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Toll-like Receptor 4 (TLR4) is Associated with Cerebral Vasospasm and Delayed Cerebral Ischemia in Aneurysmal Subarachnoid Hemorrhage

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Abstract

In the present prospective study, the Toll-like receptor 4 (TLR4) levels on peripheral blood mononuclear cells (PBMCs) were investigated in 30 patients with aneurysmal subarachnoid hemorrhage (aSAH) and in 20 healthy controls (HCs). The relationship between TLR4 levels and the occurrence of cerebral vasospasm (CVS) was also analyzed. TLR4 expression level on cell surface of PBMCs on days 1, 3, and 7 after admission was determined by flow cytometry. Results showed that patients with aSAH presented a significantly higher TLR4 levels. For patients with Hunt-Hess grades IV–V, higher TLR4 levels were also observed; higher TLR4 levels have already been seen in patients developing CVS and/or delayed cerebral ischemia (DCI). Higher TLR4 levels were also associated with modified Fisher score, occurrence of dCVS, DCI, cerebral infarction (CT), and poor neurological functional recovery. Binary logistic regression analysis indicated that high TLR4 expression on blood monocytes was an independent predictive factor of the occurrence of dCVS, DCI, and poor neurological functional recovery. Taken together, TLR4 levels on PBMCs is significantly altered in the early stage of aSAH, especially in those patients experiencing CVS and DCI. Furthermore, higher TLR4 levels in the early stage of aSAH is also associated with the neurological function outcome. As far as we know, this is the first clinical study about TLR4's significance for patients with aSAH.

Key words: aneurysmal subarachnoid hemorrhage, Toll-like receptor 4, cerebral vasospasm, delayed cerebral ischemia, peripheral blood mononuclear cells

Introduction

Cerebral vasospasm (CVS) is one of the devastating complications happening to patients with aneurysmal subarachnoid hemorrhage (aSAH), which frequently, if not necessarily, leads to delayed cerebral ischemia (DCI) and permanent neurological deficits or even death.[1–3] Unfortunately, the molecular mechanisms underlying the development of CVS remain obscure despite extensive worldwide researches and studies. The etiology and pathophysiology of CVS seem to be complex and multi-factorial.[1,4] Previous studies with animal model of aSAH have shown that, as an important player in innate immunology and regulator of inflammation process in brain, Toll-like receptor 4 (TLR4) took a crucial part in the pathogenesis of CVS and DCI.[5–10] Furthermore, whole-genome expression profiling by Kurki et al. showed that in patients with aSAH TLR4 messenger RNA (mRNA) level increased in microglia and vessel walls in the brain.[11] However, the relationship between TLR4 levels and the development of CVS and/or DCI has not been elucidated by clinical studies in aSAH patients. In the present work, TLR4 expression levels on peripheral blood mononuclear cells (PBMCs) were quantified by flow cytometry and presented as mean fluorescent intensity (MFI). What's more, its relationship with CVS and DCI was also analyzed.

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Materials and Methods

I. Study subjects

Thirty consecutive patients with aSAH admitted to the Department of Neurosurgery of Henan Provincial People's Hospital between October 2013 and October 2014 were enrolled in this prospective study. Written informed consent to participate in this study was obtained from the participants or their relatives. The study protocol was approved by the Ethics Committee of Henan Provincial People's Hospital before implementation, and was conducted in accordance with the Declaration of Helsinki and

"Good Clinical Practice" (http://www.goodclinicalpractice.com).

Inclusion criteria: aSAH confirmed by cerebral computed tomography angiography (CTA), first signs and symptoms having occurred within 48 hours before screening. Exclusion criteria: Intracerebral blood without aneurysmal bleeding source, presence of chronic infection, presence of hydrocephalus, use of interventional treatment, treated with operation within 4 weeks before admission, existing previous head trauma, neurological disease including ischemic or hemorrhagic stroke, use of antiplatelets or anticoagulant medications, and presence of other prior systemic disease including uremia, liver cirrhosis, malignancy, chronic heart or lung disease, diabetes mellitus, and hypertension.

