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C-reactive protein induced expression of adhesion molecules in cultured cerebral microvascular endothelial cells

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Abstract

Ischemic stroke can trigger an acute phase response resulting in a rise of plasma concentration of C-reactive protein (CRP). Clinical data about the relationship between CRP and prognosis suggest that CRP might be involved in the pathogenesis of cerebral ischemia. In the present work, a significant increase of circulating level of CRP was observed in an vivo rat brain ischemia model of middle cerebral artery occlusion. To determine the possible effects of CRP on brain microvessel endothelium, we performed a dose-dependent experiment in mouse brain microvascular endothelial cells (bEnd.3 cells) with emphasis on its relation to cell adhesions molecules. Incubation with CRP (1–75 mg/L) for 24 h significantly increased Lactate dehydrogenase (LDH) leakage from bEnd.3 cells ($P<0.01$) in a dose-dependent manner, and induced significant up-regulations of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expressions analyzed by Western blotting ($P<0.01$). In contrast to earlier report, CRP also induced significant increase in ICAM-1 expression in the absence of serum ($P<0.01$). In conclusion, the present results suggest that CRP may be involved directly in the development of inflammation in response to cerebral ischemia.

Keywords: Brain microvascular endothelial cells; C-reactive protein; Cell adhesion molecules; Lactate dehydrogenase

Introduction

C-reactive protein (CRP) is the first, major acute phase protein to be found and is an exquisitely sensitive systemic marker for inflammatory processes and tissue damage (Pepys and Baltz, 1983). The acute phase response comprises the nonspecific physiological and biochemical responses of endothermic animals to most forms of tissue damage and inflammation. Data from many prospective studies demonstrated that elevation of plasma CRP has strong independent predictive power for cardiovascular events in a wide variety of clinical settings. More studies which focused on the relationship between CRP and atherosclerotic disease recently suggest that the acute-phase reactant CRP, at concentrations known to predict vascular disease, elicits a multitude of effects on endothelial biology favoring a proinflammatory and proatherosclerotic phenotype (Jialal et al., 2004; Verma, 2004). The direct proinflammatory, proatherogenic effects of CRP that have been documented in large vessel derived endothelial cells include the following: decreased nitric oxide (Verma et al., 2002b) and prostacyclin, increased endothelin-1 (Verma et al., 2002a) and cell adhesion molecules (Pasceri et al., 2000).

In particular, previous data demonstrated that ischemic stroke also triggers an acute phase response resulting in a rise of circulating CRP level (Di Napoli et al., 2000, 2001; Di Napoli and Papa, 2003), but interpretation of CRP values in this situation is complicated by the fact that most stroke patients frequently have other intercurrent pathologies such as bacterial infections, developing rapidly after the acute cerebral ischemic event that can stimulate the acute phase response. However, a recent analysis of CRP and other inflammatory markers in subjects with ischemic stroke has confirmed that there is a modest but highly significant rise of CRP level, even among patients without infection (Emsley et al., 2003). Furthermore, elevated serum concentrations of CRP in stroke patients can represent a strong predictor of subsequent vascular events and a worse long-term functional outcome (Muir et al., 1999; Winbeck et al., 2002), and can reflect the extent of

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infarction (Audebert et al., 2004; Di Napoli et al., 2001). These data compelled to suggest a possible role for CRP in the evolution of stroke.

As the major components of the blood–brain barrier to prevent the entry of blood-borne substances 4 and leukocytes into the brain, brain endothelial cells express unique biochemical properties and have a fundamental role in cerebral vascular diseases such as stroke (del Zoppo and Mabuchi, 2003; Drewes, 1999). However, the interaction of CRP with brain endothelial cells remains unclear. In order to evaluate the involvement of CRP in the pathogenesis of cerebral ischemia, the present study analyzed the CRP levels in a rat cerebral ischemia model and examined the effects of CRP on brain endothelial cells with emphasis on the relation between the CRP levels and cell damage, expression of inflammatory adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).

