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BRAIN RESEARCH

Research Report

Tumor necrosis factor-α modulates glutamate transport in the CNS and is a critical determinant of outcome from viral encephalomyelitis

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ABSTRACT

Neuroadapted Sindbis virus (NSV) is a neuronotropic virus that causes a fulminant encephalomyelitis in susceptible mice due to death of motor neurons in the brain and spinal cord. We and others have found that uninfected motor neurons die in response to NSV infection, at least in part due to disrupted astrocytic glutamate transport, resulting in excitotoxic motor neuron death. Here, we examined the mechanisms of astrocyte dysregulation associated with NSV infection. Treatment of organotypic slice cultures with NSV results in viral replication, cell death, altered astrocyte morphology, and the downregulation of the astrocytic glutamate transporter, GLT-1. We have found that TNF-α can mediate GLT-1 downregulation. Furthermore, TNF-α deficient mice infected with NSV exhibit neither GLT-1 downregulation nor neuronal death of brainstem and cervical spinal cord motor neurons and have markedly reduced mortality. These findings have implications for disease intervention and therapeutic development for the prevention of CNS damage associated with inflammatory responses.

1. Introduction

Sindbis virus (SV) is an alphavirus, similar to eastern, western, and Venezuelan encephalitis viruses, arboviruses that are naturally transmitted by mosquitoes. Intracranial inoculation of susceptible adult mice with the neuroadapted strain (NSV) of Sindbis virus results in substantial neuronal death in the brain, brainstem and spinal cord, and high animal mortality and flaccid hindlimb paralysis in survivors (Jackson et al., 1987, 1988). NSV is a neuronotropic virus productively infecting only neurons since non-neuronal cells in the central nervous system (CNS) do not support viral replication (Jackson et al., 1988). We have demonstrated that motor neuron loss exceeds the number of neurons actually infected with NSV and that non-infected bystander neurons die because the NSV-induced inflammatory response triggers excitotoxic death (Darman et al., 2004; Nargi-Aizenman and Griffin, 2001; Nargi-Aizenman et al., 2004).

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Abbreviations: NSV, neuroadapted Sindbis virus; GLT-1, glutamate transporter-1; TNF-α, tumor necrosis factor-α; HEt, hydroethidium bromide

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The NSV-elicited inflammatory response involves the recruitment of activated macrophages, $CD4^+$, and $CD8^+$ T cells and results in the secretion of many cytokines (Binder and Griffin, 2003) (Binder and Griffin, 2001; Wesselingh et al., 1994). NSV infection of $CD4^+$ and $CD8^+$ deficient mice results in reduced paralysis, and NSV-infected interferon-γ deficient mice are protected from NSV-mediated mortality highlighting the potentially destructive role of the inflammatory response in NSV infection (Rowell and Griffin, 2002). We have demonstrated that NSV-infected mice treated with an inhibitor of microglial activation are protected from paralysis and mortality as well as from the downregulation of GLT-1 expression (Darman et al., 2004).

Excitotoxic motor neuron cell death may be one of the consequences of inflammation within the spinal cord and may be caused by failure of appropriate astrocytic glutamate transport away from neuronal synapses (Anderson and Swanson, 2000; Bal-Price and Brown, 2001; Bal-Price et al., 2002; Bezzi et al., 2001, 2004; Darman et al., 2004; Mander et al., 2005; Rossi et al., 2000). Additionally, cytokines have been shown to predispose neurons to excitotoxic death (Drachman et al., 2002; Fernandez-Ortega et al., 2004; Huang and O'Banion, 1998). TNF-α, in particular, has been shown to downregulate astrocyte-mediated glutamate transport either by repression of enzymes that astrocytes use to process glutamate (Chakrabarti, 1998; Huang and O'Banion, 1998) or by the direct downregulation of GLT-1 (Fine et al., 1996; Su et al., 2003; Szymocha et al., 2000; Zou and Crews, 2005).

In this study we used spinal cord organotypic slice cultures and found that NSV infection in this culture system recreates the downregulation of astrocytic GLT-1 and excitotoxic motor neuron death seen in adult rodents. TNF-α is secreted within spinal cord cultures following NSV infection and correlates with the repression of GLT-1 expression and activity. GLT-1 levels are maintained in NSV-infected cultures deficient in TNF-α demonstrating a role for TNF-α in the repression of GLT-1. Infection of TNF-α deficient mice results in neither GLT-1 downregulation nor significant motor neuron death in the brainstem and cervical spinal cord, and these animals exhibit markedly reduced mortality. These data emphasize the potentially deleterious effects of CNS inflammation and implicates TNF-α as a critical mediator of neuron death in viral encephalomyelitis.

