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Differential regulation of vascular endothelial growth factor by Akt and mammalian target of rapamycin inhibitors in cell lines derived from childhood solid tumors

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Abstract

Levels of vascular endothelial growth factor (VEGF) are regulated, in part, through activation of the phosphatidylinositol 3′-kinase/Akt pathway. Using pharmacologic inhibitors, we have examined the relative contributions of Akt and mammalian target of rapamycin (mTOR) signaling to VEGF production in neuroblastoma and rhabdomyosarcoma cells growing under normoxic (21% O_2) or hypoxic (1% O_2) conditions. Exogenous VEGF stimulated both Akt and extracellular signal–regulated kinase 1/2 phosphorylation in six of seven rhabdomyosarcoma cell lines but in only one of seven neuroblastoma cells, suggesting autocrine stimulation predominantly in rhabdomyosarcoma cell lines. In general, under normoxic conditions, neuroblastoma cells produced more VEGF (120–1,180 pg/10^6 cells/24 h) compared with rhabdomyosarcoma lines (0–200 pg/10^6 cells/24 h). Rapamycin, a selective inhibitor of mTOR, reduced VEGF production in rhabdomyosarcoma cells under normoxic conditions and partially suppressed hypoxia-driven increases in VEGF. However, it poorly inhibited VEGF production under either condition in the majority of neuroblastoma cell lines despite inhibition of mTOR signaling. Rapamycin failed to modulate levels of hypoxia-inducible factor 1α (HIF-1α) under normoxic conditions and modestly reduced hypoxia-driven increases in HIF-1α only in rhabdomyosarcoma cells. In contrast to rapamycin, inhibition of Akt by A-443654 completely blocked signaling to glycogen synthase kinase 3β and had more dramatic effects on VEGF production. Notably, A-443654 significantly inhibited VEGF production in rapamycin-refractory neuroblastoma cell lines. Importantly, whereas combining A-443654 with rapamycin had variable effect on cell proliferation, the combination essentially blocked hypoxia-driven increases in VEGF in all cell lines examined, suggesting that dual blockade at different levels in the phosphatidylinositol 3′-kinase–initiated signaling pathway may be a reasonable strategy for preventing VEGF production in cancer cells derived from pediatric solid tumors. However, this will require formal testing *in vivo* using animal models of childhood cancer. [Mol Cancer Ther 2007;6(5):1620–8]

Introduction

Several signaling pathways, such as mitogen-activated protein kinase and phosphatidylinositol 3′-kinase (PI3K)-mammalian target of rapamycin (mTOR), have been implicated in cellular hypoxic response (1–5). Support for a role of mTOR signaling in vascular endothelial growth factor (VEGF) production includes regulation of hypoxia-inducible factor 1α (HIF-1α) by mTOR signaling and increased VEGF in cells deficient in the tuberous sclerosis complex (TSC1/2) that negatively regulates mTOR via Rheb (6–8). The macrocyclic lactone antibiotic rapamycin, a highly specific inhibitor of mTOR signaling, has also been reported to have antiangiogenic activities, decreasing vessel density in several tumor models, which is linked to a decrease in VEGF production and to inhibited response of vascular endothelial cells to stimulation by VEGF (9–11). Rapamycin also targets vascular mesenchymal cells (pericytes, smooth muscle cells, and adventitial fibroblasts) inhibiting sustained VEGF and hepatocyte growth factor expression via silencing of the platelet-derived growth factor receptor-α/S6 kinase 1 (S6K1) pathway. As rapamycin showed only a minimal effect on hypoxia-related expression of VEGF in culture, these results suggest targeting of the host vasculature rather than tumor itself *in vivo* (12, 13).

Other studies support a role mainly for PI3K and, to a lesser extent, mTOR being required for insulin-induced HIF-1α expression (2). VEGF levels are decreased by PI3K inhibitors, whereas expression of constitutively active Akt reverses this effect. These data implicate Akt as a regulator of VEGF production. In contrast, serum stimulation was found to induce only a slight accumulation of HIF-1α protein in a PI3K/Akt pathway–dependent fashion, whereas hypoxia induced far higher levels of HIF-1α

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protein and HIF-1 DNA binding activity independently of PI3K and mTOR activity (14). Hypoxia causes rapid and reversible inactivation of mTOR (15) and requires the TSC1/2 tumor suppressor complex and the hypoxia-inducible gene *REDD1/RTP801* (7).

Less is known about regulation of VEGF in malignant cells derived from childhood cancers. In growth factor–deprived neuroblastoma cells *in vitro*, serum or insulin-like growth factor I (IGF-I) induced increases in HIF-1α protein that temporally paralleled increases in VEGF mRNA. VEGF and HIF-1α levels were blocked by inhibitors of PI3K and mTOR and, to a lesser extent, by the mitogen-activated protein kinase/ERK kinase-1 inhibitor PD98059 (16). However, the role of mTOR in regulating HIF-1α/VEGF is dependent on the conditions of cell culture. Pore et al. (17) recently showed that inhibition of mTOR by rapamycin only effected HIF-1α under conditions of low serum, whereas it had little effect under serum-replete conditions. There are less data available for whether mTOR signaling regulates HIF/VEGF in rhabdomyosarcoma cells, although the growth of at least one rhabdomyosarcoma xenograft model seems to be dependent on VEGF (18–20). Wan et al. (21) also reported that inhibition of rhabdomyosarcoma xenografts by the rapalog CCI-779 was linked to targeting of mTOR/ HIF-1α/VEGF signaling.

