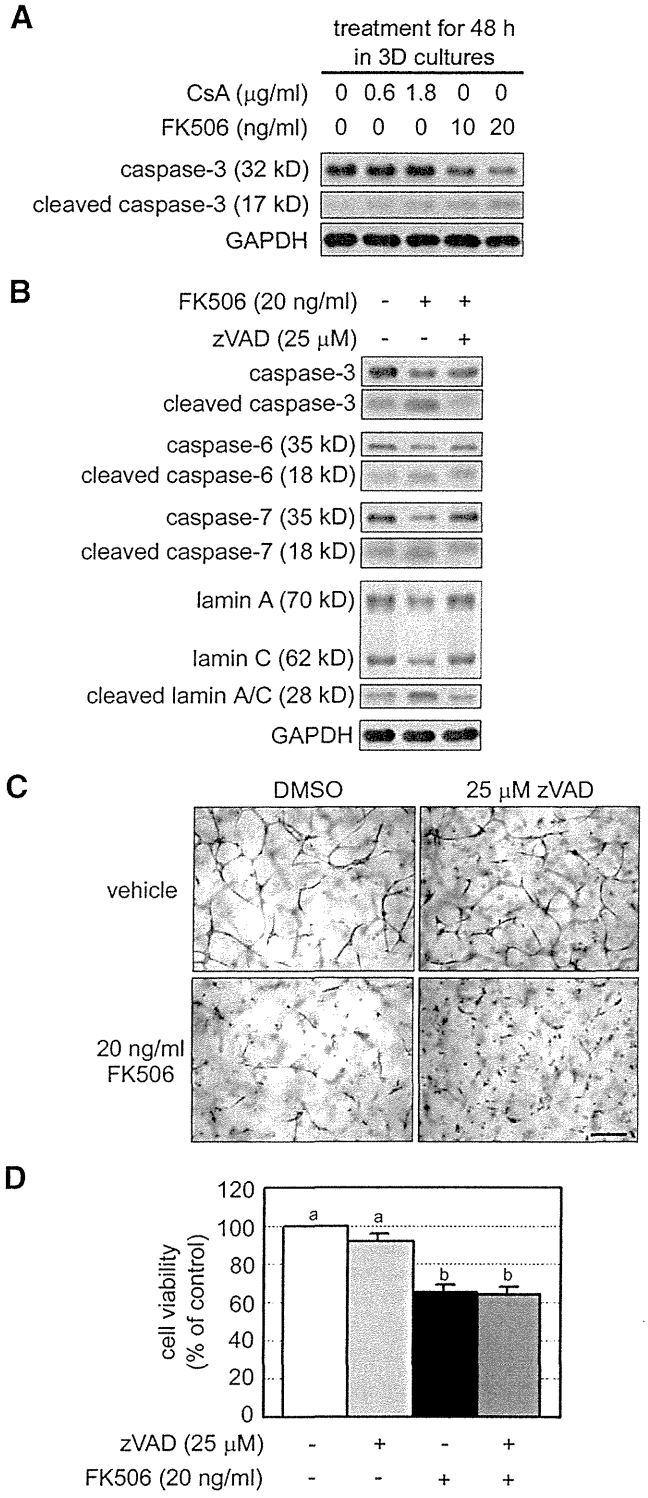


Fig. 4. FK506-induced endothelial dysfunction is not regulated by the caspase pathway in 3D cultures. (A) CsA and FK506 slightly induce caspase activation. Cell extracts were prepared from tube-forming HUVEC treated with CsA or FK506 at the indicated concentrations for 48 h. (B) zVAD inhibits FK506-induced caspase activation and its activity. Cell extracts were prepared from tube-forming HUVEC treated with 20 ng/ml FK506 together with or without 25 μM zVAD for 48 h. (C) FK506-induced tube breakdown is not suppressed by zVAD. Tube-forming HUVEC were treated with 20 ng/ml FK506 with or without 25 μM zVAD for 48 h. The bar indicates 100 μm. (D) zVAD fails to suppress FK506-induced cell death. Tube-forming HUVEC were treated with 20 ng/ml FK506 with or without 25 μM zVAD for 48 h. Each bar represents means ± SE of four independent experiments. One-way factorial ANOVA for multiple comparisons was performed for statistical differences. Values with different letters are significantly different at $P < 0.05$ (Bonferroni test). The experiments in A, B and C were performed four times and the representative data are shown.

