

# Gene therapy using viral vectors for acute neurologic insults

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**Article abstract**—Enormous knowledge has emerged concerning the cellular and molecular events underlying necrotic neuron death after seizure, hypoxia–ischemia, or hypoglycemia. This has allowed the design of rational therapies to protect neurons at such times. One of the most exciting arenas of such interventions is the use of viral vectors to deliver neuroprotective genes. This review considers the progress in this nascent discipline. Neuroprotection has been demonstrated against a variety of *in vitro* and *in vivo* rodent models of necrotic insults with vectors overexpressing genes that target various facets of injury. These have included the energetic components, calcium excess, accumulation of reactive oxygen species, protein misfolding, inflammation, and triggering of apoptosis (i.e., programmed cell death) in a subset of cells. A number of caveats, subtleties, and pressing questions concerning this literature then are considered. These include whether these gene therapy interventions actually prevent, rather than merely delay, neuron death; the extent to which the effects of such vectors on neuronal cell biology is actually understood; the potential adverse effects of the use of such vectors; and whether sparing a neuron from death with one of these interventions spares function as well. Finally, we consider the likelihood of such gene therapy becoming relevant to clinical neurology in the near future. **Key words:** Gene therapy—Hypoxia–ischemia—Seizure—Herpesvirus—Adenovirus—Energetics—Calcium—Heat-shock proteins—Reactive oxygen species—Necrosis—Apoptosis.

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There is now considerable knowledge regarding the biology of neuron death after necrotic insults such as seizure, hypoxia–ischemia, or hypoglycemia: a cascade of excessive synaptic levels of excitatory amino acid (EAA) neurotransmitters, excessive cytosolic calcium mobilization, and the generation of reactive oxygen species (ROS).

It is now possible to induce the expression of novel genes at the time of such insults in neurons, glia, and vascular endothelial cells, or in cells transplanted into the brain (such as fibroblasts), with the goal of targeting some of the steps in neuron death. In this review, we consider the use of viral vectors for gene therapy from the perspective of the pathophysiology of necrotic injury—what steps in the biology of neuron death have been targeted, and with what results, constraints, and advantages.

**Cellular cascade of neuron death following necrotic injury.** The cascade mediating necrotic and apoptotic elements of neuron death in response to necrotic insults (reviewed in references 1–3) suggests specific points to be targeted in therapy (figure).

Necrotic insults first cause synaptic concentrations of EAAs (predominately glutamate) to rise into

the excitotoxic range. With hypoxia–ischemia and hypoglycemia, there is a depletion of energy in neurons and glia. The removal of EAAs from the synapse by neuronal and glial transporters is driven by ionic gradients and is energy-dependent, and thus it fails following these insults, prolonging EAA accumulation; at an extreme, such transporters can even reverse, augmenting EAA release. Furthermore, the energy deprivation tends to depolarize the presynaptic neuron, enhancing voltage-dependent EAA release. With seizures, repeated excitation increases EAA concentrations; with prolongation of such seizures, there evolves an energy mismatch, thus impairing the activity of the transporters removing EAAs from the synapse.

The excessive EAAs exert their excitotoxic effects through NMDA and non-NMDA receptors and through increased release of calcium from intracellular stores. The energy crises of hypoxia–ischemia and hypoglycemia and the secondary energy crisis of seizures exacerbate this accumulation of cytosolic calcium by impairing the calcium extrusion and efflux mechanisms (which depend upon ionic gradients or adenosine triphosphate).

