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Studies on the role of pigment epithelium-derived factor in the involution of hemangioma

혈관종 퇴축 과정에서 pigment epithelium-derived factor 의 역할에 관한 연구

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Studies on the role of pigment epithelium-derived factor in the involution of hemangioma

by

Kyung-Jin Kim

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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ABSTRACT

Introduction: Hemangioma is a type of vascular tumor resulting from abnormal growth of blood vessels which the cause is unknown. Compared to other vascular tumors, this benign tumor is commonly found in infancy. Hemangioma is distinctive because of its proliferation during the early stage, however, followed by involution where the tumor undergoes regression. The critical onset which proceeds to involution is not well understood and only minor research are evident regarding the involution process. Therefore, this study is centered on the mechanism of the involution of hemangioma. Through this study, I have identified an anti-angiogenic factor called pigment epithelium-derived factor (PEDF) which was found to be highly expressed during the involution phase.

Methods: Endothelial cells were extracted from tissues of hemangioma patients and sorted using CD31 antibodies. Then, I observed the cellular growth rate of the hemangioma-derived endothelial cells (HemECs), and screened for pro- and anti-angiogenic factors using an angiogenesis array. Furthermore, I used hemangioma tissue samples for immunochemical staining.

Results: I have identified pigment epithelium-derived factor, (PEDF) an anti-angiogenic factor, to be associated with the growth inhibition of hemangioma-derived endothelial cells (HemECs). Also, I have examined that suppression of PEDF restored cell growth in the involuting phase of HemECs. Furthermore, I have confirmed *in vivo* by immunochemical staining that PEDF was highly expressed, mainly in the involuting phase of hemangioma tissues.

Conclusions: These results suggest that PEDF plays a role in the involution of hemangioma by inhibiting the proliferation of HemEC. The regulatory mechanism of PEDF expression as an anti-angiogenic factor could be a potential therapeutic target to treat hemangiomas.

Keywords: hemangioma; pigment epithelium-derived factor; PEDF; endothelial cell; involution; angiogenesis

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LIST OF ABBREVIATIONS

IH: Infantile Hemangioma

HemEC: Hemangioma-derived endothelial cell

HUVEC: Human umbilical vein endothelial cell

PEDF: Pigment epithelium-derived factor

VEGF: Vascular endothelial growth factor

TIMP1: Tissue inhibitor of metallopretinase-1

INTRODUCTION

Hemangioma is one of the most common tumor found in infancy but the cause is yet unknown. It is a type of benign vascular tumor resulting from abnormal growth of blood vessels where the endothelial cell lining of the vessels undergoes continuous angiogenesis and proliferation. (Phung & Hochman, 2012). Infantile hemangioma (IH) tend to occur mainly on the skin usually around the face and neck area. Additionally, being a benign tumor, IH may be considered harmless; however, depending on the location of the hemangioma, vision- or life-threatening symptoms can occur (Bartlett, Riding, & Salkeld, 1988). For example, IH resulting in the eyelids could result in visual impairment leading to blindness and recent reports of liver hemangioma are highly fatal. To date, the treatment of IH includes surgical removal, corticosteroid and propranolol treatments as a first-line therapy for intervention. However, long-term treatment with corticosteroids may cause side effects such as immune suppression and delayed skeletal growth (George, Sharma, Jacobson, Simon, & Nopper, 2004). Moreover, propranolol, a relatively new pharmacologic treatment for IH, has a vague mechanism of action and the potential to cause serious side effects such as bradycardia and hypotension (de Graaf et al., 2011). Thus, studies regarding novel therapeutic modalities to treat IH are required.

Hemangioma has a unique characteristic compared with cancer and other vascular tumor counterparts because of an intrinsic mechanism of involution (Razon, Kraling, Mulliken, & Bischoff, 1998; Ritter, Butschek, Friedlander, & Friedlander, 2007). A typical life cycle of IH consists of three stages: the

proliferating phase, involuting phase and the involuted phase. During the few months after birth, endothelial cells in IH proliferate rapidly, thus the proliferating stage. Next, spontaneous involution for several years is followed, where natural regression of the tumor occurs. This unique process of involution occurs spontaneously; the cause and onset is not well understood (Mulliken & Glowacki, 1982). In the involuting phase, the population of endothelial cells begins to decrease. This is followed by the involuted phase, in which endothelial cells are almost not present and the tumor is replaced by fatty tissue with only a minor scar remaining (Kleiman, Keats, Chan, & Khan, 2012).

Understanding this natural process of vascular regression in IH is important because it possesses the potential to treat IH through the strategy of accelerating the process of involution. Despite the importance of the induction of hemangioma involution, the mechanisms by which the involution is initiated remains largely unknown. Vascular endothelial growth factor (VEGF) (Ferrara, Gerber, & LeCouter, 2003) and platelet-derived growth factor (Battegay, Rupp, Iruela-Arispe, Sage, & Pech, 1994) are potent stimulator of endothelial cell proliferation and the expression of those factors parallels the proliferating phase of IH (Roach et al., 2012; Takahashi et al., 1994); thus, the inhibition of these factors may be effective to prevent the proliferation rather than induction of the involution of IH (Greenberger, Boscolo, Adini, Mulliken, & Bischoff, 2010; Roach et al., 2012). Although many pharmacologic approaches to treat IH target proliferating endothelial cells by inhibiting those growth factors, fewer approaches identify the novel regulators of the involution of IH. Given the spontaneous occurrence of the involuting phase (Mulliken & Glowacki, 1982)

and the possibility of the onset of an endogenous mechanism in IH (Ritter, Dorrell, Edmonds, Friedlander, & Friedlander, 2002), I hypothesized that certain autocrine factors would be associated with the inhibition of proliferating endothelial cells during the involution phase of hemangioma.