Twenty, age- and gender matched healthy volunteers were recruited from hospital workers and relatives of the study investigators.

II. Patient management

On arrival at the emergency department, a detailed history of vascular risk factors, concomitant medication, Glasgow coma scale (GCS) score, body temperature, heart rate, respiratory rate, and blood pressure were taken. At admission, clinical severity was assessed using Hunt-Hess grade.[12] The amount of blood was assessed by Hijdra score by computed tomography (CT) as previously described,[13] and the modified Fisher score was also calculated. All patients were treated by clipping within 48 hours after admission and the CT scan was performed as soon as possible after surgery. Before surgery, patients received intravenous nimodipine at a dose of 2 mg/hour from admission; and after surgery patients were treated with 60 mg nimodipine (p.o.) every 4 hours for at least 10 days. The sedative of phenobarbital was also used after operation.

Transcranial Doppler (TCD) sonography was performed daily within the first week and every other day thereafter. Recording of the mean blood flow velocities (mBFVs) were performed using the trans-temporal ultrasound window with a 2-MHz handheld transducer probe when pCO_2 levels were within normal ranges. Doppler sonographic cerebral vasospasm (dCVS) was defined as mBFV of 120 cm/s or more in the middle cerebral artery.[14] Daily clinical assessments were performed after ictus to monitor the occurrence of DCI. DCI was defined as one or more of a new focal neurological deficit, a 2-point drop in the GCS, or a new infarct on brain imaging not visible on the admission.[15] CT scan was performed whenever clinical deterioration occurred to exclude secondary complications such as hydrocephalus, further hemorrhage, or edema. CT scans were also performed at discharge and assessed by an independent radiologist.

Demographic, clinical, and laboratory values were recorded prospectively throughout the study. Participants were followed up until completion of 3 months after SAH through structure telephone interviews performed by one doctor, blinded to clinical information. Neurological function outcome was evaluated by modified Rankin scale (mRs).

III. Laboratory measurements

In the healthy control (HC), venous blood was drawn at study entry. In the aSAH patients, blood samples were prospectively collected on days 1, 3, and 7 after admission. The blood samples were immediately placed into an ethylenediaminetetraacetic acid (EDTA) test tube. The PBMCs were isolated by density-gradient centrifugation using Ficoll-Paque Premium (GE Healthcare, Milwaukee, Wisconsin, USA) according to manufacturer's manual. Flow cytometry was employed to identify TLR4/CD14 double positive PBMCs by TLR4 and CD14 specific antibody (TLR4 antibody: Cat. No. 551964; CD14 antibody: Cat. No. 555397; BD Biosciences, San Jose, California, USA); and expression level of TLR4 on TLR4/CD14 double positive PBMCs was presented as MFI.

IV. Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA). All the data were presented as mean ± standard deviation or count (percentage) unless otherwise stated. TLR4 levels were compared by Wilcoxon rank sum test. Categorical data were compared by Chi-square test or Fisher's exact test. In order to analyze the predictive value of admission TLR4 levels, the patients were divided into high TLR4 levels group and low TLR4 levels group using the mean value of MFI (9.8) as TLR4 expression cutoff. The predictive value of TLR4 levels for dCVS, DCI, and clinical outcome was evaluated through binary logistic regression analysis, adjusting for age, sex, fever, white blood cells (WBCs) count, and C-reactive protein (CRP). A P value less than 0.05 was considered statistically significant.

Results

I. Patients' characteristics

The patients were 54.7 ± 10.1 years old. There were 19 males and 11 females. Eighteen patients were assessed with Hunt-Hess grades I–III and 12 patients with Hunt-Hess grades IV–V on admission. Fourteen patients developed DCI. Ten patients

showed poor neurological function outcome with mRs 4–5. Baseline characteristics of all patients are listed in Table 1.

