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Effects of Amphotericin B on the expression of neurotoxic and neurotrophic factors in cultured microglia

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Abstract

Amphotericin B (AmB) is a polyene antibiotic and reported to have therapeutic effects on prion diseases, in which the microglial activation has been suggested to play important roles by proliferating and producing various factors such as nitric oxide, proinflammatory cytokines, and so on. However, the therapeutic mechanism of AmB on prion diseases remains elusive. In the present study, we investigated the effects of AmB on cellular functions of rat primary cultured microglia. We found that AmB, similarly as lipopolysaccharide (LPS), could activate microglia to produce nitric oxide via inducible nitric oxide synthase. Both AmB and LPS also induced mRNA expressions of interleukin-1β, interleukin-6, and tumor necrosis factor-α in microglia. AmB also changed the expression levels of neurotrophic factors mRNAs. AmB and LPS significantly down-regulated the level of ciliary neurotrophic factor mRNA. However, AmB, but not LPS, significantly up-regulated the level of glial cell-line derived neurotrophic factor mRNA in microglia. In addition, brain-derived neurotrophic factor mRNA expression level was tending upward by treatment with AmB, but not with LPS. Taken together, these results suggest that AmB regulates the microglial activation in different manner from LPS and that microglia may participate in the therapeutic effects of AmB on prion diseases by controlling the expression and production of such mediators.

Keywords: Microglia; Amphotericin B; Neurotrophic factors; Glial cell-line derived neurotrophic factor; Brain-derived neurotrophic factor; Lipopolysaccharide

1. Introduction

Microglia are brain macrophages and are major immunocompetent cells in central nervous systems (Kreutzberg, 1996). They play immunological roles in mechanisms underlying the phagocytosis of invading microorganisms and removal of cell debris or damaged cells. Moreover, microglia are also implicated in the development of neurodegenerative diseases such as Alzheimer's disease, prion diseases, multiple sclerosis, and so on (Nakamura, 2002). Most of these neurodegenerative diseases are characterized by presence of numerous activated microglia (Hofman et al., 1989; McGeer et al., 1988; Eitzen et al., 1998). When microglia are activated, their proliferative activities are up-regulated and a variety of factors including cytokines, nitric oxide (NO), and so on are released from microglia (Nakamura, 2002). The proinflammatory cytokines and NO from activated microglia increase neuronal apoptosis *in vitro* (Chao et al., 1992; Boje and Arora, 1992), suggesting that microglial activation have an unfavorable effect to neurons. On the other hand, neurotrophic factors released from microglia contribute to the maintenance of neuronal survival (Batchelor et al., 1999). It was reported that microglia produce various neurotrophic factors including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), nerve growth factor (NGF), neurotrophin (NT)-3, and glial cell-line derived neurotrophic factor (GDNF). These neurotrophic factors are expected to have therapeutic effects in various neurodegenerative diseases (Lindsay et al., 1994; Gash et al., 1996).

Microglia play important roles in the pathogenesis of prion diseases including Creutzfeldt-Jakob disease in humans, scrapie in sheep and goat, and spongiform encephalopathy in cattle. The neuropathologic features that characterize prion diseases are extracellular deposits of prion protein (PrP) amyloid fibrils, spongiform degeneration of neurons, and

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Table 1
Primers used for RT-PCR analysis

	Upstream (5′–3′)	Downstream (5′–3′)	Estimated base pair
GDNF	GGGATGTCGTGGCTGTCTGC	GCCTGGCCTACTTTGTCACTTGTT	520
CNTF	CATTTCACCCCAACTGAAGG	GGGTGCAC CATTTGCATAG	499
BDNF	GGCGCCCATGAAAGAAGTAA	CCATAGTAAGGGCCCGAACATA	614
iNOS	GGAGAGATTTTTCACGACACCCTTC	GGTTCCTGTTGTTTCTATTTCCTTTGTTAC	258
GAPDH	TGCTGAGTATGTCGTGGAGTCT	AATGGGAGTTGCTGTTGAAGTC	602
IL-1β	TGATGTTCCCATTAGACAGC	GAGGTGCTGATGTACCAGTT	378
IL-6	ATGAAGTTTCTCTCCGCA	GGGGTAGGAAGGACTATT	503
TNF-α	TTCTGTCTACTGAACTTCGGGGTAATCGGTCC	GTATGAGATAGCAAATCGGCTGACGGTGTGGG	354

gliosis including clusters of activated microglia (Williams et al., 1994). Because microglial activation was observed in mouse models of prion disease before neuronal loss and subsequent clinical signs of the disease (Williams et al., 1997), it is thought that microglial activation is a cause of neuronal loss rather than a consequence of it. *In vitro*, it was reported that microglia release NO, proinflammatory cytokines, and other factors after the treatment of microglial cell cultures with PrP106–126, a synthetic peptide corresponding to amino acid residues 106–126 of PrP (Brown et al., 1996; Peyrin et al., 1999). However, there was no report whether microglia release neurotrophic factors in the brain suffered from prion diseases, and no treatment is established for prion diseases.

