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Original Article

Co-suppression of VEGF-A and VEGF-C inhibits development of experimental hemangioma

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Abstract: Vascular endothelial growth factor A (VEGF-A) plays a critical role in the development and progression of Infantile hemangioma (IH), the most common vascular tumor occurring during infancy. However, a role of VEGF-C in IH remains unclear. Here, we addressed this question. The expression of VEGF family members in hemangiomas at involuting-phase and at proliferating-phase was compared, by RT-qPCR and by ELISA. VEGF-A and VEGF-C were suppressed by specific short-hairpin interfering RNA (shRNA), respectively. Cell growth was determined in an MTT assay. Cell proliferation was assessed by BrdU incorporation and analysis of cell-cycle regulators by Western blotting. Cell apoptosis was assessed by Annexin V assay and analysis of apoptosis-associated proteins by Western blotting. The effects of VEGF-A suppression, or VEGF-C suppression, or both, on hemangioma growth were analyzed in vivo by bioluminescence assay and by weight of the implanted tumor. Significantly higher levels of VEGF-A and VEGF-C were detected in the proliferating-phase of the hemangiomas than in the involuting-phase of the hemangiomas. Suppression of either VEGF-A or VEGF-C decreased hemangioma cell growth, likely through inhibition of proliferation and enhancement of the apoptosis, while suppression of both VEGF-A and VEGF-C had a more pronounced effect than suppression of either VEGF-A or VEGF-C alone. VEGF-A and VEGF-C seemed to regulate proliferation and apoptosis through different proteins. Suppression of both VEGF-A and VEGF-C had a more pronounced effect than suppression of either one on the growth of the implanted hemangiomas In vivo. Thus, co-suppression of VEGF-A and VEGF-C has better inhibitory effects on the growth of hemangioma.

Keywords: Vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor C (VEGF-C), infantile hemangioma (IH), proliferation, apoptosis

Introduction

Infantile hemangioma (IH) is the most common tumor occurring in vascular system during infancy, with a preponderance in early stage of infancy and in female [1]. Most of the IHs are benign, but some could become malignant after tumor cell transformation [2]. The life span of benign hemangiomas includes 3 phases, proliferating phase, involuting phase, and involuted phase [3].

Vascular endothelial growth factor (VEGF) receptor signaling plays an essential role in regulating behaviors of hemangioma-derived endothelial cells (HemECs) [4]. The VEGF family is composed of 6 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) [5-7]. There are 3 different VEGF receptors (VEGFRs), numbered VEGFR1, VEGFR2 and VEGFR3. VEGF-A binds to both VEGFR1 and VEGFR2 [7]. VEGFR2 regulates most cellular responses to VEGF, while VEGFR1 seems to modulate VEGFR2 signaling to VEGF-A [7]. VEGFR3 mediates lymphangiogenesis in response to VEGF-C and VEGF-D [7]. Interestingly, PlGF only binds to and signals through VEGFR1, while VEGF-E only binds to and signals through VEGFR2 [8]. Sharing of VEGFR2 by VEGF-A and VEGF-E indicates presence of a complex regulatory network that controls VEGFR2 signaling.

Previous studies have shown that VEGF-A-mediated angiogenesis and neovascularization are critical for hemangioma-associated vessel formation and maintenance, and thus VEGF-A has been regarded as the most important factor to be targeted during treatment for heman-

gioma [9-11]. VEGFR1 suppression was found to enhance VEGFA-dependent activation of VEGFR2, resulting in augmentation of HemEC growth [12]. Moreover, pharmacological inhibition of VEGF signaling abolishes the growth of HemECs [13]. The other members from VEGF family were also found to be associated with the pathogenesis of hemangioma. For example, a very recent study showed hemangioma-like growth of endothelial cells in a VEGF-E-transgenic model [14]. Overexpression of VEGF-E in mouse endocrine beta-cells significantly increased in number and size of the islets of Langerhans and distorted organization of insulin and glucagon-expressing cells through hyperplasia of islet endothelial cells and the formation of hemangioma-like lesions [14]. Moreover, VEGF-C and VEGFR3 were evenly detected in the hemangiomas [15]. Partanen et al. reported upregulation of VEGFR3 in human vascular malformations, including hemangiomas [16]. These studies inspired us to examine a possible role of VEGF members other than VEGF-A in the development of hemangioma.

