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Diclofenac enhances proinflammatory cytokine-induced phagocytosis of cultured microglia via nitric oxide production

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ABSTRACT

Influenza-associated encephalopathy (IAE) is a central nervous system complication with a high mortality rate, which is increased significantly by the non-steroidal anti-inflammatory drug diclofenac sodium (DCF). In the present study, we investigated the effects of DCF on brain immune cells (i.e. microglia) stimulated with three proinflammatory cytokines, namely tumor necrosis factor-α, interleukin-1β, and interferon-γ. Similar to previous findings in astrocytes, all three cytokines induced the expression of inducible NO synthase (iNOS), as well as NO production, in microglia. The addition of DCF to the culture system augmented iNOS expression and NO production. Immunocytochemical analysis and the phagocytosis assay revealed that cytokine treatment induced morphological changes to and phagocytosis by the microglia. The addition of DCF to the culture system enhanced microglial activation, as well as the phagocytic activity of cytokine-stimulated microglia. Inhibitors of nuclear factor (NF)-κB inhibited iNOS gene expression in cytokine-stimulated microglia with or without DCF, suggesting that the NF-κB pathway is one of the main signaling pathways involved. The iNOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) reduced both cytokine-induced phagocytosis and phagocytosis induced by the combination of cytokines plus DCF. Furthermore, the NO donor sodium nitroprusside induced phagocytosis, indicating that NO production is a key regulator of microglial phagocytosis. In conclusion, DCF acts synergistically with proinflammatory cytokines to increase the production of NO in microglia, leading to phagocytic activity of the activated microglia. These findings, together with previous observations regarding astrocytes, may explain the significant increase in mortality of IAE patients treated with DCF.

Introduction

Influenza-associated encephalopathy (IAE) is a central nervous system (CNS) complication associated with a high mortality rate and neurological sequelae (Wang et al., 2010). Encephalitis and/or encephalopathy associated with the influenza virus are characterized by rapid progression and a mortality rate in the range 27%–44% (Mizuguchi et al., 2007), and account for 40%–50% of patients with Reye-like syndrome or acute necrotizing encephalopathy (Mizuguchi et al., 2007). Patients with IAE have elevated levels of inflammatory cytokines (including tumor necrosis factor α [TNFα], interleukin-1β [IL-1β], and interferon γ [IFNγ]), and nitric oxide (NO) in both their serum and cerebrospinal fluid (CSF; Hasegawa et al., 2011; Kawada et al., 2006; Kawashima et al., 2005; Mizuguchi et al., 2007; Morishima et al., 2002; Yamanaka et al., 2006). Therefore, many researchers believe that the pathogenesis of IAE involves hyperimmunization and a cytokine storm in the CNS (Mizuguchi et al., 2007). Reye syndrome is a representative neurological complication associated with influenza virus infection. It is characterized by encephalopathy and fatty degeneration of the liver, triggered by viral infection. Drugs such as aspirin, a non-steroidal anti-inflammatory drug (NSAID), and valproic acid are known to be associated with encephalopathy (Belay et al., 1999; Powell-Jackson et al., 1984; Schrör, 2007). Similarly, the use of diclofenac sodium (DCF), an NSAID, is associated with increased mortality in IAE (Mizuguchi et al., 2007). Previously, we showed that DCF acts synergistically with proinflammatory cytokines by increasing the production of iNOS and NO by astrocytes, resulting in cell damage (Kakita et al., 2009). It has been suggested that DCF-induced enhancement of NO production by astrocytes may explain the significant increase in mortality in IAE patients treated with DCF (Kakita et al., 2009). Nevertheless, how DCF aggravates IAE remains to be determined. Investigating the effects of DCF on other cell types may contribute to the clarification of the pathogenesis of IAE.

The active contribution of astrocytes to the inflammatory process raises the question as to how astrocytes may interact with microglia.

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Microglia are resident immune cells of the CNS that serve as neuropathological sensors under conditions of severe neuronal injury (Walter and Neumann, 2009). After various inflammatory responses have occurred, the microglia play a crucial role in maintaining homeostasis against severe neuronal injury. Microglial activation is associated with morphological changes, proliferation, motility, phagocytosis, and the release of reactive oxygen species, reactive nitrogen species, interleukins, cytokines, and chemokines (Boscia et al., 2009; Harrigan et al., 2008). Microglia eliminate pathogens, clearing debris to support repair. However, excessive activation of microglia may destroy the surrounding healthy neurons in addition to damaged neurons (Brown and Neher, 2010; Neumann et al., 2009).

In the present study, we investigated whether DCF enhances NO production and the phagocytic function of cultured microglia stimulated with proinflammatory cytokines. To clarify the pathogenesis of IAE, primary rat microglia were treated with TNFα, IL-1β, and IFNγ, all of which are elevated in the serum and CSF of patients with IAE, to examine whether DCF affects NO production and the phagocytic activity of microglia under inflammatory conditions.

