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Identification of derlin-1 as a novel growth factor-responsive endothelial antigen by suppression subtractive hybridization ☆

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Abstract

Endothelial cells play an important regulatory role in embryonic development, reproductive functions, tumor growth and progression. In the present study, the suppression subtractive hybridization (SSH) method was employed to identify differentially expressed genes between non-stimulated endothelial cells and activated endothelial cells. Following mRNA isolation of non-stimulated and hepatocellular carcinoma homogenate-stimulated cells, cDNAs of both populations were prepared and subtracted by suppressive PCR. Sequencing of the enriched cDNAs identified a couple of genes differentially expressed, including derlin-1. Derlin-1 was significantly up-regulated by tumor homogenates, VEGF, and endothelial growth supplements in a dose-dependent manner. Knock-down of derlin-1 triggered endothelial cell apoptosis, inhibited endothelial cell proliferation, and blocked the formation of a network of tubular-like structures. Our data reveal that derlin-1 is a novel growth factor-responsive endothelial antigen that promotes endothelial cell survival and growth.

Keywords: Angiogenesis; Derlin-1; Cancer

Angiogenesis is an important biological event for a variety of physiological and pathological processes, including embryonic development, reproductive functions, tumorigenesis, and other proliferative processes [1]. Tumor angiogenesis is essential for tumor growth and metastasis, which is promoted by cytokines or chemokines that have mitogenic or chemotactic effects on vascular endothelial cells [2,3]. Over the last decade, much progress has been made in the identification of the regulators of vasculogenesis or angiogenesis [4]. VEGF and Tie 2 ligands play a central role in endothelial proliferation and in the assembly of vessel wall [5]. The dependence of tumor growth and metastasis on angiogenesis has led to the concept of antiangiogenic therapy for malignant tumors [6]. Inhibition of tumor angiogenesis as an anticancer strategy has generated much excitement among cancer researchers and clinicians [7,8]. However, realization of the full potential of antiangiogenic approaches will require a better understanding of the molecular differences between normal and tumor vessels in a variety of normal and abnormal circumstances. Many evidence indicated that there was big difference in gene expression profile between normal vascular endothelial cells and tumor endothelial cells, as well as the significant difference in vascular morphology and function [9,10].

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The formation of malignant cells that lack the ability to switch on angiogenesis rarely leads to clinically detectable cancers. Experimental evidence to support the concept of the 'angiogenic switch' already exists [3]. Accumulation of genetic alterations in tumor cells might turn on the expression or release of angiogenic factors, which may stimulate endothelial cell proliferation, migration, and tube formation.

Suppression subtractive hybridization methodology has been successfully used to identify the differentially expressed genes between two reciprocally subtracted libraries [11]. We took advantage of this approach to identify the differentially expressed genes between quiescent and tumor homogenate-activated endothelial cells. Here, we report on the identification of a novel regulator of angiogenesis, derlin-1, by suppression subtractive hybridization. Derlin-1 reportedly participated in the dislocation of misfolded proteins from ER, mediates the retro-translocation of proteins from ER lumen into cytosol [12,13]. We demonstrated here that derlin-1 was regulated by VEGF, and promoted endothelial cell survival.

Materials and methods

Cell culture. The human vascular endothelial cells derived from hepatocellular carcinoma (HCVEC) were cultured as described previously [14]. The dishes for HCVEC culture were coated with 2% of gelatin. The coated dishes were incubated at 37 °C for 15–20 min before cell plating. The HCVEC was maintained in DMEM containing 20% of fetal bovine serum, 100 μg/ml of endothelial cell growth supplements (ECGS), 2 mmol/L of L-glutamine, 100 μg/ml of sodium heparsulfate, 40 μU/ml of insulin, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Human umbilical vascular endothelial cell (HUVEC) was isolated from fresh neonatal umbillium by a recommended procedure [15].

Activation of HUVEC and suppression subtractive hybridization (SSH). To prepare the homogenate of human hepatocellular carcinoma, fresh tumor tissue was washed with sterilized PBS, cut into small pieces, and homogenized in DMEM. The homogenate was filtered and stored at −80 °C. To activate HUVECs, HUVECs were treated with bFGF, VEGF, and the homogenate of HCC. The mRNA of activated HUVECs was isolated as described. The cDNA library for activated HUVECs was constructed. Briefly, the first and second strand of cDNA were synthesized by THERMOScript RT-PCR system, cDNA was ligated into Uni-ZAP XR vector, packaged by Gigapack III gold packaging extract. The titer of primary phage cDNA library was amplified. The differentially expressed gene fragments between HUVECs and activated HUVECs were identified by suppressive subtractive hybridization (SSH) as described [11,16,17]. Briefly, the SSH was performed between unstimulated HUVEC (driver) and HUVEC stimulated with HCC homogenate, bFGF, and VEGF (tester). For preparation of polyadenylated RNA, total RNA was extracted with the TRIzol reagent and purified with the Poly(A)Quick kit (Stratagene, Heidelberg, Germany). The further steps were performed according to the PCR-Select1cDNA Substraction kit (Clontech, Palo Alto, CA, USA). It is primarily based on a technique called suppression PCR and combines normalization and subtraction in a single procedure. The differentially expressed gene fragments were amplified by PCR and labeled with ^{32}p-dATP and ^{32}p-dCTP by Primer-a-Gene Labeling system (Promega). The labeled gene fragments were used as probes to screen the established HUVECs' cDNA library.

