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TITLE: Impact of Erb-B Signaling on Myelin Repair in the CNS
Following Virus-induced Damage

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14. ABSTRACT The objective of these studies is to examine the impact of signaling by neuregulin family members in a mouse model of virus induced demyelination. These studies utilize intraspinal cord injection of Theiler's murine encephalomyelitis virus (TMEV) into mice. The hypothesis tested in these studies is that increased erbB-mediated signaling will have a protective effect in this model of multiple sclerosis (MS), and that decreased erbB-mediated signaling will have a deleterious affect on the animals. Pharmacological agents, recombinant viruses and knockout mice are being used to test this hypothesis. The data described herein suggest that within the spinal cords of mice injected with TMEV the induction of IL-11 may be a key mediator of oligodendrocyte health and well being and this may represent the mechanism for increased myelin basic protein (MBP) mRNA that is reported. Furthermore, erbB3, a receptor for the neuregulins is highly expressed by cells infiltrating the central nervous system (CNS)					
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Introduction

The immediate objective of the studies funded by this grant is to define the neuregulin-mediated interactions that enhance myelin preservation/repair in the spinal cord following TMEV injection through testing of the hypothesis that neuregulins invoke erbB signaling and protects the central nervous system (CNS) by limiting Theiler's virus-induced pathology and triggering myelin repair processes. Two animal models will be utilized in these studies. In the first model, we will utilize animal that have not been genetically engineered and examine the earliest events after injection of TMEV into the spinal cords of mice. The effect of increased or decreased erbB-mediated signaling on these early events will also be defined. The second animal model will utilize tissue-specific inducible knockout mice that are lacking either erbB2, EGFR1 (also known as erbB1) or both erbB2 and EGFR. We will examine the effects of loss of these genes on myelin gene products and repair of the demyelinating lesions. The data obtained from the studies in this proposal will provide insight into the mechanisms responsible for repair of the CNS after virus-induced damage. These studies are the first to examine the role of neuregulins and their receptors in a model of virus-induced damage of the CNS. A greater understanding of the mechanisms involved in repair of the CNS will allow us to develop and refine strategies for the treatment of humans with demyelinating disease.

Body

Myelinating cells in the spinal cords of FVB mice do not appear to undergo proliferation following TMEV injection of the spinal cord.

In order to determine whether myelinating cells proliferated at the site of TMEV infection, we performed intraspinal cord inoculation of TMEV into the FVB mice and stained the spinal cords for Ki-67, a marker of actively proliferating cells at various time-points post injection. Using dual immunohistochemical staining techniques, the majority of cells undergoing proliferation in the spinal cords of mice within the first 72 hours following TMEV infection are not CNS resident cells, but rather infiltrating immune cells (T cells predominantly). These data indicate that myelinating cells are not increasing in number within the first 72 hours after infection. This is consistent with current viewpoints on mature oligodendrocyte stability.

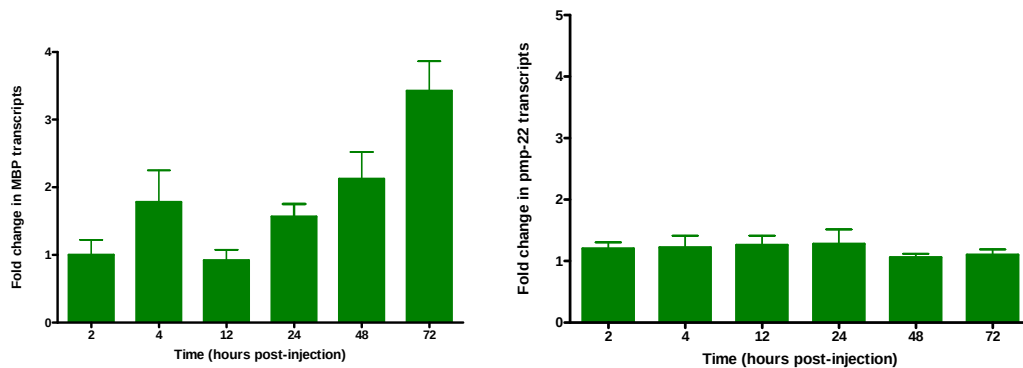
Myelinating cells at the lesion site of FVB mice undergo apoptosis following TMEV infections.

