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Interleukin-1β contributes to dopaminergic neuronal death induced by lipopolysaccharide-stimulated rat glia *in vitro*

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ABSTRACT

Inflammation is involved in the pathology of Parkinson's disease, a disorder characterised by degeneration of dopaminergic neurons. This study demonstrates that conditioned medium (CM) from lipopolysaccharide (LPS)-treated rat glial-enriched cortical cultures induced death of embryonic rat dopaminergic neurons in vitro, an effect which was additive to the toxicity of the neurotoxin 6-hydroxydopamine. Interleukin-1β (IL-1β) in the CM may mediate this neuronal death. IL-1R1 was found to be expressed on dopaminergic neurons. Blockade of IL-1R1 prevented CM-induced dopaminergic neuronal death. This study suggests that IL-1β in CM from LPS-stimulated glia contributes to dopaminergic neuronal death induced by glia-conditioned medium.

1. Introduction

Parkinson's disease (PD) is a common age-related neurodegenerative disorder. In the majority of cases, PD occurs idiopathically, whilst in the remaining 5–10% of cases, a genetic mutation is present (for review, see Toulouse and Sullivan, 2008). The cardinal symptoms of PD are bradykinesia, tremor at rest, gait disturbances, postural instability and rigidity.

The involvement of inflammation in the progression of PD has been well documented (for reviews, see Gao and Hong, 2008; Long-Smith et al., 2009). The initial evidence came from a *post-mortem* study over twenty years ago which showed the presence of activated microglia and T-lymphocytes in the substantia nigra (SN) pars compacta (pc) of a PD patient (McGeer et al., 1988). Since then, an abundance of studies have supported a role for activated microglia in the pathology of PD (Banati et al., 1998; Imamura et al., 2003; Hirsch and Hunot, 2009). The presence of activated microglia in rat brains lesioned with 6-hydroxydopamine (6-OHDA), a neurotoxin used to model PD, has been reported by numerous groups (Akiyama and McGeer, 1989; He et al., 2001; Depino et al., 2003; Crotty et al., 2008). Further evidence for a role for inflammation in PD comes from studies that revealed an increase in the expression of the pro-inflammatory cytokines, interleukin (IL)-1β, tumour necrosis factor-α (TNF-α) and IL-6, in PD patients compared with healthy subjects (Boka et al., 1994; Mogi et al., 1994a,b; Dobbs et al., 1999). Studies using animals and cells have also demonstrated the contribution of pro-inflammatory cytokines to dopaminergic neuronal demise in models of PD. In one such study, chronic expression of IL-1β in adult rat SNpc using a recombinant adenovirus caused the death of dopaminergic neurons after three weeks (Ferrari et al., 2006). A study using neutralising antibodies to IL-1β and TNF-α showed that approximately 50% of the dopaminergic neuronal cell death in primary cultures of rat midbrain, induced by the bacterial cell membrane constituent and endotoxin lipopolysaccharide (LPS), was mediated by the production of these two cytokines (Gayle et al., 2002). In support of a role for pro-inflammatory cytokines in the neuronal death induced by 6-OHDA, blockade of the soluble form of the TNF-α receptor, but not of the transmembrane form, was found to attenuate the death of dopaminergic neurons in 6-OHDA-lesioned rats (McCoy et al., 2006). A link between exposure to LPS during *pre-natal* development and the risk of developing PD-like symptoms in rats has already been shown (Ling et al., 2002, 2004). Indeed, formyl-methionyl-leucylphenylalanine, a bacterial-derived chemoattractant involved in systemic infection, has been reported to cause the death of dopaminergic neurons in mouse midbrain cultures via the activation of microglia (Gao et al. 2008). However, regardless of whether the inflammation in PD is a cause or a consequence of the disease, a vicious cycle of inflammation has been proposed to exacerbate the debilitating effects of dopaminergic neuronal loss (Block and Hong, 2007).

