Interleukin-1β is Required for the Early Evolution of Reactive Astrogliosis Following CNS Lesion

LEONIE M. HERX, BSC AND V. WEE YONG, PhD

Abstract. The CNS response to injury is characterized by the rapid activation of astrocytes in a process known as astrogliosis. The function of reactive astrocytes is controversial, in that both beneficial and detrimental properties are postulated. Identification of the molecules involved in regulating astrogliosis is an important step towards understanding astrocyte functions and establishing suitable conditions for CNS regeneration. We previously reported that inflammatory cytokines are regulators of astrogliosis but the key cytokine involved in initiating astrogliosis was unclear. We describe here that the elevation of glial fibrillary acidic protein (GFAP) transcripts follows the very early rise of interleukin (IL)-1β mRNA in a murine corticectomy model of CNS lesion. Furthermore, the injury-induced upregulation of GFAP mRNA and protein did not occur in mice genetically deficient for IL-1β compared to wild-type animals. This was correlated with an absence of an increase in GFAP-immunoreactivity (GFAP-ir) in IL-1β-null mice at 2 and 3 days of injury. However, by 5 to 7 days after the lesion, GFAP-ir was not different between cytokine-deficient and wild-type controls. Functionally, mice lacking IL-1β exhibited a significant impairment in reformation of the blood-brain barrier (BBB) following corticectomy compared to wild-type controls. These findings suggest that the rapid production of IL-1β following trauma plays a beneficial role in initiating astrogliosis in an attempt to restore the integrity of the BBB and seal off the wound site.

Key Words: Astrogliosis; Blood-brain barrier; CNS trauma; Cytokines; Inflammation; Interleukin-1β.

INTRODUCTION

Following an injury to the adult CNS, astrocytes become activated in a characteristic response known as astrogliosis. Within hours of injury, reactive astrocytes are hypertrophic, increase their content of the astroglial-specific intermediate filament (glial fibrillary acidic protein [GFAP]), and upregulate various enzyme levels (1, 2).

Historically, reactive astrogliosis has been thought to be an impediment to axonal regeneration because a long-term consequence can be the formation of a densely woven glial scar that acts as a physical barrier, preventing remyelination and regeneration (3–5). Studies have suggested that changes in the molecular properties of reactive astrocytes associated with the scar play a role in regenerative failure (6). In particular, the astrocytic upregulation of chondroitin-6-sulfate proteoglycan and cytotactin/tenascin has been correlated to an inhibition of neurite outgrowth in the injured adult CNS (7, 8).

More recent evidence suggests, however, that reactive astrogliosis is actually an attempt by the CNS to maintain homeostasis and promote recovery (9). The normal roles astrocytes play in the regulation of ionic homeostasis and formation of the blood-brain barrier (BBB) are consistent with reactive astrocytes attempting to prevent further damage to the CNS, for example, by restoring the integrity of the BBB (5, 10, 11). Moreover, reactive astrocytes have been shown to support neuronal growth and survival in vitro (12, 13), as well as acting as a preferred substrate for axon regrowth in vivo (14); these properties have been linked to the release of a range of neurotrophic factors (14–16).

The identification of the molecular mediators of astrogliosis is necessary in order to resolve the dichotomy of reactive astrocytes as either impediments or aids to recovery, as well as to gain a further understanding of the CNS reactions to injury needed to enhance recovery. With respect to mediators of astrogliosis, previous work from this laboratory has suggested a significant role for inflammatory cytokines. Several cytokines are locally and rapidly elevated following traumatic injury to the adult mouse brain (17). The application of interferon (IFN)-γ, a potent activator of the microglial production of cytokines (18), into a corticectomy injury in adults significantly enhanced the extent of astrogliosis (19). In neonatal animals, a single application of cytokine to a cortical stab wound converted minimal astroglial reactivity into extensive astrogliosis (20). Conversely, the reduction of cytokine levels by IL-10 diminished astrogliosis (21).