2. Results

To verify that NSV would behave in spinal cord organotypic slice cultures as it does in susceptible mice (Darman et al., 2004; Jackson et al., 1987; Nargi-Aizenman et al., 2004), we have characterized NSV infection of these cultures. After 12 h of incubation in serum-free media, each well (containing 5×250 μm thick slices) of spinal cord organotypic cultures was inoculated with 1×10^7 PFU/mL administered directly to the tissue slices. Culture supernatants were collected from at least 3 individual wells at each time point. Over a 48-hour period virus levels increase in culture supernatants by several log units (Fig. 1A) confirming that there is viral replication within this culture system. Since NSV is a neuronotropic virus *in vivo*, we investigated whether exclusively neurons were infected *in vitro* as well. We found that though astrocytes and microglial cells were not productively infected by a recombinant NSV-GFP construct (Figs. 1B, C), motor neurons (Figs. 1D, E) were infected, leading to the expression of GFP within these cells. Additionally, NSV infection of organotypic sections led to cellular injury as defined by LDH release (Fig. 1F) and loss of motor neurons (Fig. 1G). These findings suggested that this culture system replicated the *in vivo* tropism and neuronal injury seen with NSV infection *in vivo* and that because of the absence of the acquired immune system would allow us to determine intrinsic neural determinants of neural injury following viral infection.

We investigated the humoral inflammatory response within organotypic cultures following NSV infection from the supernatants of at least 3 wells of slice cultures using multiple cytokine/chemokine protein arrays. We found TNF-α and IL-6 consistently elevated at early time points post infection (data not shown). We have previously cytokine/chemokine arrays to screen for inflammatory factors upregulated in NSV-infected spinal cord lysates and found that there was no elevation of multiple factors including IL-2, IL-4, IFN-γ, RANTES and IL-17. We also performed quantitative ELISA for nitric oxide metabolites and MCP-1 and did not see elevation (data not shown).

We then quantitatively defined the time course and extent of this upregulation after NSV infection (Figs. 2A, B). While TNF-α could not be detected at time 0 of infection, it became significantly elevated at 12 and 24 h post infection. Peak TNF-α levels were approximately 900±0.202 pg/mL in the supernatant of infected cultures at 24 h post infection. These studies confirm that an endogenous inflammatory response is initiated within infected organotypic cultures characterized by a cytokine response similar to what has been observed *in vivo* (Binder and Griffin, 2001, 2003; Wesselingh et al., 1994). TNF-α became significantly elevated through time at 12 and 24 h following NSV infection ($p<0.01$, Figs. 2A, B).

We previously showed that downregulation of the astrocytic glutamate transporter GLT-1 was an important determinant in the outcome from NSV infection *in vivo* (Darman et al., 2004) and so we chose to investigate whether NSV infection alters astrocyte glutamate transport *in vitro*. We generated at least 3 pooled tissue lysates from NSV-infected spinal cord organotypic culture slices and carried out immunoblot analyses against GLT-1. As observed in lysates from NSV-infected adult mice (Darman et al., 2004), there was a significant decrease in GLT-1 protein expression through time over a 24-hour period of NSV-infection of spinal cord organotypic cultures (Figs. 3A, B; $p<0.05$). In order to determine if this decline in GLT-1 protein correlated with a decline in functional GLT-1-mediated glutamate transport, membrane preparations were generated from pooled tissues of organotypic slice cultures in triplicate (Fig. 3C). Membrane preparations from at least 3 sample pools were exposed to radioactive glutamate in an assay that primarily tests the ability of GLT-1 to mediate glutamate transport (Darman et al., 2004; Sepkuty et al., 2002). GLT-1-mediated glutamate transport was significantly reduced in cultures 24 h post-NSV infection (61.727 pmol/mg/min±11.8), compared to mock-treated controls (139.70 pmol/mg/min±15.7; $p<0.05$). We conclude that NSV infection of spinal organotypic cultures results in neuronal specific infection, neuron death, and repression of GLT-1 expression and function.