This excess of free cytosolic calcium is critical to

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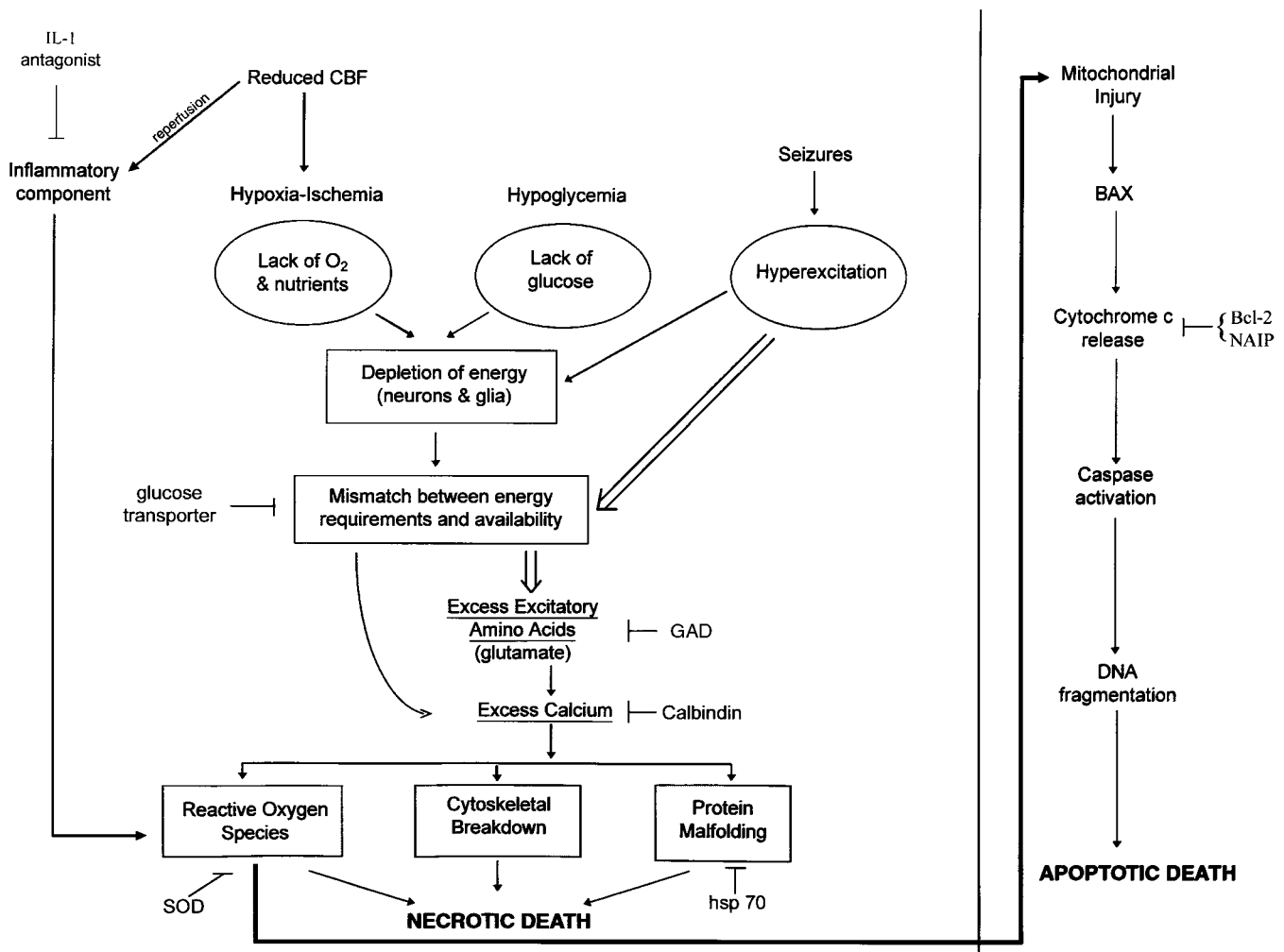


Figure. Schematic representation of the pathways mediating necrotic and apoptotic death following acute neurologic insults (arrows), and sites of demonstrated therapeutic efficacy of gene transfer approaches (T squares). IL-1 = interleukin 1; CBF = cerebral blood flow; GAD = glutamic acid decarboxylase; SOD = superoxide dismutase; hsp 70 = heat-shock protein 70; NAIP = neuronal apoptosis inhibiting protein.

subsequent neuron death. The absolute calcium load does not predict death, in that the source and subcellular compartmentalization of the cytosolic calcium are relevant. The calcium excess activates the generation of ROS, cytoskeletal proteolysis, and the mal-folding and aggregation of proteins, collectively leading to necrotic cell death. ROS are thought to be particularly damaging if reduced cerebral blood flow is followed by reperfusion.

