Expression of intestinal CD40 after experimental traumatic brain injury in rats

Yang-Chun Hu, MD, Fan Wang, MD, Ding-Ding Zhang, MD, Qing Sun, MD, Wei Li, MD, Yu-Xiang Dai, MD, Meng-Liang Zhou, MD, PhD, and Chun-Hua Hang, MD, PhD

A Department of Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, Nanjing, Jiangsu Province, People’s Republic of China
b Department of Neurosurgery, Affiliated Hospital of Guizhou Medical College, Guiyang, Guizhou Province, People’s Republic of China

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Abstract

Background: Nuclear factor kappa B (NF-κB) has been shown to be activated in the intestine after traumatic brain injury (TBI), and results in gastrointestinal mucosal injury. In addition, CD40 has a major role in the activation of NF-κB and is up-regulated in inflammatory bowel disease. However, we found no study in the literature investigating the intestinal expression of CD40 after TBI. Hence, we designed the current study to explore the intestinal expression pattern of CD40 after TBI in rats. We hypothesized that CD40 could mediate inflammation and ultimately contribute to acute intestinal mucosal injury after TBI.

Methods: We randomly divided rats into control and TBI groups at 3, 6, 12, 24, and 72 h, respectively. We assessed the expression of CD40 by quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemical study, and detected the levels of tumor necrosis factor-α (TNF-α), intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) by enzyme-linked immunosorbent assay.

Results: The mRNA and protein levels of CD40 increased by 3 and 6 h, peaked at 6 and 12 h, and remained elevated until 24 and 72 h post-injury, respectively. Levels of TNF-α, VCAM-1, and ICAM-1 also markedly increased in jejunum tissue after TBI. Interestingly, there was a positive relationship between the expression of CD40 and that of TNF-α, VCAM-1, and ICAM-1.

Conclusions: CD40 could be markedly elevated in intestine after TBI in rats, and it might have an important role in the pathogenesis of acute intestinal mucosal injury mediated by inflammatory response.

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1. Introduction

Complications are frequently seen after traumatic brain injury (TBI), not only in the central nervous system but also in the pulmonary, gastrointestinal, and psychiatric systems [1]. Specifically, the gastrointestinal dysfunction is often observed in patients with TBI [2]. Growing evidence suggests that inflammation has a vital role in the cascade of intestinal events, such as overproduction of intestinal cytokines [3], increased intestinal permeability resulting from damage of intestinal mucosa structure and impairment of barrier [4], intestinal bacteria, and endotoxin translocation [5], which might be mediated mainly by activation of the nuclear factor kappa B (NF-κB) pathway [4,6].

* Corresponding author. Department of Neurosurgery, Jinling Hospital, School of Medicine, Nanjing University, 305 East Zhongshan Road, Nanjing 210002, People’s Republic of China. Tel/fax: +86 25 80863310.
E-mail address: hang_neurosurgery@163.com (C.-H. Hang). 0022-4804/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
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Nuclear factor-κB is one of the most important modulators of inflammatory gene expression [7]. Previous studies have shown that NF-κB and proinflammatory cytokines are involved in the intestinal mucosa damage induced by ischemia-reperfusion and endotoxemia [8,9], as well as in the pathogenesis of chronic inflammatory bowel disease (IBD) [10]. Our previous studies demonstrated that acute damage and inflammatory responses occurred in the intestinal mucosa after TBI and subarachnoid hemorrhage [4,11], and a significant up-regulation of NF-κB and proinflammatory cytokines could be induced in the intestine.

