Aloe-emodin suppresses prostate cancer by targeting the mTOR complex 2

Kangdong Liu1,2,3, Chanmi Park1, Shengqing Li2, Ki Won Lee4,5, Haidan Liu1, Long He1, Nak Kyun Soung1, Jong Seog Ahn1, Ann M. Bode2, Ziming Dong3, Bo Yeon Kim1 and Zigang Dong1,2,*

1The World Class Institute and Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Ochang, Cheongpung 363-883, Republic of Korea. 2The Hormel Institute, University of Minnesota, 801 16th Avenue, Austin, MN 55912, USA. 3Basic Medical College, Zhengzhou University, Zhengzhou 450001, China and 4Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, Republic of Korea

*To whom correspondence should be addressed. Tel: +1 507 437 9600; Fax: +1 507 437 9606; Email: zgdong@hui.umn.edu

Correspondence may also be addressed to Bo Yeon Kim. Tel: +82 43 240 6163; Fax: +82 43 240 6259; Email: bykim@kribb.re.kr

Phosphatidylinositol 3-kinase (PI3-K) amplification and phosphatase and tensin homolog (PTEN) deletion-caused Akt activation contribute to the development of prostate cancer. Mammalian target of rapamycin (mTOR), mammalian target of rapamycin (mTOR), rapamycin-insensitive component of mTOR (RICTOR), mSin1, mLST8/GβL and PR55 and functions in the phosphorylation of Akt at Ser473. Herein, we report that mTORC2 plays an important role in PC3 androgen refractory prostate cell proliferation and anchorage-independent growth. Aloe-emodin, a natural compound found in aloe, inhibited both proliferation and anchorage-independent growth of PC3 cells. Protein content analysis suggested that activation of the downstream substrates of mTORC2, Akt and PKCα, is inhibited by aloe-emodin treatment. Pull-down assay and in vitro kinase assay results indicated that aloe-emodin could bind with mTORC2 in cells and inhibit its kinase activity. Aloe-emodin also exhibited tumor suppression effects in vivo in an athymic nude mouse model. Collectively, our data suggest that mTORC2 plays an important role in prostate cancer development and aloe-emodin suppresses prostate cancer progression by targeting mTORC2.

Introduction

Prostate cancer is the second most common cause of cancer-related death in men in the USA. Accumulating evidence indicates that phosphatidylinositol 3-kinase (PI3-K) and phosphatase and tensin homolog (PTEN) deletion caused Akt activation contribute to the development of prostate cancer. Mammalian target of rapamycin (mTOR), mammalian target of rapamycin (mTOR), rapamycin-insensitive component of mTOR (RICTOR), mSin1, mLST8/GβL and PR55 and functions in the phosphorylation of Akt at Ser473. Herein, we report that mTORC2 plays an important role in PC3 androgen refractory prostate cell proliferation and anchorage-independent growth. Aloe-emodin, a natural compound found in aloe, inhibited both proliferation and anchorage-independent growth of PC3 cells. Protein content analysis suggested that activation of the downstream substrates of mTORC2, Akt and PKCα, is inhibited by aloe-emodin treatment. Pull-down assay and in vitro kinase assay results indicated that aloe-emodin could bind with mTORC2 in cells and inhibit its kinase activity. Aloe-emodin also exhibited tumor suppression effects in vivo in an athymic nude mouse model. Collectively, our data suggest that mTORC2 plays an important role in prostate cancer development and aloe-emodin suppresses prostate cancer progression by targeting mTORC2.

Materials and methods

Materials

Aloe-emodin (>95% purity) and other chemical reagents, including Tris, NaCl and sodium dodecyl sulfate (SDS), for molecular biology and buffer preparation, were purchased from Sigma–Aldrich (St Louis, MO). CNBr-Sepharose 4B and glutathione-Sepharose 4B beads were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Antibodies for Western blot analysis were from Cell Signaling Technology (Beverly, MA), Santa Cruz Biotechnology (Santa Cruz, CA) or Upstate Biotechnology (Charlottesville, VA).

Cell culture

PC3 prostate cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). PC3 cells were propagated in F-12K medium (Cellgro, Manassas, VA) containing 10% fetal bovine serum (Gibco, Grand Island, NY) and 1 IU penicillin/l ml streptomycin (Cellgro) at 37°C in a humidified incubator with 5% CO2. PC3 cells were cytogenetically tested and authenticated before the cells were frozen. Each vial of frozen cells was thawed and maintained in culture for a maximum of 8 weeks. Enough frozen vials were available for each cell line to ensure that all cell-based experiments were conducted on cells that had been tested and in culture for ≤ 8 weeks.