2.11. Quantitative RT-PCR analysis

Isolation of total RNA, synthesis of first-strand complementary DNA and PCR were performed as described previously [21]. Briefly, total RNA from HUVEC in gelatin-coated dishes was isolated using TRIzol reagent (Enzo Life Sciences Inc.). First-strand cDNA was synthesized from the total RNA (1.25 μg) using the PrimeScript RT reagent kit (TAKARA Bio, Ohtsu, Japan). PCR was performed on the synthesized cDNA product using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). All reactions were carried out in triplicate. The sequences of the PCR primer pairs and fluorogenic probes used for calcineurin Aβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are available on the Applied Biosystems website (www.appliedbiosystems.com <http://www.appliedbiosystems.com>, calcineurin Aβ assay ID: Hs00917458_m1; GAPDH assay ID: Hs99999905_m1). The PCR products were analyzed using the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). Each mRNA level was normalized to the corresponding GAPDH mRNA level as an internal control.

2.12. Statistical analyses

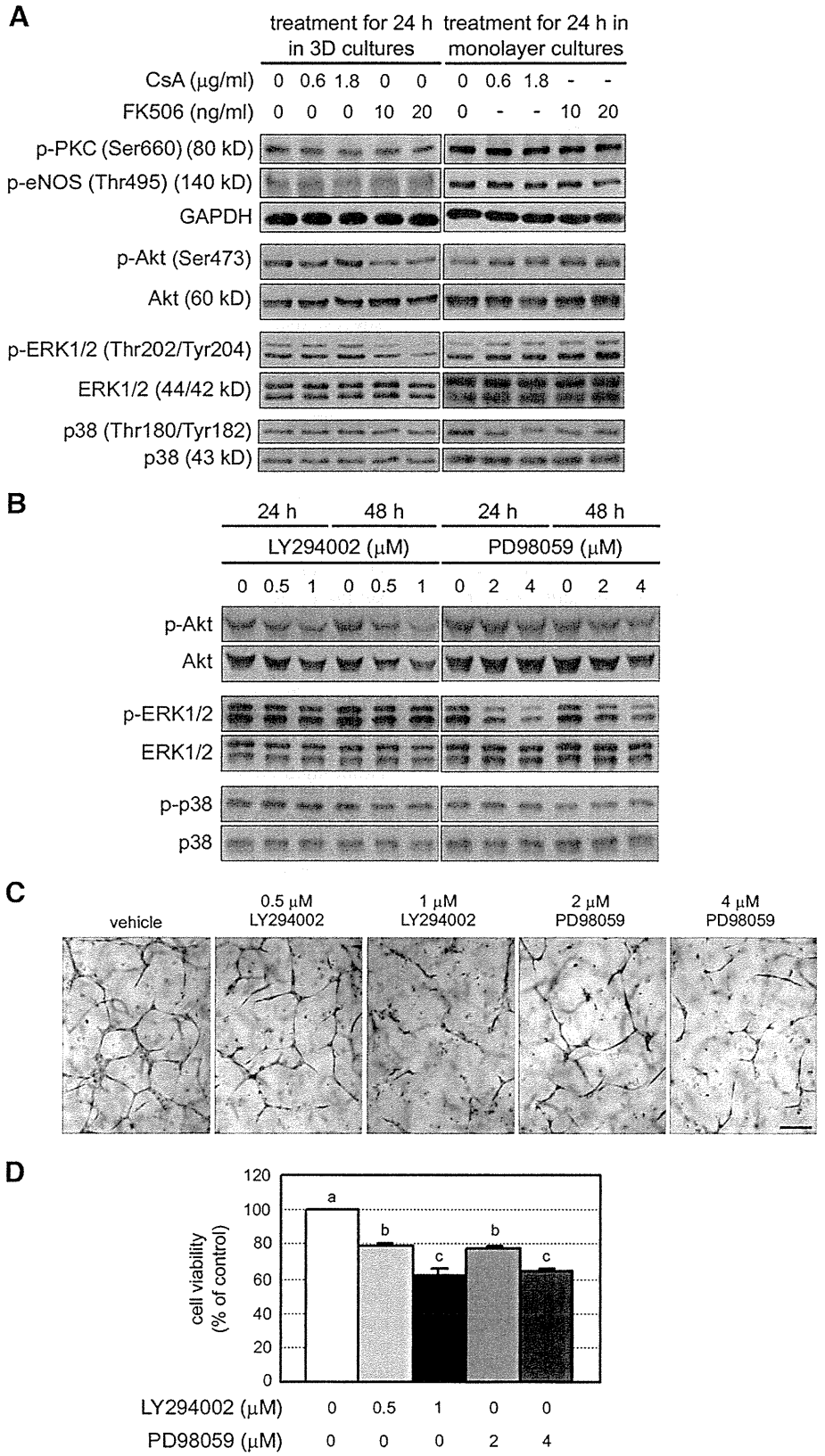
All data are presented as the mean ± standard error (SE) of four independent experiments. Differences in mean values among groups were subjected to one-way factorial analysis of variance (ANOVA) with Bonferroni test for multiple comparisons, and were considered significantly different at $P < 0.05$. Comparisons between two groups were performed using Student's unpaired *t*-test.

