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Intracerebroventricular Shiga toxin 2 increases the expression of its receptor globotriaosylceramide and causes dendritic abnormalities

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ABSTRACT

Neurological damage caused by intoxication with Shiga toxin (Stx) from enterohemorrhagic *Escherichia coli* is the most unrepairable and untreatable outcome of Hemolytic Uremic Syndrome, and occurs in 30% of affected infants. In this work intracerebroventricular administration of Stx2 in rat brains significantly increased the expression of its receptor globotriaosylceramide (Gb_3) in neuronal populations from striatum, hippocampus and cortex. Stx2 was immunodetected in neurons that expressed Gb_3 after intracerebroventricular administration of the toxin. Confocal immunofluorescence of microtubule-associated protein 2 showed aberrant dendrites in neurons expressing increased Gb_3. The pro-apoptotic Bax protein was concomitantly immunodetected in neurons and other cell populations from the same described areas including the hypothalamus. Confocal immunofluorescence showed that Gb_3 colocalized also with glial fibrillary acidic protein only in reactive astrocytic processes, and not in vehicle-treated normal ones. Rats showed weight variation and motor deficits as compared to controls. We thus suggest that Stx2 induces the expression of Gb_3 in neurons and triggers neuronal dysfunctions.

1. Introduction

Infection by Shiga toxin (Stx)-producing enterohemorrhagic *Escherichia coli* (STEC) causes hemorrhagic colitis and Hemolytic Uremic Syndrome (HUS) (O'Brien and Kaper, 1998), characterized by thrombocytopenia, microangiopathic hemolytic anemia and acute renal failure (Proulx et al., 2001; Karmali, 2004).

Central nervous system (CNS) complications are observed in around 30% of the infant population with HUS. Patients may be affected with acute seizures, coma, irritability, hemiparesis or motor disorders (Eriksson et al., 2001; Valles et al., 2005).

Stx2 is a protein composed of a 32-kDa subunit A (StxA) and five 7.7-kDa subunits B (StxB). StxA bears N-glycosidase activity, depurinates the rRNA 28S, inhibits protein biosynthesis and promotes host cell death (Lord et al., 2005). Therefore StxA must be transported to the cytosol by StxB (Johannes and Decaudin, 2005; Sandvig and van Deurs, 2005). StxB binds with high affinity to the cell membrane of the globotriaosylceramide (Gb_3) cell membrane Gb_3 receptor, allowing Stx to be internalized into endosomes towards the trans-Golgi network (Mallard et al., 1998).

Gb_3 is synthesized from lactosylceramide by α1,4-galactosyltransferase (Okuda and Nakayama, 2008). Binding of StxB to Gb_3 induces intracellular signals dependent on glycolipid-enriched membrane domains (Falguières et al., 2001; Takenouchi et al., 2004). Consequently, Stx may cause apoptosis in cells expressing Gb_3 in human brain endothelial cells (Fujii et al., 2008) and combined with either LPS (Louise and Obrig, 1992) or cytokines (Louise and Obrig, 1991) in HeLa cells (Fujii et al., 2003) or in the renal epithelium (Psotka et al., 2009). Also, TNF-α upregulates Gb_3 and confers sensitivity to different types of endothelial cells (Eisenhauer et al., 2001; Louise and Obrig, 1991).

Gb_3 cell localization and expression may vary in different cell populations in the nervous systems of different animal species, either treated or not with Stx2. It has been reported that normal rat and mouse dorsal root ganglion neurons express Gb_3 (Ren et al., 1999). Normal mouse central nervous system neurons have also been reported to express Gb_3 (Obata et al., 2008). In addition, the expression of Gb_3 in peripheral and central nervous system seems to have a correlation in humans, as it is expressed in vessels and neurons of the nervous system (Ren et al., 1999; Obata et al., 2008). For instance, it has been reported that Stx2 increases neuronal transmitter release in murine brain slices (Obata et al., 2008). Also, changes in the expression of the neurotransmitter nitric oxide and

Abbreviations: Gb_3, Globotriaosylceramide; MAP2, Microtubule-associated protein 2; GFAP, Glial fibrillary acidic protein; Stx2, Shiga toxin 2.

