Development of a Sensitive Phospho-p70 S6 Kinase ELISA to Quantify mTOR Proliferation Signal Inhibition

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Background: Drug blood levels can only serve as a surrogate because of the lack of information on the drug's direct pharmacological effects in the individual patient. Measurement of the mammalian target of rapamycin (mTOR) activity dependent on the phosphorylation status of p70 S6 kinase (p70 S6K) offers a practical way for monitoring pharmacodynamic drug activity, with the potential to better assess the state of immunosuppression in individual patients.

Material and Methods: Here, we established a novel in vitro model system by treating Jurkat cells and peripheral blood mononuclear cells with different concentrations of sirolimus after stimulation with phorbol 12-myristate 13-acetate.

Results: A dose-dependent reduction of the p70 S6K phosphorylation status was demonstrated by Western blot and a newly established enzyme-linked immunosorbent assay (ELISA). Relative phospho-p70 S6K values from ELISA and relative densities from Western blot analysis in peripheral blood mononuclear cells revealed a strong correlation (Spearman correlation coefficient r_s = 0.7, P = 0.01). Finally, parallel assays confirmed a sirolimus dose-dependent reduction of cytokine production and cell proliferation in the in vitro model.

Conclusions: Pharmacodynamic monitoring of mTOR inhibition with a p70 S6K ELISA could guide mTOR inhibitor immunosuppression therapy toward a more individualized therapy. The usage of this technique now has to be evaluated in a clinical series of patients.

Key Words: pharmacodynamic monitoring, immunosuppression, mTOR inhibitor, p70 S6 kinase, sirolimus, therapeutic drug monitoring

(*Ther Drug Monit* 2013;35:233–239)

INTRODUCTION

For eligible patients, kidney transplantation offers the best method for renal replacement therapy, but unfortunately, immunosuppressive agents, for example, calcineurin inhibitors, failed to improve long-term outcomes.[1] The proliferation signal inhibitor rapamycin (ie, sirolimus and its derivative everolimus) provides appropriate immunosuppression without significant nephrotoxicity.[2] In addition, sirolimus-based calcineurin inhibitor–free therapy is associated with a decreased incidence of de novo malignancies.[3] Despite such favorable effects, maintenance mammalian target of rapamycin (mTOR) inhibitor therapy has to be stopped in approximately 30%–50% of patients due to related side effects.[3–5] Currently, dose adjustment of the mTOR inhibitors entirely relies on measurement of their whole-blood concentration. Recommended trough levels for sirolimus range from 5 to 15 ng/mL, but sirolimus trough concentrations might not necessarily correlate with the biological effects of the drug on immune cells or other tissues. Sirolimus is extensively partitioned into solvent and formed blood elements.[6] The proportion of the drug that distributes to lymphocytes is only 1% of whole-blood concentration.[7,8] Therefore, small differences in drug partitioning might have a relevant biological impact on the immune system and produce adverse effects. P70 S6 Kinase (p70 S6K) is described as one of the best characterized downstream effectors of mTOR activation.[9] Through activation of mTOR, phospho-p70 S6K stimulates the production of ribosomal components necessary for protein synthesis and cell cycle progression.[10] Pharmacodynamic monitoring of mTOR inhibitor–based immunosuppression by assessment of the phosphorylation status of p70 S6K might represent an important advantage because it enables individualized immunosuppression. The mTOR proliferation signal inhibitor forms a complex with the FK-binding protein. This complex binds with high affinity to the mTOR and causes its inhibition. The downstream effects of mTOR activation are reduced and p70 S6K will be inactivated.[8,11] The feasibility of monitoring mTOR inhibitor–based immunosuppression has been demonstrated by Western blot analysis of the phosphorylation status at the Thr389 site of the p70 S6K in peripheral blood mononuclear cells (PBMCs).[12,13] However, Western blot analysis represents a semiquantitative method and is therefore inapplicable to further large population studies and interindividual comparison. Here, we established a novel method by using a commercial enzyme-linked immunosorbent assay (ELISA) kit that facilitates assessment of the phosphorylation of p70 S6K in a cell model and could enable the pharmacodynamic monitoring of mTOR proliferation signal inhibition.

Received for publication February 20, 2012; accepted November 28, 2012.
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MATERIAL AND METHODS

Cell Culture and Cell Treatment

Freshly isolated PBMCs and Jurkat T-leukemia cells (German Collection of Microorganisms and Cell Cultures) were used. Human PBMCs were isolated from buffy coats after Ficoll density gradient centrifugation. Buffy coat preparations of healthy donors were obtained from the German Red Cross (Deutsches Rotes Kreuz, Germany) after informed consent was obtained.