The aims of the current study were to 1) assess the effect of cytokine-rich conditioned medium from LPS-treated rat glial-enriched cortical cultures on dopaminergic neuronal death in 6-OHDA-treated

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embryonic rat neuronal-enriched ventral mesencephalon (VM) cultures, and 2) focus on the contribution of glial-derived IL-1β to the observed dopaminergic neuronal demise. The current data suggest a role for IL-1β in the death of dopaminergic neurons induced by glial-conditioned medium. This theory is supported by the presence of the IL-1 receptor type 1 (IL-1R1) on dopaminergic neurons in these cultures and the prevention of CM-induced dopaminergic neuronal death by the IL-1 receptor antagonist (IL-1RA).

2. Materials and methods

2.1. Animals

Pregnant Sprague–Dawley rats and Sprague–Dawley pups were provided by the Biological Services Unit, University College Cork. All scientific procedures were performed under a license issued by the Department of Health and Children (Ireland) and in accordance with the European Communities Council Directive (86/609/EEC). The animals were maintained in 12:12 hour light: dark cycle during which food and water were available *ad libitum*.

2.2. Primary cultures of embryonic day (E) 14 rat VM

Time-mated adult female Sprague–Dawley rats were anaesthetised by inhalation of halothane or isofluorane (both from Abbeyville Veterinary, Ireland) and were decapitated. The E14 embryos were removed following laparotomy. The crown-rump length was between 9.5–10.5 mm in accordance with the Carnegie stage of rat development (Witschi, 1962). The mesencephalic region was dissected out, then the neural tube was cut along the dorsal midline. The dorsal mesencephalon on the lateral edges was removed, as were the meninges. The VM was collected in ice-cold sterile Hank's balanced saline solution (HBSS) (Sigma, Ireland). The lateral portions of the VM were removed; medial VM was used to prepare the cultures, as it has been shown to give a higher yield of dopaminergic neurons than that obtained from whole VM (Clayton and Sullivan, 2007). Tissue from one litter was pooled and represented an individual culture. The medial VM pieces were incubated in 0.1% trypsin (Sigma) in sterile HBSS for 5 min. DNase (25 μg/ml) (Sigma) was added for 1 min at 37 °C. Soybean trypsin inhibitor (0.5 mg/ml) (Sigma) and bovine serum albumin (BSA) (1 mg/ml) (Sigma) were added and the suspension was centrifuged for 5 min at 11,000 rpm (Beckman Coulter Allegra™ 21R Centrifuge) at room temperature. The supernatant was removed and the pellet was resuspended in 1 ml of pre-warmed medium (Dulbecco's Modified Eagle's Medium (DMEM) F-12 containing 100× L-glutamine (2 mM), 100× penicillin/streptomycin (1%; 100 U/ml penicillin, 100 μg/ml streptomycin), amphotericin B (250 μg/ml), $_D$-glucose (33 mM), gentamycin (50 μg/ml), 1% fetal calf serum (all Sigma) and 2% B-27-antioxidants (AO) (Gibco Invitrogen, Ireland)). A B-27 supplement without the normal anti-oxidants present in B-27 was used. The cells were triturated using a sterile 26-gauge needle and 1 ml syringe. Cells were cultured at a density of 1×10^5 cells per poly-D-lysine (Sigma)-coated 13 mm coverslip (Alkem, Ireland), in 500 μl of medium at 37 °C with 5% CO_2. Immunocytochemical staining for the neuronal marker, β-III-tubulin, showed that 89% of the cells in culture were neurons. After 2 days *in vitro* (DIV), the medium was replaced with 300 μl medium or 6-OHDA (10 mM pre-stabilised 6-OHDA in 0.01% ascorbic acid solution; Sigma) (0, 20 or 40 μM)-containing medium for 30 min and the cells were subsequently fixed for immunocytochemical analysis. For the conditioned medium (CM) study, the cultures were pre-treated for 1 h with steri-filtered CM from LPS-treated *post-natal* day (P)2 rat glial-enriched cortical cultures or control, then treated with 6-OHDA (40 μM) for 30 min. For the IL-1β study, IL-1β (10 ng/ml) (R&D systems, UK) was added 1 h before 6-OHDA (40 μM) treatment. For the IL-1RA study, IL-1RA (1 μg/ml) (R&D systems) was added to the cultures for 30 min, followed by CM for 1 h, before treatment with 6-OHDA (40 μM) or control for 30 min.

