The Role of Pro-Inflammatory Cytokines in Neuron Apoptosis in Rats Subject to Subarachnoid Hemorrhage

Chih-Zen Chang1-3*, Shu-Chuan Wu2, Chih-Lung Lin1,2 and Aij-Lie Kwan1,2
1Department of Surgery, School of Medicine, Kaohsiung Medical University, Taiwan
2Division of Neurosurgery, Department of Surgery, Kaohsiung Medical University Hospital, Taiwan
3Department of Surgery, Kaohsiung Municipal Ta Tung Hospital, Taiwan

Abstract

Vasospasm remains a serious complication associated with subarachnoid hemorrhage (SAH). Successful management of vasospasm does not meet satisfactory outcome; therefore, our group decided to examine subarachnoid hemorrhage-induced sterile inflammation and neuron apoptosis in a rodent SAH model over the past decade.

Summary of Report: A rodent double hemorrhage SAH model was employed. CSF samplings were obtained for pro-inflammatory cytokines evaluation. NeuN immune staining was for the neuron. Mitochondria-related apoptotic factors such as cleaved caspase-3 and -9a were examined (rt-PCR). 10ng/ml IL-1β intra-thecal administration was to test the down-regulation of cytokine dependent apoptosis. Vacuolated nucleus was observed in cortex of SAH rats. The IL-1βmRNA level responded to the stimuli of SAH immediately, followed by elevated IL-6. The level of TNF-α reached a peak at 96hr after the induction of 2nd SAH. Activated caspase-3 and -9a mRNA levels were observed elevated 96hr in this model. Administration of IL-1β protein in the sham-operated group did not show the tortured lumen in basilar artery but increased active caspase-3 and -9a in the cortex as the stimuli of SAH.

Conclusions: We conclude that the surge of cytokines after the induction of SAH in rats tends to have a significant vasospasm, and induced cleaved caspase-9a mRNA. The increased IL-1β level added to the neuron apoptosis in the sham-operated rats, which indicated IL-1β partly contributes to SAH-induced apoptosis.

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a persistently major cause of high morbidity and mortality in patients with a ruptured aneurysm [1,2]. Increased evidence reveals multifaceted mechanisms contribute to the final pathogenesis and still lead to poor manifestations. For years, despite efforts to reverse vasoconstriction of cerebral arteries, therapies trying to achieve better outcomes were not observed in SAH patients [3-6]. SAH-induced early brain injury (EBI) is believed to play a significant role in deteriorated brain function among SAH patients [7,8]. These acuminated results concerning the pathogenesis of SAH-induced EBI encompass delayed neuron apoptosis contributing significantly to the high death and disability in both in vivo and in vitro studies [9].

Oxy-hemolysate in the subarachnoid space is sufficient to induce acute arteries and arteriolar constriction, passive venous obliteration and delayed arterial spasm [4,10]. An ongoing body of direct and indirect evidence publicized the mechanisms to produce EBI include oxidative stress [2], nitric oxide(NO)/nitric oxide synthase (NOS) uncoupling [6], matrix metalloproteinase 9 (MMP-9) induced blood-brain barrier disruption [11], modulation of nuclear factor erythroid-related factor 2 (Nrf2) pathway [12], activation of c-Jun N-terminal kinase pathway [6], increased proinflammatory cytokines, vascular endothelial growth factor, and mitogen-activation protein kinase [13]. Owing to a paucity of effective therapies, studies have focused on pro-inflammasomes, stress oxidative free radicals, mitochondrial dependent apoptosis, and c-Jun N-terminal kinase pathway.
Taking these findings together, we designed this study to evaluate the relationship between SAH-induced apoptosis and CSF levels of pro-inflammatory cytokines. The effects of early released cytokines (IL-1β) on the prevention of EBI after SAH and on alterations in mitochondria-related activated caspase-3 and -9a were estimated.

**MATERIAL AND METHODS**

**Materials**

Cleaved caspase-3 & -9a primers were purchased from Med Club Scientific Co., Ltd., Taoyuan 330 Taiwan authorized by Integrated DNA Technologies, Inc. Anti-rat interleukin-1β, monoclonal anti-rat interleukin -6, -8, NF-κB (p50/p65) and horseradish peroxidase-labeled rabbit anti-rat IgG antibodies were obtained from Abcam (Cambridge, MA 02139 USA), BD Transduction Lab (BD Biosciences, San Jose, CA 95128 USA) and Chemicon International (Temecula, CA 92590 USA) respectively.