In the present study, I investigated the negative regulators of hemangioma growth secreted from HemECs. I observed that pigment-epithelium derived factor (PEDF) inhibited the proliferation of HemECs in an autocrine manner and that PEDF expression was mainly up-regulated during the involuting phase of IH. To my knowledge, this is the first report highlighting the role of PEDF in the involution of IH as a therapeutic target.

MATERIALS AND METHODS

Cell culture

All of the experiments in this study were obtained from patients with informed written consent and protocols for the study approved by the Institutional Review Board of Seoul National University Hospital (IRB#H-1012-060-344). From three patients with proliferating, involuting, and involuted hemangiomas, hemangioma-derived endothelial cells were isolated. Briefly, the hemangioma tissues were extracted from the patients and washed. The blood vessel was isolated using a blade and incubated in 0.02% collagenase type II (Worthington, Lakewood, NJ, USA) for 30 min at 37℃ with gentle shaking. The digested tissues were further incised and washed in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA). Next, the samples were centrifuged at 2000 rpm for 3 min at room temperature. The supernatant was decanted and washed with DMEM. (Thermo Fisher Scientific) The pellet was resuspended in Endothelial Growth Media-2 (EGM-2; Lonza, Walkersville, USA), filtered using a cell strainer (BD Biosciences, San Jose, CA, USA) and seeded in 60-mm dishes in preparation for cell sorting. HemECs were isolated by FACS using human CD31 monoclonal antibodies (BD Biosciences) and CD31-negative non-HemEC cells were designated as HemEC(-).

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and maintained in EGM-2 with 5% FBS. All of the cells were cultured at 37℃ in an incubator with a humidified atmosphere of 95% O_2 and 5% CO_2.

Cell counting

HUVECs and HemECs (3 x 10^4 each) were seeded on separate gelatin (Welgene, Korea)-coated 12-well plates (SPL, Korea). All of the plates were incubated with EGM-2 for 72 h, while one set of plates had its media changed every 24 h. Cells were trypsinized (Gibco, Grand Island, NY, USA) and counted every 24 h using a hemocytometer (Marienfeld Superior, Lauda-Königshofen, Germany) and Trypan blue (Welgene).

MTT assay

Conditioned media were collected from HemECs after 24 h and 48 h of incubation. The conditioned media were mixed with normal EGM-2 media at a ratio of 1:1. HUVECs were incubated for 24 h in EGM-2. Media were replaced with the conditioned media and incubated for 48 h. MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to the media, and the mixture was incubated for 4 h. The resulting precipitates were dissolved in isopropanol, and formazan levels were determined at 570 nm.

For PEDF treatment, HUVECs were incubated with PEDF protein (R&D Systems, Minneapolis, MN, USA) at the indicated doses for 24 h or at 200 ng/mL for the indicated times in EGM-2 media with 1% FBS. The MTT assay was performed as described above.

Polymerase Chain Reaction (PCR)

Total RNA was extracted from the cells using the RNeasy kit (Qiagen, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized

using an oligo(dT) primer and Superscript reverse transcriptase (Invitrogen). The cDNA was then used to amplify genes of interest using GoTaq Polymerase (Promega) and a MyCycler™ system (BioRad). The PCR products were analyzed using 2% agarose gel electrophoresis and SYBR Safe (BioRad). The gel was then filmed using ChemiDoc XPS+ (BioRad).

For real-time quantitative PCR, the SYBR Green Super Mix (BioRad) and an i-Cycler PCR thermocycler (BioRad) were used. Samples containing 20 ng of cDNA were analyzed in triplicate. The values were normalized to those of β-actin expression levels. The sequences of PCR primers are summarized in Table 1.

Human angiogenesis cytokine array

The expression of cytokines in the cell culture supernatants was assessed using the Human Angiogenesis Cytokine Array kit (BD Biosciences). Conditioned media without serum were collected from HUVECs and HemECs after 48 h of incubation. The membrane blots of the conditioned media were developed according to the manufacturer's instructions and were analyzed by measuring the density of each spot compared with the negative and positive controls.

siRNA

PEDF siRNAs (siPEDF#1 and siPEDF#3) and negative-control siRNA (siControl) (IDT™ Inc., Coralville, IA, USA) were dissolved in Opti-Mem (Gibco) and Lipofectamine 2000 (Invitrogen). The mixture was added to

HemECs and incubated at 37℃ overnight. The total RNA was extracted from the cells to confirm PEDF knockdown in cells by RT-PCR. The transfected cells were then used for cell counting, the MTT assay and immunofluorescence.

Immunohistochemistry

Tissue sections from the patients of each phase of IH were deparaffinized and processed for immunohistochemical staining. The sections were blocked with 5% goat serum and then were incubated with the primary antibody against human PEDF (R&D Systems). The sections were incubated with anti-mouse IgG antibody and detected using DAB (Vector Laboratories, Burlingame, CA, USA) with hematoxylin (Sigma-Aldrich) counterstaining. Images were obtained using an Olympus BX51 microscope equipped with OlyVia software (Olympus, Germany).

Statistical analysis

The data are presented as the mean±SD. The statistical analyses were performed using Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA), and Student's *t*-test was used to compare the differences between groups; a *P* value less than 0.05 was considered statistically significant.

