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p16 downregulates VEGF and inhibits angiogenesis in breast cancer cells

Research Article

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Summary

One of the major causes of failure in the treatment of breast cancer is the occurrence of metastasis. It is thus important to intervene at a key step such as angiogenesis for breast cancer treatment and prolongation of patient survival Vascular endothelial growth factor (VEGF) plays a pivotal role in tumor angiogenesis. Tumor suppressor gene p16 is a cyclin-dependent kinase inhibitor and a negative cell cycle regulator. It was observed that the degree of tumor malignancy correlates with angiogenic capacity and the loss of p16 activity. To examine whether p16 overexpression decreases VEGF gene expression and inhibits tumor angiogenesis in breast cancer cells, human breast cancer cell line MDA-MB-231 was transduced with recombinant adenovirus expressing p16. Our study showed that p16 downregulated VEGF expression and inhibited *in vivo* angiogenesis induced by MDA-MB-231 cells in nude mice.

I. Introduction

Breast cancer is the leading type of cancer in women living in the United States today. It is estimated that there will be 211,300 new cases of breast cancer and 39,800 breast cancer death in American women in year 2003 (Jemal et al, 2003). Metastasis, the spread of tumor cells from a primary site to distant organs to form secondary tumors, is a major cause of deaths of breast cancer patients (Marshall, 1993). Metastasis is a complex process including primary tumor growth, invasion through basement membrane and extracellular matrix, dissemination to lymphatic and/or blood circulation, motility to distant organs, angiogenesis and colonization in the secondary site (Steeg et al, 1998). It is thus important to intervene at key steps of metastatic process for breast cancer treatment and prolongation of patients' survival One of the most promising avenues of breast cancer research is the development of biologically based therapies to thwart the progression of metastatic disease. However, not all aspects of the metastatic process may be equally clinical applicable. Therapies targeting angiogenesis and colonization that involve in micrometastatic outgrowth may be one of the most clinically applicable (Steeg et al, 1998).

In the angiogenesis process, endothelial cells initially respond to changes in the local environment and migrate toward the growing tumor. The endothelial cells then migrate together forming tubular structures that are ultimately encapsulated by recruiting periendothelial support cells to establish a vascular network that facilitates tumor growth and metastasis (Hanahan and Folkman, 1996). Angiogenesis is driven by a balance between different positive and negative effector molecules (or so-called angiogenic stimulators and inhibitors) that influence the growth rate of capillaries. The angiogenic stimulators include VEGF, basic fibroblast growth factor (Goldfarb, 1990), matrix metalloproteinases (John and Tuszynski, 2001), and angiopoietin-1 (Zetter, 1998). The angiogenic inhibitors include thrompbospondin-1 (TSP-1) (Tuszynski and Nicosia, 1996), angiostatin (O'Reilly et al, 1994), and endostatin (Shichiri and Jirata, 2001). Normal vessel growth results from balanced and coordinated expression of these opposing factors. A switch from normal to uncontrolled vessel growth can occur by upregulating angiogenesis stimulators or downregulating angiogenesis inhibitors (Bouck et al, 1996).

Angiogenesis is an essential prerequisite for aggressive tumor proliferation and spreading (Folkman, 1971) and it requires several angiogenic factors during the malignant transformation (Brem et al, 1978; Jensen et al, 1982; Brem et al, 1997). Among these angiogenic factors, VEGF plays a pivotal role in tumor angiogenesis

(Hanahan and Folkman, 1996; Klagsbrun and D'Amore, 1996; Risau, 1996; Grunstein et al, 1999; Neufeld et al, 1999). VEGF is a dimeric glycoprotein secreted by cells that is able to induce permeability and angiogenesis in tumor-associated blood vessels (Senger, 1983; Ferrara and Henzel, 1989). The VEGF family comprises five isoforms, including polypeptides of 121, 145, 165, 189, and 206 amino acids that are produced by the alternate splicing of a single gene containing eight exons (Leung et al, 1989; Tischer et al, 1989; Houck et al, 1991; Poltorak et al, 1997). $VEGF_{165}$ is the one most commonly secreted by tumor cells and acts most strongly on endothelial cells to lead them to form new capillaries (Keyt et al, 1996; Soker et al, 1997). The expression of VEGF, which markedly contributes to tumor-associated neovascularization, is correlated with the malignant transformation of breast cancer and the poor prognosis in the patients (Gasparini et al, 1997; Obermair et al, 1997; Linderholm et al, 1998; Hefflfinger et al, 1999; Salven et al, 1999). VEGF has been shown to be present in breast tumors at levels that are, on average, 7-fold higher than in normal adjacent tissue (Yoshiji et al, 1996). Correspondingly, two VEGF receptors, Flt-1 and KDR/Flk-1 (Shibuya et al, 1990; Terman et al, 1992; Mustonen and Alitalo, 1995), are preferentially expressed in invading and proliferating endothelial cells (Plate and Risau, 1995). Combining results from several studies have showed that angiogenesis is a necessary step for breast cancer progression and metastasis (Liotta et al, 1974; Weidner et al, 1991; McCulloch et al, 1995; Zhang et al, 1995; O'Reilly et al, 1996; Berm et al, 1997).

