Rapamycin Inhibits Ezrin-Mediated Metastatic Behavior in a Murine Model of Osteosarcoma

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Abstract
Osteosarcoma is the most frequent primary malignant tumor of bone with a high propensity for metastasis. We have previously showed that ezrin expression is necessary for metastatic behavior in a murine model of osteosarcoma (K7M2). In this study, we found that a mechanism of ezrin-related metastatic behavior is linked to an Akt-dependent mammalian target of rapamycin (mTOR)/p70 ribosomal protein S6 kinase (S6K1)/eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) pathway. Suppression of ezrin expression either by antisense transfection or by small interfering RNAs or disruption of ezrin function by transfection of a dominant-negative ezrin-T567A mutant led to decreased expression and decreased phosphorylation of both S6K1 and 4E-BP1. Proteosomal inhibition by MG132 reversed antisense-mediated decrease of S6K1 and 4E-BP1 protein expression, but failed to affect the effect of ezrin on phosphorylation of S6K1 and 4E-BP1. Blockade of the mTOR pathway with rapamycin or its analog, cell cycle inhibitor-779 led to significant inhibition of experimental lung metastasis in vivo. These results suggest that blocking the mTOR/S6K1/4E-BP1 pathway may be an appropriate target for strategies to reduce tumor cell metastasis. (Cancer Res 2005; 65(6): 2406-11)

Introduction
Ezrin is a member of the ezrin/radixin/moesin (ERM) family of proteins that link the cell membrane to the actin cytoskeleton and that are involved in cytoskeletal organization (1, 2). Ezrin is believed to be involved in intracellular signal transduction that is related to cell migration and metastasis because ezrin is reported to be a substrate for tyrosine kinases (3, 4) and binds adhesion molecules such as CD43, CD44, intercellular adhesion molecule-1, and intercellular adhesion molecule-2 (5–7). Of note, high levels of CD44 seem to be dependent on ezrin expression and are associated with invasion and metastatic behavior of tumor cells (8). The discovery that Merlin/schwannomin, the neurofibromatosis-2–associated tumor-suppressor protein, is related to the ezrin/radixin/moesin family has provided additional insights into the relationship between ezrin and tumorigenesis (9). Recently, we have found ezrin expression in murine osteosarcoma and rhabdomyosarcoma to be necessary for metastatic behavior (10–12). Suppression of ezrin protein expression by antisense transfection or stable expression of short hairpin RNA, or disruption of ezrin function by transfection of a dominant-negative ezrin significantly reduced the metastatic behavior in both murine models and was associated with decreased Akt and mitogen-activated protein kinase (MAPK) activity (11, 12). However, the specific mechanism or mechanisms by which ezrin mediates the metastatic process remains to be elucidated.

Rapamycin and analogues such as cell cycle inhibitor-779 (CCI-779) are currently undergoing clinical and preclinical evaluations as an anticancer agent. The anticancer property of rapamycin is attributed to the inhibition of mammalian target of rapamycin (mTOR) signaling pathway, which controls mRNA translation and cell proliferation. Rapamycin binds to the FK506 binding protein (FKBP-12), and this complex interacts with mTOR. This interaction inhibits mTOR kinase activity and subsequently decreases phosphorylation and activation of p70 ribosomal protein S6 kinase (p70S6K, S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) that play fundamental roles in ribosome biogenesis and cap-dependent translation, respectively (13, 14). A previous study has found that the ability of rapamycin to inhibit metastatic tumor growth and angiogenesis in in vivo mouse models is linked to reduced translational production of vascular endothelial growth factor vascular endothelial growth factor and to blockage of vascular endothelial growth factor–induced endothelial cell signaling (15). Most recently, investigators have showed that rapamycin can reverse resistance to doxorubicin in a mouse model of an Akt-driven aggressive lymphoma (16). These data suggest that blockade of the mTOR pathway might also have an inhibitory effect on both resistance to cytotoxic therapy as well as tumor metastasis.

In this study, we linked ezrin-related metastatic behavior to activation of S6K1 and 4E-BP1 signaling. We found that antisense-mediated and small interfering RNA (siRNA)–induced reduction of ezrin expression or disruption of ezrin function by transfection of a dominant-negative ezrin-T567A mutant led to decreased expression and decreased phosphorylation of both S6K1 and 4E-BP1. Proteosomal inhibition by MG132 reversed antisense-mediated decrease of S6K1 and 4E-BP1 protein expression, but failed to affect the effect of ezrin on phosphorylation of S6K1 and 4E-BP1. Blockade of the mTOR pathway with rapamycin and its analog, cell cycle inhibitor-779 led to significant inhibition of experimental lung metastasis in vivo. These results suggest that blocking the mTOR/S6K1/4E-BP1 pathway may be an appropriate target for strategies to reduce tumor cell metastasis.

