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FK506 can activate transforming growth factor-β signalling in vascular smooth muscle cells and promote proliferation

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Aims FK506-binding protein (FKBP) 12 is an inhibitor of transforming growth factor (TGF)-β type I receptors. Several lines of evidence support the view that TGF-β stimulates vascular smooth muscle cell (VSMC) proliferation and matrix accumulation. We investigated the effect of FK506, also known as tacrolimus, on cellular proliferation and on matrix protein production in human VSMCs.
Methods and results We measured cell proliferation with flow cytometry using BrdU incorporation and fluorimetrically by measuring DNA concentration with Hoechst 33258. Western blot assay of whole-cell lysates was used to measure the levels of signalling proteins involved in proliferative pathways, in particular β-catenin, pErk, pAkt, pmTOR, and cyclin D1. Collagen synthesis was also investigated by Western blotting. The TGF-β signal was studied by both Western blotting and confocal microscopy. We used the SiRNA technique for FKBP12 gene silencing. Our results show that FK506 stimulates VSMC proliferation and collagen type I production. FK506 enhanced β-catenin levels and activated the extracellular signal-regulated kinase, Akt, and mammalian target of rapamycin kinase, which are important effectors of proliferation. Accordingly, cyclin D1 expression was increased. We also demonstrate that FK506 activates the TGF-β signal in VSMCs and that, through this mechanism, it stimulates cell proliferation.
Conclusion FK506 can act as a growth factor for VSMCs.

1. Introduction

A large body of evidence suggests that transforming growth factor (TGF)-β simulates intimal growth in vascular smooth muscle cells (VSMCs) by inducing cellular proliferation and matrix accumulation.[1–6] Direct transfer of the TGF-β gene into arteries stimulates fibrocellular hyperplasia.[3] Systemic infusion of TGF-β protein in animal models of arterial injury[1–4] promotes intimal growth. Moreover, TGF-β antagonists decrease intimal growth in animal models of arterial injury.[5,6]

TGF-β is a pleiotropic cytokine important in the control of cell proliferation and differentiation, embryonic development, angiogenesis, and wound healing.[4,7,8] It can trigger a variety of biological responses by activating Smad transcription factor, depending on the cellular context and nuclear components that recruit Smad to specific target genes.[9] TGF-β signals to the nucleus by binding to a specific pair of membrane receptors, type I (TβR-I) and type II (TβR-II), that contain a cytoplasmic serine–threonine kinase domain.[10] Binding of the ligand to TβR-II results in the formation of a TβR-I/TβR-II heteromeric complex and activation of TβR-II kinase.[10] Activation of TβR-I requires phosphorylation of the glycine–serine (GS) region by TβR-II. Activated TβR-I specifically recognizes and phosphorylates Smad 2 and 3 or R-Smads.[10] This process results in the release of Smad 2 and unmasking of its nuclear import function, thereby leading to rapid accumulation of the activated Smad complex in the nucleus.[10–12] Smad 4 functions as a shared partner or Co-Smad and is required for transcriptional complexes to assemble. Once in the nucleus, activated Smad associates with partner DNA-binding co-factors that contact Smad and a specific DNA sequence, thereby resulting in transcription activation or repression.[9,11]

TGF-β receptor signalling is negatively regulated by FK506-binding protein (FKBP) 12, an abundant and highly conserved 12 kDa cytosolic protein that exerts peptidyl–prolyl isomerase activity.[13] FKBP12 regulates fundamental aspects

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of cell biology, due to specific protein–protein interactions and modulation of partner conformation and activity.[14] Among its multiple biological functions, FKBP12 is a common inhibitor of TGF-β type I receptors.[13,15] Indeed, it binds to the GS region, thereby blocking access to activators. In line with this concept, the TGF-β pathway is overactive in fibroblasts from FKBP12-knock out mice.[16] FK506, the canonical ligand of FKBP12, promoted TGF-β receptor transphosphorylation in a mink epithelial cell line.[15] FK506 is a macrolide compound isolated from *Streptomyces tsukubaensis*[17] and has potent immunosuppressive properties.[18,19] Structurally, FK506 has two domains, a domain bound by FKBP12 and an effector domain that, together with FKBP12, forms a composite surface that interacts with calcineurin, thereby inhibiting its phosphatase activity.[20] Calcineurin is activated by Ca^{2+}-dependent signal transduction events, such as T-lymphocyte activation, and in turn dephosphorylates the cytoplasmic subunit of the nuclear factor of activated T (NFAT) cells, thereby allowing its translocation to the nucleus where it associates with a nuclear subunit to form the fully active NFAT complex.[21] NFAT is an essential component of the transcriptional apparatus required for the expression of IL-2 and other cytokine genes, including interleukin-3, interferon γ, and tumour necrosis factor α.[22] As an immunosuppressive agent, FK506 is approximately 100 times more potent than cyclosporine.[23] Cytokines released in the site of arterial injury, as occurs, for example, after balloon angioplasty, play an important role in inflammation and restenosis.[24] Because some anti-restenosis devices used in interventional cardiology release FK506,[25] the aim of our study was to investigate whether such compound can activate the TGF-β signal in VSMCs and affect cell proliferation. This *in vitro* study can provide possible mechanisms governing vascular remodelling, after FK506-eluting stent implantation.