3. Results and discussion

3.1. FK506, but not CsA, directly induces endothelial dysfunction in 3D cultures

We have developed an in vitro 3D culture blood vessel model, in which human umbilical vein endothelial cells (HUVEC) at 2.86×10^6 cells/ml form and maintain capillary-like tube and lumen structures in the presence of bFGF and VEGF [8]. In this study, we made an improvement of the 3D model to mimic therapeutic conditions testing the effect of immunosuppressants. HUVEC at 1.71×10^6 cells/ml were cultured for tube formation for 48 h, replacing fresh tube-induction medium once at 24 h, that resulted in an improvement of tube formation (Fig. 1A). An electron microscope confirmed cell-to-cell adhesion and the formation of lumen structures composed of multiple HUVEC (Fig. 1B). After tube formation completed, tube maintenance followed, lasting for 24–48 h, in which effects of immunosuppressants were examined according to the schedule (Fig. 1C).

Immunosuppressants such as CsA and FK506 are known to be involved in endothelial dysfunction inducing TMA [22]. However, whether CsA and FK506 directly induce endothelial dysfunction in vitro has not been clear. We treated HUVEC in 3D cultures with CsA and FK506. The therapeutic window of CsA and FK506 is known to be 0.2–0.8 μg/ml and 10–20 ng/ml, respectively [2,23]. CsA did not induce tube breakdown at 0.6 μg/ml or even at the higher concentration of 1.8 μg/ml (Fig. 2A). In contrast, FK506 induced breakdown of the tube structures at the therapeutic window (10–20 ng/ml) in a time-dependent manner (Fig. 2A). Cell viability was also reduced by FK506 in a time- and concentration-dependent manner, but not CsA (Fig. 2B). Intriguingly, CsA and FK506 had little effect on cell proliferation in monolayer cultures (Fig. 2C). We have reported that there are significant differences in signaling pathways of cell death between 3D cultures and monolayer cultures [8]. In such 3D cultures, endothelial cells express genes/proteins in a manner observed in vivo [24,25]. FK506 has been reported to induce intestinal TMA in rats [26]. Similarly, a recent study has shown that FK506 inhibits VEGF-induced



PD98059 (μM) 0 0 0 2 4

Fig. 5. FK506-induced endothelial dysfunction is caused by attenuation of Akt and ERK1/2 without p38 activation. (A) FK506 attenuates activation of Akt and ERK1/2 in 3D cultures, but not monolayer cultures. Cell extracts were prepared from HUVEC treated with CsA or FK506 at the indicated concentrations for 24–48 h in 3D and monolayer cultures. (B) LY294002 and PD98059 at low concentrations have little effect on p38 in 3D cultures. Cell extracts were prepared from tube-forming HUVEC treated with LY294002 or PD98059 at the indicated concentrations for 24–48 h. (C) LY294002 and PD98059 induce tube breakdown in 3D cultures. Tube-forming HUVEC were treated with LY294002 or PD98059 at the indicated concentrations for 48 h. The bar indicates 100 μm. (D) LY294002 and PD98059 induce cell death in 3D cultures. Tube-forming HUVEC were treated with LY294002 or PD98059 at the indicated concentrations for 48 h. Each bar represents means ± SE of four independent experiments. One-way factorial ANOVA for multiple comparisons was performed for statistical differences. Values with different letters are significantly different at $P < 0.05$ (Bonferroni test). The experiments in A, B and C were performed four times and the representative data are shown.