Here, we found that proliferating-phase of the hemangiomas expressed significantly higher levels of VEGF-A and VEGF-C than the involuting-phase of the hemangiomas. Suppression of either VEGF-A or VEGF-C decreased hemangioma cell growth, through inhibition of proliferation and enhancement of the apoptosis, while suppression of both VEGF-A and VEGF-C had a more pronounced effects than suppression of either one. VEGF-A and VEGF-C seemed to regulate proliferation and apoptosis through different proteins. In vivo, suppression of both VEGF-A and VEGF-C had a more pronounced effect than suppression of either one on the growth of the implanted hemangiomas.

Materials and methods

Ethical issues

This study protocol was approved by the Institutional Ethical Review Board of Wenzhou Medical University. Proliferating- and involuting-phase hemangioma specimens were surgically collected from 10 (8 females and 2 males; median age, 7 months) and 10 patients (8 females and 2 male; median age, 6 months), respectively. Each patient received written informed consent for research purpose. All cases were confirmed by histological analysis at the Department of Vascular Surgery of The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China).

Culture of HemECs

Culture of HemECs has been described before [17]. Briefly, HemECs were cultured in Endothelial Basal Medium-2 (EBM-2; Invitrogen, Carlsbad, CA, USA) suppled with 10% fetal bovine serum (FBS; Invitrogen). Confluent cells were dissociated and sub-cultured using 0.05% trypsin-EDTA solution (Invitrogen). HemECs at passages 3-5 were used in this study.

AAV transduction of HemECs

A pCAG-luciferase (LUC) backbone (Clontech, Mountain View, CA, USA) was used to prepare pCAG-shVEGF-A-LUC, pCAG-shVEGF-C-LUC, and pCAG-shVEGF-A+C-LUC. Sequencing was done to verify the correct orientation of these newly prepared plasmids. HEK293T cells (American Type Culture Collection, ATCC, Rockville, MD, USA) were used for generation of adeno-associated viruses (AAV) carrying the target constructs with Lipofectamine-3000 (Invitrogen). The sequence for shVEGF-A is 5'-TGTGAATGCAGACCAAAGA-3'. The sequence for shVEGF-C is 5'-TGCAAGCATTATGTCAGCA-3'. The scrambled sequence is 5'-GGTATCTACTAGATGTACT-3'.

MTT assay

Cell growth and survival were measured in a tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) Kit (Roche, Indianapolis, IN, USA), by reading absorbance value (OD) at 570 nm.

Assessment of BrdU incorporation

For quantification of cell proliferation, bromodeoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO, USA) was added to cultured cells at a final concentration of 1 μg/ml 2 hours before analysis. Immunocytochemistry for BrdU was performed using a BrdU IHC kit (Millipore, Burlington, MA, USA). DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Thermo Scientific, Rockford, IL, USA).

Flow cytometry

For apoptosis assessment, dissociated cells were incubated with Annexin V-FITC (25 μg/ml;

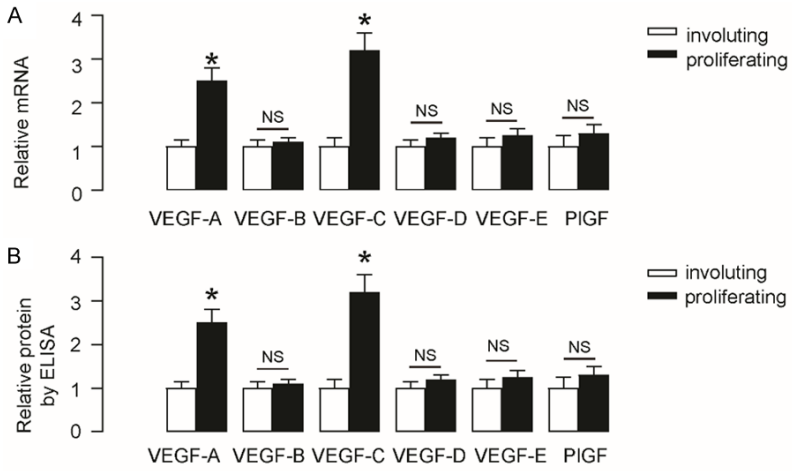

Figure 1. Proliferating-phase of the hemangiomas expresses significantly higher VEGF-A and VEGF-C than involuting-phase. A. RT-qPCR for VEGF family members in hemangiomas at involuting-phase and proliferating-phase. B. ELISA for VEGF family members in hemangiomas at involuting-phase and proliferating-phase. *P < 0.05. NS: non-significant. N = 10.

Invitrogen) and Propidium iodide (PI, 25 μg/ml; Invitrogen) for 10 minutes on ice, followed by analysis by a flow cytometer (Becton-Dickinson Biosciences, San Jose, CA, USA). Apoptotic cells were stained annexin V-FITC-positive and PI-negative. Data were analyzed and quantified using Flowjo software (Flowjo LLC, Ashland, OR, USA).

