Tumorigenesis and Neoplastic Progression

Activation of Mammalian Target of Rapamycin Signaling Promotes Cell Cycle Progression and Protects Cells from Apoptosis in Mantle Cell Lymphoma

Evangelia Peponi, Elias Drakos,
Guadalupe Reyes, Vasiliki Leventaki,
George Z. Rassidakis, and L. Jeffrey Medeiros

From the Department of Hematopathology, The University of
Texas M.D. Anderson Cancer Center, Houston, Texas

Mantle cell lymphoma (MCL) is characterized by the t(11;14) and cyclin D1 overexpression. However, additional molecular events are most likely required for oncogenesis, possibly through cell cycle and apoptosis deregulation. We hypothesized that mammalian target of rapamycin (mTOR) is activated in MCL and contributes to tumor proliferation and survival. In MCL cell lines, pharmacological inhibition of the phosphoinositide 3-kinase/AKT pathway was associated with decreased phosphorylation (activation) of mTOR and its downstream targets phosphorylated (p)-4E-BP1, p-p70S6 kinase, and p-ribosomal protein S6, resulting in apoptosis and cell cycle arrest. These changes were associated with down-regulation of cyclin D1 and the anti-apoptotic proteins cFLIP, BCL-XL, and MCL-1. Furthermore, silencing of mTOR expression using mTOR-specific short interfering RNA decreased phosphorylation of mTOR signaling proteins and induced cell cycle arrest and apoptosis. Silencing of eukaryotic initiation factor (eIF4E), a downstream effector of mTOR, recapitulated these results. We also assessed mTOR signaling in MCL tumors using immunohistochemical methods and a tissue microarray: 10 of 30 (33%) expressed ^{Ser473}p-AKT, 13 of 21 (62%) ^{Ser2448}p-mTOR, 22 of 22 (100%) p-p70S6K, and 5 of 20 (25%) p-ribosomal protein S6. Total eIF4E binding protein 1 and eukaryotic initiation factor 4E were expressed in 13 of 14 (93%) and 16 of 29 (55%) MCL tumors, respectively. These findings suggest that the mTOR signaling pathway is activated and may contribute to cell cycle progression and tumor cell survival in MCL. (*Am J Pathol* 2006, 169:2171–2180; DOI: 10.2353/ajpath.2006.051078)

Mantle cell lymphoma (MCL) is an aggressive type of B-cell non-Hodgkin's lymphoma characterized by the t(11;14)(q13;q32) and overexpression of cyclin D1.¹ This translocation, by juxtaposing *CCND-1* (cyclin D1) with the immunoglobulin heavy chain gene, places the *CCND-1* gene under the transcriptional control of immunoglobulin heavy chain gene enhancers.^{2–4} The understanding of MCL biology has been advanced considerably in recent years.⁵ Although earlier studies using transgenic mice failed to show that cyclin D1 overexpression is oncogenic,^{6,7} a recent study has shown that nuclear retention of cyclin D1 is oncogenic.⁸ Nevertheless, better biological understanding of MCL has not yet been translated into improved clinical outcome.⁹

Mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family.¹⁰ It constitutes the core of an evolutionarily conserved pathway that regulates cell growth and proliferation by integrating signals arising from growth factors, nutrients, and energy status.¹¹ In mammals, there are two mTOR protein complexes: the regulatory associated protein of mTOR (Raptor)-G protein β -subunit-like protein (G β L)-mTOR complex and the recently discovered rapamycin-insensitive companion of mTOR (Rictor)-G β L-mTOR complex. The Raptor-G β L-mTOR complex can be inhibited by the immunosuppressive macrolide rapamycin and is critical for most aspects of cell growth regulation.¹¹ The Rictor-G β L-mTOR complex is insensitive to rapamycin and regulates the organization of the actin cytoskeleton.^{12,13}

E.P. and E.D. contributed equally to this work.

G.Z.R. and J.M. contributed equally to this work.

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Current address of E.P.: Department of Radiotherapy, Saint Savvas Anticancer Center, Athens, Greece.

Address reprint requests to L. Jeffrey Medeiros, M.D., Department of Hematopathology, Unit 72, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. E-mail: ljmediros@mdanderson.org.

