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Cyclosporin A Aggravates Calcification of Vascular Smooth Muscle Cells Under High-Glucose Conditions with a Calcifying Medium

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Vascular calcification (VC) progresses substantially even after kidney transplantation, and is a predictor of morbidity and mortality. However, the effect of cyclosporin A (CsA) on VC has not been reported in diabetic kidney transplant patients. In this study, we evaluated the effect of CsA on the VC of mouse vascular smooth muscle cells (VSMCs) under high glucose (HG).

Material/Methods: To demonstrate the effect of CsA (1.0 μmol/L) and HG (30 mM) in the induction of the VC of the VSMCs, we determined alkaline phosphatase (ALP) activity, microscopic morphology of calcification, the expressions of the calcification and inflammation-related genes, and the intracellular calcium concentrations in VSMCs.

Results: Calcification was observed 14 days after exposure to a calcifying medium (sodium phosphate monobasic and dibasic mixture). On microscopic morphology, CsA alone did not induce calcification under HG conditions, but clearly increased calcification under HG with a calcifying medium. ALP activity increased under HG with CsA or a calcifying medium compared to HG conditions alone. CsA increased ALP activity under low glucose (LG, 5.5 mM) with a calcifying medium, but markedly increased under HG with a calcifying medium. CsA significantly increased the mRNA expressions of the calcification markers (core binding factor-alpha 1, bone morphologic proteins 2) as well as those of the inflammatory marker (interleukin 6), under HG with a calcifying medium. Intracellular calcium concentrations were unchanged in CsA alone but significantly increased with the presence of a calcifying medium under both LG and HG conditions.

Conclusions: Considering the effect of CsA on VC, the vascular adverse effects of CsA need to be verified in diabetic transplant patients in the future.

MeSH Keywords: **Cyclosporine • Diabetes Complications • Myocytes, Smooth Muscle • Transplantation • Vascular Calcification**

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Background

Vascular calcification (VC) is an active phenotypic transition of vascular smooth muscle cells (VSMCs) into osteoblast-like cells in a calcified environment [1–3]. Several factors are involved in VC: inflammation, oxidative stress, presence of bone morphologic proteins (BMP), high-fat diet, presence of lipid oxidized products, uremia, and hyperglycemia [4]. An increase in aortic stiffness due to VC contributes to cardiac afterload, left ventricular hypertrophy, and cardiac fibrosis, and is associated with cardiovascular mortality in the general population [5–8].

Hyperglycemia is a well-known predisposing factor for VC. Diabetes and metabolic syndrome are important causes of arterial calcification [9,10]. Several bone-related proteins such as osteopontin, type I collagen, and alkaline phosphatase (ALP) are highly expressed in the medial layer of patients with diabetes [11]. Patients with diabetes show increased VC and a high risk of cardiovascular diseases compared to the general population [12]. Coronary calcification is significantly associated with diabetes. Increased coronary calcification is associated with higher mortality in those with diabetes than in those without [13].

Patients with renal dysfunction, especially those undergoing dialysis, are vulnerable to VC due to the dysregulation of phosphorus, vitamin D, and parathyroid hormone [24]. Patients with diabetes and chronic kidney disease (CKD) are at a higher risk for coronary artery calcification compared to non-diabetic patients with CKD [15]. The risk is also increased in patients with early renal dysfunction [16]. VC is a well-known cardiovascular risk factor in patients who are undergoing dialysis or have undergone kidney transplantation [17–19]. VC progresses substantially even in cases of stable kidney transplantation and is an established predictor of morbidity and mortality [18–20].

Cyclosporine A (CsA), a fungus-derived immunosuppressant, has been clinically used to prevent organ graft rejection by inhibiting the proliferation of lymphocytes in transplant patients [21]. However, sustained CsA-immunosuppression causes endothelial dysfunction, hypertension, dyslipidemia, and nephrotoxicity. Although these adverse effects are well-known risk factors for VC, little is known about the relationship between CsA and VC. These observations suggest that CsA may induce arterial stiffness and ectopic calcification in patients after a kidney transplant.

Medial VC is clearly shown in patients with diabetes and end-stage renal disease. However, the effect of CsA on VC in diabetic kidney transplant patients has not been reported. In this study, to evaluate the effect of CsA on the calcification of VSMCs in the diabetic milieu, we sought to determine if CsA aggravates the calcification process in high glucose and calcifying environments, through microscopic examination, as well as through the estimation of intracellular calcium contents, alkaline phosphatase (ALP) activity, and calcification-related gene expressions.