RESULTS

Isolated HemECs expresses autocrine factors to inhibit their own cell proliferation

To verify that the isolated HemECs were indeed endothelial cells of each phase, I analyzed the expression of the endothelial-specific markers CD31 and VE-cadherin by quantitative RT-PCR (Fig. 1A). All HemECs expressed CD31 and VE-cadherin transcripts, whereas HemEC-negative cells did not (Fig. 1A). Because GLUT1 expression distinguishes hemangioma from other vascular anomalies (North, Waner, Mizeracki, & Mihm, 2000), I also examined the expression of GLUT1 transcripts and confirmed that the proliferating and involuting HemECs expressed GLUT1 significantly higher than HUVECs or HemEC-negative cells (Fig. 1A). Next, to determine whether HemECs exhibit phase-specific markers, I analyzed the gene expression of VEGF and E-selectin (Fig. 1B). Consistent with previous reports (Abramson et al., 2006; Greenberger et al., 2010; Takahashi et al., 1994; Zvulunov & Metzker, 2002), the proliferating HemECs expressed more VEGF and E-selectin transcripts than the involuting and involuted HemECs (Fig. 1B), providing further confirmation of a phase-specific phenotype of HemECs.

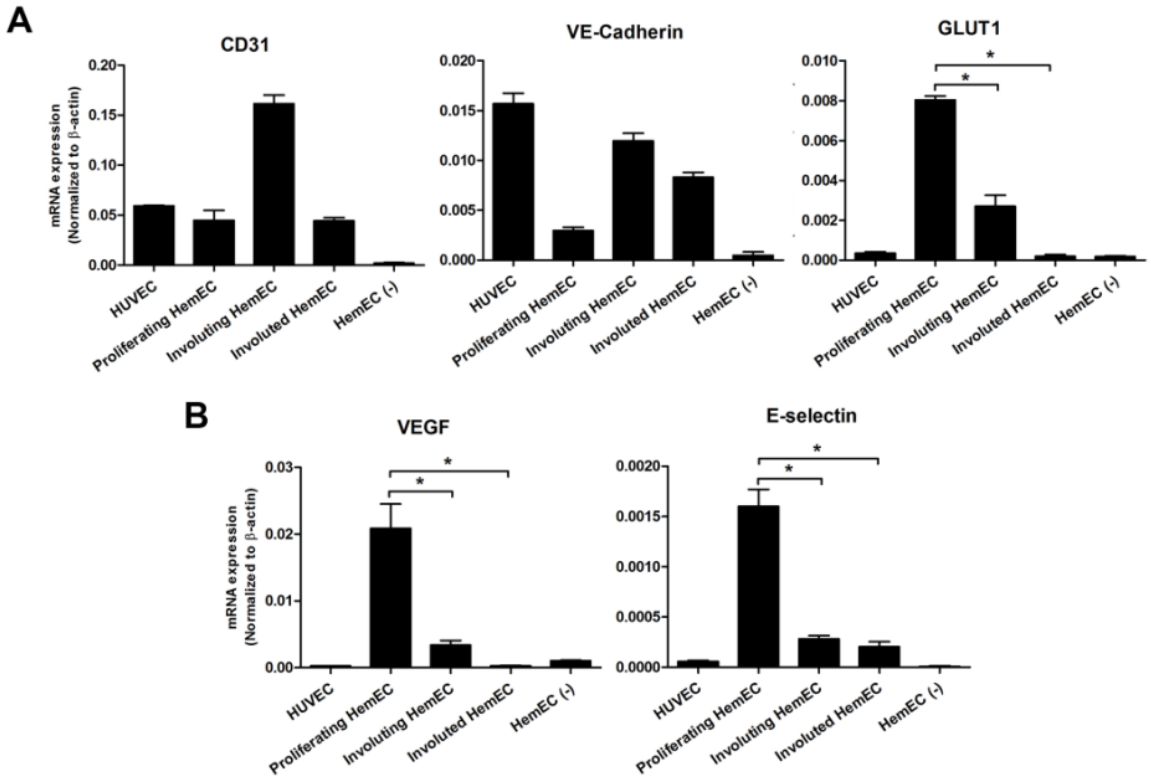

Figure 1. Gene expression of endothelial-specific and phase-specific markers in HemECs. The mRNA transcript levels of endothelial specific markers (CD31, VE-Cadherin, and GLUT1) (**A**) or phase-specific markers (VEGF and E-selectin) (**B**) were measured by quantitative RT-PCR. The mRNA expression levels were normalized to those of the β-actin control. * denotes $P < 0.05$ between the two values (n=3).

Because the objective of the present study was to identify regulators of HemEC involution, I first focused on cell growth of the involuting HemECs. To determine whether cell proliferation is impaired in involuting HemECs, I examined the cell proliferation of HemECs for 72 h and compared the cell proliferation with that of normal human endothelial cells (HUVECs). The involuting HemECs showed a relatively slower proliferation rate than HUVECs (Fig. 2A). However, when the growth medium was changed to fresh medium every 24 h, the growth rate of the involuting HemECs was restored to the level of HUVECs (Fig. 2B). Thus, I assumed that the involuting HemECs may release certain factors that reduce cell proliferation in an autocrine manner. To verify that the cultured medium of HemECs contains growth inhibitory factors, I treated HUVECs with the conditioned media from the involuting HemEC culture and measured the cell viability of HUVECs. Incubation with the conditioned media from the involuting HemECs for 48 h significantly inhibited the HUVEC viability (Fig. 2C). These results demonstrate that the involuting HemECs release certain factors that inhibit not only the involuting HemECs but also normal endothelial cell proliferation.