Among the best-characterized downstream effectors of the mTOR pathway are the translation regulators p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1).¹⁰ Phosphorylation and activation of p70S6K by mTOR leads to increased translation and ribosomal biogenesis through an incompletely understood mechanism that involves phosphorylation of ribosomal protein (rp) S6.^{11,14} Phosphorylation of 4E-BP1 by mTOR releases its inhibitory effect on eIF4E, promoting cap-dependent mRNA translation.^{14,15}

A major regulator of the mTOR pathway is the PI3K/AKT kinase cascade.^{10,16,17} Activation of PI3K/AKT has been found in a plethora of tumors, including hematological malignancies, and is believed to contribute to carcinogenesis, tumor progression, and drug resistance.^{18–21} Activation of the PI3K/AKT cascade by various growth factors leads, through TSC1/TSC2 (tuberous sclerosis complex) and Rheb (Ras homolog enriched in brain), to activation of mTOR, promoting cell growth and proliferation.^{10,22,23} Recent evidence also suggests that the rapamycin-insensitive Rictor-G β L-mTOR complex regulates AKT activation, through phosphorylation at Ser473.²⁴

Rapamycin or rapamycin analogs have been shown to have considerable *in vitro* or *in vivo* antitumor activity in many types of tumors, including hematological malignancies.^{25–28} Recent phase II clinical trials using rapamycin analogs to treat MCL patients have shown promising results.²⁹ However, the activation status of the AKT/mTOR signaling pathway and its biological significance in MCL remains unknown.

Using well-characterized MCL cell lines and tumors, we investigated the expression and activation status of AKT/mTOR pathway components. We found that mTOR signaling is activated in MCL and may contribute to tumor cell growth through cell cycle progression and inhibition of apoptosis.

Materials and Methods

Cell Lines and Reagents

Three MCL cell lines (Mino, Z-138, and Jeko-1) were used.³⁰ The Z-138 cell line was established from a patient with the blastoid variant of MCL.³¹ An ALK⁺ anaplastic large cell lymphoma (ALCL) cell line (Karpas 299) was used as a control. Karpas 299, Mino, and Jeko-1 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1% streptomycin-penicillin. Z-138 cells were maintained in Iscove's modified medium (Gibco-BRL, Grand Island, NY) with 10% horse serum (Sigma, St. Louis, MO). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂.

LY294002 (Calbiochem, San Diego, CA) was dissolved in dimethylsulfoxide and added to the cultures (10⁵ cells/ml/12-well plate) at concentrations of 0, 5, 10, and 20 μ g/ μ l. Dimethylsulfoxide was added to the control groups. LY294002 is a synthetic flavinoid that acts as a potent, competitive, and reversible inhibitor of the ATP-binding site of class I PI3K.³² Mino, Jeko-1, and Z-138

cells were also treated with rapamycin (Calbiochem, San Diego, CA) at concentrations of 0, 10, 20, and 40 nmol/L. Whole-cell lysates were prepared 48 hours after treatment.

Western Blot Analysis

Western blot analysis was performed using standard methods as previously described.³³ The antibodies used in this study were specific for Ser⁴⁷³phosphorylated (p)-AKT, total AKT, Ser²⁴⁴⁸p-mTOR, p-p70S6K, p70S6K, p-rpS6, rpS6, p-4E-BP1, 4E-BP1, eIF4E, and p21 (Cell Signaling Technology, Beverly, MA); cyclin D1 and cFLIP (Santa-Cruz Biotechnology, Santa Cruz, CA); MCL-1 and BCL-2 (Dako, Carpinteria, CA); p27 and cyclin A (BD Biosciences Pharmingen, San Diego, CA); BCL-XL (Zymed, South San Francisco, CA); and β -actin (Sigma). Detection was performed using enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ).