Such necrotic insults instead can trigger apoptotic (i.e., programmed) cell death in a subset of neurons, or a combination of both necrotic and apoptotic features of death in the same cell.<sup>4,5</sup> Necrotic cell death typically involves nonspecific DNA degradation, nuclear pyknosis, loss of membrane integrity, and mitochondrial swelling. In contrast, apoptosis involves chromosome condensation and DNA fragmentation at internucleosomal sites, membrane rounding and blebbing of the cell into apoptotic bodies, and changes in surface antigens; the advantage of such apoptotic death is that it minimizes inflammation. In general, the more slowly a necrotic insult progresses,

or the more energy stores in the damaged neurons, the greater the likelihood of apoptotic elements emerging; this is because of the protein synthesis and energy expenditure required for apoptosis. The transition point at which activation of the necrotic pathway of death diverges into apoptotic death remains controversial. Current evidence suggests that ROS-induced damage to mitochondria results in activation and dimerization of the proapoptotic protein BAX. This leads to cytochrome c release from mitochondria, which, in turn, activates proteolytic enzymes called caspases. This eventually results in the pathway of events leading to the DNA, membrane, and antigenic changes noted previously that characterize apoptosis. Although this picture of neuron death is highly simplified, it does point out the rationale for the genetic interventions.

**Neuronal protection with the viral vector approach to gene therapy.** We now review the array of studies that have used viral vectors to target steps in cell death. As discussed in the previous sec-

**Table 1** Outcomes of gene therapy studies overexpressing a glucose transporter gene

Insult	Brain region	Strongest effect presented	Reference
Excitotoxic seizures	Hippocampus	<50% Reduction in lesion size	6
		>50% Reduction in lesion size	7
Excitotoxin exposure in vitro	Hippocampal culture	>50% Reduction in neuron loss	8
	Hippocampal culture	>50% Reduction in neuron loss	7
Infarct	Striatum	>Doubling of no. of labeled neurons, relative to treatment with control vector	9
Metabolic challenge	Hippocampus	>50% Reduction in lesion size	10
	Hippocampal culture	>50% Reduction in neuron loss	8
		<50% Reduction in neuron loss	6
	Spinal culture	>50% Reduction in neuron loss	8
	Septal culture	>Doubling of no. of surviving neurons	8
	Striatal culture	>Doubling of no. of surviving neurons	McLaughlin et al. (submitted for publication 1999)

Excitotoxic damage in vitro or in vivo was induced with glutamate<sup>8</sup> or kainic acid<sup>6,7</sup> in these studies. Infarct was accomplished with middle cerebral artery occlusion. Metabolic challenge was brought about by hypoglycemia<sup>6,8</sup> or the mitochondrial poisons 3AP (3 acetylpyridine<sup>10</sup>) or 3NP (3 nitropropionic acid<sup>7</sup>). The gene was delivered with either an adenoviral vector<sup>7</sup> or a herpes simplex virus vector (all other studies). In vivo studies were carried out with either rats or mice, and vector was delivered by direct intracerebral injection. In all studies, the magnitude of protection was significant.

tion, energy availability greatly influences the efficacy with which EAA secretion and calcium mobilization are regulated; reflecting this, one of the earliest attempts to neuronal gene therapy attempted to bolster neuronal energetics during insults via overexpression of the Glut-1 rat brain glucose transporter (table 1). Such overexpression is broadly protective, reducing neuron loss in varied injury models in primary neuronal cultures from several brain regions, as well as against in vivo models of hypoxia-ischemia, seizure, and hypoglycemia. As evidence that protection arises from the transgene, rather than from some unexpected feature of the delivery system, both herpes simplex virus amplicon vectors and adenoviral vectors are protective.