Material and Methods

MOVAS-1 cell culture

MOVAS-1 cells and mouse VSMC lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Waltham, MA) supplemented with 10% foetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were seeded in 12-well plates (Costar, Bucks, UK), at a density of 1.0×10^6 cells/cm^2. At confluence, the medium was supplemented with 3.0 mmol/L sodium phosphate (Na_2HPO_4 and NaH_2PO_4) for 14 days to induce calcification and were treated with 1.0 µmol/L CsA (donated by Chongkundang Pharm, Seoul, Korea). The medium was replaced every 2–3 days for 14 days. Incubation was performed at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Induction of calcification

Calcification was induced as previously described [16]. In brief, the cells were grown to confluence (day 0) and switched to a calcifying medium, which was prepared by adding inorganic phosphate (a mixture of NaH_2PO_4 and Na_2HPO_4, pH 7.4) (Sigma-Aldrich, St. Louis, MO) to reach a final concentration of 3 mM phosphate. To determine whether CsA induced calcification, a CsA concentration of 1.0 µmol/L was maintained in the calcification medium. The MOVAS-1 cells were incubated for up to 14 days and the medium was changed every 2–3 days.

Cell viability estimation by the MTT assay

To investigate the effect of CsA on cell viability, the cells were pretreated with 1.0, 10, and 20 µmol/L CsA for 24–48 h. Cells were seeded in 96-well plates at 1×10^5 cells/well, and grown to confluence, at which point the medium was changed and fresh serum-free medium was used with 1.0 µmol/L CsA for 24–48 h. For the measurement of cell viability, the cells were incubated at 37°C for 4 h with 100 µl of 0.5 mg/mL 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), washed with phosphate-buffered saline, lysed with 100 µl dimethyl sulfoxide, and shaken for 15 min. Finally, the absorbance was measured at 570 nm using a microplate reader to determine the amount of MTT reduced to formazan (Molecular Devices, Sunnyvale, CA).

Analysis of ALP activity

To verify if CsA exacerbates VC under HG conditions, we measured the ALP activity of the treated cells. The cell layers were

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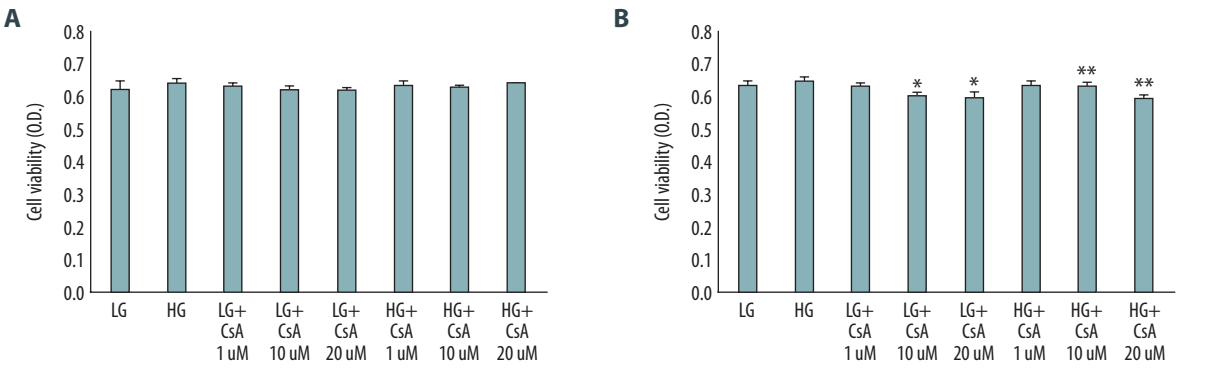

Figure 1. Toxic effects of CsA on MOVAS-1 cells. Cell viability measured by MTT assay revealed a decline in the dose of CsA of 10 μM and 20 uM at 48 h. The data was mean ±SD from 3 independent experiments. CsA – cyclosporin A; LG – low glucose; HG – high glucose. p<0.05, * *vs.* LG, ** *vs.* HG.

lysed with 0.9% NaCl and 0.2% Triton X-100, and centrifuged at 12 000×g for 20 min at 4°C. The supernatant was assayed for ALP activity. Enzyme activity was determined by measuring the cleavage of 10 mmol/L p-nitrophenylphosphate at 410 nm using a commercially available kit (Abcam, Cambridge, UK) according to the manufacturer's instruction.