Selective Inhibition of mTOR and eIF4E

Expression with Short Interfering RNA (siRNA)

The sequences of mTOR siRNA were as follows: 5'-GG-AGUCUACUCGCUUCUAUTT-3' (sense) and 5'-AUA-GAAGCGAGUAGACUCCTC-3' (antisense). The eIF4E siRNA sequences were as follows: 5'-GGAGGUUGC-UAACCCAGAAAtt-3' (sense) and 5'-UUCUGGGUAG-CAACCUCtg-3' (antisense). All siRNA sequences were purchased from Ambion, Inc. (Austin, TX). Transient transfection of Jeko-1 and Z-138 cells was performed using the Nucleofector solution "T" recommended by Amaxa Biosystems (Gaithersburg, MD). Approximately 2×10^6 cells were transfected with 0, 20, and 40 μ g of mTOR siRNA or 0 and 10 μ g of eIF4E siRNA or control siRNA at the highest equivalent dose (negative control #3 siRNA; Ambion, Inc.) using 100 μ l of Kit T solution and program T-01 (Amata Biosystems) according to the manufacturer's protocol. Cells were harvested at 48 hours after transient transfection with increasing concentrations of mTOR siRNA or eIF4E siRNA, and whole-cell lysates were prepared. Total mTOR and eIF4E protein levels were assessed by Western blot analysis to confirm adequate inhibition of mTOR and eIF4E expression in transfected cells.

Cell Viability and Apoptosis Studies

Cell viability was evaluated using trypan blue exclusion cell counts in triplicate. Annexin V staining (BD Biosciences Pharmingen), detected by flow cytometry, was used to assess apoptosis according to the manufacturer's instructions. In brief, the cells were washed in ice-cold phosphate-buffered saline (PBS) and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. Subsequently, aliquots of 100 μ l (1×10^5 cells/ml) were incubated with 5 μ l of annexin V-fluorescein isothiocyanate and 5 μ l of propidium iodide for 15 minutes in the dark at room temperature, and 1×10^4 ungated cells

were counted using a flow cytometer (FACSCalibur; Becton-Dickinson, San Jose, CA). Control cells were included in each set of experiments. All experiments were performed at least twice.

Apoptosis was also assessed morphologically by 4,6-diamidino-2-phenylindole (DAPI) staining. Cytospin cell preparations (100 μ l at a concentration of 1.0×10^6 cells/ml) were stained with DAPI (Sigma) for 5 minutes at room temperature. Subsequently, cells were examined under a fluorescence microscope. Cells were considered to be apoptotic when the nuclei showed chromatin condensation and fragmentation.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium (MTS)

Cells were treated with LY294002, rapamycin, mTOR-siRNA, or eIF4E-siRNA in 12-well plates using different concentrations as indicated. At 48 hours, a tetrazolium compound MTS was added to each well, and MTS-positive cells were counted using the CellTiter 96 AQueous cell proliferation assay (Promega, Madison, WI) and μ Quant spectrophotometer (BIO-TEK Instruments Inc., Winooski, VT) according to the manufacturer's instructions.

5-Bromo-2'-Deoxyuridine (BrdU) Incorporation Assay and Cell Cycle Analysis

Cell cycle S-phase fraction was assessed by a colorimetric BrdU incorporation assay. In brief, cells (10^4 /well) were incubated with BrdU diluted 1:100 in 96-well plates for 1 to 1.5 hours at 37°C. An anti-BrdU antibody peroxidase conjugate (Roche Molecular Biochemicals, Mannheim, Germany) was used at a 1:200 dilution according to the manufacturer's protocol. After appropriate washings, the colorimetric reaction was achieved using a substrate (tetramethyl-benzidine, TMB) and evaluated using a plate reader (μ Quant spectrophotometer; BIO-TEK Instruments). Multiple readings were obtained every 5 minutes for 30 minutes to ensure that the colorimetric reaction had reached its endpoint. Cell cycle analysis was performed using propidium iodide staining and flow cytometry (FACSCalibur; Becton-Dickinson).

Immunohistochemistry

A manual tissue microarrayer (Beecher Instruments, Silver Spring, MD) was used to construct a tissue microarray as previously described.³⁴ The tissue microarray used included duplicate tumor cores from 30 cases of MCL.

The immunohistochemical methods have been described previously.³³ The monoclonal antibodies used were specific for ^{Ser473}p-AKT, ^{Ser2448}p-mTOR, p-p70S6K, p-rpS6, total 4E-BP1, and total eIF4E (Cell Signaling Technology). For all antibodies, heat-induced epitope retrieval was performed. Cores from reactive follicular

hyperplasia included in the tissue microarray served as internal controls. The diagnosis of MCL was based on the criteria of the World Health Organization classification.³⁵ All tumor specimens were obtained before therapy. Expression levels for each marker were determined by counting at least 1000 neoplastic cells in each case. Based on the distribution of the data (histograms), we used a 10% cutoff for positivity.

