

Apoptosis of human umbilical vein endothelial cells (HUVEC) induced by IgA1 isolated from Henoch-Schonlein purpura children

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Summary

Objective: It had been shown that apoptosis of vascular endothelial cells played an important role in the pathogenesis of HSP. The present study was designed to investigate the apoptosis of vascular endothelial cells induced by isolated IgA1 from sera of HSP patients.

Methods: HUVEC were cultured in 3 different types of media with IgA1 from HSP patients, normal healthy children and simply medium (blank control). Serum IgA1 was purified by jacalin affinity chromatography. The rates of apoptosis in HUVEC incubated with IgA1 were determined by the TUNEL method and flow cytometry, respectively. The expression of bax/bcl-2 and p53 was detected with the methods of Real-time PCR and Westernblot, respectively]

Result: The results showed that the apoptosis rate of HUVEC by IgA1 isolated from HSP patients was higher than that the normal controls (14.77±2.23% vs 9.97±1.48%) and blank controls (14.77±2.23% vs 2.25±0.77%) ($P < 0.01$). Moreover the rate of HUVEC by IgA1 from normal healthy children was higher than the blank controls (9.97±1.48% vs 2.25±0.77%) ($P < 0.01$). In addition, the bax and P53 expression were up-regulated and the Bcl-2 expression was down-regulated in HUVEC co-cultured with IgA1 isolated from HSP patients for 24 hours.

Conclusions: These findings suggested that IgA1 from HSP patients could induce the apoptosis of HUVEC, which might be related to the vascular endothelial injury of HSP. (*Asian Pac J Allergy Immunol 2014;32:34-8*)

Key words: Henoch-Schonlein purpura (HSP), apoptosis, IgA1, human umbilical vein endothelial cells (HUVEC), P53, bcl/bax

Introduction

Henoch-Schönlein purpura (HSP) is a predominantly pediatric vasculitic syndrome. The classic tetrad of HSP includes palpable purpura without thrombocytopenia and coagulopathy, arthritis, abdominal pain and renal involvement. Pathological and laboratory research have indicated that widespread leukocytoclastic vasculitis caused by IgA, especially IgA1 deposition in the vessel walls, may played an important role in the pathogenesis of HSP.¹ Our previous research also showed that positive staining of IgA1 in HSP was found in vascular walls, dark brown antigen-antibody complex surrounding the vessel lumen could be seen and serum IgA1 was significantly increased in HSP children.² However, our understanding of the perpetuating mechanisms in HSP is still incomplete. Recent studies have revealed that the supernatant of peripheral blood mononuclear cells co-cultured with serum from HSP patients could induce the apoptosis of human umbilical endothelial cell.³ Our experiment also demonstrated apoptosis of VEC in skin tissue and there was correlation between the apoptosis of VEC and high serum IgA levels in HSP.⁴ All these results suggested that the apoptosis of vein endothelial cells might played an important role in the development of HSP. To further investigate the association between the apoptosis in vein endothelial cells and IgA1 in HSP patients, we isolated IgA1 from sera at the acute stage of childhood HSP and analyzed the apoptosis of HUVEC induced by IgA1 and its possible mechanism.

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Methods

Patients and preparation of IgA1

10 HSP patients (6 boys, 4 girls; mean age [\pm SD], 9.2 years [2.6]; range, 6-14 years), admitted to our hospital, were selected according to the EULAR/PReS endorsed consensus (2005) criteria for the classification of childhood vasculitides.⁵ Active sera were isolated from whole blood for the isolation of circulating IgA1 at the acute stage and before steroid or other immunosuppressant treatment. Ten age-matched healthy children were also enrolled as normal controls. Informed consent was obtained and the study was approved by the hospital institutional reviewboard of the First Affiliated Hospital of Anhui Medical University.

The circulating IgA1 was isolated from sera by Immobilized Jacalin (Pierce, Rockford, IL, USA), a commercial kit that can isolate serum-circulating IgA1 using a galactose-binding lectin. The level of IgA1 was assayed by ELISA methods.

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from the keyGEN Biotech Company (Nanjing, China). HUVECs were cultivated in DMEM culture medium containing 10% fetal calf serum until the third passage before experiments were performed. All the cell culture material was from Gibco, Oklahoma USA. Before stimulation HUVECs were plated in 6-well plates and divided into 3 groups. HUVECs from the control group and IgA1 groups were further cultured at 37°C for 24 h, in the absence (blank control) or presence of IgA1 (250ug/ml) at 37°C for 24 h. All the experiments were performed at least in triplicate and repeated at least twice.

