Experimental models of neuroprotection relevant to multiple sclerosis

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ABSTRACT Activated T cells, particularly those of the T-helper (Th) 1 subset, have the capacity to kill neurons. Strategies for preventing such damage may include deviation of activated T cells into the Th2 subset (e.g., via use of glatiramer acetate), alteration of functional properties of Th1 cells (e.g., through use of interferon [INF]-β or IV immunoglobulin), and inhibition of activated cell migration into the CNS (e.g., by employing INF-β or natalizumab). Matrix metalloproteinase-9 (MMP-9) also causes neuron death in neurotoxicity models, and examination of medications with MMP inhibitory activity indicates that minocycline is capable of preventing such damage. Minocycline also has other properties relevant to conferring neuroprotection, such as inhibition of microglial activity and apoptosis pathways. In a small pilot study in patients with relapsing–remitting multiple sclerosis, minocycline treatment produced favorable outcomes in terms of gadolinium-enhancing lesions and clinical course. Further studies are needed to establish whether experimental neuroprotection strategies involving these mechanisms may be translated into preventing neurodegeneration in multiple sclerosis.
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The deterioration and loss of axons in multiple sclerosis (MS) have been appreciated for over 100 years, and recent studies have eloquently emphasized this degenerative process.[1,2] More recently, the loss of neuronal cell bodies in MS has been noted.[3,4] Therefore, MS can be considered not only an inflammatory demyelinating disease but also a degenerative one involving significant deterioration of axons and demise of neurons. Such deterioration may be responsible for the irreversible and progressive neurologic disability evident in the disease.[5,6] Understanding the mechanisms of neurodegeneration in MS is therefore critical to devising neuroprotective strategies for the disease. Activated T cells, specifically those of the $CD4^+$ T-helper (Th1) subset but including $CD8^+$ lymphocytes,[7] and matrix metalloproteinases (MMPs), particularly MMP-9, mediate neuron injury in both tissue culture and in vivo models. The mechanisms of injury and strategies to prevent such injury are now under investigation. The findings of these investigations may have bearing on the development of neuroprotective strategies in MS.

T-CELL-MEDIATED NEURON INJURY

There is good correspondence between areas of inflammation in MS lesions and axon injury, suggesting that inflammatory cells injure neurons and axons.[2,8,9] In experimental autoimmune encephalomyelitis (EAE), areas of edema and hypercellularity in the spinal cord correspond to regions of reduced axon density and other features of axon dysfunction.[10]

We have examined the possible causal relation between inflammatory cells and neurotoxicity in in vitro paradigms. In a model involving human fetal neurons grown in tissue culture, anti-CD3–activated T cells from normal adult volunteers (allogeneic) or from fetal spleen (syngeneic) exhibited prompt and substantial neuron killing, whereas exposure of neurons to nonactivated T cells from these sources did not lead to neuron death.[11] Our findings on the characteristics of T-cell killing of human neurons can be summarized as follows[11]: (a) both $CD4^+$ and $CD8^+$ T cells are harmful; (b) destruction requires cell-to-cell contact and is associated with alignment of T cells along axons; (c)

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neurotoxicity is not confined by major histocompatibility complex (MHC) or antigen specificity; and (d) neurotoxicity is blocked by antibodies to leukocyte function-associated antigen 1, CD40 ligand, and Fas ligand (FasL), which appear to act by inhibiting association of T cells with axons and neurons. In sum, so long as T cells are activated and present in sufficient quantities, they have the potential to produce neurodegeneration.