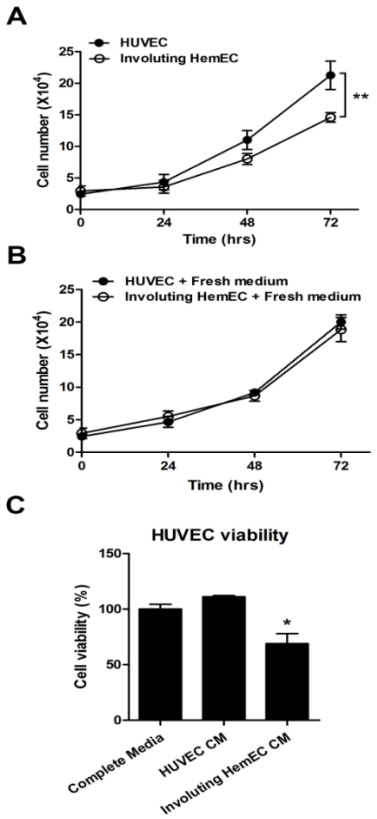

Figure 2. Effects of culture conditioned media from HemECs and HUVECs on cell proliferation. (**A**) The involuting HemECs and HUVECs were incubated for the indicated times. Cell proliferation was determined by counting the cell number. * denotes $P < 0.05$ versus the value of HUVECs (n = 5). (**B**) The involuting HemECs and HUVECs were incubated for 72 h with changing the culture media every 24 h and then were counted to determine the cell proliferation profile. (**C**) The culture conditioned media from the involuting HemECs and HUVECs cultured for 48 h were treated to HUVECs for 24 h. The complete media without incubation was used as control. Cell viability was then assessed by MTT staining. Results are given as percentages versus the control complete media value and plotted as the mean±SD. * denotes $P < 0.05$ versus the value of control complete media (n = 5).

Pigment-epithelium derived factor is identified as an anti-angiogenic molecule from HemECs

To identify the autocrine factors that induce growth inhibition of HemECs, I examined the expression profiles of angiogenesis-related proteins in the conditioned media from HemECs and HUVECs using human angiogenesis cytokine array. Among 55 angiogenesis-related proteins blotted in the array, 19 proteins were detected in the conditioned media (Fig. 3A). As observed in gene expression analysis (Fig. 1B), VEGF protein was up-regulated in the proliferating HemECs (Fig. 3A and 3B). Three anti-angiogenic factors were included in the detected protein expression: tissue inhibitor of metalloproteinase-1 (TIMP-1) and thrombospondin-1 were expressed in most HemECs and HUVECs, but PEDF was mainly expressed in the involuting HemECs (Fig. 3A and 3B).

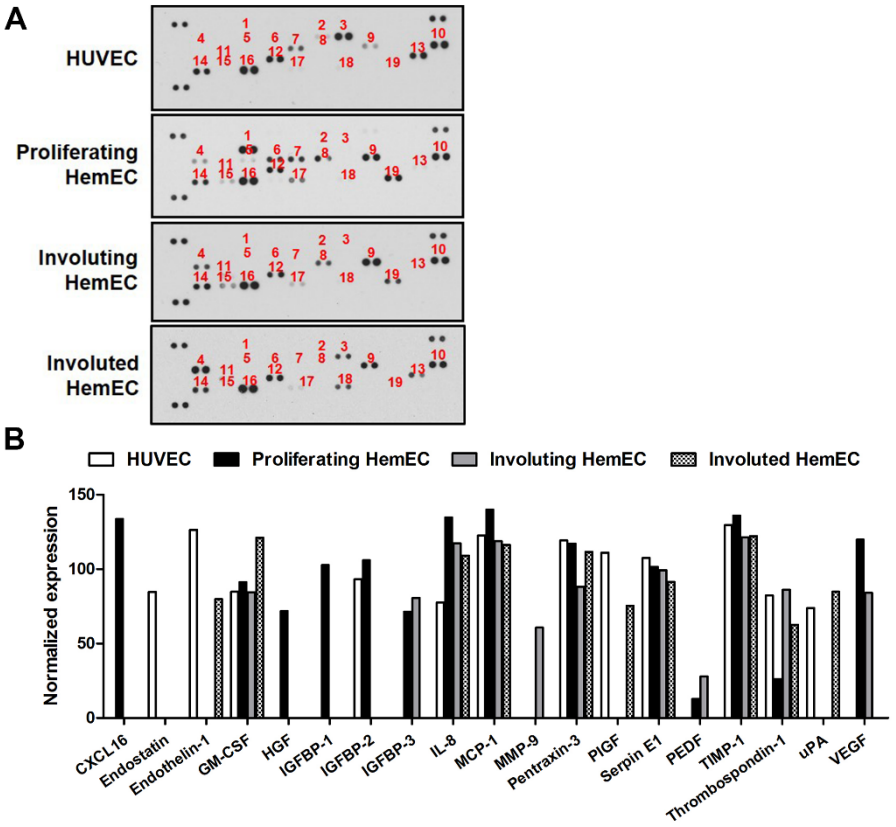

Figure 3. Identification of autocrine factors in HemECs and their culture supernatants. (**A**) Human Angiogenesis Cytokine Array evaluating pro- and anti-angiogenic protein expression changes in the culture conditioned media from the proliferating, involuting and involuted HemECs, and HUVECs after 48 h of incubation. (**B**) Quantification of the protein expression in (A). Results are given as the mean dot intensity and are expressed as a percentage of the standard intensities.