Detection of apoptosis of HUVEC

For each staining, a total number of 10^6 cells per sample were washed once with ice-cold PBS, collected and suspended in 400 μ l \times Binding buffer (1×10^6 /ml). Following the addition of 5 μ l Annexin V-FITC to each well, cells were incubated in the dark for 5 min. An aliquot of 10 μ l PI was added to

each well, followed by additional incubation in the dark for 5 min, and finally flow cytometry was performed. Cells were considered to be apoptotic when they were Annexin V+/PI-.

Apoptosis of HUVEC was also detected using TUNEL (Roche, Germany) according to the supplier's instructions, and the apoptosis index (AI) was used to evaluate cell apoptosis. Apoptotic cells were quantified by counting the number of TUNEL stained nuclei per liver tissue cross section. Cross sections of 100 liver tissues per specimen were assessed and the mean number of TUNEL positive apoptotic cells per cross-section was calculated.

Cellular RNA preparation and Real-time quantitative RT-PCR

Total RNA was isolated from HUVEC cells using Trizol reagent (Invitrogen, Shanghai, China). Reverse transcription of 1 μ g of total RNA was performed by using M-MLV reverse transcriptase (200 U; Promega, China) and the products of cDNA were used as a template for PCR. The primers were designed and synthesized by Shanghai Biology Engineering Corporation (Table 1). The PCR conditions were as follows: an initial denaturation step at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 0.5 minutes, annealing at various temperatures ranging between 50 and 60°C for 1.5 minutes, and extension at 72°C for 45 seconds. After the last cycle, the extension phase was prolonged for 10 minutes at 72°C and the samples were cooled to 15°C. The PCR products were run in an agarose gel and were in all cases confined to a single band of the expected size (data not shown). $2^{-\Delta CT}$ was used to figure the expression value of Bcl-2, Bax and P53 mRNA.

Western blot analysis

HUVEC cells were collected and the Bcl-2, Bax and P53 protein concentration of each sample was determined using the Bradford dye-binding method. Protein samples (50ug) were electrophoresed on 12%-15% SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes were

Table 1. Primer pairs

Gene	Primer sequence (5'-3')		Products size (bp)
	Forward	Reverse	
Bcl-2	GGATAACGGAGGCTGGGATGCCT	CAAGCTCCCACAGGGCCAAA	117
Bax	CTGGACCCGGTGCTCAGGA	TGGTGACAGGGCCTTGAGC	244
P53	GCACTAAGCGAGCACTGCCCA	CCCTGGCTCCTTCCCAGCCT	173
actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG	205



incubated overnight at 4°C with the rabbit antibodies Bcl-2, Bax and P53 and β -actin, followed by incubation at room temperature for 1h with an HRP-labeled goat anti-rabbit secondary antibody added dropwise concurrently with the ECL illuminating agent (Immobilon TM Western Chemiluminescent HRP Substrate, Kit No. WBKLS0100). Finally, the membranes were visualized using a BIO-Rad system.

Statistical analysis

All assays were performed in triplicate and data were expressed as mean values \pm s.d. The non-parametric Mann-Whitney U test was used to compare the means of the two groups and one-way ANOVA was used to compare the means of multiple groups. Results were considered significant at a *P*-value <0.05.

Results

Apoptosis in HUVEC induced by IgA1

To evaluate the apoptosis of HUVEC induced by the purified IgA1 by Immobilized Jacalin from HSP patients, we examined its apoptosis rate using flow cytometry and The TUNEL methods, respectively. Flow cytometry demonstrated that isolated IgA1 (250 ug/ml) from HSP markedly induced the apoptosis death of HUVEC (14.77 \pm 2.23% vs 9.97 \pm 1.48%; 14.77 \pm 2.23% vs 2.25 \pm 0.77%) compared with IgA1 (250 ug/ml) from the normal control group (*P* <0.01) and the blank control group (Table 2 and Figure 1). Moreover the rate of HUVEC by IgA1 from normal healthy children was higher than that for blank controls (9.97 \pm 1.48% vs 2.25 \pm 0.77%) (*P* <0.01). In addition, TUNEL showed that isolated

Table 2. Effect of IgA1 from different groups on the apoptosis rate of HUVEC

Group	n	Apoptosis rate % (FCM)	AI % (TUNEL)
Blank control	8	2.25 \pm 0.77	2.17 \pm 0.49
HSP	8	14.77 \pm 2.23** $\blacktriangle\blacktriangle$	13.64 \pm 2.95** $\blacktriangle\blacktriangle$
Healthy control	6	7.97 \pm 1.48**	6.32 \pm 1.41**

