Rapamycin at subimmunosuppressive levels inhibits mesangial cell proliferation and extracellular matrix production

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Lock HR, Sacks SH, Robson MG. Rapamycin at subimmunosuppressive levels inhibits mesangial cell proliferation and extracellular matrix production. Am J Physiol Renal Physiol 292: F76–F81, 2007. First published August 8, 2006; doi:10.1152/ajprenal.00128.2006.—In view of its proven antiproliferative effects, rapamycin offers potential in the treatment of mesangio proliferative disease. Previous data have shown an effect of rapamycin on mesangial cell proliferation at high doses and have not explored the mechanism of action. Therefore, we explored the effects and mechanism of action of low levels of rapamycin on mesangial cell proliferation. Primary cultures of mouse mesangial cells were grown in medium containing serum with differing concentrations of rapamycin. A rapamycin concentration of 0.1 ng/ml caused a decrease in cell number and DNA synthesis with no effect on apoptosis. Type IV collagen protein production was inhibited at 0.01 ng/ml rapamycin, although gene expression was unaffected. P70S6K phosphorylation was inhibited in parallel with the effects on mesangial cell proliferation and type IV collagen production at 0.01 ng/ml rapamycin. We suggest that further in vivo studies should explore the potential for low-dose rapamycin in the treatment of mesangio proliferative glomerulonephritis.

glomerulus; kidney; collagen

THE GROWTH AND DIFFERENTIATION of mammalian cells in response to nutrients are regulated by the mammalian target of rapamycin (mTOR) kinases. Downstream targets of mTOR kinase activity include several factors that regulate translation, such as the eukaryotic translation initiation factors 4G1 and 4B (eIF4G1 and eIF4B) and the translation inhibitors 4E binding proteins 1 and 2 (4E-BP1 and 4E-BP2). In addition, p70S6 kinase (S6K), responsible for phosphorylation of ribosomal S6 kinase, is also phosphorylated by mTOR kinase. Specificity for the mRNA encoding proteins critical for growth and proliferation results from a particular requirement by these mRNAs for this combination of translation factors (4).

Due to its immunosuppressant effects, rapamycin is increasingly used in solid organ transplantation (16). Antiproliferative effects, in cells other than lymphocytes, are being explored in a variety of clinical contexts and may extend the potential uses of this drug (2, 5, 9, 17). Mesangial proliferation is a feature of many types of glomerulonephritis, suggesting that rapamycin may be useful in this context. The antiproliferative effects of rapamycin and related drugs such as everolimus may also lead to adverse effects. Recent work has shown that everolimus worsens glomerular pathology in a model of mesangio proliferative disease, due to inhibition of endothelial cell prolifera-

tion (3). Because of these potential detrimental effects, we were interested in defining the lowest possible dose of rapamycin affecting mesangial cells.

We studied the effect of rapamycin on serum-stimulated primary cultures of mouse mesangial cells and provide a number of novel observations. We show that a remarkably low dose inhibits both proliferation and type IV collagen production. A dose-dependant inhibition of p70S6 kinase phosphorylation occurs in parallel with these effects.

MATERIALS AND METHODS

Mesangial cell isolation and characterization. C57BL/6 mice were purchased from Harlan (Bicester, UK) and kept according to local regulations and guidelines. For these studies, male mice aged 2 mo were used. Glomerular isolation was based on a previously described method (14). Glomeruli were placed in medium consisting of RPMI 1640, supplemented with 10% FCS, 1% insulin, transferrin, selenium, and 1% penicillin/streptomycin. These cell culture reagents were all from Invitrogen (Paisley, UK). After four passages, mesangial cells were characterized by positive immunocytochemistry for desmin, vimentin, and smooth muscle actin. Macrophages were excluded by negative staining for CD68, endothelial cells by negative staining for factor VIII, and epithelial cells by negative staining for cytokeratin. Cells used in these experiments were between passage 5 and 12.