2. Methods

2.1 Cell culture and reagents

VSMCs were human aortic smooth muscle cells purchased from Cambrex Bio Science (Cambrex Profarmaco, Milan, Italy). The cells were cultured in Clonetics SmGM-3 BulletKit medium, according to the manufacturer's instructions. They were starved of supplement growth factors for 48 h, after which FK506 or TGF-β was added to the medium. The experiments were performed when cells were at the 6–10 passage. FK506, TGF-β, and cyclosporin (Sigma Aldrich, St Louis, MO, USA) were used at the doses indicated under the Results section. SB 431542,[8] which inhibits receptors of the TGF-β superfamily type I activin receptor-like kinase (ALK), was purchased from Sigma Aldrich and used at the concentration of 5 μM.

2.2 Cell lysates and Western blot assay

Whole-cell lysates were prepared by homogenization in the modified RIPA buffer. The lysate was cleared by centrifugation at 14 000 rpm for 20 min. The lysate was run on SDS–polyacrylamide gel electrophoresis, transferred onto a membrane filter (Cellulose-nitrate, Schleider and Schuell, Keene, NH, USA), and incubated with the primary antibody.

The antibody against phospho (p) Smad2 (Ser 465/467) (rabbit polyclonal; Chemicon, Temecula, CA, USA), Smad4, and pErk (mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 and Smad 2/3 (rabbit polyclonal; Santa Cruz Biotechnology), pAkt, pmTOR, and extracellular signal-regulated kinase (Erk) 1/2 (rabbit polyclonal, Cell Signaling Technology, Danvers, MA, USA), FKBP12 (rabbit polycolonal; Santa Cruz Biotechnology) collagen type I (mouse monoclonal, clone SP1D8, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) were all used diluted 1:200. After a second incubation with peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology), the blots were developed with the ECL system (Supersignal West Pico, Celbio, Pierce, Rockford, IL, USA).

2.3 Immunofluorescence and microscopy

To investigate VSMC growth on bare stents, cells were plated onto 35 mm plastic dishes together with a stent in the absence or presence of 100 ng/mL FK506. After 9 days of culture, the stents were removed from the dishes, fixed in 4% paraformaldehyde in PBS for 20 min and cells were permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were then washed twice in PBS and nuclei were stained with 2′-(4-hydroxyphenyl)5-(4-methyl-1-piperazynyl)–2,5′-bi(1H-benzimidazoil)-6-(1-methyl-4piperazyl) benzimidazole trihydrochloride (Hoechst) 33258 fluorescent reagent (0.5 μg/mL in PBS) (Sigma Aldrich) for 10 min. Stents were mounted on glass slides with PBS containing 50% glycerol. Stained fluorescent nuclei were visualized on a Zeiss Axioscop 2 microscope (Zeiss, Gottingen, Germany) and images were taken with a Zeiss Axiocam.

To investigate Smad nuclear translocation, VSMCs were plated onto 12 mm glass coverslips. Cells were treated with 10 ng/mL TGF-β or with 100 ng/mL FK506 for 60 min. Cells used as control were treated with the medium alone. Cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed twice for 5 min each time, with 50 mM NH_4Cl in PBS permeabilized for 5 min in 0.1% Triton X-100 in PBS. Cells were then blocked in 1% BSA in PBS for 1 h and washed twice in PBS. A mouse monoclonal anti-Smad4 diluted 1:100 in PBS 0.5% BSA for 1 h in a humidified atmosphere served as primary antibody. Cells were then extensively washed in PBS before staining with secondary goat anti-mouse Alexa Fluor 488 conjugated (Molecular Probe, Invitrogen Corporation, Carlsbad, CA, USA). Nuclei were counterstained with Hoechst 33258 (0.5 μg/mL in PBS) (Sigma Aldrich) for 10 min. Finally, cells were washed in PBS and mounted on glass slides with PBS containing 50% glycerol.

Immunofluorescence analysis was performed using a confocal laser scanner microscope (LSM 510 Meta; Zeiss). The lambda of the argon ion laser was set at 488 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488. Nuclear Hoechst 33258-stained cells were excited with mercury lamps and images acquired in a single channel using a 460–489 band pass filter. Double-staining immunofluorescence images were acquired in the green and blue channels at a resolution of 1024 × 1024 pixels.

2.4 Proliferative assays

To measure the concentration of DNA in VSMCs that were grown on stents, cells were seeded onto six-well plates with a stent in each well, in the absence or presence of 100 ng/mL FK506. The stents were removed from wells after 9 days of culture and introduced in a tube with 1 mL of a permeabilizing solution containing Hoechst 33258 (0.5 μg/mL in 0.1% sodium citrate, w/v; 0.1% Triton X-100, v/v). After 30 min incubation at room temperature, tubes were vortexed and centrifuged at 400*g* for 10 min to collect cells. The supernatant was discarded, stents were examined using a microscope to verify the absence of cells, and the pellets were resuspended in 500 μL of RIPA buffer. The DNA in lysates was read with a fluorimeter at an excitation wavelength of 354 nm and at an emission of 461 nm.

