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Virus-Induced Damage

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14. ABSTRACT These studies examine the impact of erbB-mediated signaling on myelin destruction and repair in a novel murine model of central nervous system (CNS) demyelination. Theiler's murine encephalomyelitis virus (TMEV) injection directly into the murine spinal cord is used in these experiments. We are examining the impact of either deleting the receptor for neuregulins (erbB2 and/or EGFR; using knockout mice) or increase the availability of the ligands for these receptors (the neuregulins; using recombinant adenoviruses). We hypothesize that increased erbB-mediated signaling will protect animals from disease and that decreased signaling will negatively impact repair. The data described herein demonstrate that Schwann cells are likely one of the main mediators of repair in our model due to the large increases of P0 transcripts within the lesion site. Furthermore, the adenoviral vectors expressing various neuregulin isoforms do not appear to alter cytokine production. Furthermore, damage occurs in normal appearing white matter as indicated by apoptosis assays.					
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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

Adenovirus vectors expressing various neuregulin isoforms induce alterations in lymphocyte subset phenotype compared to untreated mice. Flow cytometry was performed on peripheral blood mononuclear lymphocytes (PBLs) to determine if treatment with adenovirus vectors expressing neuregulin isoforms altered the levels of T and B-cell subsets in animals receiving these injections. FVB mice were injected with adenovirus vectors expressing glial growth factor (GGF), glial growth factor 2 (GGF2), sensory motor neuron derived factor (SMDF) or a control insert (enhanced green fluorescent protein). The peripheral blood lymphocyte (PBL) populations from control (uninjected mice) and adenovirus-injected mice were analyzed at 9 days p.i.

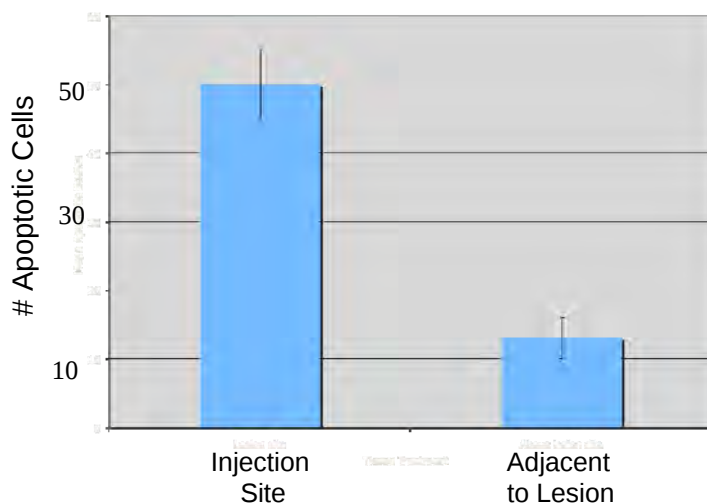
Animals receiving adenovirus vectors, regardless of the nature of the insert, had increased levels of CD8+ T cells as compared to uninjected control animals. As CD8+ T cells are increased following virus infection, it is not surprising that there is an increase in these cell types following injection with the adenovirus constructs. It is likely that the specificity of these cells is directed against the adenovirus. Furthermore, there were increased levels of CD4+ T cells in GGF and SMDF-treated animals, as well as decreased levels of B220+ B cells in the neuregulin treated animals as compared to controls. While it is likely that at least a portion of the CD4+ T cells are specific for adenovirus due to the requirement for CD4+ T cell help, it is unclear if all of the cells are adenovirus-specific. Furthermore, this explanation does not take into account the observation that mice receiving control adenovirus or GGF2-expressing construct do not have significant alterations in their CD4+ T cell compartment. Together, these data demonstrate that results generated using the adenovirus virus expression system need to carefully consider the impact of the vectors on the immune system. Our choice of using 3 different neuregulin isoforms as test vectors will further permit us to assess changes as related to either exposure to adenovirus, or insert-specific changes. We will also perform longer-term experiments using TMEV-infected animals to determine whether the mice injected with neuregulin-expressing adenoviruses can mount sufficient anti-TMEV antibody titers. This is a key experiment as the B220 compartment is severely reduced in all treatment groups. All data analysis was performed using a one-way analysis of variance (ANOVA).

