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Original Research Article

Withaferin A promotes proliferation and migration of brain endothelial cells

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Abstract

Purpose: *To investigate the effect of withaferin A (WFA) on the proliferation and migration of brain endothelial cells.*
Methods: *BALB-5023 mouse microvascular cells were treated with a range of withaferin A (WFA) concentrations from 10 to 100 ng/mL. Dojindo's CCK-8 cell proliferation kit was used for the analysis of cell proliferation. Transwell cell culture inserts were used to determine the migration potential of WFA-treated endothelial cells. Absorbance was measured at 450 nm on an enzyme-linked immunosorbent (ELISA) reader.*
Results: *The results revealed a significant increase in the proliferation and migration of endothelial cells following treatment with a low concentration (30 ng/mL) of WFA compared with the higher concentration (> 10 ng/mL). The effect was further enhanced when WFA was used in combination with soluble Fas ligand (sFasL). Autocrine signaling of vascular endothelial growth factor (VEGF) by endothelial cells was significantly increased following treatment with WFA or in combination with sFasL. WFA increased the expression of Fas on endothelial cells, suggesting the involvement of sFasL in the proliferation and migration of brain endothelial cells.*
Conclusion: *Thus, WFA promotes the proliferation and migration of endothelial cells through increase in the expression of Fas and secretion of VEGF.*

Keywords: *Endothelial cells, Vascular endothelial growth factor, Microvascular, Vascular disease, Withaferin A*

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INTRODUCTION

At present the leading cause of morbidity and mortality throughout the globe is cardiovascular diseases and stroke. Endothelial cells form the covering of the vascular lumen of peripheral organs and the central nervous system. These cells produce and release substances that relax or constrict the blood vessels, which may contribute to the development of vascular failure [1]. To maintain the necessary supply of metabolic and functional requirements to various tissues formation of new blood vessels or angiogenesis has a vital role. Angiogenesis involves the proliferation and migration of endothelial cells [2]. The immune compromised mice with stroke on administration of human cord blood-derived CD34+ cells develop neovascularization in the ischemic zone and provide a favourable environment for neuronal regeneration [3]. It is reported that circulating CD34+ endothelial progenitor cells participate in neovascularization in ischemic tissues [4,5]. But the outgrowth of pre-existing vasculature is

assumed to be indispensable in the postnatal development of neovessels. Therefore the proliferation and migration ability of endothelial cells is vital for the recovery of vascular diseases, including cardiovascular diseases and stroke.

Withaferin A [1], a steroidal lactone is isolated from *Withania somnifera*, the extracts of which are used in traditional East Indian medicine [6]. Among the withanolides isolated from *W. somnifera*, withaferin A is present as a dominant compound [7,8]. Withaferin A has anti-angiogenic effect [6, 9] at micromolar doses and inhibits soft tissue sarcoma growth and local recurrence in xenograft experiments [10]. It is reported to have proapoptotic and anti-tumor activity in breast and prostate cancers [11-13]. Withaferin A acts on NF-Kb, BCL-2, FOXO3A, Hsp90, phosphorylated STAT3 and annexin II [12,14-18]. Taking cue from the literature, we designed an experiment to study the effect of withaferin A on suppression of brain tumor growth in a nude mice model with an attempt to develop potent therapeutic agent.

EXPERIMENTAL

Cell line and culture

The mouse brain microvascular endothelial cell line, BALB-5023 was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in complete medium consisting of DMEM Gluta MAX, supplemented with 1 % penicillin/streptomycin and 10 % FCS (Gibco-BRL, Melbourne, Australia).

Endothelial cell proliferation assay

For effects on cell proliferation, Dojindo's CCK-8 cell proliferation kit (Dojindo Molecular Technologies, Japan) was used. Briefly, 1×10^5 cells in 100 µL complete medium were seeded into each well of a 96-well plate. After 24 h, different concentrations of WFA (Chinese National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) or soluble FasL (Sigma, St. Louis, MO, USA) were added to each well containing endothelial cells for 48 h. 20 µL of CCK-8 solution (5 mg/mL; Dojindo, Kumamoto, Japan) was added into each well after incubation for 48 h. The cells were then incubated for 4 h more. A Multiskan Go spectrophotometer (Thermofisher Scientific, USA) was employed to measure the cell proliferation at 450 nm.The proliferation index was calculated using the equation 1.