Tumor suppressor gene p16 (also called MTS1, CDKN2 and INK4A) is a cyclin-dependent kinase inhibitor and a negative cell cycle regulator (Shapiro and Rollins, 1996). The inactivation of p16 appears to be a common event in many cancers (Caldas et al, 1994; Hussussian et al, 1994; Jen et al, 1994; Cairns et al, 1995; Chen et al, 1996; Hatta et al, 1995; Li et al, 1995; Mao et al, 1995; Xiao et al, 1995). Angiogenic capacity correlates with the degree of malignancy and the loss of p16 activity in high-grade gliomas (Harada et al, 1999). In this study, we examined the effects of p16 expression on regulation of VEGF gene expression and vascularization of breast cancer cells.

II. Materials and methods

A. Cell culture and medium

Dulbecco's modified Eagle medium (D-MEM) and RPM1-1640 were purchased from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) was 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. Breast cancer cell line MDA-MB-231 (ATCC) was grown in RPM1-1640 medium with 10% FBS. All cell lines were grown in medium containing 100 units/ml penicillin, 100 µg/ml streptomycin at 37^0C in 5% CO_2.

B. Generation of recombinant adenovirus AdRSVp16

The construction of the adenovirus containing p16 cDNA under the control of RSV promoter (AdRSVp16) was previously described (Steiner et al, 2000). Briefly, a human wild-type p16 cDNA gene was subcloned under the control of a RSV promoter into an E1 deleted adenoviral shuttle vector pAvs6a (Genetic Therapy, Inc., Gaithersburg, MD). The resultant adenoviral shuttle vector was cotransfected into 293 cells with pJM17 (Microbix Biosystems Inc., Toronto, Canada), an adenoviral type 5 genome plasmid, by the calcium phosphate method. The individual plaques were screened by direct plaque screening PCR method (Lu et al, 1998) using primers specific for RSV promoter and p16 cDNA gene. The resultant AdRSVp16 is a replication-defective, recombinant adenoviral vector. Control virus AdRSVlacZ was generated by a similar method (Lu et al, 1999).

C. Adenovirus preparation, titration and transduction

Individual clones of AdRSVp16 and AdRSVlacZ were obtained by three times plaque purification method. Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the complete cytopathic effect was collected and adenovirus was purified and concentrated by twice $CsCl_2$ gradient ultracentrifugation. The viral titration and transduction were performed as previously described (Graham and Prevec, 1991).

D. Immunohistochemistry

The procedure followed the method as described previously (Steiner et al, 2000). Briefly, for immunohistochemical staining, culture cells were grown on SlideFlasks with bottom detachable slides (Nalge Nunc, Naperville, IL) that could be used for immunohistochemistry staining directly later. The samples (slides) were first incubated with 1% H_2O_2 for 30 min. The samples were incubated with first antibody against human p16 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 16 h at 4^0C, then by corresponding second 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 µl 50% H_2O_2 in 150 ml PBS) for 3-10 min.

