School of Pharmaceutical Sciences[1]; National Glycoengineering Research Center[2], Shandong University; Shandong Provincial Hospital Affiliated to Shandong University[3], Jinan, China

Preparation, characterization and anti-angiogenesis activity of endostatin covalently modified by polysulfated heparin

Tan Hai Ning[1,2], Cao Ji Chao[1], Mu Guo Ying[3], Xiao Min[1], Wang Feng Shan[1,2]

Received September 6, 2011, accepted October 25, 2011

Prof. Wang Feng Shan, School of Pharmaceutical Sciences, Shandong University, Jinan, China, 250012
fswang@sdu.edu.cn

Pharmazie 67: 622–627 (2012) *doi: 10.1691/ph.2012.1135*

To improve the stability and anti-angiogenesis activity of endostatin (ES), ES was modified by polysulfated heparin (PSH). SDS-PAGE and free amino group determination were employed to study purity and modification procedure. The inhibition of ES and PSH-ES on endothelial cell proliferation, chorioallantoic membrane (CAM) angiogenesis and choroidal neovascularization (CNV) were studied. Western blotting was employed to study the effects on the expression of vascular endothelial growth factor (VEGF) and pigment epithelium derived factor (PEDF). Changes of the secondary structure were analyzed by circular dichroism (CD) spectra and heat stability was also studied. Our study indicated that the modified product had a better heat tolerance than ES and its anti-angiogenesis activity in CAM model and CNV model were better than that of ES. More obvious down-regulation of VEGF and up-regulation of PEDF effects of PSH-ES than ES in chorioid tissues were detected. The result of CD analysis suggested that little secondary structure change was detected compared with that of ES. Compared with native ES, PSH-ES is a potential anti-tumor drug with better heat stability and better anti-angiogenesis activity both in CAM and CNV models.

1. Introduction

New blood vessels play an important role in nourishing tumor growth, which makes inhibition of vessel formation an excellent way for cancer therapy (Sato 2011). Endostatin (ES), a specific inhibitor of endothelial cell proliferation and angiogenesis, can inhibit the growth and metastasis of tumors, decrease drug resistance in long term repeating treatment when it is used in combination with other chemotherapeutic agents and is a novel and efficient therapeutical agent for cancer treatment (O'Reilly et al. 1997; Hu et al. 2008). However, as a protein drug, there are a lot of obstacles in clinical use (Folkman 2006), such as high dose to get its efficacy (Herbst et al. 2002; Abdollahi et al. 2005; Tan et al. 2008), short half-life, poor stability, and some other shortcomings just like other protein drugs.

Polysulfated heparin (PSH), a functional polysaccharide with a higher sulfate group content compared with heparin has also been reported to exert anti-angiogenesis and anti-tumor activity (Norrby 2006; Zugmaier et al. 1999). Thus, we conjugated PSH to ES, expecting to obtain a better derivative with higher heat stability and higher anti-angiogenesis activity. In general, after chemical modification, proteins could obtain a longer half-life (Hohenester et al. 1998), thus a better ES derivative was expected. In this study, a PHS-ES conjugate was prepared, its properties including heat stability and secondary structure were analyzed, the inhibitory effects on human umbilical vein endothelial cell (HUVEC) proliferation *in vitro* and the anti-angiogenesis activity in chorioallantoic membrane (CAM) model and choroidal neovascularization (CNV) model *in vivo* were investigated. The effects of ES and PSH-ES on vascular endothelial growth factor (VEGF) and pigment epithelium derived factor (PEDF) expressions in chorioid tissues were also studied by Western Blotting.

2. Investigations and results

2.1. SDS-PAGE of ES and PSH-ES conjugate

The molecular weight of ES was characterized by SDS-PAGE, and a single band was shown at the position of 20 kD (Fig. 1B). PSH-ES conjugates collected at different reaction time presented heterogeneity on SDS-PAGE, and protein bands near 35 kD showed increasing density with prolonged reaction time (Fig. 1A). After purification, purified PSH-ES conjugate presented single band near 35 kD (Fig. 1B).

2.2. Retained activity and the free amino group after modification

During the process of ES modification by PSH, it was found that the activity of ES decreased slightly during the first 8 h. During 8–48 h, the decrease rate was smaller than the first 8 h and there was a dramatically decrease in activity during 48–72 h. Free amino group modification increased when the time of modification prolonged and it corresponded to the changes of activity. During the preparation procedure of PSH-ES, the free amino group modification upgraded at relative stable speed. And a higher degree increase than the average was detected during 2–4 h.