(OD450 in the presence of WFA - OD450 in the blank control)/OD450 in the blank control × 100 %……1

Endothelial cell migration assay

Transwell cell culture inserts were used to determine the migration potential of WFA treated endothelial cells. The upper chambers of the inserts were seeded with 2×10^5 cells in 200 mL serum-free DMEM whereas the lower chamber was filled with 750 mL of DMEM containing 20 % FBS as a chemoattractant except in control wells which contained serum-free DMEM in both upper and lower chambers. After 24 h of incubation in a humidified incubator with 5 % CO_2 the non-migrated cells in the upper chamber were swabbed off. The plates were fixed, stained, and then observed under an inverted fluorescent microscope. Five field views were randomly counted in triplicates and averaged.

ELISA for VEGF

In 6-well plates, endothelial cells were treated for 72 h with either WFA or WFA and soluble FasL. The supernatant was collected and stored at -80 ^oC. For VEGF, ELISA (R&D Systems, Minneapolis, MN, USA) was performed as per the manufacturer's protocol. To each well 100 µl assay diluents, followed by 100 µl standard, control or cell culture supernatant was added. The plates were incubated at room temperature for 2 h followed by aspiration and washing. Thereafter, 100 µL conjugated secondary antibody was added into each well for 2 h at room temperature. After washing, 100 µL of substrate solution was added to each well for 30 min at room temperature. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm on an ELISA reader (Bio-Rad, Hercules, CA, USA) within 30 min. All readings were performed in triplicates.

Western blotting for Fas protein

The cells treated with WFA alone or combined with soluble FasL for 72 h were washed twice in PBS followed by addition of Lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10 % glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/mL leupeptin, 1 % NP-40, and 5 mM cocktail). Bicinchoninic acid assay (BCA) method was used to determine protein concentration. Equal amounts of protein were loaded and resolved by electrophoresis on a 10 % polyacrylamide gel. The semi-dry method was used to transfer proteins onto a polyvinylidene difluoride (PVDF) membrane which was then blocked with 5 % non-fat dry milk overnight. After

Tris-buffered saline Tween-20 (TBST) washing, membrane was incubated for 2 h with anti-Fas (dilution 1:2,000; KeyGen Biotech, Nanjing, Jiangsu, China) and then washed again with TBST before incubation with secondary antibodies for 2 h. Antigen was detected using enhanced chemiluminescence (Pierce Biotechnology, Inc, Rockford, IL, USA). All the samples were normalized to β-actin.

Statistical analysis

Each assay was performed in triplicate. The data are expressed as mean ± standard deviation (SD). SPSS 16 software was used for all statistical analyses. One-way analysis of variance (ANOVA) was used to determine significant differences. Statistical significance was set at $p < 0.05$.

RESULTS

Proliferation of endothelial cells stimulated by low-dosage WFA

The endothelial cells were treated with a range of WFA concentrations and analysed by CCK-8 cell proliferation assay kit. The results revealed that WFA stimulated the proliferation of the endothelial cells significantly at a low-dosage, ranging from 0.025 to 0.25 ng/mL, compared to the control cells ($p < 0.05$). However, treatment of the cells with WFA at higher dosage (> 100 ng/mL), resulted in the cell impairment (Figure 1). Thus, 0.25 ng/mL WFA with sFasL was used to analyse the combined effects.

Effect of sFasL on the WFA stimulated proliferation of endothelial cells

Treatment of the endothelial cells with WFA at 0.25 ng/mL induced the cell division. sFasL at the concentration of 0.16 ng/mL also induced the proliferation of endothelial cells through Fas-FasL pathway. However, the proliferation of endothelial cells was significantly higher on treatment with WFA and sFasL combination compared to WFA alone ($p < 0.05$; Figure 2).