In the recent past, a new subset of $CD4^+$ T cells other than Th1 or Th2 cells has been described. This is the Th17 subset in which interleukin (IL)-17 is a signature cytokine. The Th17 subset may be more important in the pathogenesis of MS or the neurodegenerative process than are Th1 cells,[12,13] although the data in MS remain to be further established. It also remains to be established whether or not Th17 cells can kill neurons.[12]

Other investigators have shown that anti-CD3-activated T cells kill human fetal neurons via soluble factors, including granzyme B,[14] and studies in murine models show that antigen-specific T cells impact neural survival through perforin,[15] Fas/FasL,[16] and tumor necrosis factor (TNF)-related apoptosis-inducing ligand.[17]

After our studies showing that polyclonal activation of T cells leads to increased neuron death, we attempted to determine whether antigen-specific T cells possess the same capacity, focusing on potential differences between $CD4^+$ Th1 and Th2 subsets. We generated myelin basic protein-specific T cells from healthy volunteers. These cells are predominantly of the Th1 subclass that produces proinflammatory cytokines. We also generated Th2 cells that produce anti-inflammatory cytokines by using glatiramer acetate (GA) as the antigen. The comparative results show a significant effect of Th1 cells in killing neurons, whereas Th2 cells were without neurotoxic activity (manuscript in preparation). The differential mechanisms of Th1 versus Th2 cells in mediating neurotoxicity remain unclear but are under investigation.

Preventing T-cell-mediated neuron damage. The findings discussed above suggest that T-cell neurotoxicity may be prevented by therapeutic application of Th2 T cells or GA-mediated deviation of T-cell populations into the Th2 subset. Consistent with this, we have determined that the previous addition of Th2 cells to neurons decreases the subsequent neurotoxicity of Th1 cells (manuscript in preparation). The diminished effect of mixed Th1 and Th2 cells in neuron killing was observed only when neurons were pretreated with Th2 cells for 24 hours before addition of Th1 cells, suggesting that the mechanism of the protective effects of Th2 cells involves gene alterations and protein expression of protective molecules. GA-reactive T cells produce neurotrophins such as brain-derived neurotrophic factor and nerve growth factor (NGF),[18-21] which are important survival factors for neurons in development and throughout life. Our own studies using focused gene arrays have shown at least a twofold increase in a number of growth factors for GA-stimulated versus non–GA-stimulated T cells, including vascular endothelial growth factors, insulin-like growth factors, and platelet-derived growth factors.[22]

In agreement with the protective effects of GA-reactive T cells in vitro, other studies have shown that GA treatment results in neuroprotection in a number of experimental models of neurologic disease, including ocular glutamate excitotoxicity,[23] facial nerve resection,[24] a mouse model of ALS,[24] EAE,[25] *N*-methyl-4-phenyl-tetrahydropyridine (MPTP) parkinsonism,[26] and a mouse model of Alzheimer's disease.[27]

Another potential strategy to decrease T-cell neurotoxicity is to alter properties of activated cells before exposure to neurons. One method for assessing the effects of altering T-cell properties involves exposure of human neurons to nonactivated T cells or to anti-CD3-activated T cells after the activated T cells are treated with test factors. Using this method, we found that treatment of activated T cells with IFN-β[10] and immunoglobulin[28] reduced killing of neurons compared with activated T cells alone.

Additional strategies to prevent T-cell-mediated neurotoxicity include reducing migration of T cells into the CNS. Such strategies could involve use of adhesion molecule inhibitors such as natalizumab, chemokine antagonists, or MMP inhibitors. The role of MMP-9 in neuron injury and the potential use of minocycline in preventing such damage are discussed below.

MMP-9-MEDIATED NEURON INJURY The MMPs constitute a family of proteolytic enzymes important in multiple processes, including turnover of extracellular matrix, cell survival and death, and signal transduction.[29] Although MMPs have important physiologic functions during homeostasis, their excessive upregulation in organs such as the brain and spinal cord contributes to pathology.[30] Multiple MMPs are elevated in human neurologic diseases.[29,30] In the setting of MS, it has been shown that serum MMP-9 levels are increased in patients with clinically isolated syndrome (CIS) compared with normal control subjects and are further elevated in patients with clinically definite MS (CDMS) com-

pared with patients with CIS.[31] In addition, serum MMP levels increase markedly between onset of neurologic symptoms and development of CDMS, whereas levels remain unchanged in subjects with CIS who do not develop CDMS. Other studies have documented elevations of MMP-9 and other MMPs in the serum, CSF, and brain of patients with MS compared with controls.[30]