Statistical Analysis

The Fisher's exact test was used to compare the expression of AKT/mTOR signaling proteins in MCL tumors. The nonparametric Spearman *r* correlation coefficient was chosen to assess the strength of association between expression of mTOR signaling proteins as continuous variables (percentage of positive MCL tumor cells). Statistical calculations were performed using StatView (Abacus Concepts, Inc., Berkeley, CA).

Results

AKT/mTOR Pathway Is Activated in MCL Cell Lines and Tumors

We assessed the baseline activation status of the AKT/mTOR pathway in the MCL cell lines Mino, Jeko-1, and Z-138. Western blot analysis revealed high levels of expression of ^{Ser473}p-AKT and ^{Ser2448}p-mTOR as well as downstream molecules such as p-p70S6K, p-4E-BP1, and total eIF4E (Figure 1a). With the exception of p-rpS6, the expression levels of these phosphoproteins were comparable with the Karpas 299 cell line (ALCL) previously shown to exhibit high levels of phosphorylated mTOR signaling proteins.³⁶

To determine the activation status of the AKT and mTOR signaling proteins in MCL tumors, we immunohistochemically assessed the phosphorylation status of AKT, mTOR, p70S6K, and rpS6 in 30 tumor specimens obtained from previously untreated patients with MCL. We also determined the expression of total eIF4E and 4E-BP1, downstream effectors of mTOR. We found that ^{Ser473}p-AKT and ^{Ser2448}p-mTOR were expressed in 10 of 30 (33%) tumors and 13 of 21 (62%) tumors, respectively, with a predominantly cytoplasmic staining pattern (Figure 1b). p-p70S6K was expressed in a variable percentage of tumor cells in all 22 tumors assessed with a predominantly nuclear staining pattern (Figure 1b). By contrast, p-rpS6 was expressed in 5 of 20 (25%) MCL tumors. Total 4E-BP1 and eIF4E were positive in 13 of 14 (93%) and 16 of 29 (55%) tumors, respectively. ^{Ser473}p-AKT expression did not correlate with mTOR expression in this study group ($P = 0.58$, Fisher's exact test). p-mTOR expression was significantly associated with total eIF4E expression as a categorical ($P = 0.006$, Fisher's exact test) or continuous (Spearman $r = 0.58$, $P = 0.02$) variable. In addition, there was a statistical trend toward a positive correlation between p-p70S6K and p-rpS6 (Spearman $r = 0.49$, $P = 0.07$) and between p-p70S6K and eIF4E (Spearman $r = 0.48$, $P = 0.059$) as continuous variables.

Figure 1. Expression of mTOR signaling proteins in MCL cell lines and tumors. **a:** The MCL cell lines Mino, Z-138, and Jeko-1 and the ALK⁺ ALCL cell line Karpas 299 (used as a positive control) were assessed by immunoblotting for expression of phosphorylated (p)-AKT, p-mTOR, p-p70S6K, p-4E-BP1, and p-rpS6. Expression of total 4E-BP1 and eIF4E was also assessed. All three MCL cell lines showed high levels of activated AKT/mTOR signaling phosphoproteins. Total mTOR was also expressed in all MCL cell lines assessed (data not shown). β-Actin was used as a control for protein load and integrity. **b:** Thirty MCL tumors were assessed immunohistochemically for expression of p-AKT, p-mTOR, p-p70S6K, p-rpS6, total 4E-BP1, and total eIF4E. p-AKT, p-mTOR, p-rpS6, total eIF4E, and total 4E-BP1 were predominantly expressed in the cytoplasm of tumor cells. Expression of p-p70S6K was predominantly nuclear in all MCL tumors studied.

Inhibition of PI3K/AKT/mTOR Down-Regulates Phosphorylation of 4E-BP1, p70S6K, and rpS6K in MCL Cells

The PI3K inhibitor LY294002 is capable of binding to the active sites of PI3K and mTOR, abolishing their activity.³⁷ Treatment of all three MCL cell lines with LY294002 resulted in decreased expression levels of Ser⁴⁷³p-AKT, Ser²⁴⁴⁸p-mTOR, and p-4E-BP1 in a concentration-dependent manner (Figure 2a). The levels of phosphorylated p70S6K and rpS6K were also decreased, whereas total p70S6K and rpS6K remained constant (Figure 2b).