WFA combined with sFasL enhances the migration of endothelial cells

The transwell cell culture inserts were used to study the effect of WFA on the endothelial cell migration. The results revealed that WFA promoted the proliferation of endothelial cells. Endothelial cell migration is also essential for angiogenesis. In the present study, WFA could also enhance the migration of endothelial cells, which is more evident if combined with sFasL ($p < 0.05$; Figure 3).

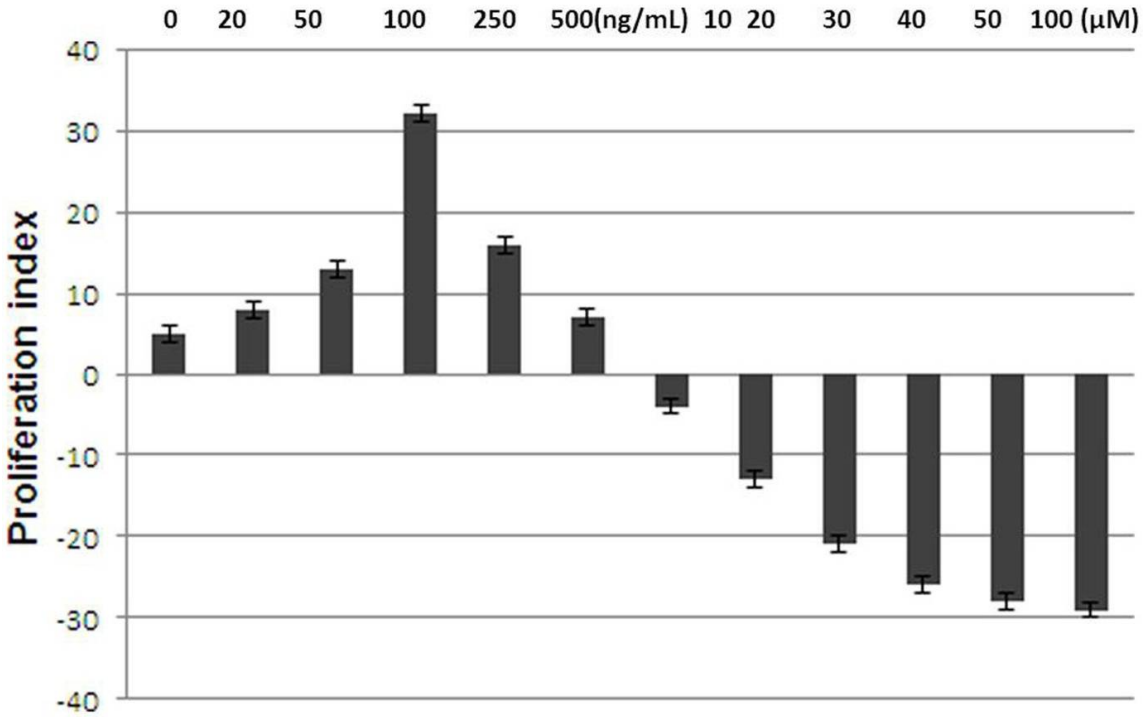

Figure 1: WFA induced proliferation of endothelial cells. Different doses of WFA (0-500 ng/mL and 10-100 ng/mL) were tested for its effect on the proliferation of endothelial cells. WFA at the lower dose (20-500 ng/mL) induced proliferation, whereas overdose (10-100 ng/mL) significantly inhibited cell growth. WFA, withaferin A

