Celastrol Suppresses Angiogenesis-Mediated Tumor Growth through Inhibition of AKT/Mammalian Target of Rapamycin Pathway

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Abstract

Understanding the molecular basis and target of traditional medicine is critical for drug development. Celastrol, derived from Trypterygium wilfordii Hook F. ("Thunder of God Vine"), a traditional Chinese medicine plant, has been assigned anticancer activities, but its mechanism is not well understood. Here, we investigated whether Celastrol could inhibit angiogenesis-mediated tumor growth and, if so, through what mechanism. When given s.c. to mice bearing human prostate cancer (PC-3 cell) xenografts, Celastrol (2 mg/kg/d) significantly reduced the volume and the weight of solid tumors and decreased tumor angiogenesis. We found that this agent inhibited vascular endothelial growth factor (VEGF)-induced proliferation, migration, invasion, and capillary-like structure formation by primary cultured human umbilical vascular endothelial cells (HUVEC) in a dose-dependent manner. Furthermore, Celastrol abrogated VEGF-induced sprouting of the vessels from articular rings and inhibited vascular formation in the Matrigel plug assay in vivo. To understand the molecular mechanism of these activities, we next examined the signaling pathways in treated HUVECs and PC-3 tumor cells. Celastrol suppressed the VEGF-induced activation of AKT, mammalian target of rapamycin (mTOR), and ribosomal protein S6 kinase (P70S6K). Additionally, we found that Celastrol inhibited the proliferation of prostate cancer cells and induced apoptosis, and these effects correlated with the extent of inhibition of AKT/mTOR/P70S6K signaling. Taken together, our results suggest that Celastrol targets the AKT/mTOR/P70S6K pathway, which leads to suppression of tumor growth and angiogenesis. Cancer Res; 70(5); 1951–9. ©2010 AACR.

Introduction

Traditional medicines provide highly fertile ground for modern drug development, but first they must proceed along a pathway of discovery, isolation, and mechanistic studies before eventual deployment in the clinic (1). Celastrol, known as a tripterine, is a functional ingredient that was originally identified and extracted from traditional Chinese medicine named Trypterygium wilfordii Hook F. ("Thunder of God Vine") and shows potential in treatment of chronic inflammatory disorders, such as arthritis (2, 3), lupus erythematosus (4), lateral sclerosis (5), and Alzheimer’s disease (6). It also potentiates apoptosis in numerous tumor cells through inhibition of IκB kinase (7, 8), proteasome (9), topoisomerase (10), heat shock protein (11–13), and vascular endothelial growth factor (VEGF) receptor (VEGFR) expression (14). Although Celastrol has already shown to be promising in tumor prevention, direct targets remain elusive. Whether Celastrol could modulate tumor angiogenesis has not been validated yet.

It is estimated that >90% cancer deaths that occur are due to angiogenesis, invasion, and metastasis of cancer to vital organs. Angiogenesis is one of the key processes that mediate metastasis, in part through the interaction of human vascular endothelial cells with VEGF. Although antibodies against VEGF (called avastin) have already been approved for human use by Food and Drug Administration (FDA), safer and more efficient inhibitors are still needed. VEGF mediates its signals through the interaction with three distinct receptors, VEGFR1, VEGFR2, and VEGFR3. These receptors mediate their signals through activation of various cell signaling intermediates, including mammalian target of rapamycin (mTOR). The latter has become an extremely important drug target in cancer therapy recently (15). The mTOR, a serine/threonine kinase, stands in a central position on the crossroad of various signal pathways (Ras, phosphoinositide 3-kinase/AKT, hypoxia-inducible factor-1, NF-κB) toward mRNA, ribosome, protein synthesis, and translation of significant molecules (16). Through activation of its downstream ribosomal p70 S6 kinase (S6K1)
and hyperphosphorylation of the 4E binding protein (4E-BP1), mTOR is critical for cellular proliferation and growth in endothelial cells and various tumor cells (17, 18). Overactivation in mTOR signaling is frequently associated with tumorigenesis, angiogenesis, tumor growth, and metastasis (15, 19, 20). In recent years, drugs known as mTOR inhibitors, rapamycin, and the newly developed rapamycin analogues have shown promise in the ongoing and recently completed cancer trials of treatments for hematologic malignancy and transplant rejection (21, 22). And temsirolimus (Torisel), an mTOR inhibitor, is currently approved by FDA as clinical anticancer therapy. Discovering additional safe and affordable inhibitors of the mTOR pathway is of great attraction. Here, we report that Celastrol effectively inhibited tumor angiogenesis and tumor growth by targeting the AKT/mTOR/P70S6K signaling pathway.

