Akt1 and Akt3 Exert Opposing Roles in the Regulation of Vascular Tumor Growth

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Abstract

Vascular tumors are endothelial cell neoplasms whose mechanisms of tumorigenesis are poorly understood. Moreover, current therapies, particularly those for malignant lesions, have little beneficial effect on clinical outcomes. In this study, we show that endothelial activation of the Akt1 kinase is sufficient to drive de novo tumor formation. Mechanistic investigations uncovered opposing functions for different Akt isoforms in this regulation, where Akt1 promotes and Akt3 inhibits vascular tumor growth. Akt3 exerted negative effects on tumor endothelial cell growth and migration by inhibiting activation of the translation regulatory kinase S6-Kinase (S6K) through modulation of Rictor expression. S6K in turn acted through a negative feedback loop to restrain Akt3 expression. Conversely, S6K signaling was increased in vascular tumor cells where Akt3 was silenced, and the growth of these tumor cells was inhibited by a novel S6K inhibitor. Overall, our findings offer a preclinical proof of concept for the therapeutic utility of treating vascular tumors, such as angiosarcomas, with S6K inhibitors. Cancer Res; 75(1): 1–11. ©2014 AACR.

Introduction

Vascular tumors are endothelial cell neoplasms with a wide spectrum of clinical presentations, ranging from benign infantile hemangiomas in children to low-grade malignant hemangioendotheliomas and highly aggressive angiosarcomas in adults. To date, the molecular pathogenesis of vascular tumors is poorly understood and current therapies, particularly those for malignant vascular tumors, do not significantly improve patient outcome (1).

Akt is a major signaling pathway activated by VEGF that regulates endothelial cell survival (2). In infantile hemangioma, hemangioma-derived endothelial cells (hemeEC) have constitutively active VEGF receptor-2 signaling with high phosphorylation levels of ERK1/2 and Akt (3). Human angiosarcoma expresses VEGF-A and the VEGF receptors (4). We have shown increased phosphorylation of Akt and 4E-BP1 in angiosarcoma (5). Hyper-activation of PI3K results in hemangiosarcoma formation in chicken chorioallantoic membrane (6). Akt1, Akt2, and Akt3 are isoforms that have shared as well as distinct functions in cancer cells. Both Akt1 and Akt2 promote cancer cell survival and growth. However, in breast and ovarian cancer, Akt1 decreases cell motility and metastasis and blocks epithelial-to-mesenchymal phenotype, whereas Akt2 enhances these processes (7–9). Akt3 is preferentially required for the growth of triple-negative breast cancer (10), and a gene fusion of Akt3 with MAG3 leads to constitutive Akt3 activation and is enriched in these tumors (11). Interestingly, there is some evidence suggesting that Akt3 exerts inhibitory effects in cancer. N-Cadherin promotes breast cancer metastasis by inhibiting Akt3, and Akt3 has been shown to inhibit lung tumor growth in mice (12–14). Studies of animal models of breast cancer with simultaneous deletion or overexpression of Akt1, Akt2, and Akt3 lend further support to Akt isoform-specific roles in cancer (8, 9).

mTOR complex-1 (mTORC1) and complex-2 (mTORC2) are composed of multiple subunits, including mTOR and Raptor (in mTORC1), and mTOR and Rictor (in mTORC2; refs. 15, 16). Akt activates mTORC1, which phosphorylates the translational regulators 4E-BP1 and p70 S6-Kinase (S6K). S6K in turn activates S6 ribosomal protein (S6; refs. 17, 18). mTORC2 directly activates Akt by phosphorylating it at serine 473, thereby exerting feedback regulation on the Akt signaling pathway (15). The S6K pathway is important in protein synthesis and cell growth, and acts as a regulator of actin cytoskeleton dynamics in cell migration (19, 20).

In this study, we showed that endothelial Akt1 drives vascular tumor growth. Importantly, we have uncovered the opposing functions of Akt1 and Akt3 in the regulation of tumor growth, which is mediated through S6K, and found a novel negative feedback regulation on Akt3 by S6K. We also demonstrated the clinical utility of a novel S6K inhibitor in the treatment of vascular lesions.
Materials and Methods

Animals

Animal studies were conducted in compliance with the Beth Israel Deaconess Medical Center (BIDMC, Boston, MA) Institutional Animal Care and Use Committee guidelines. Double transgenic myristoylated Akt1 mice in mixed FVB genetic background have been previously described (21). C57 Bl/6 Akt3−/− mice were from Argris Efstatiadis (Columbia University, New York, NY) and Morris Birnbaum (University of Pennsylvania, Philadelphia, PA; ref. 22).

