

Rapamycin Inhibits Telomerase Activity by Decreasing the *hTERT* mRNA Level in Endometrial Cancer Cells¹

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Abstract

Rapamycin exerts its biological activity by inhibiting the kinase mammalian target of rapamycin (mTOR), which regulates important cellular processes such as control of cell cycle and cell size, translation initiation, and transcription. The ability of rapamycin to inhibit cancer cell proliferation has led to efforts to develop rapamycin and related mTOR inhibitors as anticancer agents. Some investigators have hypothesized that loss of the PTEN tumor suppressor may sensitize tumor cells to the antiproliferative activity of rapamycin because PTEN loss leads to activation of the mTOR pathway. Because PTEN loss is frequent in endometrial cancer, we have characterized the effect of rapamycin in endometrial cancer cells. We show that rapamycin in the nanomolar concentration range exerts a potent growth-inhibitory effect on endometrial cancer cells through induction of cell cycle arrest. This effect is independent of PTEN status because PTEN-positive ECC-1 cells are as sensitive to rapamycin as PTEN-null Ishikawa and Hec-1B cells, suggesting that rapamycin may be effective against a broad range of endometrial cancers. We also show that rapamycin rapidly inhibits telomerase activity by decreasing the mRNA level of *hTERT*, the catalytic subunit of telomerase. This implies that rapamycin leads to inhibition of *hTERT* gene transcription. We demonstrate that rapamycin inhibits phosphorylation of downstream targets of mTOR such as p70^{S6K} kinase and 4E-BP1 translation repressor. This work suggests that rapamycin is a potentially useful targeted therapy for endometrial cancer and that loss of telomerase activity may be a good surrogate biomarker for assessing antitumor activity of rapamycin.

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Introduction

The macrolide antibiotic rapamycin exerts its immunosuppressive and antiproliferative activity in mammalian cells through its ability to inhibit a serine/threonine kinase termed mTOR³ (1). Rapamycin forms an intracellular complex with a 12-kDa immunophilin protein FK506-binding protein 12, and this complex in turn binds to mTOR with high affinity, resulting in inactivation of mTOR kinase activity. mTOR regulates many important cellular processes including control of cell cycle and cell size, translation initiation, transcription, ribosomal biogenesis, cytoskeletal organization, and autophagy (2, 3). Rapamycin has been shown to have growth-inhibitory activity against a variety of cancer cell lines *in vitro* and in preclinical animal models (4). Rapamycin and its ester analogue, CCI-779, are undergoing clinical development as anticancer agents (5). Because mTOR is activated by the PI3K/Akt pathway, several groups have made a connection between sensitivity of tumor cells to rapamycin and loss of PTEN, a tumor suppressor involved in inhibition of the PI3K/Akt pathway (6).

Telomerase has attracted much attention both as a diagnostic marker and as a therapeutic target in oncology. Telomerase is an enzyme that maintains the TTAGGG telomeric repeat units found at the distal ends of chromosomes. Without telomerase activity, somatic cells suffer from progressive erosion of telomeres that leads to cellular senescence, and therefore, activation of telomerase appears to be a critical event in carcinogenesis (7). The core telomerase holoenzyme is made up of the structural RNA hTR and the catalytic protein hTERT with reverse transcriptase activity (8). Transcriptional activation of the *hTERT* gene appears to be the limiting factor in telomerase activity because most somatic cells do not express *hTERT* mRNA. Transcription of *hTERT* is regulated by several transcription factors, such as Myc, Mad1, E2F, Sp1, p53, and steroid hormone receptors (8). However, the role of the mTOR signaling pathway in the regulation of telomerase activity is unknown.

Endometrial cancer continues to be the most common gynecologic malignancy in the United States with 39,300 new cases and 6,600 deaths estimated for 2002 (9). The molecular events that lead to endometrial cancer are beginning to emerge. Loss of PTEN occurs in 60–90% of endometrial cancers and is observed in premalignant stages of endometrial carcinogenesis, suggesting that PTEN loss is an important initiator of endometrial cancer development (10). PTEN exerts its tumor-suppressive function through its activity as a phospholipid phosphatase, leading to inhibition of

³ The abbreviations used are: mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3'-kinase; RT-PCR, reverse transcription-PCR; TPG, total product generated; TRAP, telomeric repeat amplification protocol.

