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p16 Modulates VEGF Expression via Its Interaction With HIF-1 α in Breast Cancer Cells

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Abstract

The degree of tumor progression (such as growth, angiogenesis, and metastasis) directly correlates with the expression of vascular endothelial growth factor (VEGF), but inversely correlates with the expression of tumor-suppressor gene p16, therefore we examined whether the restoration of p16 in breast cancer cells would modulate VEGF expression. Adenoviral-mediated p16 expression downregulated VEGF gene expression in breast cancer cells, and inhibited breast cancer cell-induced angiogenesis by a dorsal air sac model in mice. Moreover, p16 appears to form a complex with HIF-1 α , the transcription factor for the VEGF gene promoter. Taken together, the binding between p16 and HIF-1 α protein may alter HIF-1 α 's ability to transactivate VEGF expression.

Keywords

p16; VEGF; HIF-1 α ; Breast cancer

INTRODUCTION

Angiogenesis, the growth of new blood vessels from pre-existing vessels, is essential for tumors to grow beyond a microscopic dimension size. The new blood vessels provide nutrition and oxygen to the growing tumor, allowing tumor cells to further proliferate and break into the circulation as well as spread to other organs (tumor metastases). Solid tumors including breast cancer are angiogenesis-dependent; anti-angiogenic therapy that targets any of these components of the angiogenic switch should serve well for controlling tumor growth and metastasis. The angiogenic switch, which leads endothelial cells from a quiescent state to active proliferation, occurs when the subtle balance between angiogenic stimulators and inhibitors is

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AUTHORS' CONTRIBUTIONS

YL designed the entire study and wrote the manuscript, constructed AdRSVp16 and AdRSVlacZ, performed ELISA, dorsal air sac assay, and some immunohistochemistry work. JZ carried out some transcriptional transactivation, co-immunoprecipitation, and colocalization assays. AL constructed Ad-HIF-1 α and participated in dorsal air sac assay, reviewed, and revised the manuscript. LL participated in some transcriptional transactivation assays and immunohistochemistry. JY designed and constructed Lenti-Tet-on p16 and contributed some study design and analysis.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this paper.

altered. While the exact mechanism of the angiogenic switch is still not fully understood, vascular endothelial growth factor (VEGF), the angiogenic stimulator secreted from tumor cells which recruits both local and circulating endothelial cells, has been shown to play a critical role in promoting angiogenic switch toward tumor angiogenesis.

VEGF (also named as VPF and VEGF-A) is the most potent angiogenic factor known and plays a pivotal role in tumor angiogenesis (1–5). VEGF is a dimeric glycoprotein secreted by cells that promotes tumor-associated angiogenesis (6,7). The expression of VEGF, which markedly contributes to the tumor-associated angiogenesis, is correlated with the malignant transformation of breast cancer and the poor prognosis in patients (8). VEGF has been shown to be present in breast tumors at levels that are, on average, seven-fold higher than in normal adjacent tissue (9). Together, these studies have shown that angiogenesis is a necessary step for breast cancer progression and metastasis (10,11), and VEGF is a prominent angiogenic stimulator and a major player for tumor angiogenesis (1,2). Therefore, angiogenesis inhibitors targeting VEGF can be effective in blocking the angiogenic switch and tumor growth.

Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator for the VEGF gene promoter (12,13). HIF-1 is composed of an inducible subunit, HIF-1 α and a constitutively expressed subunit, HIF-1 β (13,14). Heterodimerization of HIF-1 α and HIF-1 β (also known as ARNT1) is required to form the transcriptionally active HIF-1 (13,15) and mediate its nuclear translocation (16) and the consequent binding of HIF-1 to the hypoxic response elements within the promoter regions of target genes (16). The basal level of the HIF-1 α subunit was found to be frequently overexpressed in advanced tumors (17). HIF-1 α is an important transcriptional factor for breast tumor progression and metastasis (18,19). The activation of HIF-1 α stimulates a group of HIF-1 α -regulated genes, including VEGF (20), one of its main downstream effectors that promotes tumor angiogenesis (21). Consequently, the cells are converted toward malignant progression.

