Interleukin-1β Promotes Oligodendrocyte Death through Glutamate Excitotoxicity

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Glutamate excitotoxicity is implicated in the progressive loss of oligodendrocytes in multiple sclerosis, but how glutamate metabolism is dysregulated in the disease remains unclear. Because there is microglia activation in all stages of multiple sclerosis, we determined whether a microglia product, interleukin-1β (IL-1β), could provide the mechanism for glutamate excitotoxicity. We found that whereas interleukin-1β did not kill oligodendrocytes in pure culture, it produced apoptosis of oligodendrocytes in coculture with astrocytes and microglia. This requirement for a mixed glia environment suggests that interleukin-1β impairs the well-described glutamate-buffering capacity of astrocytes. In support, antagonists at AMPA/kainate glutamate receptors, NBQX and CNQX, blocked the interleukin-1β toxicity to oligodendrocytes. Another microglia/macrophage cytokine, tumor necrosis factor-α, also evoked apoptosis of oligodendrocytes in a mixed glia environment in an NBQX-blockable manner. These results provide a mechanistic link between the persistent and insidious microglia activation that is evident in all stages of multiple sclerosis, with the recent appreciation that glutamate excitotoxicity leads to the destruction of oligodendrocytes in the disease.


The pathology of multiple sclerosis (MS) includes demyelination and axonal loss; oligodendrocytes (OLs) undergoing apoptosis also can be observed in some cases of the disease.¹ Thus, defining mechanisms that kill OLs is relevant to devising strategies to halt the progressive tissue loss in the disease. OLs are vulnerable to a variety of mediators of cell death, including free radicals, proteases, and inflammatory cytokines (reviewed in Casaccia-Bonnefil²). OLs are also susceptible to compounds present physiologically, such as glutamate that performs important excitatory neurotransmitter roles in the central nervous system (CNS), but becomes cytotoxic (“excitotoxicity”) when its normal concentrations are exceeded. In vitro, OLs are highly susceptible to glutamate excitotoxicity mediated through AMPA/kainate receptors.³⁻⁵ In studies of experimental autoimmune encephalomyelitis (EAE), an animal model of MS, glutamate excitotoxicity is a pathogenic factor for OL injury.⁶⁻⁷ In this regard, OL death and clinical signs of EAE were attenuated on treatment with the AMPA/kainate receptor antagonist, NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo[F] quinoxaline). In MS, enzymes that decrease or increase glutamate levels are reduced or elevated, respectively, within MS lesions.⁸ Glutamate concentrations in the cerebrospinal fluid are elevated in MS compared with controls.⁹ Nonetheless, little is known of the mechanisms that regulate glutamate excitotoxicity in MS.

In detailed studies by several groups, the neuropathology of MS is correlated with the presence of several inflammatory subsets, including T lymphocytes, macrophages, and microglia.¹⁰⁻¹¹ Microglia activation is evident in all subtypes of MS¹⁰,¹¹; therefore, microglial-derived factors must be considered candidate neurotoxins that contribute to the progressive CNS atrophy in the disease. A prominent microglial-derived cytokine is interleukin-1β (IL-1β). This proinflammatory cytokine has multiple activities that can adversely affect OL survival. For example, IL-1β is a potent regulator of the production of various matrix metalloproteinase members,¹² which are implicated in producing demyelination and axonal loss in diseases of the CNS (reviewed in Yong and colleagues¹³).

Despite these potentially adverse actions of IL-1β, and the finding of elevated IL-1β transcripts in autopsied MS brain samples,¹⁴ there are few studies that have investigated the potential injurious effect of IL-1β upon OLs in culture.¹⁵ In contrast, there is a large literature demonstrating the susceptibility of OLs to another cytokine, tumor necrosis factor-α (TNF-α).¹⁶⁻¹⁸ In this study, we have investigated the potential toxicity of IL-1β for OLs. We demonstrate that IL-1β kills
OLs but only when cells are in coculture with astrocytes and microglia. The mechanism of IL-1β toxicity is mediated in part through promoting glutamate excitotoxicity. These results link the persistent neuroinflammation in MS with the findings that glutamate contributes to OL death.