A single study has targeted another early step in

the death cascade, namely the hyperexcitation that accompanies excitotoxic insults. Specifically, the authors showed the neuroprotective effects of enhancing inhibitory GABAergic tone by overexpression GAD, the rate limiting gene in  $\gamma$ -aminobutyric acid synthesis.<sup>11</sup>

Studies also have targeted the excess of cytosolic calcium. Possible strategies would include overexpression of a calcium-binding protein, or antisense targeting of a calcium channel. To date, all relevant studies have overexpressed the calcium-binding protein calbindin D28K (table 2), chosen because of the correlation in a number of studies of its presence with neuronal survival.<sup>16</sup> Such overexpression protects against a variety of insults. Protection has been reported with both herpes simplex virus and adenoviral vectors, and from different laboratories.

**Table 2** Outcomes of gene therapy studies overexpressing calbindin D28K

Insult	Brain region	Strongest effect presented	Reference
Excitotoxic seizures	Hippocampus	>50% Reduction in lesion size	12
Excitotoxin exposure in vitro	Hippocampal culture	>Doubling of no. of surviving neurons	13
Infarct	Striatum	>Doubling of no. of labeled neurons, relative to treatment with control vector	14
Global ischemia	Hippocampus	>Doubling of no. of surviving neurons	15
Cyanide in vitro	Hippocampal culture	No significant protection	13
Metabolic challenge	Hippocampus	>50% Reduction in lesion size	12
	Hippocampal cultures	<Doubling of no. of surviving neurons	16
$\beta$ -amyloid peptide	Hippocampal cultures	>50% Reduction of neuron loss	17

Excitotoxic damage in vitro or in vivo was induced with glutamate,<sup>13</sup> kainic acid,<sup>12,13</sup> or NMDA<sup>13</sup> in these studies. Infarct was accomplished with middle cerebral artery occlusion; global ischemia was brought about with the 2-vessel occlusion model. Metabolic challenge was brought about by hypoglycemia<sup>16</sup> or the mitochondrial poisons 3AP (3 acetylpyridine<sup>12</sup>). The gene was delivered with either an adenoviral vector<sup>15</sup> or a herpes simplex virus vector (all other studies). In vivo studies were carried out with rats or mice, and vector was delivered by direct intracerebral injection. In all studies except with cyanide, the magnitude of protection was significant.

**Table 3** Outcomes of gene therapy studies overexpressing hsp70

Insult	Brain region	Strongest effect presented	Reference
Excitotoxic seizures	Hippocampus	>Doubling of no. of surviving neurons	18
Excitotoxin exposure in vitro	Hippocampal culture	No significant protection	19
Infarct	Striatum	>Doubling of no. of labeled neurons, relative to treatment with control vector	18
Anoxia	Dorsal root ganglia culture	<Doubling of no. of surviving neurons	20
Anoxia-aglycemia or aglycemia alone	Cortical astrocyte culture	>50% Reduction in cell loss	21

Excitotoxic damage in vitro or in vivo was induced with glutamate<sup>19</sup> or kainic acid.<sup>18</sup> Infarct was accomplished with middle cerebral artery occlusion. The gene was delivered with either a herpes simplex virus vector,<sup>18,19</sup> a retrovirus,<sup>21</sup> or an expression vector.<sup>20</sup> In vivo studies were carried out with rats or mice, and vector was delivered by direct intracerebral injection. In all cases except for excitotoxin exposure in vitro, the magnitude of protection was significant.

