Vitamin K Epoxide Reductase: A Protein Involved in Angiogenesis

Yibo Wang, Yisong Zhen, Yi Shi, Jingzhou Chen, Channa Zhang, Xiaojian Wang Xu Yang, Yi Zheng, Yuqing Liu, and Rutai Hui

Sino-German Laboratory for Molecular Medicine, Fuwai Hospital, Chinese Academy of Medical Sciences and National Genome Center, Beijing, P.R. China

Abstract

Vitamin K epoxide reductase (VKOR) is a newly identified protein which has been reported to convert the epoxide of vitamin K back to vitamin K, a cofactor essential for the posttranslational γ-carboxylation of several blood coagulation factors. We found that the gene is expressed ubiquitously including vascular endothelial cells, smooth muscle cells, fibroblasts and cardiomyocytes, and is overexpressed in 11 tumor tissues on microarray. Stable transfection of VKOR cDNA into tumor cell line A549 and H7402 did not promote the cell proliferation. These results promoted us to hypothesize that VKOR may also be involved in angiogenesis. To test this hypothesis, the expression of VKOR was studied in different vascular cells in developmental and pathologic heart tissues. The effects of overexpression and suppressing expression of VKOR on endothelial cell proliferation, migration, adhesion, and tubular network formation were explored. We found that VKOR expression in arteries was prominent in vascular endothelial cells and was high in the ventricular aneurysm tissue of human heart and human fetal heart. *In vitro* studies showed that overexpression of VKOR slightly but significantly stimulated human umbilical vein endothelial cell proliferation (by 120%), migration (by 118%), adhesion (by 117%), as well as tubular network formation. Antisense to *VKOR* gene inhibited the proliferation (by 67%), migration (by 64%), adhesion (by 50%), and tubular network formation. Our findings support the impact of VKOR in the process of angiogenesis; hence, the molecule may have a potential application in cardiovascular disease and cancer therapy.
(Mol Cancer Res 2005;3(6):317–23)

Introduction

Angiogenesis plays an important role in embryogenesis, tumorigenesis, and some pathologic conditions (1, 2). This statement is underscored by the fact that solid tumor growth beyond a few square millimeters in diameter requires new blood vessel growth. The angiogenic process can be divided into two phases, induction and resolution. The induction phase involves dynamic changes in endothelial cell-cell and cell-matrix interaction, including degradation of basement membrane, proliferation, migration, and adhesion of endothelial cells (3). These are the important steps for initiation of angiogenesis to restore blood supply to ischemic tissues, and to promote tumor growth (4). These changes are regulated by growth factors such as fibroblast growth factor and vascular endothelial growth factors (VEGF).

Even though the function of vitamin K epoxide reductase (VKOR) has been reported since 1974 (5), the *VKOR* gene has only been cloned for 1 year (6, 7). It has been reported that VKOR converts vitamin K 2,3-epoxide back to vitamin K, a cofactor that is essential for the posttranslational γ-carboxylation of several blood coagulation factors (8-10).

Recently, two groups have reported that mutations in the *VKOR* gene cause warfarin resistance and multiple coagulation factor deficiency (6, 7). We found that VKOR is ubiquitously expressed, and its expression in arteries is prominent in the endothelial layer. These lead us to speculate that VKOR may be involved in angiogenesis. To test this hypothesis, we studied the effects of overexpression and suppressing expression of VKOR on the proliferation, migration, adhesion, and tubular network formation of human umbilical vein endothelial cell (HUVEC) by either transfecting VKOR cDNA into HUVEC, or antisense blocking of VKOR expression in HUVEC. We also observed the VKOR expression in different developmental stages of human heart (fetal and adult), and ventricular aneurysm tissue of human heart after myocardial infarction.

Results

Ubiquitous Tissue Distribution of VKOR Gene

To determine the tissue distribution of VKOR, two human Multiple Tissue Northern blots (from Clontech, Palo Alto, CA) were hybridized with ^{32}P-labeled VKOR cDNA probe. The results showed that VKOR was expressed ubiquitously, but primarily in the liver, kidney, heart, and skeletal muscle (Fig. 1A).

VKOR Gene Up-regulated in Tumor Tissues but Overexpression In vitro *Did Not Promote Tumor Cell Line Proliferation*

Human tumor tissue cDNA microarrays indicated that *VKOR* expression was either unchanged or up-regulated (>1.5-fold) in

Received 12/29/04; revised 4/11/05; accepted 4/18/05.
Grant support: Supported by the Ministry of Science and Technology of China with grant 2002BA711A05 and National Basic Research Project (973 G2000056901) to R. Hui.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Y. Wang and Y. Zhen contributed equally to this study.
Requests for reprints: Rutai Hui, Sino-German Laboratory for Molecular Medicine and Hypertension Division, Cardiovascular Institute and Fuwai Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, 167 Beilishilu, Xicheng District, Beijing 100037, P.R. China. Phone: 86-10-6833-3902; Fax: 86-10-6833-1730. E-mail: huirutai@sglab.org