**Induction of experimental double hemorrhage SAH**

Thirty-six male Sprague-Dawley rats (bought from BioLasco Taiwan Co., Ltd., Taipei 107 Taiwan authorized by Charles River), each weighing between 300–400 g were enrolled in this study. All the experimental protocols were approved by the University of Kashihung Medicine Animal Research Committee and stand by the Declaration of Helsinki (1964). The animals received anesthesia by an intramuscular injection of a mixture of 0.9 mg/100gm xylazine and 5.5 mg/100gm ketamine hydrochloride, while 0.3ml fresh arterial blood was obtained from the central tail artery and injected into the cisterna magna via a 30-gauge needle under a stereotactic apparatus (Stoelting, Wood Dale, IL 60191 USA). At completion of the induction, the animals were placed in ventral recumbency, where they remained for 15min to allow ventral blood clot formation. They were habituated to a 12hr light-dark cycle, and given access to food and water ad libitum. Repeated procedure was carried out 48hr after the primary SAH.

**Design of experiments and treatment groups**

The rats were subdivided into five groups at random (9 animals/group): 1) Sham-operated (no SAH); 2) SAH rats received one shot SAH; 3) SAH rats receiving double shot SAH and 4) sham-operated animals received 10ng/ml IL-1β intrathecal (IT) injection. The animals were sacrificed by perfusion–fixation 72hr after 2nd SAH. CSF samplings were obtained at 48hr after 1st SAH and 72hr after 2nd SAH. Cortical tissue samples were obtained by means of placing a 22-gauge needle inserted 5mm in depth into the skull bone (N=5) through a burr hole craniectomy obtained by means of placing a 22-gauge needle inserted 5mm in 1st SAH and 72hr after 2nd SAH. Cortical tissue samples were obtained by means of placing a 22-gauge needle inserted 5mm in depth into the skull bone (N=5) through a burr hole craniectomy (300bp) was assigned as a housekeeping gene owing to its stable sequences that were employed for IL-1β (242bp), IL-6(91bp), IL-8(229bp) and TNF-α (177bp) were examined, while 18S (300bp) was assigned as a housekeeping gene owing to its stable expression under head injury. Each sample was introduced into a TaqMan Human Cytokine Card which enclosed probes and primers for specific targets as well as the 18S ribosomal RNA control[14].

**Detection of cleaved caspase -3 and -9a mRNA by rt-PCR**

The levels of cleaved caspase-3 and caspase-9a mRNA in the cortical homogenate were determined by TriPure RT-PCR Reagent (Roche Diagnostics Corp. IN 46250 USA) according to the manufacturer’s instructions. The PCR primer sequences were designed according to the caspase-3, and -9a and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences reported in GenBank. Caspase-3: (393bp; forward: 5’-GGATTGGAGACA-GACGTTG-3’; reverse: 5’-CATGGGATCTGTCTTTTG-3’); caspase-9a: (889bp; forward: 5’-GCTTCTCT TTGTTCATCTCC-3’ and a reverse: 5’-CATCTGGCTCGGTTACTGC-3’); GAPDH: (347bp; forward: 5’-GGAGCCAAAAGGTCACT-3’; reverse: 5’-CCAAGGTTCGGGTCGTC-3’). By incubation with Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega, WI 12595, USA), the caspases -3, -9a and GAPDH cDNA were amplified. The amplified cDNA fragments were detected by using agarose gel electrophoresis swirl with 1 µL of ethidium bromide. The intensity of the gene bands was measured by a comet assay method. The pro-caspase-9 was catalyzed and cleaved into caspase-9a (majority) and -9b.

**Statistics**

Group data are expressed as the means ± standard error of the means. For group comparisons, one-way analysis of variance was used for analyses followed by the Newman-Keuls post-hoc test and the Student t-test statistically. A probability level < 0.05 was assumed to indicate statistical significance (SigmaStat software, Version 4.1, San Jose, CA 95110 USA).

**RESULTS**

**Morphologic findings**

Significant increase in the vacuolated nucleus in the neurons and GFAP(+) astrocyte was observed in the SAH groups (Figure 1, upper panel (arrow head)). The changes in nucleus was observed to occur at 24hr after 2nd SAH and reached a peak 48hr later.