Tumor-suppressor gene p16 is a cyclin D kinase (CDK) inhibitor and a negative cell cycle regulator (22). The inactivation of p16 appears to be a common event in many cancers (23). Angiogenic capacity correlates with the degree of malignancy and the loss of p16 activity in gall bladder cancer (24). p16 expression was correlated to the inhibition of VEGF and angiogenesis in human gliomas (15); however, the mechanism of how p16 regulates VEGF has never been explored. In this study, we examined the effects of p16 expression on the regulation of VEGF gene expression and vascularization (angiogenesis) in breast cancer cells; and correlated with p16's binding to HIF-1 α , the transcriptional activator for VEGF gene promoter. Our hypothesis is that activity of HIF-1 α (the regulatory component of HIF-1), which is responsible for hypoxia-induced malignant progression, can be attenuated by tumor-suppressor gene p16. The interaction between HIF-1 α and p16 protein plays an important role in p16's modulation of VEGF expression, which contributes to the tumor angiogenesis and progression. To our knowledge, this is the first report to demonstrate the binding between p16 and HIF-1 α , and colocalization of both proteins in the cells.

MATERIALS AND METHODS

Cell culture and medium

Dulbecco's modified Eagle medium (D-MEM) and RPMI-1640 was purchased from Gibco BRL (Gaithersburg, MD), and fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT). Human embryonic kidney 293 cells (American Type Culture Collection, Rockville, MD) were grown in D-MEM with 10% heat-inactivated FBS. Human breast cancer cell lines MDA-MB-231 (ATCC) were grown in RPMI-1640 medium with 10% FBS. All cell lines were grown in medium containing 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in 5% CO₂.

Adenoviral vector construction

The construction of AdRSVp16, a replication-defective, recombinant adenoviral vector expressing a human wild-type p16 cDNA gene under the control of a Rous sarcoma virus (RSV) promoter, was described previously (25). The construction of the control virus, AdRSVlacZ, in which a bacterial β -galactosidase gene was under the control of the RSV promoter, was also described previously (26). The construction of recombinant adenovirus expressing the HIF-1 α , Ad-HIF-1 α , was done as follows: The HaloTag pHT2 vector (Promega, Madison, WI) was cut with EcoR V and Not I to release an 890-bp HT2 cDNA, which encodes a HaloTag reporter gene (Promega). The HT2 cDNA was subcloned into the pacAd5CMVNpA (University of Iowa, Iowa City, IA) to generate the adenoviral shuttle vector pacAd5-HT2. A 2.5-kb PCR product of human HIF-1 α cDNA was generated from HIF-1 α expression vector (pHIF-1 α , a generous gift from Dr. B. Jiang of West Virginia University; see (13)) using a set of primers specific to the human HIF-1 α cDNA gene, the primers contained an introduced Bam HI site at both ends of the PCR products. After digestion with Bam HI on both the 2.5-kb PCR product of HIF-1 α and pacAd5-HT2, the HIF-1 α cDNA was inserted into at the 5'-terminal of the HT2 gene to create an adenoviral shuttle vector pacAd5-HIF-1 α /HT2. The pacAd5-HIF-1 α /HT2 was cotransfected with adenoviral genome vector into the 293 cells (27) to generate the recombinant adenovirus Ad-HIF-1 α .

Adenoviral vector preparation, titration, and transduction

Individual clones of adenoviral vector were propagated in 293 cells. The culture medium of the 293 cells showing the complete cytopathic effect was collected, and adenoviral vectors were purified by BD Adeno-X Virus Purification Kits (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions. The viral titration and transduction were performed by standard procedures as has been described previously (28).

Construction of p16 doxycycline

An EcoR I and Bam HI I-digested 960-bp human p16 cDNA (25) was subcloned into Bam HI and Xho I sites of pLenti-Tet-pgk-puro (University of Tennessee Viral Vector Core Laboratory, Memphis, TN) to generate a doxycycline (Dox)-inducible lentiviral vector pLenti-Tet-p16-pgk-puro. In this vector, p16 gene was driven by tetracycline-regulated Tet-on promoter (Clontech, Mountain View, CA). In order to activate the p16 expression, we constructed another lentiviral vector, pcFUW-rtTA3-IERS-puro, in which the third-generation reverse Tet-transactivator (rtTA3) cDNA was placed under the control of a constitutively active ubiquitin C promoter. In brief, rtTA3 cDNA was amplified by PCR from pTRIPZ Tet-on vector (Openbiosystems, Huntsville, AL) and subcloned into XbaI and BamHI sites of pcFUW (a gift from Dr. Carlos, MIT) to generate pcFUW-rtTA3-IERS-puro. Both lentiviral vectors pLenti-Tet-p16-pgk-puro and pcFUW-rtTA3-IERS-puro, respectively, were produced in 293FT cells (Invitrogen, Carlsbad, CA) with cotransfection of ViraPower Packaging Mix (Invitrogen) that contains different expression plasmids including pLP/VSVG, pLP1/gag-pol, and pLP2-Rev to express all the essential elements for generating lentivirus. The supernatant was collected 60 hr post-transfection and centrifuged for 15 min at 3000 g at 4°C to remove cell debris, then passed through 0.45- μ m filter and collected for ultra-centrifugation at 17,000 g for 3 hr at 4°C. The pellet was dissolved in phosphate buffer solution (PBS) and kept at -80°C.