<i>Treatment</i>	<i>CD4+ % $\pm$ SEM</i>	<i>CD8+ % $\pm$ SEM</i>	<i>B220 % $\pm$ SEM</i>
GGF-adenovirus	60.350 $\pm$ 0.750*	16.600 $\pm$ 1.700*	11.400 $\pm$ 2.400*
GGF2-adenovirus	54.200 $\pm$ 1.609	17.867 $\pm$ 0.837*	11.967 $\pm$ 1.440*
SMDF-adenovirus	59.600 $\pm$ 2.498*	19.230 $\pm$ 0.882*	8.667 $\pm$ 0.593*
Control-adenovirus	48.750 $\pm$ 3.050	16.300 $\pm$ 0.000*	15.200 $\pm$ 5.800
No treatment	34.300 $\pm$ 6.300	9.350 $\pm$ 2.450	35.550 $\pm$ 5.350

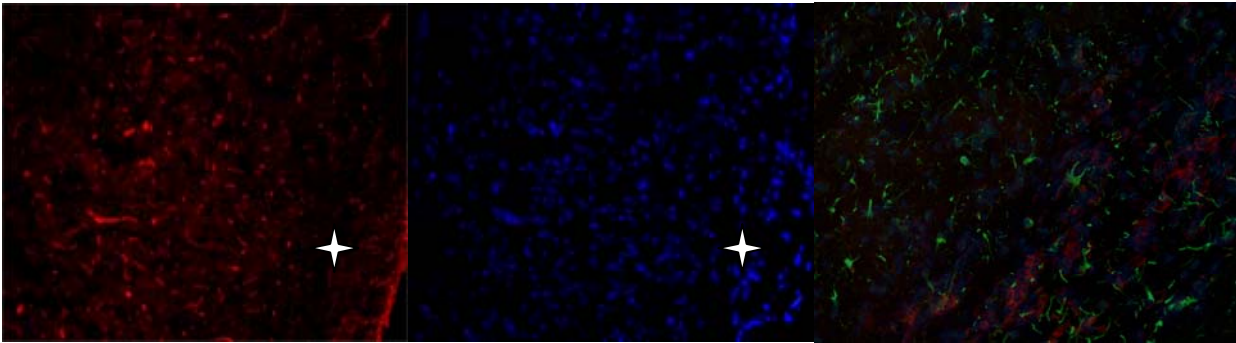
*Represent values that are significant; $p \leq 0.05$

Minimal alterations in chemokine and cytokine levels are observed in animals injected with neuregulin-expressing adenovirus constructs. To further explore the impact of the neuregulin-expressing adenoviruses on immune function (a critical experiment as the immune system is known to impact the development of demyelinating lesions), we utilized the Luminex bead array system to determine if there was a difference in the levels of 21 cytokines between in serum samples 7-days post-injection and in spleen samples 9-days after injection with adenovirus vectors expressing three different neuregulin isoforms. Control vectors contained an irrelevant insert. The cytokines tested were: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, GM-CSF, IFN γ , KC, MCP-1, and MIP-1 β , RANTES, TNF α , and VEGF. Analysis of sera at 7 days post-infection revealed that RANTES levels were significantly increased in adenovirus/GGF2-treated mice compared to control-treated mice. No other differences were observed in any of the cytokine levels between the control group and the neuregulin-expressing adenovirus groups. In the spleen (9 days post-treatment) levels of all cytokines were similar with no observed differences between treatment groups. Together, these data suggest that if there are differences observed in demyelination and remyelination in animals treated with neuregulin-expressing adenovirus vectors, the alterations are not likely to be due to alterations in immune function.

Apoptosis is increased in the lesion site of injection as compared to regions immediately adjacent to the lesion site. To assess the impact of intraspinal cord injection of TMEV on apoptosis in the spinal cord, we performed terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL) assays on the tissue. Both the lesion area and the area immediately above the lesion were examined. The area of the spinal cord immediately above the lesion was used for comparison. As shown below, there is a high level of apoptotic cells within the lesion site (data are expressed as the mean number of apoptotic cells $\pm$ SEM). While the level of apoptosis in areas adjacent to the lesion are greatly reduced (by approximately 23%), the observation that there is significant apoptosis in these uninfected areas of the spinal cord suggest that damage is occurring prior to the development of demyelination. One possible mechanism of this damage is soluble mediator production. Other potential mechanisms of damage could be the result of axonal damage and/or influx of inflammatory cells. Rarely are apoptotic cells observed in the spinal cords of uninjected animals.