PI3K signaling and inactivation of downstream kinases such as Akt and mTOR (6, 11). Because PTEN loss leads to an increase in mTOR signaling activity, rapamycin may be a potentially valuable therapeutic agent in endometrial cancer. Novel agents are needed, given that current chemotherapeutic agents have low efficacy and unfavorable side effects.

In this work, we demonstrate that rapamycin exhibits potent growth-inhibitory activity in endometrial cancer cells independent of PTEN status. In addition, rapamycin inhibits telomerase activity and *hTERT* mRNA expression and also inhibits phosphorylation of mTOR downstream targets p70^{S6K} and 4E-BP1.

Materials and Methods

Cell Culture and Reagents. Three human endometrial carcinoma cell lines were used in this study. Ishikawa and Hec-1B cells were grown in MEM supplemented with 5% fetal bovine serum, 5 μ g/ml bovine insulin, 100 units/ml penicillin, and 100 μ g/ml streptomycin under 5% CO₂. ECC-1 cells were maintained in RPMI 1640 containing 5% fetal bovine serum, 200 pg/ml estrogen, and 6 mM sodium bicarbonate. Rapamycin and LY294002 were purchased from Sigma (St. Louis, MO). Polyclonal anti-phospho-p70^{S6K}, anti-phospho-S6, anti-phospho-Akt, and anti-phospho-4E-BP1 as well as their nonphosphorylated antibodies and mTOR antibody were from Cell Signaling Technology (Beverly, MA). Anti- β -actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence Western blotting detection reagents were from Amersham (Arlington Heights, IL). All other chemicals were from Sigma.

Cell Proliferation Assay. Ishikawa, ECC-1, and Hec-1B cells (1×10^5) were plated in triplicate onto 6-well culture plates in regular medium. The next day, the medium was changed, and rapamycin was added. Cells were incubated for 7 days. Cell counts were performed with a hemocytometer.

Flow Cytometric Analysis and Detection of Apoptosis. Cultured cells were washed with a PBS solution and trypsinized. Cells were fixed in an ethanol (80%)/PBS solution at 4°C for 30 min. The cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were filtered through an 80- μ m-diameter mesh and analyzed by flow cytometry (FACScalibur; Becton Dickinson, San Jose, CA). For detection of apoptosis, the Cell Death ELISA Plus kit (Roche, Indianapolis, IN) measuring the amount of DNA fragmentation released into the cytoplasm was used. Briefly, cells were plated at a density of 2.5×10^5 cells/well in 6-well plates with regular medium. After 24 h, cells were treated with rapamycin or DMSO vehicle for 24 or 48 h. Both detached and adherent cells were harvested and processed according to the manufacturer's directions. Absorbance data are represented as a fold increase over the untreated control sample.

Telomerase Activity Assay. Telomerase activity was determined using a PCR-based TRAP assay as described previously (12). The TRAP-eze telomerase detection kit (Intergen, Purchase, NY) was used as recommended by the manufacturer, with minor modifications. Briefly, 10^5 cells

were lysed in 150 μ l of 1×3 -[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid lysis buffer supplied with the kit. Protein (0.25–0.5 μ g) was used for the TRAP assay in the presence of the ³²P-labeled TS primer in a 50- μ l reaction mixture. After 30 min of incubation at 30°C, PCR amplification was performed with 27 cycles at 94°C for 30 s and 59°C for 30 s. The PCR products were separated by electrophoresis on 10% polyacrylamide nondenaturing gels. PhosphorImager and Imagequant software from Molecular Dynamics (Sunnyvale, CA) were used to quantify the band intensities. Telomerase activity is expressed quantitatively as TPG, which reflects a ratio of the TRAP product ladder bands to the internal telomerase assay standard band and was calculated according to the formula supplied in the manufacturer's manual. The reliability and linearity of TPG as a measure of telomerase activity have been confirmed (13).

Real-Time RT-PCR for *hTERT*. Total RNA was isolated by using the RNAqueous kit (Ambion, Austin, TX) and further purified by the DNA-free kit (Ambion). The reverse transcription and PCR reactions were performed using the TaqMan Gold one-step RT-PCR kit in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Reverse transcription was carried out at 48°C for 30 min. The PCR condition consisted of a 10-min step at 95°C and 40 cycles at 95°C for 15 s and 65°C for 1 min. A housekeeping control gene acidic ribosomal phosphoprotein P0 (*RPLP0*, also known as *36B4*) was used as an internal control to correct for differences in the amount of RNA in each sample (14). Primers and fluorogenic probes for *hTERT* and *RPLP0* have been described previously (14). The standard curve for *hTERT* was generated by using dilutions of a known amount of cRNA synthesized by *in vitro* transcription of a cloned fragment. The normalized level of *hTERT* in each sample was estimated by a ratio of the *hTERT* level to the *RPLP0* level, as described previously (14).