Pigment-epithelium derived factor is mainly expressed in involuting HemECs

The predominant expression of PEDF in the involuting HemECs was also confirmed by quantitative RT-PCR analysis (Fig. 4A). Based on the findings that PEDF is anti-angiogenic (Dawson et al., 1999), and its expression is highly induced in the involuting HemECs (Fig. 3B), I hypothesized that PEDF could be induced during and could play an important role in the involution of IH.

PEDF is anti-angiogenic and associated with the growth inhibition of HemECs

To determine whether PEDF has growth-inhibiting potential in endothelial cells, I treated HUVECs with PEDF and measured cell viability using MTT analysis. PEDF decreased HUVEC viability in dose- and time dependently (Fig. 5A and 5B), indicating that PEDF can suppress normal endothelial cell growth as an anti-angiogenic factor.

Having shown that HemEC-conditioned media (Fig. 2C) and PEDF (Fig. 5) decrease the proliferation of HUVECs, I further asked whether these conditions are associated with the reduced cell proliferation of the involuting HemECs. To determine the role of PEDF in the growth inhibition of HemECs, I treated the involuting HemECs with either control siRNA (siControl) or two different PEDF siRNAs (siPEDF#1 and siPEDF#2). siPEDF#2 effectively down-regulated PEDF expression (Fig. 6A and 6B). In addition, the cell proliferation of the involuting HemECs was improved by PEDF knockdown (Fig. 7A). This result was also confirmed by the increased expression of PCNA

mRNA—a marker of cell proliferation—in siPEDF#2-treated HemECs (Fig. 7B). These results suggest that PEDF secreted from the involuting HemECs limits their own cell proliferation.

Next, to determine whether PEDF mediates the growth inhibition of HemECs by autocrine secretion rather than affecting the intracellular machinery for cell growth, I measured HUVEC viability by treatment with the conditioned media from the siRNA-treated involuting HemECs. The decreased HUVEC viability induced by the conditioned media from the involuting HemECs was abolished by that from PEDF siRNA-treated HemECs (Fig. 7C). Taken together, these results suggest that the involuting HemECs release PEDF as a growth inhibitory factor to account for the impaired cell proliferation of the involuting HemECs.

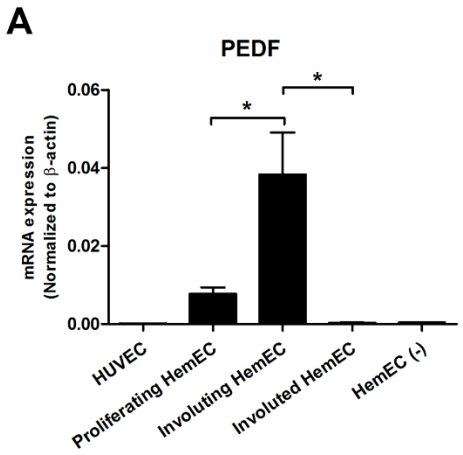

Figure 4. Quantification of PEDF mRNA expression in HemECs. (**A**) PEDF mRNA expression was measured by quantitative RT-PCR. * denotes $P < 0.05$ between the two values (n=3).

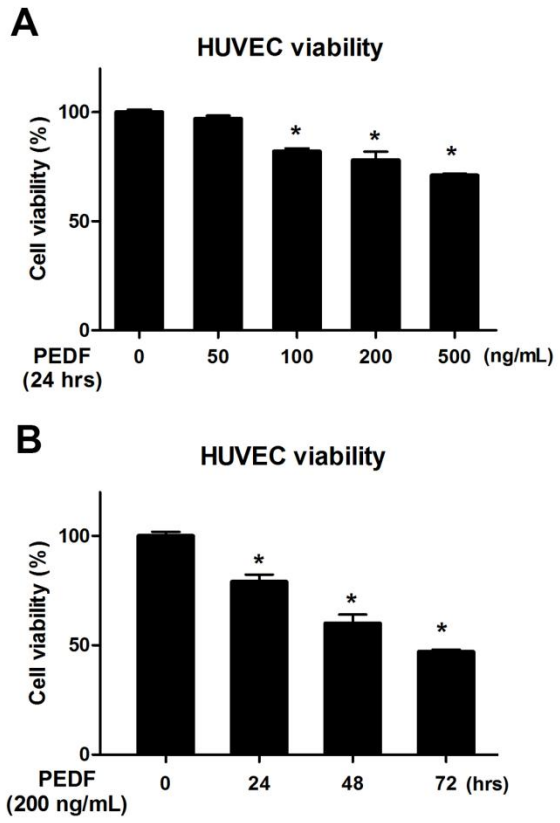

Figure 5. Effect of PEDF on HUVEC viability. (**A**) Dose- and (**B**) time-dependent effects of PEDF on HUVEC viability. HUVEC viability was assessed by MTT analysis. Results are plotted as the mean±SD. * denotes $P < 0.05$ versus the value of 0 concentration or time (n = 5).

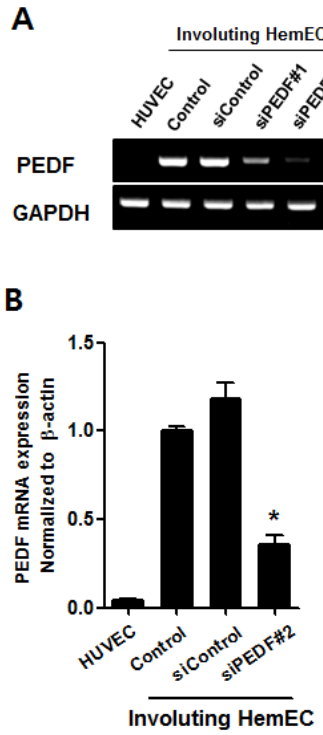

Figure 6. Suppression of PEDF on HemECs. (**A**, **B**) Transfection of siRNA for PEDF abolished PEDF gene expression. Conventional (**A**) and quantitative (**B**) RT-PCR showed the effective down-regulation of PEDF gene expression by siPEDF#3. * denote s $P < 0.05$ versus the value of the involuting HemECs transfected with control siRNA (siControl) (n = 5).