Cell lines and reagents

The use of human tissues was approved by the Institutional Review Boards at BIDMC and Baylor College of Medicine (Houston, TX). Primary human dermal microvascular endothelial cells, infantile hemangioma, and mouse endothelial cell were isolated as described in refs. 21 and 23. ASM.5 cells were from Vera Krump-Konvalinkov (Ludwig Maximilian University Munchen, Germany) and EOMA cells were from ATCC as previously published (23–25). Cell line authentication and validation by short tandem repeats was performed. HA-tagged-MyrAkt3 and constitutively active S6K (R3A) have been described (26, 27). The use of human tissues was approved by the Institutional Review Board at BIDMC and Baylor College of Medicine (Houston, TX). Cell lines were passaged every 3 to 4 days.

Mouse hemangioma skin graft model

Ten millimeter circular pieces of flank skin from donor myrAkt1 mice were grafted onto the back of recipient mice with absorbable Vicryl sutures (Ethicon). Recipient animals were maintained on 1.5 mg/mL tetracycline in the drinking water as described (21) to turn off myrAkt1 while the grafts were left to heal for 2 weeks. After this time, half of the recipients continued to receive tetracycline for 4 weeks, while the other half was taken off tetracycline to turn on myrAkt1 expression.

Immunoprecipitation and Western blots

For Akt isoform immunoprecipitations, cells were lysed in RIPA buffer with protease/phosphatase inhibitors. Cell lysates were preclarified with Protein A/G Agarose beads for 1 hour at 4°C and followed by addition of primary antibodies to 0.8 mg protein lysates and rotated overnight at 4°C. Protein A/G Agarose was then added to the lysates and rotated for 2 hours at 4°C. Immunoprecipitated proteins were denatured in Laemmli buffer and analyzed by Western blotting as described (23).

Tumor growth

EOMA cells (0.3 × 10^6) were injected subcutaneously in 6- to 8-week-old nu/nu female mice (2 sites/mouse, 4–5 mice/group). Tumor size was measured daily. For drug treatment, when tumors reached 0.01 cm^2 in size, the animals were treated with vehicle control or LY2584702 (0.25% Tween-80 and 0.05% antifoam, and administered orally to mice (12.5 mg/kg twice daily). All antibodies used were from Cell Signaling Technology, except for antibodies to smooth muscle actin, tubulin, CD31, and glucose transporter-1 (Morris Birnbaum).

Lentiviral shRNA and quantitative real-time PCR

shRNA clones used are listed in Supplementary Table S2. Quantitative PCR (qPCR) primers are provided in Supplementary Materials and Methods. Lentivirus packaging and qPCR were performed as described (23).

Results

Akt is activated in human vascular tumors

Clinical specimens of 17 benign infantile hemangioma, 6 Kaposiform hemangioendothelioma, 16 Kaposi sarcoma, and 9 angiosarcoma were stained for phosphorylated Akt Serine 473 (pAkt) (supplementary Materials and Methods). All of the tumors expressed increased pAkt levels compared with adjacent normal blood vessels in the same tissue sections (Fig. 1A). We also compared the levels of pAkt in these tumors with vessel blood vessels in 20 normal skin specimens as the normal counterpart of neoplastic vessels. The percent positively stained tumor cells (stain reactivity) and the stain intensity per group were calculated. Higher pAkt stain reactivity was seen in vascular tumors than in normal skin (73.3% ± 2.1% in normal skin vs. 92.3% ± 1.3% in infantile hemangioma, 95.5% ± 1.2% in Kaposiform hemangioendothelioma, 92.3% ± 1.3% in Kaposi sarcoma and 89.7% ± 1.9% in angiosarcoma; P < 0.001; Fig. 1B). To evaluate the stain intensity, the staining was scored using a 3-tier system (1, low; 2, moderate; and 3, high) by two pathologists and the average score was graphed. Representative pictures of low and high stain intensity are shown (Supplementary Fig. S1). Significantly higher pAkt stain intensity was seen in tumors (1.6 ± 0.1 in normal skin vs. 2.1 ± 0.2 in infantile hemangioma, 2.2 ± 0.3 in Kaposiform hemangioendothelioma, 2.5 ± 0.2 in Kaposi sarcoma and 3.0 ± 0.3 in angiosarcoma; P < 0.01; Fig. 1C and Supplementary Fig. S1). These results showed increased Akt activation across different types of human vascular tumors.

