Original Paper

VR-10 Thrombospondin-1 Synthetic Polypeptide's Impact on Rhesus Choroid-Retinal Endothelial Cells

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Key Words

Platelet reactive protein • VR-10 • Endothelial cell • Migration • Proliferation • Apoptosis

Abstract

Background/Aims: This study aimed to investigate the effects of the VR-10 TSP-1 synthetic polypeptide on cytokines and the proliferation and migration of endothelial cells, as well as exploring a new method for anti-ocular neoangiogenesis. **Methods:** We measured the proliferation of RF/6A cells by an MTT assay and investigated the migration of RF/6A cells by a Transwell chamber assay. We examined the mRNA transcript levels of TGF- β 2, VEGF, PEDF, Bcl-2 and FasL in RF/6A cells by RT-PCR and evaluated the expression of Fas and caspase-3 proteins in RF/6A cells by western blot analysis. **Results:** 1. TSP-1 (1 μ g/ml) and synthetic peptide VR-10 (0.1 μ g/ml, 1 μ g/ml and 10 μ g/ml) inhibited the proliferation of RF/6A cells in a time and dose-dependent way. 2. TSP-1 and synthetic peptide VR-10 could inhibit the migration of RF/6A cells in a Transwell chamber ($P < 0.001$). It was demonstrated that 10 μ g/ml synthetic peptide VR-10 had the strongest effect. 3. The expression of TGF- β 2 mRNA in RF/6A cells increased after treatment with 1 μ g/ml TSP-1 ($P < 0.0001$). However, there was no significant difference between the synthetic peptide VR-10 and the control group ($P > 0.05$). Expression of PEDF mRNA in RF/6A cells was increased after treatment with 1 μ g/ml TSP-1 and synthetic peptide VR-10. We demonstrated that 10 μ g/ml synthetic peptide VR-10 had the strongest effect ($P < 0.001$). There were significant differences between groups ($P < 0.001$). Expression of TGF- β 2 mRNA in RF/6A cells increased after treatment with 1 μ g/ml TSP-1 ($P = 0.000$). There was no significant difference between the synthetic peptide VR-10 and the control group ($P > 0.05$). PEDF mRNA expression in RF/6A cells decreased after 1 μ g/ml TSP-1 and synthetic peptide VR-10 therapy, among which 10 μ g/ml synthetic peptide VR-10 demonstrated the strongest effect ($P < 0.001$). There were significant differences between groups ($P < 0.001$), except for the 1 μ g/ml synthetic peptide VR-10 and 1 μ g/ml synthetic peptide VR-10 groups ($P = 0.615$). 4. Compared with the control group, FasL mRNA expression was significantly increased in the 10 μ g/ml synthetic peptide VR-10 treatment group; however, Bcl-2 mRNA expression was decreased. 5. Western blotting showed that RF/6A cells in the

control group mainly expressed the 32 kD procaspase-3 forms. For the 10 µg/ml synthetic peptide, VR-10 treatment group, it showed decreased expression of procaspase-3 (32 kD) and concomitant increased expression of its shorter pro apoptotic forms (20 kD). Compared with the control group, Fas protein expression significantly increased in the 10 µg/ml synthetic peptide VR-10 treatment group. **Conclusions:** Synthetic peptide VR-10 had an inhibitory action on the proliferation and migration of RF/6A cells. VR-10 inhibited angiogenesis by its combined actions, which included up-regulating the expression of an anti-angiogenesis gene, namely, pigment epithelium-derived factor (PEDF), down-regulating the expression of the pro-angiogenic vascular endothelial growth factor (VEGF), and mediated endothelial cell apoptosis.

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Introduction

Hypoxic-ischaemic oculopathy, which is one of the causes of blindness, has an adverse impact on visual function and patients' quality of life. This condition's common pathological changes in the advanced stage are retinal and choroid neovascularization. At present, the prevention and treatment approach in the clinic for neovascular diseases tends to be anti-vascular endothelial growth factor (VEGF) therapies, as well as anti-VEGF drugs [1, 2]. However, the application of these drugs remains a bottleneck problem due to the temporary nature and instability of any curative effect, spontaneous or secondary non-response, retinal and choroid complications caused by repeated treatment cycles, its need for long-term application and high treatment costs [3-5]. It is difficult to achieve a long-term and comprehensive perfect curative effect on the disease by applying a single therapy, and increasing numbers of studies on endogenous peptides [6-9] have introduced a new idea for comprehensive prevention and therapy for these diseases.