E. RT-PCR

Cells were extracted and total RNA was isolated by RNeasy Total RNA Kit (Qiagen, Santa Clarita, CA). After treatment of total RNA with RNase-free DNase I (Gibco BRL), reverse transcriptase reaction was carried out using Superscript II RT (Gibco BRL) according to the manufacturer's protocol. An aliquot of the RT mixture was subsequently used for the PCR reaction. The primers were specific to VEGF gene: primer 1 was $_{5'}$GGATGTCTATCAGCGCAGCTAC$_{3'}$ and primer 2 was $_{5'}$TCACCGCCTCGGCTTGTCACATC$_{3'}$. This primer set could detect mRNA encoding three molecular species of VEGF, giving rise to 322-, 454-, and 526-bp bands for $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively (Houck et al, 1991). PCR was performed in 50 µl total volume containing one fifth of above RT mixture, in a final concentration of 2 mM $MgCl_2$, 50 mM KCl, 0.2 mM each of dNTPs, 20 mM Tris-HCl (pH 8.4), 1 µM each of the primers, and 2.5 units of Taq DNA polymerase (Gibco BRL). The reaction was carried out at 94^0C for 4 min; then for 30 cycles at 94^0C for 1 min, 61^0C for 2 min, and 72^0C for 2 min; followed by at 72^0C for 10 min. To ensure the quality of total RNA samples, the same RT mixture mentioned above was used for PCR of housekeeper gene ß-actin. The primers specific to ß-actin gene were $_{5'}$TCCTGTGGCATCCACGAAACT$_{3'}$ and $_{5'}$GAAGCATTTGCGGTGGACGAT$_{3'}$ which resulted a 314-bp PCR product. The PCR conditions followed the methods described previously (Kuo et al, 2002).

F. Northern blot

Cells were extracted and total RNA was isolated by RNeasy Total RNA Kit (Qiagen, Santa Clarita, CA) according to the manufacturer's protocol. Total RNA (10 µg) was loaded on a 1.2% polyacrylamide gel and processed by electrophoresis. The standard Northern blot transfer to a Nylon membrane (Hybond-N^+, Amersham Life Science, Buckinghamshire, England) was performed. The cDNA probe (p16 and $VEGF_{165}$) was labeled by -^{32}P-dCTP using random primer method (Prime-It II Kit, Stratagene, La Jolla, CA). The membrane was hybridized with the probe in Rapid-hyb buffer (Amersham Life Science) according to the manufacturer's protocol. The membrane was exposed to a Kodak X-ray film between two intensifying screens at -80^{0}C for autoradiography. The cDNA probe of housekeeper gene -actin was labeled as described above and used as an internal control for normalization.

G. ELISA for detecting VEGF

Cells will be grown in 10-cm culture dishes and either untreated or transduced with AdRSVlacZ or AdRSVp16 (at moi=200). After 90-min viral infection, viral medium will be replaced with an exact 10 ml fresh medium to each sample dish. The cell medium (supernatant) will be collected 72 hr after viral transduction, and cell number attached on the culture dish will be counted. The supernatant will be processed to determine the secreted amount of $VEGF_{165}$ protein by VEGF immunoassay kit (Quantikine VEGF ELISA Kit, R&D Systems, Minneapolis, MN). The procedures will follow the methods according to the manufacturers' manual The results will be normalized based on the same amount of cells analyzed.

H. Matrigel in vivo angiogenesis assay

Human breast cancer MDA-MB-231 cells were transduced by AdRSVp16 at moi of 200, two days later, the cells were harvested. A matrigel (BD Biosciences, San Jose, CA) mixture containing 1x10^7 transduced cells was injected s.c. into the flank of mice (6-week-old female nude mice, Harlan). Three days later, the mice were sacrificed, the undersurfaces of the injected site of mice were examined and photographed. Untreated control group and AdRSVlacZ control virus treated group were used as controls for comparison.

I. Dorsal air sac assay

Cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. Forty-eight hours later the cells were harvested and suspended in PBS at a concentration of 1x10^8 cells/ml. This suspension (0.1 ml in PBS) was injected into a chamber (Millipore, Bedford, MA) consisting of a ring with a filter (pore size, 0.22 µm) 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 female 6-week-old nude 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 preexisting background vessels by their zigzagging characters.

III. RESULTS

A. Adenovirus AdRSVp16 expresses high level p16 protein in breast cancer cells

To facilitate induction of p16 expression, a replication-defective recombinant adenovirus expressing human wild-type p16 under the control of a Rous sarcoma virus promoter (AdRSVp16) has been generated (Steiner et al, 2000). To demonstrate that AdRSVp16 is able to transfer and express p16 protein in cancer cells, MDA-MB-231 cells were transduced with AdRSVp16 in vitro at multiplicity of infection (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 for p16 protein (**Figure. 1B**) while control untreated cells did not have the p16 staining (**Figure. 1A**).