Cell proliferation was measured with 5-bromo-2′-deoxy-uridine labelling and with a detection kit (Detection Kit II, Roche Diagnostics Corporation, Indianapolis, IN, USA) following the manufacturer's instructions. Briefly, cells were plated onto 24-well plates in the

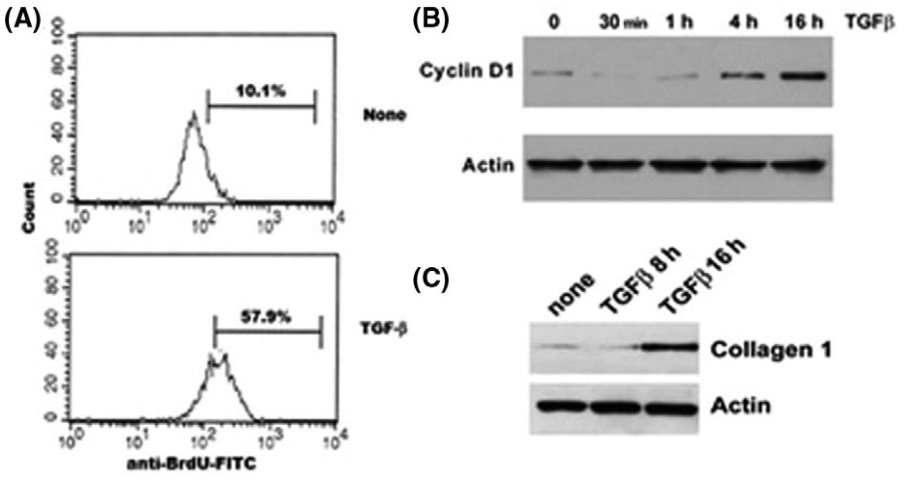

Figure 1 Transforming growth factor-β stimulates vascular smooth muscle cell proliferation and collagen production. (*A*) Flow cytometric histograms of BrdU incorporation in vascular smooth muscle cells. Cells were cultured with and without 10 ng/mL transforming growth factor-β. After incubation for 3 days, 10 μM BrdU was added to the cultures and 4 h later cells were collected, fixed with ethanol, and incubated with anti-BrdU-FITC monoclonal antibody. The bar indicates the per cent of cells incorporating BrdU in their DNA. The data presented are representative of five different experiments, each performed in triplicate. (*B*) Western blot assay of cyclin D1 levels in whole-cell lysates (30 μg) of vascular smooth muscle cells incubated with 10 ng/mL transforming growth factor-β for 0, 30 min, 3, 4, and 16 h. Similar results were obtained in other two different experiments. (*C*) Western blot assay of collagen type I levels in whole-cell lysates (30 μg) of vascular smooth muscle cells treated with 10 ng/mL transforming growth factor-β for 0, 8, and 16 h. The data of collagen upregulation was confirmed in another two independent experiments.

absence or presence of different doses of FK506 or 10 ng/mL TGF-β. After incubation for 3 days, 10 μM BrdU was added to the cultures, and, after a further 4 h, cells were collected, fixed with ethanol, and incubated with anti-BrdU monoclonal antibody. The per cent of BrdU incorporation was measured in flow cytometry (FACScan Becton Dickinson, San Diego, CA, USA).

2.5 Immunoprecipitation of membranes

Cells harvested after a 2 h incubation with FK506 were osmotically lysed in distilled water and subjected to three cycles of rapid freezing and thawing, after addition of protease inhibitors and phosphatase inhibitors. During thawing, the extract was sonicated for 10 min. After obtaining a homogeneous suspension, protein concentration was determined with the Bradford method and 500 μg of protein extract was pre-cleared for 1 h. For immunoprecipitation, 15 μg anti-TβR-I (rabbit polyclonal H-100) or anti-FKBP12 (goat polyclonal N-19; Santa Cruz Biotechnology) was added together with 25 μL protein A-Sepharose (Santa Cruz Biotechnology), and precipitation took place overnight with rotation at 4°C. Samples were separated by 14% SDS–PAGE together with a molecular weight marker and transferred onto membrane filter.

2.6 Cell transfection and short interfering RNA

Twenty-four hours before transfection of two different short interfering (si)RNAs corresponding to the target sequences GCTTGAAGATGGAAAGAAA and GAAACAAGCCCTTTAAGTT of the FKBP12 gene (Qiagen, Valencia, CA, USA) or of a scrambled duplex as control, VSMCs were incubated in medium without antibiotics at the concentration of 2.5×10^5/mL to obtain 30–50% confluence at the time of transfection. The siRNA or the scrambled oligo was transfected at the final concentration of 50 nM using Metafectene (Biontex, Munich, Germany) according to the manufacturer's recommendations, and 72 h later, cells were harvested to prepare cell lysates. The effect of siRNA on protein expression was determined by Western blot.

2.7 Statistical analysis

The results reported are the mean and the standard deviation of independent experiments. The statistical significance of differences between means was estimated using Student's *t*-test. Values of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1 Transforming growth factor-β stimulates DNA and collagen synthesis in vascular smooth muscle cells

TGF-β stimulates proliferation of VSMCs and matrix protein production.[2–6] We confirmed this observation in our experimental model of human VSMCs. Indeed, TGF-β (10 ng/mL) significantly increased VSMC proliferation as measured by BrdU incorporation (*Figure 1A*). In five experiments, each carried out in triplicate, the proportion of cells incorporating BrdU in their DNA was significantly higher ($P = 0.02$) in TGF-β-stimulated cells than in unstimulated cells (*Figure 1A*). In accordance with the results of BrdU assay, Western blot showed enhanced levels of cyclin D1 in TGF-β-cultured cells (*Figure1B*). Furthermore, collagen type I levels were increased in whole-cell lysates prepared from VSMCs cultured with TGF-β (*Figure 1C*). Taken together, these findings support the concept that TGF-β is a promoter of cell proliferation and extracellular matrix formation in VSMCs.