These somewhat conflicting data make the role of mTOR in regulating tumor-derived VEGF unclear. In many studies, one or only several cell lines have been examined. Here, we have investigated the role of mTOR and Akt, using pharmacologic inhibitors, in regulating VEGF under both normoxia and hypoxia in panels of neuroblastoma and rhabdomyosarcoma cell lines. Our results show that VEGF levels seem to be controlled via Akt in neuroblastoma cells, largely independent of mTOR, whereas in rhabdomyosarcoma cells inhibition of mTOR significantly decreases VEGF levels.

Materials and Methods

Cell Lines and Culture Conditions

Cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. The Akt inhibitor A-443654 was generously provided by Dr. S. Rosenberg (Abbott Laboratories). Cells were treated with rapamycin (100 ng/mL) and A-443654 (0.1–1 μmol/L) for 24 h or stimulated with 10 ng/mL IGF-I for 5 min and VEGF for periods up to 60 min, and then harvested to proceed with Western blotting.

Hypoxia Exposure

Tissue culture plates were placed in a hypoxia workstation (InVivo 400, Ruskinn Technology Ltd.) for 24 h with a gas mixture of 1% O_2, 5% CO_2, and balance of N_2. RPMI 1640 containing drugs was added to the cells 5 to 10 min before placing the plates in the chamber.

ELISA Assay

Cells were plated at 5×10^5/mL. After 24 h of drug treatment under hypoxia or normoxia, conditioned media were used for quantitation of VEGF secretion by sandwich enzyme immunoassay technique (Quantikine, R&D Systems). Briefly, standards and samples were pipetted into the wells of a 96-well microplate precoated with a monoclonal VEGF antibody. After washing, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash, a substrate solution was added to the wells and the intensity of color developed in proportion to the amount of VEGF bound in the initial step was measured using a microplate reader set to 450 nm (MRX Revelation Absorbance Reader, Dynex Technologies). All measurements were done in duplicate, normalized to nuclei counts from the attached cells in each well at the end of drug exposure, and plotted as picograms of VEGF per 10^6 cells.

Western Blotting

Cells were incubated, scraped off, lysed in 500 μL of 1× lysis buffer (Cell Signaling), sonicated, and centrifuged (15,000 × *g*, 15 min, 4°C). Equivalent amounts of protein were added to the same volume of 4× NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen), heated for 10 min at 70°C, resolved on 10% NuPAGE SDS-polyacrilamide gels (Invitrogen), and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were incubated with TBS containing 0.05% Tween 20 and 5% nonfat milk to block nonspecific binding and were incubated with primary antibodies, then with secondary antibodies conjugated to horseradish peroxidase (Pierce). We used primary antibodies to HIF-1α, β-actin (Santa Cruz Biotechnology), HIF-1β (Abcam), ribosomal protein S6 (rpS6), phospho-rpS6 ($Ser^{235/236}$), phospho-S6K1 (Thr^{389}), Akt, phospho-Akt (Ser^{308} and Ser^{473}), glycogen synthase kinase (GSK)-3β, phospho-GSK-3α/β ($Ser^{21/9}$), and phospho-extracellular signal–regulated kinase (ERK)-1/2 (Thr^{202}/Tyr^{204}) (Cell Signaling). Immunoreactive bands were visualized by using SuperSignal chemiluminiscence substrate (Pierce) and Biomax MR and XAR film (Eastman Kodak Co.).

Results

Differential VEGF-Induced Signaling in Rhabdomyosarcoma and Neuroblastoma Cells

VEGF can act as a paracrine or an autocrine factor. To determine whether either rhabdomyosarcoma or neuroblastoma cells were stimulated by exogenous VEGF, cells were growth factor deprived for 24 h, then stimulated with either VEGF or IGF-1 (positive control). Growth factor–induced phosphorylation of ERK1/2 and Akt was used as a read-out for stimulation. As shown, IGF-1 stimulated a robust phosphorylation of ERK1/2 in each rhabdomyosarcoma (Fig. 1A) and stimulated phosphorylation of Akt in each line except RD, where IGF-I only induced Akt phosphorylation. Interestingly, whereas IGF-I induced robust phosphorylation of both ERK1/2 (Thr^{202}/Tyr^{204}) and Akt (Ser^{308}) in all the neuroblastoma lines (Fig. 1B), VEGF failed to significantly activate either pathway in neuroblastoma cell lines with the exception of SK-N-AS and to a lesser effect in IMR-32 cells. In SK-N-AS cells,

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VEGF had similar activity to IGF-I phosphorylating ERKs, whereas the effect in IMR-32 cells was less robust. Further, in IMR-32 cells, VEGF did not significantly induce phosphorylation of Akt. These data suggest that in rhabdomyosarcoma cells, exogenous VEGF can stimulate the major signaling pathways associated with proliferation and survival. As rhabdomyosarcoma cells secrete VEGF, this factor may act both as an autocrine and paracrine factor. In contrast, VEGF robustly stimulated ERK phosphorylation in only one of seven neuroblastoma cell lines, suggesting that autocrine or paracrine stimulation by VEGF is modest or absent.