To study further the effects of selective mTOR inhibition on 4E-BP1, p70S6K, and rpS6 activation in MCL cell lines, we performed transient transfection experiments using siRNA specific for the *mTOR* gene product. Treatment of Jeko-1 and Z-138 cell lines with increasing concentrations of mTOR-specific siRNA resulted in a marked concentration-dependent decrease of Ser²⁴⁴⁸p-mTOR, p-p70S6K, p-rpS6, and p-4E-BP1 (Figure 2c). Total eIF4E levels remained constant.

Inhibition of mTOR Induces Cell Cycle Arrest in MCL Cells

Analysis of the cell cycle in MCL cells, after inhibition of mTOR by LY294002 or mTOR-specific siRNA, showed arrest. As shown in Figure 3a LY294002 substantially reduced the percentage of BrdU-incorporating tumor cells in a concentration-dependent manner. Cell cycle arrest at G₁ phase was confirmed by cell cycle analysis (Figure 3a). A similar decrease in S-phase fraction was observed after inhibition of mTOR expression using specific siRNA (Figure 3b).

To evaluate the molecular basis underlying cell cycle arrest after mTOR inhibition, we assessed a number of cell cycle-regulatory proteins after treatment with LY294002 or specific mTOR siRNA. Cyclin D1 levels were decreased with increasing concentrations of LY294002 or specific mTOR siRNA. Likewise, cyclin A levels were decreased. In contrast, the cyclin-dependent kinase inhibitors p21^{waf1} and p27^{kip1} were up-regulated, in a concentration-dependent manner (Figure 3, c–e). These results suggest that inhibition of mTOR expression or function in MCL cells induced cell cycle arrest, predominantly at G₁ phase.

Inhibition of mTOR Induces Apoptosis in MCL Cells

To determine whether inhibition of mTOR induces apoptosis in MCL cell lines, we studied cell viability and apoptosis after treatment of Jeko-1 and Z-138 cells with increasing concentrations of LY294002 or mTOR-specific siRNA.

Both treatments decreased the number of viable cells in a concentration-dependent manner (Figure 4). Treatment with 20 μg/ml LY294002 decreased the number of viable cells by 34%. Likewise, silencing *mTOR* by mTOR-

Figure 2. Inhibition of mTOR expression or function down-regulates AKT/mTOR signaling in MCL cells. **a** and **b**: Treatment with LY294002 resulted in decreased phosphorylation of AKT/mTOR signaling proteins in MCL cells. Three MCL cell lines, Jeko-1, Z-138, and Mino were treated with the PI3K inhibitor LY294002 at concentrations of 0, 5, 10, or 20 $\mu\text{g/ml}$. Whole-cell lysates were then prepared at 48 hours. After treatment of Jeko and Z-138 cells with increasing concentrations of LY294002, phosphorylation of AKT, mTOR, and 4E-BP1 (downstream effector) was decreased. In addition, rpS6 was markedly decreased, whereas total rpS6 levels remained constant. The levels of p-p70S6K were decreased at a lesser degree after treatment with LY294002. Similar results were obtained using Mino cells (not shown). **c**: Silencing of mTOR using specific siRNA also resulted in decreased phosphorylation of mTOR signaling proteins in MCL cells. Jeko-1 cells were transiently transfected with 20 μg of mTOR siRNA or 20 μg of control siRNA (Ambion), and whole-cell lysates were prepared at 48 hours. Western blot analysis demonstrated that inhibition of total mTOR expression by siRNA resulted in a substantial decrease in 4E-BP1, p70S6K, and rpS6 phosphorylation. No change in eIF4E levels was found (not shown).

specific siRNA decreased the number of viable cells by 23%.

Morphological examination of the Hoechst-stained cell preparations after treatment with 10 or 20 $\mu\text{g/ml}$ LY294002 revealed that MCL cells showed morphological evidence of apoptosis, such as nuclear condensation and fragmentation (Figure 4a). Flow cytometric analysis of annexin V staining after LY294002 treatment showed a

concentration-dependent increase in annexin V-positive cells (Figure 4b). There was an increase in annexin V-positive cells at 48 hours (28%) compared with no treatment (5%), indicating the occurrence of apoptosis. Partial inhibition of mTOR-raptor complex using rapamycin resulted in decreased growth of MCL cells (Figure 4d). However, the observed differences in cell growth or apoptosis were much smaller compared with treatment with LY294002. Silencing of mTOR also resulted in increased annexin V-positive cells in comparison with control cells (Figure 4e).