Materials and Methods

Reagents and antibodies. A 50 mmol/L stock solution of Celastrol (obtained from Cayman Chemicals) was prepared and frozen at −20°C in small aliquots until needed. Bacteria-derived recombinant human VEGF (VEGF 165) was a gift from the experimental branch of NIH. Growth factor–reduced Matrigel was purchased from BD Biosciences. The antibodies against β-actin were obtained from Santa Cruz Biotechnology. The antibodies anti-AKT, anti-mTOR, anti-p70S6K1, anti-poly(ADP-ribose) polymerase (PARP), phosphorylated-specific anti-AKT (Ser473), anti-mTOR (Ser2448), anti-p70S6K1 (Thr389), and anti-VEGFR2 (Tyr1175) were purchased from Cell Signaling Technology.

Cell lines and cell culture. Primary human umbilical vascular endothelial cells (HUVEC) were kindly provided by Dr. Xini Wang (Cardiothoracic Surgery Division of Michael E. DeBakey Department of Surgery, Baylor College of Medicine). HUVECs were cultured in endothelial cell growth medium (ECGM) as described previously (23). Human prostate cancer (PC-3) cells were purchased from American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). HUVECs and PC-3 cells were cultured at 37°C under a humidified 95%:5% (v/v) mixture of air and CO₂.

Human prostate tumor xenograft model. All the mice used in this study were maintained in a laminar airflow cabinet under specific pathogen-free condition in a 12-h light-dark cycle. Experiments and managements were followed in accordance with Texas A&M University Institutional Animal Care and Use Committee guidelines at M.D. Anderson; Animal Care and Use Committee typically approves animal protocols. Xenograft human prostate mouse model was constructed as previously described (24). Six-week-old male BALB/cA nude mice (National Rodent Laboratory Animal Resources, Shanghai Branch) weighing ~28 g were randomly divided into two groups of five each. PC-3 prostate cancer cells were s.c. injected into the mice (5 × 10⁶ per mouse). After tumors grew to ~130 mm², the mice were then s.c. treated with or without Celastrol (2 mg/kg/d). The body weight of each mouse was recorded, and tumor volume was determined by Vernier caliper everyday, following the formula of A × B² × 0.52, wherein A is the longest diameter of tumor and B is the shortest diameter. After 16 d, the mice were killed and solid tumors were removed.

Histology and immunohistochemistry. Solid tumors were fixed with 10% formaldehyde and embedded in paraffin. To identify infiltrating blood vessels, immunohistochemistry was carried out on 5-μm deparaffinized sections using a specific blood vessel staining kit (von Willebrand Factor; Millipore). Images of the stained blood vessels were taken using a Leica DM 4000B photo microscope (magnification, ×200). The Image-Pro plus 6.0 software package (Media Cybernetics, Inc.) was used to determine the mean absorbance of blood vessel staining.

Wound-healing migration assay. Wound-healing migration assay was performed as described previously (25). Briefly, HUVECs were starved to inactivate cell proliferation and then wounded by pipette tips. ECGM containing 0.5% FBS was added with or without 10 ng/mL VEGF and different dilutions of Celastrol. Images of the cells were taken after ~8 to 10 h of incubation. Migrated cells were quantified manually, and percentage inhibition was expressed using untreated wells at 100%. Three independent experiments were performed.