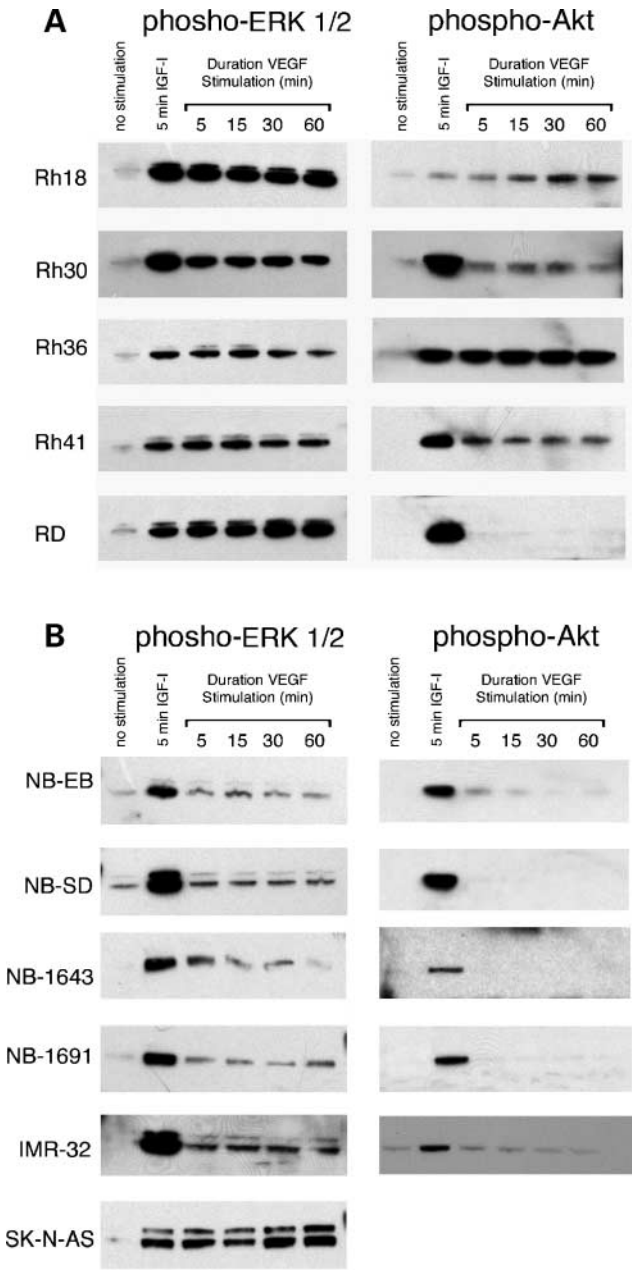

Figure 1. Determination of paracrine stimulation of rhabdomyosarcoma and neuroblastoma cells by VEGF. Cells were growth factor deprived overnight, then stimulated with IGF-1 (10 ng/mL, 5 min) or VEGF (10 ng/mL) for the indicated times. Increased phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and phospho-Akt (Ser^{308}) were used as indicators of VEGF stimulation of signal transduction pathways. **A,** rhabdomyosarcoma cells; **B,** neuroblastoma cells.

VEGF Secretion in Neuroblastoma Cells Is Not Consistently Inhibited by Rapamycin under Both Normoxia and Hypoxia

Whereas serum or IGF-1 stimulation of HIF-1α transcription and VEGF secretion may be mTOR dependent (16), such studies may not reveal the role of mTOR signaling under more physiologic conditions in the presence of exogenous growth factors (17). To examine the role of mTOR signaling in VEGF production, we measured its secretion into media for six rhabdomyosarcoma and seven neuroblastoma cell lines under ''normal'' conditions of growth (10% fetal bovine serum), under both normoxic (21% O_2) and hypoxic (1% O_2) conditions in the absence and presence of 100 ng/mL rapamycin (Fig. 2). Cells were incubated under normoxic or hypoxic conditions and then VEGF secreted into the media and cell number were determined after 24 h. Under normoxic conditions, the basal levels of secreted VEGF were lower in rhabdomyosarcoma cells (0–200 pg/10^6 cells/24 h) than in neuroblastoma cells (120–1,180 pg/10^6 cells/24 h). Thus, in rhabdomyosarcoma cells, it is probable that VEGF acts as an autocrine factor, in agreement with published data (22). Under normoxic conditions of cell culture, rapamycin reduced VEGF secretion in rhabdomyosarcoma cells (range, 49–81%), particularly in Rh-36 cells (Fig. 2A). Rhabdomyosarcoma cells incubated under hypoxia showed increased VEGF production 2- to 122-fold, although rapamycin had only a modest effect in blocking hypoxia-driven increases in VEGF. For example, rapamycin inhibited increases in VEGF by >50% in only one rhabdomyosarcoma line (Rh-36). Interestingly, whereas rapamycin reduced VEGF secretion in rhabdomyosarcoma cells, it had relatively little effect in neuroblastoma cells except in SK-N-AS and IMR-32. Under normoxia, rapamycin completely suppressed VEGF in IMR-32 and reduced VEGF by >50% in SK-N-AS, but had relatively little activity against the other neuroblastoma lines. VEGF increased between 3- and 10-fold under hypoxia, but rapamycin poorly inhibited this increase in most neuroblastoma lines. Importantly, for all cell lines where VEGF could be detected, secreted levels of this growth factor were higher under hypoxic conditions in the presence of rapamycin than in untreated cells under normoxic conditions. Thus, inhibition of mTOR signaling is insufficient to completely suppress hypoxia-driven VEGF in any of the rhabdomyosarcoma or neuroblastoma cell lines examined.