To increase the amount of phospho-p70 S6K and to have it comparable to the activated immune system after transplantation, we investigated different conditions: starving cells, incubation with insulin or insulin-like growth factor, and also unspecific stimulation with phytohemagglutinin (PHA) or phorbol 12-myristate 13-acetate (PMA). The cells were stimulated with 3 different concentrations of PMA (125, 250, 500 ng/mL) for different times (15, 30, 60 minutes). Best results were achieved after stimulation with PMA (125 ng/mL, incubation time 30 minutes).

To investigate the effect of sirolimus on phospho-p70 S6K, serial concentrations of sirolimus (0, 0.75, 1, 2, 3 nM for Jurkat cells; 0, 0.25, 0.5, 0.75, 1 nM for PBMCs) were added in presence or absence of PMA to the cells. The cells were lysed after incubation at 37°C in humidified air with 5% CO_2 for 30 minutes. These lysates were analyzed for phospho-p70 S6K with Western blot and subsequently with ELISA.

Cell Lysis

Cells were collected by centrifugation at 420*g* for 10 minutes at 4°C and washed with ice-cold phosphate-buffered saline once. Cells (2.5×10^7 Jurkat cells or 10^8 PBMCs) were lysed by incubation with 1 mL of cell extraction buffer [10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS (sodium dodecyl sulfate), 0.5% deoxycholate] supplemented with 1 mM PMSF (Phenylmethylsulfonylfluoride) and a protease inhibitor cocktail (Sigma-Aldrich) on ice for 30 minutes. The cell lysates were clarified by centrifugation at 20,800*g* for 10 minutes at 4°C and stored at −80°C.

Bicinchoninic Acid Protein Assay

Protein concentration was determined by bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Rockford, IL) according to manufacturer's instructions. Ten microliters of standards and 10 μL of diluted lysates were pipetted into a 96-well plate in triplicate. Then 200 μL freshly prepared bicinchoninic acid working reagent was added to each well. The plate was incubated at 37°C for 30 minutes. The absorbance at 562 nm was measured on the plate reader. The protein concentration of each sample was determined by using the standard curve.

Western Blot

Western blot analysis was performed as previously described.[12] Briefly, equal amounts of cell lysates were mixed with 5× sample buffer (58 mM Tris HCl/SDS, 44% glycerol, 9.8% SDS, 0.5 M 2-mercaptoethanol, 0.05% Bromophenol blue), heated at 95°C for 4 minutes. Aliquots that contained 40 mcg of protein from each lysate were subjected to 7.5% SDS polyacrylamide gel electrophoresis with 70 V for 30 minutes and then with 100 V for 60 minutes in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and then electrotransferred into a Hybond P membrane by using 100 V for 60 minutes in transfer buffer (25 mM Tris, 192 mM glycine) with 15% methanol. After blocking in freshly prepared Tris-Tween–buffered saline [TTBS (0.02 M Tris, 0.1 M sodium chloride with 0.1% Tween 20)] containing 3% nonfat dry milk at room temperature for 1 hour, membranes were blotted overnight at 4°C with polyclonal rabbit antiphospho-p70 S6K antibody targeted to threonine 389 (Cell Signaling) with 1:1000 dilution in 5% bovine serum albumin. Membranes were then washed in TTBS 3 times and incubated for 1 hour with an anti-rabbit IgG horseradish-conjugated secondary antibody (Amersham Biosciences) at a 1:5000 dilution in TTBS containing 1% nonfat dry milk. To confirm equal loading, the same membranes were immunoblotted with anti-β-actin mouse monoclonal IgG1 antibodies (Santa Cruz Biotechnology) overnight at 4°C at 1:10,000 dilution in TTBS containing 1% nonfat dry milk. Membranes were then washed in TTBS 3 times and incubated for 1 hour with an anti-mouse IgG horseradish-conjugated secondary antibody (Amersham Biosciences) at a dilution of 1:5000 in TTBS containing 1% nonfat dry milk. The bound proteins were visualized with the enhanced chemiluminescence system. The intensity of signals detected by exposure to Cronex 5 medical x-ray films was quantitated by densitometry analysis (Image J software). Results were expressed by density relative to β-actin and then normalized to basal concentrations of phospho-p70 S6K (Thr389) or phospho-p85 S6K (Thr412) in Jurkat cells or PBMCs.

