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Rapamycin Inhibits Platelet-Derived Growth Factor-Induced Collagen, but Not Fibronectin, Synthesis in Rat Mesangial Cells

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Rapamycin, a macrocyclic lactone, is effective in reducing the incidence of acute rejection after renal transplantation. The inhibitory effects of rapamycin on lymphocyte proliferation and the molecular mechanisms that were involved have been described. However, its effects on glomerular mesangial cells have not been clearly understood, and here, we examined the effect of rapamycin on platelet-derived growth factor (PDGF)-induced extracellular matrix synthesis as well as cell proliferation in mesangial cells. Rat mesangial cells were isolated from the glomeruli of Sprague-Dawley rats and cultured with Dulbecco's modified Eagles medium containing 20% fetal bovine serum. Different concentrations of rapamycin were administered 1 hour before the addition of 10 ng/ml of PDGF into growth arrested and synchronized cells. Cell proliferation was assessed by [^{3}H]thymidine incorporation, total collagen synthesis by [^{3}H]proline incorporation, and fibronectin secretion into the medium by Western blot analysis. In the mesangial cells, PDGF increased cell proliferation by 4.6-fold, total collagen synthesis by 1.8-fold, and fibronectin secretion by 3.2-fold. Rapamycin above 10 nM significantly inhibited PDGF-induced proliferation and collagen synthesis, but the treatment of rapamycin up to 1 μM did not show any significant effects on PDGF-induced fibronectin secretion. These inhibitory effects of rapamycin on PDGF-induced mesangial cell proliferation and collagen synthesis reflect the potential value of rapamycin in the prevention and treatment of glomerulosclerosis in patients with chronic allograft nephropathy.

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INTRODUCTION

Despite the improvement of kidney allograft survival, chronic allograft nephropathy (CAN) remains the leading cause of graft failure after the first year following transplantation.[1,2] CAN shows two major pathologic features. One feature is vascular remodeling (intimal hyperplasia and transplant vascular sclerosis), and the other is glomerulosclerosis. Mesangial extracellular matrix (ECM) accumulation is partly responsible for the development of glomerulosclerosis.[3-5] Early insults and the subsequent responses of vascular smooth muscle cells and mesangial cells can occur simultaneously after renal transplantation and share a common pathophysiology.[6] Therefore, any kind of measures that can inhibit vascular smooth muscle cell proliferation may also suppress mesangial cell proliferation.

Rapamycin has a similar molecular structure to FK506 (tacrolimus) and binds to FK506 binding protein (FKBP12). The rapamycin-FKBP12 complex, however, has no effect on calcineurin phosphatase, but rather, it binds to one or more proteins known as the mammalian target of rapamycin (mTOR).[7] We and others have demonstrated that rapamycin inhibits the proliferation of

rodent or human vascular smooth muscle cells, endothelial cells, and mesangial cells,[8-10] which suggest that rapamycin has the potential to prevent the development and progression of CAN. However, the effects of rapamycin on ECM synthesis have not been clearly defined.

Fibronectin and collagen are the most common proteins in the ECM and are known to be associated with transplant arteriosclerosis and glomerulosclerosis.[11-14] Rapamycin has been reported to inhibit vascular remodeling in late allograft vasculopathy through the inhibition of collagen III and matrix metalloproteinase (MMP)-2 and -9 mRNA upregulation.[15] On the other hand, the expression of collagen III, MMP-2 and -9, and transforming growth factor (TGF)-β1 mRNA in early allograft vasculopathy is not affected by rapamycin.[16] Rather, rapamycin increases and potentiates basal and chronic cyclosporine-induced TGF-β1, collagen I and IV, and biglycan mRNA and protein expression in the kidney.[17,18]

Platelet-derived growth factor (PDGF), especially the PDGF-B chain, is a potent stimulator of cell proliferation and ECM accumulation.[19,20] Renal expression of PDGF and its receptor has been increased in various CAN models.[21-24] Further, the PDGF antagonist has been shown to inhibit glomerular disease including CAN in either a TGF-β-dependent or -independent manner.[25-27] Thus, the present study examined the effects of rapamycin at a dose that inhibited cell proliferation on collagen synthesis and fibronectin secretion in rat mesangial cells in order to evaluate the effect of rapamycin on ECM accumulation.

