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Original Article

Effects of asiaticoside on human umbilical vein endothelial cell apoptosis induced by $A\beta_{1-42}$

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Abstract: This study is to investigate the potential role of asiaticoside (AS) in $A\beta_{1-42}$-induced apoptosis on the human umbilical vein endothelial cell (HUVEC). HUVEC cells were divided into $A\beta_{1-42}$ group (treated with 50 μM $A\beta_{1-42}$), AS groups (treated with 50 μM $A\beta_{1-42}$ and 10 mM, 1 mM, 0.1 mM or 0.01 mM AS), and negative control group (without treatments). Cell proliferation was detected by CCK-8 assay. Apoptosis was analyzed by Hochest33342 staining and flow cytometry. Western Blot was carried out to detect the expression of Bcl-2 and Bax protein. $A\beta_{1-42}$ treatment inhibited cell proliferation and increased cell apoptosis of HUVEC cells. Interestingly, AS at concentrations of 10 mM, 1 mM, 0.1 mM and 0.01 mM reversed the effects of $A\beta_{1-42}$ by increasing cell survival rate and reducing apoptosis of HUVEC cells. Furthermore, the expression of Bcl-2 protein was increased whereas the expression of Bax protein was decreased in AS groups. Compared with $A\beta_{1-42}$ group, the ratio of Bcl-2/Bax was significantly increased in AS groups ($P < 0.05$). These results suggested that AS may be effective in protecting cells from damage caused by aggregated $A\beta_{1-42}$. And this effect may be attributed to the increase of Bcl-2 and decrease of Bax under AS treatment.

Keywords: Asiaticoside, Beta amyloid peptide 1-42, human umbilical vein endothelial cells, apoptosis

Introduction

The β-amyloid protein (Aβ) is composed of 39-43 amino acids. The abnormal deposition of the aggregated $A\beta_{1-42}$ can cause apoptosis of neural cells and the injury of cerebral vascular endothelial cells, which may be one of the mechanisms underlying the pathogenesis of Alzheimer's disease (AD) [1]. It is known that AD is a vasocognopathy [2].

However, it is unclear about how Aβ can induce the damage of endothelial cells. The apoptosis and dysfunction of endothelial cells and the inflammation reaction induced by Aβ may be associated with the AD [3]. Aβ can induce the expression changes in Bcl-2 family, the apoptosis associated proteins, in vascular endothelial cells [4]. And the aggregation and deposition of Aβ can activate microglia cell to produce the tumor necrosis factor, interleukin-1 (IL-1), interleukin-6 (IL-6) and other cytokines [5, 6].

Asiaticoside (AS) is a triterpenoid saponins extracted from Centella asiatica with characteristics of sweet, acrid, and cool [7]. AS is widely used clinically for its pharmacological properties, such as anti-depression, anti-inflammatory, anti-oxidation, and anti-tumor properties [8, 9]. In recent years, it is reported that Centella asiatica can protect nerve cells, inhibit the apoptosis of neural cells, decrease pain and improve memory in rat model with AD [10, 11]. It can significantly repress the expression of IL-6 and cyclooxygenase-2 (COX-2) in acute lung injury induced by lipopolysaccharide (LPS) [12, 13]. However, it is still unclear about how $A\beta_{1-42}$ participates in the apoptosis of endothelial cells.

This study mainly focused on investigating the effects of AS on $A\beta_{1-42}$ induced apoptosis and Bcl-2/Bax expression. The findings may provide scientific evidences for treating AD with AS.

Materials and methods

Regents

Human umbilical vein endothelial cells (HUVEC) were purchased from Shanghai Baili Company

(Shanghai, China). AS was extracted by Purification engineering technology research center of Sichuan Province Natural Medicine (Chengdu, China) with purity more than 99% (Lot number: 20130107). The rabbit anti human IgGs of Bcl-2, Bax, and β-actin were purchased from Cell Signaling Technology Company (Danvers, *MA,* USA). Horseradish peroxidase (HRP) labeled goat anti-rabbit IgG, Hoechst3334, prodium iodide (PI) and CCK-8 assay kit were obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). $A\beta_{1-42}$ was from Beijing Biosynthesis Biotechnology CO., LTD. (Beijing, China) and Annexin V-FITC kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Cell culture and treatments