RT-qPCR

RNA was extracted with RNeasy kit (Qiagen, Hilden, Germany) to be used as templates for cDNA synthesis. Quantitative PCR (RT-qPCR) were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). Primers were Qiagen-commercial RT-qPCR primers. Data were assessed using 2-ΔΔCt method. Values of genes were first normalized against GAPDH, and then compared to controls.

Western blotting

Whole cellular extracts were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) suppled with the Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). After separation with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred onto polyvinylidenedifluoride transfer membranes to be incubated overnight at 4°C with primary antibodies: anti-p21, anti-CyclinD1, anti-CDK4, anti-CyclinB2, anti-p27, anti-Cytochrome C (CYTC), anti-cleaved caspase-3, anti-caspase 9, anti-Bcl-2, and anti-GAPDH (all from Cell Signaling, San Jose, CA, USA). After extensive washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Labs, West Grove, PA, USA) for 1 hour. Immunoreactivity was detected by the chemiluminescence method (Thermo Scientific).

ELISA

The concentration of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF in the hemangioma cells was determined by corresponding human ELISA Kit (R&D System, Los Angeles, CA, USA). ELISAs were performed according to the instructions of the manufacturer. The absorption was measured at 450 nm after comparison with the standards. The relative values to controls were presented.

Mouse model and assessment of implanted tumor

All mouse experiments were approved by the Institutional Ethical Review Board of Wenzhou Medical University. Ten-week-old male nude mice (Shanghai Laboratory Animal Center, Shanghai, China) were used in the current study. Ten mice were analyzed in each experimental condition. The murine model of IH was generated using published protocol. Briefly, 2×10^7 HemSCs and 1×10^7 Primary Human Umbilical Vein Endothelial Cells (HUVECs, ATCC) were mixed, sedimented, and then re-suspended in 200 μl Matrigel (Becton-Dickinson Biosciences, San Jose, CA, USA) to be injected subcutaneously into the backs of nude mice. The assessment of the implanted tumor was done after 4 weeks through imaging of tumor by bioluminescence assay IVIS imaging system (Xenogen Corp., Alameda, CA, USA). Images were captured 5 minutes after tail vein injection of luciferin (Sigma-Aldrich) of 50 mg/kg body weight, as a 60-second acquisition and 10 of binning. Weight of the implanted tumor was measured after dissection.

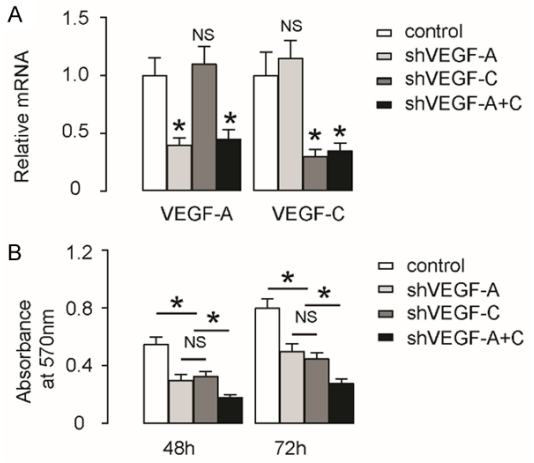

Figure 2. Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs growth than suppression of either VEGF-A or VEGF-C alone. Cultured hemangioma-derived endothelial cells (HemECs) were transduced with AAV carrying either control (scrambled), or shVEGF-A or shVEGF-C or combined shVEGF-A and shVEGF-C (shVEGF-A+C). A. RT-qPCR for VEGF-A and VEGF-C on the transduced cells, which confirmed the effects of shVEGF-A and shVEGF-C. B. An MTT assay for the transduced cells. Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs growth than suppression of either VEGF-A or VEGF-C alone. *P < 0.05. NS: non-significant. N = 5.

Statistical analysis

All values represent the mean ± standard deviation (SD). Statistical analysis of group differences was carried out using a one-way analysis of variance (ANOVA) test followed by the Fisher's Exact Test to compare two groups (GraphPad Software, Inc. La Jolla, CA, USA). A value of $P < 0.05$ was considered statistically significant after Bonferroni correction.