3.2 FK506 activates the transforming growth factor-β signalling

FK506 activates receptor I transphosphorylation, which is important for kinase activity and signal transduction.[15] We investigated whether FK506 activates the TGF-β signal in VSMCs. Using Western blot assay, we studied the kinetics of pSmad2 levels in whole-cell lysates prepared from VSMCs cultured with 10 ng/mL TGF-β or 100 ng/mL FK506. We found that the levels of pSmad2 in VSMCs cultured with TGF-β increased as early as 2 min after incubation and persisted for 3–4 h, after which they decreased (*Figure 2A*). Similar results were obtained with cells

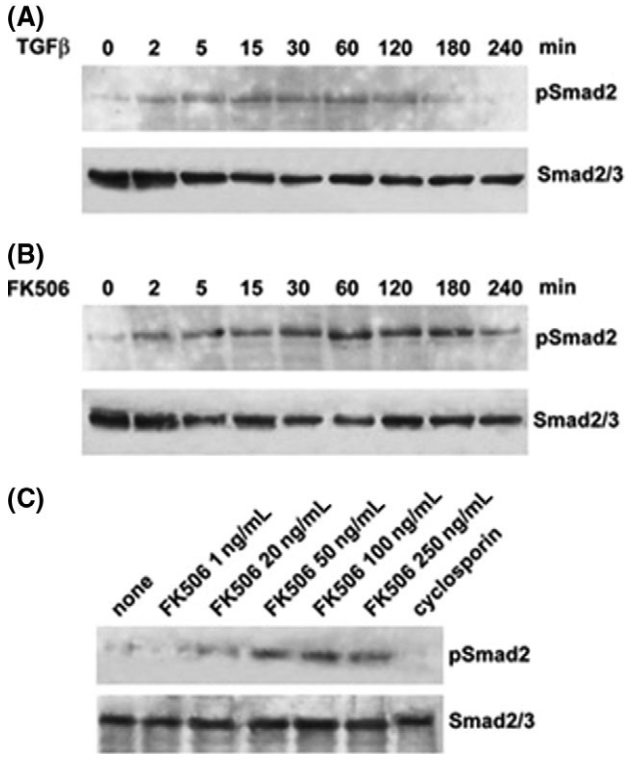

Figure 2 FK506 activates the transforming growth factor-β receptor I kinase activity in vascular smooth muscle cells. (*A*) Kinetics of transforming growth factor-β-induced Smad 2 phosphorylation. Western blot assay of pSmad 2 (Ser 465–467) levels in whole-cell lysates (30 μg) of vascular smooth muscle cells incubated with 10 ng/mL transforming growth factor-β for 0, 2, 5, 15, 30, 60, 120, 180, and 240 min. (*B*) Kinetics of FK506-induced Smad 2 phosphorylation. Western blot assay of pSmad 2 (Ser 465–467) levels in whole-cell lysates (30 μg) of vascular smooth muscle cells incubated with 100 ng/mL FK506 for 0, 2, 5, 15, 30, 60, 120, 180, and 240 min. (*C*) Dose/response effect of FK506 on Smad 2 phosphorylation. Western blot assay of pSmad 2 (Ser 465–467) levels in whole-cell lysates (30 μg) of vascular smooth muscle cells incubated with 1, 20, 50, 100, and 250 ng/mL FK506 and 300 ng/mL cyclosporin, as control, for 1 h. Smad 2/3 was used as loading control. Each experiment presented was performed at least three times.