II. TLR4 expression on PBMCs after aSAH

TLR4 expressions on PBMCs were detected by flow cytometry and its levels were presented as MFI. Time course of TLR4 expression level on PBMCs are indicated in Fig. 1. TLR4 levels were significantly increased after aSAH, with the highest levels on day 1 after ictus compared with day 3 and day 7 ($P < 0.05$) (Fig. 1). Although TLR4 levels on day 3 still differed significantly from that in HC ($P < 0.05$), on day 7 after ictus, TLR4 levels showed no significant difference with that of HC ($P > 0.05$) (Fig. 1). TLR4 levels on PBMCs was also correlated with the severity of aSAH on admission. As illustrated in Fig. 2A, patients with Hunt-Hess grades IV–V showed significantly higher TLR4 levels than those with Hunt-Hess grades I–III ($P < 0.05$). As indicated in Table 2, high TLR4 levels also significantly correlated with modified Fisher score ($P = 0.028$), the occurrence of dCVS ($P = 0.013$), DCI ($P = 0.011$), and cerebral infarction on CT ($P < 0.001$).

Table 1 Baseline characteristics of patients with aneurysmal subarachnoid hemorrhage

Parameter		dCVS		P value
		Present (n = 18)	Absent (n = 12)	
Age (years)		51.1 ± 10.3	58.3 ± 9.8	> 0.05
Gender				0.761
	Female	7	4	
	Male	11	8	
Location				0.818
	ICA	8	6	
	ACA	7	4	
	MCA	3	2	
Hijdra score		14.10 ± 3.15	13.84 ± 3.32	> 0.05
Hunt-Hess grade				0.550
	I–III	10	8	
	IV–V	8	4	
Modified Fisher score				0.029*
	1	1	3	
	2	3	2	
	3	5	6	
	4	9	1	
Delayed cerebral ischemia (DCI)				0.769
	Absent	10	6	
	Present	8	6	
Length of hospital stay in days, mean (range)		19.5 (12–48)	23.5 (11–44)	> 0.05
Modified Rankin scale (mRs)				0.437
	mRs 0–3	11	9	
	mRs 4–5	7	3	

Numerical variables are presented as mean ± standard deviation or mean (range). Categorical variables are expressed as counts. Numerical variables were analyzed by Mann-Whitney U test or unpaired Student's *t*-test. Categorical variables were analyzed by Chi-square test or Fisher's exact test. *P < 0.05. ACA: anterior communication artery, dCVS: Doppler sonographic cerebral vasospasm, ICA: internal carotid artery, MCA: middle cerebral artery.

III. dCVS, DCI, and neurological function recovery

Doppler sonography was used to identify CVS through monitoring the mean blood flow velocities (mBFVs) of the middle cerebral artery. Among 30 patients, 18 were diagnosed with CVS, namely dCVS. When diagnosing dCVS, the mBFV of the middle cerebral artery was 144 ± 17.8 cm/s in the 18 patients.

In order to analyze the association of TLR4 levels with the development of dCVS, TLR4 levels were compared between patients with and without dCVS occurrence by Wilcoxon rank sum test. As illustrated in Fig. 2B, significantly higher TLR4 levels were observed in patients with dCVS than in those without dCVS. Binary logistic regression analysis with covariates of age, sex, fever, WBCs count, and CRP demonstrated that TLR4 level on admission independently predicted the occurrence of dCVS