Phospho-p70 S6K (Thr389) ELISA

Phospho-p70 S6K (Thr389) concentrations were measured with p70 S6K (Thr389) immunoassay kits (Invitrogen, Life Technologies). This test is a solid-phase ELISA. A monoclonal antibody specific for p70 S6K (regardless of phosphorylation state) has been coated into the wells of the microtiter plate. Serial dilutions of the standard (100, 50, 25, 12.5, 6.25, 3.12, 1.6, and 0 U/mL) were prepared. Five microliters of samples were incubated with 5 μL of sample treatment buffer on ice for 20 minutes. These mixtures were then diluted in standard diluent buffer to obtain a protein concentration at 0.1 mg/mL. Standard dilutions containing phospho-p70 S6K (Thr389) and diluted lysates were pipetted into the 96-well plate in duplicate together with a rabbit polyclonal (detection) antibody specific for phospho-p70 S6K (Thr389) and incubated for 3 hours at room temperature. After a washing procedure, the horseradish peroxidase-labeled anti-rabbit IgG (anti-rabbit IgG-HRP) working solution was added to each well for further 30 minutes. Finally, after another washing procedure, a substrate solution was added to induce the color reaction. After incubation for 30 minutes in the dark, a stop solution was added to each well and the absorbance of each well at 450 nm was measured by a microplate reader. Results were expressed in units after normalization to 100 mcg total protein (U/100 mcg protein).

Proliferation Assays

Proliferation assays were performed by the CellTiter 96 AQueous One Solution Reagent (Promega) containing a tetrazolium compound [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium, inner salt; MTS]. MTS is bioreduced by cells into a colored formazan product, which is soluble in tissue culture medium. This conversion is presumably accomplished by nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt or dihydronicotinamide adenine dinucleotide produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

Cytokine Determinations

Supernatants were collected from Jurkat cells (0.3×10^6 cells/mL) and incubated with 3 μM ionomycin plus 5 ng/mL PMA and different concentrations of sirolimus (0, 0.75, 1, 2, and 3 nM) or DMSO (0.01%) at 37°C in a humidified atmosphere with 5% CO_2 for 48 hours.

Similarly, the supernatants were collected after treatment of PBMCs (0.6×10^6 cells/mL) with 20 mcg/mL PHA plus 125 ng/mL PMA. Serial concentrations of sirolimus (0, 0.25, 0.5, 0.75, and 1 nM) or DMSO (0.01%) were added at 37°C in humidified atmosphere with 5% CO_2 for 72 hours. The cytokines (IL-2, TNF, IFN-γ) in the supernatants were determined by ELISA (BD Biosciences).

Statistical Analyses

Results are presented as mean ± SD. Statistical significance ($P < 0.05$) was evaluated using 1-way analysis of variation (phospho-p70 S6K ELISA) or paired t test (proliferation assays, cytokine determinations). Statistical significance ($P < 0.01$) was evaluated using bivariate Spearman rank correlation analysis between the relative phospho-p70 S6K values from ELISA and logarithmically transformed relative densities from Western blot analysis. SPSS software (version 11.5) was used for all statistical analyses. Sigmaplot (2001) was used for semi-log scatter plot.

RESULTS

Stimulation With PMA Increased Low Basal Concentrations of Phospho-p70 S6K

Western blot analysis detected the 2 isoforms of the S6 Kinase p70 S6K and p85 S6K. The basal concentration of phospho-p70 S6K expression was very low. The phorbol ester phorbol 12-myristate 13-acetate (PMA), an activator of the mTOR pathway,[14–16] was used to increase the amount of phospho-p70 S6K. On the other hand, PBMC stimulated with PMA might also be comparable to the activated immune system after transplantation. Obvious phosphorylation of p70 S6K was achieved 15–30 minutes after stimulation with different concentrations of PMA in PBMCs. In the following experiments, 125 ng/mL of PMA was added to cells and incubated for 30 minutes. Interestingly, PMA prompted only a weak phosphorylation of p86 SK6 (Fig. 1).

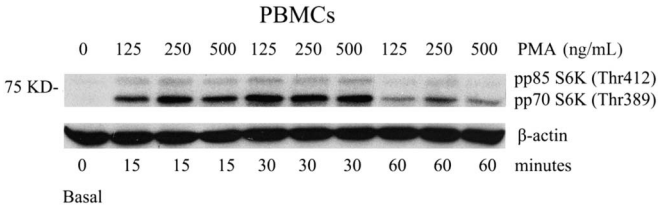

FIGURE 1. Time-course and dose–response of S6K1 phosphorylation after stimulation with PMA determined by Western blot. The position of the 2 isoforms of phospho-S6K1, phospho-p70 S6K (Thr389) and phospho-p85 S6K (Thr412), are indicated. β-actin served as the loading control.