Results

Proliferating-phase of the hemangiomas expresses significantly higher VEGF-A and VEGF-C than involuting-phase

First, we compared the expression levels of VEGF family members in hemangiomas at involuting-phase and at proliferating-phase. We found that proliferating-phase of the hemangiomas expresses significantly higher VEGF-A and VEGF-C than involuting-phase, by RT-qPCR (**Figure 1A**), and by ELISA (**Figure 1B**). On the other hand, the levels of other VEGF family members did not differ between proliferating-phase of the hemangiomas and involuting-phase of hemangioma (**Figure 1A**, **1B**). Therefore, we focused on the effects of VEGF-A and VEGF-C on hemangioma growth.

Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs growth than suppression of either VEGF-A or VEGF-C alone

AAV carrying shVEGF-A or shVEGF-C or scrambled (control) was prepared. Cultured hemangioma-derived endothelial cells (HemECs) were transduced with either control, or shVEGF-A or shVEGF-C or combined shVEGF-A and shVEGF-C (shVEGF-A+C). RT-qPCR for VEGF-A and VEGF-C was performed on these transduced cells, showing that shVEGF-A significantly reduced VEGF-A levels in HemECs, without affecting VEGF-C levels, while shVEGF-C significantly reduced VEGF-C levels in HemECs, without affecting VEGF-A levels. On the other hand, shVEGF-A+C significantly reduced both VEGF-A and VEGF-C levels in HemECs (**Figure 2A**). Next, these transduced cells were subjected to an MTT assay, showing that shVEGF-A and shVEGF-C significantly and similarly reduced cell growth in HemECs, while shVEGF-A+C more pronouncedly reduced cell growth in HemECs than either shVEGF-A or shVEGF-C (**Figure 2B**). Thus, co-suppression of both VEGF-A and VEGF-C had a more pronounced effect on HemECs growth than suppression of either VEGF-A or VEGF-C alone.

Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell proliferation than suppression of either VEGF-A or VEGF-C alone

The effects of VEGF-A/VEGF-C suppression on cell proliferation were assessed by BrdU assay. We found that shVEGF-A and shVEGF-C significantly and similarly reduced the percentage of BrdU+ cells in HemECs, while shVEGF-A+C more pronouncedly reduced cell proliferation in HemECs than either shVEGF-A or shVEGF-C, shown by representative images (**Figure 3A**), and by quantification (**Figure 3B**). Next, we analyzed the cell-cycle regulators in these transduced cells by Western blotting, and found that shVEGF-A significantly enhanced cell-cycle suppressor p21, significantly reduced cell-cycle activators CyclinD1 and CDK4, without affecting cell-cycle activator CyclinB2 and cell-cycle suppressor p27, shown by representative blots (**Figure 3C**), and by quantification (**Figure 3D**).