As noted, recent studies have emphasized that a key deleterious consequence of the calcium excess is ROS generation. Commensurate with that, overexpression of superoxide dismutase (SOD) has been reported to protect against global ischemia in the hippocampus,<sup>15</sup> but not against toxicity in hippocampal cultures induced by the  $\beta$ -amyloid peptide.<sup>17</sup>

Other adverse consequences of calcium excess following insults include incorrect intracellular targeting of nascent proteins, their failure to function resulting from misfolding, and the potential for misfolded proteins to aggregate. It is in this realm that heat-shock proteins, as molecular chaperones, are most likely to have their protective actions. Thus, heat-shock protein overexpression is also a potential strategy, complicated by the lack of consensus as to which of the numerous endogenous inducible heat-shock proteins is most physiologically protective. One possible solution is to move higher in the regulatory hierarchy and to overexpress heat-shock factor, the transcriptional factor central to induced expression of all the heat-shock proteins. No such reports have appeared. The sole published reports have instead concerned the inducible form of heat-shock protein 70, the most robustly induced of heat-shock proteins. Its overexpression failed to protect against an excitotoxic insult in primary neuronal cultures but did protect against other insults in neuronal and astrocytic cultures as well as against ischemic and excitotoxic insults in vivo (table 3).

Studies also have focused on the apoptotic branch of injury. Potentially, approaches could include overexpression of antiapoptotic genes, or antisense targeting of endogenous proapoptotic genes; only the former has been reported. Amid the variety of mammalian and viral antiapoptotic genes, studies have concerned neuronal apoptosis-inhibiting protein, which protects against global ischemia in the hippocampus; Bcl-2, protecting against excitotoxic, metabolic, and oxidative insults in vitro and in vivo; or crmA, protecting against the excitotoxin domoic acid (table 4). These results have been demonstrated with differing viral vector systems and from different laboratories.

Two groups have studied the protective conse-

quences of overexpressing interleukin (IL) 1 receptor antagonist (table 5). IL-1 $\beta$  actions are potentially protective or endangering of neurons. In agreement with pharmacologic studies,<sup>33,34</sup> overexpression of an endogenous antagonist of the IL-1 receptor resulted in neuroprotection, suggesting that the endangering actions of IL-1 predominate via this particular receptor. The mechanism of this protection is unclear. IL-1 induces proinflammatory cytokines<sup>35</sup> and activates phospholipase A2<sup>36</sup> and iNOS.<sup>37</sup> Thus, IL-1 probably contributes to the primary oxidative features of neuron death as well as mediates secondary inflammatory injury. As an implication of the inflammatory route, IL-1ra overexpression reduces neutrophil accumulation following middle cerebral artery occlusion.<sup>38</sup> Both reports of IL-1ra overexpression involved intraventricular delivery of vector (in contrast to the other cited studies, in which delivery was via direct intracerebral injection). Ventricular delivery results in infection limited to ependymal cells lining the ventricular border, which then appear to secrete the IL-1ra that accumulates diffusely throughout the extracellular space in the brain.<sup>31</sup> The efficacy of such an approach probably reflects the diffuse nature and extracellular action of secondary inflammation following necrotic brain injury.

#### Questions, qualifiers, caveats, and subtleties.

Although other steps in this degenerative cascade are potential targets, these studies represent impressive progress for this newly emerging discipline. These studies predominately have examined whether introduction of a particular transgene can be done safely and be protective. We now discuss subtler issues raised by these findings.