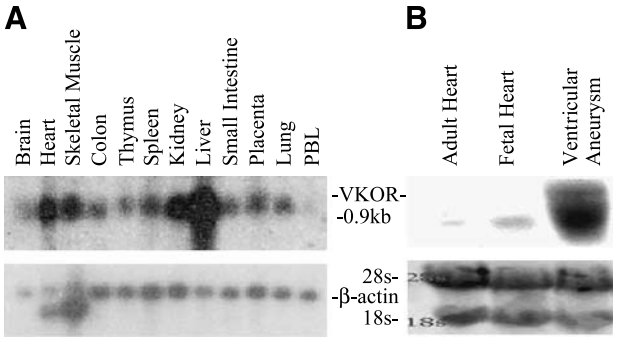


FIGURE 1. **A.** Tissue distribution of VKOR. Expression of VKOR in normal tissues was determined with Northern blot analysis by using Multiple Tissue Northern blots from Clontech. PBL, peripheral blood leukocyte. **B.** VKOR is up-regulated in the tissues with flourishing angiogenesis. Total RNA was isolated from the tissues of fetal heart and adult ventricular aneurysm and normal adult heart by using RNAgent Isolation System; 20 μg of total RNA was separated on a 1% agarose gel, transferred onto nylon membrane, and hybridized for 4 hours at 68°C in prewarmed ExpressHyb with VKOR probe.

most tumors from colon, lung, rectum, breast, uterus, stomach, cervix, thyroid, ovary, kidney, and small intestine compared with matched normal adjacent tissues (Fig. 2). To test whether up-regulated VKOR could promote tumor cell proliferation, stable transfection of VKOR cDNA into tumor cell line A549 and H7402 were done, no cell proliferation was identified in the two cell lines carrying VKOR cDNA compared with those carrying control vector (data not shown).

Up-regulation of VKOR in Fetal Heart and Ventricular Aneurysm Tissue of Human Heart

To determine the possible involvement of VKOR in heart developmental and in pathologic processes, *VKOR* gene expression was studied in ventricular aneurysm tissue of human heart after myocardial infarction and in fetal and adult hearts. The most significant overexpression of *VKOR* gene was found in ventricular aneurysm tissue, followed by that in fetal heart, and finally in the adult heart (Fig. 1B).

VKOR Expression in Arteries Is Mainly Localized in the Vascular Endothelium

Our *in situ* hybridization study showed that VKOR expression in arteries is mainly localized in vascular endothelial layers. The characteristic of endothelial cells was documented by using monoclonal antibody to Von Willebrand factor (Fig. 3). The expression of VKOR was confirmed by real-time PCR or Northern blot in HUVECs, human aortic smooth muscle cells, fibroblasts which constitute the principal vascular cellular components in the different layers of vessels, and in human fetal heart tissue, but not in human peripheral blood leukocytes. The results showed that VKOR expression was primarily in HUVEC and in fetal heart tissue (Table 1).

VKOR Participates in Angiogenesis

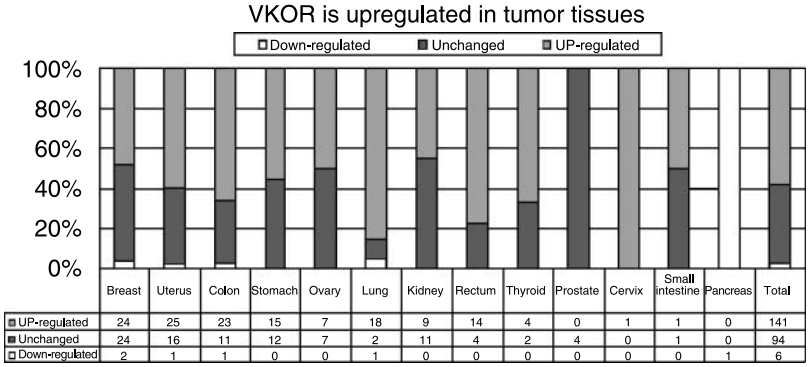
Because endothelial cells play a key role in angiogenesis, the effect of VKOR on proliferation, migration, adhesion and tubular network formation—the key steps in angiogenesis—was studied in HUVECs. Antisense oligonucleotides to *VKOR* (VKOR-AS, at 20 μmol/L) inhibited *VKOR* gene expression and VKOR activity by 50% (Table 2). The transfection of adenovirus-VKOR resulted in eight times more VKOR expression than did transfection of Ad-green fluorescent protein (GFP). Treatment of HUVECs with 10% fetal bovine serum (FBS) and VEGF (10 ng/mL) resulted in tubular network formation. Overexpressing VKOR stimulated cell proliferation by 120%, migration by 118%, and adhesion by 117%, respectively, and promoted the tubular network formation in HUVECs on Matrigel, but not in Ad-GFP control. VKOR-AS (at 10 μmol/L) inhibited HUVEC adhesion to Matrigel-coated plates by 50% (Fig. 4C) as well as tubular network formation (Fig. 5). These inhibitory effects were proportional to the concentration of the antisense from 0.2 to 10 μmol/L.