To evaluate the molecular basis underlying apoptosis after inhibition of mTOR, we assessed a number of apoptotic proteins after treatment with LY294002 or specific mTOR siRNA. There was decreased expression of the anti-apoptotic proteins MCL-1, BCL-XL, and cFLIP after silencing of mTOR (Figure 4f). Likewise, there was a concentration-dependent decrease in the anti-apoptotic protein MCL-1 with increasing concentrations of LY294002. BCL-2 levels did not change (Figure 4c). These findings indicate that inhibition of mTOR induces apoptosis and modulates the expression of proteins involved in the extrinsic and intrinsic apoptotic pathways.

eIF4E Mediates the Effects of mTOR Signaling on Cell Cycle Progression and Apoptosis in MCL Cells

Based on recently published data highlighting the biological importance of eIF4E, a downstream effector of mTOR in lymphomagenesis,^{21,38} we transiently transfected Z-138 cells with siRNA specific for eIF4E. Expression of eIF4E was significantly inhibited at a concentration of 10 $\mu\text{g/ml}$ eIF4E siRNA. As shown in Figure 5, inhibition of eIF4E expression was associated with a substantial decrease in cell viability and cell cycle S phase. These changes were associated with decreased cyclin D1 levels.

Inhibition of eIF4E expression also resulted in increased apoptotic cell death as demonstrated by annexin V binding (Figure 5c). Apoptosis was linked to a decrease in the expression levels of the anti-apoptotic protein MCL-1 and a slight decrease of BCL-XL. BCL-2 levels remained constant. These results suggest that the effects of mTOR signaling on MCL cell cycle progression and apoptosis inhibition, at least in part, are mediated by eIF4E.

Discussion

The mTOR pathway is emerging as a critical regulator of translation, coupling growth, and proliferation.¹¹ In this context, it is becoming increasingly clear that many components of signaling and biochemical pathways important for the biological behavior of cancer cells can be regulated at the level of translation.³⁹

In the present study, we show that the mTOR pathway is activated in MCL. Inhibition of total mTOR expression by using specific mTOR-siRNA resulted in considerably

Figure 3. Inhibition of AKT/mTOR signaling induces cell cycle arrest through down-regulation of cyclin D1 in MCL cells. **a:** Treatment of MCL cells with LY294002, a PI3K and mTOR inhibitor, led to growth inhibition as demonstrated by MTS assay and cell cycle arrest shown by a decrease in S-phase fraction assessed by BrdU incorporation assay in a concentration-dependent manner. Cell cycle analysis using propidium iodide (PI) staining and flow cytometry confirmed cell cycle arrest at G₁ phase, as S-phase fraction was decreased and G₁-phase fraction was increased. Results with Z-138 cells are shown. **b:** Silencing of mTOR in MCL cells using specific siRNA also resulted in decreased cell growth and decreased fraction of cells in S-phase at a concentration-dependent manner. Jeko-1 cells were transiently transfected with 20 µg (+) or 40 µg (++) *mTOR*-specific siRNA or control siRNA, and whole-cell lysates were prepared at 48 hours. Immunoblots showed inhibition of mTOR expression that resulted in decreased cell growth and proliferation as assessed by MTS assay and BrdU incorporation assay compared with cells transiently transfected with control siRNA or not transfected but electroporated cells. **c** and **d:** Western blot analysis after treatment with LY294002 demonstrated that the cyclin D1 levels were decreased and the p27 levels increased in a dose-dependent manner. **e:** Cell cycle arrest after silencing mTOR with *mTOR*-specific siRNA was associated with down-regulation of cyclin D1 and cyclin A, as well as up-regulation of two cyclin-dependent kinase inhibitors, p21^{waf1} and p27^{kip1}.