Western Blot Analysis. Ishikawa cells at a density of 2.5×10^5 cells/well in 6-well plates were incubated in serum-free medium. After 24 h of serum starvation, cells were treated with serum-containing medium and rapamycin or LY294002 or vehicle (DMSO). Cell lysates were prepared in radioimmunoprecipitation assay buffer (1% NP40, 50 mM Tris, and 150 mM NaCl). Equal amounts of protein were separated by gel electrophoresis and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk and then incubated with a 1:1000 dilution of primary antibody overnight at 4°C. The membrane was washed and incubated with a secondary peroxidase-conjugated antibody for 1 h after washing. Antibody binding was detected using the enhanced chemiluminescence detection system. Western blot films were digitized, and band net intensities were quantified using a Millipore Digital Bioimaging System (Bedford, MA). After developing, the membrane was stripped and reprobbed using antibody against β -actin or nonphosphorylated antibodies to confirm equal loading.

Results

Sensitivity of Endometrial Cancer Cells to Rapamycin.

We examined the effect of rapamycin on proliferation of three endometrial cancer cell lines. As shown in Fig. 1A, rapamycin

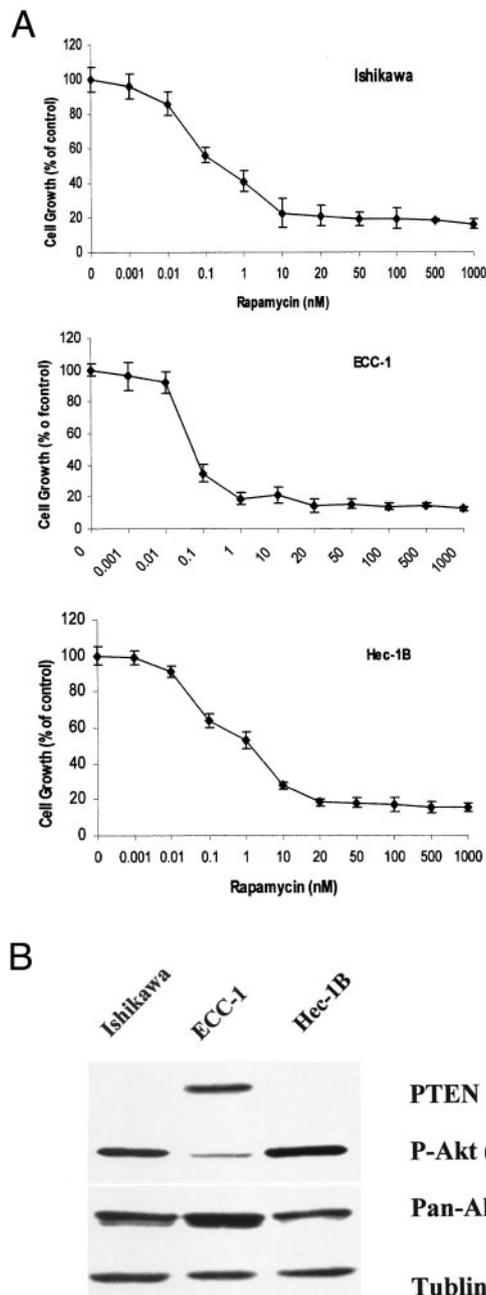


Fig. 1. Rapamycin inhibits cell proliferation. **A**, Ishikawa, ECC-1, and Hec-1B cells were cultured in the presence of varying concentrations of rapamycin. The cell number was determined after 7 days of incubation and normalized to the untreated control. The results are shown as the mean \pm SE of triplicate samples and are representative of two independent experiments. **B**, PTEN and Akt status in endometrial cancer cell lines. Total cellular proteins were analyzed by immunoblotting with antibodies specific for PTEN, phospho-Akt (Thr³⁰⁸), pan-Akt, and tubulin.