*Saving neurons versus merely delaying death.* In the studies cited, the end point (e.g., number of surviving neurons) was assessed at a single time point, raising the possibility that there was merely delay rather than prevention of death. This would be analogous to other strategies that postpone neuron death, including mild brain hypothermia under certain ischemic conditions.<sup>39,40</sup> Despite the lack of studies quantifying neuron loss at multiple time points, in most of those discussed, the time point used was

**Table 4** Outcomes of gene therapy studies overexpressing antiapoptotic genes

Insult	Brain region	Strongest effect presented	Reference
Excitotoxic seizures	Hippocampus	>50% Reduction in lesion size	McLaughlin et al., submitted for publication
Excitotoxin exposure in vitro	Hippocampal culture	>50% Reduction in neuron loss	McLaughlin et al., submitted for publication
		>Doubling of toxin dose needed for killing 50% of neurons	23
		>50% Reduction in neuron loss	24
Infarct	Cortical culture	>50% Reduction in neuron loss	25
	Cortex	<Doubling of volume of viable tissue	26
Global ischemia	Striatum	>Doubling of no. of surviving neurons	23
		>Doubling of no. of surviving neurons	27, 28
Hypoxia/aglycemia	Hypothalamic tumor cell line	>Doubling of no. of surviving neurons	29
Hypoglycemia	Hippocampal culture	>Doubling of no. of surviving neurons	23
ROS generator	Hippocampal culture	>Doubling of toxin dose needed for killing 50% of neurons	23

Excitotoxic damage in vitro or in vivo was induced with glutamate,<sup>23</sup> kainic acid (McLaughlin et al., submitted for publication, 1999), or domoic acid.<sup>24</sup> Infarct was accomplished with permanent middle cerebral artery occlusion; global ischemia was accomplished with the 4-vessel occlusion model. The gene was delivered with either a herpes simplex virus vector<sup>23–26</sup> (McLaughlin et al., submitted for publication, 1999), an adenoviral vector,<sup>27</sup> or a retrovirus.<sup>29</sup> The genes delivered were either Bcl-2,<sup>23,25,26</sup> (McLaughlin et al., submitted for publication, 1999), NAIP,<sup>27</sup> or crmA, a cowpox-derived caspase inhibitor.<sup>24</sup> In vivo studies were carried out with rats or mice, and vector was delivered by direct intracerebral injection. In all cases, the magnitude of protection was significant.

one at which maximal damage occurs. This suggests that the gene therapy interventions did indeed prevent neuron death.

*Interference with expression of vectors by necrotic insults.* Ischemia typically inhibits protein synthesis (with the exception of heat-shock proteins), including protein synthesis from viral vector DNA. For example, expression in an adenoviral vector in ischemic or reperfused tissue is blunted and delayed, relative to nonischemic controls.<sup>41</sup> Thus, the ischemic inhibition of protein synthesis likely diminishes the efficacy of a gene-therapy intervention; one possible solution is to place expression under the control of a heat-shock promoter. The fact that any transgenes have proven protective in ischemia models despite this blunted expression testifies to their potential efficacy, once this problem of inhibition is circumvented.

*Effects of gene-therapy interventions on intervening cell biology.* In the studies cited, outcome measures included some index of whether the intervention was protective—did more neurons survive? An impor-

tant issue is whether these interventions influenced survival through a logical route of action.

By this we mean, first, that if the protective action of a viral vector is to make sense, infection with it should lead to increased levels of mRNA and protein for the transgene and proper subcellular localization of the protein. It would present a serious conundrum if, for example, delivery of a vector overexpressing a glutamate transporter decreased neuron death yet was not associated with more transporter mRNA, or if increased levels of transporter protein were localized to lysosomes. The initial paper characterizing each transgene often documents that levels of mRNAs and protein do indeed increase (e.g., reference 42) but rarely documents appropriate localization.

On the next most proximal level, the transgene's protein must function logically. Thus, overexpression of a glutamate transporter should enhance glutamate transport. Along these lines, it has been shown that glucose-transporter overexpression enhances glucose transport,<sup>42</sup> and that calbindin overexpres-

**Table 5** Outcomes of gene therapy studies overexpressing IL-1 receptor antagonist

Insult	Brain region	Strongest effect presented	Reference
Excitotoxic seizures	Hippocampus	>Doubling of volume of viable tissue	30
	Striatum	>Doubling of volume of viable tissue	30
Infarct	Cortex/basal ganglia	>50% Reduction in lesion volume	31, 32

Excitotoxic damage was induced with NMDA. Infarct was accomplished with permanent middle cerebral artery occlusion. The gene was delivered with an adenovirus via intracerebroventricular infusion in all cases. Studies were carried out with rats or mice. In all cases, the magnitude of protection was significant.