**mRNA expression of IL-1β, IL-6, IL-8 and TNF-α**

The concentrations of IL-1, IL-6, IL-8 and TNF-α in the CSF were found to increase 3000- and 1000-fold at the induction of SAH, when compared with the baseline values in the sham animals. Pro-inflammatory cytokine levels by 141%, and 128% for IL-1β when compared with the baseline values in the sham animals. Pro-inflammatory cytokine levels by 141%, and 128% for IL-1β, 112.4% and 116.3% for IL-6; 82% and 74% for IL-8, and 143.4% and 134.2% for TNF-α in the SAH group respectively. (Figure 2, right column (48hr). Left column (72hr))

**Cleaved caspase-3 and -9a expression**

To examine the mitochondria-related intrinsic neuronal apoptosis following acute CNS injury, activated caspases -3 and -9a were examined. The cleaved caspase-9a was observed increased in the SAH groups when compared with the sham-operated groups (Figure 3, p<0.01). The 10ng IL-βIT significantly reduced cleaved caspase-9a to the level of SAH group (p>0.05).
DISCUSSION

In the present study, we have chosen to investigate neuro-inflammation following experimental SAH. Altered thrombocyte aggregation, leukocyte adhesion to the vascular endothelium disruption composes a seminal event in the acute stage of inflammation and is necessary for orchestrating efficient and appropriate transmigration into the vessel wall to cause SAH-related EBI [7,15,16]. Once SAH appears, inflammation-related genes are one of the most significantly altered categories of genes; this category includes a number of relevant cytokines such as: TNF-α, IL-1α, IL-1β, IL-6, and IL-8, which are known to affect the terminal outcome of SAH patients [3,4,16]. IL-1β was proven to be able to attenuate cleaved caspase-9a mRNA levels in animals subject to SAH.
As aneurismal SAH occurred, a cascade of inflammatory mediators including adhesion molecules, prostaglandin E, and IL-1, IL-6, IL-8 and TNF-α were reduced, and patients with poor outcome had significantly higher pro-inflammatory cytokines levels proportionate to those who ultimately reached a good outcome [16]. In a canine double-hemorrhage SAH model, increased expression of IL-6 was found to be maximal on Day 5 post-SAH simultaneously with maximal narrowing of the BA lumen [3]. Ibuprofen, an anti-inflammatory agent, has been shown to inhibit leukocyte endothelial adhesion by suppression of IL-1α and TNF-α induced expression of NF-κB in a rodent femoral artery vasospasm model [10]. In Sasaki et al's study [13], suppression of mitogen-activated protein kinase is useful in the prevention of vasospasm in the rabbit vasospasm model. Taking these findings together, IL-1β modulating SAH-induced inflammation is implicated in the pathogenesis of vasospasm even resulting in neuron apoptosis.

There are three major pathways to cellular apoptosis related to mitochondria found in mammals: the cell-surface receptors defined as an extrinsic pathway, the intrinsic or apoptosome pathway, and another cytotoxic lymphocyte-initiated granzyme B pathway [17]. Cepero et al [18] revealed there were 14 caspases existing in mammals, which can be subdivided into caspase-8 and -9 as the initiator caspases, the downstream effecter caspases, like caspase-3, -6, and -7, and another caspases-1, -4, -5, -11, -12, and -13, act as pro-inflammatory enzymes. Caspase-3 is believed to be the major effecter in neuronal apoptosis. In the study of caspase-3 and -7 knock-out mice, profound altered brain development, which included cellular hyperplasia and deployment disorganization, was observed [19]. The distinct roles of each caspase following cytochrome c release during the apoptosis are not well defined. It has been shown that caspases act on the mitochondria, through acting on upstream intrinsic apoptosis and also on downstream of cytochrome c release [17]. Caspase-9 inhibitor prevents vasospasm and is able to uncouple the mitochondria and increase reactive oxygen species production in a canine SAH model [20]. In this SAH study, IL-1β blocked upstream caspase-9a initiation and limited neuron apoptosis.

CONCLUSIONS

Till now, the outcome of SAH patients remained devastating, and stood still despite decades of research and treatment on cerebral vasospasm. We need to consider SAH-induced EBI and its effect dictates on the patient’s outcome. The inflammatory response accompanying SAH may be a critical and complicated pathway underlying the development and maintenance of EBI. The pilot study shows that administration of IL-1β diminishes leukocyte chemotaxis and subsequent inflammation in a rodent model of SAH. This study needs further to prove the co-relation of IL-1β, TNF-α, and related cytokines, even upwards transcription factor (NF-κB) in vitro study. However, this study also suggests that attenuation of the pro-inflammatory cytokines could help to improve SAH-induced apoptosis.

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