Rapamycin Inhibits mTOR Signaling Irrespective of Its Effect on VEGF Secretion

To determine whether failure to inhibit VEGF secretion was related to a failure to inhibit mTOR signaling, we examined signaling of mTOR by measuring the phosphorylation of two downstream targets, S6K1 (Thr^{389}) and rpS6

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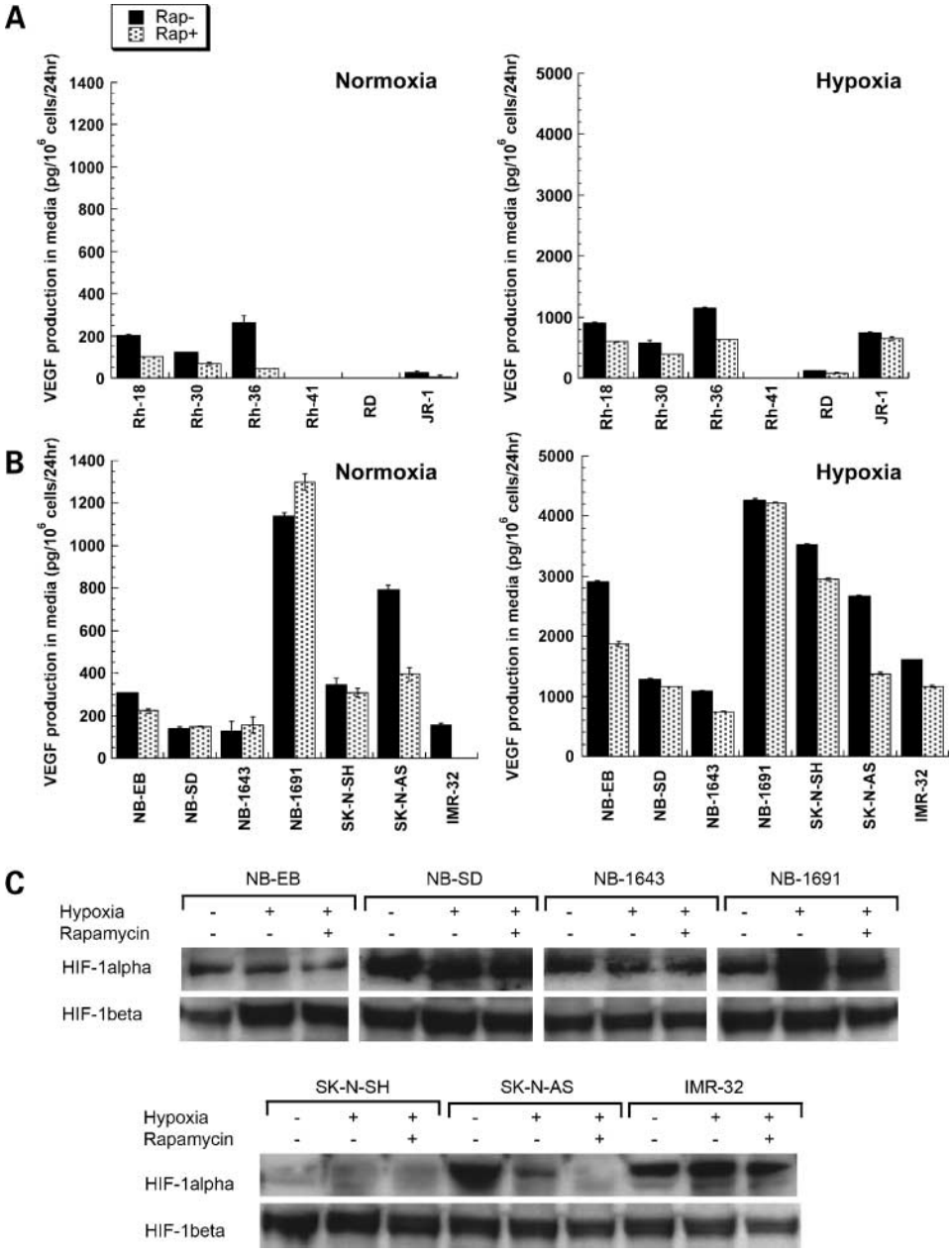

Figure 2. Secretion of VEGF by rhabdomyosarcoma and neuroblastoma cells. VEGF production was determined over 24 h in rhabdomyosarcoma (**A**) and neuroblastoma (**B**) cells without or with treatment with rapamycin (100 ng/mL) under normoxic (*left*) or hypoxic (*right*) conditions. VEGF was measured by ELISA and normalized to cell number at the end of the incubation period. Columns, mean; bars, SD.

(Ser$^{235/236}$). Cells were incubated under normoxic conditions, as described above, in the absence or presence of rapamycin (100 ng/mL) for 24 h. As shown (Fig. 3), phosphorylation of S6K1 was completely inhibited in the presence of rapamycin, whereas only ''traces'' of rpS6 phosphorylation were detected in RD, JR-1, SK-N-SH, and SK-N-AS cells. Thus, failure of rapamycin to significantly suppress VEGF secretion under these conditions is not a consequence of failure to inhibit mTOR signaling. Therefore, in most neuroblastoma cell lines, VEGF production seems to be independent of mTOR signaling whereas it is partially dependent on mTOR in some rhabdomyosarcoma cells.

Rapamycin Poorly Regulates HIF-1α in Rhabdomyosarcoma and Neuroblastoma Cell Lines

Activation of the Akt-mTOR pathway has been reported to control VEGF levels in many tumor lines, and overexpression of mTOR leads to increased VEGF production (23). Production of VEGF was generally higher in the neuroblastoma cell lines compared with rhabdomyosarcoma, with significantly higher levels in NB-1691 cells. Despite clear inhibition of mTOR signaling, under normoxic conditions, rapamycin had little effect on VEGF levels. These results argue that VEGF levels are only partly regulated via mTOR in rhabdomyosarcoma lines and to a lesser extent in most neuroblastoma lines

