Interferon regulatory factor-1 (IRF-1) regulates VEGF-induced angiogenesis in HUVECs

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A R T I C L E   I N F O
Article history:
Received 11 February 2008
Received in revised form 31 March 2008
Accepted 7 April 2008
Available online 22 April 2008

Keywords:
Interferon regulatory factor
Angiogenesis
Alternative splicing
Endothelial cell
Vessel sprouting
CAM

A B S T R A C T
Interferon regulatory factor-1 (IRF-1) is a tumor suppressor and transcriptional modulator that can regulate gene expression involved in cell growth control, induction of apoptosis, and post-translation modification. In this study, we found that IRF-1 inhibits endothelial cell angiogenesis using human umbilical vein endothelial cell (HUVECs) culture system. In addition, IRF-1 directly inhibited the tube formation of endothelial cells on Matrigel and reduced the expression of p-Akt and p-eNOS, which play a significant role in angiogenesis when stimulated by VEGF. We also demonstrate that C-terminal region including transactivation domain (TA) of IRF-1 functions as a signal for its angiostatic activity, and is spliced in human tumor tissues. These findings indicate that splicing variant involving exons 7 of IRF-1 could potentially modulate anti-angiogenic effect of IRF-1. In overall, this study provides the first evidence for anti-angiogenic role of IRF-1, which may have therapeutic values for cancer and angiogenesis-associated diseases.

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1. Introduction

Interferon regulatory factor-1 (IRF-1), the founding member of the IRF family, is a transcription factor involved in cell growth regulation, induction of apoptosis, immune responses, post-transcription modification, and cell transformation by oncogenes [1–5]. The best characterized activity of IRF-1 is as a sequence specific DNA-binding domain and transcriptional activator of certain IFN-stimulated genes [6]. Accumulating evidence supports the theory that IRF-1 functions as a tumor suppressor [7–10] and revert the transformed phenotype [9,11–13]. In human tumors, IRF-1 is inactivated to prevent cell cycle arrest and apoptosis by genetic alteration, such as gene and exon deletion [10,14–16]. IRF-1 also has been shown to play roles in regulating both ubiquitination and SUMOylation in cancer [5,17,18].

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is a crucial step in tumor growth [19,20] and progression because it enables the supply of oxygen and nutrients to the growing tumor [21]. One of the key mediators of blood vessel formation during development is vascular endothelial growth factor (VEGF), which can stimulate the proliferation and migration in endothelial cells [22]. In tumors, the activity of endothelial cells plays an essential role for the regulation of various vascular biological function and diseases.

IRF family members share significant homology with the N-terminal 120 amino acids, which comprise a DNA-binding domain (DBD) characterized by five tryptophan repeats [2,23]. The DBD also revealed as a region required for homodimerization and nuclear localization [24]. The C-terminus of IRF-1 contains a transactivation domain (TA) between amino acids 185 and 256, but this amino acid region is not present in the transcriptional repressor IRF-2. A functional antagonist of IRF-1 is IRF-2 [2], which functions as a tumor suppressor and as an oncoprotein [11,25].

In the present work, we evaluated the role of IRF-1 during angiogenesis using a human umbilical vein endothelial cell (HUVECs) culture system with purified protein of IRF-1 in vitro. We found that IRF-1 inhibited HUVECs migration and tube formation on Matrigel. Further, we defined the functional domains of IRF-1 causing angiogenesis. This study may provide evidence for the potential application of the IRF-1 as an anti-angiogenic therapy for the angiogenesis-associated disease such as cancer.

2. Materials and methods

2.1. Cell culture and antibiotics

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersville, MD), and maintained on 0.3% gelatin (Sigma, St. Louis, MO) coated dishes using the EGM-2 BulletKit medium (Clonetics) in an atmosphere of 5% CO2 in air at 37 °C. The EGM-2 BulletKit medium consists of a base medium containing 200 µM HEPES (Sigma), 5% heat inactivated fetal calf serum, and 1% Penicillin–Streptomycin (Gibco, New York, NY). HUVECs were used for experiments at passages 3–5. The following antibodies were used in this study: anti-IRF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-IRF-2 (Santa Cruz Biotechnology, Santa Cruz, CA).
2.2. Small interfering RNA (siRNA) construction