Generation of stably transfected, Dox-inducible (Tet-on), p16-expressing breast cancer cell lines

MDA-MB-231 cells were cotransduced by both viruses pLenti-Tet-p16-pgk-puro and pcFUW-rtTA3-IERS-puro at multiplicity of infection (moi) of 10 for each virus, together with 6- μ g/ml polybrene (Millipore, Bedford, MA). The MDA-MB-231 cells stably transfected with

lentivirus were enriched by incubated and transfected cells in medium containing 1- μ g/ml puromycin. The stably transfected cells are named as MDA-MB-231/Tet-on p16.

Immunohistochemistry

For *in vitro* immunohistochemistry, the culture cells were grown on SlideFlasks with detachable bottom slides (Nalge Nunc, Naperville, IL) that could be directly used for immunohistochemistry staining later. The samples (slides) were incubated with first antibody against human p16 (mouse anti-human p16 antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 16 hr at 4°C, then by a corresponding second antibody (goat anti-mouse antibody) and the Universal Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's protocol. The reaction was visualized with DAB solution (75-mg 3,3'-Diaminobenzidine and 30- μ l 50% H₂O₂ in 150-ml PBS) for 3–10 min.

Enzyme-linked immunosorbent assay (ELISA) for detecting VEGF

Cells were grown in 10-cm culture dishes and were either untreated or transduced with AdRSVlacZ or AdRSVp16 at moi of 200. After 90-min viral infection, the viral medium was replaced with exactly 10-ml fresh medium in each sample dish. The cell medium (supernatant) was collected 72 hr after viral transduction, and the cells attached on the culture dish were counted. The supernatant was processed to determine the secreted amount of VEGF₁₆₅ protein by a VEGF immunoassay kit (Quantikine VEGF ELISA Kit, R&D Systems, Minneapolis, MN). The procedures followed the methods according to the manufacturers' manual, and results were normalized based on the same amount of analyzed cells.

Transactivation assay of VEGF promoter

A chimeric construct containing a 2.4-kb VEGF promoter and a luciferase reporter gene (pVEGF/Luc) (a generous gift from Dr. B.H. Jiang of West Virginia University; see (13)) was cotransfected with or without p16 expression vector, pAvsp16 (25), or HIF-1 α expression vector, pHIF-1 α (13), or pHIF-1 α plus pAvsp16, into MDA-MB-231 cells by Eugene 6 (Roche, Indianapolis, IN) according to the manufacturer's instruction. After 48 transfections, the cells were lysed using 1x Reporter Lysis Buffer (Promega) and processed to luciferase activity assay using a Luciferase Assay Kit (Promega). The detection of luciferase activity was performed using a luminometer (Turner Designs Instrument, Model TD2020, Sunnyvale, CA), and the activity of luciferase was normalized with protein concentration. In the instance of MDA-MB-231/Tet-on p16 cells, the cells were incubated in the medium containing 1- μ g/ml Dox for 2 days prior to cotransfection with (10:1 ratio, w:w) pVEGF/Luc and phRLuc-TK (Promega), a plasmid expressing Renilla luciferase as an internal control for the normalization of transfection efficiency. After 48 hr of transfection, the cell extracts were harvested and analyzed for luciferase activity using Dual-Luciferase Assay Kit (Promega) as instructed by the manufacturer's instruction. The normalized luciferase activity was represented as the ratio of Firefly luciferase activity (pVEGF/Luc) over Renilla luciferase activity (phRLuc-TK).

In vivo angiogenic assay using a dorsal air sac model

Cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. After 48 hr the cells were harvested and suspended in PBS at a concentration of 1×10^8 cells/ml. This suspension (0.1 ml in PBS) was injected into a chamber (Millipore) consisting of a ring with a filter (pore size, 0.22 μ m) on both sides. The semi-permeable membrane chamber allowed for diffusion of growth factor, such as VEGF, but not cells. The chamber was implanted into a dorsal air sac produced by the injection of 10 ml of air in the dorsum of a 6-week-old nude female mouse (Harlan Sprague-Dawley, Indianapolis, IN). The mouse was sacrificed on day 3 and the implanted chamber was removed. A ring without filters was placed on the same site and then photographed. The newly formed blood vessels in the air

sac fascia were morphologically distinguishable from the pre-existing background vessels by their zigzagging characters.