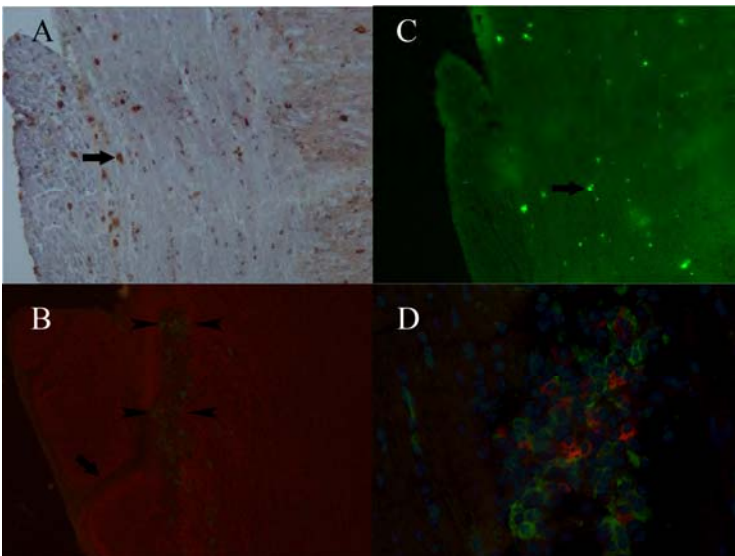
ErbB2 is expressed throughout the spinal cord of infected mice. We performed immunohistochemical staining on the spinal cords of FVB mice that have had TMEV injected into their spinal cords. At 3 days post-injection with TMEV, we utilized an antibody to erbB2 to examine the distribution of this protein in the spinal cord. As shown below (left), positive signal is found in the area of the spinal cord adjacent to the initial lesion site (star indicates lesion site). Minimal staining is observed in the lesion site, suggesting that one cell type expressing erbB2 is the myelin-producing oligodendrocyte. The DAPI-stained image is shown in the middle. Note the altered cellularity in the DAPI-stained image. The image on the far right shows a lesion from a erbB2 $^{-/-}$ -EGFR $^{-/-}$ mouse that has been stained for neuregulin (green), macrophages (red) and counterstained with DAPI (blue). Like the erbB2 staining of FVB mice, this image demonstrates that neuregulins are widely expressed in spinal cord tissue of mice following injection with TMEV.



Tamoxifen-treatment of *erbB2*^{-/-}*-EGFR*^{-/-} mice under the control of the PLP promoter experience alterations in their immune cell subsets compared to untreated mice. To determine whether tamoxifen treatment altered immune cell subset distribution, we performed flow cytometry on splenocytes isolated from tamoxifen or control treated PLP-*erbB2*^{-/-}*-EGFR*^{-/-} mice. We examined cells for the expression of CD4, CD8 and B220. As shown below, mice that were tamoxifen treated for 8 days (1 mg/day) had reduced levels of CD4⁺ T cells in the periphery 1 month after treatment, indicating that tamoxifen treatment, indicating a long-term effect on the immune system due to tamoxifen treatment.

	CD4 ⁺	CD8 ⁺	B220
Treated	11.33±3.636	8.133±2.111	35.13±3.818
Untreated	32.30±1.457	8.200±0.776	35.87±2.949
*Statistically Significant; p=0.0029			

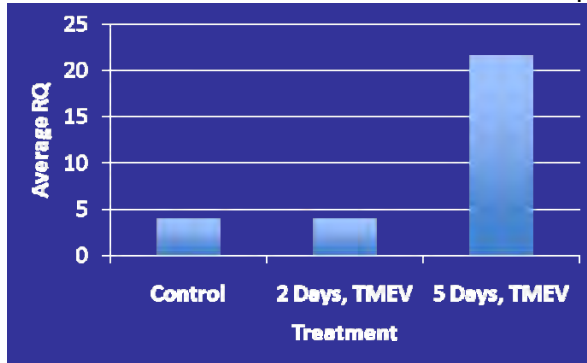
Lesion characterization of tamoxifen-induced PLP-*erbB2*^{-/-}*-EGFR*^{-/-} mice demonstrate that immune cells infiltrate the lesions. Immunohistochemical staining was performed to characterize the lesions that form in the tamoxifen-inducible knock out mice. Antibodies utilized are indicated in the legend below. All staining was performed at 7 days post injection with TMEV.