Real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR)

The genes associated with the calcification markers (core binding factor-alpha 1, Cbfα1, bone morphology peptide, and BMP2) and inflammation marker (interleukin-6 [IL-6]) were examined by real-time RT-PCR. Total RNA was extracted using a commercially available kit (Promega, Madison, WI). cDNA was synthesized from 1 μg of the total RNA using a cDNA synthesis kit (GenDEPOT, Baker, TX). Gene expression was measured by real-time RT-PCR using the SYBR Green Master. The expression levels of all the genes were normalized to that of the housekeeping gene GAPDH. Oligonucleotide primers were designed using Primer 3 software and had the following sequences:
Cbfα1, sense 5' GCCTTCCAACTTGGTTGTGAG 3',
antisense 5' TCCAGGATTTATTGGTGGGAGG 3',
BMP2, sense 5' GCTTCCGTCCCTTTCATTTCT 3',
antisense 5' AGCCTCCATTTTTGGTAAGGTTT 3',
IL6, sense 5' CCTGAGACTCAAGCAGAAATGG 3',
antisense 5' AGAAGGAAGGTCGGCTTCAGT 3',
GAPDH, sense 5' AGGTCGGTGTGAACGGATTTG 3',
antisense 5' TGTAGACCATGTAGTTGAGGTCA 3'.

Analysis of intracellular calcium content

To demonstrate the role of CsA in the induction of calcification in the MOVAS-1 cells, intracellular calcium concentrations were measured. Cells were seeded in 12-well plates at 2×10^5 cells/well, grown to confluence, and switched to a calcification medium with CsA for 14 days. The concentration of intracellular calcium was determined using a calcium detection kit (BioVision, Milpitas, CA) according to the manufacturer's instruction. Each assay was performed in triplicate and the color intensity was measured using a microplate reader at 575 nm. The intracellular calcium contents were expressed relative to the total protein concentration in each sample.

Statistical analysis

We used non-parametric analysis, as most of the variables were not normally distributed even after logarithmic transformation. The Mann-Whitney U test was used to compare the differences between the 2 groups. Statistical significance was defined as a p value <0.05. All the statistical analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL). Data are expressed as mean ± standard deviation.

Results

Toxic effects of CsA on MOVAS-1 cells

When the MOVAS-1 cells were treated with CsA at low glucose (LG, 5.5 mM) or HG concentrations for 24 h, CsA did not influence cell survival. However, the survival rate of the cells was significantly decreased at 10 or 20 μmol/L CsA in both LG and HG concentrations, at 48 h (Figure 1). Based on these results, the concentration of 1 μmol/L CsA was chosen for further study.

Morphological changes in MOVAS-1 cell calcification by CsA

We performed this step to determine whether the calcification of MOVAS-1 cells could be induced. When the cells were cultured in a calcifying medium containing a sodium phosphate monobasic

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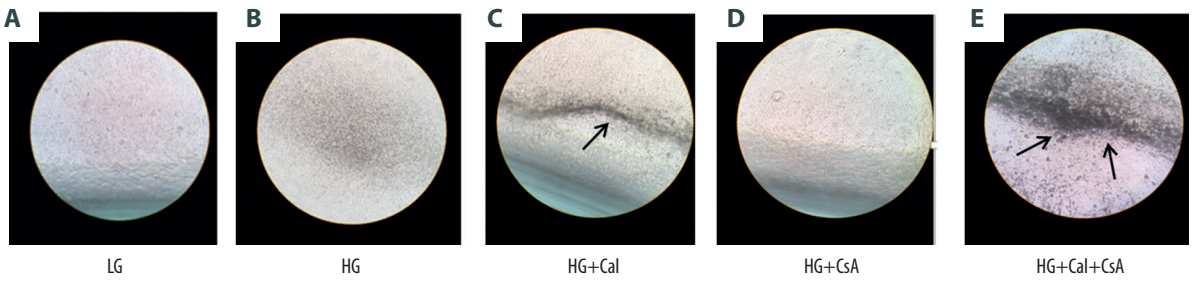

Figure 2. (**A–E**) Morphological changes in the calcification of MOVAS-1 cells due to CsA. Microscopic morphology showed calcification under HG conditions 14 days after exposure to a calcifying medium. CsA alone did not induce calcification under HG conditions, but clearly increased calcification under HG conditions with a calcifying medium. LG – low glucose; HG – high glucose; Cal – calcifying medium; CsA – cyclosporin A.