Co-immunoprecipitation assay to detect binding between p16 and HIF-1 α proteins

MDA-231 cells were seeded onto a 10-cm cell culture dish at a density of 5×10^5 cells/dish and incubated for 24 hr in complete media. The cells were either untreated or transduced with AdRSVp16 or AdRSVlacZ at moi of 200 for 24 hr, respectively. The medium was changed and the cells were then transfected with 15- μ g HIF-1 α (pCEP4 expression vector expressing human HIF-1 α ; see (13)), or empty vector pCEP4 (Invitrogen, San Diego, CA) by Fugen6 according to the manufacturer's instruction. At 48 hr after transfection, the cells were harvested and lysed at 4°C for 30 min with lysis buffer containing 1% Brij 97 (Sigma), 0.25-M Sucrose, 10-mM Tris-Cl pH 8.0, 0.05-mM CaCl₂, 0.02% azide, 0.1-mM phenylmethyl-sulfonyl fluoride, 0.2-mM sodium vanadate, and a protease and phosphatase inhibitor cocktail (Sigma). The lysates (500 μ g in 100 μ L) were precleared with 50 μ L of a combination of protein A-Sepharose and protein G-Sepharose beads (protein A+G Sepharose beads) (Amersham Bioscience) on ice for 60 min. After removing the insoluble material by 10,000 rpm for 10 min at 4°C, the supernatant was used as the cleared lysate. At the same time, 50- μ L protein A+G Sepharose beads were precleared with lysis buffer and incubated with 1- μ g anti-HIF-1 α antibody (Cat# NB100-105, Novus, Littleton, CO) for 1 hr at 4°C. These antibody-preabsorbed protein A+G Sepharose beads (50 μ L) were incubated with the cleared lysate (500 μ g in 100 μ L) overnight at 4°C. The precipitates were washed thrice with lysis buffer, dissolved at 1:1 ratio (v:v) in 2x Laemmli sample buffer (10% sodium dodecylsulfate (SDS), 20% glycerol, 1M Tris-HCL pH 6.8, and 0.01% Bromophenol Blue), heated at 90°C for 10 min, electrophoresized on a NuPage 4–12% Bis-Tris Gel (Invitrogen), and electrically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was immunoblotted with rabbit anti-human p16 antibody (Santa Cruz Biotech, Santa Cruz, CA) at 1:1000 dilution, and then followed by horseradish peroxidase-conjugated anti-rabbit IgG at 1:2000 dilution, and processed to detection with chemiluminescent reagents (ECL kit, Amersham) according to the manufacturer's protocol. The same immunoblot was stripped and reprobed with antibody against HIF-1 α (see below).

Western blot analysis

Cells were extracted and processed for gel electrophoresis as described previously (29). Cell extract lysates (100 μ g) were loaded on polyacrylamide gels and subjected to SDS gel electrophoresis, then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was treated with blocking solution (15% non-fat milk, and 0.02% sodium azide in phosphate-buffered saline) overnight at 4°C. The membrane was incubated for 1 hr at room temperature with the primary antibody, mouse anti-human HIF-1 α antibody (1:1000 dilution, BD Biosciences). The membrane was then incubated for 1 hr at room temperature with goat anti-mouse secondary antibody coupled to peroxidase, followed by detection with chemiluminescent reagents (ECL kit, Amersham).

Immunofluorescent staining of p16 and HIF-1 α and confocal microscopy

MDA-231 cells were seeded in four-chamber slide flask (BD Biosciences, Bedford, MA) at 5×10^4 cells/per chamber. Cells were either untreated or transduced with AdRSVp16 plus Ad-HIF-1 α (moi = 100 for each virus). After 72 hr of viral transduction, the cells were fixed with 3% paraformaldehyde for 15 min at room temperature. The cells were then permeabilized with 0.1% brij 98 for 2 min at room temperature, followed by blocking with 20% goat serum in 1x PBS for 60 min. The cells were incubated with mouse anti-HIF-1 α (1:1,000 dilution, BD Biosciences) and rabbit anti-p16 (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr at room temperature, respectively. After washing, the cells were incubated with goat

anti-mouse IgG FITC (1:100 dilution, Sigma), and goat anti-rabbit IgG's Rhodamine-B conjugate (1:100 dilution, Biosource, Camarillo, CA) for 1 hr at room temperature, respectively. After washing with PBS, the slides were mounted using Fluor Saver Reagent (Calbiochem, La Jolla, CA) and observed under a fluorescent microscope (BioRad 1024 Confocal Microscope, software Lasersnap 2000).

