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Role of microglia in inflammation-mediated degeneration of dopaminergic neurons: neuroprotective effect of Interleukin 10

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Summary. Inflammation in the brain has been recognized to play an increasingly important role in the pathogenesis of several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease. Inflammation-mediated neurodegeneration involves activation of the brain's resident immune cells, the microglia, which produce proinflammatory and neurotoxic factors including cytokines, reactive oxygen species (ROS), nitric oxide, and eicosanoids that directly or indirectly cause neurodegeneration. In this study, we report that IL-10, an immunosuppressive cytokine, reduced the inflammation-mediated degeneration of dopaminergic (DA) neurons through the inhibition of microglial activation. Pretreatment of rat mesencephalic neuron–glia cultures with IL-10 significantly attenuated the lipopolysaccharide (LPS) induced DA neuronal degeneration. The neuroprotective effect of IL-10 was attributed to inhibition of LPS-stimulated microglial activation. IL-10 significantly inhibited the microglial production of tumor necrosis factor α (TNF-α), nitric oxide, ROS and superoxide free radicals after LPS stimulation.

Introduction

The pathogenesis of several neurological disorders, including Parkinson's disease, Alzheimer's disease (Dickson et al., 1993; Liu and Hong, 2003), is now thought to be mediated by an inflammatory response by resident cells in the brain. Microglia, the resident immune cells of the brain, contribute to this inflammation by serving the role of immune surveillance and host defense (Kreutzberg, 1996). Activated microglia produce a variety of pro-inflammatory factors and reactive oxygen species (ROS), all of which serve immune surveillance functions by removing foreign microorganisms (Aloisi, 1999). Our previous results have shown that over-activation of microglia and overproduction of pro-inflammatory factors may lead to neuronal degeneration in the CNS (Liu et al., 2002).

Interleukin (IL)-10 is a cytokine produced by a variety of cell types including type 2 helper T cells, B cells, and macrophages. IL-10 has been shown to suppress inflammation in many experimental models of inflammatory disease. Mizuno et al. (1994) have found that cells in the CNS also produce IL-10. Microglia, which are cells in the CNS very similar both phenotypically and functionally to macrophages, express IL-10 receptor mRNA, and consequently they may be strongly regulated by IL-10. IL-10 produced in the CNS, therefore, may play an important role in the pathophysiology

of CNS disorders by inhibiting the function of microglia.

The goal of the present study is to evaluate the effects of IL-10 on LPS-induced neurotoxicity in rat primary midbrain cultures. Here, we show that IL-10 attenuate microglial pro-inflammatory cytokine and ROS production and protect DA neurons from LPS-induced neurotoxicity.

Materials and methods

Reagents

The recombinant rat IL-10 (rrIL-10; R & D system, Minneapolis, MN). LPS were purchased from Sigma-Aldrich (St. Louis, MO). All the cell culture ingredients were obtained from Invitrogen (Carlsbad, CA). The [^{3}H] DA (30 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA), The fluorescence probe DCFH-DA was obtained from Calbiochem (La Jolla, CA).

Rat mesencephalic neuron–glia cultures

Primary mesencephalic neuron–glia cultures were prepared from the brains of embryonic day 14/15 Fischer 344 rats, following our previously described protocol (Liu et al., 2002). Briefly, the ventral mesencephalic tissues were removed and dissociated by a mild mechanical trituration. Cells were seeded at 5×10^5/well to 24-well culture plates pre-coated with poly-D-lysine (20 ng/ml) and maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in 0.5 ml/well maintenance medium. The medium consisted of minimum essential medium containing 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum, 1 g/l glucose, 2 mM [SCAP]L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 50 U/ml penicillin, and 50 ng/ml streptomycin. Three days after the initial seeding, 0.5 ml of fresh maintenance medium was added to each well. Seven-day-old cultures were used for treatment. The composition of the cultures at the time of treatment was approximately 48% astrocytes, 11% microglia, 40% neurons, and 1 to 1.5% TH-immunoreactive (ir) neurons.

Primary microglia-enriched cultures

Rat microglia-enriched cultures, with a purity of >98%, were prepared from whole brains of 1-day-old Fischer 344 rat pups, following our described protocol (Liu et al., 2002). For superoxide assays, 10^5 cells/well/0.2 ml medium were grown overnight in 96-well culture plates before use.

Uptake assay

[^{3}H]DA uptake assays were performed as described previously (Liu et al., 2002). Cultures were incubated for 20 min at 37°C with 1 μM [^{3}H]DA in Krebs-Ringer buffer (16 mM sodium phosphate, 119 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.3 mM EDTA, and 5.6 mM glucose; pH 7.4). After washing three times with ice-cold Krebs-Ringer buffer, cells were collected in 1 N NaOH. Radioactivity was determined by liquid scintillation counting. Nonspecific DA uptake observed in the presence of mazindol (10 μM) was subtracted.

