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THE RETINOID, 6-[3-ADAMANTYL-4-HYDROXYPHENYL]-2-NAPTHALENE CARBOXYLIC ACID, CONTROLS PROLIFERATIVE, MORPHOLOGICAL, AND INFLAMMATORY RESPONSES INVOLVED IN MICROGLIAL ACTIVATION WITHOUT CYTOTOXIC EFFECTS

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Abstract—Activation of microglia is regulated by controlling both its population size (through modulation of proliferation/death) and the production of inflammatory mediators. Retinoids control cellular proliferation, differentiation, and death. Natural retinoids have been shown to exhibit anti-inflammatory actions against activated microglia. However, the synthetic forms, which are regarded to be more stable in their actions, have not been explored for their capacity to modulate microglial activation, proliferation, and/or trigger cell death. The aim of the current study was to address these issues by using a model, lipopolysaccharide (LPS)-activated primary cultures of rat microglia, and the stable synthetic retinoid, 6-[3-adamantyl-4-hydroxyphenyl]-2-napthalene carboxylic acid (AHPN). Morphological observations of cluster of differentiation (CD) 11b (CD11b)-positive cells suggested that low concentration of AHPN (i.e. 5 μM) reduced LPS (1 μg/ml, 24 h)-activated morphology of microglia possibly toward a lower activated state, while attenuating nitrite production and the level of its synthesizing enzyme, inducible nitric oxide synthase (iNOS), as well as the chemokine, monocyte chemotactic protein-1 (MCP-1). The mechanisms behind these anti-inflammatory actions likely involved decreased activation of nuclear factor kappa B (NF-κB) as shown by the attenuated phosphorylation of its p65 subunit. In addition, fluorescence-activated cell sorting revealed that AHPN reduced the immunophenotypic marker of activation, CD68. LPS-mediated increase in cell number was reduced by low concentration AHPN, which resulted from inhibition of proliferation, based on decreased labeling for Ki-67 and reduced protein expression of cyclin D1, and not cell death. Higher concentrations of AHPN (50–100 μM) attenuated activation and cell number; however, the release of lactate dehydrogenase and appearance of annexin V and propidium iodide-positive cells suggested that cell death was its primary cause for reduced microglial activity. Overall, the current study shows that synthetic retinoids, such as AHPN, at low concentration attenuate microglial activation-associated responses, possibly via the inhibition of their cell proliferation without triggering cell death.

Key words: anti-inflammatory, retinoid, microglia, cell division, chemokine, nitrite.

Microglial activation is a differentiating process involving proliferation and major morphological changes, an elevated secretion of inflammatory molecules such as cytokines (e.g. tumor necrosis factor alpha [TNF]-α and interleukin 1 beta [IL-1β]), production of reactive nitrogen/oxygen species, and upregulation of immunoregulatory markers (Gehrmann et al., 1995; Graeber and Streit, 2010; Lynch, 2009; Rivest, 2009). Chronic microglial activation has been implicated in neurotoxicity (Block et al., 2007; Hanisch, 2002) and thus may participate in neuronal demise, which occurs in the pathogenesis of chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) (Graeber and Streit, 2010).

Disruption of retinoid signaling has been linked to the pathological hallmarks of neurodegenerative diseases, particularly AD and ALS (Goodman, 2006; Lane and Bailey, 2005; Maden, 2007). Retinoids such as the vitamin A derivative, retinoic acid (RA), control cellular proliferation, differentiation, survival, and death (Maden, 2002, 2007) via the nuclear retinoic acid receptors (RAR-α, -β, and -γ) and retinoid "X" receptors (RXR-α, -β, and -γ) (Bastien and Rochette-Egly, 2004; Lane and Bailey, 2005). In addition, recent evidence implicates natural retinoids as a possible therapeutic option for neurodegenerative diseases with an inflammatory component. In these studies, the natural retinoids, all-trans-RA (ATRA), and 9-*cis*-RA, exhibited anti-inflammatory actions against lipopolysaccharide (LPS) or beta amyloid (Aβ)-activated microglial cells, involving reduction in the markers of activation (Dheen et al., 2005; Xu and Drew, 2006). However, whether the mechanisms behind the anti-inflammatory effect of these retinoids involve the inhibition of cellular proliferation or induction of cell