RESULTS

Induction of ectopic expression of p16 protein in breast cancer cells

Consistent with the other report (9), we observe an apparent p16 expression in normal human breast tissue and little, if any, p16 expression in breast cancer tissues; in contrast, normal breast tissue had sparse VEGF expression, whereas the cancerous breast tissue had an overwhelming high expression of VEGF (not shown). To facilitate the induction of p16 expression, a replication-defective recombinant adenovirus-expressing human wild-type p16 under the control of a RSV promoter, AdRSVp16, was used to transduce breast cancer cells. To demonstrate that AdRSVp16 is able to transfer and express p16 protein in breast cancer cells, human breast cancer MDA-MB-231 cells were transduced with AdRSVp16 at moi of 200. Three days later, the cells were processed for immunohistochemical staining for p16 protein using primary antibody against p16. As shown in Figure 1, cells transduced by AdRSVp16 expressed a positive staining (dark brown color) for p16 protein (Figure 1(b)), while control untreated cells did not have the p16 staining (Figure 1(a)). We also established a stably transfected breast cancer line expressing p16 under Dox induction, MDA-MB-231/Tet-on p16. The expression of p16 protein in this line can be turned on and off by incubating cells with (Figure 1(d)) or without Dox (Figure 1(c)), the inducer for Tet-on promoter that controls p16 expression.

p16 downregulates VEGF secretion

To determine whether p16 modulates VEGF gene expression at the protein level, MDA-MB-231 cells were either left untreated or transduced by control virus or AdRSVp16, and 72 hrs later the cell culture media were collected to analyze the secreted form of VEGF protein by ELISA assay. The ELISA results showed that AdRSVp16-transduced MDA-MB-231 cells had significantly less VEGF protein secreted into the media (about 66% reduction compared to the untreated control cells at the same amount of cells) (Figure 2(a)).

p16 appears to specifically downregulate HIF-1 α -mediated VEGF expression at the transcriptional level

To examine whether p16 regulates VEGF gene expression at the transcriptional level, we transiently cotransfected a 2.4-kb VEGF promoter/luciferase reporter chimeric construct, pVEGF/Luc (13) with either control vector (pSV40 β gal), or p16 expression vector pAvsp16 (25), into MDA-MB-231 cells. As shown in Figure 2(b), there was a moderate luciferase activity in the absence of p16 expression (lane 1, control group, Figure 2(b)), reflecting a native (basal) moderately active transactivation activity in MDA-MB-231 cells for VEGF transcription. In contrast, p16-expressing cells had a significantly reduced luciferase activity (67.2% inhibition as compared to the control group), implying that p16 downregulates VEGF expression at the transcriptional level (lane 2, Figure 2(b)). In addition, to confirm that HIF-1 α stimulates VEGF gene transcription, we also cotransfected a VEGF/Luc construct with a HIF-1 α expression vector (13). We found that ectopic HIF-1 α expression alone stimulated VEGF gene transcription by more than threefold (lane 3, Figure 2(b)), and this HIF-1 α -mediated transactivation of VEGF promoter can be neutralized by p16 when both ectopic HIF-1 α and p16 expression were introduced (lane 4, Figure 2(b)). To exclude the possibility that downregulation of VEGF gene transcription by p16 is due to an indirect effect through a p16-mediated cell-cycle arrest, we also cotransfected a pVEGF/Luc into MDA-MB-231 cells

with a vector-expressing p21Waf1, another CDK inhibitor gene that also causes cell-cycle arrest (30). p21Waf1 did not show any inhibitory effect on the VEGF gene transcription (data not shown), implying that this p16-mediated downregulation of VEGF expression is specific (possibly via regulation of HIF-1 α) and independent of p16's cell-cycle arrest effect. To avoid the possible artificial effect due to the overexpression system mediated by adenoviral transduction, we also performed the similar experiment in the stably transfected breast cancer line, MDA-MB-231/Tet-on p16. Likewise, in the MDA-MB-231/Tet-on p16 cell system, the p16-expressing cells (+Dox) had a significantly reduced luciferase activity compared to its p16-nonexpressing counterpart (–Dox) (Figure 2(c)). Together, these results indicate that p16 expression can downregulate VEGF gene expression at the transcriptional level.