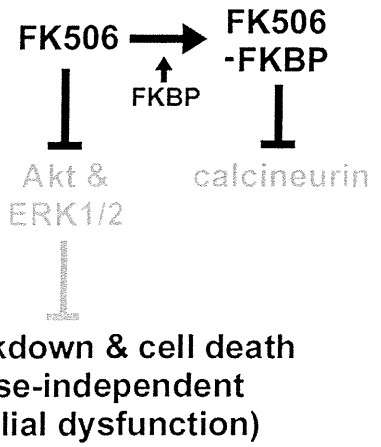


Fig. 6. FK506 induces endothelial dysfunction through attenuation of Akt and ERK1/2 independently of calcineurin inhibition and the caspase pathway. bFGF and VEGF induce activation of Akt and ERK1/2 in tube-forming HUVEC. However, FK506 induces attenuation of Akt and ERK1/2 independently of calcineurin inhibition, leading to caspase-independent endothelial dysfunction (tube breakdown and cell death).

tube formation in vitro and in vivo [27]. However, there has been an opposite result that CsA, not FK506, inhibits tube formation and induces endothelial dysfunction in vitro in the presence of bovine brain extract and EGF [28]. Our present result also provides the evidence that FK506, but not CsA, directly induces tube breakdown and cell death. Precise reasons for these conflicting results are unclear, but may depend on the experimental culture condition adopted, and are partially explained by the notion that FK506 may inhibit tube formation and maintenance induced by VEGF, but not EGF.

3.2. Calcineurin inhibition and the caspase pathway are not involved in FK506-induced endothelial dysfunction

FK506-FKBP12/12.6 complexes bind to calcineurin, inhibiting phosphatase activity of calcineurin, which in turn suppresses NFAT dephosphorylation and T cell activation [5,6]. We examined whether FK506-induced endothelial dysfunction is caused by inhibition of calcineurin activity. Western blot analysis showed that CsA and FK506 suppressed dephosphorylation of NFATc2, a member of the NFAT family, in 3D and monolayer cultures (Fig. 3A), indicating that CsA and FK506 did inhibit phosphatase activity of calcineurin. We then performed RNA interference experiments using lentivirus carrying short hairpin RNA (shRNA) targeting calcineurin Aα that regulates phosphatase activity. As shown in Fig. 3B, calcineurin Aα shRNA completely inhibited the expression of calcineurin Aα protein during tube formation. Enzyme activity of calcineurin is controlled by catalytic A subunit isoform α (Aα) and β (Aβ) [4]. In this experiment, the calcineurin A antibody we used recognized a human glutathione-S-transfecase-tagged recombinant calcineurin Aβ (arrowhead; Fig. 3B), but calcineurin Aβ protein in calcineurin Aα-abrogated HUVEC was not detected (middle lane; Fig. 3B). On the other hand, calcineurin Aα shRNA had little effect on expression of calcineurin Aβ mRNA (Fig. 3C). These findings suggest that calcineurin Aβ protein were hardly expressed in HUVEC, and that calcineurin Aα, but not calcineurin Aβ, mainly controls calcineurin activity in HUVEC. In addition, calcineurin Aα knockdown had little effect on tube formation (Fig. 3D), cell viability (Fig. 3E) and tube maintenance (Fig. 3F). However, calcineurin Aα knockdown failed to suppress FK506-induced tube breakdown (Fig. 3F) and cell death (Fig. 3G), which is consistent with the results in Fig. 2 that CsA did not induce tube breakdown and cell death. Furthermore, calcineurin Aα knockdown as well as FK506 treatment inhibited NFATc2 dephosphorylation (Fig. 3H). These results suggest that calcineurin inhibition is not involved in FK506-induced endothelial dysfunction.

We investigated whether the caspase pathway is involved in FK506-induced endothelial dysfunction. CsA and FK506 slightly induced caspase-3 cleavage in 3D cultures (Fig. 4A), while CsA did not induce endothelial dysfunction in 3D cultures (Fig. 2). To confirm whether FK506-induced endothelial dysfunction is regulated by the caspase pathway, we treated tube-forming HUVEC with FK506 together with zVAD, a broad-spectrum caspase inhibitor. FK506-induced cleavage of caspase-3, -6 and -7 and lamin A/C was inhibited by zVAD (Fig. 4B). However, zVAD failed to suppress FK506-induced tube breakdown (Fig. 4C) and cell death (Fig. 4D). These results suggest that FK506-induced endothelial dysfunction is not mainly regulated by the caspase pathway.