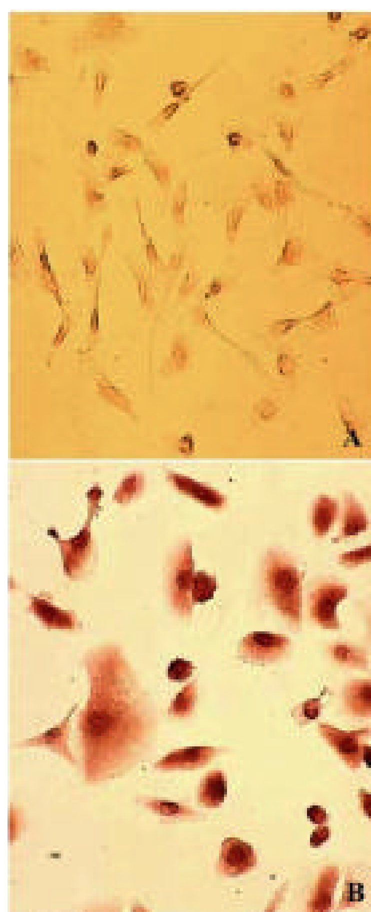

Figure 1. *In vitro* p16 expression in human breast cancer MDA-MB-231 cells after AdRSVp16 transduction. MDA-MB-231 cells were grown in culture dish and transduced by AdRSVp16 at moi=200. Seventy-two hrs later the cells were harvested and subjected to immunohistochemistry using primary antibody (mouse 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). The original magnification was 66X for both images.

To demonstrate that the adenovirus can effectively transduce and express the transgene in vivo inside the breast tumor, AdRSVlacZ, an adenovirus carrying E.coli ß-galactosidase (lacZ) reporter gene (Lu et al, 1999), was used to transduce a JygMC(A) breast tumor growing in nude mice.As shown in **Figure. 2A**, the ß-galactosidase (lacZ) transgene-expressing cells exhibit blue color after X-gal staining. A dose of $1x10^{10}$ pfu (plaque forming unit) can effectively transduce breast tumor cells in vivo. Similarly, to demonstrate that AdRSVp16 is able to transduce and express p16 protein in breast cancer cells in vivo, JygMC(A) breast tumors growing in nude mice were transduced by AdRSVp16. The immunohistochemical staining of breast tumor sections by anti-p16 antibody showed p16 expression in vivo (**Figure. 2B**). These results indicate that AdRSVp16 is able to efficiently transfer and express p16 protein in cancer cells both in vitro and in vivo.

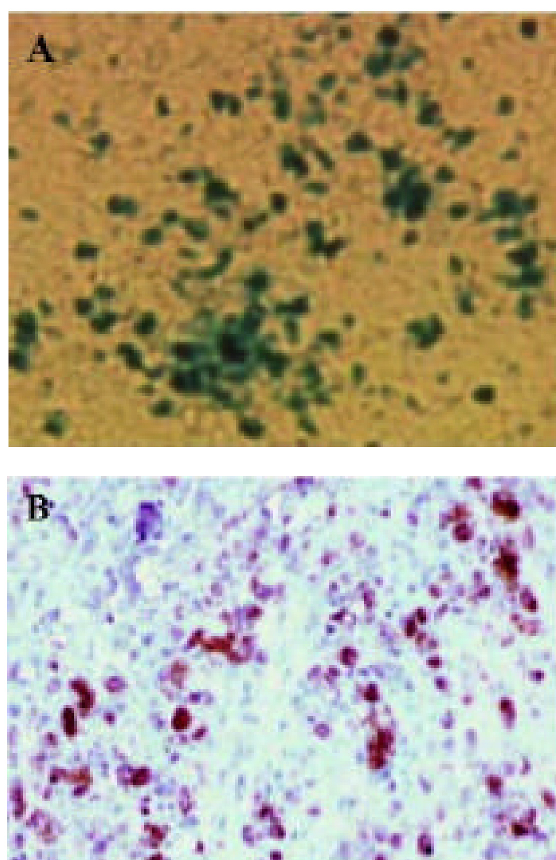

Figure 2. Adenoviral vectors effectively transduce and express transgene inside the breast tumor. Breast tumors were established in nude mice by subcutaneously injection of $1x10^7$ mouse breast cancer JygMC(A) cells in the flank of nude mice. When tumors reached about 200 mm^3, $1x10^{10}$ pfu AdRSVlacZ (A) or AdRSVp16 (B) was injected directly into the tumors, respectively. The tumors were harvested at 72 h and processed to either for X-gal staining for ß-galactosidase (lacZ) transgene expression (A, blue-color cells) or immunohistochemistry for p16 expression (B, dark brown-color cells), respectively. Untreated tumor showed neither endogenous lacZ nor p16 staining (not shown).