The siRNA oligonucleotide sequence targeting IRF-1 (CTACTCCTTTCCCTTAACT) corresponded to nucleotides 879 to 897 in the human sequence. siRNA was synthesized by using a siRNA Construction kit (Ambion, Austin, TX) and transfected by using the RNAi shuttle (Orbigen, San Diego, CA) according to the manufacturer’s protocols. HUVECs were then transfected with GFP-IRF-1 or GFP-IRF-1 with siRNA transfection. GFP images were captured using a fluorescence microscope (Zeiss, Oberkon, Germany). Total RNA was isolated using a TRIZOL Reagent (Life Technologies, Gaithersburg, MD) and reverse transcription (RT) PCR was then performed. The transfection efficiency was examined with respect to the expression level of co-transfected green fluorescent protein (GFP), which was confirmed by immunoblotting with an anti-GFP antibody.

2.3. Protein expression, purification and biochemical analysis

DNA encoding the three splicing variants of IRF-1 and full-length IRF-1 were isolated by polymerase chain reaction and subcloned into pET28a (Novagen) using EcoR1 and Xhol. Then each construct was expressed in Escherichia coli strain BL21 (DE3) grown in Luria Bertani medium supplemented with kanamycin (75 mg/ml). Culture was grown at 37 °C to an A600 = 0.4-0.5, and transferred at 30 °C, and were induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h. The induced cells were then harvested and resuspended in 20 mM Tris-HCl (pH 8.0) containing 10% glycerol, 50 mM NaCl, 0.1 mM EDTA, and 1 mM dithiothreitol. Cells were lysed by ultrasonication, and were centrifuged for 15 min to remove cell debris. The supernatant was filtered and loaded onto a nickel affinity column matrix (Invitrogen) and incubated at 4 °C for 2 h. The slurry was pelleted by centrifugation and washed with washing buffer (20 mM NaHPO4, pH 6.0, 500 mM NaCl) three times. The pellet of the gel matrix was resuspended in elution buffer (20 mM Na2HPO4, pH 8.0) containing 400 mM imidazole and incubated at 4 °C for 20 min to elute the bound His fusion proteins. The His-tag was cleaved off with thrombin, and the each protein was further purified using high performance liquid chromatography (Waters 600D). Protein concentration was determined by the Bradford assay with bovine serum albumin (Sigma) as a standard (Bio-Rad).

2.4. Transient transfection and in vitro specific activity assay

Transient transfections of 70-80% confluent HUVECs were performed using the Fugene 6 Reagent (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Luciferase reporter assay was performed as previously described [10]. The HUVECs plated on 60-mm dishes and triply transfected using Fugene 6 Reagent with full-length IRF-1 or its splicing variants (200 ng each), pGL-3 containing the interferon-stimulated response element (150 ng), and pRl containing the Renilla luciferase cDNA (50 ng). Twenty-four hours after transfection, the cells were harvested and dissolved in 40 μl of 1× passive lysis buffer (Promega, Madison, WI). After lysis, the cell extracts were incubated with luciferase substrate for 30 min at room temperature. Luminosities were then measured by centrifugation for 15 min at 14,000 rpm, and then a 10 μl aliquot of each sample was transferred to a 96-well assay luminescence plate containing 50 μl of the provided luciferase assay II. The provided stop and Glo reagent (50 μl/well) was then added to initiate Renilla luciferase activity, and the ratio of firefly luciferase activity to Renilla luciferase activity was calculated.

2.5. [3H]Thymidine uptake assay

To measure cell proliferation [26], HUVECs were seeded at a density of 9 × 103 cells per well in DMEM containing 5% heat-inactivated fetal calf serum, and 1% Penicillin–Streptomycin in the gelatinized plates on day 0. After 18 h, cells were incubated for 6 h in M199 containing 1% FBS and then stimulated with VEGF (10 ng/ml, R&D Systems, Minneapolis, MN) for 24 h in M199 containing 1% FBS. [3H]Thymidine (0.5 μCi/ml, Amersham, Arlington, IL) was then added 4 h prior to the assay. High molecular mass [3H]radioactivity was precipitated using 5% trichloroacetic acid at 4 °C for 1 h. Cells were solubilized in 0.2N NaOH and 0.1% SDS and [3H]Thymidine uptake was evaluated with a liquid scintillation counter (Beckman Instrument). Three independent experiments were conducted in triplicate; values shown represent means ± SD of total cpm.

