 *vs.* 6.1, p=0.018), vacuolar degeneration (mean rank 11.2 *vs.* 5.8, p=0.008), and in arterial hyalinosis (mean rank 10.7 *vs.* 6.3, p=0.036) in the FK506+ARB group. On the other hand, BMP-7 increased in the FK506+ARB group compared to the control group (mean rank 6.2 *vs.* 10.8, p=0.039) (figures 2 and 3). Compared to the TAC group, the TAC+ACE-inhibitors group displayed decreased vacuolar degeneration, and arterial hyalinosis without reaching statistical significance (p=0.1).

Discussion

Our observations indicate that the cardiovascular system displays significant changes at the light microscopic and immunohistochemical level in a FK506 toxicity model and these changes could be reversed by the blockade of the RAS either with an ACE-inhibitor or an ARB.

Although similar cardiovascular histopathological changes occurred as a result of diabetes mellitus and chronic renal insufficiency,[13,14] limited data exist on FK506 related cardiovascular toxicity.

Arrhythmias occur in patients after FK506 therapy.[3-5] Re-entry around the fibrotic or altered tissue histology is the common mechanism for cardiac arrhythmias. Furthermore, altered cardiac morphology has been reported after FK506 therapy[6] though cardiovascular therapeutic effects of FK506 were reported at the histopathological level in experimental autoimmune myocarditis.[10]

Most of the current knowledge about FK506 associated toxicity comes from studies performed in kidney cells. Several histopathological changes are associated with FK506 related nephrotoxicity, including tubular vacuolisation, arteriolar hyalinosis and interstitial fibrosis.[7-8] Although there may be some degree of analogy between cellular responses to tacrolimus, there may also be clear differences depending on the cell type studied.

Members of the TGF-β superfamily, comprising the TGF-β and bone morphogenic protein (BMP) family have important functions in cardiac remodelling and fibrosis.[15,16] TGF-β is a potent fibrogenic factor and also regulates the hypertrophy of smooth muscle cells. In addition, TGF-β modulates the fibrogenic actions of basic fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) period[7,8] RAS and TGF-β play pivotal roles in the development of cardiac hypertrophy and heart failure. In contrast to the deleterious effects of the TGF-β/activin subgroup, the BMP subfamily has beneficial effects in the heart.[15] BMP-7 counteracts some of the profibrogenic actions of TGF-β.[15,16]

Recent studies indicate that RAS, TGF-β, BMP-7 and VEGF modulate vascular housekeeping and fibrosis.[17,18] Previous studies report that RAS blockade has beneficial effects on cardiac remodeling in the setting of cardiac toxicity or injury. BMP-7 is previously reported to be down-regulated in cyclosporin toxicity and up-regulated with the blockade of RAS by ACE-inhibitors.[19] In cultured human mesangial cells, high glucose induces TGF-β, fibronectin, and type IV collagen accumulation and these effects were partially blocked by candesartan (an ARB).[20] Also, inhibition of the renin–angiotensin pathway by losartan, another ARB, reduced TGF-β activity and decreased fibrosis in reperfused hearts.[21] In exogenous hyperaldosteronism in the rat, aldosterone causes myocardial collagen and arterial fibrin accumulation. The aldosterone receptor antagonist, eplerenone prevents both effects.[22]

In our study, we used valsartan (ARB) and ramipril (ACE-inhibitors) to block RAS cascade at different levels. Our results indicate that cardiac expression of TGF-β is up-regulated, and BMP-7 is down-regulated with tacrolimus toxicity. Adding RAS blockade with either ACE-inhibitors or ARB partially reversed FK506 induced changes (figure 2). Therefore, part of the protective role of RAS blockade in tacrolimus related cardiac toxicity might be through down-regulation of TGF-β and up-regulation of BMP-7.

Activation of RAS, inflammation and NF-κB activation may work synergistically in the progression of FK506 toxicity. Sodium depletion, which is known to activate the RAS cascade, appeared to enhance FK506 related toxicity in experimental models.[9] Similarly, in chronic FK506 nephrotoxicity, enhanced inflammation was accompanied by enhanced renal NF-κB activity.[23] Furthermore inhibition of NF-κB markedly reduced inflammation and fibrosis in these models.[2]

Our study has several limitations. Light microscopic findings of vacuolar degeneration and hyalinisation increased with tacrolimus which could be partially reversed with RAS blockade. On the other hand, the increase in fibrosis after FK506 toxicity did not reach statistical significance. Also some of the differences in the light microscopic and immunohistochemical scores lost significance after Bonferroni correction for multiple comparisons. A longer period of tacrolimus administration with a larger sample size might be needed.

We did not have echocardiography to document any functional deterioration that can be associated with pathological changes related to FK506 toxicity. Changes in echocardiographic signals (backscatter reductions), and alterations in both systolic and diastolic function parameters may reflect tissue fibrosis or other cardiomyopathic changes.[24] Previous reports suggest that morphological changes precede functional parameters in FK506 related toxicity.[25]

In the presence of an ever growing use of tacrolimus it will become imperative to further our understanding of the underlying pathology of FK506 related toxicity, so as to develop improved pharmacological protective therapies.

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