**Table 6** Studies exploring the postinsult time window of opportunity of intervention

Timepoints examined	Brain region/insult	Reference
Glucose transporter overexpression		
Protects when vector delivered 1 hour postinsult, but not 4 hours	Hippocampus/antimetabolite	10
Protects at 1 hour postinsult, but not 4 hours	Hippocampus/excitotoxin	9
No protection 6 hours postinsult	Hippocampal cultures/excitotoxin	McLaughlin et al., submitted for publication, 1999
Calbindin D28K overexpression		
Protects 30 minutes postinsult	Hippocampal cultures/hypoglycemia	16
Bcl-2 overexpression		
Protects 30 minutes postinsult, but not 4 hours	Striatum/MCAO	54
Protects 6 hours postinsult	Hippocampal cultures/excitotoxin	McLaughlin et al., submitted for publication, 1999
Protects 5–8 hours postinsult, but not 12–18	Cortical cultures/excitotoxin	25

“Antimetabolite” = 3-acetylpyridine, an electron transport uncoupler; MCAO = transient middle cerebral artery occlusion. Excitotoxins included glutamate and kainic acid.

sion decreases free cytosolic calcium.<sup>13,16</sup> Other studies often involve a transgene for which it is not clear what constitutes the most proximal measure of function (e.g., reference 27).

A few studies have examined subsequent downstream events leading to neuronal sparing. The most detailed analysis concerns the glucose transporter, showing that during an insult, its overexpression blunts the decline in adenosine triphosphate concentrations and metabolism, decreases glutamate release and cytosolic calcium concentrations, and decreases ROS accumulation (reference 6; McLaughlin et al., submitted for publication, 1999; Gupta et al., submitted for publication). Studies examining the downstream consequences of Bcl-2 overexpression have produced evidence both for<sup>37,41</sup> and against (McLaughlin et al., submitted for publication, 1999) it having an antioxidant role.

It is important that intervening steps are filled in for other protective transgenes, linking the most primary cellular consequences with the ultimate endpoint of altered neuron death. In cases in which the pathway is well understood and linear, this functions as an internal control—something is amiss if an intervention concerning pathway A-B-C altered both A and C without changing B. In the case in which the pathway is poorly understood or multipronged, understanding the intervening steps mediating protection is heuristically valuable. For example, if in some insult model SOD overexpression decreases neuron death and oxidative damage to proteins but not to lipids, this would argue against a central role for lipid peroxidation in such neuron death. Finally, delineating the consequences of overexpression of a protective transgene might reveal novel forms of regulation. For example, overexpression of SOD, per se, may not protect unless accompanied by overexpression of a downstream antioxidant (e.g., catalase). In transgenic SOD rodents, there is upregulation of en-

dogenous catalase levels.<sup>43</sup> Thus, it would be useful to know whether the transient adenovirus-driven overexpression of SOD, protective against ischemia,<sup>15</sup> also is accompanied by upregulation of a downstream enzyme.

*Potential for unexpected adverse effects.* Amid the positive results to date are some negative findings, such as failure of heat-shock protein 72 overexpression to protect against some in vitro insults, or the failure of calbindin to protect against cyanide in vitro. These negative results may arise from a threshold problem, in which expression is sufficient to protect against only some insults, or a compartmentalization problem, in which expression is localized within neurons in a way to protect against only certain insults. Within that framework, such negative findings are temporary, awaiting the next generation vectors with stronger expression, or engineered with greater insights about protein trafficking within the cell.