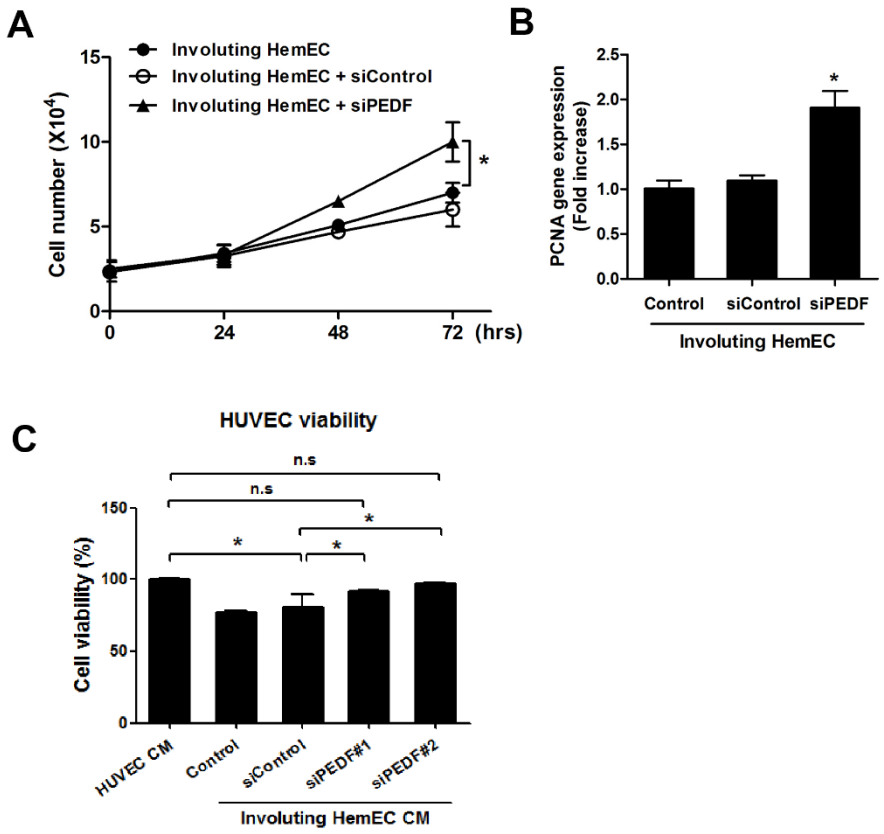

Figure 7. Effect of suppression of PEDF on HemEC proliferation. (**A**) After the transfection of control and PEDF siRNAs in the involuting HemECs, the cells were incubated for the indicated times. Cell proliferation was assessed by counting cell numbers. * denotes $P < 0.05$ between the two values (n = 5). (**B**) Quantitative RT-PCR analysis of PCNA mRNA expression in the involuting HemECs transfected with control and PEDF siRNAs. * denotes $P < 0.05$ versus the value of siControl (n = 5). (**C**) The culture conditioned media from the involuting HemECs transfected with either control siRNA or PEDF siRNA were incubated with HUVECs for 24 h. HUVEC viability was assessed by MTT analysis. Results are plotted as the mean±SD. * denotes $P < 0.05$ between the two values (n = 5).

Increased expression of PEDF coincides with the involuting phase of IH

Based on *in vitro* experiments, I next examined whether the expression of PEDF is increased in the involuting phase of IH *in vivo*. From three patient tissues per each phase of IH, I determined PEDF expression by immunochemical staining. PEDF expression was observed in all phases of IH; however, PEDF-positive cells were significantly higher in the involuting IH than in the proliferating and involuted IH phases (Fig. 8). This result suggests that PEDF is up-regulated during the involuting phase of IH tissue and could play an inhibitory role in the proliferation of HemECs.