Apoptosis rate using flow cytometry (FCM) and the TUNEL method. Flow cytometry demonstrated that isolated IgA1 (250 ug/ml) from HSP markedly induced the apoptotic death of HUVEC compared with that from the healthy and blank control groups. TUNEL also showed similar results. Data are presented as the means \pm standard deviation, ***P* <0.01, compared with blank control ; $\blacktriangle\blacktriangle$ *P* <0.01, compared with healthy control

IgA1 from HSP patients could significantly induce the apoptosis of HUVEC compared with that for the normal and blank controls (*P* <0.01) (Table 2).

Expression of Bcl-2, Bax and P53 in HUVEC estimated by Real-time quantitative RT-PCR and Western blot

To explore the apoptosis mechanism of HUVEC induced by IgA1 from HSP patients, we examined the expression of Bcl-2, Bax and P53 expression with Real-time quantitative RT-PCR and Western blot methods, respectively. PCR showed that the expression of the proapoptotic gene, bax, was significantly increased and the antiapoptotic gene, Bcl-2, were significantly downregulated in HUVEC co-cultured with IgA1 isolated from HSP patients for 24h. The P53 gene was significantly upregulated in cells treated with IgA1 for 24 h (Table 3). Western blot analysis showed that the expression of

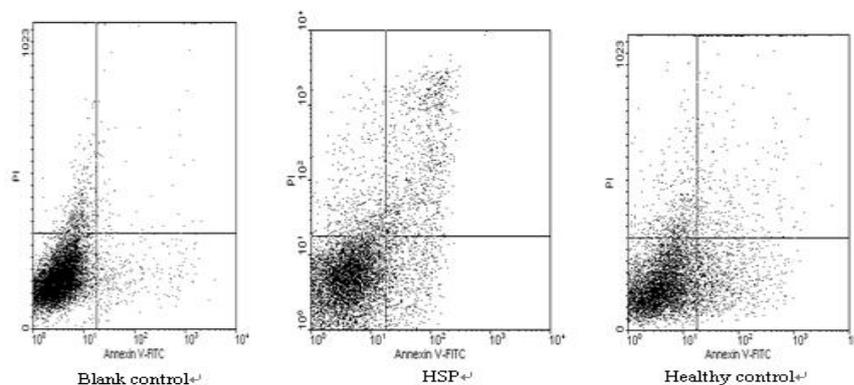


Figure 1. Effect of IgA1 from different groups on the apoptosis rates of HUVEC (FCM).

HUVEC cells were cultured in 6-well plates and treated with IgA1(250 ug/ml) from HSP patients and healthy children or left untreated for 24 h, followed by incubation with Annexin-V dye and PI. Representative histograms demonstrate the distribution of Annexin-V positive and PI positive cells.



Table 3. Expression of Bcl-2, Bax and P53 mRNA in HUVEC

Group	n	bax	Bcl-2	P53
Blank control	8	0.22±0.05	2.12±0.31	0.13±0.04
HSP	8	1.00±0.12***▲▲	1.00±0.11**▲	0.73±0.19***▲▲
Healthy control	6	0.30±0.08*	1.65±0.22*	0.18±0.06

Expression of the gene of Bcl-2, Bax and P53 examined by Real-time quantitative RT-PCR (Bcl-2, Bax and P53/ β -actin). Data are presented as the means \pm standard deviation, ** P < 0.01, compared with blank controls; ▲▲ P < 0.01, compared with healthy controls

Bcl-2 and P53 was obviously increased by incubation with IgA1 (250 μ g/ml) from HSP for 24h while bax was decreased in the HSP group (Figure 2 a). The semi-quantitative analysis results suggested that the Bcl-2, Bax and P53 expression were significantly different between the HSP group and healthy control and blank control group (Figure 2 b).