MATERIALS AND METHODS

All the chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (St. Louis, MO, USA). All tissue culture plastics were purchased from Becton Dickinson Labware (Lincoln Park, NJ, USA).

Isolation of rat glomeruli and primary mesangial cell culture

Male Sprague-Dawley rats, weighing 100-150 g, were decapitated after being anesthetized, and both kidneys were extracted. The cortex was obtained by cutting with scissors, and glomeruli isolation and mesangial cell culture were performed by a conventional sieving method with some modifications.[28] Glomeruli on a 75 μm sieve were harvested and placed in a solution that included 2 mg/ml collagenase and 0.25%/1 mM trypsin/EDTA (GIBCO BRL, Grand Island, NY, USA). The glomeruli that were obtained were centrifuged and then cultured with Dulbecco's modified Eagles medium (DMEM; GIBCO BRL) containing 20% fetal bovine serum (FBS; GIBCO BRL), 100 U/ml penicillin (GIBCO BRL), and 100 μg/ml streptomycin (GIBCO BRL). Characterization of mesangial cells was confirmed by morphology and by an immunohistochemial staining method using anti-vimentin antibody (DAKO Japan Co., Kyoto, Japan) and anti-cytokeratin antibody (DAKO Japan Co.). Cell passages 5-9 were used for the present study.

Drug treatment

Near confluent cells were incubated with serum-free medium for 24 hours to arrest and synchronize cell growth. The medium was then replaced with fresh serum-free medium containing 10 ng/ml of PDGF-BB, and cells were incubated for up to 48 hours. Rapamycin was administered 1 hour before the addition of PDGF.

DNA synthesis; [^{3}H]thymidine incorporation

One μCi/ml of [^{3}H]thymidine (Du Pont Co., Wilmington, DE, U.S.A.) was added to each well for the last 12 hours of the experimental periods. The cells were then washed twice with PBS and trypsinized before harvesting with a cell harvester (Titertek Cell Harvester 550, Flow Laboratories, Irvine, Scotland, UK) onto glass-fiber filters (Flow Laboratories). They were placed in a 3 ml scintillation cocktail solution, and their radioactivities were measured by using a β-counter (TL 5000s, Beckman Instruments Inc., Fullerton, CA, USA).

Collagen synthesis; [^{3}H]proline incorporation

One μCi/ml of [^{3}H]proline (Du Pont) was ad-

ded to each well for the last 12 hours of the experimental periods. The cells were fixed with methanol and were washed twice with PBS. Then, they were incubated for 10 minutes with 10% trichloroacetic acid (TCA) at room temperature and were subsequently reacted with 0.2 N NaOH and 0.5% SDS for 30 minutes. All the reactions were immediately stopped with 1 N HCl, and then, harvested materials were placed in 3 ml of scintillation cocktail. Their radioactivity was measured using a β-counter (Beckman Instruments Inc.).

Fibronectin secretion; Western blot analysis

After the experiments, the medium was collected, centrifuged to remove cell debris, and used for fibronectin protein assays. The cells were washed twice with PBS and lysed in a buffer containing 1.0% Triton X-100, 20 mM Tris-HCl (pH 7.0), 137 mM sodium chloride, 5 mM EDTA, 20 μM leupeptin, 10 μg/ml aprotinin, 1mM PMSF, and 1 mM EGTA (pH 8.0). Insoluble materials were removed by centrifugation.

Cellular protein was measured using a Bio-Rad protein analysis kit (Bio-Rad, Hercules, CA, USA). Protein loadings (aliquots of media) were normalized based on the cellular protein. Each sample was mixed with a sample buffer (12 mM Tris-HCL, pH 8.0, 0.5% glycerol, 0.4% SDS, 2.88 mM 2-mercaptoethanol, 0.02% bromophenol blue). The mixture was heated at 95℃ for 5 min. The proteins were resolved in SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). The peroxidase-conjugated polyclonal rabbit anti-human fibronectin antibody (DAKO Japan Co.) bound with primary and secondary antibodies was used for the detection of fibronectin secretion into the media. The antibody was detected by using an ECL kit (Enhanced Chemiluminescence, Amersham, Buckinghamshire, UK). Each density was quantified using the Tina 2.1 program and was compared to the control (density=1).