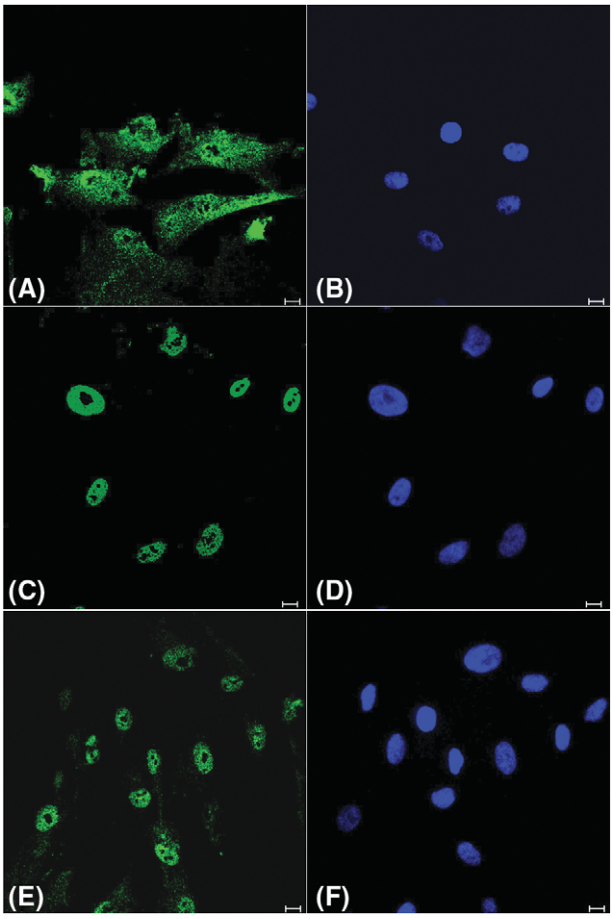

Figure 3 FK506 induces Smad nuclear translocation. Cells were incubated with control medium (*A* and *B*) or treated with 10 ng/mL transforming growth factor-β (*C* and *D*) or 100 ng/mL FK506 (*E* and *F*). After incubation for 1 h, cells were stained with Smad 4 (*A*, *C* and *E*) to visualize protein localization, or Hoechst 33258 (*B*, *D*, and *F*) to visualize nuclei. Localization of Smad4 was both cytosolic and nuclear in control cells (*A*), whereas it was clearly nuclear in cells treated with transforming growth factor-β (*C*) or FK506 (*E*). Bar 10 μm. The data were confirmed at least in five different experiments.

cultured with FK506 (*Figure 2B*). We investigated whether the effect of FK506 was dose-dependent. As shown in *Figure 2C*, the optimal dose of FK506 for Smad2 activation was $\geq$50 ng/mL. Smad phosphorylation was not found in VSMCs cultured with another calcineurin inhibitor, cyclosporine, which binds to cyclophilin A.[14] This finding confirms the specificity of the effect observed with FK506. Receptor-mediated phosphorylation increases the affinity of Smad2 for Smad4 and rapid accumulation of the complex in the nucleus.[11,12] Immunofluorescence of VSMCs stained with anti-Smad4 and confocal microscopy showed that FK506 induced nuclear translocation of Smad (*Figure 3*). Taken together, these results suggest that FK506 activates TGF-β signalling in VSMCs.

3.3 FK506 removes FKBP12 from TβR-1 thereby activating the signal

Displacement of FKBP12 from its binding to the GS region of TβR-1 is essential for the activation of the kinase activity.[13] To investigate whether FK506 displaces FKBP12 binding, we incubated the cells with or without 100 ng/mL FK506 and prepared whole-cell lysates for co-immunoprecipitation experiments. As shown in *Figure 4A*, TβR-1 co-immunoprecipitated with FKBP12 in unstimulated VSMCs but not in FK506-stimulated VSMCs, suggesting that the drug removed FKBP12. To determine whether this effect was sufficient to activate the kinase activity, we depleted the cells of FKBP12 using two different siRNAs and measured the levels of phosphorylated Smad2. We found that pSmad levels were remarkably increased in FKPB12-depleted cells (*Figure 4B*). Taken together these findings suggest that FKBP12 controls activation of the TGF-β signal in VSMCs.

3.4 FK506 promotes vascular smooth muscle cell growth

We next studied the effect of FK506 on VSMC growth. Cells were incubated with FK506 and proliferation was investigated by measuring BrdU incorporation in cell DNA. As shown in *Figure 5A*, the proportion of cells incorporating BrdU dose-dependently increased in FK506 cultures. The levels of β-catenin, Erk, protein kinase B (PkB/Akt), and the mammalian target of rapamycin (mTOR) were increased (*Figure 5B*), which indicates that FK506 activated the