potently inhibited growth of all three cell lines in a dose-dependent manner with IC₅₀ values for Ishikawa, ECC-1, and Hec-1B cells of 0.5, 0.05, and 1.1 nM, respectively. Because several reports indicate that loss of PTEN sensitizes tumor cells to rapamycin, the PTEN expression level was determined by immunoblotting. ECC-1 cells expressed PTEN,

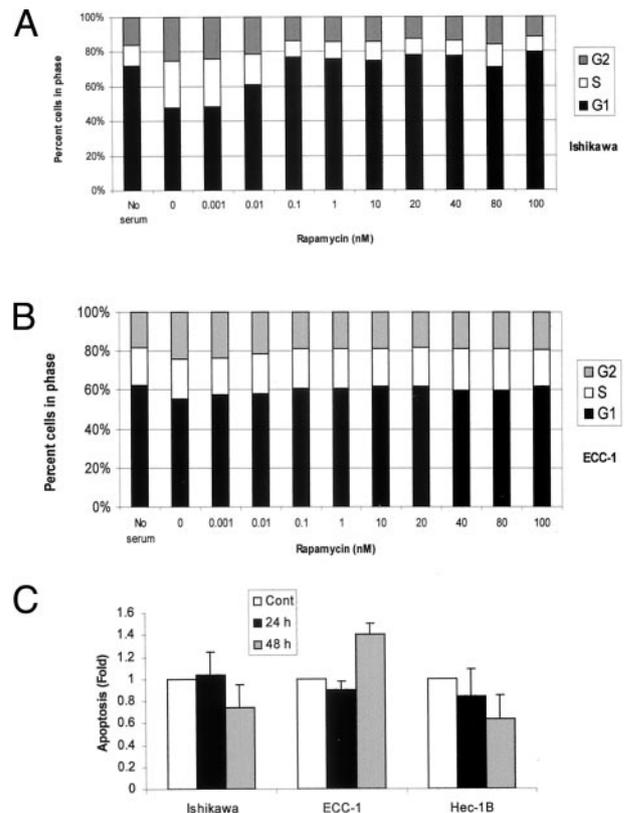


Fig. 2. Rapamycin inhibits cell cycle progression but does not induce apoptosis. **A** and **B**, after serum starvation for 24 h, Ishikawa (**A**) and ECC-1 (**B**) cells were stimulated with 15% serum and the indicated concentrations of rapamycin for 48 h. The cells were analyzed for DNA content by flow cytometry. **C**, cells were cultured in regular medium and then treated with rapamycin (20 nM) or DMSO as a control for 24 and 48 h, and the extent of apoptosis was quantified. Absorbance readings representing the amount of DNA fragmentation were normalized to the untreated control samples.

whereas there was no detectable PTEN expression in Ishikawa and Hec-1B cells (Fig. 1B). The level of activated phospho-Akt expression was inversely correlated with PTEN expression. Phospho-Akt was increased in PTEN-null Ishikawa and Hec-1B cells and decreased in PTEN-positive ECC-1 cells. PTEN-positive ECC-1 cells were equally, if not more, sensitive to growth inhibition by rapamycin, compared with PTEN-null Ishikawa or Hec-1B cells. Taken together, these data show that rapamycin inhibits proliferation of endometrial cancer cells regardless of their PTEN status.

Effect of Rapamycin on Cell Cycle and Apoptosis. To determine the mechanism of growth inhibition by rapamycin, we analyzed the cell cycle profile and induction of apoptosis after treatment with rapamycin. As expected, serum starvation resulted in accumulation of cells in G₁ phase. Serum stimulation resulted in transition of cells from G₁ to S phase by 24 h, with a concomitant decrease in G₁ phase. Rapamycin significantly blocked serum-induced entry to S phase in a dose-dependent manner in Ishikawa and ECC-1 cells (Fig. 2, **A** and **B**). In addition, there was no significant increase in apoptosis after treatment with rapamycin using an assay measuring the amount of DNA fragmentation (Fig. 2C). Fur-