To determine whether Akt is hyperactivated in neoplastic endothelial cell, we focused on infantile hemangioma, which is a common soft tissue tumor of infancy and fresh tissues are available for studies. We purified hemeEC from infantile hemangioma using CD31-magnetic bead isolation and stained for endothelial markers (Supplementary Fig. S2A). HemeEC showed a 1.9-fold increase in pAkt as compared with normal human dermal microvascular endothelial cells (HDMEC) by Western blot analysis (Fig. 1D). Consistent with a previous report of constitutive VEGFR-2 activation in infantile hemangioma (3), we observed increased phosphorylated VEGFR-2 in a subset of hemeEC samples. Interestingly, PTEN levels were also reduced in some hemangiomas. These findings showed increased Akt activation in infantile hemangioma, which is associated with decreased PTEN and increased VEGFR-2 activation.

We next examined Akt activation in malignant vascular tumors. We utilized ASM.5 cells isolated from a spontaneous human angiosarcoma (24), and EOMA cells derived from a spontaneous mouse hemangioendothelioma (25). ASM.5 and EOMA cells had increased Akt activation as compared with normal endothelial cells (Fig. 1E and F; 1.0 ± 0.2 in HDMEC vs. 2.8 ± 1.1 in ASM.5; P < 0.05, N = 3; 1.0 ± 0.5 in mouse endothelial cell vs. 6.7 ± 2.0)
Akt1 promotes the growth and migration of vascular tumor cells

HemeEC and ASM.5 cells were transduced with lentiviral shRNA to Akt1 (shAkt1). Significant Akt1 knockdown was achieved using two independent shAkt1 clones as compared with pLKO scramble shRNA control (Fig. 2A and B). Akt1 knockdown significantly reduced the growth of hemeEC and ASM.5 cells as assessed by cellular DNA content (Fig. 2C and D). As Akt1 is known to affect cell survival (28), we determined the effects of shAkt1 on apoptosis. Akt1 knockdown resulted in increased tumor cell apoptosis in response to serum starvation as determined by the apoptotic marker Annexin V (Supplementary Fig. S2B and S2C). Akt1 knockdown decreased basal and VEGF-stimulated migration of hemeEC and ASM.5 cells in Boyden chamber migration assay (Fig. 2E and F). Akt1 knockdown also significantly inhibited basal and VEGF-induced cord formation in hemeEC (Fig. 2G and H). These findings showed that Akt1 plays a key role in promoting the survival and migration of vascular tumor cells.

Endothelial Akt1 activation induces hemangioma formation in mice

To determine whether Akt1 activation in endothelial cell is sufficient to drive de novo vascular tumor formation, we utilized a double transgenic mouse model that expresses tetracycline-inducible and endothelial cell–specific activated myristoylated Akt1 (myrAkt1; ref. 21). myrAkt1 transgene induction increased Akt1 expression by 3.9-fold, but did not affect Akt2 and Akt3 levels in endothelial cells isolated from these mice (Supplementary Fig. S3A and S3B). myrAkt1 mice have systemic pathologic angiogenesis, but they do not develop vascular tumors due to shortened lifespan from systemic edema (21). To study the long-term effects of endothelial Akt1 activation, we developed a skin graft model in which 10 mm circular pieces of skin from myrAkt1 donors (FVB background) were transplanted onto the back skin of immunodeficient nu/nu mice (Fig. 3A).Recipient animals were
Akt1 promotes vascular tumor cell growth and migration. HemeEC (A) and ASM.5 (B) expressing pLKO or shAkt1 (independent clones #1 and #2) were analyzed by Western blot analysis. C and D, HemeEC (C) and ASM.5 (D) DNA content expressed as fluorescence units relative to “day 0”; *, P < 0.05; N = 3. E and F, Transwell migration assays of hemeEC (E) and ASM.5 (F) cells expressing pLKO or shAkt1 (independent clones #1 and #2) were analyzed by migration assays of hemeEC (E) and ASM.5 (F) cells expressing pLKO or shAkt1 (independent clones #1 and #2) were analyzed by Western blot analysis. C and D, HemeEC (C) and ASM.5 (D) DNA content expressed as fluorescence units relative to “day 0”; *, P < 0.05; N = 3. G, representative bright field images of HemeEC cord formation on Collagen I matrix ± VEGF (50 ng/mL) for 5 hours normalized to “pLKO-VEGF” control; **, P < 0.05 versus pLKO-VEGF; ***, P < 0.01 versus pLKO + VEGF; N = 3. H, quantitation of cord length; *, P < 0.01 versus pLKO-VEGF; **, P < 0.01 versus pLKO + VEGF; N = 3.