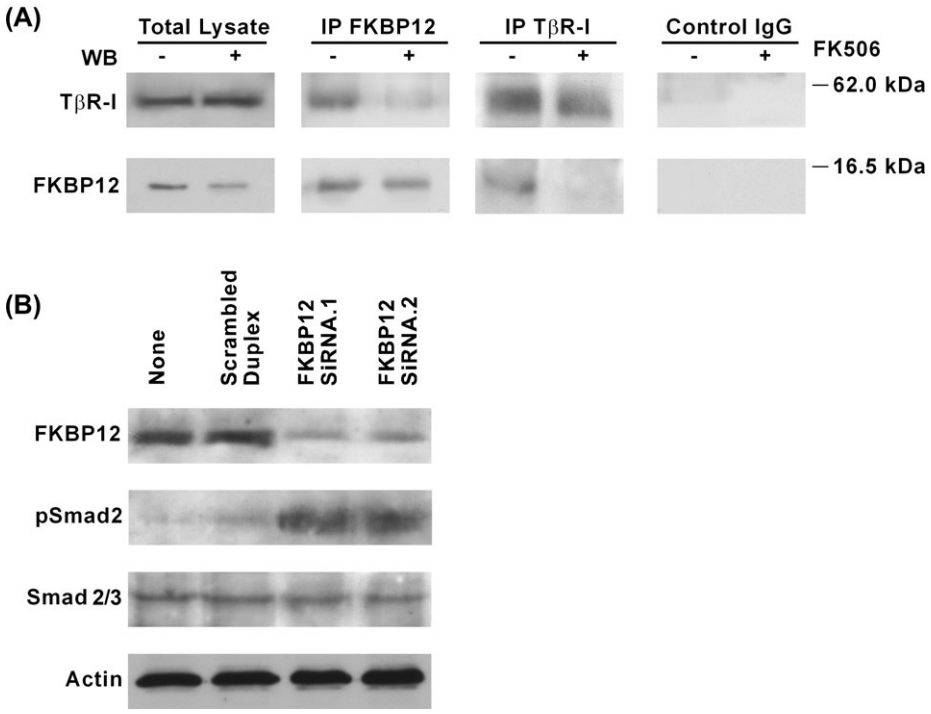

Figure 4 FK506 activates TβR-I by removing FKBP12. (*A*) FKBP12/TβR-I interaction in vascular smooth muscle cells. Total lysates, prepared from cells incubated with and without FK506 (100 ng/mL) for 2 h, were subjected to immunoprecipitation (IP) with anti-TβR-I or FKBP12 antibody. Immunoprecipitated and total lysates were then subjected to Western blot with anti-TβR-I or -FKBP12. The data were confirmed in another independent experiment. (*B*) Western blot assay of FKBP12 and pSmad 2 levels in total lysates prepared from non-transfected vascular smooth muscle cells, from vascular smooth muscle cells transfected with a scrambled duplex or with two different FKBP12 siRNAs, 3 days after transfection. Smad 2/3 and actin were used as loading control. The data were representative of three different experiments.

signalling pathways governing cell proliferation. Consistent with this finding, the cell cycle regulator cyclin D1 was induced in FK506-treated cells. Similar to TGF-β, FK506 increased collagen type I synthesis (*Figure 5C*). FK506 strikingly stimulated VSMC proliferation even in condition which does not favour cell adhesion, as it occurs in the case of metal surface.[26] *Figure 5D* shows results of a 9 day culture of cells seeded onto plates containing a metal stent and incubated with or without 100 ng/mL FK506. The metal stent was not invaded by proliferating cells in culture dishes without FK506. In fact, very few nuclei were visualized on stents removed by such dishes. Normally, once the cells reach the confluence, they detach from the plate and die. But, in the presence of FK506, cell growth continued on the metal support, suggesting that cells were refractory to death. Although the finding of growth on bare stents has no translational implication, it strengthens the hypothesis that FK506 exerts a pro-survival and proliferative effect. Stents were stained with Hoechst 33258 and examined by microscopy and fluorimetrically to evaluate DNA concentrations in the nuclei. Results of absorbance from three different experiments were 32.121 ± 2.001 and 77.126 ± 1.691 OD for cells cultured with and without FK506, respectively.

3.5 Selective inhibition of transforming growth factor-β type I receptor kinase prevents FK506-induced proliferation of vascular smooth muscle cells

To verify that the proliferative response to FK506 depended on activation of the TGF-β signal, we used SB431542, which is a selective inhibitor of TGF-β type I ALK receptors. This compound efficiently inhibited receptor kinase activity, as indicated by the reduced levels of pSmad in VSMCs cultured with FK506 plus SB431542 compared with the levels of cells cultured with FK506 alone (*Figure 6A*). Treatment of the cells with SB431542 dramatically inhibited proliferation in FK506 cultures (*Figure 6B*), which suggests that Smad 2 phosphorylation is essential for transduction of the proliferative signal stimulated by FK506.

4. Discussion

Here we demonstrate that the immunosuppressant FK506 or 'tacrolimus' acts as a growth factor for VSMCs. This compound is structurally related to rapamycin, which significantly prevents the incidence of in-stent restenosis[27] when administered coated to eluting stents in angioplastic procedures. FKBP12 is the intracellular ligand of both FK506 and rapamycin.[18–20] Although both drugs bind to the same immunophilin receptor, the resulting immunophilin–drug complexes interfere with distinct intracellular signalling pathways. The interaction between FKBP12 and FK506 results in a complex that inhibits calcineurin phosphatase,[18] which controls lymphocyte activation,[19] whereas the binding of FKBP12 to rapamycin produces a complex that inhibits mTOR[28] downstream from the phosphatidyl-inositol 3 kinase (PI3k)/Akt-PkB survival pathway.[29] There is great interest in therapeutically targeting VSMC growth with agents delivered by stents implanted in coronary vessels.[27] As a step in this direction, polymer-free stents coated with FK506 have been developed.[25]