Thrombospondin-1 (TSP-1) is a 450-kD homotrimer macromolecule glycoprotein, which is characterized by its high molecular weight and complex synthesis process [9, 10]. This study was designed to evaluate the synthetic peptide VR-10 (Val-Thr-Cys-Gly-Val-Ile-Thr-Arg-Ile-Arg), which is located in the active anti-neoangiogenesis site of TSP-1. In addition to interfering with the TSP-1 C-terminal neoangiogenesis-promoting region and other regulation regions irrelevant to angiogenesis, it also effectively solved the pharmacokinetic difficulties of macromolecular peptides application in clinic. This study was conducted to determine the effects of synthetic peptide VR-10 on the proliferation and migration of endothelial cells and cytokines, and explored a new method for anti-ocular neoangiogenesis.

Materials and Methods

Evaluation of inhibition of cell proliferation by MTT and migration by Transwell chamber assays

Experimental groups: Control group (complete medium containing 10% fetal bovine serum [FBS]), 1 µg/ml intact peptide TSP-1, 0.1 µg/ml synthetic peptide VR-10 group, 1 µg/ml synthetic peptide VR-10 group and 10 µg/ml synthetic peptide VR-10 group.

Cell viability % = (optical density [OD] of cells with drugs/OD of cells in the control group) × 100%.

Migration inhibition rate % = (migrated cell count in the control group – migrated cell count in the intervention group)/migrated cell count in the control group × 100%.

Measurement of the effects of TSP-1 and VR-10 on the expression of VEGF, PEDF and TGF-β2 in RF/6A cells by RT-PCR

Experimental groups: Control group (complete medium containing 10% FBS), 1 µg/ml of intact peptide TSP-1 group, 0.1 µg/ml synthetic peptide VR-10 group, 1 µg/ml synthetic peptide VR-10 group and 10 µg/ml synthetic peptide VR-10 group.

The first cDNA chain was synthesized by RT-PCR. The primers (Shanghai Sangon Biotech Co., Ltd.) were as follows. For TGF-β2-F: 5-TCAACACATCAGAGCTCCGA-3, TGF-

β 2-R: 5-CGGTTGCTGAGGTATCGCC-3, length of the amplified fragment was 151 bp. VEGF-F: 5-AGAGCCAGACATCCCTGAG-3, VEGF-R: 5-CTCCTCCGAAGCGAGAACA-3, length of the amplified fragment was 675 bp. PEDF-F: 5-TGGACCTGCAGGAGATCAAC-3, PEDF-R: 5-GACTTGGTGACTTCGCCTTC-3, length of the amplified fragment was 460 bp. Human β -actin-F: 5-AGCGAGCATCCCCAAAGTT-3, Human β -actin-R: 5-GGGCACGAAGGCTCATCATT-3, length of the amplified fragment was 285 bp.

VR-10 (10 μ g/ml) effect on caspase-3 and Fas protein expression in RF/6A cells by western blot

Experimental groups: 10 μ g/ml synthetic peptide VR-10 treatment group and a control group (complete medium).

First, total intracellular protein was extracted and sample loading was carried out after gel preparation. Each electrophoresis lane was loaded with 20 μ l of sample to conduct the electrophoresis at an adjusted 200 V voltage. The primary antibody was added after the membrane transfer (anti-Fas, 1:500; anti-caspase-3, 1:100). The membrane was incubated overnight at 4°C, and then we added the secondary antibodies goat anti-rabbit IgG/horseradish peroxidase (HRP, 1:40,000) and goat anti-mouse IgG+A+M (H+L)/HRP (1:500) at 37°C for 1 hour. Finally, the membrane was washed with phosphate buffered saline with Tween-20 (PBST) 4 times, and the targeted strips were inspected by enhanced chemiluminescence in the dark followed by film exposure, photographic fixation and development. The gel OD analysis software Gel-Pro Analyzer 4.0 was used to analyse the positive signals, and the integrated optical density (IOD) was measured.

Analysis of the effect of 10 μ g/ml of synthetic peptide VR-10 on the expression of Bcl-2 and FasL mRNA in RF/6A cells by RT-PCR assay

The first cDNA chain was synthesized by RT-PCR. The primers were synthesized by Shanghai Sangon Biotech Co., Ltd. FASL-F: 5-ATGCAGCAGCCCTTCAATTA-3, FASL-R: 5-ATCCTACCAAGGCAACCAG-3, length of the amplified fragment was 273 bp. BCL-2-F: 5-CTGGGAGAACAGGTACGA-3, BCL-2-R: 5-ATGACCCACCGAACTCAA-3, length of the amplified fragment was 460 bp.