Sustained endothelial Akt1 activation is necessary to maintain hemangioma growth

Skin graft transplantation was performed and myrAkt1 expression was turned on to allow hemangioma to develop. After 4 weeks, myrAkt1 expression was turned off in half of the animals while myrAkt1 expression continued to be on in the other half. Tumors in animals with sustained myrAkt1 expression continued to grow, but those in animals with myrAkt1 turned off regressed dramatically over the next 3.5 weeks and had few vessels and more fibrofatty tissue (Fig. 3E and F). Thus, sustained endothelial Akt1 activation is required to maintain the integrity of blood vessels in this vascular tumor model, and supports our previous finding of the “plasticity” of the microvasculature in response to Akt1 signaling (21).

Akt3 expression is reduced in vascular tumors

Immunostains for Akt1, Akt2, and Akt3 in 15 infantile hemangioma and 10 angiosarcoma samples showed that the levels of Akt1 and Akt2 in these tumors were similar to those present in adjacent normal blood vessels in the same tissue sections. Akt3 expression was reduced in vascular tumors.
Endothelial myrAkt1 activation drives to hemangioma formation in vivo. A, a schematic of myrAkt1 skin graft model of hemangioma (see the text for details). B, vascular tumors developed in the grafts 4 weeks following myrAkt1 induction. C, microscopic features of the tumor (scale bar, 100 μm). Arrows, tumor boundary. Graph of tumor volume in myrAkt1 mice is shown. D, immunofluorescence stains of myrAkt1 tumor for CD31 (red) and HA-tagged myrAkt1 (green); smooth muscle actin (SMA, red) and phospho-Akt (green); glucose transporter-1 (Glut-1, red) and CD31 (green); nuclei (blue). E and F, myrAkt1-induced hemangiomas regressed when myrAkt1 tumor volume in myrAkt1 mice is shown. D, immuno

We next compared the levels of Akt isoforms in vascular tumors (Fig. 4A). In contrast, Akt3 levels were reduced in tumor tissues. We next compared the levels of Akt isoforms in vascular tumors with the blood vessels in 6 normal human skin specimens (Fig. 4B). The stain reactivity and stain intensity were calculated as described for pAkt stains in Fig. 1. Representative pictures of low and high Akt isoform stain intensity are shown in Supplementary Fig. S4 at different magnifications. The stain reactivity (%) and stain intensity for endogenous Akt1 were similar in normal skin and vascular tumors (for Akt1 stain reactivity, 85.6% ± 4.4% in normal skin vs. 77.0% ± 5.4% in infantile hemangioma and 87.7% ± 1.5% in angiosarcoma; P = not significant; for Akt1 stain intensity, 2.3 ± 0.4 in normal skin vs. 1.5 ± 0.2 in infantile hemangioma and 2.1 ± 0.3 in angiosarcoma; P = not significant). Likewise, endogenous Akt2 levels were similar in normal skin and vascular tumors (for Akt2 stain reactivity, 78.0% ± 13.5% in normal skin vs. 89.1% ± 1.7% in infantile hemangioma and 86.0% ± 3.2% in angiosarcoma; P = not significant; for Akt2 stain intensity, 1.8 ± 0.5 in normal skin vs. 2.3 ± 0.2 in infantile hemangioma and 2.3 ± 0.2 in angiosarcoma; P = not significant). In contrast, endogenous Akt3 levels were reduced in vascular tumors as compared with normal skin (for Akt3 stain intensity, 2.4 ± 0.3 in normal skin vs. 1.1 ± 0.1′ in infantile hemangioma and 1.4 ± 0.2′ in angiosarcoma; *, P < 0.05). The stain reactivity for Akt3 was lower in infantile hemangioma (88.7% ± 2.3% in normal skin vs. 69.5% ± 8.3% in infantile hemangioma; *, P < 0.05), but not in angiosarcoma (88.7% ± 2.3% in normal skin vs. 87.8% ± 2.0% in angiosarcoma; P = not significant).