decreased cell growth that was a result of both cell cycle arrest and apoptosis. The effects on cell cycle arrest and apoptosis were even more prominent using the PI3K inhibitor LY294002. It must be noted that LY294002, by binding the active sites of PI3K kinase and mTOR, has

the ability to interrupt the AKT/mTOR pathway at two levels and therefore is expected to provide a more complete inhibition of the pathway.³⁷ Therefore, treatment of MCL cells with rapamycin resulted in a substantially smaller decrease in cell growth compared with that of

Figure 4. Inhibition of AKT/mTOR signaling induces apoptosis in MCL cells and modulates the expression levels of apoptosis-regulating proteins. Cell viability and apoptosis after treatment of MCL cells with increasing concentrations of LY294002 were assessed using trypan blue exclusion assay, DAPI immunofluorescence, and annexin V staining. Treatment with LY294002 resulted in decreased cell viability in a dose-dependent manner, which was associated with increased apoptotic cell death. **a:** Morphological examination of the Hoechst-stained cell preparations showed evidence of apoptosis, such as nuclear condensation and fragmentation (shown by **arrows**). **b:** Flow cytometry demonstrated an increase in annexin V-positive cells. At 48 hours after treatment with LY294002, the percentage of annexin V⁺ cells increased from 5 to 28%. **c:** Immunoblots showed a concentration-dependent decrease in the anti-apoptotic proteins MCL-1 with increasing concentrations of LY294002. BCL-2 levels remained constant. **d:** Partial inhibition of raptor-mTOR complex with increasing concentrations of rapamycin resulted in decreased growth as assessed by MTS assay in three MCL cell lines tested. However, only small differences in apoptosis (5 to 10%) were observed (not shown). **e:** Silencing of mTOR using specific siRNA also induced apoptotic cell death (increased annexin V binding) in MCL cells compared with cells treated with control siRNA at the same concentration. **f:** Immunoblots showed a concentration-dependent decrease in the levels of anti-apoptotic proteins BCL-XL, MCL-1, and c-FLIP.

treatment with LY294002 in our system. Analogous results using PI3K inhibitors (wortmannin or LY294002), rapamycin, or rapamycin analogs have been obtained in other hematological malignancies including multiple myeloma, primary effusion lymphoma, prolymphocytic leukemia, and acute lymphoblastic leukemia.^{25–28}

In our experiments, apoptosis was associated with down-regulation of cFLIP and the mitochondrial anti-apoptotic proteins BCL-XL and MCL-1. Although the links that connect the mTOR pathway and apoptosis are not fully understood and may be cell type-dependent, our findings are in agreement with previous *in vitro* studies

showing that apoptosis caused by modulation of mTOR or eIF4E activity is mediated partly by MCL-1 or BCL-XL, respectively.^{40,41} Interestingly, in a previous study, we showed that MCL-1 is expressed in a subset of MCL tumors, and its expression correlates with higher proliferative index and blastoid morphology.³³ It seems that both extrinsic and intrinsic (mitochondrial) apoptotic pathways can be modulated by inhibition of the mTOR pathway in MCL.⁴²

Cell cycle arrest at G₁ phase, after treatment of MCL cell lines with the PI3K inhibitor LY294002 or mTOR-specific siRNA, was associated with down-regulation of

Figure 5. eIF4E mediates the effects of mTOR signaling on cell cycle progression and apoptosis in MCL cells. To further study the biological importance of eIF4E as a downstream effector of mTOR, we transiently transfected Z-138 cells with siRNA specific for eIF4E or an equivalent amount of negative control #3 siRNA (Ambion). **a:** Expression of eIF4E was significantly inhibited at a concentration of 10 μ g/ml eIF4E siRNA. **b:** Inhibition of eIF4E expression was associated with a substantial decrease in cell growth and cell cycle S-phase fraction assessed by MTS and BrdU incorporation assays, respectively. **a:** These changes were associated with decreased cyclin D1 and cyclin A levels as well as increased p21 levels. **c and d:** Inhibition of eIF4E expression also resulted in increased apoptosis as demonstrated by decreased cell viability and increased annexin V binding. Apoptosis was linked to a decrease in the expression levels of the anti-apoptotic proteins cFLIP (not shown) and Mcl-1 and a slight decrease of BCL-XL.