Transwell migration assay. Transwell migration assay was performed as described previously (25). Briefly, HUVECs (2 × 10⁴ per Transwell) along with the indicated concentrations of Celastrol were seeded into the upper chambers. The bottom chambers were filled with 500 μL ECGM supplemented with 10 ng/mL VEGF. After 8 to 10 h of incubation, migrated cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. Images were taken using an Olympus inverted microscope (magnification, ×100). The percentage inhibition of migrated cells in treated groups was recorded.

Capillary-like tube formation assay. Tube formation was assessed as described previously (25). Briefly, HUVECs were pretreated with various dilutions of Celastrol for 30 min and then seeded onto the Matrigel layer in 24-well plates at a density of 4 × 10⁵ cells. ECGM was with or without 10 ng/mL VEGF. After 6 h, tubular structure of endothelial cells was photographed using an inverted microscope (Olympus; magnification, ×100). Three independent experiments were performed.

Cell viability assay. HUVECs or PC-3 cells (5–7 × 10⁵ per well) were treated with or without VEGF (10 ng/mL) and Celastrol for 24 h. Cell viability was determined by MTS (inner salt) method, as described previously (24, 25).

Rat aortic ring assay. Rat aortic ring assay was performed as described previously (26). In brief, aortas isolated from Sprague-Dawley rats (NIH) were cleaned of periadventitial fat and cut into rings at 1 to 1.5 mm in circumference. The aortic rings were randomized into Matrigel-coated wells and sealed with a 100-μL overlay of Matrigel. VEGF, with or without different dilutions of Celastrol, was added to the wells. As a control, medium alone was assayed. After 6 d, we fixed the microvessel sprouting and photographed it using an Olympus inverted microscope (Olympus; magnification, ×100).
The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner. Two independent experiments were performed.

**Matrigel plug assay.** Matrigel plug assay was performed as described previously (26). Briefly, we s.c. injected 0.5 mL of Matrigel containing 100 ng VEGF and 20 units of heparin with or without 10 μg of Celastrol into the ventral area of C57/BL/6 mice (NIH). Five mice were used for each group. After 6 d, the mice were killed and intact Matrigel plugs with different treatments were removed. H&E staining was performed to identify the formation and infiltration of new microvessels. Functional microvessels with intact RBC were quantified manually using a microscope (magnification, ×200).

**Western immunoblot analysis.** To determine the effects of Celastrol on the VEGFR2-dependent mTOR signaling pathway, HUVECs were first starved in serum-free ECGM for 6 h and then pretreated with or without Celastrol for 30 min, followed by stimulation with 50 ng/mL of VEGF for 2 min (for VEGFR2 activation) or 15 to 20 min (for mTOR pathway kinase activation). To examine the mTOR pathway in prostate tumor cells, normal cultured PC-3 cells were directly treated with indicated dilutions of Celastrol for 4 h. The whole-cell extracts were prepared in radioprecipitation assay buffer (20 mmol/L Tris, 2.5 mmol/L EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 40 mmol/L NaF, 10 mmol/L Na3P2O7, and 1 mmol/L phenylmethylsulfonyl fluoride) supplemented

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**Figure 1.** Celastrol inhibits tumor growth and tumor angiogenesis in xenograft mice. PC-3 cells were injected into 6-wk-old BALB/cA nude mice (5 × 10⁶ per mouse). After solid tumors grew to ∼130 mm³, mice were s.c. given with or without Celastrol (2 mg/kg/d). A, Celastrol inhibited tumor growth as measured by tumor volume. B, as shown by the body weight change in mice, Celastrol had little toxicity in the amount tested. C, solid tumors in the Celastrol-treated mice were significantly smaller than in the untreated mice. D, blood vessel staining revealed that Celastrol inhibited tumor angiogenesis. Arrows, blood vessels. Columns, mean; bars, SD. **, P < 0.01 versus control.
with a proteinase inhibitor cocktail (Calbiochem). About 40 μg of cellular protein of each sample were applied to immunoblot using 6% to 12% SDS-polyacrylamide gel and probed with specific antibodies. The intensity of immunoreactivity was measured using densitometer and Image J software (Version 1.34e; NIH).