VKOR-AS inhibited VEGF/basic fibroblast growth factor–induced cell proliferation in a dose-dependent manner, at 0.2 μmol/L for 11%, 2 μmol/L for 53%, and 10 μmol/L for 67%, respectively (Fig. 4A). Sense or mutant oligonucleotides to *VKOR* did not have such effects.

The chemotactic effects of VKOR on HUVECs were assessed in the presence of VEGF by using a Boyden chemotaxis chamber assay. VKOR-AS significantly inhibited the VEGF-induced migration of HUVECs in a dose-dependent manner (Fig. 4B), 64% of inhibition can be achieved with the highest dose (20 μmol/L).

Discussion

In this study, we found that VKOR was ubiquitously expressed and up-regulated in many tumor tissues, in human fetal heart, and in ventricular aneurysm tissue in human heart.



	Breast	Uterus	Colon	Stomach	Ovary	Lung	Kidney	Rectum	Thyroid	Prostate	Cervix	Small intestine	Pancreas	Total
UP-regulated	24	25	23	15	7	18	9	14	4	0	1	1	0	141
Unchanged	24	16	11	12	7	2	11	4	2	4	0	1	0	94
Down-regulated	2	1	1	0	0	1	0	0	0	0	0	0	1	6

FIGURE 2. VKOR expression in normal and cancer tissues. BD Clontech Cancer Profiling Array I was hybridized with the ^{32}P-labeled VKOR cDNA probe. Changes in VKOR expression levels were compared in normal versus cancer tissues. The data were normalized by ubiquitin. The patients were grouped based on an up-regulation of VKOR >1.5-fold, or no changes or a down-regulation of <0.7-fold. The pairs of tumor or normal tissues on the array were: breast (50 pairs), uterus (42 pairs), colon (35 pairs), stomach (27 pairs), ovary (14 pairs), cervix (1 pair), lung (21 pairs), kidney (20 pairs), rectum (18 pairs), small intestine (2 pairs), thyroid (6 pairs), prostate (4 pairs), and pancreas (1 pair).

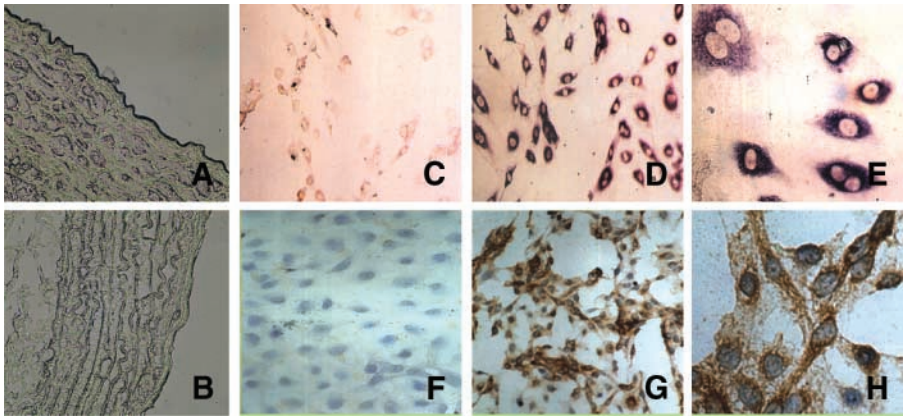


FIGURE 3. The expression of *VKOR* gene in arteries. VKOR in arteries was mainly expressed in vascular endothelium (**A,** × 400). The corresponding sense probe was used as a control (**B,** × 400; **C,** × 100). The expression of VKOR in HUVEC (**D,** × 100; **E,** × 400). HUVECs were characterized with antibody to Von Willebrand factor. The nuclei were stained by hematoxylin, PBS as a control (**F,** × 100), HUVECs (**G,** × 100; **H,** × 400).

In arteries, it was highly expressed in the vascular endothelial layers. When VKOR cDNA was transfected into tumor cell lines, no cell proliferation was promoted. We speculate that VKOR participates in the mediation of angiogenesis, and has roles in the development and angiogenesis-related pathologic conditions such as tumorigenesis and ischemic cardiovascular diseases.

Our results support VKOR as an angiogenic mediator as well. First, VKOR expression in arteries is prominently localized to endothelial cells. Its expression is significantly up-regulated in the tissues under angiogenesis-related physiologic conditions such as in fetal heart, and in pathologic conditions such as ventricular aneurysm caused by myocardial infarction and tumor tissues; however, stable transfection of VKOR into the tumor cell line did not promote cell proliferation, indicating indirect involvement in tumor growth. Second, antisense oligonucleotides to *VKOR* inhibited proliferation, migration adhesion, and tubular network formation in cultured HUVECs, the effects were dose-dependent, and not seen in cells treated with sense oligonucleotides nor in those treated with mutant oligonucleotides to *VKOR*, indicating that these effects are VKOR-specific.