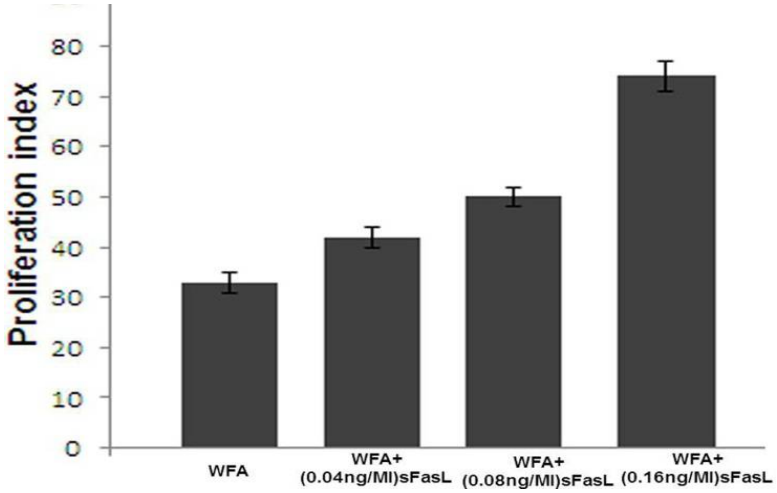

Figure 2: sFasL enhanced WFA induced proliferation of the endothelial cells. WFA at 30 ng/mL or WFA and sFasL, were used in combination for the cell culture; sFasL significantly enhanced the WFA-induced proliferation of the endothelial cells, which was dose dependent. sFasL, soluble Fas ligand; WFA, withaferin A

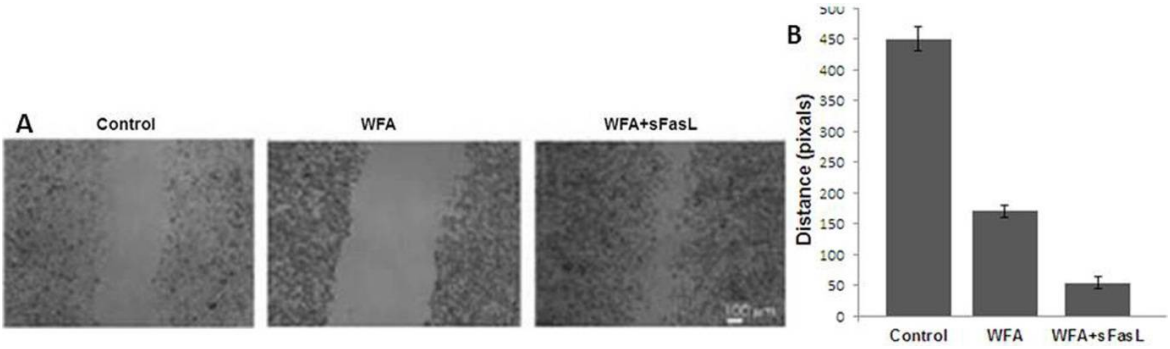

Figure 3: WFA in combination with sFasL enhanced the migration of endothelial cells. (A) WFA alone or combination of WFA and sFasL increased the migration of endothelial cells. Representative images for each group, repeated at least four times. (B) Quantification of the migration of endothelial cells. sFasL, soluble Fas ligand; WFA, withaferin A

Fas expression increases upon stimulation of WFA

WFA and sFasL could promote the proliferation and migration of endothelial cells. In addition, WFA also increased the expression of Fas on endothelial cells ($p < 0.05$; Figure 4).

WFA promotes the autocrine signaling of VEGF by endothelial cells

The proliferation and migration of endothelial cells were mediated by VEGF. Accordingly, the endothelial cells secreted significantly more VEGF upon the stimulation of WFA. sFasL combination further increased the autocrine signaling of VEGF (Figure 5).

DISCUSSION

It is reported that WFA exhibits anti-angiogenic effect [12,15] at micromolar doses and inhibits soft tissue sarcoma growth and local recurrence in xenograft experiments [16]. Withaferin A can also inhibit the NO production by modulation iNOS.

The present study demonstrates that WFA significantly enhanced the proliferation of the endothelial cells at a lower concentration (30 ng/mL). However, at a concentration > 10 µM WFA exhibited reverse effect on the proliferation of endothelial cells in the physiological condition. It was observed that overdose of WFA had a harmful effect on the endothelial cells. In addition to enhanced cell proliferation, WFA also increased the migration of the endothelial cells. Both the enhanced cell proliferation and migration were significantly promoted by sFasL. The effect of WFA on Fas-FasL pathway in the endothelial cells was also examined. It is reported that Fas-FasL ligation induces proliferation of the endothelial cells by expressing the Fas-associated death domain protein and the Flice-like inhibitory protein (FLIP).