B. p16 downregulates VEGF expression

By using primers specific to VEGF gene that would result three RT-PCR (reverse transcription polymerase chain reaction) products corresponding to isoforms $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ (Houck et al, 1991), our RT-PCR results showed that there was a decreased expression of VEGF at the mRNA level after induction of p16 (**Figure. 3**). MDA-MB-231 cells were either untreated or transduced with control virus AdRSVlacZ (AdlacZ) or AdRSVp16 (Adp16) at moi of 200. The cells were harvested at 24 hr and 48 hr after viral transduction and total RNA was isolated for detecting VEGF mRNA expression by RT-PCR. As shown in **Figure. 3**, all three isoforms of VEGF, including $VEGF_{121}$ (322-bp), $VEGF_{165}$ (454-bp), and $VEGF_{189}$ (526-bp), were dramatically reduced by p16 expression, with a more significant reduction of VEGF expression over the time. In contrast, the control virus (AdlacZ) transduced cells at both 24 hr and 48 hr (lane 3 and 5 from the left in **Figure. 3**) had no changes of VEGF mRNA expression compared to that of the untreated control (lane 2 from the left in **Figure. 3**).

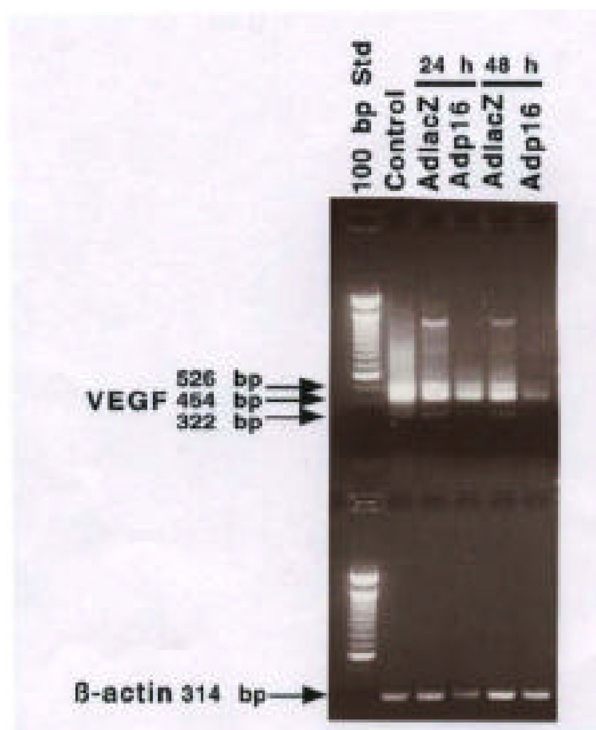

Figure 3. RT-PCR of VEGF gene in human breast cancer cell line MDA-MB-231. Total RNA were isolated from cells which were untransduced or transduced with control virus or Adp16 (moi=200) at 24 hr and 48 hr post viral transduction. Reverse transcriptase reaction using total RNA was carried out. An aliquot of the RT mixture was subsequently used for the PCR reaction. The primers specific to VEGF gene which resulted three specific RT-PCR products, 526 bp, 454 bp, and 332 bp, corresponding to $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, respectively. To ensure the quality of total RNA samples and equal measurement, the same RT mixture mentioned above was used for PCR of housekeeper gene ß-actin which resulted a 314 bp PCR product.

The similar results were also observed in another breast cancer cell line JygMC(A) by RT-PCR assay (our unpublished results). Consistently, our Northern blot analysis results also showed that there was a significant reduction of VEGF mRNA expression in MDA-MB-231 cells transduced by AdRSVp16, compared to untreated control and control virus AdRSVlacZ transduced cells (**Figure. 4**).