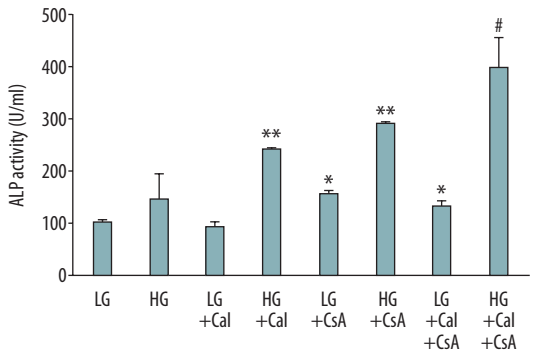

Figure 3. Changes in alkaline phosphatase (ALP) activity due to CsA. ALP activity was increased under HG conditions with CsA or a calcifying medium compared to HG conditions alone. CsA increased ALP activity under LG conditions with a calcifying medium, but markedly increased under HG conditions with a calcifying medium. The data are shown as mean ±SD. LG – low glucose; HG – high glucose; Cal – calcifying medium; CsA – cyclosporin A. $p<0.05$, * *vs.* LG, ** *vs.* HG, # *vs.* all others.

and sodium phosphate dibasic mixture, the calcification of the MOVAS-1 cells was confirmed under both LG and HG conditions at 14 days by microscopic examination (Figure 2). In the following experiments, we investigated whether CsA reduced or increased calcification during the induction period of the calcification. CsA of 1 µmol/L was introduced into a fresh calcifying medium containing MOVAS-1 cells every 2 to 3 days for 14 days. It was found that treatment with CsA alone did not induce calcification under LG or HG conditions. However, the microscopic morphology clearly revealed that CsA treatment, along with the presence of a calcifying medium, increased calcification under HG conditions.

Changes in alkaline phosphatase (ALP) activity due to CsA

Treatment with CsA significantly increased ALP activity in both the LG and HG groups. ALP activity was increased in the HG group, which was exposed to a calcifying medium. CsA further significantly increased ALP activity in the HG group with a calcifying medium (Figure 3). As a result, it was found that the exacerbation of VC caused by CsA was greater in the HG group than in the LG group. In addition, exposure to a calcifying medium or CsA alone significantly increased ALP activity in the HG group relative to the LG group.

Effects of CsA on calcification-related genes expression

There were no changes in the expression of Cbfα1 and BMP2 between the LG and HG groups. Treatment with CsA alone significantly increased the BMP2 level in both groups. Exposure to a calcifying medium alone increased the expression of Cbfα1 under LG conditions and that of Cbfα1 and BMP2 under HG conditions. In addition, CsA increased the expression in the HG group in the presence of a calcifying medium. Furthermore, this upregulation was more predominant in the HG group than the LG group, showing the effect of HG conditions on calcification-related gene changes.

The mRNA expression of IL-6 was higher under HG conditions than LG conditions. Treatment with CsA alone did not influence its expression, but its expression was upregulated under HG conditions in the presence of a calcifying medium (Figure 4).

Changes in the MOVAS-1 intracellular calcium concentrations due to CsA

The intracellular calcium concentrations were significantly increased in both the LG and HG groups that were exposed to a calcifying medium. CsA treatment did not change the calcium concentration in the absence of a calcifying medium. However, CsA significantly exacerbated intracellular calcium concentrations under HG conditions in the presence of a calcifying medium (Figure 5).