Materials and Methods

Astrocyte-Microglial Cultures

Mouse astrocytes and microglia were derived from the brains of postnatal day 3 CD1 mice (Charles River, St. Constant, PQ, Canada) using a protocol described in detail elsewhere. Upon isolation, cells were plated onto polyornithine- (10 μg/ml) coated 100mm culture dishes at an approximate density of 10 × 10^6 per dish. No attempt was made to purify for each cell type because a culture containing both astrocytes and microglia was thought to simulate the coexistence of these cells in vivo; content was mostly astrocytes and approximately 5 to 10% microglia cells, as evaluated using immunohistochemistry for Iba1, a microglia-specific marker. Culture medium was minimum essential medium containing 0.1% dextrose, 100 μg/ml penicillin-streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.2 mM glutamine, and 10% fetal calf serum (FCS; all medium constituents from GIBCO BRL, Burlington, Ontario, Canada). This culture condition of 10% FCS resulted in few OLs (<0.1% of total cell population) developing from precursor cells, and no neurons were evident.

Oligodendrocyte Cultures

Mouse OLs were derived from whole brains of 3 to 4-week-old CD1 mice. Cells were dissociated with trypsin digestion and separated from myelin and debris by Percoll gradient, as previously described. Isolated cells, consisting of OLs, astrocytes, and microglia, were plated into uncoated 25cm^2 flasks at an approximate density of 12 × 10^5 per flask and left overnight at 37°C. After the adherence of astrocytes and microglia, floating cells (mostly OLs) were collected the next day. The OLs were plated onto coated (10 μg/ml polyornithine) 16-well chamber slides (Nalge Nunc International, Naperville, IL) at a density of 5 × 10^4 cells per well. Alternatively, OLs were plated onto a semiconfluent astrocyte-microglia monolayer prepared 2 days earlier (see below). OLs were maintained in minimum essential medium containing 0.2 mM glutamine, 0.1% dextrose, 100 μg/ml penicillin-streptomycin, and 5% FCS (all medium constituents from GIBCO BRL); henceforth, this will be referred to as “OL medium.” Note that OLs isolated from brains of 3 to 4-week-old mice, or older, are predominantly mature cells as determined by immunoreactivity for galactocerebroside (GalC); less mature forms are seldom found.

Oligodendrocyte-Astrocyte-Microglia Cocultures (Mixed Glia Culture)

To establish OL-astrocyte-microglia cocultures, henceforth referred to as “mixed glia” culture, the astrocyte-microglia cells at 7 days in vitro were first detached from the dishes using 0.25% trypsin in 0.02% EDTA (GIBCO BRL). The cells were plated on polyornithine-coated 16-well chamber slides at a density of 5 × 10^4 cells per well. Two days after, enriched OLs prepared separately as described were plated at 5 × 10^4 cells per well onto the astrocyte-microglia cells. The mixed glia cultures were maintained in OL medium.

Pharmacological Agents and Treatment

Recombinant mouse IL-1β and TNF-α were obtained from R & D Systems (Minneapolis, MN). Recombinant human IL-1 receptor antagonist (IL-1ra) was from Amgen (Boulder, CO). NBQX, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and MK-801 were from Sigma RBI (St. Louis, MO).

The culture medium was changed from OL medium (containing 5% FCS) to one containing 0.1% FCS 24 hours after the OLs were plated onto the astrocyte-microglia cells. Two hours later, IL-1β (1–1,000 U/ml) was added. When potential modulators of IL-1β toxicity were used, these were added 1 hour before IL-1β. At 24 hours after cytokine stimulation, cultures were fixed with 4% paraformaldehyde, pH 7.4, for 30 minutes at room temperature.

Immunocytochemistry and Detection of DNA Fragmentation and Chromatin Condensation

The fixed cultures were stained using standard fluorescent immunocytochemistry protocols. Mature OLs were labeled by a mouse monoclonal antibody that recognizes GalC (O1). Microglia were labeled by incubating with rabbit anti–mouse Iba1 (1:500). For visualization, cells were incubated with either Cy3-conjugated goat anti–mouse or goat anti–rabbit antibodies (both 1:300; Molecular Probes, Leiden, The Netherlands).

A terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) kit (Oncogene, Cambridge, MA) was used according to the manufacturer’s instructions to detect DNA fragmentation. Cellular DNA was stained by incubation with Hoechst 33852 (Sigma) for 10 minutes at room temperature. In some experiments, the occurrence of dying OLs was detected using immunohistochemistry for cleaved caspase-3 and -7 (antibodies contained in Apoptosis Sampler kit from Cell Signaling Technology, New England BioLabs, Beverly, MA), which are produced from their proenzymes in cells that are undergoing apoptosis.

Using a Leitz immunofluorescence microscope, OLs with DNA fragmentation (GalC and TUNEL double-positive cells) were identified as dying OLs. Apoptotic cells were confirmed in most cases by condensed chromatin assessed by Hoechst staining. The percentage of OL death per well was expressed as the number of TUNEL-positive OLs divided by the number of OLs counted times 100%. To achieve consistency in quantitation between samples, we assessed each well along the midportion of its vertical axis, until greater than 200 OLs were counted per well. Most experiments were replicated at least three times with n = 4 wells per treatment group each time. Statistical analyses were performed using Graphpad InStat (San Diego, CA).

Results

Interleukin-1β Causes Oligodendrocyte Death in Mixed Glia Culture

We have reported previously that a significant proportion (10–25%) of mature mouse OLs cultured on
polyornithine-coated plates undergoes apoptotic cell death and that this can be attenuated by coculturing OLs in a mixed glia environment consisting of astrocytes and microglia. In correspondence, less than 5% of OLs cocultured with astrocytes and microglia in this study were undergoing apoptosis as assessed using TUNEL immunoreactivity (Fig 1 and 2A); the presence of cleaved caspase-3 and -7 was also indicative of apoptosis of OLs (see Fig 1B). Treatment with IL-1β (100U/ml) in mixed culture for 24 hours resulted in a significant 2.5 to 2.9-fold increase in the percentage of mature OLs displaying features of apoptosis compared with the control cocultures (see Fig 2A). A dose-related effect was observed for IL-1β with concentrations as low as 3U/ml producing OL death (see Fig 2B). In several experiments, the toxic effect of IL-1β on OLs in mixed glia culture plateaued at 100U/ml (data not shown). Finally, the specificity of IL-1β was confirmed by the ability of IL-1ra to negate the toxic effects of IL-1β (see Fig 2C).

We addressed whether the toxicity of IL-1β required the presence of non-OL glia cells. Figure 2D shows that the stimulation of OLs cultured alone with IL-1β (100U/ml) did not affect the basal rate of OL apoptosis. Thus, IL-1β is not directly toxic to OLs, and astrocytes and/or microglia were necessary for the OL cytopathic effect of IL-1β to manifest.

Interleukin-1β Toxicity in Mixed Glia Culture Is Mediated through Glutamate Excitotoxicity

Because the toxicity of IL-1β required the presence of other cell types, it appeared likely that IL-1β was acting indirectly on astrocytes and/or microglia, producing a cytotoxic outcome to OLs. We considered that glutamate was involved downstream of IL-1β toxicity principally because astrocytes are important in the uptake and the metabolism of glutamate, and IL-1β has been reported to impair the glutamate-buffering capacity of astrocytes. To test the hypothesis that the toxicity of IL-1β involved glutamate excitotoxicity, we treated mixed glia cultures with the AMPA/kainate receptor antagonists NBQX (30µM) or CNQX (30µM).

Fig. 1. Detection of oligodendrocyte (OL) apoptosis in vitro. (A) An adult mouse OL cultured on an astrocyte monolayer assumes a healthy highly branched structure (GalC immunofluorescence, ×3,000 magnification). (B) An OL on an astrocyte monolayer undergoes apoptosis, as demonstrated using immunoreactivity for cleaved caspase-3 (red, ×4,000 magnification); several other cells illuminated by Hoechst staining of nuclei (blue) are not apoptotic. Cleaved caspase-7 provides for a similar result (data not shown). Panels C and D are overlapping frames of many OLs that are undergoing apoptosis as shown by cells labeled with GalC (red, C) and TUNEL (green, D) (×800 magnification). Apoptotic OLs are indicated by arrows in C.
As a result, there was a significant attenuation of IL-1β-mediated OL apoptosis (Fig 3). In contrast, treatment of the cocultures with N-methyl-D-aspartate receptor antagonist MK 801 (30 μM) did not affect IL-1β-mediated OL toxicity, consistent with the lack of N-methyl-D-aspartate receptor expression by OLs.3,24,25

In summary, the findings indicate that AMPA/kai-
nate receptor–mediated glutamate excitotoxicity contributes to the lethal effect of IL-1β on mature mouse OLs.