Characterization of a lesion site in tamoxifen-induced PLP-*erbB2*^{-/-}*-EGFR*^{-/-} mice at 7 days post-injection with TMEV. Despite intra-spinal cord injection, the animal had a normal gait at sacrifice. **A:** CD8⁺ cells (arrow) are present and concentrated in the lesion. 20x magnification. **B:** Foci of CD4⁺ cells are associated with demyelination in the lesion (arrowheads). The site of needle insertion is indicated by the arrow. **C:** Scattered apoptotic cells in the lesion and in tissue surrounding the lesion (arrow). **D:** F4/80⁺ phagocytic cells (green) are concentrated in an area of myelin producing cells (red). Nuclei are stained with DAPI (blue).

Real time PCR for P-zero transcripts indicate an increase in mRNA at 5 days post-injection with TMEV.

Real time PCR was performed using primers specific for P0, a myelin transcript made in abundance by Schwann cells. Minimal amounts of P0 are produced by oligodendrocytes. At 2



days post-injection, minimal changes in P0 transcripts were observed at the lesion site. By 5 days post-injection with TMEV, there is a large increase in the levels of P0 levels, indicating that there is an increase in myelin products. Of note however, it is unlikely that this increase in P0 can be attributed to oligodendrocyte-mediated repair. It is our hypothesis that there is an influx in Schwann cells from the peripheral nervous system that are attempting to repair the demyelinated central nervous system.

Key Research Accomplishments

1. The effects on the immune response of the recombinant adenoviral vectors expressing three isoforms of neuregulins have been characterized both in FVB mice as well as the tamoxifen-treated erbB2-/-EGFR-/- mice.
2. TMEV lesion sites have been characterized in the tamoxifen-treated erbB2-/-EGFR-/- mice.
3. Real-time RT-PCR experiments suggest that there is Schwann cell-mediated repair of the central nervous system occurring. Further characterization of this process is underway.
4. Apoptosis assays demonstrate that damage to the spinal cord occurs prior to the spread of demyelination and virus. These data indicate that there are other mechanisms (that is non-viral mediated) of damage occurring to the spinal cord.
5. Expression of erbB2 and neuregulins have both been shown to be widespread throughout the spinal cord, indicating that the components required for signaling are intact in mice with spinal cord lesions.

Reportable Outcomes:

Presentations:

Drescher KM and Tracy SM (2008) Development of a model allowing study of the early stages of demyelination. International Society of Neuroimmunology, Fort Worth, TX October 2008. Oral Presentation.

Rasband-Lindquist, A and Drescher KM (2009) Role of neuregulins in myelin preservation and repair in the central nervous system. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Lindquist JD and Drescher KM (2009) THE ROLES OF ERBB2 AND EGFR IN THE DEVELOPMENT OF TMEV-INDUCED LESIONS. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Conclusion

Our focus on the adenoviral constructs have provided us with some interesting data. We were surprised to find that there were not widespread alterations in the cytokines produced by the injected animals. This is very promising, in that any changes in myelination that we observe would unlikely be the result of immune system – soluble mediator production. This permits us to hypothesize that the changes are directly related to changes in the nervous system. Furthermore, the alteration in P0 transcripts is significant as Schwann cells, the primary producer of P0, are known to be very active in

the remyelinating process in the peripheral nervous system. Studies have also shown that Schwann cells may be recruited into the central nervous system and induce repair processes that are capable of preserving function (1).

We currently have two manuscripts in progress.

References

Rivera-Quinones, C., D. McGavern, J. D. Schmelzer, S. F. Hunter, P. A. Low, and M. Rodriguez. 1998. Absence of neurological deficits following extensive demyelination in a class I-deficient murine model of multiple sclerosis. *Nature Medicine* 4: 187-193.

Appendices

Drescher KM and Tracy SM (2008) Development of a model allowing study of the early stages of demyelination. International Society of Neuroimmunology, Fort Worth, TX October 2008. Oral Presentation.

Rasband-Lindquist, A and Drescher KM (2009) Role of neuregulins in myelin preservation and repair in the central nervous system. Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

Lindquist JD and Drescher KM (2009) THE ROLES OF ERBB2 AND EGFR IN THE DEVELOPMENT OF TMEV-INDUCED LESIONS . Midwest Medical Student Biomedical Research Forum, Omaha, NE, February 2009, Poster Presentation.