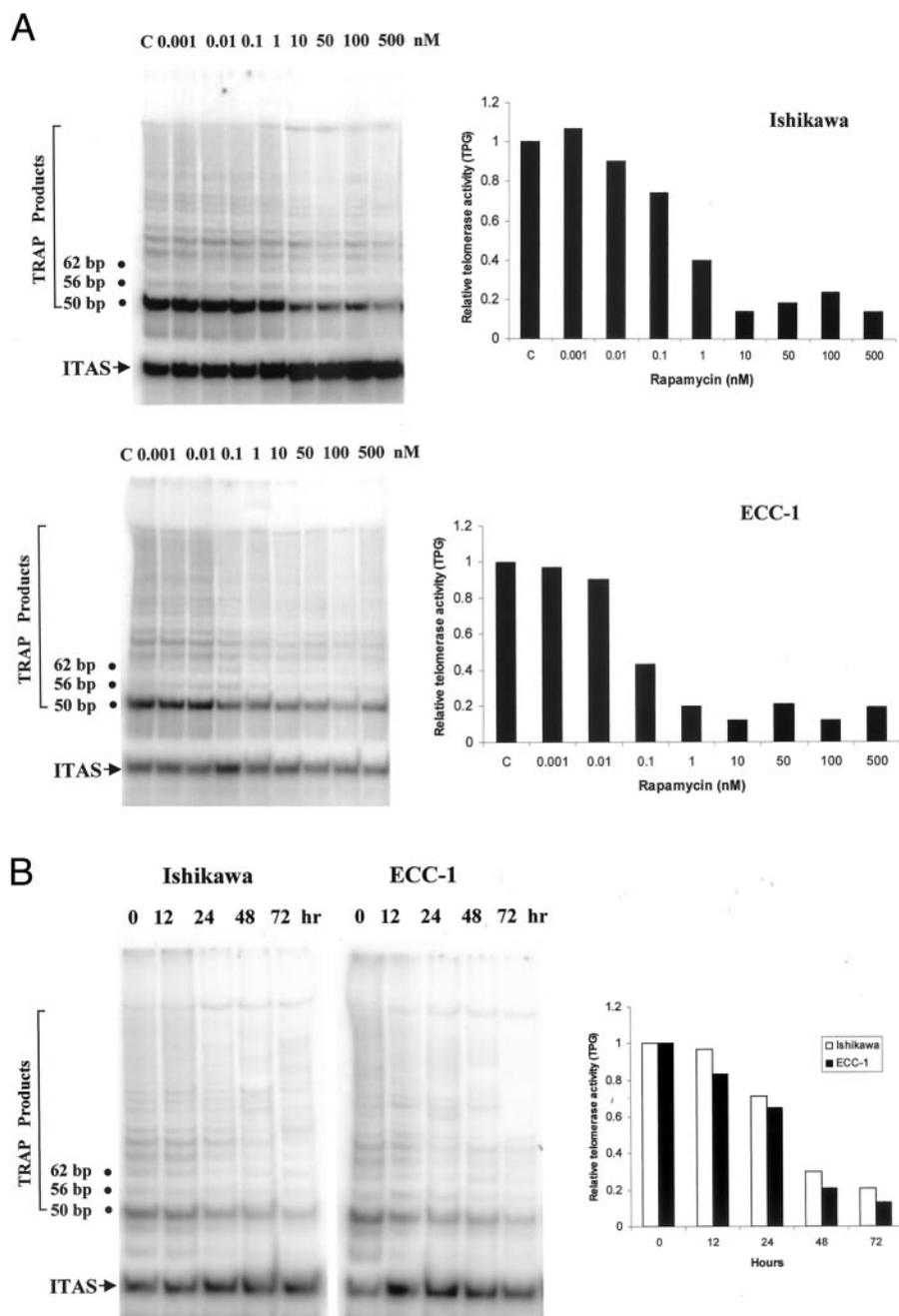


Fig. 3. Rapamycin inhibits telomerase activity. **A**, Ishikawa and ECC-1 cells were cultured for 24 h and then treated with the indicated concentrations of rapamycin or control (Lane C) for 48 h. Cell lysates were assayed for telomerase activity using the TRAP assay. *Left panels*, TRAP assay products separated by gel electrophoresis. *ITAS*, internal telomerase assay standard. *Top left panel*, Ishikawa cells; *bottom left panel*, ECC-1 cells. *Right panels*, relative telomerase activity is derived from TPG, which is calculated from the ratio of TRAP product bands to the internal telomerase assay standard as described in "Materials and Methods." **B**, Ishikawa cells and ECC-1 cells were treated with rapamycin (20 nM) for the indicated times, and telomerase activity was assayed as described in **A**. *Left panel*, TRAP assay products separated by gel electrophoresis. *Right panel*, relative telomerase activity. Data shown are representative of at least two independent experiments.

thermore, rapamycin did not increase the amount of cells in the sub- G_1 fraction, confirming no increase in apoptosis (data not shown). These results suggest that rapamycin inhibits cell growth via cell cycle arrest and not by induction of apoptosis in endometrial cancer cells.