MTS assay

PC3 cells (3 × 104) were seeded into 96-well plates in 100 µl of 10% fetal bovine serum F-12K medium and incubated in a 37°C in a 5% CO2 incubator. After culturing for 12 h, different concentrations of aloe-emodin were added to each well. After incubation for another 24, 48, 72 or 96 h, 20 µl of the CellTiter 96 Aqueous One Solution Promega, Madison, WI) was added to each well and cells were then incubated at another 2 h at 37°C. Absorbance was measured at 490 and 690 nm.
Western blotting
For Western blot analysis, cells (2 × 10^6) were cultured in a 10-cm dish for 24 h. The cells were then treated with various concentrations of aloe-emodin (0, 2.5, 5, 10 or 15 μM) for 24 h. The protein concentration was determined and lysate protein (30 μg) was subjected to 10% SDS-polyacrylamide gel electrophoresis. After transferring proteins, membranes were incubated with a specific primary antibody at 4°C overnight. Protein bands were visualized by a Chemiluminescence Detection Kit (Amersham Pharmacia Biotech) after hybridization with a horseradish peroxidase-conjugated secondary antibody.

mTOR in vitro kinase assay
The PI3-K/4-kinase assays were conducted as described (25). In brief, purified Akt fusion proteins (1 μg) were used for an in vitro kinase assay. Reactions were conducted in kinase buffer containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 mM potassium acetate, 1 mM MgCl₂ and 50 μM ATP at 30°C for 30 min. Reactions were terminated and proteins resolved by 8% or 6% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

mTORC2 in vitro kinase assay
mTORC2 was pulled down with a Rictor antibody as described by Sarbassov et al. (11). Purified Akt1 fusion proteins (1 μg) were used for an in vitro kinase assay. Reactions were performed in kinase buffer containing 50 μM unlabeled ATP with or without 10 μCi of [γ-^32P]ATP at 30°C for 30 min. Reactions were terminated and proteins resolved by 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Pull-down assays
For the ex vivo pull-down assay, a cellular supernatant fraction of PC3 cells (500 μg) was incubated with aloe-emodin-Sepharose 4B (or Sepharose 4B alone as a control) beads (100 μl, 50% slurry) in reaction buffer (50 mM Tris pH 7.5, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 M phenylmethylsulfonyl fluoride and 1 μg protease inhibitor mixture). After incubation with gentle rocking overnight at 4°C, the beads were washed five times with buffer (50 mM Tris pH 7.5, 5 mM ethylenediaminetetraacetic acid, 150 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet P-40 and 0.02 M phenylmethylsulfonyl fluoride) and proteins bound to the beads were analyzed by Western blotting.

In vivo tumor growth assay
Athymic nude mice (BALB/c nude mouse, 6 weeks old) were from Orient Bio (Jungwon-gu, Gyeyonggi-Do, Republic of Korea). Animals were maintained under ‘specific pathogen free’ conditions and all animal studies were conducted according to guidelines approved by the KIBIB-IBACUC (Korea Research Institute of Bioscience & Biotechnology—Institutional animal care and use committee). Animals were maintained for 2 weeks before the study and had free access to food and water. The animals were housed in climate-controlled quarters with a 12-h light/12-h dark cycle. Animals were randomly assigned to the following groups: vehicle group (n = 12); 10 mg/kg aloe-emodin group (n = 12); 50 mg/kg aloe-emodin group (n = 12) and 50 mg/kg aloe-emodin control group (n = 12). Each mouse was administered aloe-emodin (10 or 50 mg/kg body weight in 100 μl of 20% PEG 400 in autoclaved phosphate-buffered saline as vehicle) or only vehicle five times per week by intraperitoneal injection. After 3 days of treatment, PC3 cells (1 × 10^7) were injected subcutaneously into the right flank of mice in the respective groups. Following injection, mice continued to be administered aloe-emodin or vehicle. Mice in the 50 mg/kg aloe-emodin control group were not injected with cells but maintained for comparison of body weight and tumor development. Mice were weighed and tumors measured by caliper three times per week. Tumor volume was calculated from measurements of two diameters of the individual tumor according to the following formula: tumor volume (mm³) = (length × width × width)/2. Mice were monitored until day 28 and at that time, mice were euthanized and tumors extracted.