Statistical analysis. Data were presented as mean ± SDs, and statistical comparisons between treated group and untreated group were performed using one-way ANOVA followed by Dunnet’s test. P values of ≤0.05 were considered statistically significant.

Results

The goal of this study was to investigate whether Celastrol, a triterpene from traditional Chinese medicine, can suppress angiogenesis and, if so, through what mechanism. For this, we used a wide variety of in vitro and in vivo assays. These included tumor angiogenesis in vivo, cell proliferation, invasion, and migration of VEGF-induced endothelial cells in vitro, VEGF microvessel tubule formation, VEGF-induced microvessel sprouting, and VEGF-induced blood vessel formation ex vivo. We also investigated the effect of Celastrol on VEGF-induced cell signaling pathways and on the survival of prostate cancer cells.

Celastrol inhibits tumor growth and tumor angiogenesis in a xenograft mouse model. To investigate the effect of Celastrol on tumor growth and tumor angiogenesis in vivo, we applied different concentrations of Celastrol in a prostate tumor (PC-3) xenograft model. We found that administration of 2 mg/kg/d Celastrol for 16 days substantially suppressed tumor volume (Fig. 1A) and reduced tumor weight (Fig. 1B). The average tumor volume in the control mice increased from 130.63 ± 37.62 to 330.74 ± 72.20 mm³ after 16 days, whereas the average tumor volume in the Celastrol-treated mice decreased from 129.36 ± 37.35 to 35.49 ± 9.71 mm³. Additionally, the average tumor weight in the control group was 110.16 ± 47.73 mg, whereas the average tumor weight in Celastrol-treated group...
was only 9.46 ± 8.46 mg (Fig. 1B), a significant inhibition in tumor growth. However, the same dose of Celastrol had no significant effect on the body weight of mice (Fig. 1C), suggesting low toxicity of Celastrol at the test dosage and conditions. A lower dose of Celastrol (0.5 mg/kg/d) had little effect on the tumor growth (data not shown).

To investigate whether Celastrol inhibited tumor growth through the inhibition of tumor angiogenesis, we used a blood vessel staining kit to stain solid tumor sections in the xenograft mouse model. We found that the mean absorbance of blood vessels in Celastrol-treated group was 29.96 ± 15.29/HPF compared with 100 ± 17.87/HPF in the control group (Fig. 1D), indicating that Celastrol significantly inhibited tumor angiogenesis.

Celastrol inhibits VEGF-induced chemotactic migration, capillary structure formation, and viability of HUVECs in vitro. To assess the antiangiogenic property of Celastrol in vitro, we examined its inhibitory effects on the chemotactic motility of endothelial cells using the wound-healing migration assay and Transwell assay, respectively. We showed that Celastrol at 1 μmol/L significantly inhibited VEGF-induced HUVEC migration in the wound-healing migration assay (Fig. 2A). Using the Transwell assays, we further showed that Celastrol dramatically reduced cell invasion (Fig. 2B). Both effects were dose dependent, with significant inhibition first occurring at ∼1 μmol/L of Celastrol.