Sirolimus Induced a Dose-dependent Depression of p70 S6K Phosphorylation in PMA-stimulated PBMCs

Cells were incubated with different concentrations of sirolimus (0–1 nM) together with PMA (125 ng/mL) stimulation for 30 minutes. In Western blot analysis (Fig. 2A), the phospho-S6K1 phosphorylation was measured at Thr389 (p70 S6K) and Thr412 (p85 S6K) as previously described.[16] In PMA-stimulated PBMCs, sirolimus prevented phosphorylation of p70 S6K in a dose-dependent manner (Fig. 2B). A PMA-induced increase in phospho-S6K1 was dose-dependently downregulated by sirolimus, although the extent of downregulation was lower than that of phospho-p70 S6K. In contrast, the PMA-induced slight increase in phospho-p85 S6K did not show a significant downregulation by sirolimus. In PMA-stimulated cells, the downregulating effect of sirolimus on the elevated concentrations of phospho-S6K1 was similar to that on phospho-p70 S6K.

Contrary to the results in PMA-stimulated cells, in unstimulated cells, the phospho-p85 S6K band was stronger than the phospho-p70 S6K band, suggesting that in unstimulated cells phospho-p85 S6K contributed much more to the concentrations of phospho-S6K1 than phospho-p70 S6K. Densitometry analysis of Western blots from 20 buffy coats (Fig. 2C) demonstrated the same tendency as that from the representative buffy coat (Fig. 2B).

Parallel Measurements of Cell Proliferation and Distinct Cytokine Production Confirmed Efficacy of Sirolimus Treatment

To analyze whether sirolimus-induced downregulation of phospho-p70 S6K parallels to the downregulation of the cellular immune functions, MTS proliferation assays were performed and concentrations of selected cytokines (IL-2, TNF and IFN-γ) were determined (Fig. 3).

In PBMCs, PMA and the costimulator PHA were used to activate cytokine production. Sirolimus (0.25–1.0 nM) inhibited PMA/PHA-activated cytokine production (IL-2 and TNF) in PBMCs (Fig. 3). The sirolimus-induced downregulation of IFN-γ was not statistically significant, probably because of high individual variations.

Additionally, cell proliferation after stimulation with PHA was measured (data not shown). Sirolimus (0.25–1.0 nM) inhibited PHA-activated proliferation in PBMCs.

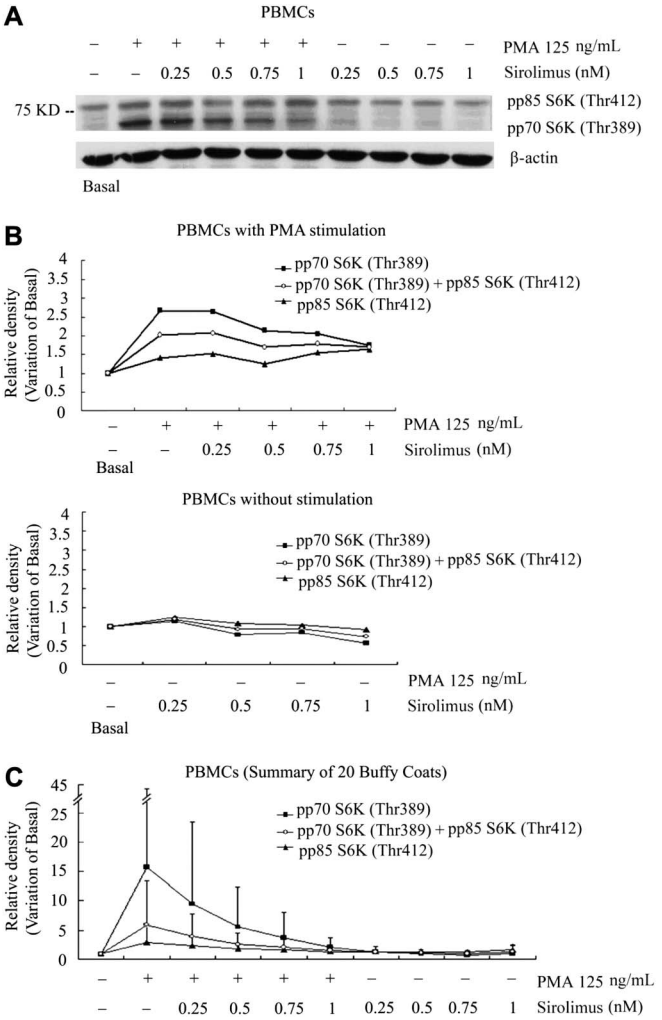

FIGURE 2. Effect of sirolimus on phospho-S6K1 in PMA-stimulated and unstimulated PBMCs determined by Western blot. A, Western blot analysis. B and C, Densitometeric analysis of Western blot results. Results were expressed by density relative to β-actin and then normalized to basal concentrations of phospho-p70 S6K (Thr389) and/or phospho-p85 S6K (Thr412) in PBMCs. Results from a representative buffy coat in PMA-stimulated cells (B, upper part) and unstimulated cells (B, lower part) are shown. The densitometeric analysis of 20 buffy coats are summarized as a mean ± SD in (C).