Immunoblots for Akt3 in ASM.5 and EOMA cells showed that they had significantly lower Akt3 levels than normal endothelial cells, which is consistent with the findings in patient tumor tissues (Supplementary Fig. S5). We did not observe a significant change in Akt3 in hemeEC. To examine the phosphorylation status of Akt isoforms, ASM.5 cells were immunoprecipitated with antibodies specific for Akt1, Akt2, and Akt3, and immunoblotted for each Akt isoform and phospho-Akt. Akt1 and Akt3 were phosphorylated at both threonine 308 (T308) and S473 residues, sites that are required for full Akt activation (Fig. 4C). Interestingly, Akt2 did not appear to be phosphorylated in these studies, indicating that only Akt1 and Akt3 are the two active isoforms in these tumor cells.

Akt1 promotes, whereas Akt3 inhibits vascular tumor growth

We assessed the effects of loss of each Akt isoform on sprouting angiogenesis in infantile hemangioma. As Akt2 does not appear to be activated in vascular tumor cells (Fig. 4C), we chose to focus on Akt1 and Akt3. Western blot analysis and quantitation by densitometry showed effective knockdown of each Akt isoform in hemeEC by shRNA (Supplementary Fig. S6A). Of note, the levels of Akt3 appeared higher than Akt1 and Akt2 in the blots. However, because the antibodies to detect Akt1, Akt2, and Akt3 were different antibodies, it would not be possible to cross compare the levels of one Akt isoform to another by Western blots.

In spheroid sprouting assay, loss of Akt1 reduced basal and VEGF-A–stimulated sprout formation as compared with pLKO

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However, loss of Akt3 significantly increased sprout formation under basal conditions and with VEGF-A using independent shRNAs. Similar findings were observed in ASM.5 and EOMA cells. Effective knockdown of each Akt isoform was achieved as shown by densitometric quantitation of Western blots (Supplementary Fig. S6B and C). Compared with pLKO, loss of Akt1 reduced tumor cell migration in scratch wound assays (Fig. 5C and D). In contrast, loss of Akt3 significantly increased cell migration. Akt1 and Akt3 also exert opposing effects on cell growth—loss of Akt1 reduced, whereas loss of Akt3 increased EOMA growth in vitro (Fig. 5E).

To evaluate the functions of Akt isoforms in vivo, Akt1, Akt2, and Akt3 were knocked down in EOMA cells using independent shRNA clones for each isoform (Supplementary Fig. S6D). Cells were then injected subcutaneously in nu/nu mice and tumor size was monitored for 12 days. Loss of Akt1 reduced tumor growth and loss of Akt2 had no effect, whereas loss of Akt3 enhanced tumor growth (Fig. 5F and Supplementary Fig. S6E). These findings demonstrate the opposing roles of Akt1 and Akt3 in vascular tumor growth.

The distinct functions of Akt1 and Akt3 are mediated through p70 S6K

To evaluate downstream effectors of Akt isoforms, Akt1 and Akt3 were knocked down in hemeEC and EOMA cells and analyzed for Akt1, Akt2, and S6. pAkt levels were normalized to total Akt levels and calculated relative to that in pLKO. Knockdown of either Akt1 or Akt3 decreased pAkt in hemeEC (Fig. 6A; 1.0 ± 0.0 in pLKO vs. 0.6 ± 0.1* in shAkt1 and 0.7 ± 0.04* in shAkt3; *, P < 0.05, N = 4). Similar reduction in pAkt was found in EOMA cells (1.0 ± 0.0 in pLKO vs. 0.4 ± 0.02* in shAkt1 and 0.7 ± 0.1* in shAkt3; *, P < 0.05, N = 3). These results indicate that both Akt1 and Akt3 contribute to the total pool of phosphorylated Akt in these cells.