Materials and Methods

Cell Culture. The K12, K7M2 murine OSA cell lines, ezrin-antisense clones 13, 1.46 and 1.52 cells, and dominant-negative ezrin (T567A mutant) clones T567A-GFP-7, and T567A-GFP-8 as well as empty-GFP clones empty-GFP-2.5, empty-GFP-2.7 cells generated from K7M2 have been previously described (13, 14). These cells were maintained in DMEM containing 10% fetal bovine serum, l-glutamine (2 mmol/L), penicillin (100 units/mL), and streptomycin (100 units/mL, BioSource International Inc, Camarillo, CA) at 37°C in a humidified CO2 incubator.
Antibodies and Reagents. Anti-ezrin monoclonal antibody was purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to phospho-S6K1 (Thr389), S6K1, 4E-BP1 and Akt (Ser473), Akt, phospho-4E-BP1 (Ser2448), phospho-p44/42 MAPK, and phospho-S6K1 (Thr389), S6K1, phospho-4E-BP1 (Thr37), 4E-BP1, phospho-p44/42 MAPK were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-actin antibody was from Amersham Pharmacia Biotech (Piscataway, NJ). MG132 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega Corp. (Madison, WI). LY294002 was purchased from LC Laboratories (Woburn, MA). CCI-779 was obtained from Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD) and Wyeth Laboratories (Philadelphia, PA).

Transfection. Myc-tagged, activated Akt, dominant-negative Akt (Akt K179M), and empty vector (pUSE) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). K7M2 cells were transfected with dominant-negative Akt and empty vector by using electroporation in a gene Pulsar (0.22 kV/cm; capacitance, 960 μF; Bio-Rad, Richmond, CA). After selection in medium containing G418, single clones were isolated and expanded. Ezrin-antisense clones 1.46 and 1.52 were transfected with activated Akt and empty vector by using electroporation in a Bio-Rad gene Pulsar (0.22 kV/cm; capacitance, 960 μF). After 72 hours, these cells were harvested and subjected to Western blot analysis.

Western Blot Analysis. Confluent cells were lysed in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L sodium orthovanadate, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin). Protein lysates (20-50 μg per lane), as determined by Bio-Rad protein assay, were separated in 10% to 12% SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech). Membranes were blocked with 5% nonfat dried milk in TBS-T (20 mmol/L Tris-HCl, pH 7.5, 8 g/L of sodium chloride, 0.1% Tween 20) and then incubated with primary antibodies. Horseradish peroxidase conjugated anti-rabbit IgG (Cell Signaling) was used as secondary antibody. Protein was visualized using enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

S6K1 Assay. S6K1 activity was determined in vitro as described previously (17).

Gene Silencing with siRNAs. We obtained annealed, 21-bp siRNA duplexes from Dharmacon Research Inc. (Lafayette, CO). The target sequence for ezrin was 5′-AAGGAUCCUUAGCGAUGA-3′, corresponding to position 440 to 460 in the human ezrin mRNA. A siRNA targeting a nonspecific sequence was purchased from Dharmacon Research Inc. and served as a negative control. We applied siRNA duplexes at a final concentration of 100 mmol/L using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

In vivo Experimental Metastasis Assay. Four- to 5-week-old female beige severe combined immunodeficient (SCID) mice (Charles River Laboratories, Wilmington, MA) were inoculated with 1 × 10⁶ K7M2 cells per mouse via the tail vein and then randomly assigned to treatment groups. Mice were treated i.p. daily × 5 days for 5 to 6 consecutive weeks with 5 mg/kg rapamycin, 5 mg/kg CCI-779, 20 mg/kg CCI-779, or vehicle alone. All mice underwent complete necropsy for visual inspection and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These data suggest that S6K1 and 4E-BP1 are associated with ezrin-mediated signaling.