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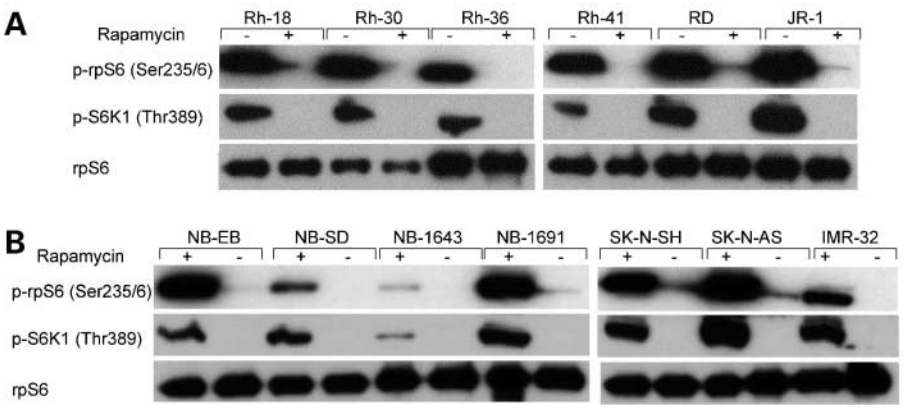

Figure 3. Rapamycin inhibits mTOR signaling independent of inhibition of VEGF production. Phospho-rpS6 ($Ser^{235/236}$) and phospho-S6K1 (Thr^{389}) were determined following incubation with or without rapamycin (100 ng/mL) for 24 h in rhabdomyosarcoma (**A**) and neuroblastoma (**B**) cells. Total rpS6 was used as a loading control.

under these conditions. Previously, it has been shown that in growth factor–deprived neuroblastoma cells, exogenous IGF-1 stimulated VEGF transcription and increased VEGF production and stabilized HIF-1α in a mTOR-dependent manner (16). We were interested, therefore, on whether rapamycin regulated HIF-1α levels under normoxic or hypoxic conditions of cell growth. At concentrations of rapamycin that inhibit mTOR signaling, there was no decrease in HIF-1α levels under normoxic conditions in either rhabdomyosarcoma or neuroblastoma cell lines. However, rapamycin did reduce slightly the hypoxia-driven increase in HIF-1α in several embryonal rhabdomyosarcoma lines (Rh-18, Rh-36, and RD) or in NB-1691 cells (Fig. 4).

Hypoxia-Driven VEGF Is Regulated More by Akt than by mTOR in Neuroblastoma Cell Lines

That inhibition of mTOR by rapamycin failed to suppress VEGF in most neuroblastoma cell lines suggested that mTOR signaling plays a minor role under normoxic or hypoxic conditions in these cell lines. However, it has consistently been shown that signaling via PI3K regulates VEGF in numerous cell systems. As forced expression of constitutively active Akt can abrogate the effect of PI3K inhibitors, it suggests that Akt inhibition may be a target for pharmacologic intervention to suppress VEGF. However, the effect of Akt pharmacologic inhibitors on VEGF has not been reported. To test if inhibiting Akt had a significant effect on tumor-derived VEGF in rhabdomyosarcoma or neuroblastoma cells, we used a specific Akt inhibitor, A-443654. Consistent with reported data (24), this inhibitor increased phospho-Akt, but blocked downstream signaling, suppressing phosphorylation of rpS6 and/or GSK-3α/β in each cell line examined (Fig. 5A and B). As previously reported, rapamycin induced phosphorylation of Akt (Ser^{473}) in each rhabdomyosarcoma line and in NB-1691 cells (29, 32) and increased GSK-3α/β phosphorylation,

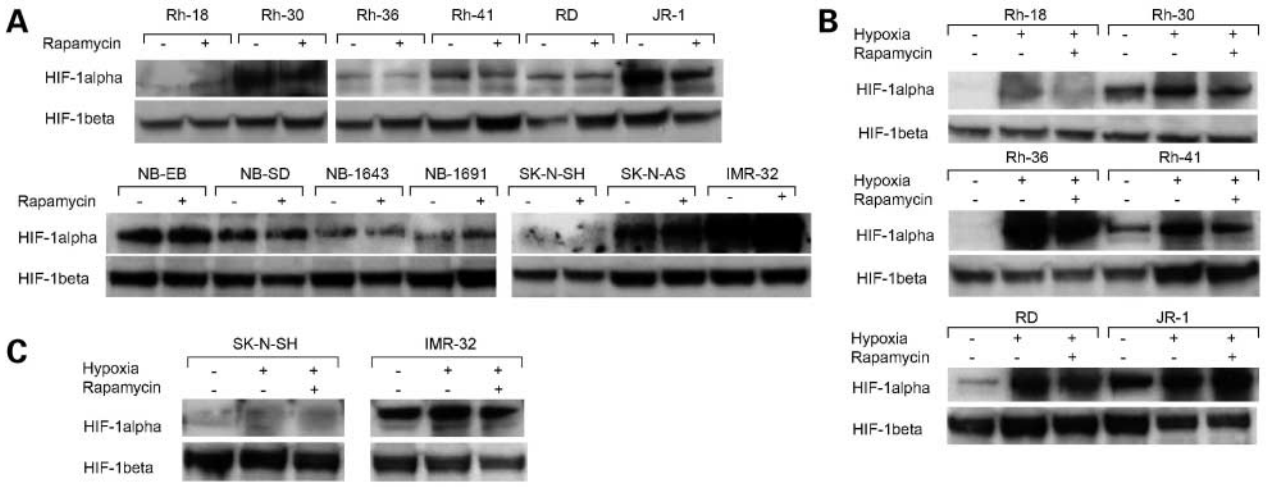

Figure 4. The effect of rapamycin on HIF-1α expression under normoxia and hypoxia. **A,** HIF-1α levels were determined by Western blot analysis under normoxic conditions of growth, without or with treatment with rapamycin (100 ng/mL) for 24 h. **B** and **C,** comparison of HIF-1α levels under normoxic conditions or after 24 h of hypoxia stimulation without or with rapamycin treatment in rhabdomyosarcoma and neuroblastoma cells, respectively. Expression of HIF-1β was used to normalize for protein loading.