HUVEC were cultured in RPMI 1640 contained 10% fetal bovine serum (FBS), 0.5% penicillin and streptomycin, at 37°C, 5% CO_2. According to different treatments, cells were divided into $A\beta_{1-42}$ group, AS groups, and negative control group. For $A\beta_{1-42}$ group, cells were treated with 50 μM $A\beta_{1-42}$ for 24 h. For AS groups, cells were treated with 50 μM $A\beta_{1-42}$ and different concentrations of AS (10 mM, 1 mM, 0.1 mM and 0.01 mM AS) for 24 h. For the negative control group, HUVEC were not treated with $A\beta_{1-42}$ or AS.

CCK-8 assay

HUVEC at the logarithm growth phase were seeded in 96-well plate (1×10^5/L cells/well). After culturing for 24 h, HUVEC were treated with $A\beta_{1-42}$ or AS and were divided into negative control group, $A\beta_{1-42}$ group and AS groups, as above described. Three duplicate wells were set for each group. After culturing for 24 h, 10 μl of CCK-8 reagent was added and incubated at 37°C for 1 h. The optical density (OD) was read at 450 nm using a Model 550 microplate reader (BioRad, Hercules, CA, USA).

Hochest33342/PI staining

Cells from negative control group, $A\beta_{1-42}$ group and AS groups were re-suspended in 1 ml medium. Then 10 μl of Hochest33342 (100 mg/L) were added. After incubation for 15 min, cells were centrifuged and re-suspended in 1 ml PBS. Finally, 5 μl of PI (1 g/L) was added and incubated for 30 min. The apoptosis of cells was observed by a fluorescence microscope (CKX41, Olympus, Tokyo, Japan).

Annexin V-FITC staining and flow cytometry analysis

Annexin V-FITC staining was performed according to the instructions provided by the kit. Briefly, cells from negative control group, $A\beta_{1-42}$ group and AS groups were mixed with 5 μl Annexin-FITC and 5 μl PI and were incubated in the dark at room temperature for 15 min. The cell apoptosis was detected within 1 h by flow cytometry (SBK-YLQX-003552; BD Biosciences, San Jose, CA, USA).

Western blot

Total proteins were extracted and separated by SDS-PAGE. Then proteins were transferred onto PVDF membrane. After blocking with non-fat milk, the membrane was incubated with primary antibodies against Bcl-2, Bax, and β-actin overnight. After washing with TBST for 3 times, the appropriate HRP conjugated secondary antibodies were added and incubated at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence plus reagent. The developed film was scanned using the AlphaImager gel imaging systems (AlphaImager, Santa Clara, California, USA). And the Western blot images were analyzed using Image Pro-Plus software (Media Cybernetics, Inc., Rockville, MD, USA). The β-actin was used as an internal control.

Statistical analysis

Experimental data were expressed as mean ± standard deviation (SD) and analyzed by SPSS 16 statistical software (SPSS Inc., Chicago, IL, USA). All data were analyzed by one-way ANOVA, and SNK method was used to do comparison among groups. $P < 0.05$ was regarded as statistical significance.

Results

AS treatment reverses the inhibitory effect of $A\beta_{1-42}$ on proliferation of HUVEC

To investigate the impact of AS on proliferation of HUVEC, CCK-8 assay was performed. As shown in **Figure 1**, the proliferation of HUVEC was decreased significantly in $A\beta_{1-42}$ group compared to negative control group ($P < 0.05$), which indicated that 50 μM $A\beta_{1-42}$ could decrease the proliferation of HUVEC. Compared with $A\beta_{1-42}$ group, cell proliferation ability of