We found that VKOR may have roles in physiologic and pathologic angiogenesis. VKOR was highly expressed in human fetal heart and extremely up-regulated in tissues from human ventricular aneurysm caused by myocardial infarction, indicating that VKOR may mediate angiogenesis in late fetal and early postnatal periods of development. During fetal development, rapid growth of cardiac myocytes is accompanied by a proportional growth of capillaries and some degree of growth in larger vessels. In myocardial infarction, local angiogenesis compensating for the tissue hypoxia has been documented (11). Our *in vitro* study showed that overexpression of VKOR in HUVEC led to the cell proliferation, migration, adhesion, and tubular network formation. Antisense to *VKOR* could block VEGF/basic fibroblast growth factor–induced cell proliferation, migration, adhesion, and tubular network formation, the key early steps for angiogenesis.

The mechanism of VKOR mediating angiogenesis is presently unclear. VKOR is a cytoplasmic protein (6), the molecular features of VKOR indicate that the protein has an endoplasmic reticulum membrane retention signal (KKXX-like motif): KAKRH. This motif serves as a retention and retrieval signal that brings proteins back from a sorting compartment such as the Golgi complex to the endoplasmic reticulum (12). The p24 family (p24/gp25L/emp24/Erp) of proteins have been shown to be critical components of the coated vesicles that are involved in the transportation of cargo molecules from the endoplasmic reticulum to the Golgi complex (13). They all have the GOLD domain, but not VKOR. The mechanisms responsible for endoplasmic reticulum retrieval and retention are not well-understood, but it has been shown that yeast and mammalian dilysine-tagged endoplasmic reticulum–resident transmembrane proteins interact with the coatomer in cell lysates (14, 15). Previously, our yeast two-hybridization results did not support that VKOR bound to the coatomer, but indicated that VKOR may interact with the domain EMP24_GP25L,[1] and all these proteins (Tmp21, CGI100, gp25L2), which contain the domain EMP24_GP25L, have a KKXX motif. We speculate that VKOR may be involved in the transportation of cargo molecules related to angiogenesis from the endoplasmic reticulum to the Golgi complex.

The key function of VKOR is converting the epoxide of vitamin K back to vitamin K so that the other enzyme of the vitamin K cycle, the γ-glutamyl carboxylase, can accomplish the posttranslational γ-carboxylation modification of some

Table 1.VKOR Expression Analysis by Real-time PCR

Cells or Tissues	Normalized VKOR Amount Relative to HSMC $2^{-\Delta\Delta CT}$
Human aortic smooth muscle cell	1.0 (0.9-1.1)
HUVEC	3.9 (3.6-4.3)
Human fetal heart	7.7 (7.3-8.0)
Human dermal fibroblast	0.6 (0.5-0.7)
Human peripheral blood leukocyte	~0

NOTE: Total RNA was isolated from human aortic smooth muscle cell, HUVEC, human dermal fibroblast, human peripheral blood leukocyte, and human fetal heart, and reverse-transcribed to cDNA by using reverse transcriptase. Aliquots of cDNA were used as a template for real-time PCR reactions containing primers for VKOR or for β-actin. Each reaction contained cDNA derived from 10 ng total RNA. Four replicates of each reaction were done.

[1] Unpublished data.

Table 2.Effect of the Antisense Oligonucleotide to VKOR on VKOR Activity and Expression

VKOR	Relative Enzyme Activity	Relative Amount of mRNA
Control	100	1.0
Antisense (20.0 μmol/L)	46	0.5
Sense (20.0 μmol/L)	90	0.9
Mutant antisense (20.0 μmol/L)	88	0.9

NOTE: HUVECs were treated with the antisense oligonucleotide to VKOR (20.0 μmol/L) for 1 day. VKOR activity was measured as the ratio of the percentage of substrate VKOR converted into quinine. The mRNA of VKOR was analyzed by real-time PCR, with β-actin as a control. All tests were done in duplicate.

coagulation factors, and the blood coagulation system can be a tool to coordinate angiogenesis and vascular development. VKOR may be involved in angiogenesis through this pathway.

Expression of VKOR in angiogenic endothelium is consistent with our data, which suggests that VKOR is elevated in tumor tissues, ischemic ventricular aneurysm tissue, fetal heart, and arteries.

Materials and Methods

The study protocol was approved by the Fuwai Hospital Review Board. Animal use was confined to the regulations of the NIH and Chinese Academy of Medical Sciences, Ministry of Public Health. Human heart tissues were obtained from patients who suffered spontaneous abortions (fetal heart), or from surgical patients of either myocardial infarction, complicated with ventricular aneurysm, or traffic accidents. Umbilical veins were obtained from cesarean sections. Consent was obtained from the patient's legal representatives.

Molecular Cloning and Constructing pGEMTeasy-VKOR

The open reading frame (ORF) of *VKOR* was amplified by PCR using the aorta cDNA library as template with plaque-forming unit polymerase (Promega, Madison, WI), sense primer (5′-TCG GGC GGA ACC TGG AGA TAA TA-3′), and antisense primer (5′-GGT GTA AAA AAG AGC GAG CGT GTG-3′). The resultant PCR products were ligated into pGEMTeasy vector (Promega) by T4-ligase (New England Biolabs, Beverly, MA), sequenced. The ORF was documented as intact before use.