Statistical analysis

The data analysis was performed using the SPSS 16.0 software package. Data were presented as the mean $\pm$ standard deviation. Significance tests were performed by one-way analysis of variance (ANOVA). The differences between the two groups were analysed by least-significant difference (LSD)-t tests. The significance level was $\alpha = 0.05$. $P < 0.05$ was considered to be statistically significant.

Results

MTT assay was used to analyse the viability of RF/6A cells treated with TSP-1 and synthetic peptide VR-10

Both TSP-1 and synthetic peptide VR-10 had an inhibitory effect on RF/6A cells. As the incubation time increased, cell viability became lower. The higher the concentration of synthetic peptide VR-10 was, the lower the cell viability was. After 48 hours of incubation, minimum cell viability was 78% in the 10 μ g/ml synthetic peptide VR-10 group (Fig. 1).

Impact of TSP-1 and synthetic peptide VR-10 on migrating RF/6A cell count and migration suppression rate

TSP-1 and synthetic peptide VR-10 had an inhibitory effect ($P < 0.001$) on RF/6A cell migration. The migration suppression rate of

Fig. 1. Viability of RF/6A cells under different concentrations and exposure time to TSP-1 and VR-10 synthetic peptides.

Fig. 2. Inhibition rates of TSP-1 and VR-10 synthetic polypeptides on the migration of RF/6A cells. 1: VR-10 synthetic polypeptide (0.1 $\mu\text{g/ml}$); 2: VR-10 synthetic polypeptide (1 $\mu\text{g/ml}$); 3: VR-10 synthetic polypeptide (10 $\mu\text{g/ml}$); 4: TSP-1 intact peptide (1 $\mu\text{g/ml}$); The difference between 1 and 2 was evaluated using the least significant difference (LSD) test, $P = 0.114$.

Fig. 3. TGF- β 2, PEDF and VEGF mRNA expression by RF/6A cells detected by RT-PCR after treatment with TSP-1 and VR-10 synthetic peptide. M: marker; 1–5: TGF- β 2 mRNA; 6–10: PEDF mRNA; 11–15: VEGF mRNA; 1, 6, 11: controls; 2, 7, 12: TSP-1 intact peptide (1 $\mu\text{g/ml}$); 3, 8, 13: VR-10 synthetic polypeptide (0.1 $\mu\text{g/ml}$); 4, 9, 13: VR-10 synthetic polypeptide (1 $\mu\text{g/ml}$); 5, 10, 15: VR-10 synthetic polypeptide (10 $\mu\text{g/ml}$).

synthetic peptide VR-10 was the highest at 10 $\mu\text{g/ml}$ followed by 1 $\mu\text{g/ml}$ TSP-1. The higher the concentration of synthetic peptide VR-10 was, the higher ($P < 0.001$) the suppression rate was. However, the difference in suppression rate on RF/6A cells was not statistically significant ($P = 0.114$) between the cells treated with 0.1 $\mu\text{g/ml}$ synthetic peptide VR-10 and 1 $\mu\text{g/ml}$ synthetic peptide VR-10 (Fig. 2).

Fig. 4. Ratios of the optic density (OD) values of VEGF, TGF- β 2 and PEDF mRNA expression by RF/6A cells after treatment with TSP-1 and VR-10 synthetic peptides detected by RT-PCR. Relative to the OD value of the β -actin internal control band.

Fig. 5. Caspase-3, FAS and GAPDH expression by RF/6A cells after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic peptide detected by western blotting. M: marker; 1: Bcl-2 mRNA expression after treatment with 10 $\mu\text{g/ml}$ VR-10 synthetic polypeptide; 2: Caspase-3 mRNA expression in the control group; 3: FasL mRNA expression after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic polypeptide; 4: FasL mRNA expression in the control group; 5: β -actin mRNA expression after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic polypeptide; 6: β -actin mRNA expression in the control group.

Evaluation of TSP-1 and synthetic peptide VR-10 impact on TGF- β 2, PEDF and VEGF mRNA expression in RF/6A cells by RT-PCR

TGF- β 2: The mRNA expression level of TGF- β 2 was elevated in RF/6A cells in the 1 $\mu\text{g/ml}$ TSP-1 group compared with the

control group ($P = 0.000$), but the difference was statistically insignificant ($P > 0.05$) for all other groups compared with the blank control group (Fig. 3–4).