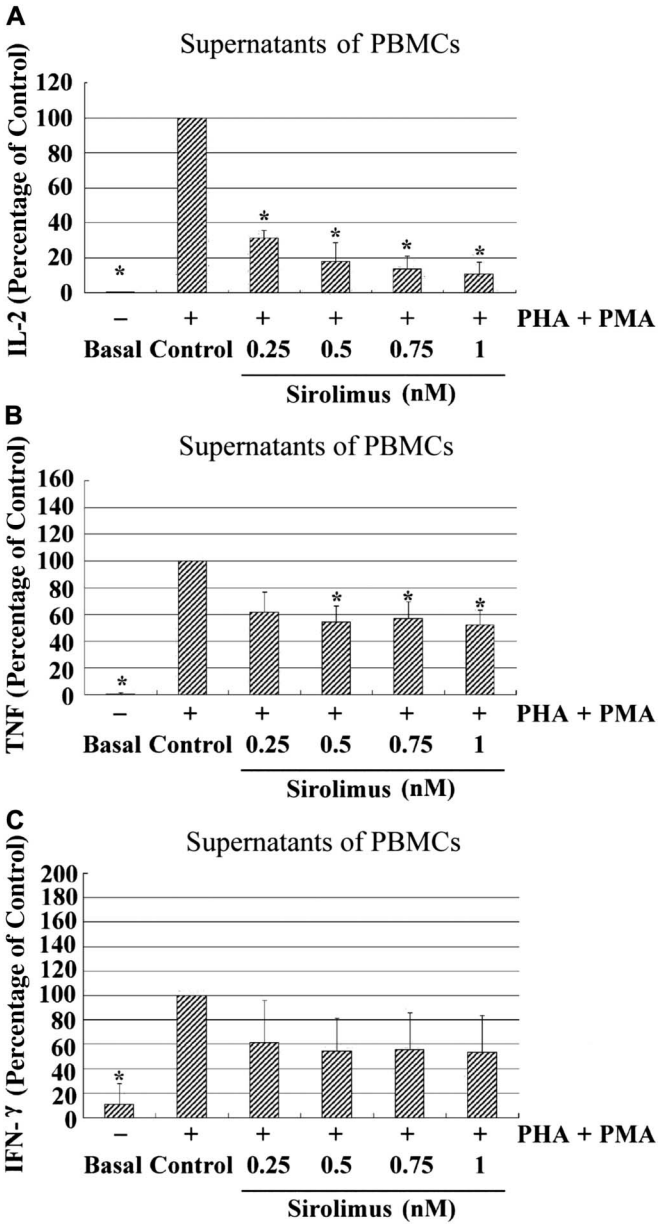

FIGURE 3. Sirolimus inhibited PHA/PMA-activated cytokine secretion of PBMCs. The supernatants were quantified for IL-2 (A), TNF (B), and IFN-γ (C) by Human IL-2, TNF, and IFN-γ ELISA. Results were expressed as the percentage of the values in PHA/PMA-activated sirolimus-untreated PBMCs (Control). Results from PBMCs without stimulation were taken as basal value. Data were presented as a mean ± SD. Differences between quantitative variables were analyzed by paired *t* test (*$P < 0.05$, versus Control). Three independent experiments were performed.

The inhibitory effects of sirolimus on cell proliferation and cytokine production were not influenced by dimethyl sulfoxide (DMSO), which was used as the solvent of sirolimus. These results confirmed the efficacy of sirolimus treatment in vitro and demonstrated that reduction of phospho-p70 S6K paralleled the reduction of proliferation and immune competence of lymphocytes as indicated by the IL-2, TNF, and IFN-γ production. This observation supports the hypothesis that phosphorylated p70 S6K serves as a promising biomarker of the effect of sirolimus on mTOR.

A Novel p70 S6K ELISA Analysis Showed a Dose-dependent Downregulation of Phospho-p70 S6K by Sirolimus in PMA-stimulated PBMCs

To quantitatively determine phospho-p70 S6K, the concentrations were measured with a p70 S6K ELISA in the

same samples that had been previously analyzed by Western blot (Fig. 4). The average concentrations of phospho-p70 S6K were 3.09 U/100 mcg protein in unstimulated PBMCs, which were increased to 12.39 U/100 mcg protein in PMA-stimulated cells, respectively (buffy coats, n = 20). Similar to Western blot, ELISA detected a dose-dependent depression of p70 S6K phosphorylation induced by sirolimus in PMA-stimulated cells. The inhibition of phospho-p70 S6K detected by ELISA was lower than that of phospho-p70 S6K detected by Western blot; moreover, the inhibition was more similar to that of phospho-S6 K1 (phospho-p70 S6K plus phospho-p85 S6K) detected by Western blot (data not shown).