In general, the activity decreased while the modification degree of the free amino group increased over time. But during 48–72 h,

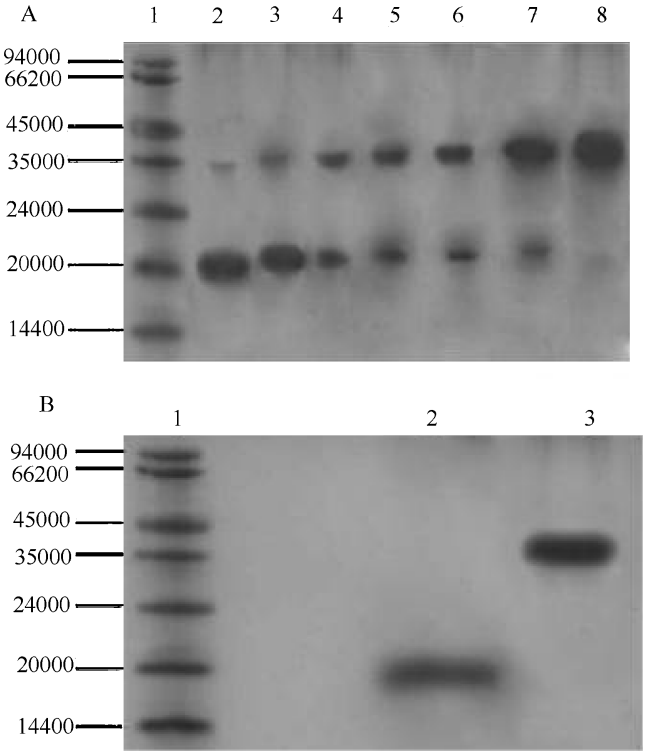

Fig. 1: SDS-PAGE analysis of ES and PSH-ES. (A) PSH-ES collected at different reaction time: lane 1, markers; lane 2–8, samples modified after 1, 2, 4, 8, 12, 24 and 48 h. (B) Purified ES and PSH-ES: lane 1, markers; lane 2, purified ES; lane 3, purified PSH-ES

there was a dramatical decrease in activity. Therefore, the ideal reaction time of the ES modification by PSH was defined to be 48 h (Fig. 2A).

2.3. Effect on cell proliferation

MTT assay was performed to analyze the anti-proliferative effects of ES, PSH-ES, and a mixture of PSH and ES on HUVEC (Fig. 2B). ES could significantly inhibit the HUVEC proliferation in a dose dependent manner. The inhibitiory rates of ES at 5 μg/ml, 10 μg/ml and 15 μg/ml were 49.8%, 58.3% and 66.4% respectively. In terms of PSH-ES, the inhibitiory rates were 38.7%, 43.6% and 50.8% at 5 μg/ml, 10 μg/ml and 15 μg/ml, respectively. The inhibitiory rates of mixture of PSH and ES were 54.1%, 59.1% and 67.7% at 5 μg/ml, 10 μg/ml and 15 μg/ml, respectively. ES has a higher inhibition on HUVEC proliferation compared with PSH-ES ($P < 0.05$), but there was no significantly difference between ES and PSH + ES. Because of the effect on the activity domain after modification, the PSH-ES group performed a decrease of inhibition on cell proliferation and it was coincide with the regular pattern of protein modification.

2.4. Heat stability of PSH-ES conjugate

The effects of heat on the activities of PSH-ES conjugate and native ES were evaluated by incubating them at 25 °C or 37 °C. The results shown in Fig. 2C (25 °C) and Fig. 2D (37 °C) indicated that the PSH-ES conjugate is more stable than native ES.

2.5. Secondary structure analysis

CD spectra data of ES and PSH-ES were processed by CDNN software. Results of ES from CD were similar with the previous report on recombinant endostatin (Sasaki et al. 2008). Relative percentage of conformation of ES was α-helix 11.9%, β-sheet 70.2%, β-turn 16.2% and β-antiparallel 1.17%, and that of PSH-ES was α-helix 10.45%, β-sheet 69.95%, β-turn 16.08% and β-antiparallel 3.52%. The result of secondary-structure analysis suggested that the percentage of β-turn, an important factor on the activity and stability, in the modified product (PSH-ES) had little change compared with that of ES. And the whole relative percentage of conformation change of PSH-ES was also not obvious compared with that of ES.