2.6. Migration and invasion assay

Migration and invasion were assayed using Transwells (Costar, 8 µm pore size) as previously described [26]. For migration assays, the lower surface of a filter was coated with 10 μg of gelatin. M199 containing 1% FBS with VEGF (25 ng/ml) was placed in the lower wells. Cells were fixed and stained with H&E. Non-migrating cells on the upper filtersurface were removed by wiping with a cotton swab. The numbers of cells that migrated to the lower side of the filter were counted under a light microscope and the mean values of eight fields were determined. For the invasion assay, the lower and upper surfaces of a filter were coated with 10 μg of gelatin and 10 μg of Matrigel (BD biosciences), respectively. The fixation and quantification methods used were the same as described for the migration assay. Three independent experiments were conducted in triplicate; and the values shown represent means ± SD.

2.7. Tube formation assay

Growth factor-reduced Matrigel (200 μl of 10 mg/ml) was added to a 24-well plate and polymerized for 30 min at 37 °C. Untransfected, pEGFP, pEGFP-IRF-1, or siIRF-1-transfected HUVECs (1×103 cells) were seeded on the surface of the Matrigel. Cells were then incubated for 48 h with or without 10 ng/ml of VEGF in M199 containing 1% FBS. Morphological changes were photographed at ×40 magnification. HUVEC tube lengths were determined using an inverted microscope equipped with a digital CCD camera (Zeiss) and quantified using ImageJ imaging software (MC/M Design).

2.8. Ex vivo angiogenesis assay

A novel ex vivo angiogenesis assay using an explant culture of skeletal muscle on Matrigel was done with some modifications, according to Jang et al. [27]. Six-week-old BALB/c mice were anesthetized and the legs were shaved. The tibialis anterior muscle was extracted and the cross-sections taken from the muscle were washed thrice with PBS. The washed muscle was placed in a 24-well plate containing 200 μl of growth factor-reduced Matrigel and polymerized for 30 min at 37 °C. M199 containing 1% FBS with or without 10 ng/ml of VEGF was added. After 6 days, outgrowths of capillary-like structures were observed. The observed structures were then treated with Mock, IRF-1, or IRF-1 siRNA for 5 days. The mean area of the microvessels was measured by employing an optical imaging technique and the measurements were then quantified using ImageJ imaging software. Independent experiments were repeated thrice and each value represents the mean ± SD of triplicate samples.

2.9. Chick chorioallantoic membrane (CAM) assay

The CAM assay was performed with minor modifications [28]. Fertilized chick embryos were pre-incubated at 37 °C with 70% humidity in order to conduct the chorioallantoic membrane assay. After 3 days, a square window was opened after the removal of 2–3 ml of albumin to detach the developing CAM from the shell. A 1.5 × 1.5 cm window in the shell was made to expose the CAM. Clear tape was used to seal the windows that were formed and the eggs were incubated for 60 h. On day 8, CAMs were implanted, under sterile conditions within a laminar flow hood, with sterilized Thermannox discs. Thermannox discs were loaded with 100 ng Mock, IRF-1, or

![Fig. 1. The effect of human IRF-1 on endothelial cell migration. (A) Various concentrations of IRF-1 were used for the assays. Serum-starved HUVECs were plated onto the Transwell plate with different doses of IRF-1 (25–200 ng/ml). After 24 h incubation, HUVECs migrated to the 96-well chamber were calculated by a fluorescence staining as quantification. The data are expressed as the means ± SD of three independent experiments which were performed in duplicate. (B) In the time course, serum-starved HUVECs were plated onto the Transwell plate with or without 100 ng/ml IRF-1. HUVECs migration was investigated at 4, 6, 12, and 24 h. Each bar represents the means ± SD, *, P < 0.05 and **, P < 0.01 as compared to untreated cells.](image-url)
siRNA-treated recombinant protein as negative and positive controls, respectively. The CAM was examined daily until day 12 and photographed in ovo with an Axioskop2 plus microscope (Zeiss) equipped with color CCD camera (ProgResC14, Jenoptik, Germany). Recombinant human VEGF (100 ng), incorporated into Thermanox discs, induced branching of blood vessels. At a dose of 100 ng/disk, IRF-1 inhibited these responses in 90% (n = 9/10) of CAMs. At similar dose, both Mock or siRNA-treated group did not show angiogenesis inhibition (0%, n = 0/10). Two independent, blinded investigators performed the count of blood vessels for each group.