Statistical analysis

All experimental results and measurements are expressed as means ± standard deviation (SD), and statistical comparisons were done using ANOVA followed by the Tukey method for the multiple comparison. P values below 0.05 were considered statistically significance.

RESULTS

Effect of rapamycin on PDGF-induced cell proliferation (Fig. 1)

Basal [^{3}H]thymidine incorporation in mesangial cells cultured under a serum-free condition for 48 hours was 57.61 ± 13.25 cpm. 10 ng/ml of PDGF stimulated [^{3}H]thymidine incorporation up to 270.72 ± 90.10 cpm (4.6-fold). Rapamycin inhibited PDGF-induced, but not basal, mesangial cell proliferation in a dose-dependent manner. The IC50 of rapamycin on mesangial cell proliferation was 10 - 100 nM.

Effect of rapamycin on PDGF-induced total collagen synthesis (Fig. 2)

Collagen synthesis of mesangial cells after 48 hours in the presence of 10 ng/ml of PDGF was measured by [^{3}H]proline incorporation. The measurement of basal [^{3}H]proline incorporation in mesangial cells that were cultured under serum-free conditions for 48 hours were 47.67 ± 12.52 cpm. 10 ng/ml of PDGF stimulated [^{3}H]proline incorporation up to 86.48 ± 19.34 cpm (1.8-fold).

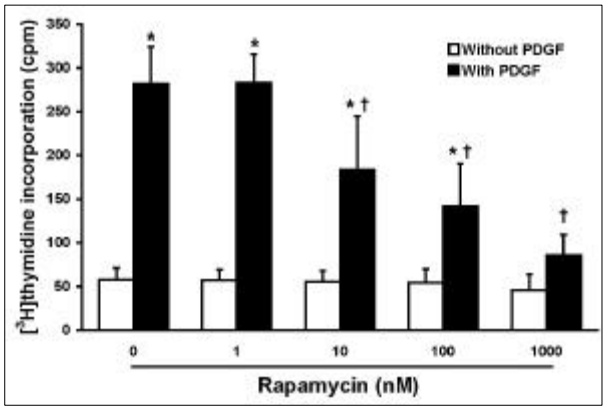

Fig. 1. Effect of rapamycin on PDGF-induced mesangial cell proliferation. Cell proliferation was measured by [^{3}H]thymidine. The experimental protocol is detailed in Materials and Methods. Data are presented as means ± SD of five experiments. $^*p<0.05$ versus rapamycin 0 without PDGF, $^\dagger p<0.05$ versus rapamycin 0 with PDGF.

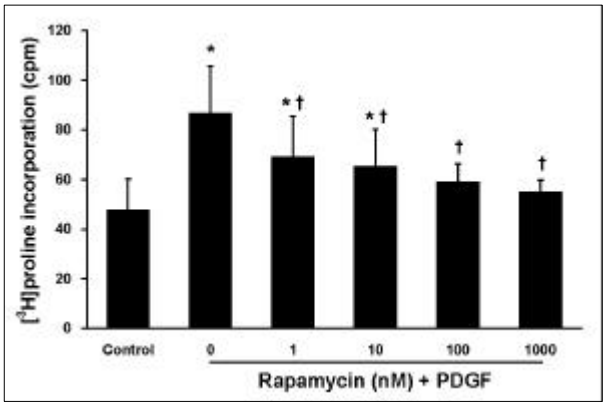

Fig. 2. Effect of rapamycin on PDGF-induced collagen synthesis. Total collagen synthesis was measured by [3H]proline incorporation. The experimental protocol is detailed in Materials and Methods. Data are presented as means ± SD of five experiments. *$p<0.05$ versus control, †$p<0.05$ versus rapamycin 0.