Statistical analysis
All quantitative data are expressed as means ± SE or SD as indicated. The one-way analysis of variance was used for statistical analysis. A probability of P < 0.05 was used as the criterion for statistical significance.

Results
Akt activity is increased in prostate cancer cell lines and the mTOR complex2 regulates Akt phosphorylation and is required for PC3 prostate cancer cell growth
To determine whether Akt activity is directly associated with the tumorigenic properties of cancer cells, we measured the phosphorylation level of Akt at Ser473 and Thr308 in several human cancer cell lines. Akt phosphorylation at Ser473 was increased in prostate cancer cell lines and in some lung cancer cell lines (Figure 1A). These data suggested that Akt phosphorylation at Ser473 might be associated with tumorigenic potential of prostate cancer. Based on this finding, we investigated whether knocking down the upstream kinase of Akt would affect proliferation of prostate cancer cells. We established a PC3 cell line stably expressing short hairpin RNA targeting Rictor.

Fig. 1. Akt is activated in prostate cancer cell lines and knocking down Rictor expression suppresses proliferation and anchorage-independent growth of PC3 prostate cancer cells. (A) Western blot analysis of Akt phosphorylation in various cancer cell lines. PC3 and LNCaP prostate carcinoma; HCT116 and HT29: colorectal carcinoma; A549, Calu3, H1650 and H1795: lung adenocarcinoma; H520: lung squamous cell carcinoma. (B) PC3 cells were transfected with an sh-mock or sh-rictor plasmid and stable colonies were selected with puromycin. Knockdown of Rictor expression and Akt phosphorylation were analyzed by Western blotting. (C) Proliferation was determined at 24-h intervals up to 96 h in sh-mock or sh-rictor stably transfected cells. Data are shown as means ± SD from three independent experiments. The asterisk indicates a significant (P < 0.05) decrease in proliferation compared with the control group. (D) Colony formation in soft agar using sh-mock or sh-rictor stably transfected cells. Cells were grown in soft agar for 14 days and then colonies were counted using a microscope and the Image-Pro PLUS software program (v.4). Data are shown as means ± SD from three independent experiments and the asterisk indicates a significant (P < 0.05) decrease in colony formation compared with sh-mock cells.
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A component of mTORC2. The specificity of sh-rictor was confirmed by Western blotting (Figure 1B). Knocking down Rictor expression suppressed the phosphorylation of Akt at Ser473, but not at Thr308. Cell proliferation results showed that proliferation was delayed after knocking down Rictor (Figure 1C). We thus examined whether knocking down Rictor would affect cell growth under anchorage-independent conditions. Our results indicated that knocking down Rictor in PC3 cells resulted in fewer colonies being formed in soft agar compared with control cells (Figure 1D).

Aloe-emodin suppresses proliferation and anchorage-independent growth of PC3 cells

Aloe-emodin is an anthraquinone present in the aloe plant (Figure 2A). Aloe-emodin exhibits antitumor effects against gastric cancer and hepatoma cell lines (28,29). Our data indicated that PC3 cell proliferation was inhibited by aloe-emodin in a dose-dependent manner with a maximal concentration at 15 μM (Figure 2B). Furthermore, we examined the effect of aloe-emodin on anchorage-independent growth of PC3 cells. Aloe-emodin-treated cells showed an impaired anchorage-independent growth capability, leading to a dose-dependent reduction of colony formation (Figure 2C). However, the effect was not due to toxicity (Supplementary Figure 1, available at Carcinogenesis Online).

Aloe-emodin attenuates mTORC2-mediated downstream signaling

To identify the mechanism and molecular target of aloe-emodin, we treated PC3 cells with various amounts of aloe-emodin for 24 h. The data indicated that Akt phosphorylation at Ser473 was inhibited by aloe-emodin in a dose-dependent manner. The phosphorylation of GSK3β at Ser9, directly downstream of Akt, was also inhibited, but the phosphorylation level of p70S6k at Thr389 was not affected (Figure 3). In addition, aloe-emodin had no effect on the mitogen-activated protein kinase pathway, including phosphorylation of extracellular signal-regulated kinases and total protein level of p38, c-Jun N-terminal kinases or extracellular signal-regulated kinases (Supplementary Figure 2, available at Carcinogenesis Online).