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indicating rapamycin-induced activation of Akt. Combining A-443654 and rapamycin completely inhibited phosphorylation of rpS6 in all cell lines and markedly reduced phosphorylation of GSK-3α/β.

We next examined the effect on VEGF secretion of inhibiting Akt, mTOR, or both targets under normoxic and hypoxic conditions of cell growth. Cells were exposed for 24 h with or without inhibitors, and culture supernatant was removed for quantitation of VEGF. Adherent cells were quantified at the end of drug exposure, thus normalizing for drug effects on cell growth or survival, and VEGF was quantified as picograms produced over 24 h per 10^6 cells. For the rhabdomyosarcoma cells, inhibiting Akt had similar or greater effects than rapamycin under normoxic conditions, and this difference was enhanced under hypoxia (Fig. 6A). Similarly, inhibition of Akt was more effective in reducing VEGF secreted into the medium by neuroblastoma cells. For example, under normoxia, A-443654 markedly reduced VEGF in NB-EB and NB-1691 cell lines where rapamycin had either a modest effect (NB-1691) or no effect (NB-EB). The effect of inhibiting Akt compared with mTOR (both under normoxic and hypoxic conditions) was more dramatic against NB-EB and NB-1691, whereas SK-N-SH and SK-N-AS cells were relatively resistant to either inhibitor (Fig. 6B).

Thus, rapamycin seemed to have greater effect on VEGF production in rhabdomyosarcoma cell lines compared with the neuroblastoma lines, whereas two neuroblastoma lines were highly sensitive to the Akt inhibitor. It was therefore of interest to examine the effect on VEGF production of inhibitors Akt and mTOR when combined. VEGF produced over 24 h was normalized to cell number at the end of the exposure period. The effect of combining A-443654 and rapamycin was essentially additive against each cell line examined, and greater than additive against SK-N-SH cells. Of note, however, is that under hypoxic conditions in the presence of both inhibitors, the level of VEGF produced in each cell line was even lower than that in the respective control cells growing under normoxic conditions. Thus, combining Akt and mTOR inhibition completely suppressed hypoxia-induced increases in VEGF secretion in all cell lines examined. The effect on cell proliferation of combining A443654 with rapamycin was slightly less than additive under normoxic conditions and essentially additive in most cell lines under hypoxia (Table 1).

Discussion

Although ultimately assessing the effect of signaling inhibitors against tumor models *in vivo* is essential, dissecting the mechanism(s) for their activity is complex in such systems. Inhibition of mTOR, for example, may have direct effects on cancer cell proliferation and survival; indirect effects via inhibition of HIF-1α, thus reducing tumor-elicited VEGF; or direct effects on vascular endothelial cells, both on their proliferation and migration and their response to tumor-derived growth factors (13, 25). Here, we have used pharmacologic inhibitors of Akt and mTOR to determine their role in tumor-derived secretion of VEGF.

Results reported recently by Gee et al. (22) and Das et al. (26) showed VEGF-driven autocrine stimulation in some rhabdomyosarcoma and neuroblastoma cells, respectively.

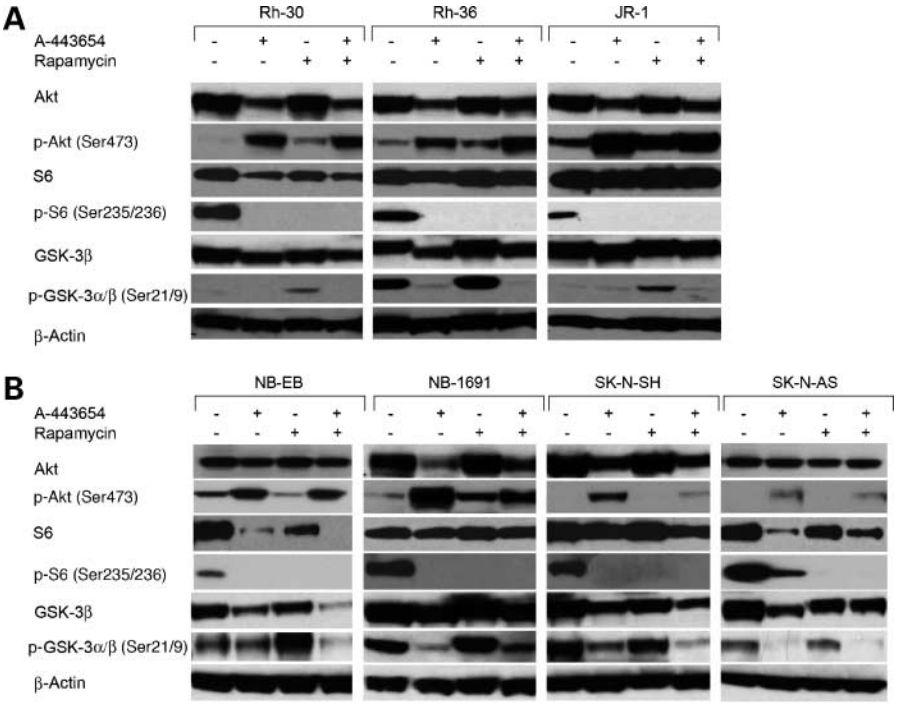

Figure 5. Inhibition of Akt or mTOR blocks downstream signaling. The effect of A-443654, rapamycin, or their combination (24 h incubation) on signaling in rhabdomyosarcoma (**A**) or neuroblastoma (**B**) cell lines. Equal protein was loaded for determination of β-actin.