Until now, many drugs and agents have been attempted for the therapy in experimental animal models; including hormones, antibiotics, antiviral drugs, antifungal agents, and so on (Ehlers and Diringer, 1984; Ingrosso et al., 1995). Amphotericin B (AmB), a polyene antibiotic, has been reported to delay the appearance of experimental scrapie in hamsters infected intracerebrally or intraperitoneally with the 263 K scrapie strain (Pocchiari et al., 1987). AmB is an effective antifungal agent and has side effects of therapy such as nephrotoxicity, fever, and so on (Varlam et al., 2001; Brajtburg et al., 1990). However it was reported that AmB also delayed PrP accumulation and inhibited gliosis in the brain (Adjou et al., 2000), its mechanism remains unclear.

In prion diseases, it has been suggested that microglial activation cause astrocytic proliferations and result in gliosis (Brown et al., 1996). However, the effect of AmB on microglia has not been reported. In the present study, we investigated whether and how AmB affects microglial cellular functions as a basic study to develop the therapeutic mechanism of AmB in prion diseases. We treated cultured rat microglia with AmB, and examined the productions of NO, proinflammatory cytokines, and neurotrophic factors as an index of microglial cellular functions.

2. Experimental procedure

2.1. Materials

AmB, lipopolysaccharide (LPS; from *Salmonella* serovar Enteritidis or *Escherichia coli*), deoxyribonuclease I (DNase I), trypsin, and peroxidase-labeled isolectin b4 (*Bandeiraea simplicifolia* bs-I isolectin b4) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal calf serum (FCS) was from Biosource International (California U.S.A.). Dulbecco's modified Eagle's medium (DMEM) and horse serum were from GibcoBRL (Grand Island, NY, USA). 2,3-Diaminonaphthalene (DAN) was from Dojindo (Kumamoto, Japan). Western Lightning Chemiluminescence Reagent Plus was from PerkinElmer (U.S.A.). Hyperfilm was from Amersham Biosciences (U.S.A.). Immunoblock was from Dainippon Sumitomo Pharma (Osaka, Japan). Anti glyceraldehydes-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon (California, U.S.A.). SV Total RNA Isolation System was obtained from Promega (Madison, WI. U.S.A.). Omniscript Reverse Transcription, HotstarTaq Master Mix Kit, and Taq PCR Master Mix Kit were from Qiagen (Hilden, Germany). Mouse monoclonal anti-mouse inducible NO synthase (iNOS) antibody was kindly provided by Dr. Shunji Kosaki and Dr. Hideshi Ihara of Osaka Prefecture University.

2.2. Preparation of microglia cultures

The present study was carried out in compliance with the Guide for Animal Experimentation at Osaka Prefecture University. Primary cultures of rat microglia were prepared as described previously (Nakamura et al., 1999). Briefly, the whole brains of neonatal Wistar rats were homogenized with DMEM using a Pasteur pipette, and treated with 0.25% trypsin in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline containing 5.5 mM glucose for 15 min at 37 °C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin, and the tissues were centrifuged at 200 × *g* for 5 min. The tissue sediments were triturated through a blue-tip-mounted pipette with DMEM containing 10% FCS, 100 mg/l streptomycin, and 5×10^4 unit/l penicillin. The cells were plated on polyethyleneimine-coated plastic bottles, and cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every week. On day 14, microglia were harvested from glial mixed cultures with shaking bottles for 1 h at 80 rpm, and replated onto 96- or 24-well plates (Sumiron, MS-8096R or MS-8024R) at a density of 4×10^4 or 5×10^5 cells/well, respectively. After 1 h, the medium was changed to remove non-adherent cells, and the remaining microglia were allowed to stabilized for 1 day in DMEM containing 10% FCS before the experiments. In our culture, more than 90% of the cells were positive to a microglia marker, lectin from *Bandeiraea simplicifolia* bs-I isolectin b4.

2.3. Limulus test

To confirm whether AmB used in the present study is not contaminated with bacterial endotoxin or LPS, limulus test (Limulus HS-F Single Test Wako; Wako Pure Chemical Industries, Osaka, Japan) was carried out. The endotoxin in the sample (0.2 ml) induces the gel formation of liquid in the vials. Addition of 100 ng/ml LPS caused the complete gel formation; however, AmB solution did not cause the gel formation at all, up to 9.0 μg/ml (data not shown).

2.4. Nitrite assay

NO release from microglia was determined by assaying the levels of nitrite (NO_2^-), a relatively stable metabolite of NO. The concentration of NO_2^- accumulated in the medium was assayed by a fluorometric method using DAN reagent as described previously (Murakami et al., 2003). Microglia replated