Log-Linear Correlation Between the Relative Phospho-p70 S6K Values From ELISA and the Relative Densities From Western Blot Analysis Was Significant

To study the correlation between ELISA and Western blot measurements, we compared relative values of phospho-p70 S6K (normalized to basal concentrations) obtained from ELISA with relative densities of phospho-p70 S6K (variation of basal) obtained from Western blot by bivariate Spearman rank correlation analysis. The semi-log scatter plot is shown in Figure 5A. The Spearman correlation coefficient was $r_s = 0.70$. Correlation was significant at the 0.01 level (2-tailed). These results demonstrate a strong correlation between the phospho-p70 S6K ELISA and Western blot phospho-p70 S6K measurements, although the inhibition of phospho-p70 S6K detected by ELISA was lower than that detected by Western blot.

The Stability of the ELISA-based System Showed a Low Interassay Variation

To evaluate the stability of the whole system, a Jurkat cell line was chosen as a control. Protein extracts from PMA-stimulated (125 ng/mL for 30 minutes) Jurkat cells were obtained from 8 independent experiments. The average

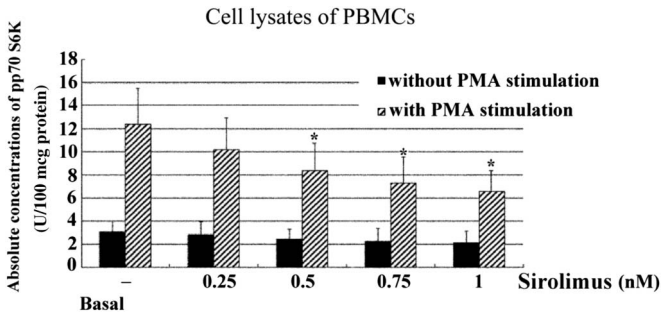

FIGURE 4. Effect of sirolimus on phospho-p70 S6K in PMA-stimulated and unstimulated PBMCs determined by the ELISA-based method. The absolute concentrations of phospho-p70 S6K were measured in the same samples analyzed by Western blot. Results were expressed in units after normalization to 100 mcg total protein (U/100 mcg protein). Data shown were from 20 buffy coats and presented as mean ± SD. In PMA-stimulated cells, differences between quantitative variables were analyzed by 1-way analysis of variance test. *$P < 0.05$, versus sirolimus-untreated PBMCs.

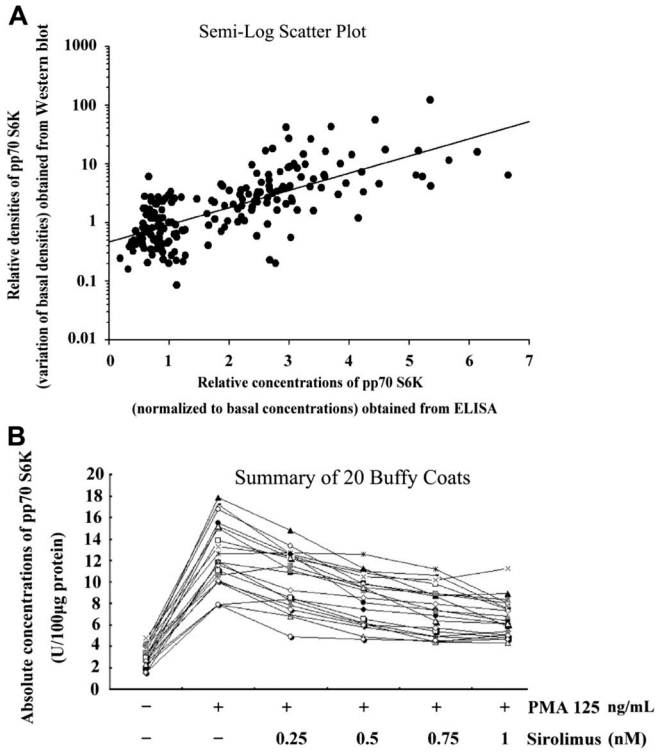

FIGURE 5. A, Spearman rank correlation analysis ($r_s = 0.70$, $P = 0.01$) between the relative phospho-p70 S6K values from ELISA and the log-transformed relative densities from Western blot analysis in PBMCs. PBMCs isolated from 20 individuals were incubated with serial concentrations of sirolimus (0, 0.25, 0.5, 0.75, and 1.0 nM) alone or together with PMA (125 ng/mL). Two hundred data points are shown and the trend line is indicated. B, Similar tendency in responses of PBMCs to serial concentrations of sirolimus observed by the ELISA-based method. The absolute values of phospho-p70 S6K from 20 buffy coats were summarized in the presence of PMA.

phospho-p70 S6K value was 9.25 U/100 mcg protein, and coefficient of variation was marked with 23%, which represented the whole system variation except the interassay variation between ELISAs. One cell lysate from PMA-stimulated PBMCs isolated from one buffy coat was analyzed in 5 assays using ELISA kits with 3 different lot numbers to determine precision between assays. The interassay variation was 6.7%.