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reactive astrocytes have also been observed in rat brains (Boccoli et al., 2008). In rabbit brains, Stx2 might damage neuronal cells by the inflammatory response that occurs in endothelial cells expressing Gb_3 (Takahashi et al., 2008). In addition, expression of Gb_3 has been found in neurons and endothelial cells of normal motor cortex from brains of human cadavers (Obata et al., 2008).

As an attempt to determine whether Stx2 could affect rat brains by possessing a direct neurotoxic involvement, in the present work we studied the role of Stx2 in brain injury. Our group has previously investigated the effects of the i.c.v. administration of Stx2 at the ultrastructural level (Goldstein et al., 2007). This approach discarded the synergistic effect of Stx2 with the pro-inflammatory cytokines TNF-α and IL-1β produced in circulatory endothelial cells (Paton and Paton, 1998). Accordingly, (in that work) we observed the highest incidence of brain damage after 8 days of i.c.v. administration of Stx2. This event included neuronal apoptosis, hypertrophic and demyelinated axons, the immunoelectron localization of Stx2 in these axons and in the nuclei of astrocytes, astrocytic cytoplasmic edema and gliosis, and demyelinated sheaths in oligodendrocytes. It has been postulated that Stx2B may be involved in the inhibition of rRNA assembly occurring in the nucleus (Falguieres and Johannes, 2006). Since neuronal apoptosis may be caused by the action of endocytosed Stx2, rat brain neurons may express a Gb_3 receptor for the toxin.

In the present work we seek to obtain new knowledge for the role of the Stx2 receptor Gb_3, in an attempt to further address the neurological damage caused by Stx2 from enterohemorrhagic *E. coli*.

Stx2 may produce changes in the expression of its Gb_3 receptor in neurons, and concomitantly alter the expression of the proteins involved in neuronal plasticity and synapsis. Apoptotic neurons found at the ultrastructural level (Goldstein et al., 2007) may express pro-apoptotic proteins. These events may occur in a reactive astrocytic scenario, a characteristic of the area of brain damage.

To address these issues, the aims of the present study were: (i) to assess whether Stx2 can induce changes in the expression of the Gb_3 receptor in the rat brain, (ii) to investigate the effects of Stx2 on the expression of the cytoskeletal protein MAP2 related to dendritic structure and synaptic plasticity (for review refer to Sánchez et al., 2000), (iii) to determine whether Gb_3 induction by Stx2 occurs in a reactive astrocytic area, and (iv) to correlate these events as a result of the expression of the pro-apoptotic Bax protein in neurons.

2. Materials and methods

2.1. Stx2 protein purification

Stx2 was purified by affinity chromatography under native conditions. The Stx2 purification procedures have been previously described and characterized (Goldstein et al., 2007). Briefly, recombinant *E. coli* DH5α containing pStx2 were cultured overnight in LB supplemented with 100 μg/ml ampicillin. The culture was centrifuged and the supernatant was precipitated in 60% $SO_4(NH_4)_2$ 1 mM PMSF following precipitation at 12,000 rpm at 4 °C for 20 min. The concentrate was dialized to remove the $SO_4(NH_4)_2$ overnight with 4 volumes of PBS. The pellet obtained was resuspended in PBS with a cocktail of protease inhibitors (20 mM leupeptin, 1 mM aprotinin, 10 uM pepstatin A, 1 mM PMSF) and incubated with the Globotriose Fractogel Resin (IsoSep AB, Tullinge, Sweden) for 4 h at 4 °C. The resin was washed and eluted with $MgCl_2$. The protein concentration in all the eluates was followed measuring the absorption at 280 nm, and the toxin was aliquoted and stored at −70 °C. Protein content of all the fractions was monitored by Silver Coomassie Blue (Candiano et al., 2004), and the presence of Stx2 was confirmed by Western Blot, which showed a 7.7-kDa band corresponding to Stx2B and a 32-kDa band corresponding to Stx2A. The cytotoxic capacity of Stx2 was assessed in Vero cells by neutral red assay for 72 h and the cytotoxic dose 50 (CD_{50}) found was about 1 pg/ml (Fig. 1A). This effect was