p16 inhibits angiogenesis in vivo

To determine whether p16 inhibits tumor cell-induced angiogenesis, the effect of p16 on neovascularization of tumor-surrounding cells were examined by dorsal air sac assay (31). MDA-MB-231 cells were transduced with AdRSVp16. After 48 hr, the cells were harvested and injected into a chamber that was wrapped with a semi-permeable membrane allowing for the diffusion of growth factor, such as VEGF, but not cells. The chamber was implanted into a dorsal air sac in nude mice, and the newly formed blood vessels on the undersurface of the chamber were examined 3 days later. As shown in Figure 3, PBS-treated mice (as a negative control) did not have any obvious neovascularization (Figure 3(a)). However, the mice injected with MDA-MB-231 cells developed tumor cell-induced neovascularization as evidenced by the newly formed “zigzagging-shape” small vessels in the air sac fascia (exampled by arrow, Figure 3(b)). A similar high neovessel formation was observed in MDA-231 cells transduced with control virus (exampled by arrow, Figure 3(c)). In contrast, mice with AdRSVp16-transduced MDA-MB-231 cells developed much less newly formed blood vessels (Figure 3 (d) and (f)), compared with mice injected with MDA-MB-231 cells alone (Figure 3(b)), or mice injected with control viral-transduced MDA-MB-231 cells (Figure 3(c) and (e)); both of the latter two groups induced a more extensive capillary network. When mice were injected with MDA-MB-231 cells along with neutralized anti-VEGF antibody, this tumor-induced angiogenesis nearly disappeared (Figure 3(g)). By using matrigel plug assay in another experiment to analyze the *in vivo* angiogenic ability of tumor cells in the mice, the angiogenic ability of MDA-MB-231 cells can be measured and quantitated as microvessel density. In comparison with the untreated control, the control virus-treated group had a 5% inhibition of angiogenesis and AdRSVp16-treated group had a 72% inhibition (not shown). Taken together, these results demonstrate that breast cancer cells can induce neovascularization around the tumor by secreting VEGF to the surrounding environment, and p16 can inhibit this tumor cell-induced neovascularization.

p16 appears to form a complex with HIF-1 α in MDA-MB-231 cells

To explore how p16 regulates VEGF gene expression, we examined the possibility that p16 may regulate VEGF expression by modulating its transcriptional factor HIF-1 α . To study whether p16 forms a complex with HIF-1 α (as one potential mechanism to alter HIF-1 α 's ability to either locate its normal cellular location or its transactivation ability to VEGF promoter), MDA-MB-231 cells were transduced with AdRSVp16 and transfected with HIF-1 α expression vector (13) for 72 hr. The nuclear extracts were immunoprecipitated with either anti-HIF-1 α (lane 1–3, Figure 4) or with preimmune IgG as a negative control (lane 4, Figure 4). The immunoprecipitates (lane 1–4) were then analyzed by Western blotting using anti-p16. As a positive control for the p16 band, cell extracts over-expressing p16 protein (we used AdRSVp16-transduced PC-3 cells, see (25)) were also used *directly* for Western blotting by anti-p16. Indeed, we saw a complex of HIF-1 α and p16 (lane 3, Figure 4), demonstrating that p16 binds to HIF-1 α in cells expressing both p16 and HIF-1 α .

Colocalization

To further confirm the interaction between p16 and HIF-1 α , we performed colocalization in MDA-MB-231 cells that were transduced by both AdRSVp16 and Ad-HIF-1 α . For every cell transduced by both AdRSVp16 (red, Figure 5(a)) and Ad-HIF-1 α (green, Figure 5(b)), there is a yellow color (the merged color of red and green, Figure 5(c)). Some cells may be transduced only by AdRSVp16 but not by Ad-HIF-1 α (as demonstrated by an arrow, Figure 5(a)), correspondingly, no yellow color was shown in that particular cell. Thus, there is a clear colocalization for both protein p16 and HIF-1 α in cells expressing both (yellow color, Figure 5(c) and (f)), strongly implying that p16 may interact with HIF-1 α protein in the cells. Together with result from Figure 4, these results demonstrate that p16 binds HIF-1 α inside the cells, which may provide one mechanism for how p16 modulates VEGF gene expression: via interaction of HIF-1 α , the transcriptional activator for VEGF, may somehow affect HIF-1 α 's ability to transactivation of its targeted gene VEGF.