2.10. Western blot analysis of PI3K, Akt, and eNOS phosphorylation

A Western blot analysis of PI3K, Akt, and eNOS was conducted. VEGF was used to stimulate the HUVECs for 20 min. The cells were then lysed, and equal amounts of protein were separated using SDS-PAGE. The separated protein was then transferred to a nitrocellulose membrane. After incubation in blocking solution, membranes were incubated with anti-PI3K, anti-p-Akt, and anti-p-eNOS antibody for 1.5 h at room temperature. An ECL system (Amersham) was used for detection.

2.11. Total RNA extraction and RT-PCR

Total cellular RNA was isolated using TRIzol Reagent according to the manufacturer’s instructions, and 2 μg of total RNA was reverse transcribed in a reaction volume of 20 μl using the Superscript II enzyme (Invitrogen). The resulting cDNA was used as the template for PCR amplification of the full-length IRF-1 gene (forward 5′-GGCTCCGGCCGCCCTCAT-3′ and reverse 5′-CCAGGTTCTGCTGCTGCTG-3′), or two fragments of the IRF-1 encoding gene (N-terminal half forward 5′-GGCTCCGGCCGCCCTCAT-3′ and reverse 5′- TAGCTGGCTGCTGCTGCTG-3′; comprising exons 2, 3, 4, and 5; C-terminal half forward 5′-AACTTCTGGGGATTCAG-3′ and reverse 5′-GAGGTTCTGCTGCTGCTG-3′ comprising exons 6, 7, 8, 9, and 10). The three splicing variants were amplified using the C-terminal half forward primer and specific reverse primers (Δ7, 5′-TGTAGCTTCAGAGGTGGA-3′; Δ8, 5′-CTCCAAGAGCTGGAGTCA-3′; Δ9, 5′-CCCAATATCCCCCCCTGG-3′). The PCR conditions consisted of the indicated number of cycles at 95 °C for 30 s, 61 °C for 30 s (but 58 °C for Δ9), and 72 °C for 30 s. The resulting fragments were resolved by 1% agarose gel electrophoresis and confirmed by automatic sequencing (ABI 373, PerkinElmer Life Sciences).

Fig. 2. Activity of human IRF-1 domain on HUVECs. (A) Ablation of GFP-IRF-1 protein and mRNA expression by siRNA in HUVECs. Bar, 50 μm. After transfection, the cells were visualized under fluorescence microscope, and analyzed the mRNA or protein expression levels by RT-PCRs and immunoblots. (B) Inhibition of DNA synthesis in endothelial cells by human IRF-1. Human IRF-1 (100 ng) was assayed on HUVECs in the presence of VEGF165 in a 72h proliferation experiment. cpm value of [3H]thymidine was determined with a liquid scintillation counter. (C) and (D) To investigate whether overexpressed IRF-1 modulates the effects of VEGF on endothelial cell migration and invasion, we tested using Transwells for the migration assays (C) or on Matrigel-coated Transwell for the invasion assays (D) followed by stimulation with or without VEGF (25 ng/ml) for 48 h. Numbers of migrated or invaded cells were counted under a light microscope and mean values were determined. *, P<0.05; **, P<0.01 compared with VEGF alone.
2.12. Yeast two-hybrid analysis and coimmunoprecipitation