Aloe-emodin binds with the mTOR complex2

mTORC2 plays a pivotal role in PTEN loss and prostate cancer development and it phosphorylates Akt at Ser473 and PKCα at Thr638 (12,30). Based on our Western blotting data, we speculated that mTORC2 might be a molecular target of aloe-emodin. To test this idea, we performed an ex vivo pull-down assay using aloe-emodin-conjugated Sepharose 4B beads. Results revealed that aloe-emodin-conjugated beads, but not Sepharose beads alone, can pull down endogenous Rictor and mTOR together. Furthermore, the Akt protein can also be pulled down with weak binding compared with input cell lysate (Figure 4A).

To further identify the direct binding target of aloe-emodin with mTORC2, we constructed myc-tagged mammalian expression...
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plasmids containing Rictor, mSin1.1, mSin1.2, mSin1.5 and PRR5. These plasmids were, respectively, transfected into HEK 293 cells and the overexpressed proteins were analyzed by Western blot. The same amounts of overexpressed proteins were used for an \textit{ex vivo} binding assay with CNBr or CNBr-aloe-emodin conjugated Sepharose 4B beads. The pulled down proteins were visualized by a myc-specific primary and horseradish peroxidase-conjugated secondary antibodies.

\textbf{Aloe-emodin inhibits mTORC2 kinase activity in vitro}

Based on our results showing that aloe-emodin can directly bind with mTORC2, we determined whether aloe-emodin can inhibit mTORC2 kinase activity \textit{in vitro}. We first examined whether aloe-emodin can inhibit mTOR kinase by using glutathione-S-transferase-Akt1 as substrate and found that mTOR kinase activity could not be inhibited by aloe-emodin \textit{in vitro} (Figure 5A). Next, we evaluated the effect of aloe-emodin on mTORC2 kinase activity by using glutathione-S-transferase-Akt1 as a substrate. The phosphorylation of Akt1 at Ser473 was suppressed by aloe-emodin in a dose-dependent manner (Figure 5B).

\textbf{Discussion}

An accumulation of evidence reveals that PI3-K activation and PTEN loss play an important role in prostate tumorigenesis. Both these alterations strongly activate Akt, which plays a vital role in cell survival and cell growth. mTORC2 is known to be responsible for phosphorylation of Akt at Ser473 resulting in its full activation. Furthermore, mTORC2 is not required for survival of mouse embryonic fibroblasts and...
development of *Drosophila* embryos but is very important for transformed human prostate epithelial cells lacking PTEN to form tumors (12,23). Consistent with these findings, our observations showed that Akt is strongly activated in androgen-dependent cells (LNCaP) and androgen-refractory cells (PC3) (Figure 1A).

Rictor is a unique binding partner of mTORC2 and is known to play an important role in cell proliferation and tumor metastasis (31,32). Knocking down Rictor in PC3 cells inhibited proliferation and impaired anchorage-independent colony formation (Figure 1C). Phosphorylation of Akt at Ser473 was decreased, but not totally blocked, which is consistent with a recent observation that IkappaB kinase epsilon and TANK-binding kinase 1 (IKKepsilon/TBK1) also play a role in Akt phosphorylation (33). Taken together, these studies indicate that mTORC2 might be a promising target for both chemoprevention and chemotherapy of prostate cancer.

Aloe-emodin is a natural compound from aloe, which exhibits antitumor effects (34). Indeed, we found that aloe-emodin can inhibit PC3 cell proliferation and anchorage-independent growth (Figure 2A and B, Supplementary Figure 1, available at *Carcinogenesis* Online). Phosphorylation of Akt at Ser473 was decreased, but not totally blocked, which is consistent with a recent observation that IkappaB kinase epsilon and TANK-binding kinase 1 (IKKepsilon/TBK1) also play a role in Akt phosphorylation (33). Taken together, these studies indicate that mTORC2 might be a promising target for both chemoprevention and chemotherapy of prostate cancer.

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Our results herein are noteworthy in that prostate cancer cell growth is suppressed by aloe-emodin in vivo. Moreover, the low in vivo toxicity and tumor inhibitory activity of aloe-emodin observed in nude mice suggest that aloe-emodin is an effective chemopreventive agent against prostate cancer (Figure 6A and B). In conclusion, we showed here that mTORC2 is closely associated with prostate cancer cell growth. We also provided clear evidence showing that aloe-emodin effectively suppresses anchorage-independent cell growth and in vivo tumor growth in PC3 cancer cell-bearing nude mice by inhibiting Akt activity. Collectively, these findings support the anticancer efficacy of aloe-emodin through its targeting of mTORC2 for the inhibition of prostate cancer progression.

**Supplementary material**

Supplementary Figures 1–3 can be found at http://carcin.oxfordjournals.org/

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