Neuroscience 229 (2013) 164–175

NEUROTOXINS RELEASED FROM INTERFERON-GAMMA-STIMULATED HUMAN ASTROCYTES

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Abstract—Astrocytes become activated in degenerative neurological diseases. In order to gain a greater understanding of the inflammatory factors released upon activation, we stimulated adult human astrocytes with interferon-gamma and examined the resultant conditioned medium (CM) for toxicity against differentiated human neuroblastoma SH-SY5Y cells. Cell death was measured by lactate dehydrogenase release assay. We then used various treatments of the media to determine the distribution and nature of the toxic components.

Removal of interleukin-6 by a specific antibody reduced the toxicity by 22%. Blockade of proteases with an inhibitor cocktail reduced it by a further 22%. When oxygen-free radical production was blocked with NADPH oxidase inhibitors, the toxicity was reduced by 15.4%. When prostaglandin production was blocked by cyclooxygenase inhibitors, the toxicity of the CM was reduced by 14.5%. When glutamate was removed by treatment with glutamate decarboxylase, the toxicity was reduced by 10.3%. When the inhibitors were added together to the astrocyte culture, the total toxicity of the CM was reduced by 91%. This was in reasonable agreement with the 85.37% total obtained by adding the individual components. The data show that activated astrocytes release a specific combination of neurotoxic compounds. They suggest that effective anti-inflammatory treatment of such neurodegenerative diseases as Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis could be improved by using an appropriate combination of anti-inflammatory agents instead of relying on any single agent.

Key words: interleukin-6, NADPH oxidase, cyclooxygenase, glutamate, proteases, neurotoxicity.

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Abbreviations: AD, Alzheimer disease; cDNA, complementary DNA; CM, conditioned medium; COX, cyclooxygenase; DMEM, Dulbecco's-modified Eagle's medium; DPI, diphenylene iodonium; EDTA, ethylene diamine tetra-acetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; GAD, glutamate decarboxylase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBU, ibuprofen; IFNγ, interferon-gamma; IL-6, interleukin-6; INT, p-iodonitrotetrazolium; LDH, lactate dehydrogenase; NADP(H), nicotinamide adenine dinucleotide phosphate-oxidase; PD, Parkinson's disease; PGE_2, prostaglandin E_2; PBS, phosphate-buffered saline; RA, retinoic acid; ROS, reactive oxygen species; RT-PCR, reverse-transcriptase polymerase chain reaction.

INTRODUCTION

Astrocytes are the most abundant glial cells in the brain. They supply nutrients to neurons, maintain an ionic balance between extracellular milieu and neurons, scavenge toxic molecules such as glutamate, release gliotransmitters and help to maintain the blood–brain barrier. They also coordinate with microglia to maintain an optimal extracellular environment (Stevens, 2008; Belanger and Magistretti, 2009; Perea et al., 2009; Sofroniew and Vinters, 2010). When they sense an abnormal environment, they rapidly change their morphology to a reactive state and function to protect neurons. For example, they release pro- and anti-inflammatory cytokines, neurotrophic factors, chemokines, complementary factors and reactive oxygen species (ROS), all of which potentially mediate neuroprotective and/or neurotoxic effects. They insulate inflammatory sites to prevent additional damage to neighboring cells (Liberto et al., 2004; Farina et al., 2007; Sofroniew and Vinters, 2010). There has been considerable debate as to whether the contribution of reactive astrogliosis in the neuroinflammatory processes should be regarded as beneficial or detrimental. Furthermore, astrocytes are known to be a heterogenous cell population based on their morphology and the expression of different sets of receptors, transporters, ions channels and other proteins (Matyash and Kettenmann, 2010). This raises the intriguing possibility that different subtypes of astrocytes are implicated in distinct metabolic homeostatic functions.