Proteosomal Inhibition Reverses Ezrin-Antisense-Mediated Suppression of S6K1 and 4E-BP1 Expression, but Does Not Affect S6K1 and 4E-BP1 Phosphorylation. We sought to determine whether S6K1 and 4E-BP1, two major downstream targets of mTOR that play critical roles in translation regulation, were involved in ezrin-mediated metastatic signaling because our previous data showed ezrin-mediated effects on Akt activity. We first examined the phosphorylation and expression status of S6K1 and 4E-BP1 in K12 and K7M2 cell lines. Our data revealed that S6K1 and 4E-BP1 are both more highly phosphorylated and expressed in K7M2 cells compared with K12 cells (Fig. 1B and D). Furthermore, S6K1 activity, as evaluated by in vitro kinase assays, is significantly elevated in K7M2 cells compared with K12 cells (Fig. 1C). Down-regulation of ezrin expression decreased phosphorylation and expression of S6K1 and 4E-BP1 as well as S6K1 activity (Fig. 1B–D). To further test the effects of ezrin on the regulation of S6K1 and 4E-BP1, we next targeted ezrin by siRNA. Inhibition of phosphorylation by siRNA led to decreased phosphorylation of S6K1 and 4E-BP1, with minimal reduction of S6K1 and 4E-BP1 expression (Fig. 1E). These data suggest that S6K1 and 4E-BP1 are associated with ezrin-mediated signaling.
S6K1 and 4E-BP1 protein expression but failed to influence the levels of Akt protein. These data are consistent with our findings in ezrin-antisense transfection cells (Fig. 1). We have previously shown phosphorylation and activity of p44/42 MAPK are reduced when ezrin protein expression is suppressed by stable transfection of ezrin antisense (11). However, p44/42 MAPK phosphorylation is increased, not decreased, in cells expressing ezrin-T567A (Fig. 3), whereas the total expression of p44/42 MAPK is not significantly changed in these clone cells (Fig. 3A). Thus, phosphorylation of ezrin-T567 residue seems to be a crucial site for ezrin-mediated activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway, but not for ezrin-mediated activation of the p44/42 MAPK signaling pathway.

Ezrin-Linked S6K1 and 4E-BP1 Phosphorylation Is Rapamycin Sensitive. To further determine whether the ezrin-linked S6K1 and 4E-BP1 phosphorylation is mTOR dependent, K7M2 cells were treated with an mTOR inhibitor rapamycin, as well as a PI3K inhibitor LY294002, and a MAPK inhibitor U0126, for 1 hour. As shown in Fig. 3B, rapamycin inhibited S6K1 and 4E-BP1 phosphorylation but did not affect Akt phosphorylation. LY294002 completely inhibited not only Akt phosphorylation but also S6K1 and 4E-BP1 phosphorylation. U0126 affected neither Akt phosphorylation nor S6K1 and 4E-BP1 phosphorylation. These inhibitors failed to alter the expression of Akt, S6K1, and 4E-BP1. Taken together, these data suggest that the ezrin-related S6K1 and 4E-BP1 phosphorylation in K7M2 cells is rapamycin sensitive and downstream of PI3K.

Suppression of Experimental Metastasis in the K7M2 Murine Osteosarcoma Model by Rapamycin and Its Analogue CCI-779. To determine the potential effect of mTOR inhibition on experimental metastases in the K7M2 murine osteosarcoma model, we evaluated the effect of rapamycin and its analogue CCI-779. Mice were treated i.p. daily × 5 days every week for 5 to 6 weeks with 5 mg/kg rapamycin, 5 mg/kg CCI-779, 20 mg/kg CCI-779, or vehicle alone. Treatment with rapamycin and CCI-779 significantly prolonged the survival (moribidity associated with pulmonary metastasis) of SCID beige mice (Fig. 4). In the control group, only 25% (2 of 8) mice survived beyond 38 days, whereas 100% (9 of 9) in both 5 mg/kg rapamycin- and 20 mg/kg CCI-779–treated groups were alive at 38 days. Two of 9 mice treated with 5 mg/kg CCI-779 died, but no gross pulmonary metastasis were detected in the two dead mice. At necropsy, no evidence of tumor was observed. Therefore, the cause of death in these mice is uncertain. All eight mice in the control group after 38 days of injection developed multiple lung metastases, whereas 1 of 7 mice in the 5 mg/kg CCI-779–treated group and 2 of 9 mice in the 5 mg/kg rapamycin–treated group developed single lung metastases. We failed to detect gross lung metastasis in any mouse treated with 20 mg/kg CCI-779.
(Fig. 4B). Histopathologic examination of H&E-stained sections of lungs was done in all animals. In contrast to multiple, large pulmonary metastases seen in all control-treated mice (Fig. 4C, left), we found single small micrometastases in 6 of 9 mice treated with 5 mg/kg rapamycin (not shown), 6 of 9 mice treated with 5 mg/kg CCI-779 (Fig. 4C, middle, micrometastasis indicated by arrow), and in only 2 of 9 mice treated at 20 mg/kg CCI-779 (Fig. 4C, right, indicate normal lung only).