The cDNAs encoding human VEGFR1 or VEGFR2 were constructed into pGilda expression vector, respectively. The resulting plasmid pGilda-VEGFR1 or VEGFR2 were introduced into yeast strain EGY48 [MATa, his3, trp1, leu2:GAL1-LacZ, ura3-52, trpl:GAL1-T整合] by a modified lithium acetate method. The cDNAs encoding B42-IRF-1 fusion protein were introduced into the competent yeast cells that already contained pGilda-VEGFR1 or VEGFR2 and the tryptophan prototroph (plasmid marker) transformants were selected on a synthetic medium (Ura-, His+, Trp-), containing 2% glucose. The interaction between the VEGFR1 or VEGFR2 and IRF-1 was compared by measuring the expression level of the two reporter genes. The β-galactosidase activity was determined according to the method as described [29]. Yeast cells containing each of the constructions were cultured in SD media until they reached a mid-log phase. The culture broth (0.4 ml) was taken and mixed with Z buffer (1.4 ml) containing 2-mercaptoethanol. Chloroform (100 μl) and 0.1% SDS (100 μl) were added to the mixture, and the cells were vortex mixed for 45s. The reaction substrate O-nitrophenyl-β-D-galactopyranoside (ONPG) (0.32 ml) was added and the reaction was carried out at 30 °C until a yellow color appeared, and was then quenched by adding 1M Na2CO3. Samples were then centrifuged briefly to remove cell debris, and the absorbance of the supernatant was measured at 420nm.

For co-immunoprecipitation, HUVECs were co-transfected with cDNA constructs of pEFGPC1-IRF-1 and pcDNA4/HisMax-VEGFR1 or VEGFR2 using Fugene 6 Reagent. As a negative control, pEFGPC1-IRF-1 and empty vector pcDNA4/HisMax were also co-transfected. Two days after transfection, cells were harvested by trypsinization and centrifugation. Cell pellets were washed in PBS, resuspended in cell lysis solution (50 mM Tris, pH 7.2, 150 mM NaCl,1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 200 μg/ml PMSF). Lysates were incubated with anti-His antibody (Santa Cruz) and precipitated with protein A-agarose beads. Immunoprecipitates were resolved by SDS-PAGE, and immunoblotted with anti-GFP antibody or anti-His antibody (Santa Cruz). An ECL system (Amersham) was used for detection.

2.13. s.c. tumor models and immunohistochemistry

Specific pathogen-free BALB/c and nude mice were purchased from Biogenomics (Seoul, Korea) and Charles River Labs (Wilmington, MA), respectively. To establish s.c. tumor models and immunohistochemistry (Santa Cruz). An ECL system (Amersham) was used for detection.

3. Results

3.1. IRF-1 decreases the VEGF-induced migration of HUVECs in a dose-dependent manner

In order to confirm the effect of IRF-1 on HUVECs migration, cells were treated with several concentrations of IRF-1 (25–200 ng/ml). We observed that IRF-1 decreased the VEGF-induced migration of HUVECs in a concentration-time-dependent manner and maximum effect was at 100 ng/ml (Fig. 1A). To confirm the above finding, we investigated a time course at 4, 6, 12, and 24 h and observed an inhibitory effect of IRF-1 (100 ng/ml) on cell migration (Fig. 1B). After 24 h of treatment with increasing concentrations (25–200 ng/ml) of IRF-1, no cell migration change was observed in the treated sample (data not shown). The results indicate that VEGF-induced endothelial cell migration is specifically inhibited by IRF-1, whereas was not caused by its cytotoxic effect.

3.2. IRF-1 inhibits VEGF-induced proliferation, migration, and invasion of HUVECs

To further confirm the effect of IRF-1 on endothelial proliferation, we used RNA interference. As shown in Fig. 2A, siRNA markedly inhibited the expression of GFP-IRF-1 protein and mRNA in HUVECs (lane 2). However, siRNA did not affect the expression of an irrelevant gene (GAPDH). HUVECs were either untreated or treated with Mock, IRF-1 or body weight was measured every other day. Mice were sacrificed on day 24 after final injection. Tumors were then excised and prepared for immunohistochemistry.