Of several inflammatory cytokines, IL-1β transcript levels have been shown to increase first, within 15 minutes (min) of injury (22). In this study, we have examined whether IL-1β could be the initiator of the activation of astrocytes and, if so, whether the extent of astrogliosis could be manipulated to reveal some of its functional consequence.

From the Neuroscience Research Group and Departments of Clinical Neurosciences and Oncology, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada.

Correspondence to: V. Wee Yong, PhD, 3330 Hospital Drive N.W., Calgary, Alberta T2N 4N1, Canada.

This work was supported by an operating grant from the Canadian Institutes for Health Research (CIHR). VWY is a Senior Scholar of the Alberta Heritage Foundation for Medical Research for Medical Research (AHFMR) and a CIHR Scientist.
Fig. 1. GFAP transcript elevation following corticectomy injury in adult mice. Each point represents mean ± SEM of 4 mice. *p < 0.05 compared to unoperated controls. Note that GAPDH levels remain relatively constant to indicate similarity of RNA loading in all groups. Previously, total brain RNA from the same mice revealed a rapid increase in IL-1β transcripts within 15 min of injury, which peaked by 3 h (22).

MATERIALS AND METHODS

Mice

Adult retired female breeders (4–6-months-old) of the CD1 strain (Charles River, Montreal, Canada), IL-1β -/- mice (129Su/C56BL6, back-crossed for over 3 generations with the B10RIII strain as previously described) (23), and the B10RIII genetic controls (“wild-type”) (Jackson, Bar Harbor, ME) were housed on a 12-hour (h) light/dark cycle with ad libitum access to food and water. All experimental procedures were approved by the institution’s animal care committee and were in accordance with the guidelines instituted by the Canadian Council of Animal Care. CD1 mice were used in the experiments summarized in Figure 1, while the IL-1β-deficient mice and their wild-type controls were used in the experiments depicted in Figures 2 to 7.

Corticectomy Injury

Corticectomy, in which a 12–15 mm³ volume of parietal-occipital cortex is removed by gentle aspiration, was used to invoke CNS injury. This model has been previously detailed (21, 22) and mimics similar procedure in humans for the resection of brain tumors, epileptic foci, etc. The corticectomy model is advantageous in that the large insult allows for a substantial area of the surrounding brain parenchyma to be analyzed; previous studies have determined that the corticectomy injury produces astrogliosis in a similar temporal kinetic as that seen after a brain stab injury (20, 21, 24). In brief, animals were anesthetized with ketamine (200 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and immobilized in a stereotaxic frame. A midline incision was made, followed by a unilateral circular (2-mm diameter) craniectomy over the left hemisphere, 1 mm lateral of the midline and midway between lambda and bregma. Following removal of the dura mater, corticectomy was performed by aspiration of the cortex just down to the dorsal aspect of the corpus callosum. All animals were killed by cervical dislocation.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The levels of transcripts encoding GFAP and GAPDH were determined by semi-quantitative RT-PCR. Total RNA was isolated from tissue resected from around the lesion site using the
Trizol reagent (Gibco BRL, Burlington, Ontario). Four samples were collected for each set of controls and experimental time points. RNA (0.5 μg) was reverse-transcribed and amplified in a single-step process, using oligonucleotide primers designed for murine GFAP (5'-CTCAGGTCATCTTACC-3', 3'-CCTTCTGAACCTCGGT C-5') and GAPDH (5'-CGAGTTGAC-ACGGAATTTGTCGTAT-3', 3'-CAGAAGTGCTGGTGGT CCTTCCGA-5'), previously described in detail (17). All primers were purchased from Gibco BRL. The number of cycles was predetermined to be in the linear range of amplification for GFAP (35 cycles) and GAPDH (25 cycles). cDNA products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide incorporation under UV light. NIH Image Analysis software was used to quantify the size of each cDNA product, which was then expressed as a ratio to the mean size of the cDNA products of unoperated controls. GAPDH housekeeping gene was used as an internal control to demonstrate that equivalent amounts of RNA were loaded per sample for all samples. Statistical analysis was performed using ANOVA and Bonferroni post-test.