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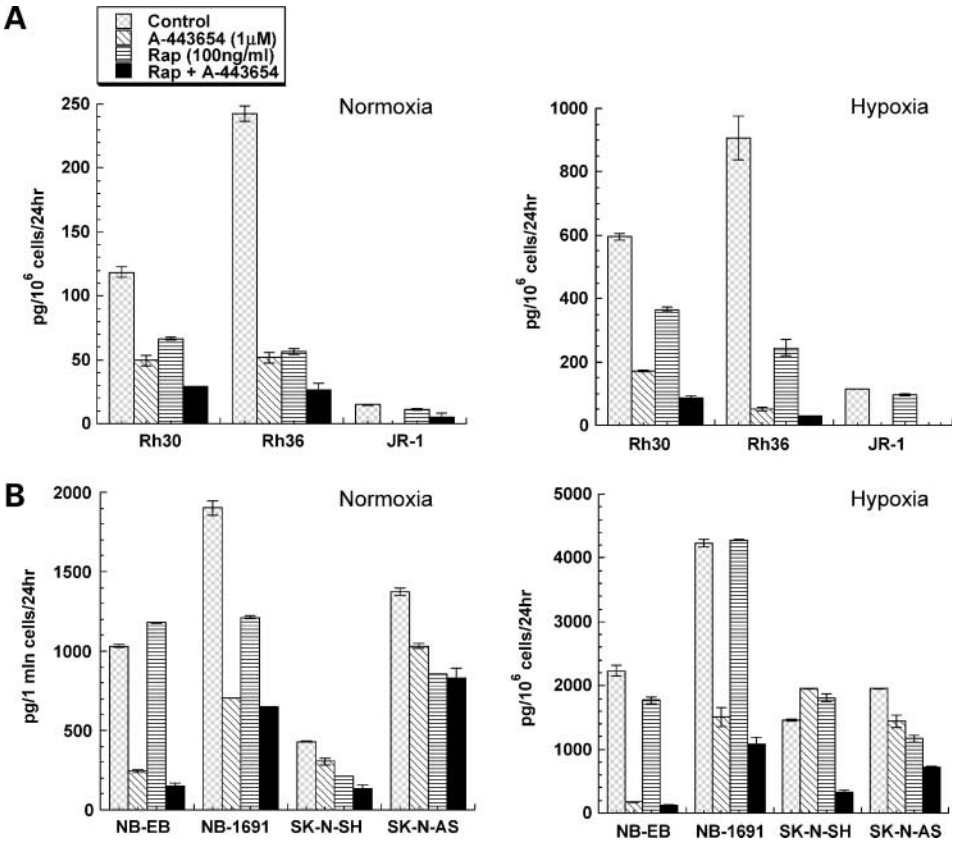

Figure 6. The effect of rapamycin (100 ng/mL), A443654 (1 μmol/L), or combination of both inhibitors on VEGF production under normoxic and hypoxic conditions of cell growth. VEGF secreted into media over 24 h by rhabdomyosarcoma (**A**) or neuroblastoma (**B**) cells under normoxic (*left*) or hypoxic (*right*) conditions (by ELISA). Secreted VEGF was normalized to the number of cells harvested at the end of drug exposure. Columns, mean; bars, SD.

We were interested, therefore, in determining whether tumor-derived VEGF was also acting in an autocrine loop. In each rhabdomyosarcoma and neuroblastoma cell line, exogenous IGF-1 stimulated phosphorylation of Akt and ERK1/2 mitogen-activated protein kinases. In rhabdomyosarcoma cells, exogenous VEGF induced a robust phosphorylation of ERK1/2 in all lines and phosphorylation of Akt in six of seven lines, which is equivalent to that induced by IGF-1. These results indicate the potential for autocrine signaling by VEGF. As reported by Gee et al. (22), exogenous VEGF stimulated growth of rhabdomyosarcoma cell lines, except Rh-30.[1] In contrast, VEGF induced a modest phosphorylation of ERK1/2 (~10% of that induced by IGF-1) in all but SK-N-AS cells where the response was equivalent to that induced by IGF-1. Further, exogenous VEGF did not significantly activate Akt in neuroblastoma cell lines. Thus, these results support the idea that in these neuroblastoma lines, VEGF acts primarily as a paracrine factor.

Activation of the PI3K-Akt-mTOR pathway has been reported to control VEGF levels in many tumor lines, and overexpression of mTOR leads to increased VEGF production (23). We examined VEGF secretion in a panel of six rhabdomyosarcoma and seven neuroblastoma cell lines. Secretion of VEGF into media was generally higher in the neuroblastoma cell lines compared with rhabdomyosarcoma. In Rh-41 and RD cell cultures, VEGF was not detected under normoxic conditions of growth. VEGF was readily

Table 1. Effect of inhibiting Akt or mTOR on proliferation of pediatric cell lines

	A443654 (% growth)	Rapamycin (% growth)	A443654 + rapamycin [% growth (additive)*]
Normoxia			
Rh-30	42	56	25 (25)
Rh-36	21	23	11 (5)
JR-1	0	77	37 (0)
NB-EB	24	100	15 (24)
NB-1691	37	64	34 (24)
SK-N-SH	71	50	31 (35)
SK-N-AS	75	61	61 (46)
Hypoxia			
Rh-30	29	61	15 (18)
Rh-36	6	27	3 (2)
JR-1	0	84	0 (0)
NB-EB	8	79	6 (6)
NB-1691	36	100	26 (36)
SK-N-SH	100	100	22 (100)
SK-N-AS	74	60	37 (44)

*Additivity defined as the product of fractional growth for each agent (% growth rapamycin treated × % growth A443654 treated).