DISCUSSION

In summary, our study showed that adenoviral-mediated overexpression of p16 decreased VEGF expression at protein levels in human breast cancer MDA-MB-231 cells (Figure 2(a)). Our study also showed p16 can reduce and neutralize HIF-1 α —mediated transactivation of VEGF promoter in MDA-MB-231 cells (Figure 2(b) and (c)). *In vivo* angiogenesis assay (dorsal air sac assay) on nude mice showed that p16 inhibited angiogenesis of MDA-MB-231 cells (Figure 3). Our co-immunoprecipitation assay (Figure 4) revealed that p16 appears to bind to HIF-1 α , a transcriptional activator for the VEGF gene promoter, and p16 and HIF-1 α colocalize in the cells. This is quite exciting because this is the first report to demonstrate that p16 forms a complex with HIF-1 α and colocalization of p16 and HIF-1 α at the protein levels. Taken together, these results demonstrated that p16 modulates VEGF expression and inhibits tumor-induced angiogenesis, the binding between p16 and HIF-1 α protein may alter HIF-1 α 's ability to transactivate VEGF expression. This study reveals a less explored function of p16, namely, p16's anti-angiogenesis function by its interaction with HIF-1 α and modulation of VEGF gene expression.

Accumulated evidences indicate that tumor hypoxia plays an important role in tumor progression and metastasis (32–34). Studies both *in vitro* and *in vivo* demonstrated that transient hypoxic exposure of tumor cells enhanced the cells' metastatic ability (35,36). Although the mechanisms by which tumor hypoxia increases metastatic potential are not fully understood, it is likely that HIF-1, the main mediator of hypoxic response and a master-regulator at the transcription level, drives expression of a set of downstream genes that control metastatic processes, such as proliferation, angiogenesis, survival, migration, and invasion, in order to transform the tumor toward a malignant phenotype (37,38). Many human neoplastic cells, including colon, breast, lung, endometrium gastric, bladder, and prostate, have been recently reported to overexpress HIF-1 and VEGF transcripts. As VEGF is correlated to vascular density, malignant stage of the disease, and poor prognosis (39–41), targeting HIF-1 (the upstream regulator of VEGF) or its pathway, have become a strategy for effectively blocking tumor progression and metastasis.

Several other tumor-suppressor genes, such as p53 (42) and Rb2/p130 (43), were reported to downregulate VEGF expression and inhibit angiogenesis in colon and lung cancer cells, respectively. However, the link between tumor-suppressor genes and angiogenesis remains obscure. Our study is the first to explore the mechanism of how p16 may actually regulate VEGF. We have demonstrated that p16 forms a complex with HIF-1 α , a transactivator of the VEGF gene. This may provide a foundation to elucidate how p16 exactly downregulates the VEGF gene expression. Based on our study, it is speculated that p16 may regulate VEGF gene

expression at the transcriptional level via affecting VEGF transcriptional factor, HIF-1 (HIF-1 α).

In this study, a new function of tumor-suppressor gene p16 that interacts HIF-1 α and modulates VEGF gene expression is presented and its potential anti-HIF-1 α therapeutic effect on the inhibition of tumor angiogenesis is discussed. We hypothesize that p16 downregulates VEGF gene expression at the transcriptional level by forming a complex with HIF-1 α (via either direct or indirect binding), which somehow prevents HIF-1 α 's cellular localization toward the nucleus, its usual place to act as a transcription factor. Consequently, it prevents HIF-1 α 's binding (and its transactivation) to the VEGF promoter in the nucleus. However, whether p16's binding to HIF-1 α causes disruption of the binding between HIF-1 α and 1 β (thus preventing the translocation of HIF-1, the complex of HIF-1 α and 1 β , to the nucleus), or p16's binding to HIF-1 α *per se* directly affects HIF-1 α translocation into the nucleus, or HIF-1's ability to transactivate VEGF promoter, remains unclear at the present time. Experiments are currently in the process to examine whether p16 affects transactivation of the pVEGF/Luc construct in which the VEGF promoter containing either a wild-type or mutant hypoxia response element (HRE), in both HIF-1 α wild-type and HIF-1 α knockout breast cancer cell lines.

The development of tumor hypoxia is intrinsically linked to the formation of neovasculature by the process of angiogenesis. Therefore, targeting and inhibiting the activity of HIF-1 α , the main mediator of hypoxia response and the regulator of VEGF (37), should provide a novel and effective strategy to suppress tumor angiogenesis and metastasis. Small-molecule inhibitors of HIF-1 α are being screened and tested for their efficacy as anticancer agents (37, 44,45). However, an anti-HIF-1 α approach through biologically based therapies may be the most promising and clinically applicable act (46). Our study may reveal p16's novel function as an effective anti-HIF-1 α protein. The advantage of our study that employs p16 gene transfer in breast cancer therapy is two-fold: first, p16 inhibits tumor angiogenesis by downregulating VEGF gene expression via its anti-HIF-1 α ability; second, p16 itself is a CDK inhibitor that suppresses cell division. Therefore, with the combination of p16's effect on both anti-HIF-1 α and anti-proliferation, p16 gene transfer should have a significantly therapeutic potential in the treatment of breast cancer patients.