cyclin D1 and up-regulation of the cell cycle inhibitors p21 and p27. The mechanisms by which the mTOR pathway regulates cell cycle progression are likely to be complex.²² However, recent studies have shown that such mechanisms may involve the downstream mTOR effectors p70S6K1, 4E-BP1, and eIF4E.¹⁵ We report here that eIF4E may regulate the levels of cyclin D1 expression. Previous experiments also have demonstrated the ability of eIF4E to regulate cyclin D1 levels via transcriptional, and more importantly, posttranscriptional mechanisms.⁴³ Therefore, activation of the mTOR pathway seems to contribute to cell cycle progression through up-regulation of cyclins and down-regulation of cyclin-dependent kinase inhibitors.²²

As the network of mTOR-interacting proteins is unraveling, it is becoming apparent that this network is more complex than previously thought. The upstream PI3K/AKT pathway, which regulates mTOR activity, can act

independently of mTOR, exerting control on many critical apoptotic and cell cycle proteins.¹⁶ As a result, agents similar to LY294002 that can independently inhibit both pathways can be expected to have a comparative advantage in tumors expressing activation of both AKT and mTOR. There are also several points along the mTOR pathway that can be influenced independently of PI3K/AKT.¹⁰ Recently, the link between the PI3K/AKT and mTOR pathways, TSC1/TSC2, was shown to be regulated by the p53 pathway, known to exert master control on apoptosis, cell cycle, and DNA safeguard functions.⁴⁴ Of note, the p53 pathway is known to be deregulated in a subset of MCL.⁴⁴ Another important piece of the puzzle is that a second Rictor-mTOR complex was characterized recently: it is rapamycin-insensitive, and its functions, aside from actin cytoskeleton regulation, are largely unknown. Interestingly, Rictor-mTOR complex was shown to be critical for the activation of AKT, by phosphorylating

AKT at position Ser473 and facilitating the second phosphorylation at position Thr308 by PDK1.²⁴ Because it is known that AKT can generally down-regulate the cell cycle inhibitor p27, the link Rictor-mTOR→AKT could explain the up-regulation of p27 after inhibition of mTOR by mTOR-specific siRNA in our experiments.⁴⁵

Overexpression of Rictor-mTOR could play a role in rapamycin-resistant tumors exhibiting activation of the AKT and mTOR pathways. This is particularly important because there is a negative feedback loop between the downstream mTOR effector p70S6K1 and PI3K/AKT activation in normal cells, and this seems to operate in cancer cells as well.⁴⁶ Accordingly, recent studies have shown that rapamycin treatment can augment AKT activation, whereas additional inhibition of AKT seems to overcome rapamycin resistance in some tumor types.⁴⁷ However, further molecular studies involving selective inhibition of Rictor-mTOR complex are needed to investigate this hypothesis.

We also assessed for expression of activated AKT, mTOR, and downstream effectors of the mTOR pathway in MCL tumors. This is the first study showing expression of AKT/mTOR signaling proteins in MCL. We found that AKT is activated in approximately one-third of MCL tumors. A previous immunohistochemical survey of non-Hodgkin's lymphomas for expression of eIF4E, a downstream mTOR target, found that eIF4E is overexpressed in a subset of MCL.⁴⁸ In addition, our data confirm recent gene and protein expression studies of MCL that showed overexpression of AKT pathway components.^{49,50}

With the exception of activated p70S6K, all of the other mTOR pathway molecules were detected primarily in the cytoplasm of MCL cells. Notably, localization of mTOR and its activated form has been reported predominantly in the cytoplasm or the nucleus, depending on the cell type and conditions, and it seems to be shuttled between the two compartments with yet unknown functional consequences.^{51,52} Activated p70S6K was detected primarily in the nucleus, and such localization has been reported in a subset of Hodgkin's lymphomas.⁵³ Interestingly, activated p70S6K expression was widely expressed in MCL. This is not unexpected, because p70S6K can be activated independently of mTOR by other pathways, such as the mitogen-activated protein kinase pathway.²⁰ This, in combination with the fact that p-p70S6K probably has a positive feedback effect on mTOR, makes p70S6K a critical node of the mTOR pathway network.^{54,55}

In conclusion, mTOR signaling proteins are activated in MCL cell lines and in a subset of MCL tumors. mTOR activation may contribute to tumor cell proliferation and survival of MCL cells. Our results also suggest that components of the AKT/mTOR pathway may provide novel therapeutic targets for the treatment of MCL patients.

References

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