CD40, first identified and functionally characterized on B-cells, is a member of tumor necrosis factor receptor (TNFR) family, and is known as a transmembrane receptor. CD40 can convey signals regulating diverse cellular responses ranging from proliferation and differentiation to growth suppression and cell death. Numerous studies have shown that CD40 can activate both canonical and noncanonical NF-κB signaling pathways [12,13], and finally result in the release of proinflammatory cytokines. CD40 may exert a key action in intestinal inflammation in ischemia-reperfusion, endotoxemia, and IBD, resulting in amplification of tissue injury [14]. To date, however, no research in literature has been found studying the expression of CD40 in the intestine after TBI. Thus, we hypothesized that CD40 may mediate inflammation and result in acute intestinal mucosal injury after TBI. Accordingly, the present pilot study aimed to explore the expression pattern of intestinal CD40, and to indicate the potential role of CD40 in regulation of intestinal inflammatory response after TBI in rats.

2. Materials and methods

2.1. Animal model of TBI

We purchased male Sprague-Dawley rats, weighing 250–300 g, from the Experimental Animal Center of Jinling Hospital (Nanjing, China). We housed the rats in temperature- and humidity-controlled animal quarters according to a 12-h light/dark cycle. The temperature was maintained at about 25°C. All procedures were approved by Animal Ethics Committee of Nanjing University and conformed to the guidelines of the National Institutes of Health on the care and use of animals.

We developed the TBI models following previously described methods in our laboratory [6]. Briefly, we induced anesthesia with 10% chloral hydrate and then placed the animals on a heat pad to keep body temperature at 37°C during the experimental procedure. The local hair on the skull was shaved. Subsequently, the scalp was sterilized with 75% alcohol. Rats were mounted in a stereotaxic device with the head in a horizontal position. We then made a bone window about 5 mm in diameter on the right skull just behind the cranial coronal suture and beside the midline with a dental drill, with the dura kept intact. We induced trauma by letting a copper hammer weighing 40 g with a flat-end diameter of 4 mm fall onto a piston resting on the dura from a height of 25 cm. The piston was allowed to compress the tissue a maximum of 5 mm. Rats with cortical contusion were divided into five subgroups and killed by ventricle perfusion at 3, 6, 12, 24, and 72 h post-injury, respectively (n = 6/subgroup). Rats in control group received a sham operation and right parietal craniotomy without cortical contusion. In our pilot study, we found no statistical difference in all detected variables among the control and sham groups at each time point (data not shown). Therefore, we killed animals in control group at 24 h after the sham operation.

After we killed the animals, we took a 3-cm segment of the mid-jejunum was taken, flushed it with ice-cold saline, and opened it longitudinally. Half of the jejunum tissue was immersed in 4% buffered formalin for histopathologic studies and the other half was stored at −80°C until use.

2.2. Western blotting analysis

We extracted total protein and performed Western blotting following our previous methods [15]. The primary antibodies were rabbit anti-CD40 antibody (1:100; Santa Cruz Biotechnology Inc, Santa Cruz, CA) and rabbit anti-β-actin antibody (1:1000; Sigma, St Louis, MO), respectively. The optical density of the resultant bands was determined by UN-SCAN-IT (Skill Scientific Inc, Salt Lake City, UT), with normalization of densitometry measures to β-actin.

2.3. Quantitative real-time polymerase chain reaction

We extracted RNA from each sample using the TRIZOL Reagent (TaKaRa Biotechnology, Dalian, China) following the manufacturer’s recommendations. We then performed reverse transcription and amplification following our previous methods [16]. Sequence-specific primers used for the reaction are presented as follows: CD40: FWD: 5’-CAGCAAGGGATGCGGACGAC-3’; REV: 5’-ACGGAGGAATGACCAGCAG-3’; GAPDH: FWD: 5’-AGTGGCCAGCTCGTTCAAG-3’; and REV: 5’-CTTTGAACTTGCGGTTGATA-3’. We normalized total RNA concentrations from each sample by quantity of glyceraldehyde-3-phosphate dehydrogenase mRNA and determined the relative quantification of mRNA expression using the 2−ΔΔCt method. All samples were analyzed in triplicate.