PEDF: PEDF mRNA expression in the RF/6A cells treated with all concentrations of TSP-1 and synthetic peptide VR-10 was enhanced compared with the control group. The expression was higher as the synthetic peptide VR-10 levels increased, among which the synthetic peptide VR-10's effect was the strongest ($P < 0.001$) in the 10 $\mu\text{g/ml}$ group. The comparative differences among all groups were statistically significant ($P < 0.001$) (Fig. 3–4).

VEGF: VEGF mRNA expression in the RF/6A cells treated with all concentrations of TSP-1 and synthetic peptide VR-10 was decreased compared with the control group. The expression level was higher as the synthetic peptide VR-10 levels decreased, among which the synthetic peptide VR-10 effect was the strongest ($P < 0.001$) in the 10 $\mu\text{g/ml}$ group. The difference was not statistically significant ($P = 0.615$) between the 1 $\mu\text{g/ml}$ synthetic peptide VR-10 group and the 1 $\mu\text{g/ml}$ TSP-1 group, but the other differences were all statistically significant ($P < 0.001$) in pairwise comparisons among the other groups (Fig. 3–4).

Apoptosis-related gene expression

The caspase-3 and FAS protein expression in RF/6A cells treated with 10 $\mu\text{g/ml}$ synthetic peptide VR-10 (Fig. 5–6)

Caspase-3 western blot bands appeared in the areas corresponding to 20 kD and 32 kD in extracts from RF/6A cells in each group. Caspase-3 protein expressed by RF/6A cells in controls was primarily observed as an inactive zymogen (32 kD). Caspase-3 proteins expressed by RF/6A cells treated with 10 $\mu\text{g/ml}$ synthetic peptide VR-10 were primarily active small molecular fragments (20 kD).

Fig. 6. Ratios of the optic density (OD) values of caspase-3 and FAS expression of RF/6A cells after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic peptide detected by western blotting. Relative to the OD value of the GAPDH internal control band.

Fig. 7. Bcl-2, FasL and β -actin mRNA expression of RF/6A cells after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic peptide detected by RT-PCR. M: marker; 1: BCL-2 mRNA expression after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic polypeptide; 2: BCL-2 mRNA expression in the control group; 3: FasL mRNA expression after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic polypeptide; 4: FasL mRNA expression in the control group; 5: β -actin mRNA expression after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic polypeptide; 6: β -actin mRNA expression in the control group.

Fig. 8. Ratios of the OD values of Bcl-2 and FasL mRNA expression to the OD value of β -actin mRNA of RF/6A cells after treatment with 10 $\mu\text{g/ml}$ of VR-10 synthetic peptide detected by RT-PCR.

The Fas protein western blot band appeared in the area corresponding to 48 kD in RF/6A cells in each group, including the control group. Fas protein expression was higher after treatment with 10 µg/ml synthetic peptide VR-10 ($P = 0.000$) compared with the control group.

2.4.2 Determination of Bcl-2 and FasL mRNA expression in RF/6A cells by RT-PCR (Fig. 7–8)

Using β -actin mRNA as the internal reference, Bcl-2 mRNA expression decreased ($P = 0.000$) in the 10 µg/ml synthetic peptide VR-10 group compared with the control group. Furthermore, FasL mRNA expression increased ($P = 0.001$) in the 10 µg/ml synthetic peptide VR-10 group compared with the control group.

Discussion

Anti-neoangiogenesis active fragments of TSP-1

TSP-1 is divided into seven structural domains, among which thrombospondin type 1 repeats (TSRs) consist of three groups of a repeated properdin-like motif. These domains are known to be the structural basis [11] for TSP-1's activity in suppressing endothelial cell proliferation, inducing endothelial cell apoptosis [12] and inhibiting neoangiogenesis by binding to CD36, heparin sulphate glycoprotein and integrin to regulate intercellular contacts, axon growth, and the activation of various protein factors such as TGF- β [13]. At present, the key points of TSP-1 structure and function research have focused on this segment. Scholars have explored these actions through chemically synthesized peptides in order to provide a theoretical basis for drug development. There are three major active fragment sequences, as follows: (1) The CSVTCG sequence [14] that can suppress fibroblast growth factor-2 (FGF-2) and VEGF-induced capillary formation in chicken chorioallantoic membrane. (2) The WSHWSPW sequence located at the 2th and 3th TSR. Prior to the CSVTCG sequence, it can suppress FGF-2-induced angiogenesis but has no effect on VEGF-induced angiogenesis. Since the WSHWSPW sequence can bind to heparin, we speculate that it plays a role in anti-angiogenesis by competing against FGF-2 to bind to the proteoglycan binding site on the surface of endothelial cells. However, a study conducted by Panetti et al [15], revealed that recombinant peptides containing the TSR sequence could only bind to heparin under lower ionic concentrations, thereby raising queries as to whether the affinity is sufficient between the WSHWSPW sequence and proteoglycans on the surface of endothelial cells in order to compete with FGF-2. (3) A study conducted by Dawson *et al* [16], discovered that the amino acid sequence, GVITRIR, which is located in the 2nd TSR, is the 3rd amino acid sequence with an anti-angiogenesis effect. This sequence lies behind the CSVTCG sequence mentioned above, and has an inhibitory effect on both FGF-2- and VEGF-induced endothelial cell migration.