Effect of Rapamycin on Telomerase Activity and *hTERT* mRNA Level. Constitutive expression of telomerase activity is vital to the ability of cancer cells to maintain telomere length and unlimited proliferative potential. We investigated the effect of rapamycin on telomerase activity. Treatment of cells with rapamycin resulted in inhibition of telomerase activity in a dose-dependent manner in Ishikawa

and ECC-1 endometrial cancer cells (Fig. 3A). Inhibition of telomerase activity occurred as early as 24 h after rapamycin treatment (Fig. 3B). To understand the mechanisms underlying loss of telomerase activity, we quantified by real-time RT-PCR the mRNA level of the *hTERT* gene encoding the catalytic subunit of telomerase because *hTERT* mRNA expression is usually the rate-limiting determinant of telomerase enzymatic activity. Treatment with rapamycin reduced the *hTERT* mRNA level in a dose-dependent manner (Fig. 4). These data suggest that rapamycin rapidly leads to loss of telomerase activity and that this inhibition of telomerase activity is through the decreased *hTERT* mRNA level.

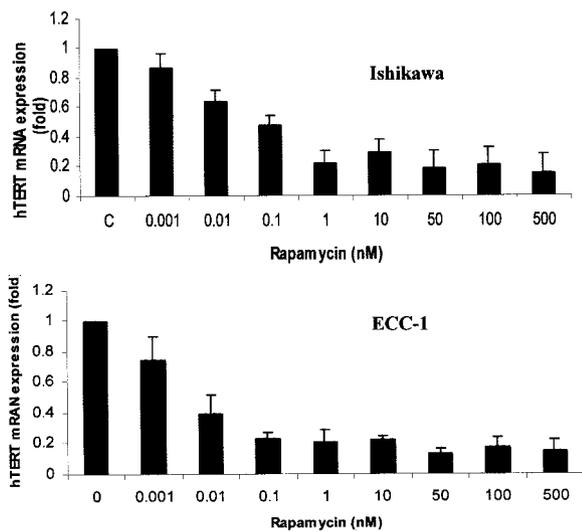


Fig. 4. Rapamycin decreases the *hTERT* mRNA level. Ishikawa cells and ECC-1 cells were cultured for 24 h and then treated with the indicated concentrations of rapamycin or control (C) for 48 h. The *hTERT* mRNA level was determined by real-time RT-PCR. The results are shown as the mean \pm SE of three independent experiments, each done in triplicate.

Effect of Rapamycin on mTOR Signal Transduction Pathway.

To investigate the mechanisms underlying inhibition of proliferation and telomerase activity, we characterized the effect of rapamycin on relevant cell signaling pathways. To determine how rapamycin affects the function of Akt, we examined the effect of rapamycin and LY294002, an inhibitor of PI3K, on serum-induced activation of Akt. LY294002 inhibited serum-induced Akt phosphorylation at Thr³⁰⁸ and Ser⁴⁷³, whereas rapamycin did not, consistent with the model that rapamycin functions downstream of Akt in these cells (Fig. 5). Previous studies suggest that p70^{S6K} is a downstream target of mTOR (15). p70^{S6K} kinase directly phosphorylates the 40S ribosomal protein S6, which results in enhanced translation of proteins that contain a polypyrimidine tract in the 5'-untranslated region (16). Therefore, we studied the phosphorylation of p70^{S6K} and S6 induced by serum in Ishikawa and ECC-1 cells. Because p70^{S6K} activity involves a hierarchical phosphorylation of several sites by different kinases, we selected the anti-phospho-p70^{S6K} antibody specific for the Thr³⁸⁹ site, which has been shown to correlate most clearly with p70^{S6K} activity (17). S6 protein is phosphorylated on Ser^{235/236} by p70^{S6K} (18). As shown in Fig. 5, serum stimulation induced p70^{S6K} phosphorylation within 15 min, and the p70^{S6K} phosphorylation remained at this level for at least 90 min. Both rapamycin and LY294002 blocked serum-stimulated phosphorylation of p70^{S6K}. Ishikawa cells maintained a low level of basal S6 phosphorylation even after 48 h of serum starvation. In contrast, serum stimulation increased both phospho-S6 and total S6 levels, suggesting that phosphorylation of S6 may lead to stabilization of the protein. Further work is required to understand the mechanisms underlying this process. Both rapamycin and LY294002 blocked serum induction of phospho-S6 and total S6, which confirms that the PI3K/Akt and mTOR pathways