**Tumor Necrosis Factor–α but Not Interleukin-12 Toxicity to Oligodendrocytes in Mixed Glia Cultures also Involves Glutamate Excitotoxicity**

We tested whether other inflammatory cytokines implicated in MS also may mediate OL death through glutamate excitotoxicity. Stimulation of cells in mixed glia culture with TNF-α (100U/ml) for 24 hours in serum-deficient medium resulted in a significant increase in the proportion of apoptotic OLs (Fig 4A), and this was attenuated by NBQX. Thus, TNF-α promotes mature OL apoptosis through excitotoxic mechanisms in a similar manner to IL-1β. We did not observe any further increase in the extent of OL apoptosis when both IL-1β and TNF-α (100U/ml each) were combined (see Fig 4B).

IL-12 is another proinflammatory cytokine that has been implicated in MS. We found that IL-12 increased OL death in a mixed glia culture; however, unlike IL-1β or TNF-α, this was not blocked by NBQX (see Fig 4A).

**Discussion**

The presence and activation of various inflammatory cell types in active lesions of MS are well described by several authors and have been extensively reviewed. In inactive lesions, and in secondary progressive MS, the degree of neuroinflammation generally has been underappreciated because the infiltration of leukocytes into the CNS is less obvious than in earlier stages of relapsing-remitting MS. However, significant microglia activation exists in all stages of MS, including the secondary progressive form, and microglia activity may provide the insidious and persistent inflammation that mediates the progressive loss of CNS tissue in MS. A prominent microglia product is IL-1, and a limited literature has documented immunoreactivity and in situ hybridization signal for IL-1β in sections taken from MS lesions. Recently, IL-1β transcripts were found to be elevated by approximately threefold in MS brain samples compared with control.

In MS and EAE, OLs may undergo either apoptotic or cytolytic cell death. As mentioned previously, an accumulating literature implicates glutamate excitotoxicity in contributing to OL demise. An important means to remove excessive glutamate from the extracellular environment is by the activity of glutamate transporters. At least five different glutamate transporters have been cloned: GLT-1, GLAST, EAAC1, EAAT4, and EAAT5. GLT-1 and GLAST are principally expressed on astrocytes, and the decrease of their levels through antisense oligonucleotides produces elevated extracellular glutamate levels and excitotoxic neu-

Fig. 4. Tumor necrosis factor (TNF–α) but not interleukin (IL–12) killing also involves glutamate excitotoxicity. IL-1β and TNF-α toxicity are not additive. (A) TNF-α (100U/ml) results in the death of oligodendrocytes (OLs) in mixed glia culture, which was blocked by NBQX. In contrast, the killing of OLs by IL-12 (100U/ml) was not inhibited by NBQX. Each bar is the mean ± SEM of four to eight wells, in which, on each well, more than 200 oligodendrocytes (OLs) were analyzed. (B) The toxicity of IL-1β and TNF-α (100U/ml each) is not additive. Furthermore, the result of IL-1β plus TNF-α is not different from either alone. *p < 0.05; **p < 0.01 and ***p < 0.001 compared with control cocultures (one-way ANOVA with Tukey–Kramer post hoc analysis). N.S. = not significantly different.

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rodedefication. In EAE, the levels of GLT-1 and GLAST are downregulated in the spinal cord at the peak of disease symptoms. Of particular relevance to the results of this study, IL-1 has been found to reduce the expression of glutamate transporters in astrocytes and to inhibit their activity. Thus, IL-1 would have a net effect of increasing the levels of glutamate in the extracellular space.