**Qualitative Assessment of GFAP Immunoreactivity (GFAP-ir)**

At 1, 2, 3, 5, 7, 10, and 14 days (d) following corticectomy, animals were anesthetized and transcardially perfused with 0.1 M phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed in 4% PFA, embedded in paraffin, and cut into 4-6-μm coronal sections. Mounted sections were heated for 1 h at 60°C, deparaffinized in xylene, and rehydrated through a graded series of ethanol solutions to H2O and PBS. For GFAP immunohistochemistry, sections were first treated with 0.25% Triton X-100 in PBS for 30 min at room temperature (RT), washed in PBS, and then blocked in 1% ovalbumin (Sigma, St. Louis, MO) in HHG (10% goat serum, 2% horse serum, 1 mM HEPES buffer) for 4 h at RT. Sections were then incubated overnight at 4°C with a rabbit anti-GFAP polyclonal antibody (1:100; Dako, Carpenteria, CA) diluted in HHG, washed with PBS, and incubated with a goat anti-rabbit immunoglobulin conjugated to Alexa 488 (1:500; Molecular Probes, Eugene, OR) for 1 h at RT. Following washing, sections were counterstained with nuclear yellow (500 ng/mL; Sigma) for 5 min, rinsed in water, and mounted with Gelvatol. Replacement of the primary antibody with HHG was used as negative control.

**Quantitative Assessment of GFAP Protein Levels by Western Blot Analysis**

GFAP protein levels were analyzed by Western blotting. Frozen brain tissues from around the lesion site were thawed and homogenized in 6 M urea. Samples were boiled for 5 min, put on ice, and then quantified using the Bradford Assay. One μg of total protein for each sample was electrophoresed on 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked overnight at 4°C with 5% skim milk powder in PBS and then incubated with rabbit anti-GFAP (1:100 Dako) in 1% milk for 1
Fig. 5. GFAP-ir 7 days postcorticectomy. Comparison of representative sections from IL-1β -/- and wild-type animals: (A) lesion site (magnification, ×400); (B) corpus callosum directly below the lesion (magnification, ×800); (C) cortex lateral to the lesion (magnification, ×800). Note that GFAP-ir in IL-1β -/- animals is indistinguishable from wild-type at this time point.
h at RT. Blots were washed 3 times (5 min each) with 0.1% Tween 20 in PBS (PBS-T), and incubated with HRP-conjugated goat anti-rabbit IgG (1:10,000 in 1% milk) for 1 h at RT. After washing 3 times with PBS-T, protein bands detected by the antibody were visualized using the ECL western blotting method (Amersham Pharmacia Biotech). Ponceau S staining was used to confirm equal amounts of protein loading.

**Assessment of BBB Disruption**

The extravasation of endogenous IgG was used to examine the extent of BBB disruption following corticectomy in the IL-1β -/- animals (n = 10) compared to their wild-type controls (n = 9). Following corticectomy, anesthetized animals were perfused with PBS followed by 4% PFA in PBS. Brains were removed, postfixed for 4–6 h in 4% PFA, and then cryoprotected in 15% sucrose in PBS overnight at 4°C. Frozen brains were mounted and cut on a sliding microtome (Leica) into 20-μm coronal sections from the olfactory bulb to the end of the medulla. Every sixth section was mounted onto gelatin-coated slides. Sections were hydrated and incubated in 3% H2O2 for 10 min to quench endogenous peroxidase activity, washed in PBS, blocked in HHG for 4 h at RT, and incubated with biotinylated anti-mouse IgG (5 μg/mL; Vector Laboratories, Burlingame, CA) in HHG overnight at 4°C. Sections were washed again, incubated with an avidin-biotin-peroxidase complex (Vectastain ABC elite kit, Vector Laboratories), washed, and then reacted in 0.05% diaminobenzidine (DAB; Sigma). Sections were then rapidly dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with Acrytol (Surgipath, Richmond, IL). HHG replacement of the biotin anti-mouse IgG was used as a negative control.