Materials and methods

Microglial culture. The protocols used in the present study were approved by the Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences. Microglia were prepared from the cerebral cortex of postnatal day 1 Wistar rats, as described previously (Nagano et al., 2010). Briefly, the cerebral cortex was dissected, trypsinized, and dissociated in high-glucose (4500 mg/L) Dulbecco's modified Eagle's medium (h-DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Dissociated cells were plated in 75-cm^2 culture flasks (Corning Incorporated Life Sciences, Lowell, MA). After 10 days, the culture flasks were gently agitated on an orbital shaker at 100 r.p.m. for 30 min to yield loosely attached cells. Supernatants containing detached cells were collected and re-plated on non-coated plates. Twenty-four hours before treatment experiments, the culture medium was replaced with h-DMEM containing 1% N-2 supplement (Invitrogen). Immunocytochemistry with ionized calcium-binding adaptor protein 1 (Iba-1) antibodies (Wako, Osaka, Japan) revealed that >95% of the cell population was microglia.

Cell stimulation. After the culture medium had been replaced with a fresh medium, recombinant rat TNFα (20 ng/mL; Wako), IL-1β (5 ng/mL; Wako), and IFNγ (5 ng/mL; Wako) were added to the culture medium, as described previously (Kakita et al., 2009; Kozuka et al., 2005, 2007). When used, DCF (1 μg/mL; Wako), *N*-acetyl-*p*-aminophenol (APAP; 1 μg/mL; Sigma, St. Louis, MO), indomethacin (IND; 1 μg/mL; Sigma), or ibuprofen (IB; 15 μg/mL; Sigma) were added to the culture medium as COX inhibitors. The concentration of each COX inhibitor used in the present study was chosen on the basis of the maximum therapeutic concentration of that COX inhibitor in serum. Cells were maintained in a humidified chamber at 21% O_2.

Measurement of PGE_2 and NO production. After treatment, culture media were collected to evaluate PGE_2 and NO production. PGE_2 levels in the medium were measured using a commercially available enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions. NO production was evaluated by measuring the accumulation of nitrates and nitrites using a colorimetric reaction kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction. Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) was performed on selected genes using the 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was obtained from total RNA samples. Reverse transcription was performed using random primers and Ready-To-Go You-Prime First Strand Beads (GE Healthcare Bio-Science, Piscataway, NJ) and the resulting cDNA was subjected to PCR-based amplification. The primer pairs used for amplification were as follows:

iNOS: forward, 5′-CCTTGTTCAGCTACGCCTTC-3′,
iNOS: reverse, 5′-GGTATGCCCGAGTTCTTTCA-3′;
β-Actin: forward, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′,
β-Actin: reverse, 5′-CCTAGAAGCATTTGCGGTGCAGGATG-3′.

Real-time PCR was performed using SYBR Green Master Mix Reagents (Applied Biosystems). Amplifications were performed by activation of AmpliTaq Gold polymerase at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The amount of RNA was calculated using relative standard curves for each mRNA of interest and β-actin. mRNA expression of the genes of interest was normalized against that of β-actin to account for variability in the quality and total concentration of RNA, as well as RT efficiency.

Immunocytochemical staining analysis. Cells were plated on glass coverslips coated with BD Matrigel Matrix, Growth Factor Reduced (BD Bioscience, San Jose, CA) and treated as described previously (Kakita et al., 2009). After a 6 h treatment, cells were washed with phosphate-buffered saline (PBS), fixed in 3% paraformaldehyde at room temperature for 30 min, permeabilized with 0.2% Triton X-100 for 5 min, washed with PBS, and blocked for 1 h at room temperature with blocking solution (3% bovine serum albumin, 0.1% glycine in PBS). After cells had been washed with PBS, they were incubated with a polyclonal antibody against iNOS (1:500; BD Bioscience) for 1 h at room temperature, followed by incubation in goat anti-rabbit IgG fluorescence-conjugated Alexa Flour 488 secondary antibody (1:1000; Invitrogen) for another 1 h at room temperature. After cells had been washed with PBS, they were mounted on glass slides using ProLong Gold antifade reagent with 4′,6′-diamidino-2-phenylindole (DAPI; Invitrogen). Stained cells were examined under an AX70 fluorescence microscope (Olympus, Tokyo, Japan). For quantification, the number of iNOS-positive cells was counted at ×400 magnification in each field (~100 000 μm^2). To avoid counting of non-specifically stained cells, only those cells that were stained precisely were counted as iNOS-positive cells. In addition, the number of microglia exhibiting amoeboid morphology (i.e. a larger cell body relative to the nucleus) was determined at ×400 magnification in each field. Observers who were blinded to the treatment conditions performed the cell counts. Counts from five fields, chosen at random, were averaged, with experiments replicated at least three times.

Phagocytosis assay. Microglial activation was examined using the phagocytosis of latex beads (1 μm particular carboxylate-modified yellow-green florescent; Invitrogen), as described previously (Koizumi et al., 2007; Uesugi et al., 2012). The uptake of fluorescent particles was demonstrated using time-lapse imaging, with confocal microscopy used to confirm that the fluorescent beads had been taken up into the cells by phagocytosis (Koizumi et al., 2007; Uesugi et al., 2012). The latex beads were diluted with the medium used in the assay system to a concentration of 5×10^7 beads/well. After the extracellular beads had been washed away with PBS, they were mounted on glass slides using ProLong Gold antifade reagent with DAPI (Invitrogen). Microglia with florescent beads were examined under an AX70 fluorescence microscope (Olympus). For quantification, the number of phagocytosing microglia, which contained more than one fluorescent bead per cell, was determined in each field at ×400 magnification. Furthermore, the number of extracellular beads per field was counted at ×400 magnification. Observers blinded to the treatment conditions performed the cell counts. Counts from five fields, chosen at random, were averaged and experiments were replicated at least three times.