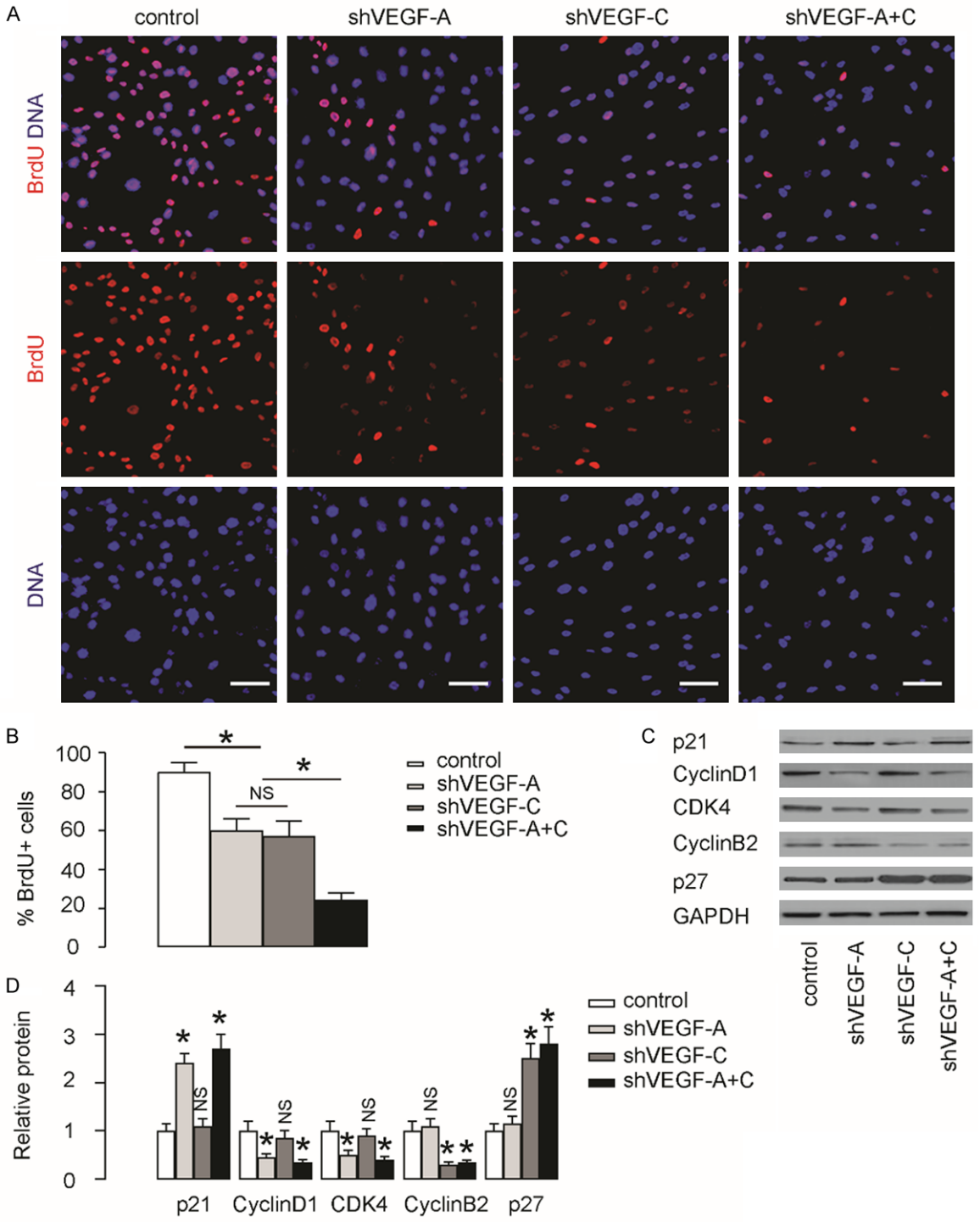

Figure 3. Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell proliferation than suppression of either VEGF-A or VEGF-C alone. (A, B) BrdU assay on control (scrambled), or shVEGF-A or shVEGF-C or combined shVEGF-A and shVEGF-C (shVEGF-A+C) cells, shown by representative images (A), and by quantification (B). BrdU in red and DNA in blue. Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell proliferation than suppression of either VEGF-A or VEGF-C alone. (C, D) Western blotting for cell-cycle regulators in transduced HemECs, shown by representative blots (C), and by quantification (D). shVEGF-A significantly enhanced cell-cycle suppressor p21, significantly reduced cell-cycle activators CyclinD1 and CDK4, without affecting cell-cycle activator CyclinB2 and cell-cycle suppressor p27. On the other hand, shVEGF-C significantly enhanced cell-cycle suppressor p27, significantly reduced cell-cycle activator CyclinB2, without affecting cell-cycle activators CyclinD1 and CDK4 and cell-cycle suppressor p21. Co-suppression of both VEGF-A and VEGF-C significantly enhanced both p21 and p27, and significantly reduced CyclinD1, CDK4 and CyclinB2. *P < 0.05. NS: non-significant. N = 5. Scale bars are 20 μm.