Differences in immunostaining for IgG extravasation were quantified using Image Pro Plus (Media Cybernetics, Silver Spring, MD). The region of stained area was measured based on a threshold intensity generated from an equivalent area in the contralateral hemisphere, and the area above this intensity expressed as a proportion of the dorsal field area of the ipsilateral hemisphere (equivalent to approximately 1/2 of the ipsilateral hemisphere). Two sections were analyzed for each animal from midway through the lesion site (with the exception of 2 wild-type and 1 IL-1β -/- in which only 1 section was assessed). Statistical differences between groups were analyzed using the unpaired Student t-test.

**RESULTS**

**Relationship of IL-1β Transcript Elevation to GFAP Following Corticectomy Injury**

The elevation of GFAP mRNA following injury is a reliable early indicator of astrogliosis (24). In this study, a temporal profile of GFAP transcript elevation was prepared from 15 min to 10 days following corticectomy. GFAP first became detectable above basal levels from 6 to 12 h postinjury, peaked at 12 to 24 h, and dropped slowly back to basal levels by 10 days postinjury (Fig. 1). In contrast, we have previously reported that there was a rapid increase in IL-1β transcripts within 15 min following injury, which peaked by 3 h (22). Therefore, IL-1β elevation occurs prior to the rise and peak in GFAP levels, thus establishing a temporal relationship for IL-1β in regulating the levels of GFAP and astrocyte reactivity.

**GFAP Transcript Levels in IL-1β -/- Mice**

We examined the mRNA levels of GFAP in mice genetically deficient for IL-1β in comparison to their wild-type controls. At 24 h following corticectomy, a time point corresponding to peak transcript elevation in CD1 animals (Fig. 1), the IL-1β animals showed no increase in GFAP levels above control while the corticectomized wild-type animals had nearly a 2-fold increase (Fig. 2A, B). Since the IL-1β -/- mice were competent in upregulating another inflammatory cytokine, TNF-α, in response to corticectomy (22), these results highlight the selectivity of IL-1β in initiating a rapid astroglial response to injury.

We compared the basal level of GFAP transcript in the uninjured brain of wild-type and IL-1β-deficient mice, to obviate the possibility that differential basal expression accounts for the apparent lack of an increase in GFAP in the IL-1β-deficient mice. Figure 2C demonstrates that in the brain of uninjured mice, levels of GFAP transcripts were similar in both groups.

**GFAP-ir in Wild-Type Versus IL-1β -/- Animals**

To demonstrate whether the changes in mRNA levels of IL-1β -/- mice translated into differences at the protein level of GFAP expression, and to define the spatial distribution of reactive astrocytes, we examined the immunoreactivity (ir) of GFAP through a time course of 1–14 days.

GFAP-ir was first discernible at 1 day following injury in wild-type animals, but only readily detected at 2 days postinjury. GFAP-ir astrocytes were present bordering the lesion site, in the corpus callosum below the lesion, and in the adjacent cortex within the ipsilateral hemisphere. GFAP-ir remained present throughout the 14-day period.
Fig. 7. IgG staining at 7 days postcorticectomy as an indication of BBB permeability. A: DAB visualization of extravasated IgG consistently showed greater permeability of the BBB in IL-1$\beta$ -/- mice compared to wild-type. Note the darker and more profuse staining localized around the lesion and extending further into the brain of IL-1$\beta$ -/- animals. B: Quantification of the percent area IgG stained (over total area shown by box in inset) for IL-1$\beta$ -/- was significantly different from wild-type animals ($p < 0.0001$). Each point represents 1 animal: $n = 10$ for IL-1$\beta$ -/-; $n = 9$ for wild-type.
examined. In the IL-1β−/− animals, the increase in GFAP-ir lagged that of their wild-type controls; indeed, a rise in GFAP-ir did not occur in the cortex at 2 days (Fig. 3) and 3 days (Fig. 4) following injury. However, GFAP-ir appeared indistinguishable from wild-type staining by 5 days (unpublished observations) or 7 days (Fig. 5) of injury. Thus, the lack of IL-1β resulted in a delayed response of the astrocytes to upregulate GFAP, but not an absolute impairment since GFAP levels appeared similar at 5–7 days between both groups of mice. It is noted that GFAP-ir provides for a qualitative rather than a quantitative measure of protein expression.