Manual cell number assay. Mesangial cells were seeded into 24-well plates, at 4 × 10^4 cells/ml in a volume of 0.5 ml medium. The following day, medium was changed for medium containing varying concentrations (0.01–1 ng/ml) of rapamycin or an equivalent amount of vehicle. Rapamycin was from Sigma (Poole, UK), dissolved in DMSO (final concentration was 0.1% in all wells). Cells from some wells were used for baseline counting on the day that rapamycin was added. Medium was changed on day 3 (to contain the same concentration of rapamycin or vehicle), and on day 6 supernatants were taken and stored at −20°C until used for collagen IV ELISA, and cells were counted. Cells were detached using trypsin, diluted in an equal volume of Trypan Blue (Sigma) to exclude dead cells, and loaded into a hemocytometer.

Colorimetric cell number assay. Mesangial cells were seeded into 96-well plates at 2 × 10^4 cells/ml in a volume of 100 µl. Two days later medium was changed for medium containing varying concentrations of rapamycin or an equivalent amount of vehicle. At time points after this, a Celltiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Southampton, UK) was performed according to the manufacturer’s instructions.

Cytotoxicity assay. Mesangial cells were seeded in 96-well plates in medium with 20% FCS. When cells were at least 50% confluent, medium was changed to contain 2% FCS with varying concentrations of rapamycin (at least 3 wells per group). The low serum level was to avoid interference with the assay. Twenty-four hours later, LDH

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release (relative to complete cell lysis) was measured using a Cytotox 96 Assay (Promega) according to the manufacturer’s instructions.

**Apoptosis assay.** Glass coverslips in 24-well plates were coated with gelatin (Sigma), and mesangial cells were seeded onto them in a volume of 0.5 ml. Three days later, TUNEL staining was performed using a FITC Apoptag kit (Chemicon) according to the manufacturer’s instructions. Coverslips were mounted on microscope slides with Shandon Permaflour (Thermo Electron, Basingstoke, UK), to which Hoechst 3342 dye (Sigma) had been added at 0.3 µg/ml. Apoptotic cells were identified by a combination of characteristic nuclear morphology due to Hoechst 3342 dye and positive fluorescence for TUNEL staining. Staurosporin (10 µM)-treated cells served as a positive control.

**Thymidine uptake proliferation assay.** Mesangial cells were seeded into 96-well plates at 2 x 10⁵ cells/ml in a volume of 100 µl. Two days later medium was changed for medium containing varying concentrations of rapamycin or an equivalent amount of vehicle. [³H]thymidine (Amersham Biosciences, Chalfont St. Giles, UK) was added at 1 µCi/well on days 0–2, and 6 h later, plates were frozen at −20°C until analyzed. Plates were harvested using a Mach-3 Harvester96 (Tomtec) and counted using a Packard Topcount microplate scintillation counter (Perkin Elmer, Beaconsfield, UK).

**Total protein assay.** Cells were cultured with rapamycin exactly as described in the manual counting experiment as described above. A sandwich ELISA was performed. The capture antibody was J3–2, a gift of Nirmala Sundaraj (13), purified from ascites on an IgM column (Amersham Biosciences) and coated at 5 µg/ml. Standard concentrations of type IV collagen were from BD Biosciences (Oxford, UK). Detection was with rabbit anti-mouse type IV collagen (Cedar Lane, Ontario, Canada), followed by horseradish peroxidase-conjugated goat anti-rabbit (Dakocytomation, Ely, UK). The lower limit of detection was 78 ng/ml.

**Type IV collagen assay.** Supernatants were collected from the manual counting experiment as described above. A sandwich ELISA was performed. The capture antibody was J3–2, a gift of Nirmala Sundaraj (13), purified from ascites on an IgM column (Amersham Biosciences) and coated at 5 µg/ml. Standard concentrations of type IV collagen were from BD Biosciences (Oxford, UK). Detection was with rabbit anti-mouse type IV collagen (Cedar Lane, Ontario, Canada), followed by horseradish peroxidase-conjugated goat anti-rabbit (Dakocytomation, Ely, UK). The lower limit of detection was 78 ng/ml.