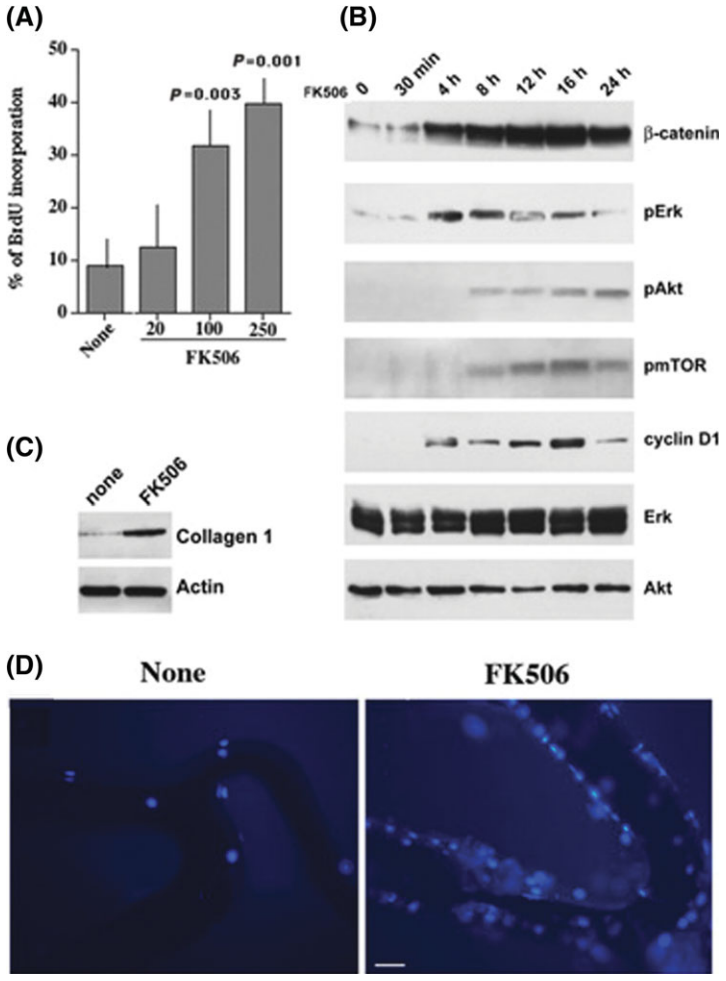

Figure 5 FK506 stimulates vascular smooth muscle cell proliferation. (*A*) Dose/response effect of FK506 on vascular smooth muscle cell proliferation. Graphic representation of mean values and standard deviations of per cent of cells incorporating BrdU in their DNA, in five different experiments, each performed in triplicate. Cells were cultured with and without 20, 100, and 250 ng/mL FK506. After incubation for 3 days, 10 μM BrdU was added to the cultures and 4 h later cells were collected, fixed with ethanol, and incubated with anti-BrdU monoclonal antibody. The per cent of BrdU incorporation, measured by flow cytometry, was significantly increased by FK506 at the doses of 100 ng/mL ($P = 0.003$) and 250 ng/mL FK506 ($P = 0.001$), compared with that found in cells cultured with medium alone. (*B*) FK506 activates signalling kinases involved in cell proliferation. Western blot assay of β-catenin, pErk, pAkt, pmTOR, and cyclin D1 levels in vascular smooth muscle cell lysates prepared from cells cultured with 100 ng/mL FK506, for 0, 30 min, 4, 8, 12, 16, and 24 h. Erk 1/2 and Akt were used for loading control. Comparable results were obtained in two other different experiments. (*C*) Western blot assay of collagen type I levels in whole-cell lysates of vascular smooth muscle cells treated with 100 ng/mL FK506 for 16 h. The result confirmed two other independent experiments. (*D*) FK506 stimulates vascular smooth muscle cell growth on a metal stent. Vascular smooth muscle cells were cultured in dishes containing a metal stent with and without 100 ng/mL FK506. After 9 days of culture, the stent was removed from the dish and nuclei were stained with Hoechst 33258 and visualized with a Zeiss Axioscop 2. Bar 25 μm. In three different experiments, the mean absorbance was 32.121 ± 2.001 and 77.126 ± 1.691 OD for cells cultured with and without FK506, respectively.

It is noteworthy that migration and proliferation of smooth muscle cells in injured vessels are stimulated by cytokines released by infiltrating mononuclear cells.[24] Therefore, FK506, which is a potent inhibitor of lymphocyte activation,[19,20,30] appeared to be a good candidate to counteract this phenomenon. Nevertheless, contrary to rapamycin, which exerts immunosuppressant,[20,30] anti-proliferative,[29] and pro-apoptotic[31] effects, little is known about the effect of FK506 on cell growth. Here we show that FK506 activates the TGF-β signal in VSMCs. In fact, FK506 increased levels of phosphorylated Smad and nuclear translocation of the Smad complex in VSMCs.

This finding is in accordance with studies showing that FKBP12 ligands, i.e. FK506 and rapamycin, promote TGF-β receptor transphosphorylation.[15] We found that FK506 provoked FKBP12 release from the cytoplasmic tail of the TGF-β receptor. This displacement was apparently sufficient to trigger the receptor kinase activity, as suggested by the appearance of phosphorylated Smad in cells depleted of FKBP12.

TGF-β plays an important role in promoting excess intimal hyperplasia after vascular injury, thereby concurring in restenosis after coronary angioplasty.[1–6] Consistent with this notion, TGF-β enhanced proliferation and extracellular matrix production in our specific cellular context of human VSMCs, as indicated by increased BrdU incorporation, cyclin D1 upregulation, and induction of collagen type 1 synthesis.