Neuroinflammation is a normal defense mechanism aimed at protecting the CNS against insults such as infection, injury or disease. It is usually a beneficial process that resolves on its own restoring homeostasis (Glass et al., 2010). However, when an insult may persist or the inflammatory process may become seriously out of balance the result, in combination with activated microglia, may be the induction of chronic neuroinflammation, a deleterious process contributing to the pathogenesis of several neurological diseases including Alzheimer's disease (AD) and Parkinson's disease (PD) (Allan and Rothwell, 2003; Whitton, 2007; Rossi and Volterra, 2009; Glass et al., 2010; Li et al., 2011).

In a previous study, we reported that stimulated monocytes and their surrogate THP-1 cells release a combination of neurotoxic factors (Lee et al., 2011a). In the present investigation we studied the nature of neurotoxic factors released by human astrocytes. For these studies, we stimulated them with interferon-gamma

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.10.033

(IFNγ) since our laboratory recently established that human astrocytes express IFNγ receptors (IFNγR) and IFNγ-stimulated astrocytes induce neurotoxicity through the IFNγR–STAT3 pathway (Hashioka et al., 2010, 2011).

EXPERIMENTAL PROCEDURES

Materials

Unless stated otherwise, reagents were purchased from Sigma (St. Louis, MO).

Cell culture

The human neuroblastoma cell line SH-SY5Y was a gift from Dr R. Ross, Fordham University, NY. Human astrocytes were isolated from surgically normal temporal lobe tissue that was resected in order to gain access to an epileptic focus in patients. Protocols, which were approved by the ethics committee in the University of British Columbia, were performed as described previously in detail (Lee et al., 2009; Hashioka et al., 2011). Briefly, tissues were dissected and incubated in 0.25% trypsin solution. They were pelleted, resuspended in Dulbecco's-modified Eagle's medium (DMEM)/F12 medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and then passed through a 100-μm nylon cell strainer. They were resuspended in the same medium, and plated onto tissue culture plates to achieve the adherence of microglial cells. The non-adherent astrocytes, along with myelin debris, were transferred onto other culture plates. Adherent astrocytes were grown in an incubator under humidified 5% CO_2 and 95% air conditions with the medium being replaced once a week. This procedure has previously been shown by GFAP immunostaining to produce astrocytic cultures of more than 99% purity (Hashioka et al., 2011). To establish the purity of astrocytes used in this study, the cultures were examined by immunostaining with a monoclonal anti-GFAP antibody (1/4000, DAKO, Mississauga, Ontario). To rule out the presence of contaminating microglia and oligodendrocytes, the cultures were immunostained with the microglial-specific monoclonal CR3/43 antibody (1/2000, DAKO, Mississauga, Ontario) and polyclonal anti myelin basic protein antibody (1/1000, Millipore, Billerica, MA) and to rule out neuronal contamination they were immunostained with the monoclonal anti-200 kDa neurofilament heavy antibody [RT97] (1/2000, Abcam, Cambridge, MA). The cells were then incubated with Alexa Fluor 546 goat anti-mouse (Invitrogen, 1:500) or Alexa Fluor 546 goat anti-rabbit (Invitrogen, 1:500) in the dark for 3 h at room temperature to yield a positive red fluorescence. To visualize all cells, the slides were washed twice with phosphate-buffered saline (PBS) and counterstained with the nuclear dye DAPI (100 μg/ml, Sigma) to give a blue fluorescent color. Images were acquired using an Olympus BX51 microscope and a digital camera (Olympus DP71). Fluorescent images were colocalized with ImagePro software (Improvision Inc., Waltham, MA). There was no evidence of any microglia, oligodendrocytes or neurons in the cultures (data not shown). Astrocytes did not change their morphology and viability in the presence of incubation with IFNγ for 2 days (data not shown).