Nitrite and TNFα assays

The production of NO was determined by measuring the accumulated levels of nitrite in the supernatant with the Griess reagent, and release of TNFα was measured with a rat TNFα enzyme-linked immunosorbent assay kit from R & D Systems (Minneapolis, MN).

Superoxide assay

The production of superoxide was determined by measuring the superoxide dismutase (SOD)-inhibitable reduction of the tetrazolium salt WST-1. Microglia-enriched cultures in 96-well culture plates were washed twice with Hanks' balanced salt solution without phenol red (HBSS). Cultures were then incubated at 37°C for 30 min with vehicle control (water) or IL-10 in HBSS (50 μl/well). Afterward, to each well was added 50 μl of HBSS with and without SOD (50 U/ml, final concentration), 50 μl of WST-1 (1 mM) in HBSS, and 50 μl of vehicle or LPS (10 ng/ml). Thirty minutes later, absorbance at 450 nm was read with a SpectraMax Plus microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). The difference in absorbance observed in the absence and presence of SOD was considered to be the amount of superoxide produced, and results were expressed as percentage of vehicle-treated control cultures.

Statistical analysis

The data were expressed as the mean ± S.E.M. Statistical significance was assessed with an analysis of variance followed by Bonferroni's t test using the Stat View program (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was considered statistically significant.

Results

Effect of IL-10 on LPS-induced degeneration of DA neurons

Mesencephalic neuron–glia cultures were pretreated with IL-10 for 1 h and then stimulated with LPS for 7days. The degeneration of DA neurons was then determined by [^{3}H] DA uptake assay. The [^{3}H] DA uptake assay showed that LPS treatment reduced the capacity of the cultures to take up DA to approximately 40% of the vehicle control (Fig. 1A). At 30 ng/ml IL-10, the LPS-induced decrease in DA uptake was completely restored, and IL-10 alone at this concentration range did not affect DA uptake levels in the cultures.

IL-10 treatment inhibits LPS-induced production of NO, TNFα and intracellular and extracellular reactive oxygen species

The LPS-stimulated activation of microglia was suppressed by pretreatment with IL-10 in neuron–glia cultures. Accumulation of nitrite, an indicator of LPS stimulated production of NO, was determined 24 hrs and 48 hrs after LPS stimulation. As shown in Fig. 1B, pretreatment with 30 ng/ml IL-10, completely blocked LPS-stimulated NO production. As shown in Fig. 1C, pretreatment with 30 ng/ml IL-10 significantly reduced LPS-induced production of TNFα determined at 3 h after LPS stimulation.

To test the effect of IL-10 on the microglial generation of ROS, enriched-microglial