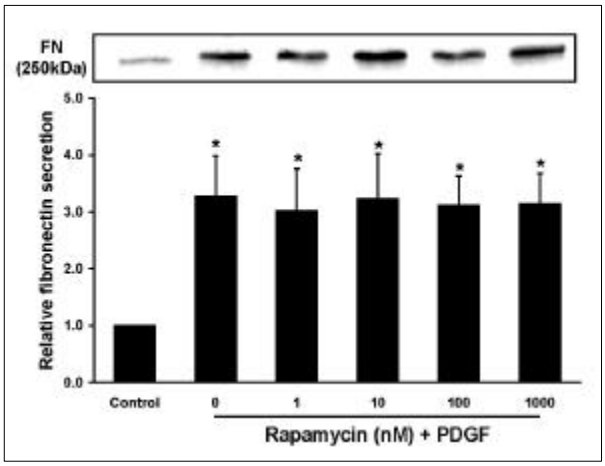

Fig. 3. Effect of rapamycin on PDGF-induced fibronectin secretion. Fibronectin (FN) secretion into the medium was measured by Western blot analysis. The experimental protocol is detailed in Materials and Methods. Data are presented as a representative Western blot and means ± SD of five experiments. *$p<0.05$ versus control.

Rapamycin above 1 nM significantly inhibited PDGF-induced collagen synthesis in a dose-dependent manner. PDGF-induced [^{3}H]proline incorporation in the presence of 0.1 or 1 μM of rapamycin was not different from the control.

Effect of rapamycin on PDGF-induced fibronectin secretion (Fig. 3)

Fibronectin secretion by mesangial cells after 48 hours in the presence of 10 ng/ml of PDGF was measured by Western blot analysis. PDGF increased fibronectin secretion by 2.8-fold compared to the control at 48 hours. Rapamycin up to 1 μM did not affect PDGF-induced fibronectin secretion.

DISCUSSION

In this study, we found that rapamycin, at the dose of inhibiting PDGF-induced mesangial cell proliferation, down-regulated the collagen synthesis but did not affect fibronectin secretion.

We used PDGF-induced mesangial cell activation as an *in vitro* model of progressive glomerulosclerosis, since renal expression of PDGF is up-regulated in CAN, and PDGF induces both proliferation and ECM synthesis in mesangial cells.[29] Rapamycin above 10 nM significantly inhibited the proliferation of PDGF-induced mesangial cells in a dose-dependent manner. This result agrees with a previous report by Wang et al.[10] The dose of rapamycin that inhibited PDGF-induced mesangial cell proliferation was relatively higher than the inhibitory concentration of rapamycin (1 nM) for VSMC proliferation.[8] A molarity of rapamycin up to 1 μM did not affect basal [^{3}H]-thymidine incorporation in this study. The clinically available concentration of rapamycin as an immunosuppressive agent was reported between 10 and 60 ng/ml (10.9 to 65.6 nM).[30]

Rapamycin above 1 nM significantly inhibited PDGF-induced collagen synthesis in mesangial cells, but rapamycin up to 1 μM did not show any significant impact on the PDGF-induced fibronectin secretion. The inhibitory effect of rapamycin on the collagen synthesis started to occur at a relatively lower concentration compared to the anti-proliferative concentration of rapamycin. The present results of the effects of rapamycin on ECM synthesis are in line with a previous report by Svelgliati-Baroni et al.[31] They demonstrated that rapamycin inhibits acetaldehyde-induced type I collagen, but not fibronectin, mRNA expression in hepatic stellate cells. Considering that mTOR are cellular targets of the rapamycin effect, collagen synthesis appears to be dependent on mTOR, but fibronectin synthesis does not. However, it should be noted that rapamycin increases basal, as well

as cyclosporine-induced collagen mRNA up-regulation, in the kidney.[17,18]

In conclusion, this study demonstrates that, in rat mesangial cells, rapamycin inhibits PDGF-induced mesangial cell proliferation and collagen synthesis, but not fibronectin secretion. The inhibitory effects of rapamycin on PDGF-induced mesangial cell proliferation and collagen synthesis suggest that rapamycin has potential for being used in the prevention and treatment of glomerulosclerosis in renal transplant recipients.

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