Tissue Distribution

Tissue distribution of VKOR mRNA was analyzed by Northern blot on normal Multiple Tissue Northern blots purchased from Clontech. ^{32}P-labeled cDNA probes were synthesized according to the ORF of *VKOR* using a Prime-a-Gene Labeling System (Promega) based on supplier's protocol. After hybridization with ^{32}P-labeled *VKOR* probe and a sequential washing, the membranes were subjected to autoradiography.

The mRNA expression profile of VKOR in tumor tissue array was analyzed by using Cancer Profiling Array I (Clontech) and compared with matched normal adjacent tissue.

Stable Transfection and Cell Counting

To construct pcDNA3.1/Myc-His(−)B-VKOR, the plasmid of pEGFP-C3-VKOR was digested with *Xho*I and *Kpn*I. The fragment containing ORF of *VKOR* was ligated into *Xho*I and *Kpn*I sites of pcDNA3.1/Myc-His(−)B vector (Invitrogen, Carlsbad, CA). H7402 and A549 cells were plated in six-well plates at a density 10^6 cells/mL (0.5 mL cells per well). After 20 hours, the cells were transfected with 2 μg of pcDNA3.1/Myc-His(−)B (empty vector) or 2 μg of pcDNA3.1/Myc-His(−)B-VKOR using LipofectAMINE 2000 reagents (Invitrogen) according to the manufacturer's protocol. Stable clones were selected by G418 resistance (200 μg/mL, Life Technologies, Gaithersburg, MD), 48 hours later, cultured at 37°C under 5% CO_2, and counted after 4 days. Each experiment was done in triplicate and repeated four times.

Isolation and Characterization of HUVECs

HUVECs were obtained from human umbilical cord according to the protocol (http://www2.cbm.uam.es/bc-015/english/protocols/huvec.html). In brief, all manipulations were done under sterilized tissue culture hood. The umbilical cords were placed on Petri dishes, filled with 0.1% collagenase solution and incubated for 10 minutes at 37°C for isolation of the endothelial cells. The isolated endothelial cells were collected into a falcon tube and spun down at 1,200 rpm at room temperature for 10 minutes. The supernatant was discarded carefully. The cells were resuspended in Medium 199 containing 10% FBS and plated onto a 25 mL flask

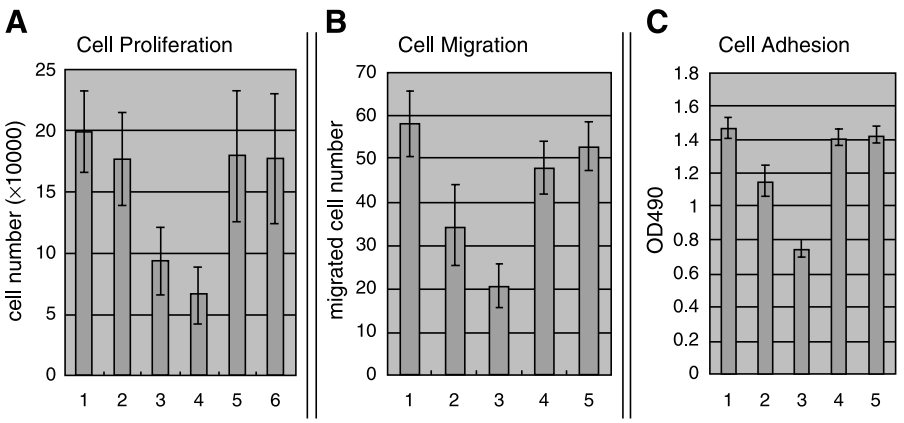


FIGURE 4. The effects of VKOR on HUVEC proliferation, migration, and adhesion. HUVECs were treated with the oligonucleotides to VKOR (VKOR-AS, VKOR-S, or VKOR-MUT) or infected with adenovirus carrying VKOR or GFP. **A.** Effect of antisense VKOR cDNA on HUVEC proliferation: (1) control; (2) VKOR-AS (0.5 μmol/L); (3) VKOR-AS (2.0 μmol/L); (4) VKOR-AS (10.0 μmol/L); (5) VKOR-S (10.0 μmol/L); (6) VKOR-MUT (10.0 μmol/L). **B.** Effect of VKOR-AS on HUVEC migration: (1) control; (2) VKOR-AS (10.0 μmol/L); (3) VKOR-AS (20.0 μmol/L); (4) VKOR-S (20.0 μmol/L); (5) VKOR-MUT (20.0 μmol/L). **C.** Effect of VKOR-AS on HUVEC adhesion: (1) control; (2) VKOR-AS (5.0 μmol/L); (3) VKOR-AS (10.0 μmol/L); (4) VKORS (10.0 μmol/L); (5) VKOR-MUT (10.0 μmol/L).