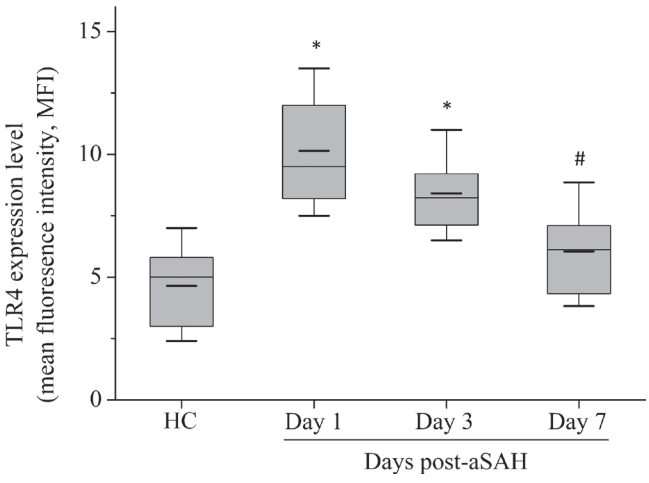

Fig. 1 Toll-like receptor 4 expression on peripheral blood mononuclear cells in healthy controls (HCs) and patients with aneurysmal subarachnoid hemorrhage (aSAH). *P < 0.05 vs. HC; #P > 0.05 vs. HC.

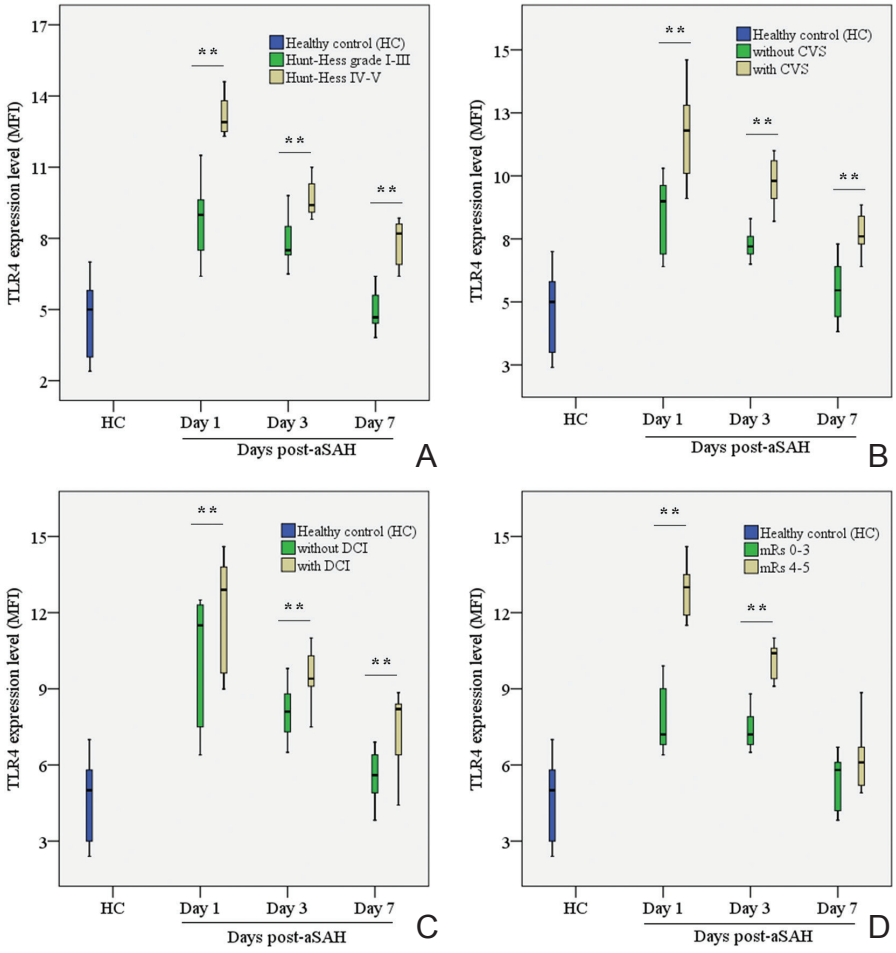

Fig. 2 Toll-like receptor 4 (TLR4) expression levels in different patient groups. A: TLR4 levels in patients with Hunt-Hess grades I–III compared to Hunt-Hess grades IV–V. B: TLR4 levels compared between patients with and without the occurrence of Doppler sonographic cerebral vasospasm (CVS). C: TLR4 levels compared between patients with and without the occurrence of delayed cerebral ischemia (DCI). D: TLR4 levels in patients with modified Rankin score (mRs) 0–3 compared to mRs 4–5. **P < 0.05, aSAH: aneurysmal subarachnoid hemorrhage.