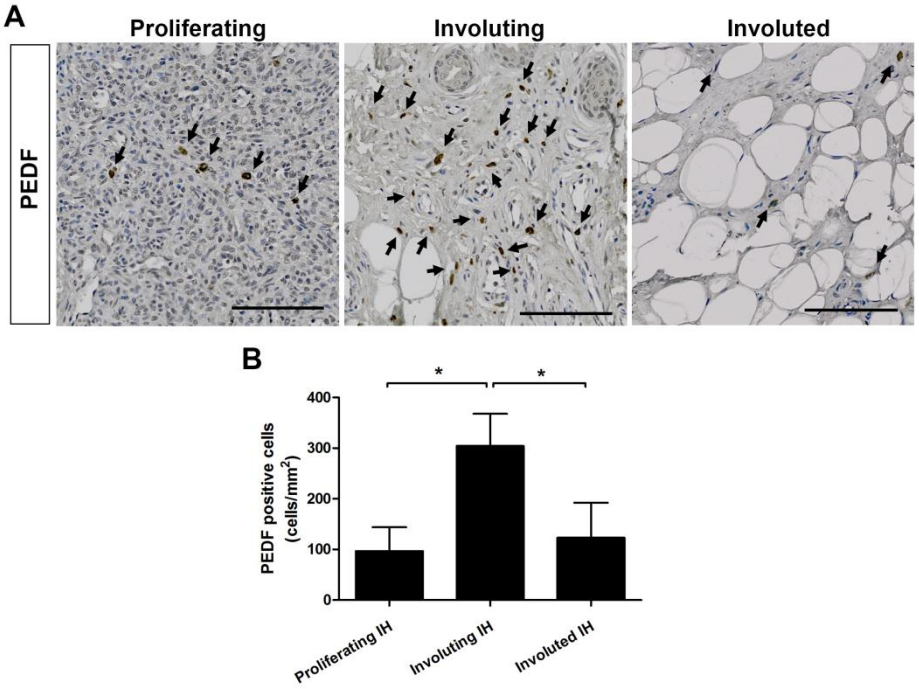

Figure 8. Expression of PEDF in hemangioma tissue sections. (**A**) Representative immunohistochemical staining of PEDF in the proliferating, involuting and involuted phase of IH tissue sections. The arrows indicate PEDF-positive cells. Bar = 100 μm. (**B**) Quantification of PEDF-positive cells in each phase of the IH tissue sections. Results are plotted as the mean±SD. * denotes $P < 0.05$ between the two values (n = 3).

Table 1.

Nucleotide sequences of primers used in experiments

Targets of PCR	Forward primers (5’ to 3’)	Reverse primers (5’ to 3’)
PEDF	TTCCTGCCCCTGAAAGTGAC	GGACTTGGTGACTTCGCCTT
CD31	GCTCTCTTGATCATTGCG	GAGGACACTTGAACTTCC
VE-cadherin	GGCAAGATCAAGTCAAGCGTG	ACGTCTCCTGTCTCTGCATCG
VEGF	GCAGAAGGAGGAGGGCAGAAT	GCACACAGGATGGCTTGAAGA
E-selectin	CCGAGCGAGGCTACATGAAT	GCCACATTGGAGCCTTTTGG
GLUT1	CTTTTCTGTTGGGGGCATGAT	CCGCAGTACACACCGATGAT
PCNA	AGGGCTCCATCCTCAAGAAGG	TGGTGCTTCAAATACTAGCGC
β-actin	TCCCTGGAGAAGAGCTACGA	AGCACTGTGTTGGCGTACAG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

DISCUSSION

In the present study, I screened the expression of anti-angiogenic factors to identify proteins that may be important for the involution of IH. I found that PEDF, an inhibitor of angiogenesis (Dawson et al., 1999), was highly expressed in the involuting phase of HemECs and IH tissues. PEDF limited the proliferation of HemECs in an autocrine manner.

Pharmacological approaches to treat IH have focused more on the proliferating phases because angiogenic growth factors such as VEGF and FGF were expressed in the proliferating phases and subsequently diminished during the involution phases (Takahashi et al., 1994). Moreover, the increased apoptosis and decreased proliferation of endothelial cells during the involution of IH emphasize anti-angiogenic therapy in the treatment of these tumors (Hasan, Ruger, Tan, Gush, & Davis, 2000; Razon et al., 1998). Recent studies have shown that VEGF is a major target of corticosteroid treatment by the inhibition of both the proliferation of HemECs and vasculogenic activity of hemangioma-derived stem cells (Claesson-Welsh, 2008; Greenberger et al., 2010; Jinnin et al., 2008). However, given that 30% of IHs do not respond to corticosteroid treatment (Zvulunov & Metzker, 2002) and other therapies may be necessary, the induction of an intrinsic pathway of endothelial apoptosis could provide a novel therapeutic strategy for IH treatment. Thus, I focused on the proteins with an anti-angiogenic potential induced during the involuting phase that could cause the acceleration of the involution of IH.

The determination of the IH phase is largely dependent on clinical observations and the age of the patients: the proliferating phase spans the first

year of age, the involuting phase spans the ages of 2~5 years, and the involuted phase occurs thereafter (Mulliken & Glowacki, 1982). An accurate assessment of the phases is required to understand the pathogenesis of IH. The latter could be accomplished by characterizing cellular markers. Many approaches have revealed the cellular markers of the proliferating phase of IH: for example, VEGF (Greenberger et al., 2010; Takahashi et al., 1994) and E-selectin (Kraling et al., 1996; Smadja, Mulliken, & Bischoff, 2012) are highly expressed in the proliferating phase but are down-regulated in the involuting phase. Consistently, I observed the increased expression of VEGF and E-selectin in the proliferating rather than the involuting HemECs (Fig. 1B). Conversely, less is known concerning the cellular markers of the involuting phase of IH. I demonstrated here that PEDF is exclusively expressed in HemECs rather than in normal endothelial cells (Fig. 5A) and predominantly in the involuting HemECs (Fig. 3C). These findings provide additional support for us to isolate the phase-specific HemECs and propose PEDF as a novel marker of the involuting HemECs.

PEDF is a member of the serine protease inhibitor superfamily without an inhibitory function toward serine protease (Becerra, Sagasti, Spinella, & Notario, 1995). It was first discovered in human retinal cells (Tombran-Tink, Chader, & Johnson, 1991; Tombran-Tink & Johnson, 1989) and was later found to have potent anti-angiogenic activity that prevented angiogenesis in the eye and tumors (Alberdi, Aymerich, & Becerra, 1999; Dawson et al., 1999; Mori et al., 2002). A growing body of evidence has shown the involvement of PEDF in the regulation of vascular integrity and angiogenesis. For example, PEDF

promotes endothelial cell apoptosis through p38 MAPK signaling (Chen, Zhang, Barnstable, & Tombran-Tink, 2006) and/or PPARδ-induced p53 activation (Ho et al., 2007). PEDF also performs an anti-angiogenic function in VEGF- or bFGF-induced endothelial cell migration (Dawson et al., 1999). Furthermore, PEDF depletion in mice demonstrated the importance of PEDF in the vasculature (Doll et al., 2003). In accordance with these data, I also found that PEDF exerted anti-proliferating activity in HemECs as well as HUVECs (Figs. 4 and 5). Likewise, the administration of PEDF to Wilms' tumors suppressed not only the growth of tumors but also that of the surrounding cells in the cancer microenvironment such as the endothelial cells (Abramson et al., 2006). Based on the previous reports and our findings, I assume that PEDF could promote the involution of IH by suppressing the proliferation of HemECs. Although the induction mechanism of PEDF expression during the involuting phase remains to be studied, these findings highlight a novel mechanism of PEDF to accelerate the regression of IH.