Table: Effects of ES and PSH-ES on CNV (area, $\bar{x} \pm s, n = 10$)

Groups	Area (μm^2)	
	Day 3	Day 7
PBS	18.14 ± 0.14	23.28 ± 0.10
ES	$10.65 \pm 0.16^{*}$	$14.20 \pm 0.14^{*}$
PSH + ES	10.31 ± 0.38	13.19 ± 0.17
PSH-ES	$8.93 \pm 0.21^{*\S\square}$	$11.81 \pm 0.93^{*\S\square}$

$^{*}P < 0.01$ vs. PBS group; $^{\S}P < 0.01$ vs. ES group; $^{\square}P < 0.05$ (PSH-ES vs. PSH + ES)

2.6. Effect on CAM blood vessel formation

It was reported that ES potently inhibited the neovascularization in chick embryo in a dose-dependent manner with ED50 of 0.5 μg/embryo (You et al. 1999). Thus, the protein concentration of ES, PSH + ES and PSH-ES used in this experiment were both 0.5 μg/embryo. CAM blood vessels of bFGF group grew productively and the number of vessels was 25.2 while the number of vessels of ES group, PSH + ES group and PSH-ES group were 11.3, 10.4 and 8.6, respectively (Fig. 3). Obvious inhibition of ES, PSH + ES and PSH-ES on CAM blood vessels was shown and a higher inhibition effect of PSH-ES was seen compared with that of ES and PSH + ES. When embryos were treated with more than 10 mg of ES, PSH + ES or PSH-ES, new blood vessel formation in CAM was completely inhibited and some chick embryo was not able to grow up to the next developmental stage and died thereafter.

2.7. Effect of ES and PSH-ES on the size of laser-induced CNV lesions

The areas of CNV lesions are shown in the Table. Three days after treatment, the CNV areas of PBS, ES and PSH-ES treated groups were 18.14×10^3 μm^2, 10.65×10^3 μm^2 and 8.93×10^3 μm^2, respectively. Compared with PBS, ES and PSH-ES showed significant inhibitory effects on CNV ($P < 0.05$). PSH-ES were significantly more effective than ES ($P < 0.05$). Seven days after treatment, the CNV areas of PBS, ES and PSH-ES treated groups increased and became 23.28×10^3 μm^2, 14.20×10^3 μm^2 and 11.81×10^3 μm^2, respectively. Just like the results after 3 days, ES and PSH-ES could significantly inhibit CNV ($P < 0.05$), and PSH-ES had better effects than ES ($P < 0.05$). PSH + ES can also inhibit CNV, but the effect was lower than that of PSH-ES.

2.8. Effects of ES and PSH-ES on VEGF and PEDF expression in choroid tissue

The VEGF and PEDF expressions in choroid tissue were determined by western blotting (Fig. 4). Three days after treatment, compared with control group, ES and PSH-ES showed significant down-regulating effects on VEGF expression and up-regulating effects on PEDF expression in choroid tissue.