**Discussion**

Ezrin is known to be involved in a variety of cellular functions, such as cell cytoskeletal organization, cell motility, and morphogenesis. The high levels of ezrin expression in cell lines of endometrial (18), colorectal (19), and pancreatic carcinoma with high metastatic potential (20) have suggested that its expression has been associated with events that may promote tumor progression and metastasis. Consistent with that, our recent studies found that high expression of ezrin in K7M2 murine osteosarcoma cells is associated with highly metastatic behavior (10). Suppression of ezrin protein by antisense transfection and disruption of ezrin function significantly reduced lung metastases in two distinct mouse tumor models (11, 12), providing an excellent experimental model to investigate the mechanisms of ezrin-mediated metastasis. In this report, we show that both blockade of ezrin expression either by antisense transfection or by siRNA and disruption of ezrin function by stable transfection of dominant-negative ezrin (T567A) led to inhibition of S6K1 and 4E-BP1 phosphorylation (Figs. 1B, D, and E and 3A), which both lie downstream of mTOR and play fundamental roles in ribosome biogenesis and cap-dependent translation, respectively (21, 22). These results indicate that ezrin signaling is involved in regulating mRNA translation and provide, for the first time, a linkage between ezrin and mTOR signaling.

Recent studies reported that S6K1 and 4E-BP1 also are regulated through the PI3K/Akt-signaling pathway (23). These studies raise the possibility of a direct signaling pathway from PI3K/Akt to mTOR. Our previous studies show that the inhibition of ezrin expression resulted in markedly reduced Akt phosphorylation and activity (11). Both S6K1 and 4E-BP1 phosphorylation were completely inhibited by the PI3K inhibitor LY294002 in K7M2 cells (Fig. 3B). Furthermore, stable transfection of the dominant-negative Akt (K179M mutant) into K7M2 cells led to reduction of Akt phosphorylation as well as S6K1 and 4E-BP1 phosphorylation (data not shown). On the other hand, transient transfection of activated Akt into ezrin-antisense clones 1.46 and 1.52 led to up-regulation of S6K1 and 4E-BP1 phosphorylation (data not shown). These data are consistent with a PI3K/Akt/mTOR pathway in these cells. Phosphorylation of ezrin at T567 has been identified to play an important role in its conformational activation. Inactive, cytosolic ezrin, in a closed conformation through head-to-tail interaction between the amino- and carboxyl-terminal domains, requires phosphorylation at residue T567 and interaction with phosphatidylinositol 4,5-bisphosphate to cause unfolding, translocation to the plasma membrane, and cross-linking between integral membrane proteins and cytoskeleton (24–27). Disruption of ezrin function by transfection of ezrin-T567A mutant significantly reduced lung metastases in two distinct mouse tumor models (11, 12). In this study, we found that transfection of ezrin-T567A mutant into K7M2 cells not only inhibited Akt phosphorylation but also inhibited S6K1 and 4E-BP1 phosphorylation (Fig. 3), which is consistent with our findings in ezrin-antisense transfected cells (Fig. 1). As noted, ezrin has been found to directly bind PI3K (28). Thus, ezrin-mediated regulation of mTOR targets S6K1 and 4E-BP1 seems to be indirect through a direct interaction of ezrin with PI3K or phosphatidylinositol 4,5-bisphosphate leading to sequential activation of PI3K/Akt/mTOR signaling cascades. The role of Akt in the regulation of mTOR activation is complex. Although Ser2448 in mTOR has been identified to be a direct phosphorylation target of Akt (29), substitution of Ser2448 by alanine

![Figure 3](image-url)
failed to alter the ability of mTOR to activate S6K1 (30). We examined the phosphorylation of serine residue 2448 of mTOR in ezrin-antisense transfected cell lines. Down-regulation of ezrin failed to affect mTOR phosphorylation on Ser2448 (data not shown). Recent study has shown that phosphorylation of Ser2448 does not seem to modulate in vitro 4E-BP1 phosphorylation by mTOR (31). Moreover, mutation of Ser2035 in mTOR inhibited the abilities of mTOR to phosphorylate S6K1 and 4E-BP1 in vitro (32). Furthermore, in our study ezrin-associated phosphorylation of S6K1 and 4E-BP1 is rapamycin sensitive, suggesting that these observed ezrin effects occur through a mTOR signaling pathway. However, the specific mechanism remains to be elucidated.

To further determine the functional significance of ezrin-regulated mTOR/S6K1/4E-BP1 pathways, we studied the effect of mTOR inhibition on in vitro and in vivo metastatic pathways. Suppression of S6K1 and 4E-BP1 by rapamycin led to decreased K7M2 cell migration and invasion compared with untreated cells (data not shown). Treatment of tumor-inoculated SCID beige mice with rapamycin and CCI-779 resulted in prolonged survival and inhibition of pulmonary metastasis (Fig. 4 A and B). These results suggest that mTOR/ S6K1/4E-BP1 pathways play an important role in ezrin-mediated metastatic behavior. Recently, rapamycin has been reported to inhibit metastatic tumor growth in other murine models (15, 33). Thus, inhibition of the mTOR/ S6K1/4E-BP1 pathway by rapamycin or other inhibitors may be worthy of clinical evaluation as an antimetastatic intervention. The challenge will be to develop schedules of rapamycin and its analogues that can be chronically administered without causing significant immunosuppression.

Acknowledgments

References


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