ELISA Confirmed Individual Variability of p70 S6K Phosphorylation Among 20 Buffy Coats

The variability of p70 S6K phosphorylation was evaluated in 20 distinct buffy coats. Response of PBMCs to serial concentrations of sirolimus (0–1 nM) after stimulation with PMA (125 ng/mL) is shown (Fig. 5B). PMA elevated the absolute concentrations of phospho-p70 S6K in all specimens; moreover, sirolimus downregulated the concentrations of phosphorylated kinase in a dose-dependent fashion in PMA-stimulated cells. The downregulation of phospho-p70 S6K in unstimulated PBMCs was not as evident as that in PMA-stimulated cells. The similarity of the results among distinct

individuals confirms that our procedure for PMA stimulation and sirolimus treatment on PBMCs is stable and reproducible.

Nonetheless, we observed interindividual variabilities of p70 S6K phosphorylation not only in PBMCs without any treatment but also in PBMCs with PMA stimulation and/or inhibition with sirolimus. The interindividual variability in absolute values of phospho-p70 S6K in the absence of PMA was between 31% and 50% and that in the presence of PMA was between 25% and 31%. In PMA-stimulated PBMCs, we further calculated the relative phospho-p70 S6K concentrations in sirolimus-treated cells to that in untreated cells and assessed interindividual variability. Interestingly, the interindividual variability was reduced to 15%–25%. Thus, the relative phosphorylation concentrations among the individual cells were more comparable than the absolute phosphorylation concentrations.

DISCUSSION

Pharmacodynamic monitoring has long been a main objective to improve the use of immunosuppressive medication. P70 S6K is one of the best characterized downstream effectors of mTOR[8,9] and might reflect at best differential sensitivity of the mTOR raptor complex 1 to inhibition by sirolimus.[8,17,18] Measuring the phosphorylation status of p70 S6K offers a possible method for pharmacodynamic monitoring of mTOR proliferation inhibitor.[12,13,19–23] In a previous study, we demonstrated the clinical relevance of measurement of the p70 S6K phosphorylation status by Western blot.[12] Our observation that there is no correlation of sirolimus trough level and the p70 S6K phosphorylation status was confirmed in a subsequent study.[13] Furthermore, a distinct stimulation of the p70 S6K phosphorylation status was necessary to obtain a more reliable measurement. Although measurement of the specific phosphorylation status of the p70 S6K at the Thr389 site in cell lysates is challenging, it was our aim to establish an ELISA to facilitate monitoring of mTOR inhibitor treatment.

Similar to the Western blot, the ELISA detected a dose-dependent suppression of p70 S6K phosphorylation by sirolimus in PMA-stimulated cells. The inhibition rate of phospho-p70 S6K detected by ELISA was lower than that of phospho-p70 S6K detected by Western blot. One explanation for this finding is that both isoforms share the same antibody-binding site. The larger isoform of S6Kl, the p85 S6K, is uniquely distinct from the shorter isoform in which it contains an amino-terminal 23-amino acid extension.[11] Even so, in our experiments, the effects of a dose-dependent downregulation from sirolimus treatment were not influenced by phospho-p85 S6K (Thr 412) in PMA-stimulated cells because the contribution of phospho-p70 S6K to the concentrations of phospho-S6K1 exceeded that from phospho-p85 S6K by far. In addition, a log-linear correlation was observed between relative phospho-p70 S6K values from ELISA and relative densities from the Western blot analysis.

Leogrande et al[13] found that with Western blot analysis, sirolimus (10 ng/mL, even higher than ours) did not modify basal p70 S6K phosphorylation in control PBMCs; instead, the mTOR inhibitor significantly downregulated insulin-stimulated phosphorylation of p70 S6K in a dose-dependent fashion. These findings and ours demonstrate that both Western blot and ELISA-based method were not sensitive enough to detect the downregulation by sirolimus of the basal phospho-p70 S6K activity. The PMA stimulation in vitro might mimic the activation of mTOR in vivo by a transplant or a tumor, respectively. As well as the molecular effects, a parallel functional impact of p70 S6K inhibition on the cytokine production was detected, that is, a significant downregulation of IL-2 and TNF by sirolimus in a dose-dependent fashion (Fig. 3).