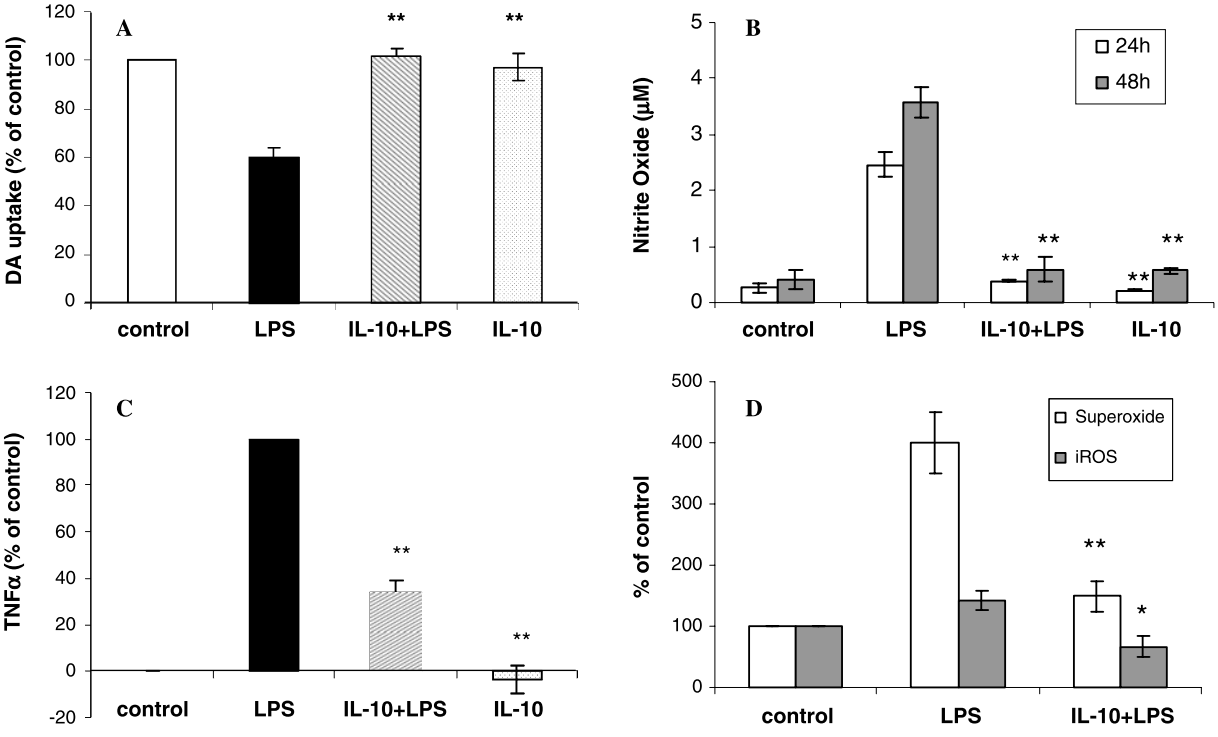

Fig. 1. IL-10 is neuroprotective against LPS-induced neurotoxicity and inhibits LPS-induced microglia activation. Rat primary mesencephalic neuron–glia cultures seeded in a 24-well culture plate at 5×10^5 cells were pretreated with IL-10 (30 ng/ml) for 1 h before the addition of 10 ng/ml LPS. Eight days later, the LPS-induced dopaminergic neurotoxicity was quantified by the [^{3}H] DA uptake assay (**A**); Effects of IL-10 on LPS-induced production of nitrite oxide (**B**); TNF-α (**C**); superoxide and iROS (**D**) as % of control. The results are the mean ± SE of 4 individual experiments in triplicate in each experiment. $^*P < 0.05$, $^{**}P < 0.01$, compared with LPS culture

cultures were pretreated with IL-10, then exposed to LPS. IL-10 significantly inhibited intracellular ROS production and microglial superoxide response to nearly control levels. (Fig. 1D). Based on our previous data that indicates a central role for ROS in microglial-mediated destruction of DA neurons, it appears that the neuroprotective effect of IL-10 is at least partially due to a reduction in LPS-induced oxidative stress.

Discussion

Degeneration of the nigrostriatal DA pathway is a hallmark of Parkinson's disease. LPS-induced degeneration of DA neurons in mesencephalic neuron–glia cultures is a useful *in vitro* model for the identification of potential therapeutic agents. Our data confirms that microglia play an important role in Parkinson's disease by secreting pro-inflammatory mediators such as TNFα, nitric oxide, and ROS. And this inflammatory response can be inhibited by the anti-inflammatory cytokine, IL-10. Concomitantly, IL-10 also can protect the DA neurons from LPS-induced DA neuronal degeneration. This further supports the notion that pro-inflammatory products from micorglia are responsible for all or most of the neurodegenerative phenotype seen in Parkinson's patients.

IL-10 is predominantly an immunosuppressive and anti-inflammatory cytokine, and plays a critical role in limiting tissue injury during infections by limiting the duration and intensity of immune and inflammatory reactions (Moore et al., 2001; Berg et al., 1995), including the response to LPS. LPS has been shown to activate macrophages and microglial cells through the TLR-4-mediated signaling pathway (Olson and Miller, 2004), leading to the production of pro-inflammatory mediators such as cytokines, reactive oxygen species, reactive nitrative species, NO, and eicosanoids. IL-10 has been shown to strongly regulate that pathway in macrophages, also to predominantly inhibit the NF-κB pathway through interference in transcriptional regulation (Schottelius et al., 1999), and it is likely that activation of this pathway is a major player in the inflammatory response seen in Parkinson's patients. Therefore, it is likely that this pathway could serve as an excellent target for therapy directed at decreasing neuronal destruction in Parkinson's disease.

We show here that IL-10 regulates a large number of pro-inflammatory mechanisms in microglia that may have a role in the destruction of DA neurons, including TNFα, NO, and ROS. However, the exact mechanism by which the neuroprotective effect of IL-10 on activated microglia is unclear, since each of these neurotoxic factors may have similar effects on DA neurons. Our previous results have shown that microglial NADPH oxidase plays a crucial role in causing DA neuronal death by over-activated microglia through the direct or indirect induction of ROS and TNFα production (Qin et al., 2004). Therefore, it appears that IL-10 may mediate its neuroprotective effect either directly or indirectly through the inhibition of NADPH oxidase activity, perhaps by regulating the NF-κB-dependent activation or function of PHOX. Experiments to test these hypotheses are currently underway.

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