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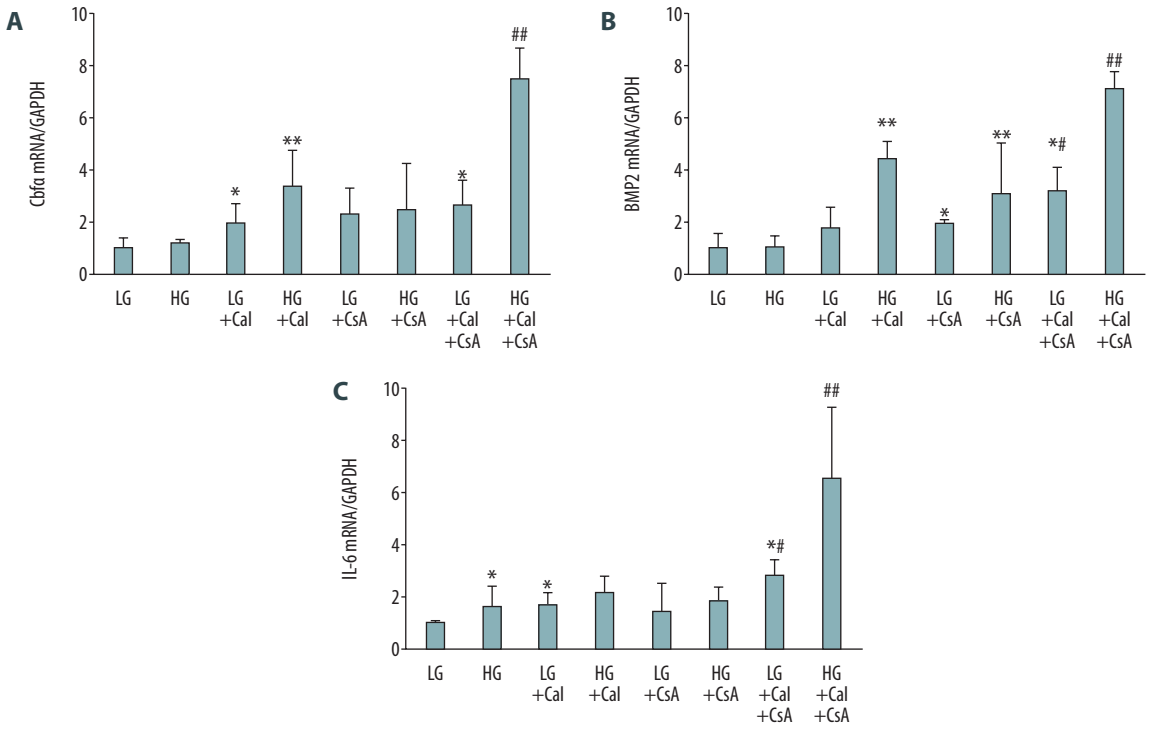

Figure 4. (**A–C**) Effects of CsA on calcification-related genes expression. The mRNA expressions of Cbfα1 and BMP2 showed similar patterns. CsA significantly increased their levels under HG conditions with a calcifying medium. CsA alone did not affect IL-6 under LG or HG conditions, but significantly increased the level under HG conditions with a calcifying medium. The data was mean ±SD. CsA – cyclosporin A; LG – low glucose; HG – high glucose. p<0.05 * *vs.* LG, **, *vs.* HG, # *vs.* LG+Cal and LG+CsA, ## *vs.* all other groups.

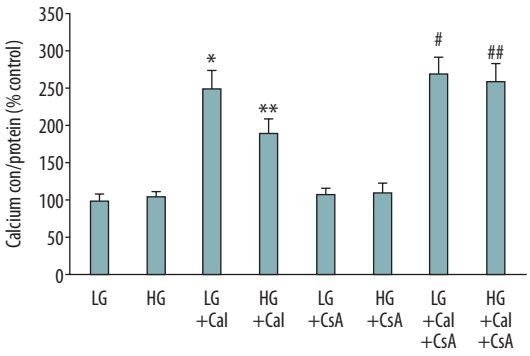

Figure 5. Changes in intracellular calcium concentrations due to CsA. Intracellular calcium concentrations were significantly increased in both the HG and LG groups exposed to a calcifying medium. CsA significantly increased intracellular calcium concentrations under HG and LG conditions with a calcifying medium. The data are shown as mean ±SD. LG – low glucose; HG – high glucose; Cal – calcifying medium; CsA – cyclosporin A. p<0.05, * *vs.* LG, ** *vs.* HG, # *vs.* LG+CsA, ## *vs.* HG+Cal and HG+CsA.

Discussion

The findings of the present study demonstrate that CsA induces calcification in the diabetic milieu. Treatment with CsA increased the expression of calcification markers (Cbfα1 and BMP2) and the inflammatory marker (IL-6). In addition to gene expression, increases in the intracellular calcium concentrations and ALP activity were also observed after CsA treatment. Treatment with CsA causes an increase in intracellular calcium concentrations and ALP activity, which further aggravate VC under HG conditions. These calcification changes were confirmed by microscopic examination, and the results suggest that CsA evokes a vascular pathology when used in diabetic transplant patients.

