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A purine antimetabolite attenuates toll-like receptor-2, -4, and subarachnoid hemorrhage-induced brain apoptosis

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ABSTRACT

Background: Upregulation of high-level toll-like receptors (TLRs) is observed in the serum of animals following experimental subarachnoid hemorrhage (SAH) and is highly related to SAH-induced early brain injury (EBI). The present study was of interest to examine the effect of 6-mercaptopurine (6-MP) on alternation of TLR-2, -3, and -4 in this model.

Methods: A rodent SAH model was used. Administration with 6-MP (0.5/1/2 mg/kg/d) was initiated 1 h after the induction of SAH via an osmotic minipump. Cerebral cortex was harvested to measure TLRs messenger RNA and protein (reverse transcription polymerase chain reaction [rt-PCR] and Western blot). Cerebral cortex was harvested for activated caspases (rt-PCR) measurement.

Results: Cellular evaluation revealed increased neuronal nuclei(+) neurons with vacuolated nuclear and glial fibrillary acidic protein(+) astrocytes in the SAH group, but absent in the 6-MP treatment and healthy controls. The TLR-3 levels were not significantly increased in animals subject to SAH, compared with the controls (no SAH). The levels of TLR-2 and -4 in the SAH only and SAH plus vehicle groups were significantly elevated ($P < 0.01$), and treatment with 6-MP reduced TLR-2, -3 (at 2 mg/kg), and -4 (dose-dependently) protein expression following SAH. Likewise, the TLR-4 messenger RNA levels were also significantly reduced in the 6-MP (at 1 mg/kg and 2 mg/kg) groups. Cleaved caspase-3 and caspase-9a were reduced at 2-mg/kg 6-MP treatment group.

Conclusions: These results show that 6-MP attenuates the expression of TLR-2, -4, especially TLR-4, which play an antiapoptotic effect on SAH-induced EBI. This finding supported that through modulating TLRs, 6-MP can attenuate SAH-induced EBI. Those results offer credit to the neuroprotective effect of 6-MP.

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1. Introduction

Even cerebral vasospasm following subarachnoid hemorrhage (SAH) has been recognized as a multifaceted mechanism, and although the optimal therapeutic menus have aimed at correcting this condition for >50 y, persistent poor outcome is observed in patients suffering from ruptured aneurism [1–9]. As effective therapies to halt this condition are lacking, increased evidence shows early brain injury (EBI) [10–16] that stimulates cortical spreading depression, early cortical depolarization waves, and impaired neurovascular coupling, play a key role in neurologic deterioration after SAH. The accumulated results arouse interest to consider the pathogenesis of EBI concerning the SAH patient's outcome. Till now, EBI-induced obstacles have included cortical spreading ischemia [11], oxidative stress [12], a cascade of inflammatory reaction [17–19], blood-brain barrier breakdown [20], and excitotoxicity [10]. These, all or less, contribute to the neurologic deterioration in SAH patients. Efforts heading toward correcting the cellular and molecular disturbance of EBI are important to set up logical therapeutic targets.

Previous studies have revealed that the levels of cytokines have risen in cerebrospinal fluid (CSF)-mediated SAH-induced vasospasm [3]. It is believed inflammasome induced by SAH also plays a key role in EBI [6,21]. However, the mechanism of the development of inflammatory cascade after the induction of SAH remains unclear [1]. Toll-like receptor (TLR) is a family of pattern recognition receptors that act as a signal transducer for microbial lipoproteins, peptidoglycan, and lipoglycan to initiate a series of innate immunity [8,19,22–26]. Recent studies also indicate severe neurologic deterioration was found in organotypic hippocampal slice cultures of TLR-2 knockout mice in the presence of β-amyloid deposit or not [27]; in the study of Cole *et al.*, TLR-3 [28] displays a protective role in mouse atherosclerosis models through activating interferon regulatory factor 3 and the induction of type I interferons. Likewise, via acting with endogenous molecules, such as oxidized low-density lipoprotein, heat shock protein 60 and 70, fibrinogen, and fibronectin in the CSF or brain after SAH, TLR-4 has been able to initiate the inflammation related to stroke, Alzheimer's disease, Huntington's disease, and Parkinson's disease [26,29,30]. It is reasonable to postulate that TLRs are involved in inflammation in the brain and play a putative role in brain damage after SAH.