3.3. FK506 induces endothelial dysfunction through attenuation of Akt and ERK1/2

FK506 bind to FKBP12/12.6, leading to detachment of FKBP12/12.6 from ryanodine receptors on the endoplasmic reticulum, which resulted in Ca^{2+} leaks from the endoplasmic reticulum [29,30]. FK506-induced Ca^{2+} release is known to activate protein kinase C (PKC), which phosphorylates Thr-495 in endothelial nitric oxide synthase (eNOS) to down-regulate NO production, leading to FK506-induced hypertension and endothelial dysfunction in mice [31]. However, we found that CsA and FK506 did not increase phosphorylation of PKC and Thr-495 in eNOS in 3D and monolayer cultures (Fig. 5A). Akt has been reported as another regulator of eNOS activity [32]. We found that FK506, but not CsA, attenuated Akt phosphorylation in 3D cultures (Fig. 5A). It is possible that FK506-induced hypertension is mediated by the Akt/eNOS pathway, not by the PKC/eNOS pathway.

Using our previous 3D culture blood vessel model, we have shown that potent inhibition of either Akt or ERK1/2 induces apoptosis accompanied by p38 activation in tube-forming HUVEC [33]. FK506, but not CsA, attenuated ERK1/2 phosphorylation in 3D cultures (Fig. 5A). However, CsA and FK506 did not attenuate phosphorylation of Akt and ERK1/2 in monolayer cultures (Fig. 5A), which is compatible with the Fig. 1C result that CsA and FK506 allowed cell proliferation in monolayer cultures. These findings are in line with the notion that endothelial cells in monolayer cultures may be in predominant proliferating status, and may not reflect physiological condition in vivo. Unexpectedly, FK506 as well as CsA did not activate p38 in 3D and monolayer cultures (Fig. 5A). To investigate whether FK506-induced mild suppression of Akt and ERK1/2 induces endothelial dysfunction without p38 activation, we treated tube-forming HUVEC with LY294002, an inhibitor of phosphatidylinositol 3-kinase/Akt signaling, or PD98059, a selective MAPK/ERK kinase inhibitor, at low concentrations in long-term. We found that LY294002 at 0.5–1 μM attenuated Akt phosphorylation and had little effect on ERK1/2 for 24–48 h (Fig. 5B). Similarly, PD98059 at 2–4 μM attenuated ERK1/2 phosphorylation and had little effect on Akt for 24–48 h (Fig. 5B). Intriguingly, neither inhibitor at the low concentrations increased p38 phosphorylation (Fig. 5B). Under these conditions, we observed that LY294002 and PD98059 induced tube breakdown (Fig. 5C) and cell death (Fig. 5D) in a concentration-dependent manner. These results showed that mild suppression of either Akt or ERK1/2 induces tube breakdown and cell death without p38 activation. Taken together, these results suggest that FK506 induced tube breakdown and endothelial cell death through attenuation of Akt and ERK1/2.

4. Conclusions

Our 3D culture blood vessel model demonstrated that FK506, but not CsA, induces tube breakdown and endothelial cell death (Figs. 1, 2). Calcineurin inhibition and the caspase pathway were not involved in

the FK506-induced endothelial dysfunction (Figs. 3, 4). On the other hand, FK506 attenuated activation of Akt and ERK1/2, and attenuation of Akt or ERK1/2 by each inhibitor led to tube breakdown and cell death (Fig. 5). These results suggest that FK506 induces endothelial dysfunction through attenuation of Akt and ERK1/2 independently of calcineurin inhibition and the caspase pathway (Fig. 6).

We need to further investigate the precise mechanism that FK506-induced attenuation of Akt and ERK1/2 induce tube breakdown and endothelial cell death, and the effective strategy to block the mechanism for prevention of FK506-induced endothelial dysfunction.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

We are grateful to Ms. Ritsuko Fujimoto and Ms. Hiromi Takeda for their excellent technical assistance. This work was supported by JSPS KAKENHI Grant Number 24790985 to R. Eguchi.

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