3.4. Data and statistical analysis

All data values are presented as the mean ± SD or mean ± SEM. Statistical comparisons were carried out using the Student’s t-test. P < 0.05 were considered relevant.
3.3. IRF-1 inhibits tube formation in vitro and vessel sprouting ex vivo

As proliferation, migration, and invasion of endothelial cells inhibited key events of the angiogenic process induced by VEGF, such as invasion of HUVECs. Therefore, the overexpression of IRF-1 potently by siRNA maintained the stimulatory effects of VEGF on migration and migration and invasion of HUVECs. The ablation of overexpressed IRF-1 organized network of endothelial cells on Matrigel (Fig. 3A). In contrast, untreated or empty Mock-treated cells incubated with VEGF formed an extensive network of vessels compared with that of unstimulated cells [30]. Overexpression of IRF-1 markedly inhibited VEGF-induced migration and invasion of untreated HUVECs and of empty Mock-treated HUVECs when compared with those of unstimulated cells [30]. Overexpression of IRF-1 markedly inhibited VEGF-induced DNA synthesis. This inhibitory effect was not due to the cytotoxicity of IRF-1 in endothelial cells, since IRF-1 had no effect on the viability of HUVECs in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). In addition, IRF-1 did not exhibit cytotoxic effects on other normal cell types tested, such as normal fibroblasts, MRC-5 and IMR-90 (data not shown). The inhibitory effect of IRF-1 on VEGF-induced endothelial cell proliferation was completely recovered by siRNA infection. The results indicate that IRF-1 specifically inhibits VEGF-induced endothelial cell proliferation.

To investigate whether overexpressed IRF-1 modulates the effects of VEGF on endothelial cell migration and invasion, we conducted Transwell migration and invasion assays. VEGF enhanced the migration (Fig. 2C) and invasion (Fig. 2D) of untreated HUVECs and of empty Mock-treated HUVECs when compared with that of unstimulated cells. However, overexpression of IRF-1 significantly reduced VEGF-induced migration and invasion of HUVECs. The ablation of overexpressed IRF-1 by siRNA maintained the stimulatory effects of VEGF on migration and invasion of HUVECs. Therefore, the overexpression of IRF-1 potently inhibited key events of the angiogenic process induced by VEGF, such as proliferation, migration, and invasion of endothelial cells in vitro.

3.4. Angiostatic activity of IRF-1 in the chicken chorioallantoic membrane (CAM) assay

Chick CAM assays were performed to test angiostatic activity of IRF-1 on in vivo angiogenesis. As shown in Fig. 4, VEGF-induced angiogenesis in CAMs was clearly inhibited by about 80%. Again, activity was completely recovered by IRF-1-siRNA treatment. These observations suggest that IRF-1 effectively suppressed the formation of blood vessels in vitro and in vivo.

3.5. IRF-1 interacts with VEGFR2

We next tried to determine the mechanism involved in the inhibition of angiogenesis by identifying proteins which bind to IRF-1.
Thus, we investigated for IRF-1 binding proteins using the yeast two-hybrid system. One prominent gene identified was VEGFR2. The interactions between IRF-1 and VEGFR2 were examined by measuring the relative expression levels of β-galactosidase and by co-immunoprecipitation. As shown in Fig. 5A, β-galactosidase activity indicated interactions between IRF-1 and VEGFR2, which were fully observed, whereas little β-galactosidase activity was observed from the interactions between IRF-1 and the empty vector (vector only). VEGFR1 was used to conclude the binding specificity of IRF-1 to VEGFR2. IRF-1 was shown to bind VEGFR2 but not VEGFR1 as illustrated in Fig. 5A.

For co-immunoprecipitation, cDNA constructs of IRF-1 (pEGFPC1-IRF-1) and VEGFR1, 2 (pcDNA4/HisMax-VEGFR1, 2), along with pEGFPC1-IRF-1 and vector only (pcDNA4/HisMax) were co-transfected into HUVECs. Likewise, as above, IRF-1 was used to confirm the specificity of VEGFR2's binding to IRF-1. Subsequently, immunoprecipitation was also performed using an anti-GFP antibody with lysates from both transfected cells. After immunoprecipitation, precipitated proteins were immunoblotted using anti-VEGFR1, 2 and anti-IRF-1 antibodies. As shown in Fig. 5B, pcDNA4/HisMax/VEGFR2 was co-immunoprecipitated with pEGFPC1-IRF-1 (lane 2, upper left panel), whereas no interaction was observed between pcDNA4/HisMax (vector only) and pEGFPC1-IRF-1 (lane 1, upper left panel). Immunoblotting using anti-IRF-1 antibody confirmed that an equal amount of IRF-1 was precipitated in both samples (middle left panel). Whole cell lysates from both samples contained equivalent amounts of protein as determined through immunoblotting using anti-β-actin antibody (lower left panel). Also shown in Fig. 5B (upper right panel), is the comparison made with VEGFR1. The panel clearly shows the absence of binding between VEGFR1 and IRF-1. Taken together, our results strongly suggest that the interaction between IRF-1 and VEGFR2 is highly specific.