**GFAP Protein Quantification by Western Blot Analysis**

For a more quantitative analysis of protein expression, the levels of GFAP protein were examined at 2 days following corticectomy in wild-type and IL-1β−/− animals. This time point corresponded to a significant difference in GFAP immunostaining as described above. When compared to the wild-type controls, IL-1β−/− animals exhibited 3-fold lower GFAP levels at 2 days postinjury (Fig. 6).

**BBB Disruption**

To assess whether the reduction in GFAP upregulation following corticectomy in IL-1β−/− animals had any functional consequence, we compared the permeability of the BBB of these animals versus their wild-type controls. Astrocyte end feet comprise an important part of the BBB and drive its reformation following injury. BBB permeability was quantified by examining the extravasation of IgG into the brain. IL-1β−/− animals consistently showed greater permeability of BBB, exhibited by a darker and more profuse staining compared to wild-type mice (Fig. 7A). The staining pattern of detected IgG was localized around the area of corticectomy and extended further into the brain. Upon quantification, it was found that the percent area of IgG stained was 14.6% ± 2.7% (mean ± SEM) for IL-1β−/− animals and 4.4% ± 1.2% for wild-type animals (Fig. 7B).

**DISCUSSION**

The function of reactive astrocytes following CNS injury remains unclear. Astroglia has traditionally been considered undesirable, serving to inhibit axonal growth and regeneration. However, accumulating evidence suggests that reactive astrocytes have important beneficial effects, including neurotrophic activity, reformation of the BBB, and restoration of ionic homeostasis (9, 25). Identification of the molecules involved in mediating astrogliosis would help resolve this dichotomy of functions and may allow us to enhance the regenerative capacity of reactive astrocytes.

The local production of proinflammatory cytokines IL-1 and TNF-α is well documented as a normal and early feature of the CNS response to injury (17, 22, 26–28). Previous work from this laboratory has suggested that these inflammatory cytokines could be mediators of astrogliosis. In this regard, the application of interferon (IFN)-γ, a potent activator of the microglial production of IL-1 and TNF-α (18), into a corticectomy injury in the adult mouse brain significantly enhanced the extent of astrogliosis over that produced by the injury alone (19). In neonatal animals, where astrogliosis seldom develops following a penetrating brain injury, the levels of various inflammatory cytokines produced locally in the CNS parenchyma are significantly lower than those found after similar insults to the adult brain (17). In addition, the single application of cytokine to the neonatal cortical stab wound converted minimal astrogliosis reactivity into extensive astrogliosis (20). Furthermore, the inhibition of cytokine synthesis by IL-10 following adult brain trauma reduced the number of reactive astrocytes by over 60% (21). Other laboratories have shown similar roles for cytokines in regulating astrogliosis (2, 9). For example, the injection of IL-1 into the adult rodent brain significantly increased the extent of astrogliosis (29).

If inflammatory cytokines regulate astrogliosis, is any one cytokine particularly important in the early course of injury? In this study we examined the role of IL-1β in the process of astrogliosis, given our recent finding that it is the first inflammatory cytokine to be upregulated (within 15 min) following injury (22). Also, astrocytes are known to express receptors for IL-1β (30). A significant finding is that the early rise of GFAP transcripts that follows corticectomy did not occur when IL-1β is genetically absent. In support, GFAP-positive astrocytes were less detectable in areas surrounding the injury of IL-1β−/− mice within the first 3 days after injury. By 5 to 7 days following injury, no difference in GFAP staining was discernible. This assessment corresponded to a significant difference in the quantification of GFAP protein levels at 3 days as measured by Western blot analyses. Collectively, the results implicate the necessity of IL-1β in regulating the early evolution of reactive astrogliosis.