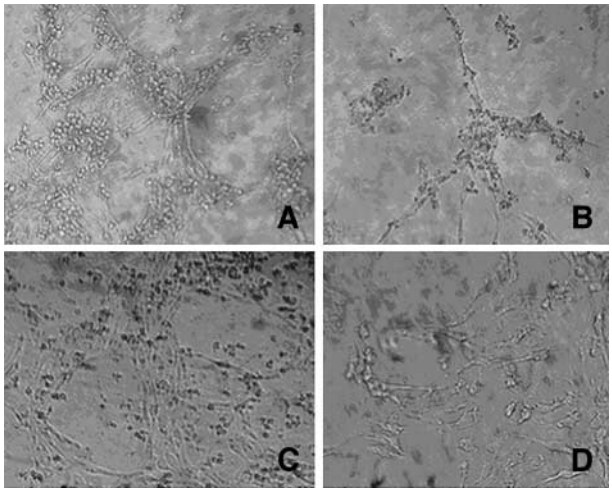


FIGURE 5. Effect of VKOR on HUVEC tubular network formation *in vitro*. The tubular network formation was visualized under an inverted phase contrast microscope. **A,** control; **B,** cells treated with VKOR-AS (10.0 μmol/L); **C,** cells infected with Ad-VKOR; **D,** cells infected with Ad-GFP (all ×100 magnification).

(three cords of cells per flask). Characterization of HUVECs was documented by using immunocytochemical staining with antibody to Von Willebrand factor (Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of Human Peripheral Blood Leukocytes

Human peripheral blood (3 mL) was collected aseptically into the 50 mL falcon tube containing 60 μL of 15% EDTA, and incubated on ice for 3 minutes. After diluting with 20 mL cold PBS, the sample was spun down at 1,000 × *g* at 4°C for 5 minutes. The supernatant was discarded carefully. The cells were resuspended in 2 mL of cold PBS and further diluted with 18 mL of 0.2% NaCl, then incubated on ice for 30 seconds, to which 6 mL of 2.8% NaCl was immediately added and mixed gently, followed by centrifugation at 1,500 × *g* for 5 minutes. The supernatant was discarded and the cell pellets were washed with 20 mL of cold PBS, and resuspended in 100 μL cold PBS.

Expression Analysis of VKOR by Using Real-time PCR

Total RNA was isolated by using RNAgent Isolation System (Promega), and reverse-transcribed to cDNA by avian myeloblastosis virus transcriptase XL (Takara, Dalian, China). Real-time PCR was done on an opticon MJ DNA Engine (MJ Research, Waltham, MA). Aliquots of cDNA were used as the template for real-time PCR reactions containing VKOR primers or β-actin primers. Four replicates of each reaction were done. β-Actin was used as an internal reference of the amount of mRNA in each sample. The following primers were used: VKOR, (TTCTGTCTACCTGGCCTGGATC, CACGTTGATAGCATAGGTGGTGA); β-actin (CAGCAAGCAGGAGTATGACGAG, AAGAAAGGGTGTAACGCAACTA). HASMC and HDF were purchased from Cascade Biologics (Portland, OR). VKOR expression was assayed in the HUVECs after 1-day treatment with oligonucleotides.

In situ *Hybridization*

In situ hybridization was done according to the method described by Solban et al. (16). A total of 10 Sprague-Dawley rats were used in the experiment. Briefly, the fresh aorta tissues from the rats were cut into slices 8-μm-thick and mounted on microscope slides, treated with aminopropylthioethoxysilane, dried at 37°C, and then at 60°C for 10 minutes prior to use. The HUVECs were plated in six-well flat-bottomed plates at 4.8×10^5 cells per well in 2 mL of Medium 199 containing 10% FBS, and cultured at 5% CO_2 and 37°C for 48 hours. A unique 248-bp fragment, amplified with sense primer (5′-GCT CGC TCT TTG CCT GAC-3′) and antisense primer (5′-CTA ACA ATA GCT GTA GTG TGT A-3′), corresponding to *VKOR* ORF, was cloned into pGEM-Teasy vector which has T7 and SP6 promoters. The sense riboprobes were transcribed by using T7 polymerases and the antisense riboprobes by using SP6 polymerases. The resultant probes were labeled with digoxigenin-UTP by using DIG RNA Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany). *In situ* hybridization was done according to DIG Luminescent Detection Kit (Roche Molecular Biochemicals).

Design of Oligonucleotides to VKOR

The sense oligonucleotide to VKOR (VKOR-S), 5′-GAGATAATGGGCAGCACC was synthesized according to VKOR cDNA sequence −6 to +12; antisense (VKOR-AS), 5′-GGTGCTGCCCATTATCTC; mutant VKOR-AS (VKOR-MUT), 5′-GG***A***GC***A***GC***G***CA***A***TA***A***CT***G***, by Sangon Company (Shanghai, China). The inhibition of VKOR expression by VKOR-AS was determined by VKOR activity measurement and quantitative real-time PCR (7).