To determine whether p16 modulates VEGF gene expression at the protein level, MDA-MB-231 cells were either untreated or transduced by control virus or AdRSVp16, and 72 hrs later the cell culture medium 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 medium (about 66% reduction compared to the untreated control cells at the same amount of cells) (**Figure. 5**).

These data indicate that p16 decreases VEGF expression at both mRNA and protein levels in MDA-MB-231 cells, implying that p16 downregulated VEGF gene expression in breast cancer cells.

C. p16 inhibits angiogenesis

To determine whether p16 inhibits tumor cell-induced angiogenesis, the effects of p16 on tumor cell neovascularization were assessed by "Matrigel in vivo angiogenesis assay" (see Materials and Methods section), in which MDA-MB-231 cells were either untreated or transduced with control virus AdRSVlacZ and AdRSVp16, 48 hrs later, the cells were harvested and injected subcutaneously (s.c.) into the flank of the nude mice. Three days after the tumor cell injection, the mice were sacrificed and the blood vessels of undersurface of the injection site were examined and photographed. AdRSVp16-treated MDA-MB-231 cells induced much less newly formed blood vessels (**Figure. 6B**) compared to its control-virus treated (**Figure. 6A**) and untreated control (not shown) counterparts. The latter two induced significantly higher amount of newly-formed blood vessels, as demonstrated by their characteristic zigzag and bifurcation/trifurcation forms (arrows in **Figure. 6A**). These results demonstrate that p16 inhibits angiogenesis induced by injected breast cancer cells.

The effects of p16 on neovascularization of tumor surrounding cells were examined by dorsal air sac assay. MDA-MB-231 cells were transduced with AdRSVp16. Forty-eight hrs later the cells were harvested and injected into a chamber that was wrapped with semi-permeable membrane allowing for 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 in the undersurface of the chamber will be examined 3 days later after chamber implantation. As shown in **Figure. 7**, PBS-treated mice (as a negative control) did not have any obvious neovascularization (**Figure. 7A**). 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 (**Figure. 7B**).

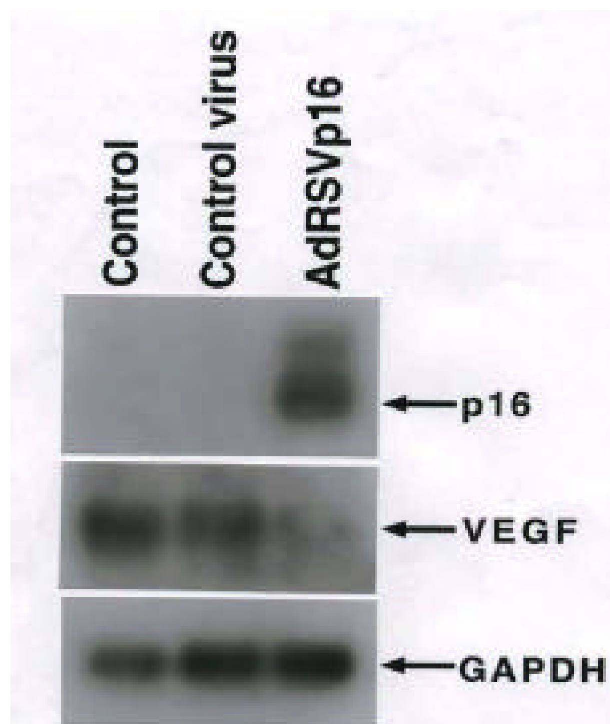

Figure 4. p16 overexpression decreased VEGF expression at mRNA level in MDA-MB-231 cells. MDA-MB-231 cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. The cell extracts were harvested at 48 hrs after viral transduction and mRNA expressions of $VEGF_{165}$, p16 and internal control GAPDH were determined by Northern blot analysis by using corresponding cDNA probes.