We found that, similar to TGF-β, FK506 stimulated cell growth. We thus investigated the pathways governing vascular remodelling and VSMC proliferation in cells cultured with FK506. Our data show that FK506 increased the levels of β-catenin, a key regulatory molecule of the highly conserved Wnt pathway[32] that controls the dynamic process of vascular remodelling.[33] Several lines of evidence implicate β-catenin in the development of intimal thickening.[33–35]

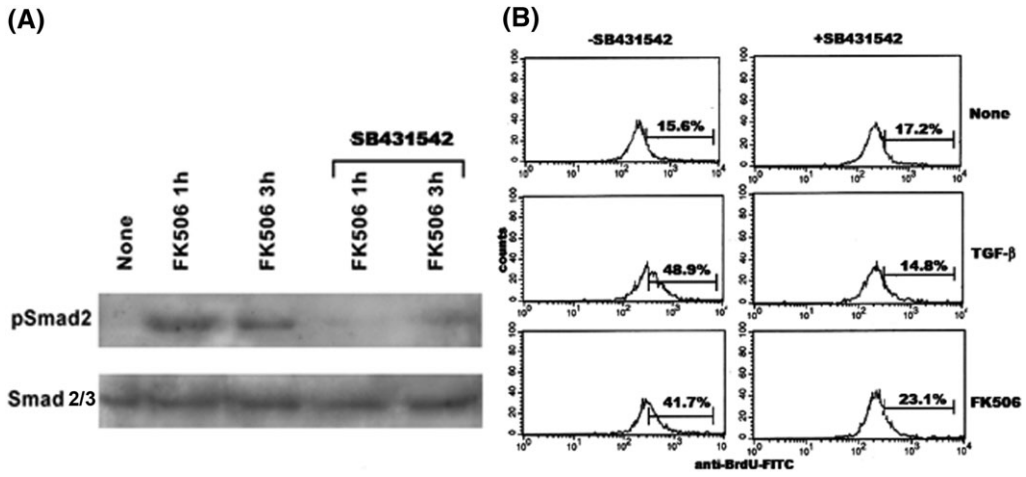

Figure 6 Effect of transforming growth factor-β type I receptor kinase inhibition on FK506-induced proliferation of vascular smooth muscle cell. (*A*) Western blot assay of pSmad2 levels in whole-cell lysates prepared from vascular smooth muscle cells incubated with 100 ng/mL FK506 for 1 and 3 h in the absence or the presence of 5 μM SB431542. Comparable results were obtained in two other different experiments. (*B*) Flow cytometric histograms of BrdU incorporation in vascular smooth muscle cells. Cells were cultured with 10 ng/mL transforming growth factor-β or with 100 ng/mL FK506 in the absence or the presence of 5 μM SB431542. After incubation for 3 days, 10 μM BrdU was added to the cultures and 4 h later cells were collected, fixed with ethanol, and incubated with anti-BrdU-FITC monoclonal antibody. The bar indicates the per cent of cells incorporating BrdU in their DNA. The experiment was representative of three independent experiments, each performed in triplicate. The per cent of BrdU incorporation was significantly decreased by SB431542 both in FK506 ($P = 0.05$) and in transforming growth factor-β ($P = 0.003$) cultures.

In an *in vivo* model of intimal thickening, β-catenin expression co-localized in proliferating VSMC within the developing intima.[34] β-catenin regulates VSMC proliferation in response to stimulation by growth factors via the regulation of the cell cycle genes cyclin D1 and p21.[34] Interestingly, the Wnt/β-catenin pathway can be activated in response to various stimuli,[36] including TGF-β.[32,37,38] In agreement with this concept, we found that FK506-induced proliferation was prevented when the TGF-β type I receptor kinase was inhibited. The increase in β-catenin levels was accompanied by the activation of Erk and Akt, which are important mediators of β-catenin signalling.[39,40] Furthermore, consistent with reports that mTOR can be activated through tuberin phosphorylation by both Erk[41] and Akt,[42] mTOR levels were also increased in FK506-treated VSMCs.

It is noteworthy that all these signalling kinases drive biochemical networks that directly regulate the expression of genes responsible for cell cycle progression.[41] In accordance with this finding, we found that the expression of cyclin D1 was enhanced in FK506 cultures. The Akt pathway also exerts a primary role, synergistically driven by mTOR, in cell survival.[41,43] Indeed, stimulation of Akt produces phosphorylation of downstream targets involved in apoptosis control.[43] Phosphorylation of FOXO3A, a member of the forkhead family of transcription factors, leads to inhibition of its transcription activity. This results in the down-regulation of the pro-apoptotic BH3-only molecule, Bim.[43] Moreover, Akt-induced phosphorylation of another pro-apoptotic Bcl-2 family member protein, namely Bad, causes its dissociation from the complex with Bcl-xL, thereby allowing cell survival.[43] Therefore, Akt activation may result in resistance to death-inducing signals possibly released in the site of vascular injury.

Although rapamycin also activates TGF-β receptor signalling because of the binding to FKBP12 (not shown), it did not increase the levels of cyclin D1 and collagen type 1 (not shown). This finding suggests that the proliferative and pro-fibrotic stimuli are inhibited downstream the pathway consequent to mTOR blockage by FKBP12/rapamycin complex itself. Also in the case of immunosuppression, rapamycin acts on the signalling pathway of T-cell activation downstream to FK506. In fact, while FK506 blocks transcription of the IL-2 gene, rapamycin has no effect on gene transcription but rather has a potent inhibitory effect on growth factor-induced proliferation.

Our findings support the conclusion that FK506 is a potential stimulus of neointima formation when administered coated to intravascular stents. This implies that, although FK506 dampens cytokine-induced VSMC migration and proliferation through its immunosuppressant mechanism, it exerts a direct proliferative effect on these cells. Therefore, the clinical outcome of patients receiving FK506-eluting stents depends on the balance between these opposite effects. Curiously, the stent name, 'Janus', who is the Roman god with two faces looking in opposite directions, reflects our conclusions.

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