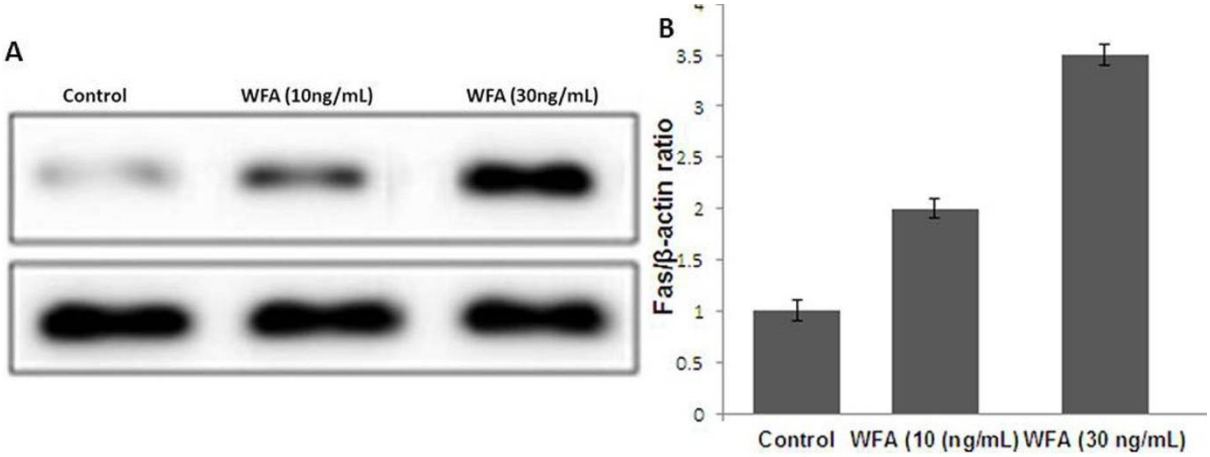

Figure 4: Fas expression increased upon the stimulation of WFA. BALB-5023 endothelial cells were treated with WFA (30 ng/mL) and the cell lysate was collected for the quantification of Fas. (A) Western blotting for the blank control or the WFA group; β-actin was used as an internal control. (B) Quantification of Fas was normalized to β-actin and is expressed as the mean ± SEM (n = 3). WFA, withaferin A

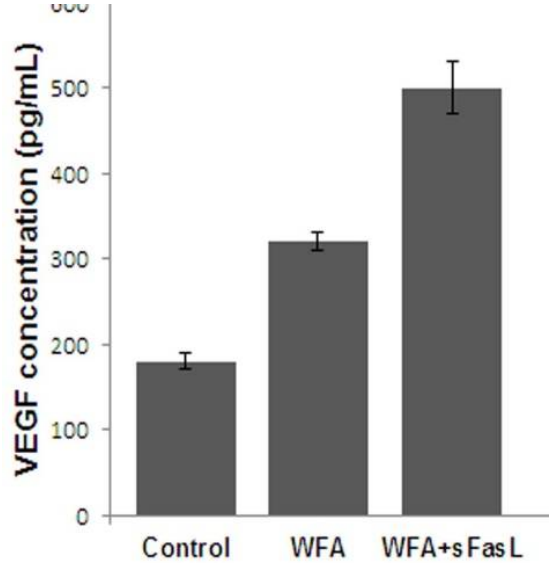

Figure 5: VEGF secretion by endothelial cells. WFA increased the autocrine signalling of VEGF by endothelial cells, which was further improved by the combination with sFasL. WFA, withaferin A; VEGF, vascular endothelial growth factor; sFasL, soluble Fas ligand

FLIP recruits and activates the downstream molecules TNF-receptor-associated factor and nuclear factor κB (NF-κB). This down regulation finally induces the proliferation of the cells. Among the downstream molecules of the Fas-FasL pathway NF-κB plays a vital role. In the present study, WFA directly up-regulated the expression of Fas, suggesting the underlying mechanisms of WFA on endothelial cells.

CONCLUSION

WFA promotes the proliferation and migration of endothelial cells and the effect is enhanced by combining it with sFasL. The effect of WFA on the endothelial cells is dependent on the increased expression of Fas and enhanced secretion of VEGF.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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