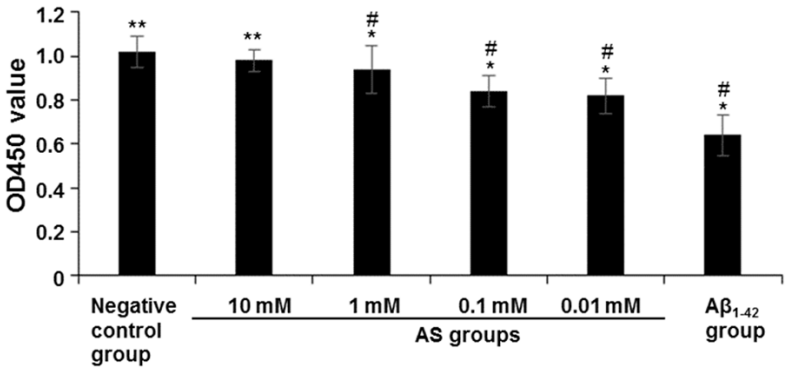

Figure 1. Analysis of proliferation of HUVEC cells. According to different treatments, cells were divided into $A\beta_{1-42}$ group (treated with 50 μM $A\beta_{1-42}$), AS groups (treated with 50 μM $A\beta_{1-42}$ and 10 mM, 1 mM, 0.1 mM or 0.01 mM AS), and negative control group (without treatments). Cell proliferation was detected with CCK-8 assay. OD value was measured at 450 nm. Compared with negative control group, $^{*}P < 0.05$. Compared with $A\beta_{1-42}$ group, $^{\#}P < 0.05$.

cells in AS groups was significantly increased ($P < 0.05$), suggesting that 10 mM, 1 mM, 0.1 mM, and even 0.01 mM of AS can recover the impact of $A\beta_{1-42}$ on the proliferation. This result indicates that AS could reverse the effect of $A\beta_{1-42}$ on HUVEC proliferation.

AS treatment alleviates the apoptosis of HUVEC induced by $A\beta_{1-42}$

To further examine the impact of AS on cell apoptosis, we first used Hochest33342 staining to detect cell apoptosis. As shown in **Figure 2A**, no apoptotic and dead cells were found in the negative control group. In $A\beta_{1-42}$ group, several cells were stained red with the bright blue nuclei, which mean that these cells were apoptotic cells. This result indicates that $A\beta_{1-42}$ can cause apoptosis of HUVEC cells. After treating with 10 mM, 1 mM, 0.1 mM, and 0.01 mM of AS, the numbers of apoptotic cells were decreased. This data showed that the effect of $A\beta_{1-42}$ on apoptosis of HUVEC cells was reduced by AS treatment.

To further verify the effect of AS on $A\beta_{1-42}$ induced apoptosis, Annexin V-FITC staining and flow cytometry analysis was then used to measure the apoptosis of HUVEC. Quantitative flow cytometry results were shown in **Figure 2B**. The apoptosis rate of negative control group was 0.82 ± 0.05 while in $A\beta_{1-42}$ group was 28.69 ± 5.92. Statistically, there was significant difference between negative control group and $A\beta_{1-42}$ group ($P < 0.05$). However, with the increase of AS concentration, the apoptosis rate of HUVEC cells gradually decreased in AS groups. The apoptosis rate of AS groups at concentration of 10 mM, 1 mM, 0.1 mM and 0.01 mM was 1.89 ± 0.19, 3.43 ± 0.25, 5.63 ± 0.21 and 12.18 ± 0.88. Compared with $A\beta_{1-42}$ group, the apoptosis rates of AS groups (10 mM, 1 mM, 0.1 mM and 0.01 mM) were all significantly lower ($P < 0.05$).

Collectively, these results indicate that $A\beta_{1-42}$ could induce apoptosis of HUVEC cells and that this effect is alleviated by AS treatment.

AS treatment increases Bcl-2/Bax ratio in HUVEC treated by $A\beta_{1-42}$

The ratio of Bcl-2 and Bax plays important roles in regulating the apoptosis of cells. In order to reveal the molecular mechanism of AS in the protection of HUVEC apoptosis induced by $A\beta_{1-42}$, we detected the expression of Bcl-2 and Bax protein in negative control group, $A\beta_{1-42}$ group and AS groups by Western Blot. As shown in **Figure 3A**, the expression level of Bax protein in negative control group was less than that in $A\beta_{1-42}$ group, while the expression level of Bcl-2 protein in the negative control group was more than in $A\beta_{1-42}$ group. As shown in **Figure 3B**, the ratio of Bcl-2/Bax was significantly higher in the negative control group than that $A\beta_{1-42}$ group ($P < 0.05$). Compared with $A\beta_{1-42}$ group, in AS group, the expression of Bax protein was decreased whereas the expression of Bcl-2 protein was increased at 10 mM, 1 mM, and 0.1 mM of AS (**Figure 3A**). The ratio of Bcl-2/Bax increased with the concentration of AS. Statistically, the concentration of 10 mM, 1 mM, and 0.1 mM significantly increased of the ratio of Bcl-2/Bax compared with $A\beta_{1-42}$ group ($P < 0.05$) (**Figure 3B**). Thus, Bcl-2/Bax ratio is decreased by $A\beta_{1-42}$ whereas this decrease could be reversed by AS treatment.