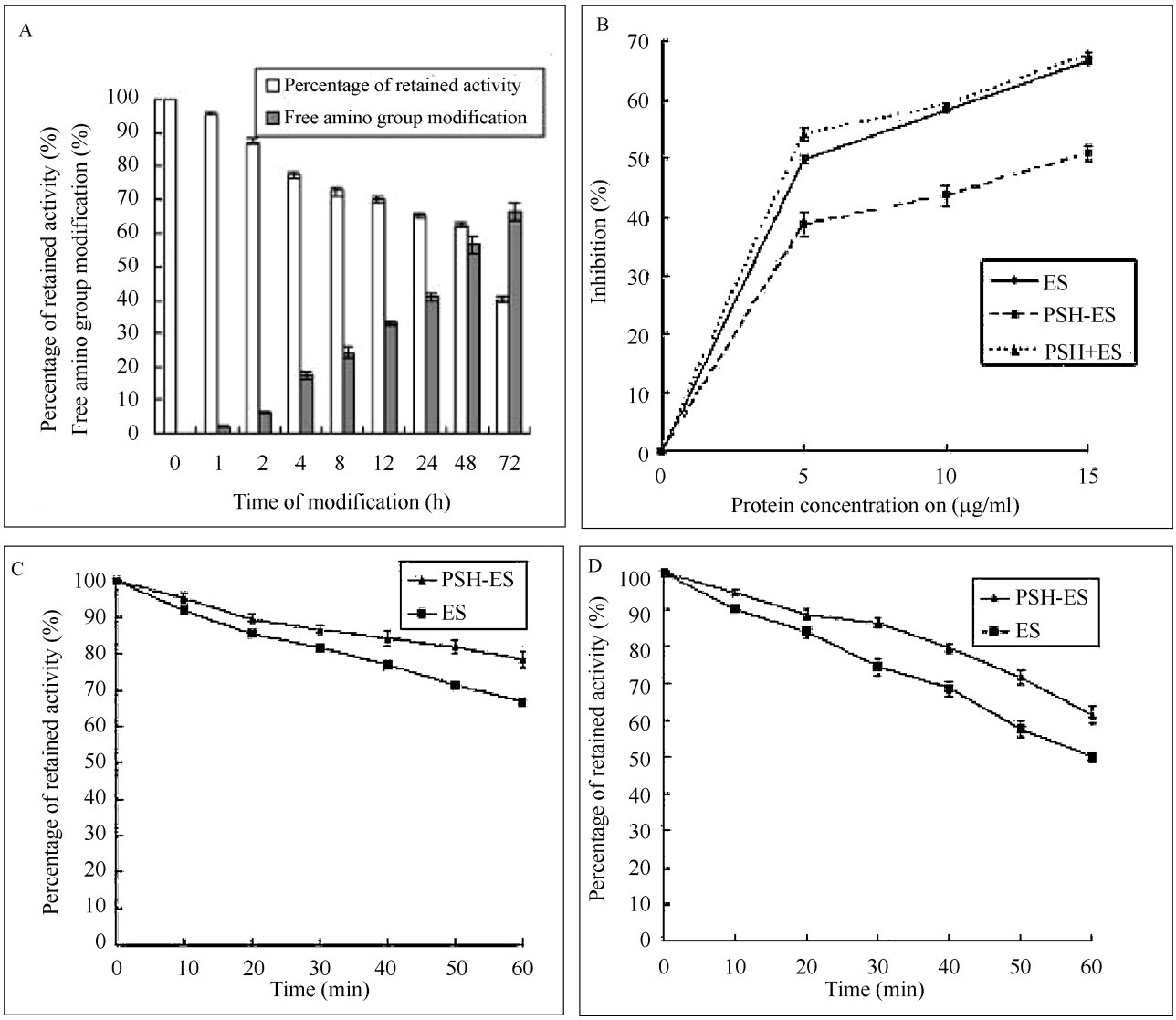

Fig. 2: Free amino group modification, bioactivity and stability analysis of ES and PSH-ES. (A) Activity assayed by MTT method with different reaction time and the degree of free amino group modification, determined using TNBS method; (B) Purified ES, PSH + ES and PSH-ES showed comparable inhibition effects on HUVEC, significant difference between the inhibition rates of ES and PSH-ES groups was observed ($P<0.05$); (C) and (D) Heat stability at 25 °C and 37 °C. Samples (protein concentration: 100μg/mL) were dissolved in 0.2 M phosphate buffer (pH 7.0) and incubated at 25 °C or 37 °C for 1 h

PSH-ES showed higher activity in down-regulating VEGF expression and up-regulating PEDF expression than ES. Seven days after treatment, the same pattern was shown.

3. Discussion

In this study, we modified ES using PSH for the first time in order to achieve better anti-angiogenesis activity. The purified PSH-ES was obtained and its heat stability, inhibition on HUVEC and angiogenesis in CAM were studied. SDS-PAGE showed that the molecular weight of PSH-ES was 35 kD. The molecular weight of the modifier PSH was studied by GPC method, and it was 5.2 kD. At the same time, the molecular weight of ES is 20 kD, so we presumed that one molecule ES conjugated with three molecules of PSH in average. The retained activity assay indicated that the activity of ES was affected by prolonging reaction time when it was modified by PSH. This indicated that purified PSH-ES had a decreased inhibitory activity on HUVEC compared with ES, which was coincide with the regular pattern of protein modification, but a more than 60% of retained activity was obtained, with an ideal reaction time of 48 h.