VKOR Activity Assay

VKOR activity assay was done according to previously described methods (6, 7). The cells which were treated with the oligonucleotides once a day for 4 days were trypsinized and washed twice with cold PBS. The cells (1.5×10^7) were taken for each VKOR assay. A total of 200 μL buffer D [250 mmol/L Na_2HPO_4-NaH_2PO_4, 500 mmol/L KCl, 20% glycerol, and 0.75% CHAPS (pH 7.4)] was added to the cell pellet, followed by sonication of the cell lysate. VKOR (at 5 μmol/L) and DTT (at 4 mmol/L) were added to initiate the reaction. The reaction mixture was incubated in yellow (or no) light at 30°C for 30 minutes and stopped by adding 500 μL of 0.05 mol/L $AgNO_3$/isopropanol (5:9). A total of 500 μL hexane was added and the mixture was vortexed vigorously for 1 minute to extract the vitamin K and vitamin K epoxide. After 5 minutes of centrifugation, the upper organic layer was transferred to a 5 mL brown vial and dried with N_2. A total of 150 μL buffer for high-performance liquid chromatography, acetonitrile/isopropanol/water (100:7:2) was added to dissolve the vitamin K and vitamin K epoxide, and then analyzed by using high-performance liquid chromatography with a C-18 column. Measurements were run in duplicate and the relative activity is given as the ratio of the percentage of substrate converted into quinone. Vitamin K 2,3-epoxide was prepared by the oxidation of vitamin K quinone (Sigma-Aldrich, St. Louis, MO) with H_2O_2.

Constructing the Recombinant Adenovirus Expression Vectors

The recombinant adenoviral expressing vectors were constructed as a protocol (17) for expressing human VKOR (Ad-VKOR) or Ad-GFP. To obtain pAdTrack-cytomegalovirus-VKOR, the plasmid of pEGFP-C3-VKOR was digested by *Bgl*II and *Sal*I, and then the fragment containing the ORF of VKOR was ligated into the *Bgl*II and *Sal*I sites of pAdTrack-cytomegalovirus. Then pAdTrack-cytomegalovirus-VKOR and pAdEasyI were used to cotransform BJ5183 electrocompetent cells for obtaining Ad-VKOR. To obtain Ad-GFP (as a control), pAdTrack-cytomegalovirus and pAdEasyI were used to cotransform BJ5183 electrocompetent cells. Large-scale viral preps were harvested from 293A cells after postinfection for 36 to 40 hours, resuspended in PBS, lysed by freeze-thawing, purified by CsCl equilibrium density gradient centrifugation, and quantitated by spectrophotometry. Infectious titer was estimated by an end point plaque assay on 293 cells. The concentrated virus was dialyzed against PBS plus 10% glycerol, and then aliquoted, and stored at −80°C. The overexpression of VKOR by the infection of Ad-VKOR was determined by Northern blot.

Endothelial Cell Proliferation Assay

The HUVECs were plated onto 24-well flat-bottomed plates precoated with gelatin at 0.6×10^5 cells per well in 500 μL of Medium 199 containing 10% FBS. After 24 hours of starvation (1% FBS) at 37°C, the cells were rinsed twice with serum-free medium and then fed with fresh medium containing 2% FBS, $VEGF_{165}$ and basic fibroblast growth factor (10 ng/mL; PeproTech, London, United Kingdom) replaced every 48 hours. The cells were either treated with oligonucleotides (VKOR-AS, VKOR-S, or VKOR-MUT) every 24 hours for 4 days, or infected with adenovirus carrying VKOR or GFP. After 4 days, the cells were counted by using an inverted microscope (Nikon, Tokyo, Japan). Each experiment was done in triplicate and repeated thrice.

Endothelial Cell Migration Assay

To determine the effect of VKOR on HUVEC migration, migration chambers of 24-transwell (Costar, Cambridge, MA) with an 8 μm pore size were coated with 10 μg/mL of type 1 collagen (BD Biosciences, Bedford, MA) from rat tail for 1 hour at 37°C (18). Cells were synchronized by serum starvation overnight in Medium 199, treated with the oligonucleotides every 24 hours or infected with adenovirus carrying VKOR or GFP for 2 days, the cells were plated at 4×10^4 on the top chamber and allowed to migrate to the bottom chamber containing VEGF (10 ng/mL) and 1% FBS for 6 hours at 37°C. After incubation, cells on the top surface of the membrane (nonmigrated cells) were scraped with a cotton swab. Cells on the bottom side of the membrane (migrated cells) were stained with 0.2% crystal violet dye (Fisher Scientific, Springfield, NJ) in 70% ethanol for 30 minutes, then destained in PBS (pH 7.4); the cell numbers on the bottom chamber were counted as migrated cells by using a microscope (Nikon) in six fields per filter and a total of four wells per group.

Endothelial Cell Adhesion Assay

Flat-bottomed tissue culture plates of 96 wells (Costar) were coated with Matrigel (10 μg/mL, BD Biosciences) overnight at 4°C, then blocked with 1% bovine serum albumin in PBS (pH 7.4) for 2 hours at 37°C. The HUVECs, either treated with the oligonucleotides every 24 hours, or infected with adenovirus carrying VKOR or GFP for 2 days, were trypsinized, washed, and resuspended in serum-free media containing 1% bovine serum albumin at 1.5×10^5 cells/mL, replated to each well, and incubated at 37°C for 45 minutes in 5% CO_2, unattached cells were removed by washing thrice with serum-free medium, and attached cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) assay. The adhesion cell numbers were compared between HUVECs treated with VKOR-AS, VKOR-S, and VKOR-MUT; or between HUVECs either infected with Ad-VKOR or with Ad-GFP.