Discussion

AD is one of the most common diseases in senile dementia, and the roles of vascular factors in the initiation and progression of AD have raised much attention. The vascular abnormalities in AD included irregular angiogenesis,

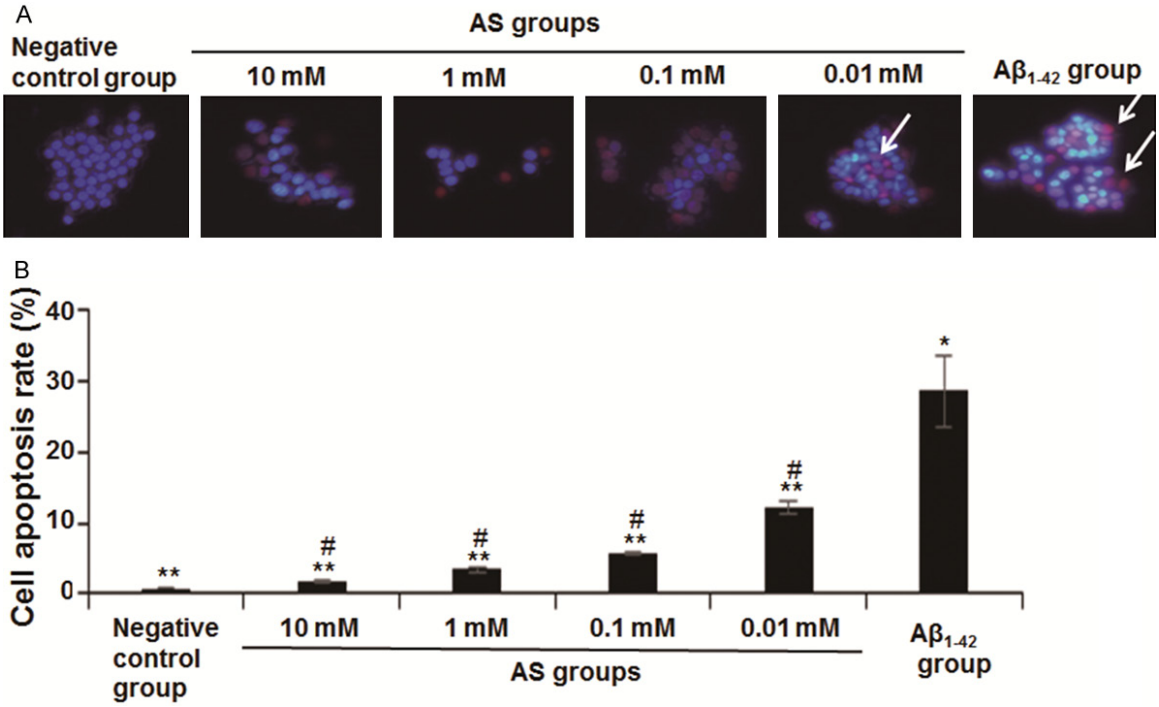

Figure 2. Analysis of apoptosis of HUVEC cells. According to different treatments, cells were divided into $A\beta_{1\text{-}42}$ group (treated with 50 μM $A\beta_{1\text{-}42}$), AS groups (treated with 50 μM $A\beta_{1\text{-}42}$ and 10 mM, 1 mM, 0.1 mM or 0.01 mM AS), and negative control group (without treatments). A. Cell apoptosis was detected with Hochest33342 staining. White arrows indicate apoptotic cells. B. Cell apoptosis was detected by Annexin V-FITC staining and flow cytometry analysis. Quantitative results were shown. Compared with negative control group, $^{*}P < 0.05$. Compared with $A\beta_{1\text{-}42}$ group, $^{\#}P < 0.05$.