The synthetic peptide VR-10 designed in this study is a decapeptide molecule containing the GVITRIR sequence, as well as the VTC amino acids. The synthetic peptide VR-10 is located in the active anti-angiogenesis fragment of TSP-1. This property not only excludes the interference of the C-terminal neoangiogenesis-promoting region of the TSP-1 and other regulation regions irrelevant to neoangiogenesis and the treatment effect but also effectively solves the pharmacokinetic difficulty in the application of macromolecular peptides in the clinic.

Anti-angiogenesis pathway of synthetic peptide VR-10

Inhibition of endothelial cell proliferation: A major step in angiogenesis is endothelial cell proliferation. TSP-1 can directly suppress the *in vitro* proliferation of rabbit corpus luteum, bovine adrenal cortex, pulmonary artery and human umbilical vein endothelial cells. This inhibitory effect is neutralized [17] by anti-TSP-1 monoclonal antibodies. This study evaluated the inhibiting effect of TSP-1 intact peptide and synthetic peptide VR-10 on RF/6A cells. Moreover, the synthetic peptide was dose-dependent.

Inhibition of endothelial cell migration: Endothelial cell migration occurs in response to cytokine chemotaxis and plays an important role in abnormal neoangiogenesis. The present study adopted the Transwell chamber assay to verify whether both TSP-1 and the synthetic peptide VR-10 have an inhibitory effect on RF/6A cell migration. Several studies have shown that TSP-1's inhibition of endothelial cell migration can be mediated [18] by the receptor CD36. CD36 expression is extensive, including by microvascular endothelial cells (except in large vessels) [19]. Therefore, microvascular RF/6A cells can be regulated by the receptor CD36. The CSVTCG sequence of TSP-1 mediates the interaction [18] with CD36. Synthetic peptide VR-10 contains the VTCG sequence, which may be determined to be a binding site to CD36. However, this property has to be validated through further studies.

Interaction between TSP-1 and cytokines: The action of cytokines is not isolated. This action is associated with the differences in the existing environment and the interactions among cytokines.

VEGF: The study results show that exogenous TSP-1 intact peptide and synthetic peptide VR-10 suppressed secretion of VEGF by RF/6A cells, which was decreased to a certain extent at the transcriptional and post-transcriptional level. TSP-1's mechanism has to be further studied. However, this peptide's effects may be involved in the anti-angiogenesis mechanism of TSP-1. The study conducted by Mousa et al [20]. revealed that exogenous TSP-1 increases VEGF secretion by RPE cells. Moreover, this action is blocked by anti- $\alpha 5$ or anti- $\alpha 5\beta 3$ antibodies but not anti- $\alpha 5\beta 5$ antibodies. This finding indicates that the TSP-1-induced RPE cell secretion of VEGF goes through the $\alpha 5\beta 3$ and $\alpha 5\beta 5$ signalling pathways. Therefore, it is believed that TSP-1 plays an angiogenesis-promoting role under the retina. The impact of exogenous TSP-1 on different cells that express VEGF may vary. This finding may explain how TSP-1 inhibits or promotes angiogenesis in different environments.

PEDF: The study on human umbilical vein endothelial cells conducted by Aparicio et al [21]. revealed that exogenous PEDF can prevent the decrease in TSP-1 caused by hypoxia and kept the inhibitors of angiogenesis at a certain level. It is believed that PEDF can block angiogenesis via blocking a hypoxia-induced decrease in other anti-angiogenesis factors such as TSP-1, rather than directly reducing the transcriptional level of VEGF-A expression, thereby decreasing and controlling angiogenesis.