[1] P.J. Houghton, unpublished data.

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detected in all neuroblastoma lines, with significantly higher levels in NB-1691 cells. Despite clear inhibition of mTOR signaling, under normoxic conditions, rapamycin had little effect on VEGF levels in five of seven neuroblastoma lines, although rhabdomyosarcoma lines seemed to be somewhat more responsive. Similarly, rapamycin treatment only slightly reduced hypoxia-induced VEGF in rhabdomyosarcoma cells and in only two of seven neuroblastoma cell lines. These results argue that VEGF levels are only partly regulated via mTOR in rhabdomyosarcoma lines and to a lesser extent in most neuroblastoma lines under these conditions. Further, hypoxia-driven induction of VEGF is poorly inhibited by rapamycin in these cells. Previously, it has been shown that in growth factor–deprived neuroblastoma cells, exogenous IGF-1 stimulated VEGF transcription, increased VEGF production, and stabilized HIF-1α in a mTOR-dependent manner (16). However, regulation by mTOR is highly dependent on the conditions of cell culture (17). Our results suggest that, under more ''physiologic'' conditions, mTOR plays a minor role in regulating VEGF in most of the cancer cell lines studied. Indeed, rapamycin failed to modulate levels of HIF-1α under normoxic conditions in any cell line and only modestly decreased hypoxia-driven HIF-1α in rhabdomyosarcoma lines, but not in neuroblastoma lines examined. HIF-1α was readily detected under normoxic conditions in neuroblastoma cell lines, consistent with reduced levels of von Hippel-Lindau protein (27). In contrast, HIF-1α was not detected under normoxic conditions in embryonal rhabdomyosarcoma lines. The high basal level of HIF-1α in neuroblastoma lines probably contributes to the higher VEGF production.

Although mTOR signaling is implicated in the regulation of VEGF, there are data to suggest that VEGF may be regulated through PI3K/Akt signaling independent of mTOR (14). Further, inhibition of mTOR in many cancer cell lines results in the anecdotal activation of Akt (28, 29). We observed the similar effect of rapamycin stimulating the phosphorylation of Akt and GSK-3β in three rhabdomyosarcoma lines examined but in only one neuroblastoma line (NB-1691). Enhanced phospho-Akt was associated with increased phosphorylation of GSK-3β, indicating Akt activation. However, we have not determined whether this is a consequence of dysregulation of IRS-1 (30, 31) or activation of the TOR complex 2, which can also phosphorylate Akt (Ser^{473}) (32). To determine whether Akt inhibition had a greater effect on VEGF production, we used an Akt selective inhibitor, A-443654. As previously reported (24), phospho-Akt (Ser^{473}) increased in the presence of the inhibitor, but there was a concentration-dependent abrogation of downstream signaling, as determined by decreased phospho-GSK-3α/β, phospho-S6K1, and phospho-rpS6. A443654 also decreased the level of Akt (~50%) in NB-1691 and SK-N-SH neuroblastoma lines and in each of three rhabdomyosarcoma lines. The mechanism for decreasing Akt is not known but could be related to increased proteasomal degradation. Of note, inhibition of Akt caused very striking inhibition of VEGF in rapamycin-refractory NB-EB and NB-1691 cell lines, especially under hypoxic conditions. In contrast, Rh-36 cells were sensitive to both Akt and mTOR inhibition, whereas SK-N-SH cells were relatively refractory to inhibitors of both Akt and mTOR. When inhibition of Akt was combined with mTOR inhibition, the effect on cell proliferation was cell line dependent. Under normoxia, the effect of the combination was less than additive in JR-1 and SK-N-SH, but slightly greater than additive in NB-EB. Under hypoxic conditions, the combination seemed to have greater than additive effects against SK-N-SH cells but essentially additive activity against the other cell lines. However, there was very significant reduction in VEGF in both rhabdomyosarcoma and neuroblastoma cell lines. VEGF production in both rhabdomyosarcoma and neuroblastoma cells under hypoxia in the presence of dual inhibitors was lower than VEGF in control cells under normoxia; hence, dual inhibition essentially ablated hypoxia-driven VEGF production.

Of importance, when considering the use of agents such as rapamycin analogues for potential antiangiogenic use, is the observation that whereas rapamycin inhibits VEGF in rhabdomyosarcoma cells, it poorly inhibits VEGF in neuroblastoma cells and only marginally inhibits hypoxia-driven VEGF in most cell lines. Conversely, inhibition of Akt seems to be more effective in reducing VEGF, particularly in cells that are unresponsive to inhibitors of mTOR signaling. Combining inhibitors at two steps in the same pathway (Akt and mTOR) results in at least additive effects on VEGF production and severely attenuates hypoxia-driven increases in VEGF. Thus, combining inhibitors in the same pathway seems to be a reasonable strategy to inhibit VEGF production by pediatric tumor cells. Initial evaluation of rapamycin against a large panel of childhood solid tumors and acute lymphocytic leukemia xenograft models by the Pediatric Preclinical Testing Program revealed broad-spectrum activity of rapamycin.[2] Pertinent to this study, rapamycin induced significant growth delay and regressions of sarcoma models and significantly inhibited growth of several neuroblastoma xenografts.

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Differential regulation of vascular endothelial growth factor by Akt and mammalian target of rapamycin inhibitors in cell lines derived from childhood solid tumors

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