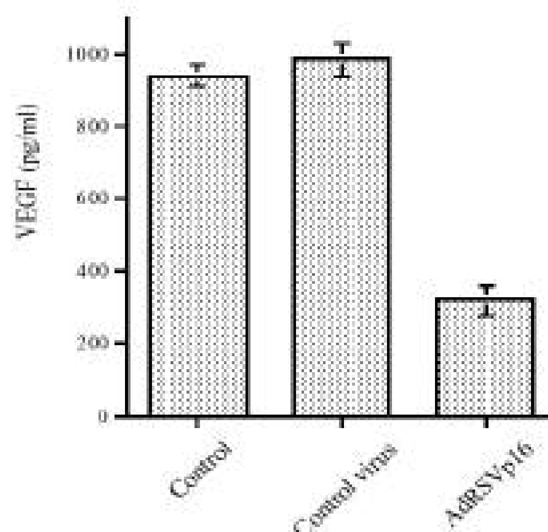

Figure 5. p16 overexpression decreased VEGF secretion of MDA-MB-231 cells. MDA-MB-231 cells were grown in medium containing charcoal-stripped serum. Cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. The cell medium were collected 72 hrs after viral transduction and subjected to VEGF determination by ELISA assay using a kit designated for human $VEGF_{165}$ immunoassay (Quantikine VEGF ELISA Kit, R&D Systems). The data represent the results from two independent experiments, each performed in triplicate.

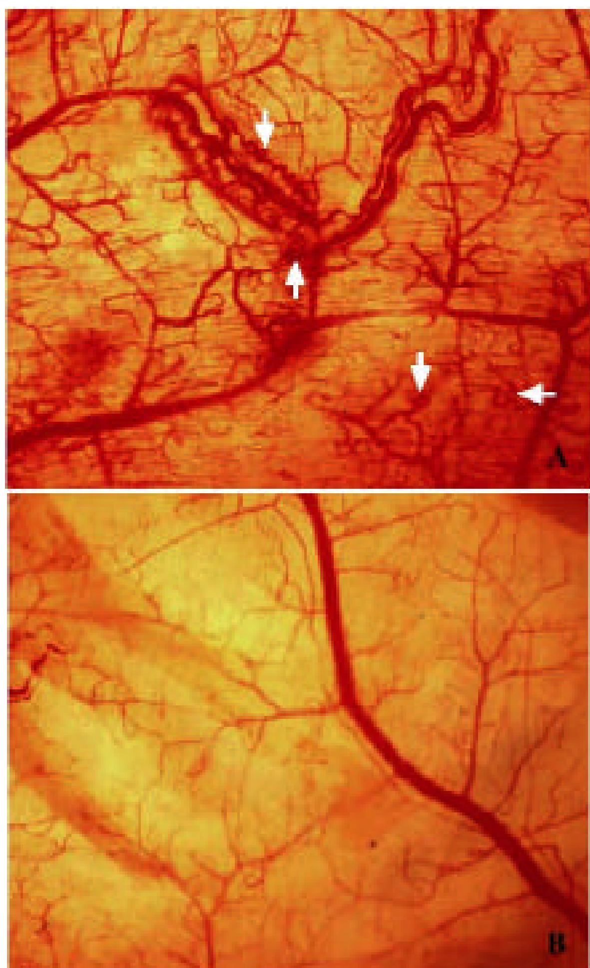

Figure 6. p16 inhibited angiogenesis. MDA-MB-231 cells were either untreated or transduced with control virus AdRSVlacZ or AdRSVp16 at moi of 200. Cells were harvested 48 hrs post viral transduction, and 1x10^{7} cells were mixed with Matrigel in 1:1 volume and s.c. injected into the flanks of 6-week-old female nude mice. Three days later, the mice were sacrificed, the undersurfaces of the injected site of mice were examined and photographed. Shown are undersurface blood vessels of mice injected with (A) AdRSVlacZ-treated cells, and (B) AdRSVp16-treated cells. Mouse injected with untreated MDA-MB-231 cells gave the similar results as (A) (not shown). The newly formed blood vessels are morphologically distinguishable from the preexisting background vessels by their zigzag characters, some of them are representatively pointed by the arrows (A). Each figure represents a typical image from 3 mice in the same group.

In contrast, mice with AdRSVp16-transduced MDA-MB-231 cells induced much less newly-formed blood vessels (**Figure. 7D**) compared to mice injected with MDA-MB-231 cells alone (**Figure. 7B**) or mice injected with control viral transduced MDA-MB-231 cells (**Figure. 7C**); both of the latter two induced a more extensive capillary network. These results suggest that breast cancer cells can induce neovascularization around the tumor by molecules (such as VEGF) secreted from tumor cells to the surrounding environment; and p16 can inhibit this tumor cell-induced neovascularization to the surrounding environment by impairing or blocking this secreted angiogenesis-inducer from the tumor cells.