DEVELOPMENT OF A MODEL ALLOWING STUDY OF THE EARLY STAGES OF DEMYELINATION

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Studying the earliest phases of the development of demyelinating lesions can be difficult given the current animal models available. We have modified the currently existing Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis to permit us to study the lesion site hours to days after insult. In this model, we directly inject virus into the spinal cord of mice, thereby allowing for precise localization and aging of the lesion site

Following surgical exposure of the spinal cord, female SJL/J mice were injected with 2×10^4 pfus of TMEV (Daniel's strain) directly into the spinal cord white matter. Using antibodies directed against myelin proteins, immunohistochemistry revealed large areas of demyelination within three days of virus injection. Staining with a polyclonal antiserum to TMEV revealed that the area of demyelination overlapped with the region that was positive for TMEV antigens. Animals injected with DMEM (virus vehicle) did not experience demyelination. Tissue was isolated from the injection site, and plaque assay revealed the presence of replicating virus at levels approximately 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 at the lesion site, as well as F4/80 reactive macrophages. Real-Time PCR was used to assay the levels of various myelin gene transcripts from lesion sites dissected from the spinal cords over time. Alterations in gene transcript levels over time were unique for myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and myelin associated glycoprotein (MAG).

Together, these data represent a model of TMEV-induced demyelination which permits the study of the earliest phases of lesion development. The events that occur in the hours and days after insult are largely undefined, in particular with regards to the exact sequence of mediators and events that are triggered. Studies are ongoing to determine the earliest immune system participants in the demyelination process in this model.

Presented as an oral presentation at the International Society of Neuroimmunology Meeting, October 2008, Fort Worth, Texas

ROLE OF NEUREGULINS IN MYELIN PRESERVATION AND REPAIR IN THE CENTRAL NERVOUS SYSTEM

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The precise role of neuregulins in myelin preservation and repair in the central nervous system is not well defined, particularly in disease processes such as multiple sclerosis. The role of neuregulins, specifically glial growth factor (GGF), glial growth factor 2 (GGF2), and sensory and motor derived factor (SMDF), as well as their primary receptor (erbB2) was evaluated in the Theiler's murine encephalomyelitis virus (TMEV) infection. The role of neuregulins in TMEV was assessed through measurement of cytokines, flow cytometry, RT-PCR, immunostaining and apoptotic cell detection. The concentration of 21-cytokines did not statistically differ between TMEV-infected and uninfected control mice 7 days and 10 days after infection. However, after 18 hours, animals that received adenovirus vectors expressing GGF, GGF2, or SMDF had increased numbers of T-cells, both helper and cytotoxic, and decreased numbers of B-cells. The level of myelin basic protein (MBP) transcripts was statistically decreased in spinal cord tissue from areas near TMEV-injection sites 3-days post-injection compared to uninfected spinal cord tissue. The expression of proteolipid protein (PLP) transcripts was statistically decreased in the spinal cords of mice following infection of TMEV and treatment with SMDF-expressing adenovirus when compared to uninfected spinal cord tissue. Fluorescent immunostaining was performed for macrophages, myelin basic protein, erbB2, and coxsackie and adenovirus receptor (CAR). Immunostaining revealed that NRG3 and CAR were localized to the demyelinated lesion. Taken together, these results show that growth factors impact the environment of demyelinated lesions. Additional experimentation done on neuregulins and growth factors will help to clarify the role of all of these relationships in demyelination/remyelination.

Acknowledgements: This research was supported by the Department of Defense (W81XWH-07-1-0223)

Presented as a poster at the Midwest Medical Student Biomedical Research Forum, Feb 2009

THE ROLES OF ERBB2 AND EGFR IN THE DEVELOPMENT OF TMEV-INDUCED LESIONS Jonathan D. Lindquist and Kristen M. Drescher, Department of Medical Microbiology and Immunology, Creighton University School of Medicine, Omaha, NE 68178

The roles of ErbB2 and EGFR in remyelination of the adult spinal cord are poorly defined. ErbB2 and EGFR inducible knockout mice in the Theiler's murine encephalomyelitis virus (TMEV) model of multiple sclerosis were used to address the role of these receptors in spinal cord repair. Following induction of the knockout, CD4+ T cell populations were altered in B6/129 mice. Immunostaining showed infiltration of spinal cord lesions with CD4+, CD8+, and F4/80+ cells in both treated (ErbB2, EGFR knockout) and control (B6/129) mice 7 days post-infection. Staining also revealed foci of preserved myelin-producing cells associated with F4/80+ macrophages. Real-time PCR demonstrated alterations in P0 transcripts, but not PLP and MBP transcripts in ErbB2, EGFR knockout mice. Together, these findings suggest that the increase in P0 is due to Schwann cell infiltration of the CNS lesion.

Acknowledgements: This research was supported by the Department of Defense (W81XWH-07-1-0223)

Presented as a poster at the Midwest Medical Student Biomedical Research Forum, Feb 2009