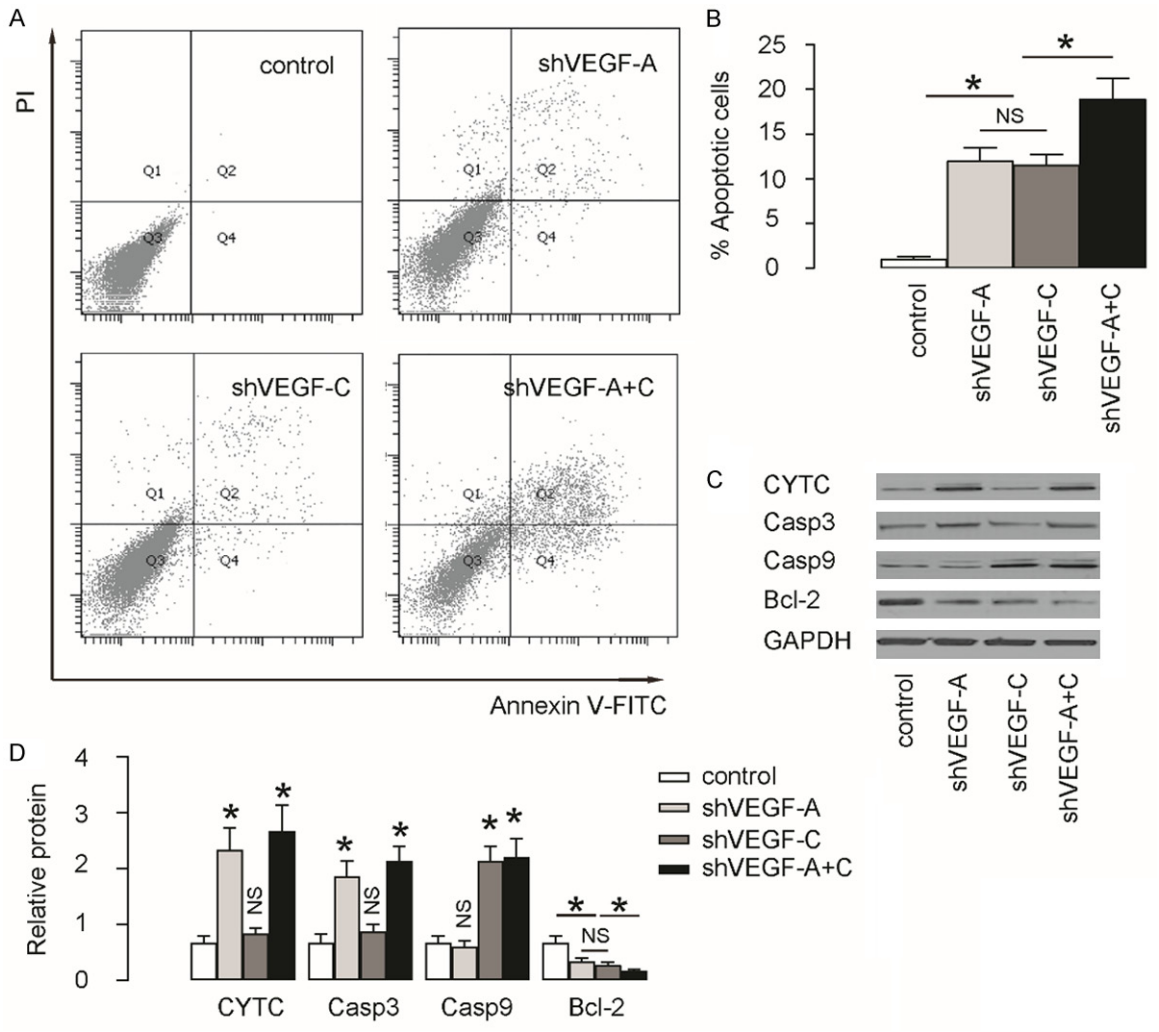

Figure 4. Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell apoptosis than suppression of either VEGF-A or VEGF-C. (A, B) Annexin V assay on control (scrambled), or shVEGF-A or shVEGF-C or combined shVEGF-A and shVEGF-C (shVEGF-A+C) cells, shown by representative flow charts (A), and by quantification (B). (C, D) Western blotting for apoptosis-associated proteins in transduced HemECs, shown by representative blots (C), and by quantification (D). shVEGF-A significantly enhanced pro-apoptosis protein CYTC and caspase3, significantly reduced anti-apoptosis protein Bcl-2, without affecting pro-apoptosis protein caspase9. On the other hand, shVEGF-C significantly enhanced pro-apoptosis protein caspase9, significantly reduced anti-apoptosis protein Bcl-2, without affecting pro-apoptosis protein CYTC and caspase3. Co-suppression of both VEGF-A and VEGF-C significantly enhanced CYTC, caspase3 and caspase9, and more pronouncedly reduced Bcl-2. *$P < 0.05$. NS: non-significant. $N = 5$.

On the other hand, shVEGF-C significantly enhanced cell-cycle suppressor p27, significantly reduced cell-cycle activator CyclinB2, without affecting cell-cycle activators CyclinD1 and CDK4 and cell-cycle suppressor p21, shown by representative blots (**Figure 3C**), and by quantification (**Figure 3D**). Co-suppression of both VEGF-A and VEGF-C significantly enhanced both p21 and p27, and significantly reduced CyclinD1, CDK4 and CyclinB2, as an explanation for the more pronounced suppressive effect on HemECs growth (**Figure 3C**, **3D**). Hence, co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell proliferation than suppression of either VEGF-A or VEGF-C alone. VEGF-A and VEGF-C seemed to regulate proliferation through different proteins.