3.6. IRF-1 inhibits PI3K/Akt signaling pathway

Based on the findings presented, we first investigated whether the human IRF-1 inhibits Akt upstream phosphatidylinositol 3-kinase (PI3K). PI3K pathway is implicated in upregulating VEGF [31]. As shown in Fig. 6A, VEGF-stimulated PI3K activation was markedly reduced by overexpressed IRF-1. Akt is one of the important downstream targets of PI3K. We also determined whether IRF-1 inhibits the phosphorylation of Akt (Ser-473) and eNOS (Ser-1172). For example, eNOS, endothelial NO synthase, inhibitors block endothelial cell migration, proliferation, and tube formation that are induced by VEGF in vitro as well as in vivo. As a result, the conclusion that VEGF induced phosphorylation of Akt and eNOS plays a key role in VEGF-stimulated angiogenesis can be made. As presented in Fig. 6B, VEGF-stimulated Akt and eNOS phosphorylation were dramatically reduced by the IRF-1. Therefore, it seems that IRF-1 inhibits the PI3K/Akt signaling pathway in HUVECs.

3.7. The exon 7 is essential for angiogenesis in vitro and reduces VEGF expression in endothelial cells

We next used RT-PCR to examine splicing variants in 17 human uterine cervical tissue samples (5 normal and 12 cancerous samples). Previously, our results revealed that all tested cervical cancer tissue samples showed a trio of lower bands in addition to the major wild-type band [16]. In contrast, the uterine myometrial tissue samples did not yield any IRF-1 splicing variants. Thus, this alternative splicing may be tissue specific. To facilitate characterization of the IRF-1 splicing variants in cervical cancer, we subcloned the Δ7, Δ8, and Δ9 variants and the wild-type into pEGFP expression vectors, which were then transfected into HUVECs. To confirm IRF-1's direct anti-

Fig. 6. IRF-1 interferes with VEGF-mediated PI3K-Akt signaling in HUVECs. (A) HUVECs were transfected with Mock, IRF-1, or siIRF-1 for 48 h followed by treatment with VEGF (50 ng/ml) for 20 min. PI3K (p85) were detected by Western blot analysis. Detection of the tubulin protein served as a loading control (Akt, eNOS). Each band index was calculated by computer-assisted densitometric signal intensities of PI3K, p-Akt, and p-eNOS (lower panel). Results are representative of three separate experiments. Data is shown as means ± SEM. *, **, ***, ***, **, **, and ***P < 0.05; **, **, **, **, **, and ***, **, **, **, **, **, and ***, **, **, **, **, and ***, **, **, **, **, **, and ***, **, **, **, **, and **, **, **, **, **, and ***, **, **, **, **, and *** compared with VEGF alone.
angiogenic effects, we investigated whether overexpression of IRF-1 splicing variants could alter endothelial tube formation. Empty Mock-treated cells incubated with VEGF formed an organized network of endothelial cells on Matrigel (Fig. 7A). In contrast however, overexpression of IRF-1 and IRF-1(Δ7) markedly inhibited VEGF-induced tube formation. The inhibitory effects of IRF-1 and IRF-1(Δ7) on VEGF-induced tube formation were completely recovered by IRF-1(Δ8) and IRF-1(Δ9) transfection. In order to find the key exons in the IRF-1, all of the IRF-1 splicing variants revealed the protein expression in HUVECs. The protein encoded by Δ7, Δ8, and Δ9 could be visualized using an anti-IRF-1 antibody (Fig. 7B). Consistent with these findings, overexpression of IRF-1(wt) and IRF-1(Δ7) is shown to completely inhibit VEGF expression in HUVECs, the IRF-1(Δ8) and IRF-1(Δ9) splicing variants did not (Fig. 7B). These indicate that IRF-1(wt) and IRF-1(Δ7) inhibits the autocrine effect of VEGF in endothelial cells and thus, a direct anti-angiogenic effect. These results strongly suggest that the exon 7 within the C-terminal region of IRF-1 is involved in the activation of angiogenic signaling in vitro and in vivo.