2.4. Enzyme-linked immunosorbent assay

We measured the protein levels of TNF-α, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in the tissue supernatants using an enzyme-linked immunosorbent assay (ELISA) kit (Diacalone, Bescanc, France) for rats. We measured TNF-α, VCAM-1 and ICAM-1 based on the specifications of the ELISA kit. The cytokine contents in the jejunal tissue are expressed as nanograms of cytokines per gram of protein.

2.5. Immunohistochemical staining

We performed immunohistochemical staining following our previous methods [15]. The primary anti-CD40 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was diluted at 1:100 with 1% bovine serum albumin. Six views from each section were randomly selected and observed under a light microscope (×400). We used the mean number of positive cells in the six views for statistical analysis.
2.6. Statistical analysis

We used SPSS 11.0 (SPSS Inc, Chicago, IL) in the statistical analysis. Each parameter is expressed as means ± standard deviation (SD) and was compared using one-way analysis of variance followed by Dunnett T3 post hoc test. In the semiquantitative analysis of immunohistochemistry, we determined differences between experimental groups Student t-test. We analyzed the relationship between variables using a linear regression model. P < 0.05 was considered statistically significant.

3. Results

3.1. Expression of CD40 mRNA levels

Figure 1 shows intestinal CD40 mRNA expression as detected by real-time polymerase chain reaction. We found a markedly low level of CD40 mRNA in the control group, whereas we detected significantly increased mRNA in the TBI groups compared with that in the control group (3 and 24 h, P < 0.05; 6 and 12 h, P < 0.01). Results showed that the intestinal CD40 mRNA increased by 3 h, peaked at 6 h, and remained elevated at 24 h after TBI.

3.2. Expression of CD40 protein levels

We measured CD40 protein expression by Western blotting. As shown in Figure 2, CD40 protein was at a low level in the control group, whereas in the TBI groups, CD40 protein levels significantly increased, with a peak at 12 h post-injury. There was a statistical difference between the control and TBI groups (6 h, P < 0.05; 12, 24, and 72 h, P < 0.01).

3.3. Expression and distribution of CD40

We assessed the distribution of intestinal CD40 by immunohistochemical study at the peak of activation (12 h after TBI, according to Western blot). In the control group, weak expression of CD40 was observed in the jejunum, whereas increased CD40-positive cells could be found in the 12-h group (Fig. 3). Semiquantitative analysis showed a low level of CD40-positive cells in the control group (17.0% ± 7.7%), whereas CD40-positive cells significantly increased (69.0% ± 11.8%) in the 12-h group. Compared with the control group, intestinal CD40 immunoreactivity was significantly higher in the TBI group (P < 0.01).

3.4. Concentrations of TNF-α, VCAM-1, and ICAM-1

We performed ELISA to detect the concentrations of TNF-α, VCAM-1, and ICAM-1 in the jejunal tissue. Our results showed that TNF-α, VCAM-1, and ICAM-1 protein levels were low in the control group. Compared with those in the control group, TNF-α, VCAM-1, and ICAM-1 protein levels significantly increased in each TBI group, respectively (P < 0.01) (Figs. 4–6). The results also showed that peak activation of TNF-α was at 24 h, whereas VCAM-1 and ICAM-1 reached their peaks at 12 h after TBI. In addition, there was a positive relationship between CD40 expression and TNF-α, VCAM-1, and ICAM-1 expression, respectively (r = 0.616, P < 0.01; r = 0.654, P < 0.01; and r = 0.644, P < 0.01).

4. Discussion

In the current study, we demonstrated for the first time that both the protein and mRNA levels of CD40 significantly increased...
increased in the jejunum after TBI in rats. Results showed that the intestinal CD40 mRNA expression was up-regulated at 3 h, peaked at 6 h, and persisted up to 24 h, and the protein expression increased at 6 h and peaked at 12 h after TBI. In the present study, we detected two isoforms of the CD40 protein (45 and 27 kDa), which were the results of alternative splicing [17]. In addition, the concentrations of TNF-α, VCAM-1, and ICAM-1 in the jejunum were also significantly elevated, showing a positive relationship with the protein levels of CD40.