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Abbreviations: AD, Alzheimer's disease; AHPN, 6-[3-adamantyl-4-hydroxyphenyl]-2-napthalene carboxylic acid; ALS, amyotrophic lateral sclerosis; ATRA, all-trans-RA; CD11b (OX-42), cluster of differentiation 11b; CD68 (ED1), cluster of differentiation 68; DAB, 3,3′-diaminobenzidine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; ICC, immunocytochemistry; IL-1β, interleukin 1β; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor kappa B; (p)-NF-κB, phosphorylated NF-κB; NO_2^-, nitrite; PB, permeabilization buffer; PBS, phosphate buffered saline; PI, propidium iodide; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoic acid "X" receptor; RT, room temperature; SNAP, (S)-nitroso-*N*-acetylpenicillamine; TNF-α, tumor necrosis factor alpha; +ve, positive.

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death was not thoroughly explored. This issue is important to consider in the context of neuroinflammation because controlling optimal size and function of the microglial cell population may be vital for the maintenance of brain homeostasis (Jones et al., 1997). Therefore, reducing the activation of microglia by regulating their proliferation may be more beneficial than inducing cytotoxicity to these cells, which may severely compromise their functional ability to protect the brain.

Synthetic retinoids are regarded as being more stable and active than their endogenous counterparts, primarily because of the addition of more robust pharmacophores that reduce photo-induced isomerization hence decreasing degradation, thus improving RAR/RXR activity (Barnard et al., 2009). As synthetic retinoids potently modulate both proliferation and survival, they are currently used as therapeutic agents for cancer and proliferative skin disorders (Altucci et al., 2007). One of these analogs, 6-[3-adamantyl-4-hydroxyphenyl]-2-napthalene carboxylic acid (AHPN/CD437), has received much attention as a potential chemotherapy agent (Holmes et al., 2000; Mologni et al., 1999; Zhao et al., 2001) because of its ability to regulate cellular differentiation (via RAR-γ), potently inhibit cellular proliferation, and induce cell death in various cancer/tumor cell populations (Li et al., 1998; Zhao and Spanjaard, 2003). In view of these findings, the current study used primary microglia cultures to directly address the mechanism of action of AHPN on the activation state of these cells. To achieve this goal, we used LPS-exposed microglia as a model to investigate AHPN effects on the following: (1) the population size as determined by the balance between proliferation and death, and (2) the activation-associated responses, such as the secretion of inflammatory molecules (e.g. NO_2^- and cytokines/chemokines), expression of immunoregulatory markers (e.g. CD68), and morphological alterations.

EXPERIMENTAL PROCEDURES

Animals

Pregnant Sprague–Dawley rats (20–21 days) were purchased from Charles River Laboratories (Quebec, Canada) and housed at the Douglas Research Centre's animal facility. Neonatal rats, age 1–2 days, were used for tissue culture. All work was conducted in accordance to the guidelines approved by McGill University Animal Care Committee and the Canadian Council for Animal Care. Therefore, every effort was taken to minimize the number of animals and their suffering.