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**Real-time PCR for type IV collagen.** Mesangial cells were grown in 25-cm² tissue culture flasks. Twenty-four hours after medium was changed to contain rapamycin or vehicle, RNA was extracted using RNeasy (Qiagen, Crawley, UK) according to the manufacturer’s instructions. This was reverse transcribed to cDNA using oligodT and M-MLV-RT from Promega. Quantitative real-time PCR was performed using a PCT-200 thermocycler with a Chromo4 detector and Optichon software from MJ Research (Waltham, MA). Primer sequences for type IV collagen and β-actin were as published previously (1). Primers for GAPDH were ACCACAGTCCATGGCAATCATC (sense) and TCCACACCTGTGCTGTA (antisense). Annealing temperatures were 60°C for all reactions. A DyNAmo HS SYBR Green qPCR kit from Finnzymes (Espoo, Finland) was used according to the manufacturer’s instructions. A published method for relative quantification of cDNA was used (11).

**Immunoprecipitation and Western blotting for p70S6 kinase.** Mesangial cells were seeded in 75-cm² tissue culture flasks. Twenty-four hours after medium was changed to contain rapamycin or vehicle, cells were lysed in 1 ml of TBS, pH 7.4, containing 0.1 mM sodium vanadate, 2 mM PMSF, 0.05% SDS, 1% NP-40, and 1% Pierce Halt TM Protease inhibitor cocktail (Pierce). Immunoprecipitation and blotting for phosphorlated p70S6K were performed using a Phospho-p70S6 kinase antibody kit (Cell Signaling Technology, Beverly, MA), with protein A (Sigma), and nitrocellulose membranes and ECL detection reagents from Amersham. Membranes were stripped in Restore Western blot stripping buffer (Pierce), blocked as before and reprobed using an antibody to total S6K.

**Statistics.** Statistics were performed using Student’s t-test and Prism software (Graphpad Prism, San Diego, CA). Where the F-test suggested unequal variances, data were analyzed after a logarithmic transformation.

**RESULTS**

Rapamycin at a concentration of 0.1 ng/ml inhibits an increase in mesangial cell number. To explore the effects of rapamycin on mesangial cells, we began by measuring the increase in cell number that occurred when mesangial cells were grown in the presence of various concentrations of rapamycin. Direct counting and a colorimetric method gave similar results. Figure 1 shows that, with cell number assessed by direct counting at 6 days after treatment with rapamycin, a significant effect was seen at a dose of 0.1 ng/ml, with complete inhibition of the increase in cell number at 1 ng/ml. Figure 2 shows that, using the colometric assay at 1, 2, and 4 days after treatment with rapamycin, a significant effect was also seen at 0.1 ng/ml, with a greater effect at 1 ng/ml. There was a transient effect of 0.01 ng/ml seen at day 1, but this was not sustained. In addition, we tested the reversibility of the inhibitory effect on cell number. After 4 days of culture in medium with the above doses of rapamycin, medium was changed in all wells to medium containing no rapamycin. Six days later, cell number was measured by the colorimetric assay, and results (OD at 450) were 1.31 ± 0.03, 1.28 ± 0.05, 1.05 ± 0.04, and 1.02 ± 0.05 for 0, 0.01, 0.1, and 1 ng/ml rapamycin, respectively. The differences between the highest two doses and no rapamycin were still significant (P = 0.01 and 0.019), but there was no longer a significant difference between these doses. This demonstrated a degree of reversibility of the effect of rapamycin. By these two methods, the data showed that at a dose of 0.1 ng/ml caused inhibition of the increase in mesangial cell number. We also demonstrated that this effect was not caused by a toxic effect of rapamycin on mesangial cells. The cytotoxicity assay results for 0, 0.01, 0.1, and 1 ng/ml rapamycin were 6.8 ± 0.9, 4.5 ± 0.6, 5.0 ± 0.5, and 6.0 ± 0.9 (% of maximum), respectively.
Effect of rapamycin on cell number is not caused by increased apoptosis. One possible explanation for the effect of rapamycin on cell number was that rapamycin caused an increase in apoptosis, as this had been described for other cell types (8). We therefore cultured mesangial cells on coverslips in the presence or absence of rapamycin (0.01, 0.1, or 1 ng/ml). Three days later, cells were analyzed by TUNEL staining counterstained with Hoechst 3342 dye. The proportion of apoptotic cells was counted and fewer than 1 in 100 of treated (with any concentration of rapamycin) or untreated cells were apoptotic, as shown in Fig. 3. This showed that the effect of rapamycin on cell number was not due to increased apoptosis.