Due to the difficulty in obtaining a single crystal after combination with a high molecular material, such as PSH, the X-ray crystal structure of PSH-ES have not been obtained, but for the research on the structure after modification, the secondary structure of the PSH-ES was analyzed by CD. The analysis of the CD data showed that the relative conformation percentage change of PSH-ES was not obvious compared with that of ES. *In vitro*, PSH-ES presented a retained activity of about 60%, but the secondary structure change was not that obvious compared with ES. Thus, the activity decrease was not only caused by the structure change. Another reason was thought to the coating effect of the modifying agent (PSH) on ES molecular surface.

In the CAM assay, at the dose of 0.5 μg/embryo (the published ED50 of ES), PSH-ES showed the best inhibitory effect on CAM compared with ES or PSH + ES, which seems quite different from the activity of PSH-ES *in vitro*. In the heat stability assay, a better heat stability of purified PSH-ES was shown at 25 °C and 37 °C compared to ES, this may be one reason of the higher activity of PSH-ES *in vivo*. More obvious down-regulation of VEGF and up-regulation of PEDF effects of PSH-ES than ES in CNV model were also detected, this may be another reason of the higher activity of PSH-ES *in vivo*. After modification, PSH-ES may obtain a longer half-life than ES (Zhu et al. 2010), this was thought to be the third reason of the higher activity of PSH-ES *in vivo*.

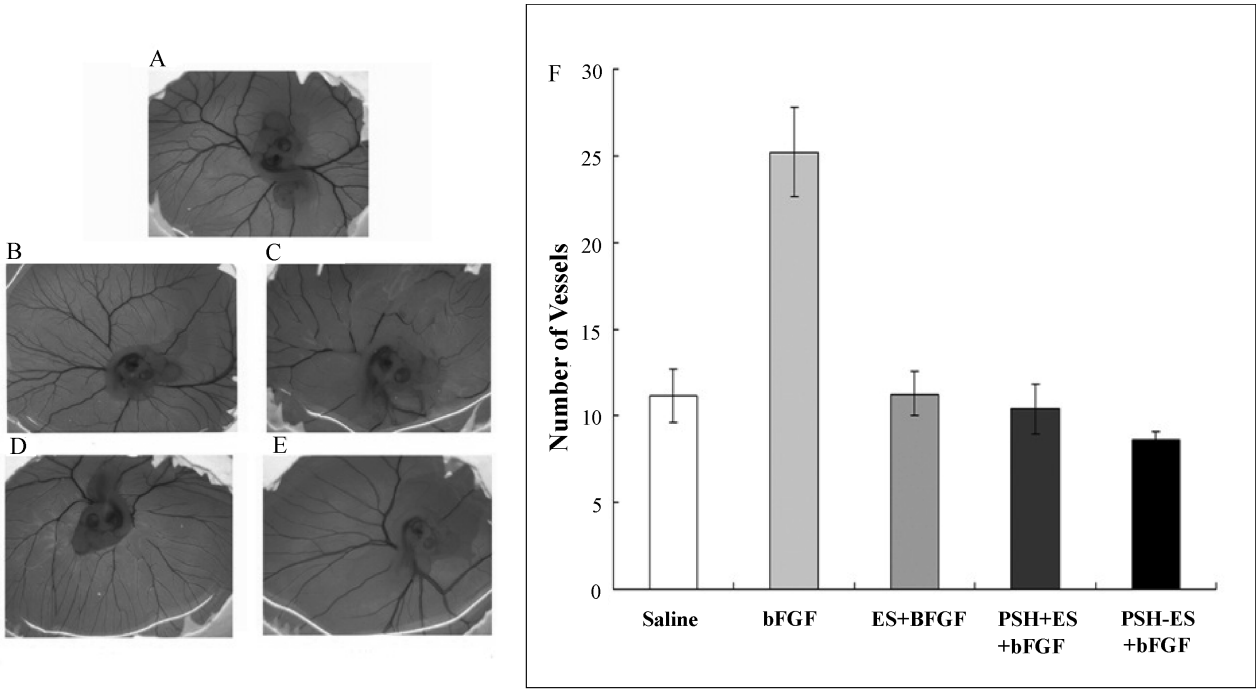

Fig. 3: Effect of ES and PSH-ES on angiogenesis of CAM. (A) saline group; (B) bFGF group; (C) ES (0.5 μg/embryo) + bFGF group; (D) PSH + ES (0.5 μg/embryo) + bFGF group; (E) PSH-ES (0.5 μg/embryo) + bFGF group; (F) Vessels number of saline, bFGF, ES + bFGF, PSH + ES + bFGF and PSH-ES + bFGF. ES and PSH-ES showed comparable inhibition effects on CAM. Higher inhibition effect of PSH-ES was seen compared with that of ES and PSH + ES