Studies in vitro have shown a dose-dependent reduction in neuron numbers with increasing concentrations of active MMP-9.[32] In vivo, the injection of MMP-9 into rat cortical white matter increases axon injury as assessed by amyloid precursor protein immunocytochemistry.[33] We have used a model of intracerebral hemorrhage (ICH) in mice to determine the contribution of MMP-9 to neuron injury. In this model, injection of 10 μL of autologous blood into the striatum produced extensive injury and neuron death compared with 10 μL of saline. MMP-9 expression was upregulated at 6 and 24 hours after ICH. The contribution of MMP-9 to neuron death in ICH was then assessed by comparing cells positive for Fluoro-Jade (as a marker for dying neurons) from MMP-9-deficient animals and wild-type animals. We found that ICH caused by use of autologous blood was associated with significantly greater killing of neurons in wild-type animals (containing MMP-9) than in MMP-9-deficient animals. These findings therefore suggest that MMP-9 plays a direct role in neuron damage.[32] Although our experimental studies were conducted in the context of ICH injury, the results are pertinent to MS because they indicate that excessive increase of MMP-9 in MS lesions may contribute to the neurotoxicity seen in the disease.

Preventing MMP-9-mediated neuron damage. There is considerable interest in assessing the potential of minocycline, which has MMP-inhibitory activity, in inhibiting neuron damage. It should be noted, however, that minocycline has many other actions,[34] so it would not be possible to ascribe its outcome in vivo solely to an MMP-mediated effect.

Initial investigation of the effects of minocycline on MMPs showed that it exhibits a dose-dependent effect in reducing MMP-9 activity and also decreases MMP-9 level.[35] In addition, minocycline decreases clinical severity, inflammation, and neuropathology in EAE in mice[35] and rats.[36] We also demonstrated that minocycline attenuates axon loss and improves histologic and behavioral outcomes in spinal cord injury in mice.[37] The combination of GA and minocycline produced a significant reduction in disease severity and disease burden with attenuation of inflammation, axon loss, and demyelination in EAE in mice.[38] The effects of minocycline in such models may be associated with a variety of activities, including those that reduce neuroinflammation and potential direct effects in attenuating cell death.[39]

On the basis of experimental findings and in light of experience with safe use of minocycline as an acne medication, our group undertook a small open-label study of minocycline treatment in patients with relapsing–remitting MS.[40] In total, 10 patients underwent clinical, MRI, and patient-based assessments at baseline. MRI was repeated monthly during an initial 3-month run-in phase without treatment and during a 6-month treatment phase in which patients received minocycline 100 mg BID. After completion of this initial 6-month treatment phase, MRI was performed at months 12, 24, and 36. Serum was collected at regular intervals for analysis. We found that the enzymatic activity of MMP-9 was markedly reduced throughout 18 months of treatment compared with baseline levels in these patients.[41] MRI findings show that in the 5 of 10 patients with gadolinium (Gd)-enhancing lesions before the treatment phase, Gd-enhancing lesions were no longer detectable by 3 months on therapy. Clinical assessments in this small sample of 10 patients indicate a reduction in the number of relapses over 24 months of treatment compared with pretreatment levels.[41] Additional analysis in this group has indicated a trend toward reduced annualized rate of brain atrophy with increased time on minocycline treatment (Zhang et al., unpublished data). Confirmation and extension of these findings require study in larger patient groups.

We must emphasize that although we first tested minocycline in an animal model of MS[35] based on its MMP-inhibitory activity, and although minocycline reduced MMP-9 activity in the serum of patients with MS,[41] we cannot be certain that a therapeutic effect in vivo would be solely due to MMP inhibition. Minocycline possesses multiple actions,[34] and it would be difficult to ascribe particular clinical outcomes to specific mechanisms in vivo. Finally, because there is good correspondence between inflammation and neurodegeneration, it is virtually impossible to dissociate a purely neuroprotective effect from an indirect effect on neuroinflammation that then modulates toxicity. In this regard, because minocycline has effects on multiple components of the immune system,[34] it may achieve its neuroprotective outcome by dampening detrimental inflammation, rather than by boosting an endogenous neuronal protective response. It is likely that both direct and indirect mechanisms contribute to its efficacy in vivo. However, this remains to be estab-

lished. We emphasize that minocycline crosses the blood–brain barrier (BBB) into the CNS, and this distinguishes it from many immunomodulators, such as IFN-β formulations, that act largely in the periphery. The central actions of minocycline are relevant to consideration of its use in combination with immunomodulators that do not enter the CNS.