Tissue culture

Dissociated rat microglia cultures were prepared from primary cortical glial cultures according to a previously established protocol (Farso et al., 2009), with some modifications. Briefly, cortices were dissected, exposed to trypsin followed by soybean trypsin inhibitor and then triturated to a single cell suspension. The dissociated cells were seeded in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada) supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin/100 μg/ml streptomycin, 0.25% Fungizone™, 50 μg/ml insulin, and 10% fetal bovine serum; and maintained in a humidified incubator (95% O_2 with 5% CO_2, 37 °C). Media changes with supplemented DMEM were performed on 1 day *in vitro (div)* and 5 *div*. By 10 *div* confluent cultures of astrocytes contained a significant number of microglia, either freely flowing in the media or lightly attached to the surface of the astrocytic layer. Microglial cells were collected, centrifuged (1500 rpm, 5 min), and the pellet was re-suspended in supplemented DMEM followed by trituration. After counting with trypan blue, viable microglial cells were seeded at either 0.1×10^6 cells/cm^2 in 48-microwell plates for biochemical, immunocytochemical, and fluorescence-activated cell sorting (FACS) analyses or 0.9×10^6 cells/cm^2 in six-well plates for Western immunoblots. Microglial cultures were maintained in the humidified incubator. Cells were washed 1 h after seeding and freshly supplemented DMEM was added. Approximately 16 h later, the medium was removed and Neurobasal Medium™ (NBM; Invitrogen, Burlington, ON, Canada) containing 2% B27 (without antioxidants) was added. Based on the original observations by abd-el-Basset and Fedoroff (1995) that has been confirmed by Rohl et al. (2008), ramification of microglia indicated that these cells were in a surveying state (Nimmerjahn et al., 2005), which was reached 6–7 h later. Microglial cells were subsequently exposed to either vehicle (NBM without B27) or 1 μg/ml LPS (Sigma-Aldrich, Oakville, ON, Canada) (Dheen et al., 2005; Xu and Drew, 2006) with or without AHPN (Tocris BioScience, Ellisville, MI, USA) for 24 h (Xu and Drew, 2006). Treatments were performed in triplicate or quadruplicate in a minimum of three independent cultures. All drugs were prepared and stored according to the manufacturer's guidelines. Immunocytochemistry for astrocytes (anti-Glial Fibrillary acid protein (GFAP); Invitrogen, Burlington, ON, Canada), microglia (anti-CD11b; AbD Serotec, Raleigh, NC, USA), and oligodendrocytes (anti-O4; Abcam, Cambridge, MA, USA) indicated that the microglial culture consisted of approximately 95% microglia (data not shown) (Farso et al., 2009).

Characterization of microglia by immunocytochemistry using 3,3′-diaminobenzidine (DAB)

After microglial cultures were washed (with 0.2 M phosphate buffered saline [PBS]) and fixed with 4% paraformaldehyde (5 min, room temperature, RT), they were subjected to the immunocytochemistry (ICC) procedure according to Farso et al. (2009), with slight modifications. Briefly, cells were permeabilized and nonspecific binding blocked simultaneously with permeabilization buffer (PB; 10% normal goat serum, 0.3% Triton X-100 in 0.2 M PBS) for 1 h at RT. Once endogenous peroxidase activity was quenched (by exogenous peroxide), the primary antibody, mouse anti-CD11b (AbD Serotec, Raleigh, NC, USA), was added (1:500, 4 °C, overnight) followed by biotinylated anti-mouse IgG (1:200, 1 h at RT). Immunoreactivity was developed by using the DAB substrate kit (Vector Laboratories, Burlingame, CA, USA), and the reaction terminated by washing with 0.2 M PBS. Immunopositive labeling for CD11b was visualized using the Zeiss Axio Observer microscope, under bright-field microscopy. Digital photographs were taken and visualized using Zeiss Axio Vision software (version 4.7.1.0).

Cell counts

Fixed cells were incubated with Hoechst dye 33342 (Invitrogen, Burlington, ON, Canada) (1 μg/ml, 5 min at RT) and then washed. Under ultraviolet (UV) microscopy, Hoechst-positive cells were counted (irrespective of nuclei condensation state) from 12 to 18 random fields per treatment, a total of approximately 1500 cells. From each individual culture, the mean from the total number of cells counted per treatment was expressed as a percentage of the mean of the LPS treatment. The mean percentages per treatment from each individual culture were then pooled and graphed using the GraphPad Prism software (Version 3).