Our results suggest that the impairment of glutamate metabolism or uptake accounts for the IL-1β–induced toxicity to OLs, because its mode of action requires the presence of other glia cell types such as astrocytes that are endowed with glutamate-metabolizing enzymes and transporters, and because antagonists at AMPA/kainate receptors could ameliorate its effects. Thus, the results provide a mechanistic link between the chronic
microglia activation in MS, with resultant IL-1 production, and the loss of OLs through glutamate excitotoxicity. Can the finding that IL-1β affects glutamate metabolism to result in OL loss be extrapolated to other proinflammatory cytokines, such as TNF-α that has been commonly associated with OL demise? TNF-α inhibits the activity of glutamate transporters, and promotes the exocytotic release of glutamate by astrocytes. Figure 4 demonstrates that TNF-α induced death of OLs in a mixed glia environment and this was blocked by NBQX. Thus, akin to IL-1β, TNF-α produces death to OLs and this involves glutamate excitotoxicity. This similarity of IL-1β and TNF-α is not extended to another proinflammatory cytokine, IL-12, because the toxicity of IL-12 to OLs is not prevented by NBQX. To the best of our knowledge, this is the first demonstration of IL-12 being toxic to OLs, and its mechanism of cytotoxicity requires further examination.

We are uncertain why the effect of IL-1β consistently reaches a plateau at 100U/ml, and why only up to 15% of OLs are susceptible to glutamate excitotoxicity. One possible explanation is that many OLs express the GluR2 form of glutamate receptors, thereby preventing receptor permeability to Ca^{2+} and rendering most OLs resistant to toxicity mediated through calcium-dependent glutamate toxicity; these possibilities deserve further investigations.

Although IL-β may be produced by a variety of cell types, its prominent sources are microglia and macrophages. Both cell subsets are present in active lesions, whereas chronic lesions continue to show an abundance of activated microglia. Strategies exist to neutralize the activity of IL-1β, including the use of soluble IL-1 receptor that has been shown to ameliorate EAE, but another approach may be to decrease microglia activation and thereby reduce the expression of IL-1β. There are several means to deactivate microglia, but one that may be of particular relevance to MS is minocycline, a semisynthetic derivative of tetracycline. Minocycline has been found to inhibit the activation of microglia, and we and others have reported that it attenuates the severity of EAE in rodents. It would be important to address whether minocycline could inhibit microglia activation in EAE and MS, leading to a decrease in IL-1β expression and an attenuation of subsequent glutamate excitotoxicity.

In summary, the results of this manuscript emphasize that IL-1β is not directly cytotoxic to OLs in isolation, but that it kills OLs when these are in coculture with astrocytes or microglia. The mechanism of IL-1β involves glutamate dysregulation, likely through the inhibition of glutamate uptake by astrocytes, and thus exposing OLs to excessive glutamate levels (Fig 5). Another cytokine associated with OL demise, TNF-α, also kills OLs in part through glutamate excitotoxicity. As IL-1β and TNF-α are both released by microglia, we propose that our results provide a mechanistic link between persistent microglia neuroinflammation, even in the absence of obvious leukocytic infiltration, and progressive tissue atrophy through glutamate excitotoxicity.

This work was supported by the Multiple Sclerosis Society of Canada and the Canadian Institutes of Health Research (MT H246, V.W.G.).

We acknowledge the initial contributions of S. Corley and U. Ladiwala, two previous trainees in the Yong Laboratory, to this work. The skilled technical assistance of T. Wilson is gratefully acknowledged. We thank T. Giroux for excellent secretarial help.

References


Fig. 5. A view of how interleukin (IL)–1β and tumor necrosis factor (TNF)–α kill oligodendrocytes (OLs) in a mixed glia environment through glutamate excitotoxicity. It is proposed that there is increased generation of glutamate in MS, due to increased synthesis of glutamate (elevated glutaminase activity) and decreased activity of glutamate catabolic enzymes (glutamate dehydrogenase and glutamine synthetase). The elevation of glutamate is normally buffered through uptake by glutamate transporters located on astrocytes, but the presence of IL-1β and TNF-α released by activated microglia impairs the uptake process, leading to glutamate excitotoxicity of OLs.