To further determine the effect of Celastrol on angiogenesis, we examined how Celastrol regulates capillary tubule formation of HUVECs. When HUVECs were seeded on growth factor–reduced two-dimensional Matrigel, robust tubular-like structures were formed in the presence of VEGF. However, treatment with 1 or 2 μmol/L Celastrol abolished the VEGF-induced tubule formation of HUVECs (Fig. 2C), suggesting the potential effect of Celastrol on angiogenesis. Angiogenesis requires the proliferation of endothelial cells. We next examined whether Celastrol modulates VEGF-induced proliferation of endothelial cells by the MTS assay. As shown in Fig. 2D, 1 μmol/L Celastrol significantly decreased the VEGF-induced proliferation of HUVEC (Fig. 2D). Together, these results indicated that Celastrol could block VEGF-induced angiogenesis in vitro by inhibiting cell motility, cell proliferation, and endothelial cell tubular structure formation.

Celastrol inhibits VEGF-induced microvessel sprouting ex vivo. To study whether Celastrol affects VEGF-induced angiogenesis ex vivo, we examined the sprouting of vessels from aortic rings in the presence or absence of Celastrol. VEGF (20 ng/mL) significantly stimulated microvessel sprouting, leading to the formation of a network of vessels around the aortic rings (Fig. 3A). Addition of different concentrations of Celastrol antagonized the VEGF-induced sprouting in a dose-dependent manner, and 2 μmol/L Celastrol completely blocked microvessel sprouting (Fig. 3B).

Celastrol inhibits VEGF-induced angiogenesis in vivo. Matrigel plug assay has been used to evaluate the effects of Celastrol on VEGF-induced angiogenesis in vivo. As shown in Fig. 4A, Matrigel plugs containing VEGF alone appeared dark (Fig. 4A) and were filled with intact RBC, indicating that
functional vasculatures had formed inside the Matrigel via angiogenesis triggered by VEGF. In contrast, addition of different concentrations of Celastrol dramatically inhibited vascular formation. As a result, the color of the Matrigel plugs of Celastrol-treated group became much lighter (Fig. 4A). H&E staining of the functional vasculature in Matrigel plugs (Fig. 4B) showed that Celastrol at a dose of 10 μg dramatically blocked VEGF-induced vasculature formation in vivo (Fig. 4C).

Celastrol inhibits the activation of AKT/mTOR/P70S6K pathway in endothelial cells. Interaction of VEGFR2 with VEGF led to the activation of various downstream signaling molecules responsible for endothelial cell migration, proliferation, and survival. To further delineate the mechanism that underlies the antiangiogenic effect of Celastrol, we examined the signaling molecules and pathways using Western blotting assays. As shown in Fig. 5A, the phosphorylation of VEGFR2 was suppressed by Celastrol in a dose-dependent manner (Fig. 5A). Thus the antiangiogenic property of Celastrol may be at least partially due to VEGFR2 inhibition.

When examined for the key pathway components that regulate the endothelial cell function in angiogenesis, we found that Celastrol effectively suppressed VEGF-triggered activation of mTOR signaling cascade, including AKT, mTOR, and S6K kinase in HUVECs in a concentration-dependent manner (Fig. 5B), suggesting that Celastrol inhibit tumor angiogenesis through blocking of the mTOR signaling pathway.

Celastrol induces cell apoptosis and inhibits AKT/mTOR/P70S6K pathway in PC-3 prostate cancer cells. As shown in our xenograft prostate cancer model, tumor growth was strongly suppressed by Celastrol administration, suggesting Celastrol also have direct cytotoxic effects on cancer cells besides its antiangiogenic effect on endothelial cells. To test this hypothesis, we examined tumor cell viability and the potential signaling pathways in prostate cancer cells. Our results show that Celastrol significantly decreased PC-3 cell viability (Fig. 6A) and induced tumor cell apoptosis by detecting full-length PARP1 (116 kDa) and its large cleavage fragment (89 kDa; Fig. 6B). Furthermore, Celastrol also inhibited the activation of AKT, mTOR, and P70S6K in a concentration-dependent manner in normal cultured PC-3 cancer cells (Fig. 6C), suggesting that the mTOR pathway is also a possible target of Celastrol in tumor cells.