Knockdown of Akt1 in hemeEC decreased levels of phosphorylated S6K (pS6K) and its downstream effector phosphorylated S6 (pS6). In contrast, loss of Akt3 increased pS6K and pS6 (Fig. 6A). Quantitative analysis of Western blots showed that pS6K levels in hemeEC were 1.0 ± 0.0 in pLKO versus 0.5 ± 0.2* in shAkt1 and 3.3 ± 0.5* in shAkt3; *, P < 0.05, N = 3. Similarly, the levels of pS6 were 1.0 ± 0.0 in pLKO versus 0.5 ± 0.1* in shAkt1 and 1.9 ± 0.3*
in shAkt3; P < 0.05. We also evaluated EOMA cells and found similar opposing effects of Akt1 and Akt3 on S6K and S6 activation (Fig. 6A). pS6K levels in EOMA cells were 1.0 ± 0.0 in pLKO versus 0.3 ± 0.1* in shAkt1 and 3.2 ± 0.3* in shAkt3; *, P < 0.05, N = 3. Similarly, levels of pS6 were 1.0 ± 0.0 in pLKO versus 0.3 ± 0.2* in shAkt1 and 2.2 ± 0.7* in shAkt3; *, P < 0.05. Endothelial cells from Akt3−/− mice similarly showed increased levels of pS6K and pS6 (Fig. 6A). pAkt levels were 1.0 ± 0.0 in wild-type (WT) cells versus 0.8 ± 0.03* in Akt3−/− cells; *, P < 0.05, N = 3. pS6K levels were 1.0 ± 0.0 in WT cells versus 4.8 ± 0.8* in Akt3−/− cells; *, P < 0.05. Levels of pS6 were 1.0 ± 0.0 in WT cells versus 1.8 ± 0.3* in Akt3−/− cells; *, P < 0.05. Thus, we have observed in vascular tumor cells and confirmed in Akt3−/− cells that Akt1 and Akt3 exert opposite effects on S6K signaling pathway, in which Akt1 promotes, whereas Akt3 inhibits S6K activation.

To determine whether S6K mediates the biologic effects observed in knockout studies, we rescued Akt1 knockdown cells with overexpression of constitutively activated S6K R3A, and rescued Akt3 knockdown cells with concurrent knockdown of S6K. S6K rescued shAkt1 and shAkt3 effects on the migration and proliferation of EOMA and ASM.5 cells (Fig. 6B–E and Supplementary Fig. S7). These findings showed that S6K is a mediator of the inhibitory effects of Akt3 in vascular tumor cells.

We also utilized a small-molecule inhibitor of S6K, LY2584702. LY2584702 is highly selective for S6K1 when tested against 83 different kinases and 45 cell surface receptors. In S6K1 enzyme assay, the IC50 of the drug was 2 nmol/L. For pS6 inhibition in cells, the IC50 = 100 nmol/L (Supplementary Table S1). The drug has some activity against the S6K-related kinases MSK2 and RSK at high concentrations (enzyme assay IC50 = 58–176 nmol/L). LY2584702 inhibits S6K activity in EOMA cells, as determined by the phosphorylation of its downstream effector S6, in a dose-dependent manner (Fig. 6F). To examine the role of S6K in vivo, EOMA cells expressing shAkt3 were implanted in nu/nu mice, then treated for 14 days with LY2584702 or rapamycin, which is a potent inhibitor of mTORC1 and S6K activation (30). Analysis of tumors removed after 14 days showed that LY2584702 inhibited S6 phosphorylation almost as effectively as rapamycin (Fig. 6G). Loss of Akt3 increased tumor growth as compared with pLKO (Fig. 6H). LY2584702 treatment alone did not significantly increase tumor growth.
Affect the growth of pLKO tumors. However, it significantly reduced the growth of tumors with shAkt3. These findings showed that downregulation of Akt3 increased S6K activation in vascular tumors, and enhanced the antitumor efficacy of S6K inhibition with LY2584702.

Akt3 modulates Rictor levels

We evaluated the effects of Akt3 knockdown on the protein components of mTOR complexes in EOMA and ASM.5 cells. The levels of mTOR and Raptor (a key component of mTORC1) were not affected by loss of Akt1 or Akt3 (Fig. 7A and B). However, the levels of Rictor (a key component of mTORC2) were significantly reduced in cells with loss of Akt3, but not Akt1. Rictor immunoblots were quantified by densitometry, normalized to β-actin and calculated relative to pLKO. In EOMA cells, Rictor levels were 1.0 ± 0.0 in pLKO versus 1.4 ± 0.2 in shAkt1 and 0.4 ± 0.2 in shAkt3; *P < 0.05, N = 9. Similarly in ASM.5 cells, Rictor levels were 1.0 ± 0.0 in pLKO versus 1.1 ± 0.2 in shAkt1 and 0.5 ± 0.03 in shAkt3; *P < 0.05, N = 3. We observed that knockdown of Akt3, but not Akt1, significantly reduced Rictor mRNA (Fig. 7C). These findings indicate that Akt3 positively regulates Rictor levels, at least in part by modulating Rictor mRNA expression.