Last year, we demonstrated that there was significant apoptosis in the lesions of FVB mice infected into the spinal cord with TMEV using terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL). Since then, we have determined that the majority of cells undergoing apoptosis within the spinal cord white matter (>50%) are oligodendrocytes, indicating that the host is susceptible to a high degree myelin loss within the spinal cord. It is not surprising that the majority of cells experiencing apoptosis are oligodendrocytes, as this population comprises the majority of cell types in the spinal cord white matter.

Changes in the levels of MBP but not pmp-22 transcription occur within the lesioned area of the spinal cord.

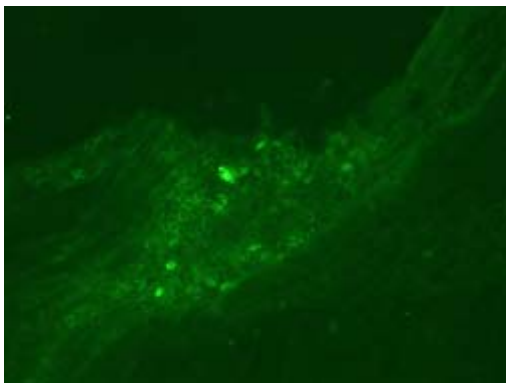
Previously, we reported that we developed a series of real-time RT-PCR primers to detect various myelin transcripts including myelin basic protein (MBP) and peripheral myelin protein-22 (pmp-22) mRNA. We have since performed real time RT-PCR assays

on the spinal cord lesions from mice infected via the spinal cord to determine whether there were alterations in the level of MBP and/or pmp-22 following injection with TMEV. As shown below, there were increased levels of MBP transcripts detected following virus injection, but no change in pmp-22 levels. This is consistent with our expectations, as pmp-22 is largely localized to the peripheral nervous system. Furthermore, MBP is known to be upregulated very quickly after CNS insult. It may be of interest to examine later time points to determine when MBP transcript levels return to baseline. Data shown below are expressed as the level of myelin gene transcript relative the level observed in control injected mice. All data were normalized using a housekeeping gene (GAPDH).



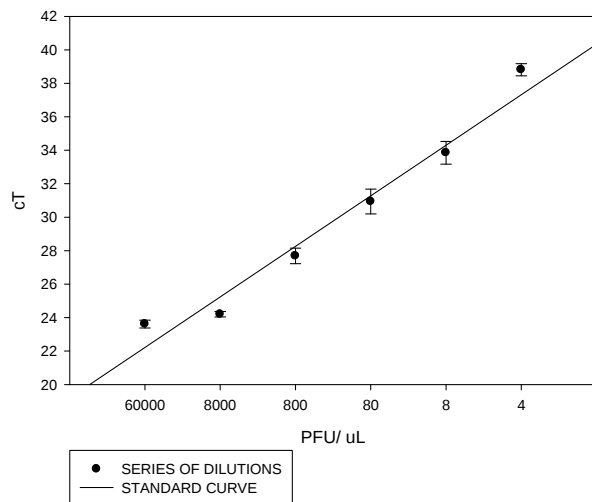
IL-11 is localized into the lesion area of TMEV-infected FVB mice.

Because we observed apoptosis of oligodendrocytes in the lesion and minimal proliferation, we were curious to know what other mechanisms could be invoked that would provide some degree of protection to the spinal cords of TMEV infected mice. One mechanism by which protection could be afforded is via the production of IL-11, a mediator known to be involved in oligodendrocyte survival and maturation (1). Immunohistochemical staining (below) revealed immunoreactivity (bright green) to IL-11 within the lesion, suggesting that one mechanism that could be involved in functional preservation (in the absence of oligodendrocyte proliferation) is via the maturation of oligodendrocyte precursor cells. Further analysis is required to determine whether there are alterations in the number of oligodendrocyte precursors in these animals. Reduced levels of oligodendrocyte precursor cells would support the hypothesis that CNS protection is due to enhanced maturation of myelinating cells, versus *de novo* generation of myelinating cells.