Discussion

The endothelial cells (ECs) have a key role in the regulation of local immune and inflammatory reactions and the processes of vascular injury.^{6,7} Vascular ECs can interact with complement, chemokines, or humoral components,^{6,7} express receptors for blood leukocytes or modify immune reactions,⁷ generate and respond to cytokines⁸, and modulate local vascular reactions at inflammatory sites.^{7,8} Because of the ECs' strategic location in the lining of blood vessels, they are major targets for immune-mediated injury. EC injury or death results in endothelial dysfunction that is associated with the development of atherosclerosis and its subsequent vascular events, as well as several systemic autoimmune diseases, such as vasculitis, inflammatory bowel disease, and multiple sclerosis.⁸

HSP is one of the most common small vessel forms of autoimmune vasculitis in children caused by IgA1-mediated inflammation.^{2,9} Recently, it has been shown that apoptosis in vascular endothelial cells play an important role in the VEC injury of HSP^{3,4}. In the present study we found that IgA1 isolated from HSP patients could significantly induce the apoptosis of HUVEC in contrast with IgA1 isolated from normal healthy children and controls without IgA1 using flow cytometry and the TUNEL method, which indicates that IgA1 from HSP patients has an effect on the apoptosis of endothelial cells.

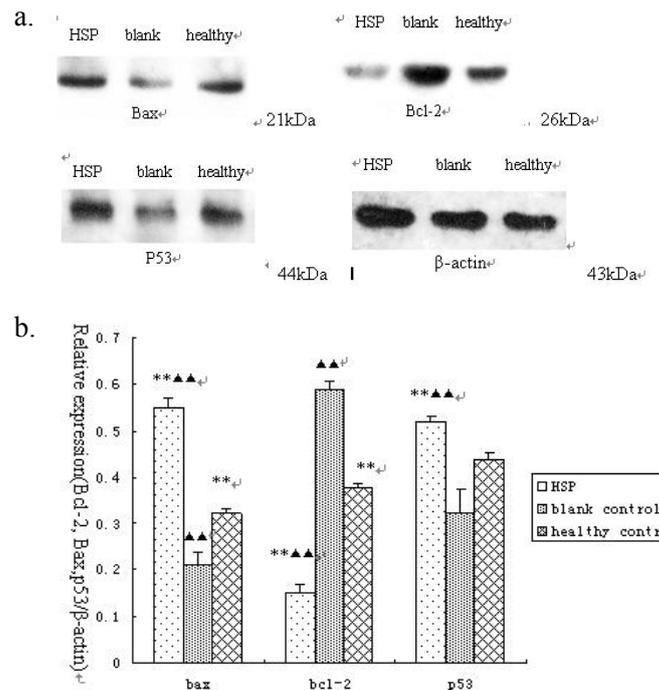


Figure 2. Expression of Bcl-2, Bax and P53 protein in HUVEC by Western blot method HUVEC cells were cultured in 6-well plates and treated with IgA1(250 μ g/ml) from HSP patients, healthy children or left untreated for 24 h, collected and determined by the Western blot method. (a) Protein electrophoresis images demonstrate the expression of Bcl-2, Bax and P53 protein. (b) Representative histograms demonstrate the result of semi-quantitative analysis of Bcl-2, Bax and P53 protein. Data are presented as the means \pm standard deviation, ** P < 0.01, compared with blank controls; ▲▲ P < 0.01, compared with healthy controls

It is known that apoptosis involves two pathways, either the death receptor pathway (Fas mediated) or the mitochondrial pathway.^{10,11} The mitochondrial pathway of apoptosis is mainly initiated by down-regulation of anti-apoptotic proteins e.g., Bcl-2 and Bcl_xL and up-regulation of pro-apoptotic proteins e.g., Bax, Bak and Bid. If pro-apoptotic protein is present in sufficient amounts to bind to and overwhelm anti-apoptotic protein, sequestered Bax and Bak are released, allowing the escape of mitochondrial cytochrome.^{11,12} This in turn activates apoptotic protease-activating factor-1 and procaspase 9 forming the apoptosome, which cleaves downstream

effector caspases 3, 6, and 7, resulting in DNA fragmentation and the characteristic morphological changes of apoptosis.¹² P53 is a tumor suppressor gene that accumulates when DNA is damaged and arrests the cell cycle to allow additional time for repair. Thus, P53 normally stimulates apoptosis, but when it is mutated or absent it favors cell survival. Many exogenous stimuli, including genotoxic agents, promote the accumulation of the p53 protein in the nucleus, which induces growth arrest and apoptosis.¹³ Our data showed that the up-regulation of Bax expression and down-regulation of Bcl-2 was observed in HUVEC co-cultured with IgA1 from HSP patients. Moreover p53 expression increased in HUVEC co-cultured with IgA1 from HSP patients. This mechanism seems to be responsible for the apoptosis of HUVEC induced by IgA1 from HSP.

In conclusion, our findings indicated that IgA1 isolated from HSP patients could induce the apoptosis of HUVEC and bax/bcl-2 and p53 signaling pathways may be related to this apoptosis.

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