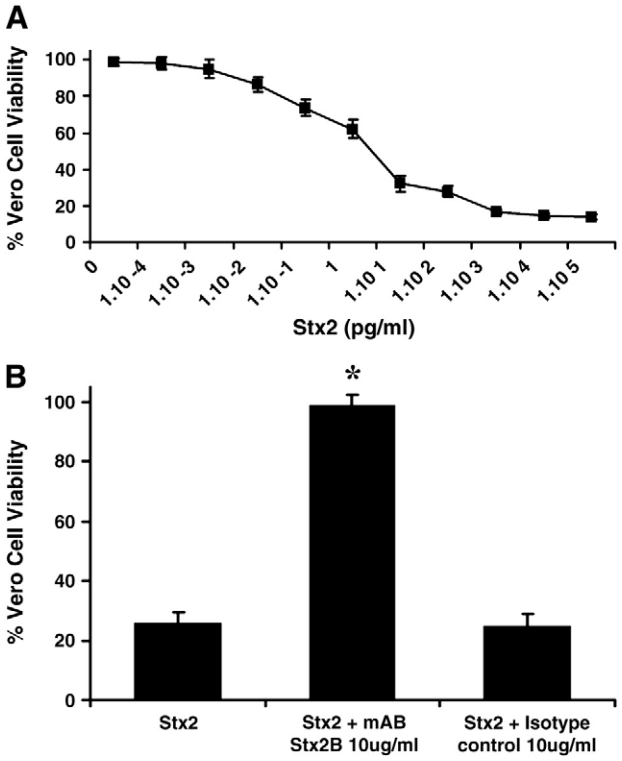

Fig. 1. Stx2 obtained by affinity chromatography purification was cytotoxic to Vero cells. The Stx2 cytotoxic capacity was confirmed on a Vero monolayer cell culture (A). Preincubation of the toxin with a monoclonal anti-Stx2B antibody resulted in a significant increase in Vero cell viability, while that with an isotype monoclonal antibody had no neutralizing effect (B). Data are reported as means ± S.E.M. of at least three triplicate experiments (*$p<0.05$).

neutralized by means of preincubation with an anti-subunit 2B monoclonal antibody (Sifin, Berlin, Germany), and not neutralized when using an isotype antibody instead (Fig. 1B).

Another set of control experiments was performed to demonstrate that an i.c.v. administration of 50 ng/ml of lipopolysaccharide (LPS) alone (without Stx2) does not increase the (levels of) the Gb_3 receptor. Consequently, LPS was removed from the Stx2 solution to use it for additional experiments aimed to demonstrate that Gb_3 neuronal expression was increased only by the i.c.v. of Stx2. LPS was removed from the Stx2 solution by using Detoxi-gel (Pierce, Rockford, USA). This Stx2 solution contained less than 0.03 EU/ml.

2.2. Vero cell culture

Vero cells were maintained in DMEM supplemented with 10% fetal calf serum. Cells were seeded in 96-well plastic microplates for cytotoxicity assays to 90% confluency. Cytotoxicity experiments were carried out without serum.

2.3. Animals

Male Sprague–Dawley rats (250–300 g) were housed in an air-conditioned and light-controlled (lights between 06:00 and 18:00 h) animal facility. Rats were provided with food and water *ad libitum*. They were daily monitored for weight and observed for neurological manifestations from the beginning of the experiment until the last day, always at the same time. After 8 days of i.c.v. Stx2, Stx2 free of LPS, LPS (Sigma, St. Louis, MO, USA) or vehicle infusions, the rats were killed for confocal immunofluorescence studies. Rats were anesthetized with Chloral hydrate (350 mg/kg) and perfused transcardially with 0.9% NaCl solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) [fixative per animal weight (ml/g)]. Brains were removed from the skull, and post-fixed in the same fixative solution for 2 h. Brain