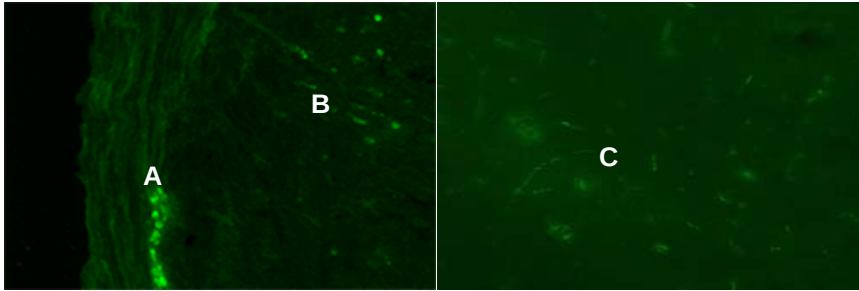
Real time PCR for TMEV has been optimized and can detect 4 to 8 plaque forming units (PFUs) of TMEV in a sample.

Because we are using very small pieces of tissue in our assays, it became necessary to determine what the sensitivity of our assay was despite the fact that we are using relative quantification as our output. To test the sensitivity of our assay, we spiked spinal cord samples with a known numbers of PFUs of TMEV. Our dilution series ranged from 0.5 to 60,000 PFUs. Using this assay, we can routinely detect between 4 and 8 PFUs of TMEV, and feel very comfortable stating that the limit of detection of this assay is <8 PFUs per sample. An example of the data obtained from these optimization experiments is shown below. This assay will allow us to detect even very small amounts of virus in our samples, which we expect to see in some of the samples collected from areas distal from the spinal cord injection site. This assay is being used to substitute for our viral plaque assay that has a detection limit of 200 PFUs per mg tissue (~50X more sensitive).



ErbB3 expressed constitutively at low levels in the spinal cords of control mice, but highly expressed on infiltrating cells in TMEV infected mice.

We examined whether erbB3, a receptor for neuregulin, was expressed in the spinal cords of control (uninjected; right panel) and TMEV-injected mice (left panel). As shown on the next page, TMEV infected mice express erbB3 at high levels on cells infiltrating the CNS (A). Based on our knowledge of the model, these cells are likely to be T cells. In addition, there are immunoreactive cells in the gray matter of the spinal cord (B) that are likely resident cells (neurons). These immunopositive cells are also localized in uninjected spinal cords (C).



Other grant activity:

1. The dose of inhibitors capable of blocking erbB signaling (AG1478, AG825) have been optimized. The experiments using these pharmacological inhibitors will commence shortly.
2. We have collected a large number of tissues from the transgenic models of intraspinal cord injection. Analysis of these tissues is ongoing. We expect to complete this phase of the study over the summer
3. Western blotting studies have begun but no conclusions can be made at this time regarding alterations in phosphorylation of EGFR, erbB2 and erbB4.
4. Real-time PCR array studies are being tested in the laboratory. These studies will be used to determine whether kinases are activated following phosphorylation.

Additional data relevant to these studies (but not funded by this DOD grant):

This DOD funded grant does not examine whether clinical parameters of disease are altered by exogenous neuregulin expression. We received a pilot grant from Creighton University to study whether exogenous administration of neuregulin isoforms (in particular sensory motor derived factor, glial growth factor, or glial growth factor 2) delivered via intraperitoneal injection with alters clinical function in TMEV infected mice using an objective measure of function (the CatWalk Gait Analysis System). Interestingly, we found that administration of glial growth factor slows the loss of clinical function in the TMEV-induced model of demyelination relative to control-treated animals. The mechanism of action of this improvement appears to be related to the downregulation of 2 specific microRNAs. This finding is likely relevant to the third specific aim of this DOD proposal and in the course of the studies, levels of these regulatory molecules (miR-684 and miR-466g) will be measured.

Key Research Accomplishments

1. Optimized real-time RT-PCR assay for TMEV so that 4-8 PFUs of virus can be detected per sample.
2. Myelinating cells undergo minimal proliferation, but significant apoptosis following TMEV injection into the spinal cord.
3. The identification of IL-11 within the TMEV-induced lesion may represent a key mechanism for preservation of myelin in this model, and may account for the increased levels of MBP that are observed within the spinal cords of infected animals. In addition, if we examine the lesions at a time-point later than 3 days post-infection, we may detect changes in the level of oligodendrocyte proliferation.

4. Determined that glial growth factor administration can improve clinical function in a mouse model of multiple sclerosis.

Reportable Outcomes

Medical Student Fellowships:

One medical student fellowship was awarded to pursue a novel aspect of this research. This student will be working in my laboratory on the project during the summer of 2010.