Interestingly, PEDF is expressed as an adipokine in adipocytes differentiated from mesenchymal stem cells (Chiellini et al., 2008; Famulla et al., 2011). Because the involution of IH accompanies the appearance of adipocytes and the completely involuted hemangioma tissues are filled with fibrofatty tissues, the regulatory mechanism of the onset of IH involution may coincide with the onset of adipogenesis. Actually, the inhibition of β-adrenergic signaling with propranolol is effective in IH treatment by inducing both the apoptosis of HemECs and differentiation of hemangioma-derived stem cells into adipocytes (Wong et al., 2012). These reports and our findings that PEDF

was induced during the involuting phase of IH and inhibited the proliferation of HemECs suggest the possible involvement of PEDF not only in restraining the proliferation of HemECs but also in initiating the adipogenic commitment.

Currently, there are efforts to use PEDF for the treatment of cancer. These include developing variant forms of recombinant PEDF (Subramanian et al., 2012) and substitutions that can mimic the functions of PEDF (Konson, Pradeep, & Seger, 2010). Other method administering PEDF along with microparticles that can assist the delivery of PEDF potentiates the anti-cancer effect of PEDF (Dass, Contreras, Dunstan, & Choong, 2007). Accordingly, PEDF itself or an unidentified signaling molecule(s) for the induction of PEDF in IH could be developed for therapeutic purposes for the treatment of IH.

One of the limitations of this study is that I could not generalize PEDF expression in the involuting HemECs *in vitro* because of the limited availability of human patient-derived HemECs. Instead, I confirmed the increased expression of PEDF in the involuting phase of IH by immunohistochemical staining of various tissue sections from each phase of IH patients (Fig. 6); however, it remains to be addressed how and by what PEDF expression is induced. Thus, further studies are warranted to observe PEDF expression in multiple HemECs and identify the mechanisms regulating PEDF expression in HemECs. Nevertheless, our study provides the first step toward understanding the inhibitory role of PEDF in the involution of IH.

In conclusion, I found that PEDF was highly expressed in the involuting phase of IH and plays a role in the involution of hemangioma. PEDF, as an anti-angiogenic factor, can decrease the proliferation of HemECs, a representative

phenomenon during the process of involution of IH. I suggest that the regulatory mechanism of PEDF expression could be a potential therapeutic target to accelerate the regression of IH.

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국문 초록

서론: 혈관종은 양성종양으로서 혈관의 비정상적인 성장으로 인해 생겨난다. 가장 대표적인 혈관종은 유아 혈관종이며 주로 태어난 후에 발생하고 발병 원인은 아직 알려져 있지 않다. 다른 종양과 달리 혈관종은 초기 단계에 증식을 하고 후에는 퇴축기를 거치면서 서서히 사라지게 된다. 이러한 혈관종 퇴축 과정은 잘 알려져 있지 않은 상태이며 연구가 진행이 되고 있다. 본 연구의 가설은 혈관종 퇴축기의 원인으로서 특정 요소가 혈관내피세포에 작용할 것으로 예상된다. 연구 목적은 퇴축기의 작용하는 그 특정 요소를 확인하고 혈관종 내피세포의 어떠한 작용을 일으키는지 관측하는 것이다.

방법: 혈관종 환자로부터 혈관종 샘플을 얻고 혈관내피세포를 추출을 한 후, 증식기 및 퇴축기 간의 세포 증식률과 PCR 및 단백질 array 를 통해 각종 혈관생성 및 혈관생성억제 요소들의 발현량의 차이를 관측하였다. 추가로 혈관종 조직으로부터 면역조직화학염색을 하였다.

결과: 혈관종 내피세포에서 자신의 증식을 억제하는 자가분비 요소를 발견하였다. 여러 혈관생성 및 혈관생성억제 요소들 중에서 pigment epithelium-derived factor (PEDF)라는 요소가 혈관종 퇴축기에서 상대적으로 많은 발현량을 관측하였다. 이 PEDF 는 혈관종 내피세포의 증식을 억제하는 효과를 보였고 PEDF 의 knockdown 을 통해 억제되었던 세포 증식이 다시 회복하는 현상을 관찰하였다. 또한 혈관종 조직염

색을 통해 PEDF 가 퇴축기에서 상대적으로 많이 발현하는 것을 재차 확인 하였다.

결론: PEDF 는 혈관종 퇴축기에 많은 발현량을 보이며 혈관생성억제 요소로서 혈관내피세포의 증식을 억제한다. 따라서 PEDF 는 혈관종 퇴축 과정에서 중요한 역할을 맡고 있으며 추후 혈관종의 원인 또는 치료제 연구에 적합한 연구대상이라고 생각된다.

주요어 : 혈관종, 퇴축, 퇴행, PEDF, 혈관 내피세포

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