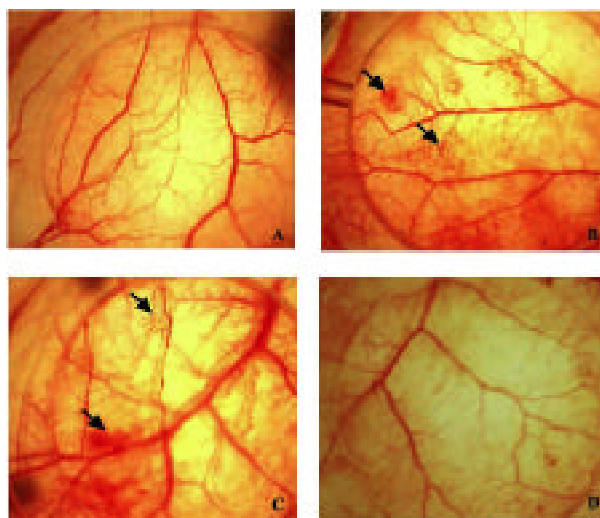

Figure 7. p16 suppressed neovessel formation in air sac model. The mouse in air sac model was sacrificed on day 3 after chamber implantation and the implanted chamber was removed from the s.c. air fascia, a ring without filters was placed on the same site and then photographed. The newly formed blood vessels were morphologically distinguishable from the preexisting background vessels by their zigzagging characters (see representative arrows). Shown are undersurface images of sites from chamber contains PBS only as negative control (A), MDA-MB-231 cells (B), AdRSVlacZ-transduced MDA-MB-231 cells (C), and AdRSVp16 transduced MDA-MB-231 cells (D).

IV. Discussion

In summary, our studies showed that adenoviral-mediated overexpression of p16 decreased VEGF expression at both mRNA and protein levels in human breast cancer MDA-MB-231 cells. In vivo angiogenesis assay and dorsal air sac assay on nude mice showed that p16 inhibited angiogenesis of MDA-MB-231 cells. These results together strongly demonstrate that p16 downregulates VEGF gene expression and suppresses tumor cell angiogenesis and neovascularization, suggesting that p16 expression may have a potential to suppress metastasis in breast cancer cells. Thus, AdRSVp16 may be useful to suppress breast cancer metastasis as a gene therapy approach. Likewise, other tumor suppressor genes p53 (Bouvet et al, 1998) and Rb2/p130 (Claudio et al, 2001) were reported to downregulate VEGF expression and inhibit angiogenesis in colon and lung cancer cells, respectively. Rb2/p130 seems to downregulate VEGF expression at the transcriptional level (Claudio et al, 2001). p53 was also shown to inhibit angiogenesis by stimulating TSP-1 gene and positively regulate TSP-1 promoter (Dameron et al, 1994). Despite all these associations, however, the link between tumor suppressor genes and angiogenesis remains obscure, in particular, how p16 exactly regulates VEGF expression is not clear. It is speculated that p16 may regulate VEGF gene expression at the transcriptional level or via stabilization of VEGF mRNA, or both. Our ongoing study of evaluation of VEGF promoter activity in cells, that are transiently cotransfected with p16 expression vector and a series of VEGF promoter/CAT

(chloramphenicol acetyltransferase) reporter gene chimeric constructs (a generous gift from Dr. M. Kuwano, Kyushu University, Japan) (Ryuto et al, 1996), will determine whether p16 modulates VEGF gene expression at the transcriptional level. If p16 indeed regulates VEGF expression at the transcriptional level, the transactivation response element within VEGF promoter will be defined. Together with the gel shift assay, we may also find out whether it is due to a direct p16 binding to VEGF promoter or by an indirect p16 regulation, i.e., by binding of a p16-regulated component to the promoter for this transactivation.

While research studies focusing on breast cancer treatment have been increased dramatically in recent years and some therapies of local control appear to be effective, there is still no effective approach to prevent and cure tumor metastasis -- the fatal cause for the death of breast cancer patients. The relative success at local control has been confounded by a general failure to progressively and substantially reduce breast cancer death rates. Thus, a critical need exists to understand and develop effective treatments for those parameters contributing to breast cancer metastasis. This study has provided an innovative approach to combat and prevent breast cancer metastasis by using tumor suppressor gene p16, which downregulates VEGF gene expression, suppresses angiogenesis and may have a potential inhibition on secondary tumor formation of breast cancer.

Acknowledgments

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