Protocols

Human primary-cultured astrocytes (5×10^5 cells) were seeded into 24-well plates in 800 μl of DMEM/F12 medium containing 5% FBS and 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells were treated with IFNγ at the final concentration of 150 U/ml. After incubation for 2 days at 37°C in humidified 5% CO_2 and 95% air conditions, their conditioned medium (CM) (400 μl) was transferred to differentiated human neuroblastoma SH-SY5Y cells (2×10^5 cells per well). The cells were incubated with CM for a further 72 h and cell death evaluated by lactate dehydrogenase (LDH) release assays.

To achieve SH-SY5Y differentiation, the undifferentiated cells were treated for 4 days with 5 μM retinoic acid (RA) in DMEM/F12 medium containing 5% FBS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Singh et al., 2003). The RA-including medium was changed in every 2 days. Differentiated SH-SY5Y cells demonstrated neurite extension, indicative of their differentiation (Fig. 1). During the differentiation process, the SH-SY5Y cells did not divide as evidenced by MTT assay data. After 4 days of RA treatment there was no significant change (100.00 ± 2.34% before vs 99.95 ± 3.75 after in total mitochondrial activity, $n = 4$ independent experiments).

For mRNA assessment, total RNA was extracted from cell lysates. For pharmacological assessment, human astrocytes were exposed for 2 days to IFNγ in the presence or absence of various pharmacological agents. These agents included diphenylene iodonium (DPI) and apocynin as inhibitors of NADPH oxidase (Williams and Griendling, 2007), ibuprofen (IBU) plus acetylsalicylic acid (aspirin) as inhibitors of cyclooxygenase (COX)-1 and COX-2 (Klegeris et al., 1999; Klegeris and McGeer, 2002, 2005) and protease cocktails (aprotinin, pepstatin and leupeptin, 1 ng/ml to 3 μg/ml each). Their supernatants (CM) were transferred to differentiated SH-SY5Y cells (Fig. 2B–D). For interleukin-6 (IL-6) studies, astrocytes were stimulated with IFNγ for 2 days and their CM was transferred to wells which had been coated with IL-6 antibody. After 3 h of incubation the CM was transferred to SH-SY5Y cells (Fig. 2A). Glutamate decarboxylase (GAD 65, 1 ng/ml–1 μg/ml, Novus Biologicals, Littleton, CO) was added to the astrocytic CM for 1 h to remove the glutamate (Fig. 2E).

In all cases, control experiments were conducted in which the agents were added to supernatants from unstimulated cells prior to SH-SY5Y exposure. This procedure was designed to determine if there was any direct effect of these agents on differentiated SH-SY5Y cells. None was observed following the addition of any of these agents. Experiments were performed with four independent cultures.

Neurotoxicity assays and data normalization

The viability of differentiated SH-SY5Y cells following incubation with stimulated astrocytic CM was evaluated by the LDH release assay (Lee et al., 2009). Briefly, cell culture supernatants (100 μl) were pipetted into the wells of 96-well plates, followed by the addition of 15 μl lactate solution (36 mg/ml in PBS) and 15 μl p-iodonitrotetrazolium violet (INT) solution (2 mg/ml in PBS).

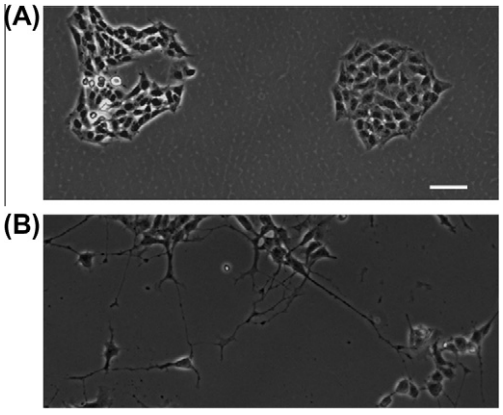

Fig. 1. Differentiation of SH-SY5Y cells with 5 μM retinoic acid for 4 days. (A) Undifferentiated cells and (B) differentiated cells. Photography in bright field microscopy. The calibration bars in (A) = 20 μm.