Materials and methods

Reagents

Dulbecco's Modified Eagle Medium Dulbecco (DMEM) and fetal bovine serum (FBS) were produced by Gibco RBL (Grand Island, NY, U.S.A.). Purified human CRP was purchased from CHEMICON International (CHEMICON, CA, U.S.A.). Goat polyclonal anti-mouse ICAM-1 and VCAM-1, peroxidase-conjugated rabbit anti-goat antibody immunoglobulin IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Lactate dehydrogenase (LDH) reagent kits and all other reagents were from local commercial sources. All CRP preparations and media used in cell culture were tested for Lipopolysaccharide (LPS) and found to be under 25 pg/mL using limulus assay. These amount of LPS in our study are under the limit of <55 pg/mL which was considered to have no positive effects in a recent report (Taylor et al., 2005).

Cerebral ischemia model

Male Sprague–Dawley (SD) rats weighing 250–280 g provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China) were housed in controlled conditions, and received a standard rat chow and tap water ad libitum. All the animals used in this work received humane care in compliance with institutional animal care guidelines, and were approved by the Local Institutional Committee.

Transient focal cerebral ischemia in the SD rats was conducted according to procedures described by Zea-Longa et al. (1989). Briefly, each animal was anesthetized with 400 mg/kg chloral hydrate intraperitoneally, a 30-mm segment of 4-0 ethylon monofilament which tip rounded by heating was gently introduced from the external carotid artery into the internal carotid artery lumen approximately 17–18 mm distal to the carotid bifurcation until a mild resistance was felt. Reperfusion was accomplished by withdrawing the filament 120 min after induction of ischemia. In sham-operated rats, the external carotid artery was surgically prepared for insertion of the filament, but the filament was not inserted. Rectal temperature was monitored continuously and maintained between 36.5 and 37.5 °C by surface heating and cooling. Animals were then returned to their cages and closely monitored until they recovered from anesthesia.

Serum was separated from blood sample collected before surgery operation and 24 h after reperfusion and CRP was measured by immunoturbidimetric assay by using commercial Randox kit (UK) with standards provided by the same firm (Valtchanova-Matchouganska et al., 2004). The rats were then decapitated, and serial coronal slices (2 mm thickness) from the brain were stained with a 2% solution of 2,3,5-triphenyltetrazolium (TTC) dye at 37 °C for 20 min. Stained sections were then recorded using a digital camera and the volume of total brain and infarct area were measured by an image analysis software (MedBrain 4.0, Medease Science and Technology, China).

Cell culture

Mouse brain microvascular endothelium-derived bEnd.3 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were maintained in DMEM containing 10% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 mg/L), in a humidified 5% CO_2, 95% air incubator at 37 °C. At confluency, cells were trypsinized, counted and seeded in 12-well flat-bottomed plates with a density of 10^5 cells/cm^2. After the cells grew for 24 h, culture fluid was changed and cells were washed by D-Hank's solution (NaCl 8 g, KCl 0.4 g, Na_2HPO_4 0.06 g, KH_2PO_4 0.06 g, $NaHCO_3$ 0.35 g in 1 L) for three times and then was further cultured in DMEM with free FBS for 12 h. Then cells were treated with human CRP (0–100 mg/L) for 24 h at 37 °C. The medium was collected and used for determination of LDH. Cells between passages 6 and 10 confirmed by an immunohistochemical stain for factor VIII were used for all the experiments.

Determination of released LDH activity

The quantity of LDH released by the cells into the medium was determined by monitoring the oxidation of pyruvate coupled with the reduction of NAD at 340 nm using a commercial LDH reagent kit according to the manufacturer's instructions. The LDH activity was expressed as units per liter culture medium.

Gel electrophoresis and Western blotting to detect the adhesion molecules ICAM-1 and VCAM-1

After rinsing with phosphate-buffered saline (PBS), the cells were lysed with SDS-PAGE protein loading buffer containing 5% 2-mercaptoethanol. Cell lysates with equal amounts of total protein were then separated on 8% SDS-polyacrylamide gels with a 4% polyacrylamide stacking gel, and the proteins were transferred to nitrocellulose paper. The resulting Western blots