Rapamycin at a concentration of 0.1 ng/ml inhibits mesangial cell proliferation as measured by thymidine uptake. To assess whether the effect of rapamycin on cell number that we had observed was due to inhibition of proliferation, we used a thymidine uptake assay. Cells were cultured in the presence of varying doses of rapamycin and pulsed with [3H]thymidine. As shown in Fig. 4, the effect on proliferation mirrored that which we had seen on cell number. At days 1 and 2 after treatment with rapamycin, there was a clear effect at 0.1 ng/ml, and a greater effect at 1 ng/ml. These data showed that the effect of a concentration of rapamycin as low as 0.1 ng/ml on cell number was due to a direct inhibition of proliferation.

Rapamycin at a concentration of 0.01 ng/ml inhibits mesangial cell collagen translation independently of the effect on cell number. We also measured the effect of rapamycin on mesangial cell production of type IV collagen, a major component of mesangial matrix. In the experiment shown in Fig. 1, medium was changed at day 3. Type IV collagen was measured in supernatants collected on day 6, therefore representing collagen IV production between days 3 and 6. Results are shown in Fig. 5A (graph). Concentrations of rapamycin as low as 0.01 ng/ml inhibited collagen IV production. The data in Fig. 5A (graph) are from the same experiment as the cell number data in Fig. 1. We did not detect any effect on cell number at 0.01 ng/ml rapamycin. Therefore, the decrease in collagen IV production cannot be due to the presence of fewer cells. In addition, the effect on collagen IV production at 0.1 and 1 ng/ml is not simply a reflection of the presence of fewer cells. Collagen IV production is decreased ~5- and 30-fold by 0.1 ng/ml rapamycin.
and 1 ng/ml rapamycin, respectively. At day 6, the difference in cell number in the presence of 0.1 and 1 ng/ml rapamycin was about two- and fourfold, respectively. To differentiate between an effect on gene expression or translation, we measured mRNA for collagen IV using quantitative real-time RT-PCR, with results expressed in relation to the amount of GAPDH. There was no difference in collagen IV mRNA between mesangial cells that were untreated, and those that were treated with rapamycin concentrations up to 1 ng/ml. These data are shown in Fig. 5B. Data were also normalized with respect to β-actin expression, with no differences between groups seen. To show that this effect on collagen IV did not represent a nonspecific effect on protein synthesis, we measured the total protein content of cells grown in varying concentrations of rapamycin, after adjusting all cell numbers to 3 × 10^4/ml. The results were (mg/ml) 2.98 ± 0.14, 3.25 ± 0.12, 3.2 ± 0.11, and 3.34 ± 0.15 for 0, 0.01, 0.1, and 1 ng/ml rapamycin, respectively. This indicated that rapamycin specifically inhibits collagen IV secretion.