3.8. IRF-1 suppresses tumor growth and associated angiogenesis

To explore whether IRF-1 has direct antitumor activity, we tested the effects of overexpressed IRF-1 on tumor cell growth in vitro. We found that IRF-1 markedly decreased 2774 ovarian cancer cell growth when compared with controls (data not shown).

The anti-angiogenic activity of IRF-1 was then evaluated in vivo. 2774 cells were implanted s.c. in nude mice. We allowed the tumors to grow until they reached a mean volume of 100 mm³. On day 11, an intratumoral injection of IRF-1 was done and repeated every 3 days for 9 days. Tumors from IRF-1-treated mice were excised on day 24 after the final injection. The volume of IRF-1-treated tumors was 80% smaller than those from Mock mice (Fig. 8). Moreover, immunohistochemical staining of endothelial cells in the IRF-1-treated mice showed an 85% decrease in the number of blood vessels stained with anti-CD31. Taken together, these results show that overexpressed IRF-1 potently inhibits tumor growth and angiogenesis in vivo.

4. Discussion

IRF-1 was the first member of the IRF family, and originally identified as a regulator of IFN α/β and its homolog IRF-2 [3]. Functional antagonist of IRF-1 as a tumor suppressor is IRF-2. IRF family diverse expressions could be due to their multifunctional properties with respect to cell
growth control, induction of apoptosis, and susceptibility to transformation by oncogenes [32,33]. Recently, it was reported that SUMOylated IRF-1 interferes with IRF-1-mediated apoptosis in tumor cells. The SUMOylated protein represses IRF-1-mediated transcriptional activation and apoptosis [5]. Thus, IRF-1 of tumor suppressor can enhance the tumor cell evasion of immune system. It is known that IRF-1 is frequently inactivated to prevent apoptosis by genetic alteration such as exon deletion in human cancer populations [10,14–16].

In this study, IRF-1 significantly inhibited endothelial cell migration in dose–time-dependent manner. In addition, IRF-1 directly inhibited the tube formation of endothelial cells on Matrigel and reduced the expression of VEGF, p-Akt, and p-eNOS in HUVECs. Akt and eNOS play a significant role in angiogenesis when stimulated by VEGF. VEGF is known to stimulate Akt dependent phosphorylation of eNOS, therefore activating eNOS. The observations from our study show that IRF-1 has little to no effect on unphosphorylated Akt and eNOS. Once phosphorylated though, p-Akt produced a faint expression of protein, and it was observed that protein expression became fainter when p-Akt was associated with IRF-1. Phosphorylation of eNOS also showed protein expression like p-Akt, and the expression decreased as it was associated with IRF-1. These data indicate that IRF-1 has a directly inhibitive effect on angiogenesis in vitro. We also observed that exogenous IRF-1 has a direct inhibitive effect on endothelial cell migration and tube formation, and using a CAM model. Endothelial cell proliferation and migration are initial steps critical to the angiogenesis process.

To further examine the biological significance of the splicing variants of IRF-1 in cancer, we performed two types of methods relevant to its physiological functions of tube formation and expression level of VEGF via Western blot. Our results revealed that the variants differed in their abilities to angiogenic effect (Fig. 5A). In addition, VEGF protein expression followed an induction pattern in dose –time dependent manner. Furthermore, VEGF is known to stimulate Akt dependent phosphorylation of eNOS, therefore activating eNOS. The observations from our study show that IRF-1 has little to no effect on unphosphorylated Akt and eNOS. Once phosphorylated though, p-Akt produced a faint expression of protein, and it was observed that protein expression became fainter when p-Akt was associated with IRF-1. Phosphorylation of eNOS also showed protein expression like p-Akt, and the expression decreased as it was associated with IRF-1. These data indicate that IRF-1 has a directly inhibitive effect on angiogenesis in vitro. We also observed that exogenous IRF-1 has a direct inhibitive effect on endothelial cell migration and tube formation, and using a CAM model. Endothelial cell proliferation and migration are initial steps critical to the angiogenesis process.

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