It should be noted that in the IL-1β−/− mice, a corticectomy injury elicits the generation of other inflammatory cytokines. Thus, TNF-α elevation occurs promptly (within 1 h) following trauma to the IL-1β−/− mice as we previously reported (22). This indicates that at least for the initial generation of astrogliosis, other inflammatory cytokines (e.g. TNF-α) cannot substitute for IL-1β. The lack of IL-1β becomes irrelevant to astrogliosis by 5 to 7 days after CNS trauma, possibly because other factors then compensate for IL-1β as mediators of astrogliosis. IL-1β may thereby serve as a key early regulator of the astroglial response to injury, but not the sole mechanism since its absence can be compensated for later. In this regard, we have noted that
endothelial cells were expected to be activated following GFAP in IL-1 specially to wild-type levels, given the later upregulation of response and, more specifically, to the production of IL-1 repair following traumatic injury to the inflammatory response through microglial production of IL-1.

An important consideration, given the putative role of IL-1 as an initial trigger for astrogliosis, is its cellular source. We have previously shown that the very early production of IL-1 following traumatic injury was 100% colocalized to microglia (22). This invites the idea that the initial trigger for astrogliosis is a CNS-intrinsic inflammatory response through microglial production of IL-1.

As outlined earlier in this paper, the impact of reactive astrocytes on CNS recovery is very controversial. An important function of astrocytes is their role in the normal maintenance of BBB integrity (35, 36), as well as in the reformation of the BBB following CNS injury (1, 2). A recent study by Bush et al (37) confirmed the essential role for astrocytes in BBB repair. Mice expressing the herpes simplex virus thymidine kinase gene (HSV-Tk) were treated with ganciclovir following a forebrain stab injury to selectively ablate reactive astrocytes adjacent to the injury site. In the absence of reactive astrogliosis, Bush et al (37) found there was a failure of BBB repair, as well as increased neuronal degeneration. In this paper, we have assessed whether the earlier difference in GFAP levels (and the implied difference in activation state of the astrocytes) translated into a functional difference. Analyses of BBB integrity were carried out on mice 7 days after injury, in line with other laboratories (38), and in view of the findings that BBB repair following CNS lesion in rodents begins to be noticeable only from 5–7 days thereafter (39–41). Thus, we found that animals lacking IL-1β and showing less reactive astrogliosis exhibited a significantly greater permeability of the BBB. Our results thereby link the activation of astrocytes and their subsequent role in BBB repair following traumatic injury to the inflammatory response and, more specifically, to the production of IL-1β.

It is not known whether the greater BBB permeability in IL-1β-/- mice at 7 days postinjury will recover subsequently to wild-type levels, given the later upregulation of GFAP in IL-1β-deficient astrocytes to a level that is indistinguishable from the wild-type.

It is reasonable to postulate that the role of IL-1β in limiting BBB breach is due to its effect on endothelial cells rather than on astrocytes, since endothelial cells can also respond to IL-1β (42, 43). While this is possible, we noted that in mice in which reactive astrocytes have been ablated (37) there was a failure of BBB repair, although IL-1β and endothelial cells were expected to be activated following the CNS lesion. Thus, we favor the view that the genetic lack of IL-1β results in a greater breach of the BBB because there is a delay in the reactivity of astrocytes.

In summary, this manuscript links the occurrence of astrogliosis in CNS trauma with cytokine upregulation in a functional manner. Specifically, we demonstrate that IL-1β is required for the initial evolution of astrogliosis, since its absence delays the evolution of astrogliosis following CNS trauma. Finally, we demonstrate an important role for reactive astrocytes in the reformation of the BBB. These results have implications for studies aimed at limiting damage and promoting recovery in CNS pathologies.

ACKNOWLEDGMENT

LMH gratefully acknowledges Alberta Heritage Foundation for Medical Research (AHFMR) and the Canadian Institutes for Health Research (CIHR) for studentship support.

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Received March 14, 2001
Revision received June 26, 2001
Accepted July 2, 2001