Table 2 Correlation of Toll-like receptor 4 expression on monocytes with other clinicopathological parameters

Parameters		Admission TLR4 expression		P value
		High (n = 16)	Low (n = 14)	
Modified Fisher score				0.028*
	1 + 2	2	7	
	3 + 4	14	7	
Delayed cerebral ischemia (DCI)				0.011*
	Absent	5	11	
	Present	11	3	
dCVS				0.013*
	Absent	3	9	
	Present	13	5	
Cerebral infarction (CT)				< 0.001*
	Absent	2	11	
	Present	14	3	

CT: computed tomography, dCVS: Doppler sonographic cerebral vasospasm, TLR4: Toll-like receptor 4, *P < 0.05.

[odds ratio (OR): 4.729; 95% confidence interval (CI): 2.882–12.831; P = 0.001] (Table 3).

TLR4 levels were also associated with DCI. Significantly higher TLR4 levels were observed in patients developing DCI compared with those without DCI (P < 0.05) (Fig. 2C). Binary logistic regression analysis with covariates of age, sex, fever, WBC count, and CRP indicated that TLR4 level on admission independently predicted the occurrence of DCI (OR: 1.303, 95% CI: 0.934–2.424; P = 0.028) (Table 3).

CVS and ischemia frequently lead to poor clinical outcome. In the present study, the relationship between TLR4 levels and neurological function recovery after 3 months was also analyzed. As showed in Fig. 2D, patients with poor neurological function recovery (mRs 4–5) presented significantly higher TLR4 levels compared to those with mRs 0–3 (P < 0.05). Binary logistic regression analysis with covariates of age, sex, fever, WBC count, and CRP showed that TLR4 level on admission independently predicted the poor 3-month outcome (OR: 2.364, 95% CI: 1.722–6.008; P = 0.023) (Table 3).

Table 3 Logistic regression analysis

	OR (95%CI)	P value
dCVS		
Age	3.202 (1.596–9.842)	0.634
Sex	2.137 (1.194–4.002)	0.914
Fever	3.191 (1.026–10.023)	0.903
WBC	4.001 (1.294–8.895)	0.428
CRP	3.134 (2.080–16.668)	0.029*
Admission TLR4 expression	4.729 (2.882–12.831)	0.001*
DCI		
Age	3.062 (2.007–11.588)	0.725
Sex	2.366 (1.332–12.557)	0.924
Fever	1.737 (0.980–3.406)	0.231
WBC	2.034 (1.384–4.723)	0.019*
CRP	2.661 (1.327–6.729)	0.008*
Admission TLR4 expression	1.303 (0.934–2.424)	0.028*
Clinical outcome		
Age	1.252 (0.970–1.389)	0.304
Sex	1.066 (0.726–1.511)	0.474
Fever	1.068 (0.909–2.105)	0.100
WBC	4.083 (2.901–7.003)	0.045*
CRP	1.292 (1.002–4.683)	0.022*
Admission TLR4 expression	2.364 (1.722–6.008)	0.023*

CI: confidence interval, CRP: C-reactive protein, DCI: delayed cerebral infarction, dCVS: Doppler sonographic cerebral vasospasm, OR: odds ratio, TLR4: Toll-like receptor 4, WBC: white blood cell, *P < 0.05.