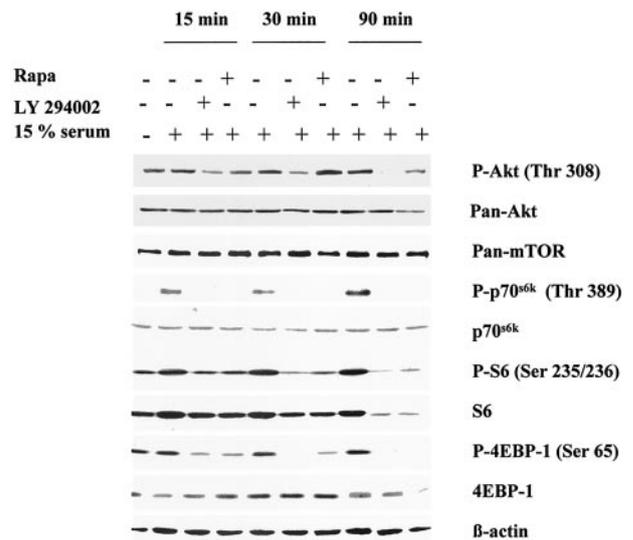


Fig. 5. Effect of rapamycin on Akt, p70^{S6K}, S6, and 4E-BP1 phosphorylation. Ishikawa cells were serum-starved for 24 h and then exposed to 15% serum for varying times in the presence of rapamycin (20 nM) or LY294002 (20 μ M) as indicated. Total proteins were analyzed by immunoblotting with antibodies against phospho-Akt (Thr³⁰⁸), pan-Akt, total mTOR, phospho-p70^{S6K} (Thr³⁸⁹), total p70^{S6K}, phospho-S6 (Ser^{235/236}), total S6, phospho-4E-BP1 (Ser⁶⁵), and total 4E-BP1 as indicated. Data shown are representative of three independent experiments.

are important in p70^{S6K} kinase regulation and S6 phosphorylation.

mTOR kinase regulates protein translation by phosphorylating the translation repressor 4E-BP1 on multiple sites (19). The phosphorylation status of 4E-BP1 regulates binding to eukaryotic initiation factor 4E. Hyperphosphorylation of 4E-BP1 disrupts its binding to eukaryotic initiation factor 4E, activating cap-dependent translation. Addition of serum to starved cells strongly induces phosphorylation of Ser⁶⁵ and Thr⁷⁰ of 4E-BP1 (20). To determine whether the mTOR/4E-BP1 pathway is involved in serum-induced cell proliferation, we performed immunoblotting using anti-phospho 4E-BP1 (Ser⁶⁵) antibody in serum-induced Ishikawa and ECC-1 cells in the absence or presence of rapamycin and LY294002. As expected, serum significantly increased phosphorylation of 4E-BP1. This effect was abrogated by both rapamycin and LY294002 despite stable protein levels of 4E-BP1 (Fig. 5). These experimental data suggest that rapamycin exerts its effect through the PI3K/Akt/mTOR pathway by regulating phosphorylation of both S6 and 4E-BP1 proteins.

Discussion

In this work, we demonstrate that rapamycin potently suppresses proliferation of endometrial cancer cells by induction of cell cycle arrest and concomitantly causes rapid loss of telomerase activity by decreasing the *hTERT* mRNA level. In addition, we show that rapamycin inhibits phosphorylation of downstream targets of mTOR such as p70^{S6K} and 4E-BP1. Because PTEN loss results in constitutive activation of the PI3K/Akt pathway and mTOR is a downstream target of this pathway, mTOR is expected to be constitutively active in