Compared with other ES derivatives (Tan et al. 2011), PSH-ES has a better heat stability than LMWH-ES and almost the same heat stability with PEG-ES. PSH-ES has very little secondary structure change, while that of PEG-ES and LMWH-ES was more obvious. The number of blood vessels of PSH-ES was 8.6, while the numbers of PEG-ES and LMWH-ES were 11.6 and 9.8 (detailed data not shown), separately. So, a better anti-angiogenesis activity of PSH-ES than LMWH-ES and PEG-ES in CAM model was detected. Better anti-CNV activity and more obvious down-regulation of VEGF and up-regulation of PEDF effects of PSH-ES than PEG-ES and LMWH-ES in CNV model were also obtained. In all, PSH-ES presented better effects than LMWH-ES and PEG-ES in these described models.

In conclusion, for the first time we successfully carried out a modification of ES by PSH. The data reported herein demonstrated that PSH-ES has small change in the secondary structure, better heat stability than ES, better anti-angiogenesis activity in CAM and CNV models and more obvious down-regulation of VEGF and up-regulation of PEDF effects than ES in chorioid tissues. Therefore, PSH-ES may be a good derivative of ES and an ideal candidate for preclinical and clinical development. Our studies are on the way to study the specific modification (Nie et al. 2006; Kodama et al. 2010) by polysaccharide PSH or analyze the modified position and confirm the half-life, immunogenicity and toxicity when they were used in animal model systems.

4. Experimental

4.1. Agents

Coomassie brilliant blue R-250 (Fluka, Germany); heparin (Dongcheng Biochemical Co. Ltd., China); PSH (Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, China); *Pichia* yeast strain containing human ES gene (Institute of Immunol-

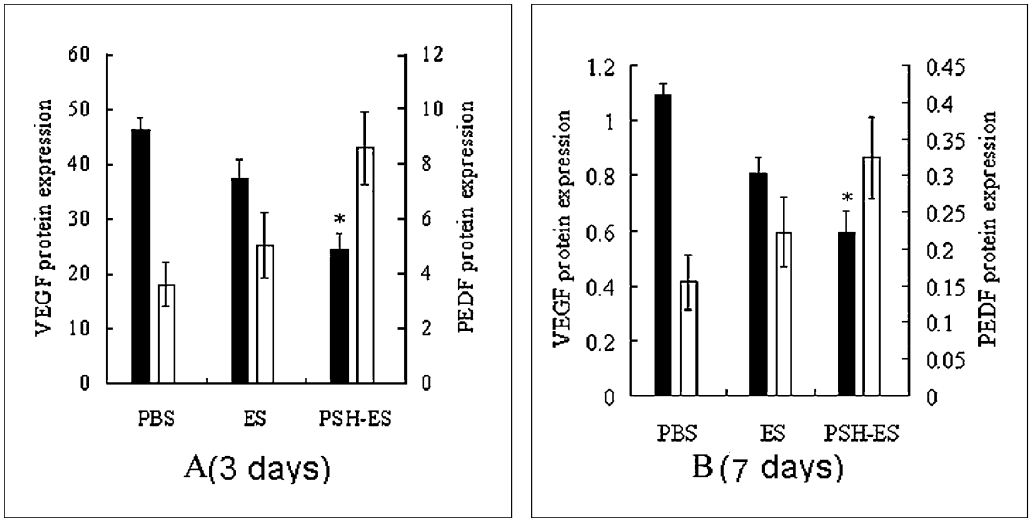

Fig. 4: The protein expression levels of VEGF and PEDF in chorioid tissue. (A) and (B) The VEGF and PEDF protein level of 3 days and 7 days, quantified by densitometric scanning and expressed as the ratio to β-actin

ogy, Medical School of Shandong University, China); Human umbilical vein endothelial cells (HUVEC, Institute of Pharmacology, School of Pharmaceutical Sciences, Shandong University, China); carboxymethylcellulose-II (Whatman, America); superdex 75 (Pharmacia, Sweden); Protein molecular weight standard (Tiangen Biotech Co. Ltd., China); 2,4,6-trinitrobenzene sulfonic acid (TNBS) and MTT(Sigma, America); β-actin (Santa Cruz, U.S.A.); horseradish peroxidase-conjugated immunoglobulin G (IgG) (Biosune Biotechnology, China); Other chemicals and reagents were of analytical grade.