The effect of CsA on medial VC is not clear. Calcineurin inhibitors induced endothelial dysfunction, hyperlipidemia, and diabetes in transplant patients, resulting in an increased risk of cardiovascular diseases [22,23]. High trough levels of calcineurin inhibitors were associated with the calcification model in kidney transplant patients [24]. However, there are other contradictory results. In an animal study, CsA was found to reduce the Cbfα1 expression and ALP activity [25]. CsA treatment

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prevented the calcification of homograft valves by inhibiting immune response in the early stages after transplantation [26]. Although the VC of VSMCs is important, as one of the mechanisms behind cardiovascular diseases, little is known of the effect of CsA on VC under HG conditions. In our study, treatment with CsA augmented intracellular calcium concentrations and ALP activity under HG conditions in the presence of a calcifying medium. These changes were also consistent with the molecular changes in the calcification-related genes and inflammatory gene. Interestingly, treatment with CsA alone did not induce morphological calcification. However, it induced the ALP activity and mRNA expression of BMP2, leading to molecular changes before morphological changes in calcification.

VC was stabilized in patients with good allograft function after kidney transplantation [27]. The stabilization could be due to the restoration of the dysregulation of vitamin D, phosphorus, and calcium metabolism. However, several contradictory reports point to progressive calcification after transplantation. VC was found to progress with time after transplantation [28,29]. Mazzaferro et al. [30] compared the 2-year coronary artery calcification changes between transplant and dialysis patients. In 12.2% of the transplant patients, VC worsened, although the prevalence was lower than the 56.6% observed in dialysis patients. Slow progression was observed in 8.3% of the patients who did not present with calcification at the time of the transplantation. These findings show that transplantation lowered, but did not halt, the progression of VC. Marechal et al reported the rate of VC for 4 years in kidney transplant patients: coronary artery calcification progressed at 11% per year and aortic calcification at 4% per year [20]. Taking into consideration the fact that VC progresses even after a kidney transplant and aortic endothelium-dependent relaxation response due to immunosuppressants [31], the vascular changes in transplant patients need to be closely monitored.

Arterial stiffness is related to aging, blood pressure, and inflammation. Pulse wave velocity, which is the most widely used measure for assessing arterial stiffness, is predictive of cardiovascular events and survival in transplantation patients as well as in the general population [32,33]. The PWV was higher in patients with CsA than those with tacrolimus, and was attenuated after conversion from CsA to mTOR inhibitor [34,35]. Arterial stiffness measured by PWV is reported to be correlated to aortic calcification [36]. These findings suggest that CsA is an important factor in VC in transplant patients.

Considering that diabetes is the most common cause of metabolic derangement due to uremic conditions, and drives the need for dialysis, VC could be more problematic in diabetic transplant patients. In most diabetic patients with hyperglycemia, the blood vessels undergo calcification; therefore, these patients need to be cautious in the use of drugs. Although many studies have focussed on the adverse effects of post-transplant drugs, to the best of our knowledge, the present study is the first to report that cyclosporine aggravates VC in the diabetic milieu. Our study shows that CsA, one of the most commonly used immunosuppressants, should be used with caution for the treatment of patients with hyperglycemia.

Currently, kidney transplantation is the best method to correct uremic complications. However, most patients who undergo transplantation have to take immunosuppressants for the rest of their lives. Corticosteroid, one of the main immunosuppressants, aggravates hyperglycemia in transplant patients with diabetes, and induces new onset of diabetes after transplantation. Corticosteroid induces insulin resistance as well as hyperglycemia [37]. Insulin resistance is also a well-known risk factor for VC [38,39]. Therefore, it would be possible that aggravated hyperglycemia and insulin resistance aggravates further VC in transplant patients with CsA.

There are some limitations in this study. First, the results of intracellular calcium concentrations are not consistent with other results. It is not clear why intracellular calcium concentration under HG was not different from that of LG only. Considering other findings, such as microscopic morphology, ALP activity, and the mRNA expressions of calcification-related genes, the effect of CsA on calcification would be clear under HG condition. Second, this was an *in vitro* study; therefore, systemic immune modulating effect of CsA could not be elucidated. Further studies including animals or humans are needed to clarify this difference. Third, it is not clear whether the effect of calcification is due to CsA itself or calcineurin inhibitor. Although CsA was reported to be more associated with calcification than is tacrolimus, the calcification effect of tacrolimus needs to be clarified [40].

Conclusions

Treatment with CsA induced the calcification of VSMCs under HG conditions in the presence of a calcifying medium, which are common conditions in post-transplant diabetic patients. Considering the effects of CsA on VC, the adverse effects of the drug must be verified in diabetic transplant patients in future studies.

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