The purine antimetabolite 6-mercaptopurine (6-MP; Sigma Laboratory, Taipei, Taiwan) is one of the most widely used drugs for the treatment of acute myelocytic leukemia. As a hypoxanthine analogue, 6-MP with its by-product, 6-thioguanine, is incorporated into the DNA and the RNA of humane bone marrow cells [3]. It inhibits the conversion of inosinic acid to xanthylic acid that is necessary for the synthesis of guanylic acid, prohibiting the resulting nucleic acids to direct proper protein synthesis [31,32]. Moreover, many of 6-MP's antiproliferative effects can be specifically attributed to *de novo* inhibition of purine synthesis and incorporation into nucleic acids, which is present in most cells involved in acute and chronic inflammation, including neutrophils, macrophages, platelets, smooth muscle, and endothelial cells [3]. Many recent studies show that purine antimetabolites have biological functions outside of the purine biosynthesis pathway, which include telomerase [31], protein kinase N [27], and apoptosis in B cells through the regulation of the B-cell lymphoma 2 (Bcl-2)/Bax ratio and gene expression [33], such as with the orphan nuclear receptor Nurr1 [32,34], which has been shown to regulate a small number of genes related to proinflammatory cytokine synthesis.

Taking these findings together, we designed this study to evaluate the relationship between SAH-induced EBI and expressed levels of TLR-2, -3, and -4, which are believed to play a critical role in a sterile inflammation. The effect of 6-MP on SAH-induced EBI and alterations of TLRs messenger RNA (mRNA) was estimated.

2. Methods

2.1. Materials

6-MP has been previously characterized as a potent agonist of the purine analogues and was bought from Sigma Laboratory. Horseradish peroxidase-labeled goat antimouse IgG antibodies were obtained from Abcam (Cambridge, MA), BD Transduction Lab (BD Biosciences, San Jose, CA), Upstate Biotech (Lake placid, NY), Santa Cruz Biotech (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and Chemicon International (Temecula, CA), respectively. CNM protein extraction kits were from BioChain (Hayward, CA). 6-MP was prepared by S.-C.W. (Kaohsiung Medical University Hospital, Kaohsiung, Taiwan). 0.9% saline solution was used as a vehicle.

2.2. Induction of experimental SAH

Fifty-four male Sprague–Dawley rats, weighing between 320 and 420 g (BioLasco Taiwan Co, Ltd, authorized by Charles River Laboratories International, Inc), were enrolled in this study. All the experimental protocol was approved and under the supervision of the University of Kaohsiung Medicine Animal Research Committee and conducted compliant with the Declaration of Helsinki (1964). The animals were anesthetized by an intramuscular injection of a mixture of 0.9 mg/100 g of xylazine and 5.5 mg/100 g of ketamine HCL. Measures of 0.3-mL fresh arterial blood were withdrawn and injected into the cisterna magna under a stereotactic apparatus (Stoelting, Wood Dale, IL) [3]. Rats were placed in the ventral recumbent position for 15 min to allow ventral blood clot formation. After close monitoring for respiratory distress and giving mechanical ventilation if necessary, the animals were returned to the vivarium until fully awake. They were habituated to a 12-h light-dark cycle, and given access to food and water *ad libitum*.

2.3. General design of experiments and treatment groups

The rats were randomly divided into the following six groups (nine animals per group): (1) sham operated group (no SAH); (2) SAH only; (3) SAH plus vehicle; SAH plus 6-MP treatment of (4) 0.5 mg/kg/d, (5) 1 mg/kg/d, and (6) 2 mg/kg/d. The first injections given to animals were administered 1 h after