Co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell apoptosis than suppression of either VEGF-A or VEGF-C alone

The effects of VEGF-A/VEGF-C suppression on cell apoptosis were assessed by Annexin V

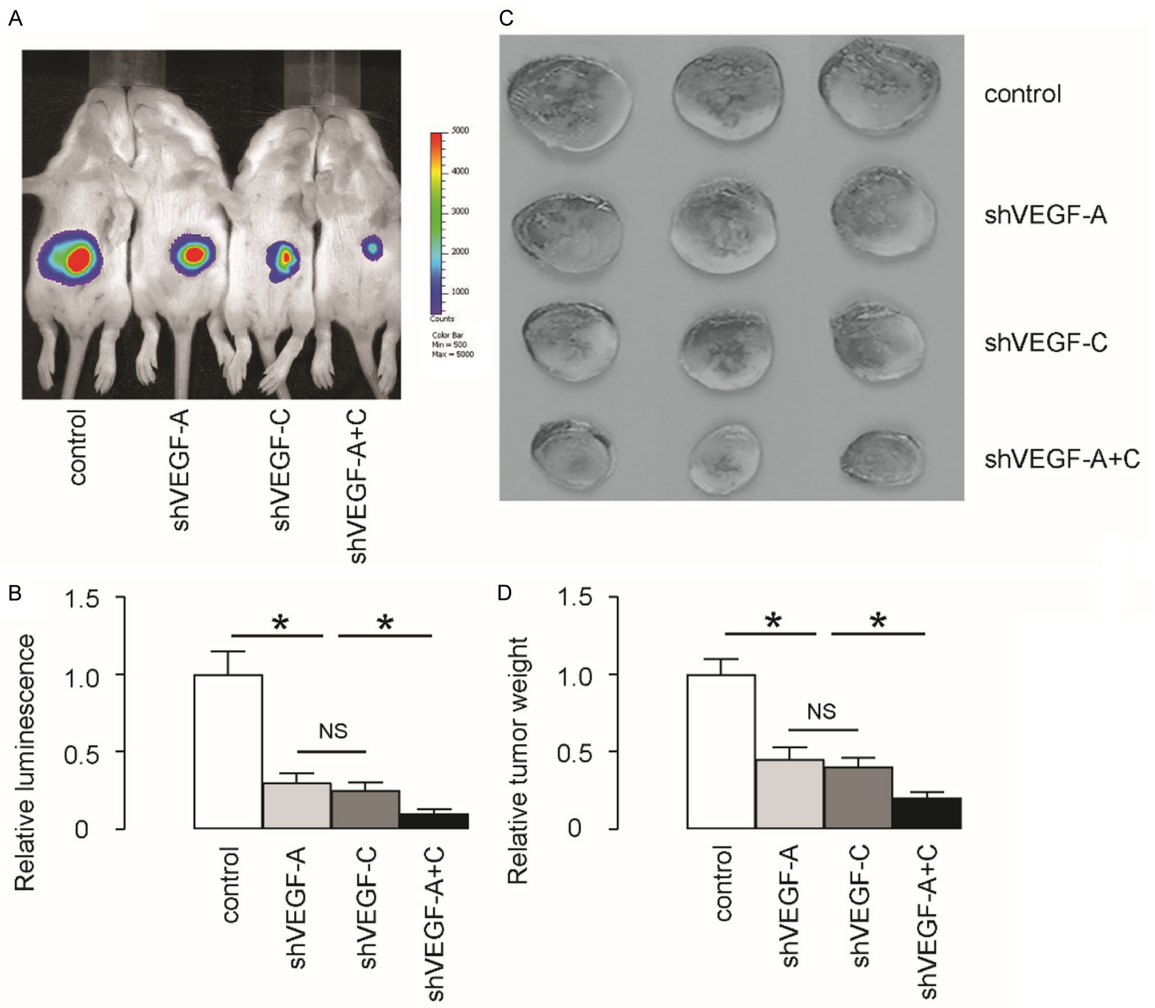

Figure 5. Suppression of both VEGF-A and VEGF-C has more pronounced effects on the growth of the implanted hemangiomas than suppression of either VEGF-A or VEGF-C HemECs transduced with control or shVEGF-A or shVEGF-C or shVEGF-A+C were implanted into nude mice with HUVECs and the tumor growth was assessed after 4 weeks. (A, B) Bioluminescence assay, shown by representative images (A), and by quantification (B). (C, D) Tumor size, shown by gross view (C), and by quantification (D). *P < 0.05. NS: non-significant. N = 10.

