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Full-length Article

Platelet CD40L induces activation of astrocytes and microglia in hypertension

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ABSTRACT

Studies have demonstrated separately that hypertension is associated with platelet activation in the periphery (resulting in accumulation and localized inflammatory response) and glial activation in the brain. We investigated the contribution of platelets in brain inflammation, particularly glial activation *in vitro* and in a rat model of hypertension. We found that HTN increased the expression of adhesion molecules like JAM-1, ICAM-1, and VCAM-1 on brain endothelium and resulted in the deposition of platelets in the brain. Platelet deposition in hypertensive rats was associated with augmented CD40 and CD40L and activation of astrocytes (GFAP expression) and microglia (Iba-1 expression) in the brain. Platelets isolated from hypertensive rats had significantly higher sCD40L levels and induced more prominent glial activation than platelets from normotensive rats. Activation of platelets with ADP induced sCD40L release and activation of astrocytes and microglia. Moreover, CD40L induced glial (astrocytes and microglia) activation, NFκB and MAPK inflammatory signaling, culminating in neuroinflammation and neuronal injury (increased apoptotic cells). Importantly, injection of ADP-activated platelets into normotensive rats strongly induced activation of astrocytes and microglia and increased plasma sCD40L levels compared with control platelets. On the contrary, inhibition of platelet activation by Clopidogrel or disruption of CD40 signaling prevented astrocyte and microglial activation and provided neuroprotection in both *in vivo* and *in vitro* conditions. Thus, we have identified platelet CD40L as a key inflammatory molecule for the induction of astrocyte and microglia activation, the major contributors to inflammation-mediated injury in the brain.

1. Introduction

A number of experimental studies and clinical trials demonstrate that hypertension is one of the major risk factors for some of the central nervous system disorders like stroke and memory impairment (Stumpf et al., 2005; Fogari et al., 2004, 2006; Saavedra, 2012). The relationship between hypertension and memory impairment is further strengthened when antihypertensive agents like angiotensin converting enzyme inhibitors and AT1 receptor blockers, improved memory functions in hypertensive subjects (Fogari et al., 2004, 2006; Saavedra, 2012). Apart from memory impairment, hypertension is also associated with glial activation and neuroinflammatory changes in the cardio-regulatory brain regions like paraventricular nucleus (Shi et al., 2010; Xia et al., 2013) and nucleus tractus solitary (NTS; Waki et al., 2010; Shan et al., 2013). Glia (astrocytes and microglia) upon activation induce neuroinflammation by the release of inflammatory mediators like TNF-α, IL-1β, IL-6 and ROS (Tansey et al., 2007; Guadagno et al., 2013; Wang et al., 2015), thereby promote neuronal injury and neurodegeneration (Saavedra, 2012; Guadagno et al., 2013).

Hypertension is also a major risk factor for cerebrovascular dysfunction and endothelial activation leading to the recruitment of blood cells (platelets and leukocytes) in the brain (Iadecola and Davisson, 2008). Waki et al. (2010) demonstrated that hypertension increased the expression of adhesion molecule JAM-1 in the NTS, resulting in platelet and leukocyte accumulation in hypertensive rats, but not in normotensive rats. Likewise, earlier reports have demonstrated endothelial activation (increased expression of adhesion molecules) is a critical step in the process of blood cell (platelets and leukocytes) recruitment during brain inflammation (Thornton et al., 2010; Rossi et al., 2011).

Platelets are probably the first cell type to appear at the site of vascular dysfunction and have the ability to induce vascular

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inflammation, thus initiating the disease progression (Massberg et al., 2002; Thornton et al., 2010; Liu et al., 2012). Apart from peripheral vasculature, the involvement of platelets is documented in the brain in response to cerebral ischemia (Jafar et al., 1989; Karakantza et al., 2003; Marquardt et al., 2002; Ishikawa et al., 2004), cerebral malaria (Grau et al., 2003) and multiple sclerosis (Sheremata et al., 2008). Platelet derived interleukin-1β (IL-1β), IL-1α, or CD40 ligand (CD40L) can activate peripheral endothelial cells (Gawaz et al., 2000; Liu et al., 2012) and contribute to leukocyte infiltration and inflammation in peripheral tissues (Lievens et al., 2010; Thornton et al., 2010; Liu et al., 2012). In brain, CD40, the cognate receptor of CD40L, is mainly expressed on astrocytes and microglia and to a lesser extent on endothelium (Giunta et al., 2010). CD40L is expressed on a number of cell types (Henn et al., 1998; Schonbeck and Libby, 2001; Lievens et al., 2010; Thornton et al., 2010); however, more than 95% of the circulating CD40L exists in platelets (Andre et al., 2002). These observations suggest the involvement of platelet CD40L in the biological and pathological context of inflammatory diseases.