This study has observed that TSP-1 intact peptide and synthetic peptide VR-10 increased secretion of PEDF by RF/6A cells. Therefore, this interaction exists between PEDF and TSP-1 as both cause and effect. The anti-angiogenesis of exogenous TSP-1 intact peptide and synthetic peptide VR-10 play either a direct role via autologous anti-angiogenesis activities or an indirect role via increasing PEDF.

TGF- β : Iruela-Arispe *et al* [22]. considers that the anti-angiogenesis activity of TSR is independent of potential TGF- $\beta 1$ activation, which is based on the replacement of necessary phenylalanine in TSR by tryptophan without affecting the capacity of the anti-angiogenesis of the peptide. In this study, synthetic peptide VR-10 had no significant effect on the TGF- $\beta 2$ mRNA and protein expression in RF/6A cells, which may be associated with the lack of the RFK and WSHWSPW sequence. Synthetic peptide VR-10 inhibits neo-angiogenesis via the TGF- $\beta 2$ -independent mechanism.

Cell apoptosis: Apoptosis is a process induced by *in vivo* and *in vitro* factors, triggering the intracellular pre-stored death procedure, and thereby resulting in cell death, also known as programmed cell death. Many anti-angiogenesis actions are implemented by inducing endothelial cell apoptosis.

Bcl-2: Bcl-2 prolongs cell survival time by inhibiting apoptosis. Therefore, Bcl-2 gene over-expression may prolong the lifespan of cells by promoting cell survival rather than stimulating cell division and proliferation and by blocking apoptosis.

Fas/FasL: Fas is also known as Apo-1 and CD95. Fas exists mainly as a membrane receptor and plays a role in signal transduction during apoptosis. FasL is a Fas ligand. Fas-FasL binding induces Fas molecules to cross-link on the cell surface. The apoptosis signals are sent to the interior of the cell to activate caspase-8 (FLICE) via regulating proteins and begin a cascade reaction that results in cell death. Therefore, FasL is also known as a death factor [23]

The study conducted by Volpert *et al* [24]. revealed continuous low expression of Fas and FasL in skin microvascular endothelial cells. TSP-1 can induce the external activation of apoptosis by up-regulating FasL expression. In Fas/FasL-deficient mice, TSP-1 was unable to suppress bFGF-induced corneal neovascularization. This finding indicates that the Fas/FasL apoptosis receptor pathway plays an important role in TSP-1-induced apoptosis.

In this study, 10 µg/ml synthetic peptide VR-10 increased FasL mRNA and Fas protein expression in RF/6A cells compared with the control group. This finding suggests that this synthetic peptide induces apoptosis through the Fas/FasL pathway.

Caspase-3: Caspase-3 is a 32 kD cysteine protease that is expressed in the non-activated state. The occurrence of apoptosis is a complicated protease cascade reaction process mediated by caspase-3 family members. Caspase-3 is a downstream effector molecule of Fas and FasL. When FasL expression is enhanced, caspase-3 activity is significantly increased [25]. The study conducted by Nör *et al.* revealed [26] that exogenous TSP-1 significantly decreased pro-caspase-3 expression in HDMEC cells. Meanwhile, the short-term pro-apoptosis state also increased. Caspase-3 inhibitor (z-DEVD-FWK) suppresses TSP-1-induced dose-dependent caspase-3 activation. Therefore, TSP-1 down-regulates angiogenesis, relying on caspase-3 activation-mediated endothelial cell apoptosis. In this study, the caspase-3 proteins in RF/6A cells were almost all inactive zymogens (32 kD) in the control group. Under the 10 µg/ml synthetic peptide VR-10 treatment, both FasL mRNA and Fas protein expression increased, thereby inducing caspase-3 proteins to become activated and produce active small molecular fragments (20 kD). It can be concluded that synthetic peptide VR-10 also down-regulates angiogenesis via caspase-3 activation-mediated endothelial cell apoptosis.

Conclusion

Synthetic peptide VR-10 plays a number of roles in inhibiting endothelial cell proliferation and migration. Synthetic peptide VR-10 participates in anti-angiogenesis by up-regulating anti-angiogenesis factor PEDF and down-regulating pro-angiogenesis factor VEGF expression through a TGF-β2-independent mechanism. The increase in apoptosis-promoting gene Fas/FasL expression activated caspase-3, while the survival gene Bcl-2 expression decreased, which mediated the apoptosis of endothelial cells, thereby inhibiting angiogenesis through their combined action.

Disclosure Statement

No conflict of interests exists.

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