Discussion

In the present report, we have shown that Celastrol, a component of traditional Chinese medicine, can inhibit the growth of human prostate cancer in a xenotransplant mouse model. One of the major mechanisms by which Celastrol mediates its effects against prostate cancer seems to be through suppression of angiogenesis. We found that Celastrol targeted AKT/mTOR/P70S6K (S6K1) signaling pathway in a relatively specific manner both in endothelial cells and prostate cancer cells.
in prostate tumor cells. Celastrol effectively inhibited microvessel sprouting \textit{ex vivo} and angiogenesis \textit{in vivo}. Once angiogenesis was blocked by Celastrol, tumor growth was substantially suppressed.

Previous studies have shown that Celastrol suppresses the growth of human tumors in nude mouse models. Yang and colleagues showed that Celastrol could inhibit the growth of human prostate cancer through suppression of proteasomal pathway (9). Zhang and colleagues, however, showed the inhibition of human pancreatic cancer growth and metastasis by Celastrol through the suppression of heat shock protein Hsp90 (12). These studies differ from that of Huang’s group, which showed the suppression of human gliomas by Celastrol through the downregulation of expression of mRNA and protein for VEGFR1 and VEGFR2 (14). The effect on VEGFR2 is in agreement with our studies, as shown in Fig. 5A, that Celastrol inhibits VEGFR2 activation in endothelial cells. However, in the present study, we further found that triterpenoid mediates antitumor effects through suppression of both angiogenesis and tumor growth. Additionally, our results suggested that VEGF receptor is not the principle target of Celastrol in suppression of tumor angiogenesis and tumor growth, because PC-3 prostate cancer cells express little Flt-1 (VEGFR1) and KDR (VEGFR2), as reported previously (27).

Importantly, our study is the first to show the effect of Celastrol on AKT/mTOR/S6K pathway. We showed Celastrol similarly and consistently reduces signaling from AKT pS473 in a relatively specific manner in both prostate tumor cells and endothelial cells, which suggests the possibility that Celastrol is competing directly with TORC2 (the mTOR-rictor complex that phosphorylates Akt at Ser473). Other potential targets of Celastrol include HSP90-cdc (28), topoisomerase II (10), Kir 2.1 and hERG potassium channels (29), downregulation of expression of adhesion molecules (30), and suppression of transforming growth factor-b1 expression (31). A contribution of these targets to the antitumor effects of Celastrol cannot be ruled out in our studies. Additionally, we have shown that Celastrol is a potent inhibitor of NF-κB activation (8). The latter is known to regulate the expression of antiapoptotic and angiogenic gene products. Thus, the suppression of AKT/mTOR/S6K pathway may also contribute to the antitumor effects of the tripterine. Probably, all the above mechanisms might lead to synergic effect on angiogenesis \textit{in vitro} and \textit{ex vivo}.

Dysregulation of mTOR signaling frequently leads to tumorigenesis (16, 19, 32). mTOR inhibitors are currently being applied to malignant tumors in clinical trials (33), suggesting that agents targeting mTOR signaling are...
powerful and potential agents in cancer therapy. Recently, several studies have shown the role of p70S6K1, a direct downstream target of the mTORC1 (mTOR, GβL, and raptor), in modulating cell migration (34, 35). Phosphorylation of p70S6K1 is sufficient to induce actin filament remodeling, form lamellipodia and filopodia structures, and decrease actin stress fibers (36). In accord with this evidence, our results in the VEGF stimulation models in vitro showed that Celastrol at lower concentration of 1 μmol/L could effectively inhibit endothelial cell migration, invasion, and capillary structure formation.

In conclusion, our results have shown that Celastrol can suppress tumor growth through inhibition of tumor angiogenesis in vitro and in vivo via targeting AKT/mTOR/S6K kinase signaling pathway. Our discovery of this novel mechanism of Celastrol in anticancer actions not only confirms its ethnopharmacologic value but also contributes to modern drug developments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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