The interindividual variability of basic p70 S6K phosphorylation in PBMCs measured by our ELISA-based method was 31%. The result is similar to the interindividual variability in healthy controls using immunoblot analysis (39%).[13] Possible explanations for the high interindividual variability are substantial individual variations. Furthermore, the low absolute basal concentration values of phosphorylation in PBMCs have a greater variability because they are located in the very beginning of the standard curve. Interindividual variability in PMA-treated cells was between 25% and 31%. The substantial variation in stimulated PBMCs observed among individuals emphasized the future need to use each patient as his or her own control in the analysis of pharmacodynamic markers.

We presume that PMA-stimulated cells corresponded to the original state of basal activation. The phosphorylation ratio indicates phosphorylation relative to PMA-stimulated cells. The phosphorylation ratio was downregulated when sirolimus concentration increased. Under the same sirolimus concentration, the buffy coat from different individuals had different p70 S6K phosphorylation ratios. Previously, the same phenomenon was observed.[12,13] Clinically, patients with similar sirolimus trough levels may show different clinical outcomes. This finding implies that some patients with the same sirolimus blood levels develop relevant rejection episodes as a consequence of insufficient suppression of p70 S6K phosphorylation, whereas others have serious adverse events due to oversuppression. Our results have shown that the ELISA technique enables the use of phospho-p70 S6K in PBMCs as a biomarker of mTOR.

We are aware of the principal limitations of our system: (1) First of all, the conclusions drawn from our cell culture system have to be translated and further evaluated in the clinical setting. A cell culture system, although consisting of PBMCs from individual donors, cannot be able to mimic in every respect the complex clinical situation after transplantation. For example, here we constantly measured cytokine production after an incubation period of 72 hours, and also we are aware that, for example, IL-2 production by CD4-positive T cells in vivo is more rapid. (2) Furthermore, even though ELISA needs less blood than Western blot, the amount is still more than that needed for trough concentration measurement. This might be a problem with frequent testing in anemic patients. (3) Although the assay is easier to perform than Western blot, the whole ELISA-based system is a multistep procedure including PBMCs isolation, activation of cells with PMA, cell lysis, protein concentration assay, and performing the ELISA itself. Failing in any step will affect accuracy. (4) Moreover, to calculate the phosphorylation ratio (percentage of state without sirolimus), it is necessary to obtain blood from

patients before sirolimus administration and to activate cells in vitro with PMA. Then, after starting sirolimus therapy, this procedure has to be repeated to evaluate the p70 S6K phosphorylation response to sirolimus.

The utility of ELISA methodology extends well beyond transplant immunosuppression. Further clinical applications are oncology medicine and treatment of renal and cerebral tumors in tuberous sclerosis. Conferring a proliferative advantage, the sirolimus-sensitive signal transduction pathway is a potential target for cancer therapy in several types of neoplasms. Consequently, the definition of a recommended dose range associated with optimal biological activity is an important challenge. Different approaches have been undertaken to establish a biochemical monitoring of mTOR inhibition, especially in the field of antitumor therapy.[19–22] Overactivation of basal p70 S6K in PBMCs from kidney transplant recipients was associated with the presence of Kaposi sarcoma dermal lesions; conversely, kinase inhibition was linked to regression of skin cancer lesions.[23] Thus, monitoring of the p70 S6K phosphorylation status in PBMCs might help to predict and monitor the biological effectiveness of sirolimus in patients with Kaposi sarcoma and potentially other carcinoma and might help to adjust the biologically appropriate mTOR inhibitor doses.

Recently, the technique of phospho-flow cytometry has been validated for the detection of an mTOR-signaling–specific pharmacodynamic biomarker in peripheral human blood.[24] In addition, the feasibility of real-time, direct pharmacodynamic monitoring by flow cytometry was demonstrated during clinical trials combining intensive chemotherapy and signal transduction inhibitors,[25] but further validation in patients after solid organ transplantation is urgently needed.

In summary, we successfully established an ELISA-based method to investigate the immunosuppressive properties of sirolimus by assessing the phosphorylation status of p70 S6K. Although this method needs activation of the cells by PMA and the results may be influenced by p85 S6K phosphorylated at its analogous sites, the dose-dependent downregulation effect of sirolimus on the phosphorylated kinase was not altered by PMA or p85 S6K. Future large-scale studies now have to translate this technique into clinical practice and should determine the phospho-p70 S6K interindividual and intrasubject variability over time, the effect of interaction between mTOR inhibitors and other immunosuppressive drugs, and the target value of the phosphorylation ratio in renal transplant recipients.

ACKNOWLEDGMENTS

B. H. received a financial grant for scientific work from Wyeth Pharma GmbH, now Pfizer Pharma GmbH. The authors thank Ms. Rosa Herzog for her excellent technical assistance.

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