Preparation of polyclonal antibodies. Anti-derlin-1 antisera were generated by immunizing rabbits with peptides coupled to keyhole-limpet haemocyanin through an added Cys residue. The derlin-1 sequence used was (C)RHNWGQGFRLGDQ. The titer for anti-derlin-1 antisera was more than 1×10^9. Antibodies specific to the C-terminus of human derlin-1 were affinity-purified with Sepharose 4B which was conjugated with the C-terminus peptide of derlin-1 as described [18]. The affinity-purified anti-derlin-1 polyclonal antibody was used for immunohistochemical and Western blot analysis.

Western blot analysis. Cells were washed twice with phosphate-buffered saline and lysed with cold RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 0.5 mM DTT) containing protease inhibitors (phenylmethylsulfonyl fluoride (PMSF) 1 mmol/L and leupeptin 0.1 g/L). Cell lysates were collected from culture plates using a rubber policeman, and protein collected by centrifugation. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Aliquots of 20 μg proteins were boiled in 2× loading buffer (0.1 M Tris–Cl, pH 6.8, 4% SDS, 0.2% bromophenyl blue, and 20% glycerol) for 10 min, then loaded into 10% Tris–HCl–polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred electrophoretically to PVDF membrane (Pierce). Membranes were incubated with primary antibodies against derlin-1 and appropriate HRP-secondary antibodies. Membranes were additionally probed with an antibody against actin (Sigma) to ensure equal loading of protein between samples. Detection was performed with enhanced chemiluminescence reagent (Pierce).

Cell proliferation assay. For HCC homogenates assay, fresh HCC tissues were homogenized in DMEM and desterilized. HUVECs were seeded in 12-well plate at 15,000 cells/well. The cells were treated with or without HCC homogenates, VEGF, and bFGF. Cell number was counted for 6 days. For the in vitro cell proliferation assays, exponentially growing cells were seeded in quadruplicate at 1000 cells/well into 96-well plate. Cell numbers were determined every other day for 7 days by MTT assay. Briefly, 20 μL of MTT (5 g/L) was added into each well and cultured for another 4 h, the supernatant was discarded, then 100 μL DMSO was added. When the crystals were dissolved, the optical absorbent density (*A*) values of the slides were read on the minireader II at the wavelength of 490 nm. Each assay was repeated three times.

Construction of shRNA expression vector. Two shRNAs targeting derlin-1 and one negative control shRNA were synthesized. shRNAs were cloned into pSilencer downstream of human U6 promotor. The expression cassets are as follows: Derlin-1shRNA1, 5′-GATCCCAGAGACATGATTGTATCATTCAAGAGATGATACAATCATGTCTCTGTTTTTTGGAAA-3′; derlin-1shRNA2, 5′-AGCTTTTCCAAAAAACACGATTTAAGGCCTGCTATCTCTTGAATAGCAGGCCTTAAATCGTGG-3′; negative control shRNA, 5′-AGCTTTTCCAAAAAAAGGCTTAGGAATCATACTATCTCTTGAATAGTATGATTCCTAAGCCTG-3′.

Gene transfections. The expression vectors for derlin-1 shRNA and negative control shRNA were transfected into human HCVECs as described [19]. Briefly, Subconfluent proliferating cells in 12-well plate were incubated with 2 μg of expression vectors in 1 ml of serum-free medium containing LipoFECTAMINE for 5 h. Culture was washed to remove the excess medium to allow the expression of shRNAs. Transfected cells were selected with G418 for 2 weeks. Isolated clones were picked up. The expression of derlin-1 was checked by RT-PCR and flow cytometry.

RT-PCR. Total RNA from HCVECs was isolated using Trizol reagent. Approximately 4 μg of total RNA was subjected to reverse transcription by M-MLV, followed by semiquantitative PCR analysis. The primer sequence for derlin-1 is as follows: sense, 5′-TCGGCAAACTCGGCCTCATC-3′; antisense, 5′-GAATGGCGGAGGCGGGAGA-3′. *Gapdh* was also amplified as internal control.

Flow cytometry. Replicate cultures ($n = 5$) of 1×10^6 cells were plated in cell culture wells. The cells were harvested, washed with PBS, and fixed in 70% ethanol for 30 min at 4 °C, then treated with 50 μg/ml RNase A (Sigma), stained with 50 μg/ml of propidium iodide for 20 min at 4 °C without light, and analyzed by flow cytometry for DNA synthesis and cell cycle status.

Tube formation assay. To prepare the gelatin-coated plates, 96-well plates were coated with DMEM supplemented with 3% of gelatin, incubated at 4 °C overnight. 1×10^4 cells/well were plated in gelatin-coated