The discussion so far has advocated that chronic hypertension is associated with platelet deposition (Waki et al., 2010) and glial activation (Shi et al., 2010; Saavedra, 2012) in the brain. However, the contribution of platelets in glial activation during the hypertensive state in brain regions associated with memory functions (cortex and hippocampus) has not been explored. Therefore, in the present study, we hypothesised that in the hypertensive state, platelets might induce activation of astrocytes and microglia in the brain. We, for the first time, show that platelet CD40L resulted in brain endothelial activation (increased expression of adhesion molecules), deposition of platelets, activation of microglia and astrocytes and neuronal injury in brain in the hypertensive state. Further, inhibition of platelet activation by Clopidogrel or disruption of CD40 signaling prevented glial activation and neuroinflammation.

2. Materials and methods

2.1. Reagents and antibodies

Clopidogrel and ADP were purchased from Sigma-Aldrich (USA). Recombinant CD40L was purchased from Peprotech (USA). Primary antibodies, anti-GFAP (Sigma, USA), anti-eNOS, anti-TIMP2, anti-MMP9, anti-GFAP, anti-TRAF6, anti-p65NFκB, anti-IκB-α, anti-Iba-1, anti-CD40, anti-CD40L (Abcam, USA), phospho-p38 mitogen-activated protein kinase (p38MAPK), total p38MAPK, phospho-ERK1/2, total-ERK1/2 antibodies (Cell Signaling Technology, USA), anti-ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, USA), JAM-1 (Millipore, UK) and Alexa Fluor 594 and Alexa Fluor 488 IgG conjugate (Invitrogen), secondary HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (USA).

2.2. Animals

The experiments were carried out with adult male Sprague-Dawley (SD) rats (RGD, 10395233) procured from the Laboratory Animal Services Division of CSIR-Central Drug Research Institute (CDRI), Lucknow, India. Experiments were performed according to internationally followed ethical standards and approved by Institutional Animal Ethics Committee (IAEC) of CSIR-CDRI and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Rats were maintained under standard housing conditions (room temperature 24–27 °C and humidity 60–65%) with a 12 h light and dark cycle. Food and water were available *ad libitum*. This study adheres to the ARRIVE guidelines for animal research.

2.3. Model of chronic hypertension

Chronic hypertension was induced in male SD rats (180–200 g), by partial occlusion of the left renal artery, while in sham animals; renal artery was only exposed but not occluded (Kharin and Krandycheva, 2004). Briefly, animals were anaesthetized with pentobarbital (50 mg/kg i.p.). After, a midline abdominal incision, the left renal artery was exposed and isolated over a short segment by a blunt dissection. After isolation, renal artery was constricted by making the loop of folded-in-two cotton thread tunneled through a plastic tube (0.5 mm internal diameter, 1.5–2.0 mm long). The artery was slowly inserted into the tube by pulling the ends of thread. The thread was tied such that it did not constrict the artery but prevented its slippage from the plastic tube (Kharin and Krandycheva, 2004; Lorenz et al., 2011; Shih et al., 2016). After surgery, neosporin, an anti-fungal powder and betadine, a topical antiseptic, were applied at the site of the incision. The animals were left for 5 weeks and allowed food and water *ad libitum*.

2.4. Drug administration

To study the involvement of platelets in HTN induced glial activation and neuroinflammation, Clopidogrel, anti-platelet drug, was administered orally at a dose of 10 mg/kg/day for 5 weeks in hypertensive animals. This dose of Clopidogrel was selected from previous studies (De La Cruz et al., 2003; Giachini et al., 2014; Osmond et al., 2014) and approximately matches the normally prescribed dose of 75 mg/day in humans (Osmond et al., 2014).

2.5. Measurement of hemodynamic parameters

After 5 weeks, rats were anesthetized with urethane (1.25 g/kg, i.p.) and placed on an isothermal pad to maintain normal body temperature during surgical procedures. The hemodynamic parameters like systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR) were recorded by inserting fluid filled catheters in the carotid artery. The catheter was attached to the pressure transducer coupled to Data Acquisition System. All animals were allowed to stabilize for 20 min before the final readings.

After taking hemodynamic parameters, the animals were sacrificed by transcardiac perfusion with ice cold saline. The brain was immediately removed and kept on ice-cold plate to dissect cerebral cortex and hippocampus.

2.6. Cerebral microvessels (CMVs) isolation

CMVs were isolated by following the protocol of Yamakawa et al. (2003). In brief, the brains were removed and rinsed in cold isotonic sucrose buffer (0.32 mol/L sucrose, 3 mmol/L HEPES, pH 7.4). Cerebellum, pia mater and choroids plexus were discarded. The rest of the brain was homogenized in 3 vol of sucrose buffer, twice, each followed by centrifugation at 4 °C for 10 min at 1000*g*. Sediments were resuspended in sucrose buffer and centrifuged twice for 30 sec at 100*g*. The final pellet was resuspended in 1 ml sucrose buffer followed by centrifugation at 14,000*g*. Finally, the precipitate containing microvessels was stored at −80 °C until further use (Yamakawa et al., 2003).

The microvessels purity was evaluated by light microscopy and estimation of gamma-glutamyl transpeptidase activity, an enzyme predominantly localized to brain microvessels (Yamakawa et al., 2003).

2.7. TUNEL for assessment of apoptosis

TUNEL (TdT-mediated dUTP Nick-End Labeling) assay was done on brain sections by using a commercially available kit (Dead End