4.2. Preparation of ES and PSH

ES was prepared as previously described (Tan et al. 2008). Briefly, ES was expressed in engineered *Pichia* yeast containing human ES gene. The culture supernatant of the *Pichia* yeast was purified by CM-II ion exchange column chromatography and Superdex 75 column chromatography.

PSH was prepared according to a previous method (Ji et al. 2004). Briefly, heparin was dissolved in formamide at 60 °C under constant stirring. A mixture of formamide and chlorosulfonic acid with a volume ratio of 2:1 was added into the heparin solution at 25 °C, and constantly stirred for 6 h. The reaction was stopped by alcohol precipitation. Then, 0.5% $NaHSO_3$ was added into a 10% solution of the precipitate and the pH was adjusted to 9.0 by adding Na_2CO_3. After that, the solution was kept at 65 °C for 3 h, followed adding 1.0% NaCl and adjusting the pH to 6.5. The sulfated heparin was obtained by dialysis against water and alcohol precipitation. The degree of substitution was calculated according to the ratio of carbon to sulfur by element analysis (Yoshida et al. 1995).

4.3. Preparation of PSH-ES

ES was dissolved in 0.3 M sodium carbonate buffer (pH 9.5) and PSH activated by periodate oxidation was added (Tam et al. 1976), then the solution was agitated slowly in dark at 4 °C for 48 h. During the reaction, aliquots of the solution were pipetted out at the time of 1, 2, 4, 8, 12, 24 and 48 h, respectively, for analysis. The reaction was terminated by adding glycine (Zhang et al. 2006). The solution was subjected to Superdex 75 column chromatography. The eluent containing PSH-ES was collected, desalted, concentrated and lyophilized.

4.4. SDS-PAGE

Samples collected at the time of 1, 2, 4, 8, 12, 24 and 48 h were analyzed by SDS-PAGE at ambient temperature as previously described with little modification (Li et al. 2011). Briefly, a discontinuous system was employed, the total monomer concentration of the stacking gel was 3% (w/v) and the separation gel was 15% (w/v). Dimensions of the gel immersed in running buffer (0.1% SDS, 0.05 M Tris/0.384 M glycine buffer, pH 8.3) were 8 cm × 8 cm × 0.1 cm. Samples in loading buffer (1% SDS, 1% mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, 0.01 M Tris-HCl, pH 8.0) were incubated at 100 °C for 5 min before electrophoresis. Electrophoresis was programmed to a two-step mode with applying constant current of 10 mA in stacking gel and 20 mA in the separation gel. After approximately 2 h, gels were stained with Coomassie brilliant blue R-250 overnight and destained afterwards.

4.5. Free amino group determination

The free amino group was determined by TNBS method (Habeeb 1966). To 1 ml of protein solution (0.6–1 mg/ml) 1 ml of 4% $NaHCO_3$, pH8.5, and 1 ml of 0.1% TNBS were added. The solution was allowed to react at 40 °C for 2 h. Then 1 ml of 10% SDS was added to solubilize the protein and prevent its precipitation on addition of 0.5 ml of 1 mol/L HCl. The absorbance of the solution was read at 335 nm against a blank treated as above but with 1 ml of water instead of the protein solution. Absorbances before (A_0) and after (A_X) different reaction time were read. $A_X/A_0 \times 100\%$ was the percentage of the remaining free amino group and $(1\text{-}A_X/A_0) \times 100\%$ was the modification degree of the free amino group.

4.6. Cell proliferation assay

Cell culture was performed as previously described (Han et al. 2011). HUVE cells were maintained in DMEM containing 10% heat-inactivated BCS, 3 ng/ml bFGF (UBI), and 1% antibiotics. The inhibitoy effect of ES and its modified derivatives on HUVEC proliferation *in vitro* were assayed by a MTT colorimetric assay (Tan et al, 2011; Chen et al. 2011). The endothelial cells in the exponential phase were collected and plated in a 96-well plate format at 1.0×10^4cells/well, in a volume of 200 μl. Then cells were incubated at 37 °C with humidified 95% air/5% CO_2. After ES, PSH-ES or a mixture of PSH and ES (5 μg/ml, 10 μg/ml and 15 μg/ml) was added into the wells respectively, the cells were incubated for 48 h at 37 °C in DMEM media with 10% calf serum. Then the supernatant was removed and cells were washed with PBS twice. After that, the cells were resuspended in DMEM media with 10% calf serum and incubated for 4 h at 37 °C with 20 μl of 5 mg/ml MTT solution in each well. After incubation, the supernatant was removed, 150 μl of DMSO was added to each well and the plate was agitated for 10 min. Then the absorbance at 570 nm was read, and the inhibitiory ratio (IR) was calculated according to the formula:

IR (%) = (1-Absorbance of experimental group/Absorbance of blank control group) × 100%

4.7. Heat stability determination

The unmodified ES and PSH-ES conjugates were dissolved in 0.2 M phosphate buffer (pH 7.0) and incubated at 25 °C or 37 °C for 1 h. Samples were collected at 0, 10, 20, 30, 40, 50, 60 min and their activities were determined by MTT assay. Their percentages of retained activity were calculated.

4.8. Secondary structure analysis

The secondary structure of ES and PSH-ES were analyzed by circular dichroism (CD) spectra (Whitmore et al. 2008). The spectra were measured using an Applied Photophysics Chirascan spectropolarimeter. CD spectra of ES and PSH-ES were measured using a 0.1 mm path length cell at room temperature, under the following conditions: bandwidth is 1 nm; time-per-point is 1 s. Proteins were dissolved in 50 mM sodium phosphate, pH 7.4 at the final concentration of 0.15 mg/ml-0.2 mg/ml, and filtered with 0.45 mm pore size filter unit before use. Protein concentration was measured using Lowry method (You et al. 1999). The data were processed using CDNN software.

4.9. CAM assay

To determine anti-angiogenic activity *in vivo*, CAM assay was performed as previously described with slight modification (Cao et al. 2004; Cui et al. 2009). Fertilized 3-day old eggs were incubated at 37 °C, 70% of humidity. After 2-day incubation, a small hole was made on the top of the eggs and a second hole was made directly in the middle of the eggs over embryonic blood vessels. A drop of saline was dropped on the second hole and then sucked by suction bulb from the hole on the top. CAM sagged and the artificial air chamber formed. After 48 h incubation, test samples and bFGF were added to CAM by a microsyringe. After 3-day incubation, embryos and CAMs were observed using a stereomicroscope.

4.10. CNV Assay

CNV model was generated as previously described (Tan et al. 2011; Cui et al. 2009). Mice were randomly divided into 8 groups: 1, PBS control group (3 days); 2, ES group (3 days); 3, PSH + ES group (3 days); 4, PSH-ES group (3 days); 5, PBS control group (7 days); 6, ES group (7 days); 7, PSH + ES group (7 days); 8, PSH-ES group (7 days). Samples were dissolved in PBS at a final concentration of 10 μg/μl and were injected into the cavum vitreum with a volume of 1 μl.

Days after treatment, the size of CNV lesion was evaluated by measuring the area of CNV in choroidal flat mounts. Mice used for the flat-mount technique were anesthetized and perfused as previously described (Mori et al. 2001). The eyes were removed and fixed for 1 h in a 10% formalin solution. Cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts were made from the edge of the eyecup to the equator and the eyecup was flat-mounted in Aquamount with the sclera facing down and the choroid facing up, flat mounts were examined.

4.11. Western blotting

Eyes were rapidly removed and fixed in a 10% formalin solution. Cornea and lens were removed and the entire retina was carefully dissected from the eyecup. After that, the chorioid tissue was separated from the entire retina and each chorioid tissue was used for western blotting.

Protein extracts were obtained by homogenizing frozen tissues in a pH 7.5 buffer, followed by a centrifugation at 15,000 rpm for 20 min. Chorioid tissue extracts (50 μg per lane) were separated via 15% gel electrophoresis and electroblotted onto cellulose nitrate (CN) membranes. Then CN membranes were incubated for 1 h in PBS containing 5% low-fat dry milk and incubated with a primary mouse multiclonal anti-VEGF antibody or anti-PEDF antibody, followed by a secondary horseradish peroxidase (HRP)-conjugated IgG antibody. Subsequently, specific bands were visualized.

Acknowledgements: This work was supported by National Natural Science Foundation of China (81001018) and China Postdoctoral Science Foundation funded project (20100471540).

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