assay. We found that shVEGF-A and shVEGF-C significantly and similarly increased the percentage of apoptotic cells in HemECs, while shVEGF-A+C more pronouncedly increased cell apoptosis in HemECs than either shVEGF-A or shVEGF-C, shown by representative flow charts (**Figure 4A**), and by quantification (**Figure 4B**). Next, we analyzed the apoptosis-associated proteins in these transduced cells by Western blotting, and found that shVEGF-A significantly enhanced pro-apoptosis protein CYTC and caspase3, significantly reduced anti-apoptosis protein Bcl-2, without affecting pro-apoptosis protein caspase9, shown by representative blots (**Figure 4C**), and by quantification (**Figure 4D**). On the other hand, shVEGF-C significantly enhanced pro-apoptosis protein caspase9, significantly reduced anti-apoptosis protein Bcl-2, without affecting pro-apoptosis protein CYTC and caspase3, shown by representative blots (**Figure 4C**), and by quantification (**Figure 4D**). Co-suppression of both VEGF-A and VEGF-C significantly enhanced CYTC, caspase3 and caspase9, and more pronouncedly reduced Bcl-2, as an explanation for the more pronounced promoting effect on HemECs apoptosis (**Figure 4C**, **4D**). Hence, co-suppression of both VEGF-A and VEGF-C has a more pronounced effect on HemECs cell apoptosis than suppression of either VEGF-A or VEGF-C alone. VEGF-A and VEGF-C seemed to regulate apoptosis through different proteins.

Suppression of both VEGF-A and VEGF-C has more pronounced effects on the growth of the implanted hemangiomas than suppression of either VEGF-A or VEGF-C alone

Finally, HemECs transduced with control or shVEGF-A or shVEGF-C or shVEGF-A+C were implanted into nude mice with HUVECs and the tumor growth was assessed after 4 weeks. First, bioluminescence assay was done, showing that shVEGF-A and shVEGF-C significantly and similarly decreased the size of the implanted tumor, while shVEGF-A+C more pronouncedly decreased tumor size than either shVEGF-A or shVEGF-C, shown by representative images (**Figure 5A**), and by quantification (**Figure 5B**). These data were further confirmed by measurement of the weight of the dissected tumor (**Figure 5C**, **5D**). Thus, suppression of both VEGF-A and VEGF-C has more pronounced effects on the growth of the implanted hemangiomas than suppression of either VEGF-A or VEGF-C alone.

Discussion

The VEGF family is composed of 6 secreted amino acids, among which VEGF-A may play the most important role in the physiological pathological angiogenesis [18]. However, the exact effect of VEGF-A may be coordinated with other members, since there is a complex ligand-receptor binding map of VEGF members and receptors [19]. Of note, VEGF-C exclusively binds to VEGFR3, through which it promotes lymphoangiogenesis, which is different from VEGF-A/VEGFR2-mediated angiogenesis [20]. Since our study found that co-suppression of VEGF-A and VEGF-C appeared to have more pronounced effects on HemECs cell growth, it is apparent that both vascular angiogenesis and lymphoangiogenesis contribute to the malignant growth of IH.

Interestingly, we found that the suppression of VEGF-A or VEGF-C seemed to affect cell proliferation via different signaling cascades. For example, VEGF-A suppression inhibited CyclinD1 and CDK4, which form a regulatory complex to control the G1/S transition [21]. VEGF-E suppression inhibited CyclinB2, which primarily controls the G2/M transition [22]. VEGF-A suppression did not alter CyclinB2, and VEGF-E suppression did not alter CyclinD1 and CDK4. Moreover, since suppression of VEGF-A and VEGF-E increased 2 G1-checkpoint CDK inhibitors - p21 and p27 [23-26], respectively, these data suggest that VEGF-A may enhance proliferation of HemECs mainly via promotion of G1/S transition, while VEGF-E may enhance proliferation of HemECs via promotion of both G1/S transition and G2/M transition. It is hence comprehensible that suppression of both VEGF-A and VEGF-E has more pronounced antiproliferative effects on HemECs.

Similarly, we found that the suppression of VEGF-A or VEGF-C seemed to affect cell apoptosis via different signaling cascades. CYTC generates apoptosome with Apaf1 and pro-caspase9, which cleaves pro-caspase9 into active dimer caspase9 to mediate the cleavage of caspase3 [27]. Apparently, VEGF-A and VEGF-C signaling controls different stages of apoptosis. Hence, suppression of both VEGF-A and VEGF-E has more pronounced pro-apoptotic effects on HemECs.

To summarize, our study shows a model in that VEGF-A and VEGF-C regulate proliferation and apoptosis of HemECs through different proteins in the molecular pathway. Hence, the combined suppression appeared to be more potent in restriction of hemangioma growth in vitro and In vivo.

Acknowledgements

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Disclosure of conflict of interest

None.

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