To determine whether Rictor affects S6K phosphorylation, we knocked down Rictor and immunoblotted for pS6K and pS6 (mTORC1 activity) and pAkt S473 (mTORC2 activity). Rictor knockdown decreased pAkt levels in both EOMA and ASM.5 cells (Fig. 7D and Supplementary Fig. S8A). Importantly, loss of Rictor resulted in increased pS6K in both cell lines. Densitometric analysis showed that in EOMA cells, pS6K levels were 1.0 ± 0.0 in pLKO versus 1.6 ± 0.2 in shRictor; *P < 0.05, N = 4. pS6 downstream of S6K also increased with Rictor knockdown. pS6 levels were 1.0 ± 0.0 in pLKO versus 1.7 ± 0.3 in shRictor; *P < 0.05, N = 4. Rictor knockdown appeared to recapitulate the effects of Akt3 knockdown on S6K and S6, suggesting that Akt3 regulates S6K activation by modulating Rictor levels.
To more definitely demonstrate that the inhibitory effects of Akt3 on S6K is mediated by Rictor, we performed a rescue experiment in which constitutively activated myristoylated Akt3 (myrAkt3) was overexpressed in EOMA cells. Expression of HA-tagged myrAkt3 was confirmed by immunoblotting (Fig. 7E). Overexpression of myrAkt3 increased pAkt (T308 and S473) and Rictor, but decreased pS6K. Concurrent knockdown of Rictor in cells with myrAkt3 overexpression rescued the inhibitory effects of myrAkt3 on S6K phosphorylation. Densitometric analysis showed that pS6K levels were 1.0 ± 0.0 in vector versus 0.3 ± 0.04* in HA-myrAkt3 and 1.5 ± 0.5* in HA-myrAkt3 + shRictor; *, P < 0.05, N = 3. These findings further support our hypothesis that Akt3 is a negative regulator of S6K signaling pathway and Rictor is a potential mediator of the effects of Akt3.

S6K exerts negative feedback regulation on Akt3

It has been shown that S6K exerts negative feedback regulation on Akt signaling via downregulating IRS-1 and receptor tyrosine kinase signaling (31). Given the observed differential effects of Akt1 and Akt3 on S6K pathway, we investigated whether S6K exerts differential feedback regulation on Akt isoforms. Knockdown of S6K increased pAkt (T308 and S473) in EOMA cells, consistent with the relief of feedback inhibition of Akt signaling by S6K (Fig. 7F). S6K knockdown increased Akt3, but not Akt1 levels, in EOMA and ASM.5 cells (Fig. 7F and Supplementary Fig. S8B). Akt3 levels in EOMA cells were 1.0 ± 0.0 in pLKO versus 1.9 ± 0.5* in shS6K; *, P < 0.05, N = 3. Inhibition of S6K activation with rapamycin showed a significant increase in Akt3, but not Akt1 (Supplementary Fig. S8C). In immunoprecipitation experiments, more Akt3 was pulled down in cells with S6K knockdown as compared with pLKO (1.0 in pLKO vs. 1.8 in shS6K, N = 2; Fig. 7G). No changes were seen with Akt1 (1.0 in pLKO vs. 0.9 in shS6K). Conversely, overexpression of constitutively active S6K reduced levels of Akt3 (Fig. 7H). These findings show that Akt3 inhibits S6K activation, which in turn exerts negative feedback regulation on Akt3 itself.

Discussion

Our studies showed increased Akt activation and decreased PTEN levels in both benign and malignant vascular tumors. These tumors are composed of multiple cell types, including neoplastic endothelial cells, inflammatory, and stromal cells. It is not known whether the endothelial cellular component alone is sufficient for vascular tumor development. We showed in myrAkt1 animal model that de novo hemangioma formation is driven by endothelial Akt1 activation, and is "endothelial-cell autonomous."
Tumor regression was observed upon loss of endothelial myrAkt1 in our animal model, indicating that sustained Akt signaling is required for tumor maintenance. Spontaneous regression is a distinctive characteristic of infantile hemangioma that is biologically programmed in the natural progression of the tumor. It is conceivable that a preprogrammed network that switches off downstream VEGF signaling pathways, such as Akt, may be a mechanism of hemangioma regression.