OTHER MECHANISMS OF NEURON DEATH In addition to T-cell– and MMP-9–mediated neurotoxicity, several other mechanisms may contribute to neuron and axon injury in MS (table 1). These mechanisms can be considered to be largely inflammation-derived, such as through cytokines, complement, or free radicals elaborated by inflammatory cells, or they may be noninflammatory in nature. The latter include loss of myelin, given the recognition that there is an intimate functional relation between axons and myelin, so that the persistent loss of one would compromise the integrity of the other. In animals, the subtle change of myelin components eventually gives rise to axon disruption.[42] Alteration of ion fluxes, resulting in internal calcium overload, is another means to promote neurotoxicity, and both inflammatory and noninflammatory mechanisms may contribute to this.

Other neuroprotective agents. Table 2 lists several agents that have been reported to have neuroprotective activity for axons and neurons, either in tissue culture or in animal models of MS. At least one sodium channel blocker, which targets downstream calcium elevation, is in clinical trials for its potential neuroprotective effect in MS. An inhibitor of glutamate neurotransmission, riluzole, has been tested for neuroprotective outcomes in a small study of patients with primary-progressive MS.[43] Larger and longer-term studies need to be performed to address whether these agents have neuroprotective effects, either directly and/or indirectly, by affecting components of the immune system. The growing list of medications considered for neuroprotective potential augurs well for eventually conferring neuroprotection against the increasingly recognized neurodegenerative insults of MS. One of the challenges in the field is the development of validated and practical outcome measures suited for trials of neuroprotective agents.

CONCLUSION Neuron damage characteristic of MS may be caused by activated T cells, particularly Th1 cells, and by activity of MMP-9. Strategies for preventing T-cell damage may include deviation of T cells into the Th2 subset (e.g., via exposure to GA), modulation of activated T-cell properties (e.g., through exposure to INF-β, immunoglobulin, or minocycline), and inhibition of T-cell migration into the CNS (figure). Given the increasing appreciation of neurodegeneration in MS and the correspondence of axon and neuron loss to progression of disability, it is important to continue investigations into derivation and evaluation of better neuroprotective approaches to treat the disease.

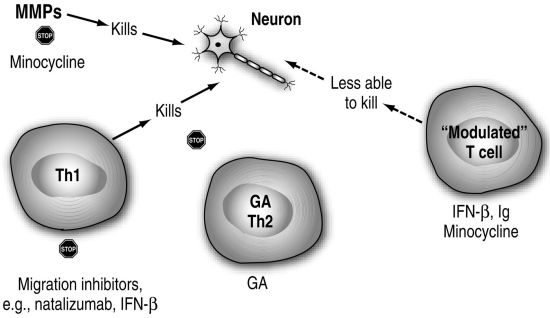
Table 2 Agents that have potential neuroprotective effects in MS

Erythropoietin[45]
Immunophilin ligands[46]
Sodium channel blockers[47]
Glutamate receptor antagonists[48]
Cannabinoids[49]
Statins[50]
Promotors of remyelination

Table 1 Summary of mechanisms that may contribute to axon and neuron injury in MS[34,44]

Products of inflammation	Others
Cytokines	Demyelination
Complement	Ion dysregulation and calcium excitotoxicity
Free radicals	
Nitric oxide	
Matrix metalloproteinases and other proteases	
Direct injury by $CD4^+$ and $CD8^+$ T cells	
Persistent activation of microglia	

Figure Mechanisms of neuronal injury and means to confer neuroprotection. GA, glatiramer acetate; IFN-β, interferon β; Ig, immunoglobulin; MMP, matrix metalloproteinase; Th, T helper.



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