Discussion

TLRs are composed of 13 different receptors that take part in innate immunity. Among them, TLR4 is the first identified member, which is widely expressed in the brain, can recognize endogenous ligands called alarmins or danger associated molecular patterns (DAMPs),[16] and has been widely investigated in various central nervous system (CNS) injury models, especially stroke.[7–10,17] In animal models of aSAH, TLR4 is significantly increased in brain, primarily in microglia and endothelium.[7,9,10] TLR4 signaling via pathway involving myeloid differentiation primary response gene 88 (MyD88) culminates in the activation of nuclear factor kappa B (NF-κB) and of mitogen-activated protein kinases, which is increasingly recognized as an crucial player in the pathogenesis of neuronal damage after aSAH.[6–11] As one of the most important downstream molecules in TLR4 signaling pathway, NF-κB is a transcriptional factor required for the gene expression of many inflammatory mediators, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), IL-6, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), all of which have been illustrated to show significant association with complications of aSAH, such as vasospasm, ischemia, and neuronal apoptosis.[18–24] Furthermore, studies in rabbit model of aSAH show that TLR4 expression upregulation correlates with the occurrence of CVS,[9] and administration with peroxisome proliferator-activated receptor (PPAR) γ agonist, rosiglitazone, inhibits both TLR4 upregulation and vasospasm.[25] Recent work with gene knockout mice has confirmed the vital role of TLR4 in SAH-induced vasospasm and neuronal cell death.[6] Still, TLR4 is not extensively investigated in aSAH patients so far, and the relationship between TLR4 and CVS is unknown at present.

In this study, TLR4 levels on PBMCs in aSAH patients were determined and expressed as MFI. Our data showed that significantly higher TLR4 levels were observed in aSAH patients compared with HC. Admission TLR4 level was highest and thereafter TLR4 level decreased continuously, and on day 7 after ictus TLR4 levels showed no significant difference between aSAH patients and HCs. TLR4 levels on PBMCs also correlated with the severity. The more severe the disease was on admission, the higher TLR4 level as assessed by Hunt-Hess grade. Significant higher TLR4 levels were also identified in those patients developing CVS or DCI, or with poor clinical outcome. Results of binary logistic regression analysis further demonstrated that TLR4 levels on admission independently predicted the occurrence of CVS or DCI or poor clinical outcome.

Our work firstly demonstrated the close association of TLR4 levels on PBMCs with the occurrence of dreaded complications and poor clinical outcome in aSAH patients, and primarily proved its predictive value, which further illustrated the importance of TLR4 mediated inflammation in the pathophysiology of aSAH, as proved in animal experiments.[6,19–21,24,26–30] However, it should be noted that in current study the diagnosis of CVS was based on TCD evaluations and not on digital subtraction angiography. Although the observed incidence of dCVS was within the known ranges,[31] we might have missed some patients with CVS since the sensitivity of TCD in detecting angiographic CVS in not 100%.[32,33] Furthermore, among patients enrolled in this study, 18 patients were evaluated as Hunt-Hess grades I–III on admission, and 12 patients with Hunt-Hess grades IV–V, and DCI occurred in 14 patients; the

relatively high incidence of DCI in this study might be caused by the fact that the ratio of patients with Hunt-Hess grades IV–V was too high. And also, there were 6 patients among 12 who had suffered from DCI without spasm; we thought that the high incidence of DCI without spasm might be caused by hemodynamic change after clipping surgery which might unstabilize the vascular plaque and make it come off. Finally, this is a single-center study with limited number of subjects. Large-sampling and multi-centered study should be performed in the future in order to confirm our results and conclusions.

In conclusion, this is the first report of the temporal dynamics of TLR4 levels on PBMCs and its relationship with CVS, DCI, and poor clinical outcome. TLR4 levels on PBMCs showed an initial acute increase after ictus and continuous decline to the baseline values in patients with aSAH. Admission TLR4 level on PBMCs is associated with CVS, DCI, and poor clinical outcome, and can be used as an independent predictor for their occurrence. However, at present, no data were obtained to determine whether elevation of TLR4 expression on blood monocytes reflected the TLR4 expression status in the brain. Whether elevation of TLR4 expression on monocytes was the cause of dCVS and DCI or just the result of initial brain damage was also unknown. To answer these questions further investigations should be performed in the future.

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Conflicts of Interest Disclosure

The authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices in the article.

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