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Figure 1. Ectopic p16 expression in human breast cancer MDA-MB-231 cells either by AdRSVp16 transduction or induction by Dox in a Tet-on regulatory system

(a) and (b) MDA-MB-231 cells were grown in culture dish and transduced by recombinant adenovirus-expressing p16 (AdRSVp16) at multiplicity of infection (moi) of 200. After 72 hr, the cells were harvested and subjected to immunohistochemistry using primary antibody (mouse anti-human p16 antibody), followed by goat anti-mouse secondary antibody coupled with horseradish peroxidase. Shown are p16-immunostaining for control untreated cells (a), and cells transduced by AdRSVp16 (b). (c) and (d) MDA-MB-231 cells stably transfected by lentivirus-expressing inducible p16 protein under the control of Tet-on promoter (MDA-MB-231/Tet-on p16) were treated either without (c) or with (d) 1 μ g/ml Dox for 72 hr. The cells were then IHC stained by anti-p16 antibody as described above. The dark brown color indicated p16 protein (b) and (d).

Figure 2. p16 downregulates VEGF expression at the protein and transcriptional levels

(a) p16 expression decreased VEGF secretion. MDA-MB-231 cells were grown in medium containing charcoal-stripped serum. Cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. The cell medium was collected 72 hr after viral transduction and subjected to VEGF determination by ELISA assay. (b) p16 neutralizes HIF-1 α -mediated transactivation of VEGF gene promoter. pVEGF/Luc was cotransfected into MDA-MB-231 cells with control plasmid DNA or pAvsp16 DNA or pHIF-1 α (pHIF-1 α), or pAvsp16 plus pHIF-1 α . (c) Tet-on inducible p16 expression inhibits the transactivation of VEGF gene promoter. MDA-MB-231/Tet-on p16 cells were treated with or without 1- μ g/ml Dox and cotransfected with pVEGF/Luc and pRLuc-TK. All data represent the results from two independent experiments, each performed in triplicate. Error bars represent standard error ($p < .05$). Some error bars are too small to be shown in this scale.

Figure 3. p16 inhibited tumor-induced angiogenesis and neovessel formation in the air sac model

The mouse in the air sac model was sacrificed on day 3 after chamber implantation and the implanted chamber was removed from the subcutaneous air fascia, a ring without filters was placed on the same site, and then photographed. The newly formed blood vessels were morphologically distinguishable from the pre-existing background vessels by their zigzagging characters (see representative arrows). Shown are undersurface images of sites from chambers containing PBS only as negative control (a), MDA-MB-231 cells (b), AdRSVlacZ-transduced MDA-MB-231 cells (c), AdRSVp16-transduced MDA-MB-231 cells (d), and MDA-MB-231 cells with 0.1 $\mu\text{g/ml}$ neutralized anti-VEGF antibody (g). (e) and (f) are the magnified pictures of (c) and (d), respectively. Original magnifications: (a), (b), (c), (d), and (g) 7.5 $\times$; (e) and (f) 30 $\times$. Each group uses at least five mice and the representative photos were shown.

Figure 4. p16 forms a complex with HIF-1 α

MDA-MB-231 cells were transfected with HIF-1 α expression vector (13) (lane 1–4) and transduced with either no virus (lane 1), control virus (lane 2), or AdRSVp16 (lanes 3 and 4) for 72 hr. Cell lysates were immunoprecipitated (IP) with anti-HIF-1 α (lane 1–3) or preimmune IgG (as a negative control, lane 4); the immunoprecipitates (lane 1–4) were then analyzed by Western blot (WB) with anti-p16. The same blot was also immunoblotted with anti-HIF-1 α . As a positive control for p16 protein, cell extracts overexpressing p16 protein (AdRSVp16-transduced cells) were used directly for Western blot by anti-p16 (lane 5).

Figure 5. Colocalization of p16 and HIF-1 α protein

MDA-MB-231 cells were cotransduced with Ad-HIF-1 α and AdRSVlacZ at moi of 100 for each virus for 3 days. The cells were then fixed and proceeded for immunofluorescent staining using anti-p16 and anti-HIF-1 α as primary antibodies as described in Materials and Methods section. The images were recorded by a confocal microscope: (a) p16 expression, (b) HIF-1 α expression, and (c) merge of (a) and (b).