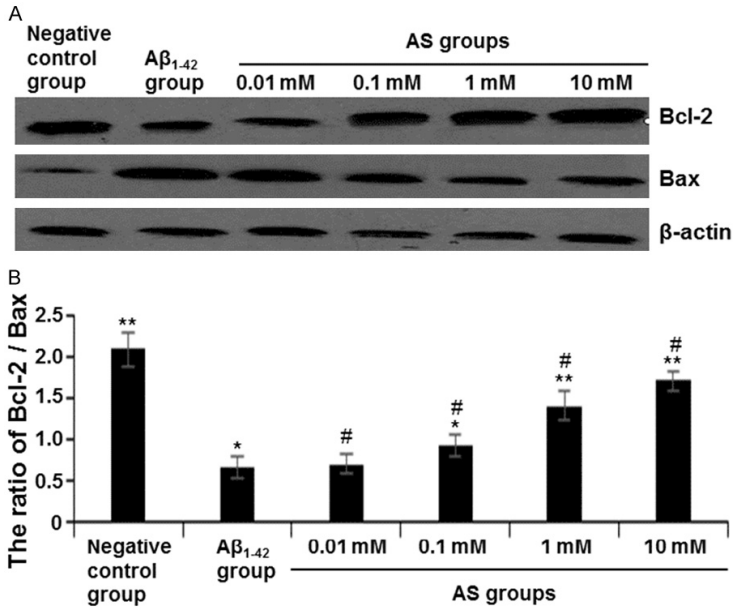

Figure 3. Analysis of Bcl-2 and Bax expression in HUVEC cells. According to different treatments, cells were divided into $A\beta_{1\text{-}42}$ group (treated with 50 μM $A\beta_{1\text{-}42}$), AS groups (treated with 50 μM $A\beta_{1\text{-}42}$ and 10 mM, 1 mM, 0.1 mM or 0.01 mM AS), and negative control group (without treatments). A. Bcl-2 and Bax expression was measured with Western Blot. Representative results were shown. B. The ratio of Bcl-2 to Bax. Compared with negative control group, $^{*}P < 0.05$. Compared with $A\beta_{1\text{-}42}$ group, $^{\#}P < 0.05$.

microvessel density decrease, atrophy of small arteries and capillaries, degeneration of vascular endothelial function, and changes in brain vascular structures [14]. The mechanism underlying Aβ-induced endothelial injury is not completely clear. Currently, it is considered that the mechanism underlying Aβ-induced endothelial injury is mainly associated with apoptosis, inflammation and endothelial dysfunction [15-17].

This study used the HUVEC as an experimental model as this cell line was more stable and easy to operate. CCK-8 assay indicated that different concentration (10 mM, 1 mM, 0.1 mM and 0.01 mM) of AS significantly increased the survival rate of HUEVC, which reversed the inhibitory effect of Aβ on cell proliferation of HUEVC. The recovery was concentration dependent. Through Hochest-

33342 staining, the number of apoptosis and death cells decreased significantly in AS groups compared with $A\beta_{1-42}$ group. The number of apoptosis and death cells also decreased significantly with the increase of AS concentration. This result was further verified by flow cytometry analysis.

The Bcl-2 family plays key roles in regulating apoptosis of cells. Bcl-2 can inhibit the apoptosis and promote the survival of cells [18]. Bax can counterwork the roles of Bcl-2 and promote the apoptosis of cells [19]. The ratio of Bcl-2/Bax determines the apoptosis status of cells [20].

The over-expression of Bax will promote apoptosis while the over-expression of Bcl-2 will promote cell survival [21]. So the apoptosis of HUVEC can be reflected by the ratio of Bcl-2/Bax. Western blot analysis showed that Bax protein expression level decreased and Bcl-2 protein expression level increased after AS treatment in HUVEC cells. And the ratio of Bcl-2/Bax increased significantly. The decrease of Bax and the increase of Bcl-2 was correlated with the concentration of AS. At 0.01 mM concentration, there was no significant difference in the ratio of Bcl-2/Bax between $A\beta_{1-42}$ group and AS group, which may indicate that small dose could not completely recover the apoptosis induced by $A\beta_{1-42}$.

In conclusion, AS could protect HUVEC from the injury induced by $A\beta_{1-42}$. And, this effect was acted through promoting cell proliferation, inhibiting apoptosis, and increasing Bcl-2/Bax ratio.

Disclosure of conflict of interest

None.

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