Rapamycin causes an inhibition of mesangial cell p70S6 kinase phosphorylation. We performed Western blotting for phosphorylated forms of S6K on immunoprecipitates of mesangial cell lysates to explore the mechanisms by which rapamycin causes decreased proliferation and collagen IV secretion. There was no visible effect at 0.01 ng/ml, but at 0.1 ng/ml there is a shift in the band for total S6K consistent with decreased phosphorylation and the band for 389Thr S6K was just visible. At 1 ng/ml rapamycin, there was a further shift in the band for total S6K, and the band for 389Thr S6K was not visible. Representative blots are shown in Fig. 6. These results show a dose-dependant effect of rapamycin on S6K phosphorylation in mesangial cells and demonstrate these effects at the same dose that inhibited proliferation. We could detect no effect on S6K phosphorylation at 0.01 ng/ml, a dose which inhibited collagen IV translation.

DISCUSSION
In this study, we demonstrated that serum-stimulated mesangial cell proliferation is inhibited by 0.1 ng/ml rapamycin. We showed an inhibition of the increase in cell number by both manual counting, and with a colorimetric assay. The colorimetric assay also showed a decrease in cell number with 0.01

Fig. 4. Rapamycin inhibits mesangial cell proliferation measured by thymidine uptake. Mesangial cells were grown in 96-well plates, and medium was changed to contain varying concentrations of rapamycin or vehicle. At days 0–2, cell proliferation was measured using a thymidine uptake assay. Each dot represents a separate well. On day 1, P < 0.0001 for all comparisons except between 0.1 and 0.01 ng/ml rapamycin (P = 0.0029) and between 0 and 0.01 ng/ml rapamycin (not significant). On day 2, P < 0.0001 for all comparisons except between 1 and 0.1 ng/ml rapamycin (P = 0.0036) and between 0 and 0.01 ng/ml rapamycin (not significant). Data are representative of 3 independent experiments.

Fig. 5. Rapamycin inhibits mesangial cell type IV collagen production. Collagen IV was measured by ELISA (A). The data represent collagen production between days 3 and 6 of growth in varying concentrations of rapamycin. Each dot represents a separate well. The data given are from the same experiment as the cell number data in Fig. 1, showing that the differences in collagen production are not simply a reflection of differences in cell number, as explained in the text. For all comparisons, P < 0.0001 except between 0 and 0.01 ng/ml rapamycin (P = 0.0007). Data are representative of 3 independent experiments. B: real-time RT-PCR data for collagen IV gene expression in the presence of varying doses of rapamycin. Each dot represents a separate flask.

Fig. 6. Rapamycin inhibits p70S6 kinase (S6K) phosphorylation. Mesangial cells were grown in the presence or absence of 0, 0.01, 0.1, or 1 ng/ml rapamycin (3 flasks per dose). Each lane represents a sample from a separate flask. Lysates were immunoprecipitated with an antibody against total S6K and probed with antibody against 389Thr S6K. The bands in the bottom blot labeled antibody represent detection of the antibody used for immunoprecipitation. This has been shown as it makes the band shifts in total S6K, due to rapamycin, easier to appreciate.
ng/ml rapamycin, but this was not sustained. We showed that the effect of rapamycin was not due to a toxic effect, or to enhancement of apoptosis, and that it was directly due to an effect on proliferation using a [3H]thymidine uptake assay. The data shown in Fig. 1 show a small decrease in cell number at day 6, compared with baseline, after treatment with rapamycin at 1 ng/ml. We did not demonstrate any toxic or proapoptotic effect of rapamycin at these doses. However, if proliferation was markedly inhibited, then the small decrease in cell number could be explained by necrosis and/or apoptosis, even if these effects occurred to the same degree as in untreated cells. Type IV collagen secretion was inhibited independently of any effect on cell number by 0.01 ng/ml of rapamycin. This was demonstrated for protein by ELISA, with differences in protein concentration greater than could be accounted for by cell number, as assessed manually in the same experiment. The lack of an effect on gene expression suggested that this was due to an inhibition of translation of type IV collagen. This was observed at 0.1 ng/ml rapamycin. There was no difference in total protein content of cells treated with rapamycin, suggesting that we demonstrated a specific effect on collagen secretion. We showed that rapamycin was acting on mesangial cells, at least in part, through inhibition of S6K phosphorylation.