CD40 is a type 1 transmembrane protein that belongs to the TNFR superfamily for its homology with the TNF receptor [18]. More important, CD40 has been implicated in the pathophysiologic process in many human diseases, especially in autoimmune and inflammatory diseases [19,20]. CD40 is expressed on the surface of immune cells including B-cells, monocytes, macrophages, and dendritic cells, as well as nonimmune cells such as epithelial, endothelial, and mesenchymal cells and platelets [21]. CD40 ligand (CD40L) has been regarded as a member of the TNF gene superfamily [18,22]. Increasing evidence has found that cognate interactions between CD40 and CD40L could generate signals that affect both humoral and cellular immunity, and eventually have a key role in inflammation. Recent experimental evidence suggests that the CD40-CD40L pathway could augment mucosal inflammatory responses via mucosal primary colonic lamina propria fibroblasts [23]. During inflammation, CD40 and TNF-α influence each other’s biologic activity. In brief, CD40L leads to TNF-α elevation in various cell types [24], whereas TNF-α up-regulates expression of CD40, promotes CD40 downstream signaling pathways [25], results in the release of proinflammatory cytokines, and activates its potent proinflammatory activity. Moreover, CD40 engagement enhances expression of VCAM-1, ICAM-1, and E-selectin on the surface

![Image](image-url)
of endothelial cells, which in turn facilitates the recruitment and activation of leukocytes under the condition of inflammation [26]. CD40 has been found to be up-regulated in IBD, and it could promote the recruitment of leukocytes into the inflamed mucosa [27]. Moreover, blockade of the CD40-CD40L pathway could down-regulate both CD40 and VCAM-1 [28,29]. In the present study, the levels of CD40, VCAM-1, and ICAM-1 were increased, which shows that the CD40 pathway might be involved in the intestinal inflammatory response.

It is well established that CD40 signaling activates both canonical and noncanonical NF-κB signaling pathways [12,13]. Nuclear factor-κB has a key role in regulating cytokine-mediated inflammation [30]. Our previous studies showed that NF-κB DNA-binding activity increased significantly in the intestine after TBI [6,15]. In the canonical NF-κB signaling pathway, CD40 activates the inhibitor of the NF-κB kinase complex, and finally induces the rapid inhibitor of the NF-κB kinase-α degradation and subsequent nuclear translocation of associated NF-κB dimers [12,31]. The non-canonical pathway depends on proteolytic cleavage of the precursor p100, liberating mainly p52/RelB heterodimers for nuclear translocation. Upon ligation with CD40L, CD40 interaction leads to proteolysis of both TNFR-associated factor (TRAF) 2 and 3 [32]. The receptor recruitment of these TRAF members is important for triggering their degradation, a critical step leading to the activation of NF-κB−inducing kinase and induction of p100 processing [33]. Ultimately, both canonical and non-canonical NF-κB signaling pathways induce the formation and nuclear translocation of distinct NF-κB heterodimers that activate distinct target genes [34].

As mentioned above, CD40 is essential in the up-regulation of TRAF proteins and NF-κB−dependent proinflammatory cytokines in vivo [35]. CD40 deficiency profoundly inhibits the expression of TRAF proteins and activation of NF-κB. Furthermore, blocking down the CD40 pathway could effectively dampen inflammation in vitro [24] as well as in vivo [36]. Based on these observations and our findings, we hypothesized that CD40 might have an important role in regulating the NF-κB−dependent inflammatory response in acute gut mucosal injury after TBI.

Our study shows that the expression of CD40 significantly increased in the jejunum after TBI in rats. The findings indicated that CD40 pathway might have a key role in intestinal inflammation after TBI. However, this is a pilot study and the specific mechanisms remain unclear. Future studies are warranted to ascertain whether genetic deletion or pharmacologic inhibition of CD40 confers protection against CD40-induced acute gut mucosal injury after TBI.

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