Endothelial Cell Tube Formation Assay

Endothelial tube formation assays were done as described previously (18, 19). Briefly, the HUVECs, treated with the oligonucleotides every 24 hours, or infected with adenovirus carrying VKOR or GFP were cultured for 2 days, trypsinized, washed with PBS (pH 7.4), resuspended in Medium 199 containing 1% FBS supplemented with 10 ng/mL $VEGF_{165}$, and seeded (2.5×10^4 cells per well) onto solidified Matrigel in 96-well plates, incubated at 37°C for 1 day. Tubular networks were visualized with an inverted phase contrast microscope (Nikon). The tubular networks were captured by using a Kodak digital camera, analyzed by using Photoshop 7.0, and compared either between HUVECs with VKOR-S/VKOR-MUT and those with VKOR-AS or between HUVECs with VKOR and those with GFP.

mRNA Expression of VKOR in Fetal Heart and Ventricular Aneurysm

To verify whether VKOR is up-regulated in the physiologic development and pathologic angiogenesis, we determined the expression of VKOR in right ventricles in human fetal and adult heart, and ventricular aneurysm tissues of human heart (11). Total RNA was isolated by using RNAgent Isolation System (Promega), and 20 μg was separated on a 1% agarose gel, transferred onto nylon membrane (Schleicher & Schuell, Keene, NH), and hybridized in prewarmed ExpressHyb (Clontech) with *VKOR* probe for 4 hours at 68°C, after washing, the blots were mounted on X-ray film, and developed at −70°C for 2 days.

References

1. Carmeliet P, Jain RK. Angiogenesis in cancer and other disease. Nature 2000; 407:249–57.

2. Gorski DH, Walsh K. The role of homeobox genes in vascular remodeling and angiogenesis. Circ Res 2000;87:865–72.

3. Madri JA. Extracellular matrix modulation of vascular cell behavior. Transpl Immunol 1997;5:179–83.

4. Webster KA. Therapeutic angiogenesis: a complex problem requiring a sophisticated approach. Cardiovasc Toxicol 2003;3:283–98.

5. Nelsestuen GL, Zytkovicz TH, Howard JB. The mode of action of vitamin K. Identification of γ-carboxyglutamic acid as a component of prothrombin. J Biol Chem 1974;249:6347–50.

6. Rost S, Fregin A, Ivaskevicius V, et al. Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. Nature 2004; 427:537–41.

7. Li T, Chang CY, Jin DY, Lin PJ, Khvorova A, Stafford DW. Identification of the gene for vitamin K epoxide reductase. Nature 2004;427:541–4.

8. Cain D, Hutson SM, Wallin R. Assembly of the warfarin-sensitive vitamin K 2,3-epoxide reductase enzyme complex in the endoplasmic reticulum membrane. J Biol Chem 1997;272:29068–75.

9. Begent LA, Hill AP, Steventon GB, Hutt AJ, Pallister CJ, Cowell DC. Characterization and purification of the vitamin K1 2,3-epoxide reductases system from rat liver. J Pharm Pharmacol 2001;53:481–6.

10. Wallin R, Hutson SM, Cain D, Sweatt A, Sane DC. A molecular mechanism for genetic warfarin resistance in the rat. FASEB J 2001;15:2542–4.

11. Ho HV, JJ Jang, Kaji S, et al. Developmental endothelial locus-1 (Del-1), a novel angiogenic protein: its role in ischemia. Circulation 2004;109:1314–9.

12. Jackson M, Nilsson T, Peterson PA. Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J 1990;9:3153–62.

13. Anantharaman V, Aravind L. The GOLD domain, a novel protein module involved in Golgi function and secretion. Genome Biol 2002 ;3:research0023.1–0023.7.

14. Cosson P, Letourneur F. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science 1994;263:1629–31.

15. Lowe M, Kreis TE. *In vitro* assembly and disassembly of coatomer. J Biol Chem 1995;270:31364–71.

16. Solban N, Jia HP, Richard S, et al. HcaRG, a novel calcium-regulated gene coding for a nuclear protein, is potentially involved in the regulation of cell proliferation. J Biol Chem 2000;275:32234–43.

17. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A 1998; 95:2509–14.

18. Kamphaus GD, Colorado PC, Panka DJ, et al. A novel matrix-derived inhibitor of angiogenesis and tumor growth. J Biol Chem 2000;275:1209–15.

19. Dhanabal M, LaRochelle WJ, Jeffers M, et al. Angioarrestin: an antiangiogenic protein with tumor-inhibiting properties. Cancer Res 2002; 62:3834–41.

Molecular Cancer Research

Vitamin K Epoxide Reductase: A Protein Involved in Angiogenesis

Yibo Wang, Yisong Zhen, Yi Shi, et al.

Mol Cancer Res 2005;3:317-323.