PTEN-null cancer cells. This has led some investigators to hypothesize that PTEN may be a good predictor of rapamycin sensitivity in cancer cells. Using a genetically defined mouse model system or PTEN-null cancer cells, several groups have demonstrated that loss of PTEN function confers enhanced sensitivity to rapamycin or its ester analogue, CCI-779 (21–24). However, our data in endometrial cancer cells clearly show that PTEN-positive ECC-1 cells are as sensitive to growth inhibition by rapamycin as PTEN-null Ishikawa and Hec-1B cells. PTEN protein expression in ECC-1 cells is capable of suppressing Akt activation, whereas loss of PTEN protein in Ishikawa and Hec-1B cells leads to constitutive expression of activated phospho-Akt. These results agree with previously published data on PTEN mutations in endometrial cancer cell lines and also provide confirmation of the PTEN status at the functional level in these cells (25). Our observation that the PTEN status does not predict sensitivity to rapamycin agrees with data from a breast cancer model (26). Although the factors responsible for these disparate conclusions are unclear, it is possible that sensitivity of cancer cells to rapamycin may still be linked to Akt activation. Although ECC-1 cells express lower levels of phospho-Akt than PTEN-null Ishikawa and Hec-1B cells, ECC-1 cells may express more activated Akt than normal endometrial tissue and thereby be differentially sensitized to rapamycin. This hypothesis requires further investigation. In any case, the ability of rapamycin to inhibit endometrial cancer cell proliferation, regardless of PTEN status, suggests that rapamycin and its analogues may have useful clinical activity against a broad range of endometrial cancers and provides a rationale for its further development in clinical trials.

The major novel finding from this work is that rapamycin inhibits telomerase activity. Because both telomerase activity and the *hTERT* mRNA level are proportionately decreased, our experimental data would imply that rapamycin exerts its effect on telomerase at the level of *hTERT* gene transcription. A potential connection between rapamycin and telomerase regulation is through its effect on the cell cycle. Regulation of telomerase during the cell cycle is controversial. One group of investigators reported that telomerase activity is high during S phase but undetectable in G₂-M phase (27). Another group showed that telomerase activity does not change during the cell cycle but that exit from the cell cycle and quiescence produced by serum starvation lead to loss of telomerase activity (28). However, serum starvation did not inhibit telomerase activity in cell lines used in our work (data not shown). This suggests that the decreased *hTERT* transcription and loss of telomerase activity observed in our study are not merely a nonspecific consequence of cell cycle arrest but rather a specific downstream effect caused by the ability of rapamycin to inhibit the mTOR signaling pathway. Observations that support this hypothesis include experiments demonstrating that many differentiation-inducing agents such as IFN- α , transforming growth factor β , vitamin D₃, and retinoids repress *hTERT* transcription (8). Rapamycin-sensitive pathways are known to regulate transcription in yeast and mammalian cells. In yeast, rapamycin rapidly changes the global transcription program through

regulating nuclear localization of certain transcription factors (29–31). In mammalian cells, mTOR modulates the activity of transcription factors such as signal transducers and activators of transcription 3, E2F, and hypoxia-inducible factor 1 α (32–35). Identification of transcription factors targeted by the mTOR pathway, which are able to regulate *hTERT* gene expression, will be the focus of future investigation.

Another implication of this work is that telomerase activity may serve as a biomarker for assessing the cellular response to rapamycin *in vivo*. Because many novel targeted agents currently in development act as cytostatic agents, a rapid assessment of their cellular and biochemical effects on tumor specimens that predict antitumor activity would be desirable. For rapamycin, the phosphorylation status of 4E-BP1 has been suggested as a surrogate marker of mTOR kinase inhibition in tumors (36). Similarly, we suggest that inhibition of telomerase activity may be a useful molecular marker for inhibition of proliferation of tumor cells after treatment with rapamycin.

To gain insight into mechanisms by which rapamycin inhibits cell cycle progression and *hTERT* transcription, we characterized the signaling pathways affected by rapamycin. Using LY294002 and rapamycin, we show that the PI3K/Akt pathway is upstream of mTOR. Our data are consistent with reports showing that Akt directly or indirectly controls phosphorylation and activity of mTOR (37, 38). mTOR regulates two parallel pathways, p70^{S6K} kinase and 4E-BP1, which regulate initiation of translation and cell cycle progression through downstream effectors such as cyclin D (39, 40). Whether mTOR modulates telomerase through these or additional pathways will require further study. In conclusion, our work suggests that rapamycin is a potentially useful targeted therapy for endometrial cancer and that loss of telomerase activity may be a good surrogate biomarker for assessing antitumor activity of rapamycin.

Acknowledgments

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