Rapamycin has been shown to inhibit both proliferation and extracellular matrix production in a variety of cell types. The inhibition of vascular smooth muscle proliferation (2, 10) is an effect that is being exploited to prevent restenosis of coronary artery stents. In addition, the antiproliferative effects of rapamycin have been explored in a variety of tumor cell lines (reviewed in Ref. 5) and nontumor cells such as hepatic stellate cells (17). A recent in vivo study explored the effect of a rapamycin analog on disease severity in Thy-1 nephritis (3). This suggested that, despite an antiproliferative effect on mesangial cells, the rapamycin derivative exacerbated disease through inhibition of endothelial cell proliferation. At lower doses, these adverse effects were reduced, however. Limited in vitro data were presented, suggesting inhibition of serum-stimulated mesangial cell proliferation at levels four times higher than in the current study. This difference may be due to differences in culture conditions or to differences between mouse and rat cells. It may also reflect different effects of everolimus and rapamycin. There were no in vitro data in this study on extracellular matrix production.

Two other in vitro studies have shown that rapamycin inhibits platelet-derived, growth factor-stimulated rat mesangial cell proliferation at far higher doses than those used in the current study (6, 15). Both of these found an effect at 10 nM, which is ~100-fold higher than the dose that produced a clear effect on proliferation in our study. The second of the above studies also found an effect on total collagen synthesis, although this also occurred at 1 nM rapamycin which is 100 times the dose that we found inhibited type IV collagen secretion. In addition, the effect described in this paper is small. Even at doses of 1,000 nM an inhibition of only 25% was seen, despite a profound effect on cell proliferation and presumably cell number at this dose. There was no clear demonstration that the effect on collagen production was not simply a reflection of differences in cell number. We found that 1 ng/ml (approximately equals 1 nM) almost totally inhibited collagen secretion, and a clear effect was seen at 0.01 ng/ml. None of these effects were explained by differences in cell number due to the antiproliferative effects of rapamycin. The reasons for the greater sensitivity of mesangial cells to rapamycin in our study are of interest and may be due to the method of stimulation used. We used serum-stimulated mesangial cells rather than PDGF stimulation. The concentrations of PDGF used in other studies were high and may not reflect physiological concentrations even in disease states. Since the mesangium is constantly bathed in serum in vivo, our stimulation with serum may be more physiological.

In addition to the finding of extreme sensitivity of mesangial cells to rapamycin, we explored the mechanism of this effect. Studies in other cell types have shown that one of the major downstream targets of mTOR is S6K. These studies have inferred inhibition of S6K phosphorylation from changes in band intensity when blots are probed with antibodies for S6K phosphorylated at specific residues. Other evidence of inhibition of phosphorylation has included changes in electrophoretic mobility that may be seen with antibodies either to total S6K or to phosphorylated residues on S6K. For example, a study examining the effect of rapamycin on neutrophils found that the band for 389Thr S6K disappeared with rapamycin (7). A shift in mobility of total S6K was not described in this study. However, another study exploring the effect of rapamycin on CD8 cells did show a shift in mobility of total S6K (12). In the present study, we showed a clear dose-dependant effect of rapamycin on S6K phosphorylation that occurs in parallel with the effects of rapamycin on proliferation. However, no effects were seen on S6K phosphorylation at 0.01 ng/ml, a dose at which collagen IV secretion was inhibited. This probably reflects the differing sensitivities of these assays, and we cannot exclude an effect of 0.01 ng/ml rapamycin on S6K phosphorylation.

We showed that both proliferation and extracellular matrix production in mesangial cells are inhibited by very low levels of rapamycin. These levels are below those that are used to produce clinically immunosuppressive effects (4.5–14 ng/ml). These levels would also minimize other potential adverse effects. Our data suggest that further in vivo studies in mesangiproliferative models are warranted to assess the role of low-dose rapamycin.

GRANTS

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DISCLOSURES

The authors have no potential conflicts of interest.

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