Little is known about Akt3 function. Limited studies on Akt3 showed that it is required for VEGF stimulation of mitochondrial biogenesis and autophagy in endothelial cell, and is important for growth-factor induced angiogenic responses (32). Akt3 is the dominant isoform in melanoma and ovarian cancer and regulates cellular senescence, VEGF secretion and angiogenesis in these tumors (33–35). Our studies of vascular tumors and the emerging literature on Akt3 provide a new perspective on Akt signaling: there is a “check-and-balance” by different Akt isoforms to modulate overall Akt signaling output and limit unchecked growth signals downstream of Akt. Thus, one Akt isoform may regulate growth-promoting biological output, whereas another isoform may regulate growth-inhibitory output to ensure homeostatic regulation of Akt signaling. We have found that Akt1 and Akt3 have unique opposing roles, in which Akt1 promotes, whereas Akt3 inhibits tumor endothelial cell growth. It is possible that the net balance of Akt signaling output drives vascular tumors. Akt3 is expressed at lower levels in vascular tumors than in normal blood vessels. Such a scenario has also been observed in malignant glioma, in which the expression of Akt3 is lower in the tumor than in normal brain tissue; however, the remaining Akt3 has kinase activity (36). This suggests that although the level of endogenous Akt3 is reduced, it still retains the functional capacity as an active kinase.

Different Akt isoforms can signal via distinct downstream pathways that may vary depending on the cellular context and subcellular localization (37). Akt1 and Akt3, but not Akt2 activation in mice predominantly influences GSK-3 α/β signaling in a mouse lung tumor model (14). Akt3, but not Akt1, is crucial for the activation of the mTORC1–S6K signaling pathway in the brain, thus reinforcing the differential effects of Akt isoforms on the S6K pathway (22). We have found that Akt1 and Akt3 exert distinct effects on S6K signaling: Akt1 stimulates, whereas Akt3 inhibits S6K activation. To determine how Akt3 regulates S6K activation, we showed that knockdown of Akt3 leads to reduced Rictor protein levels, which is due at least in part to a reduction of Rictor mRNA expression.

Overexpression of myrAkt3 increases Rictor levels and reduces S6K activation. Importantly, knockdown of Rictor in cells with myrAkt3 overexpression rescues the effects of myrAkt3 on S6K. Taken together, these findings suggest a potential mechanism by which Akt3 regulates S6K activation through Rictor. We postulate that by regulating Rictor levels, Akt3 can affect the formation of mTOR complexes and the balance of mTORC1 and mTORC2 activities in the cell. Published studies lend support to this hypothesis. The formation of TOR complexes in Caenorhabditis elegans is controlled by semaphorin–plexin signaling, in which semaphorin promotes the formation of TORC1 and inhibits TORC2 by promoting a shift of TOR from Rictor toward Raptor, thereby altering the ratio of TORC1 and TORC2 (38). Another mechanism of regulation of mTOR complex assembly involves the mTOR inhibitor rapamycin. Rapamycin mainly inhibits mTORC1, but long-term drug treatment can lead to the inhibition of mTORC2, in which rapamycin may sequester mTOR and interfere with the assembly of mTORC2 (39).

Emerging studies have highlighted the importance of negative feedback loops that normally operate to dampen various types of signaling, and therefore ensure homeostatic regulation of signals in the cell. We have found that S6K exerts negative feedback regulation on Akt3. This is consistent with the known S6K-mediated negative feedback on the PI3K–Akt pathway (31). The selective feedback effects of S6K on Akt3 may reflect the collective pressure in tumor cells to lower Akt3 levels as a way to counteract the inhibitory effects of active Akt3.

Improved understanding of the unique roles of Akt isoforms has led to the recent development of Akt inhibitors that preferentially block Akt1 and Akt2, such as Akti-1/2 (40). We have found that inhibition of S6K activity with LY2584702 was more effective in reducing the growth of vascular tumors with loss of Akt3 than tumors with normal levels of Akt3. These findings highlight the potential clinical utility of treating vascular tumors, such as angiosarcoma, with agents that block S6K signaling. While our studies are selective for vascular tumors at this time, the findings provide an impetus for further investigation of Akt isoforms in other tumor types, which could potentially improve our ability to integrate molecular data with therapeutic treatment regimens.

Disclosure of Potential Conflicts of Interest

S. Nhek is a scientist III at Eli Lilly and Company. S. Geeganage owns ownership interest (including patents) in Eli Lilly. No potential conflicts of interest were disclosed by the other authors.

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