Thomas Hendricks was awarded a \$3,000 fellowship from the Nebraska Medical Foundation for his proposal entitled "Expression of miR684 in a mouse model of multiple sclerosis."

Presentations:

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Poster Presentation. Military Health Research Forum. Kansas City, Missouri. September 1, 2009

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Oral Presentation. Military Health Research Forum. Kansas City, Missouri. September 2, 2009

Other:

Served on Abstract Selection Committee for the Military Health Research Forum held in Kansas City, Missouri (August 31 – September 3, 2009).

Conclusions

Because myelin basic protein transcripts increased despite the increase in apoptotic cell death, we postulated that myelinating cells were undergoing proliferation. This is unlikely based on the data that we have obtained. Rather it currently appears that precursors to myelinating cells are undergoing a maturation process due to the increase in IL-11 levels. Further studies will be performed to confirm this. The sensitivity of the real-time RT-PCR assay that we developed will greatly enhance our analysis of the spinal cord tissues. This assay will better be able to detect whether some of the apoptosis that we observe in areas distal to the injection site are in fact, negative for virus or have virus burdens below the level of detection of the plaque assay.

References

1. Zhang Y, Taveggia C, Melendez-Vasquez C, Einheber S, Raine CS. 2006. Interleukin-11 potentiates oligodendrocyte survival and maturation, and myelin formation. *J Neurosci* 26:12174-85

Appendix

Drescher, K.M., Thal-Jantzen, H. (2009). Development of a Model of MS Permitting Study of Early Stages of Lesion Development. Military Health Research Forum. Kansas City, Missouri

Development of a Model of MS Permitting Study of Early Stages of Lesion Development

Kristen M. Drescher and Helene Thal-Jantzen

Background and Objectives: The cause of multiple sclerosis (MS), the most common central nervous system (CNS) demyelinating disease, is unknown although it is likely that both host genetics and environmental influences are involved in disease progression. The cellular immune response however, is partially responsible for the ongoing demyelination of the CNS. Because diagnosis occurs well after lesion formation, the earliest stages of lesion formation in humans have not been well-characterized. We have modified the existing Theiler's murine encephalomyelitis virus (TMEV) model of MS to permit the study of the lesion site within hours to days after the initial insult. In this model, TMEV is directly injected into the spinal cord of mice, thereby allowing for precise localization and aging of the lesion site

Methodologies: The DA strain of TMEV was utilized in all studies. Viral load was assayed by plaque assay. Immunohistochemistry and real time RT-PCR were used to characterize protein and gene expression within the lesion, as well as in areas distant from the lesion. TUNEL assays were also performed to determine the extent of apoptosis in the CNS relative to the lesion site.

Results: After surgical exposure of the spinal cord, female SJL/J mice were injected with 2×10^4 pfus of TMEV directly into the spinal cord white matter. Immunohistochemistry was performed using an antibody raised against myelin proteins and large areas of demyelination were observed within 3 days of injection. This demyelination was not observed in animals injected with vehicle (Hanks Balanced Salt Solution). Staining for viral proteins with a polyclonal antisera to TMEV revealed that demyelinated regions overlapped in areas where TMEV antigens were localized. The injection site was dissected out of the spinal cord and infectious virus was assayed. Replicating virus levels were nearly 3 logs greater than the initial inoculum. Immune cells were recruited to the site of the demyelination within 5 days post-injection: CD4+ and CD8+ T cells were all found within the lesion, as well as F4/80 reactive cells. Morphology of these cells was consistent with macrophages. Real-time RTPCR was used to assay levels of various myelin gene transcripts from lesion sites dissected from the spinal cords as a function of time post-injection. Alterations in myelin gene transcript levels over time were unique for myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG), indicating a differential impact of infection on each of these genes.

Conclusions: Together, these data describe a new model designed to examine the earliest phases of demyelinating lesion development. This will permit definition of events that occur within minutes of initial CNS damage, thereby permitting insight into the earliest pathogenic (and possibly protective) responses invoked by the host. Studies are ongoing to determine the earliest immune system participants in the demyelination process in this model.

Impact: These studies permit, for the first time, insight into the spatiotemporal development of CNS demyelinating lesions. Knowledge of these events may assist in the development or refinement of therapies for MS.

Presented at the Military Health Research Forum, Kansas City, MO in both poster and oral presentations.