2.3. Primary cultures of P2 rat cortex

P2 Sprague–Dawley rats were anaesthetised by hypothermia on ice and were killed by decapitation. Brains were removed and transferred to sterile Petri dishes containing ice-cold HBSS. The cerebral hemispheres were separated and the cortex was dissected out. The meninges were carefully removed and cortical tissue was placed in ice-cold sterile HBSS. The dissected cortical tissue was incubated in 0.1% trypsin solution in sterile HBSS for 15 min at 37 °C with 5% CO_2. This was replaced with pre-warmed dissociation medium (10% heat-inactivated (50 °C, 30 min), normal horse serum (NHS) (Sigma) and DNase (100 μg/ml) in DMEM F12). The suspension was inverted gently for 1 min, triturated with a flame-polished Pasteur pipette and passed through a 70 μm cell strainer to remove cellular debris. The suspension was centrifuged for 5 min at 1000 rpm at room temperature, the supernatant was removed and the pellet was resuspended in 1 ml of medium (10% NHS and 100× penicillin/streptomycin (1%; 100 U/ml penicillin, 100 μg/ml streptomycin) in DMEM-F12). The cells were cultured at 1×10^5 per poly-D-lysine-coated 13 mm glass coverslip, in 24-well plates at 37 °C with 5% CO_2. Immunocytochemical analysis confirmed that the culture was composed of cells immunopositive for GFAP (astrocytes): 33%; nestin (progenitors): 32%; OX-42 (microglia): 17% and β-III-tubulin (neurons): 18%. After 3 DIV, half of the medium was replaced with fresh medium. After 4 DIV, half of the medium was replaced with fresh medium, or with LPS (final concentrations of 50, 100, 500 or 1000 ng/ml; Sigma)-containing medium. The cultures were incubated with LPS for 24 h, the supernatant was removed and stored at −20 ºC. Supernatant from individual wells was stored separately for analysis of cytokine concentrations by enzyme-linked immunosorbant assay (ELISA), while supernatant which was to be used as CM was pooled. Before the CM was used, it was defrosted thoroughly and filtered through a 0.20 μm filter.

2.4. ELISA analysis of IL-1β and TNF-α

The concentration of IL-1β and TNF-α was assessed in supernatant obtained from LPS-treated glial-enriched cortical cultures using ELISA (R&D Systems). Antibody-coated (0.84 μg/ml goat anti-rat IL-1 antibody or 4 μg/ml mouse anti-rat TNF-α antibody in PBS, pH 7.3) 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20, blocked for 1 h at room temperature with blocking buffer (1% bovine serum albumin (BSA) in PBS), and then incubated with standards (0–2000 pg/ml) or samples for 2 h at room temperature. Wells were washed with PBS, incubated with secondary antibody (350 ng/ml biotinylated goat anti-rat antibody for IL-1β or 100 ng/ml biotinylated goat anti-rat antibody for TNF-α, diluted in PBS containing 1% BSA and 2% normal goat serum) for 2 h at room temperature, washed again and incubated in horseradish peroxidase-conjugated streptavidin (1:200 dilution in PBS containing 1% BSA) for 20 min at room temperature. Wells were washed and substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) (R&D Systems) was added, incubation continued at room temperature in the dark for 30 min and the reaction stopped using 2 N H_2SO_4. Absorbance was read at 450 nm with a wavelength correction of 540 nm, a standard curve was constructed by plotting the standard concentrations against the absorbance, and the unknown concentrations of the samples were calculated from the standard curve. Data are expressed as pg/ml.