In contrast, there are also likely to be adverse consequences of gene therapy because of manipulation of something that, in effect, should not be manipulated. One possibility concerns the numerous feedback loops of end-product inhibition in neurons. Thus, for example, overexpression of an exogenous heat-shock protein might exert a sufficient feedback signal as to cause an overcompensatory downregulation of expression of the endogenous heat-shock protein, the result being lower total heat-shock protein levels.

This issue may manifest itself on the subcellular level of compartmentalized local feedback loops. For example, the mobilization of cytosolic calcium during a necrotic insult triggers a number of feedback loops that decrease subsequent calcium influx.<sup>44,45</sup> Heavily localized overexpression of calbindin, for example, could sufficiently disrupt these feedback loops as to result in a greater cumulative calcium load. Such a

mechanism was thought to explain the report of calbindin overexpression worsening glutamatergic death in cultured hippocampal neurons.<sup>46</sup>

As another complication, a transgene may have an adverse consequence because of an unanticipated secondary function. For example, it might initially appear desirable to overexpress the glutamate transporter, with the goal of enhancing reuptake of synaptic glutamate. However, the collapsing sodium gradient during necrotic insults often causes these pumps to reverse direction, thereby extruding cytosolic glutamate.<sup>47,48</sup> As such, an abundance of such transporters would exacerbate this effect.

Finally, some transgenes may disrupt neuronal function in the absence of an ongoing necrotic insult. For example, calbindin overexpression disrupts hippocampal plasticity and blocks post-tetanic potentiation.<sup>49</sup> Thus, the protective effects of a transgene might be outweighed by the adverse actions before or after an insult.

*Potential for clinical applications.* One goal of the studies reviewed is to use viral vectors as tools for gaining heuristic insight into the mechanisms of neuron death. The other is to ultimately use some version of a vector system therapeutically in a clinical setting. A few issues have moved the field in the direction of fulfilling that second goal.

First, expression driven by many types of viral vectors (especially using herpes simplex virus), declines precipitously within days, and viral DNA itself is eliminated soon after (Tsai et al., submitted for publication, 1999). This is a drawback for contemplating therapy for chronic neurodegenerative disorders. This time course, however, is sufficient to guarantee expression during the few days after a necrotic insult (during which delayed neuron death occurs), and such transient expression decreases the chances of any adverse effects of virally derived DNA.

Second, reporter-gene expression has been demonstrated in the nonhuman primate brain with a herpes simplex-1 vector<sup>50</sup> and in human hippocampal slices with adenoviral and adenoassociated viral vectors delivered in situ.<sup>51,52</sup> Furthermore, gene therapy with retroviral vectors against malignant brain tumors has been successfully performed in humans.<sup>53</sup>

Finally, protective genes can be introduced up to a few hours after the onset of a necrotic insult and still protect (table 6). (In contrast, studies in tables 1–5 involved introduction of the transgene either prior to or at the time of the insult). This postinsult window of opportunity suggests that such interventions ultimately might be efficacious, even given the typical clinical constraint of not being able to predict the occurrence of a necrotic insult. The examples in table 6 are too few to provide any patterns, but there should be differences in the duration of the window, depending on the transgene delivered: protection from overexpression of a potassium channel meant to hyperpolarize a neuron during a seizure, for example, is likely to be have a shorter window than is protection from overexpression of a neurotrophin

meant to enhance elaboration of processes for weeks postinsult.

Despite these salutary findings, impediments exist to clinical application of viral vectors for necrotic insults in the near future. These have been the focus of recent reviews<sup>55-59</sup> and are discussed briefly here.

One problem is the route of delivery of vectors. Most studies cited utilized local intracerebral stereotaxic injections of vector, which is of limited clinical applicability and results in only localized expression. Vector has been delivered intraventricularly in some studies. The resulting expression is limited to ependyma, apparently a route that results in considerable extracellular secretion and diffusion of the protein. This is useful for overexpressing proteins that exert their effect extracellularly (e.g., IL-1 receptor antagonist). This route, however, is unlikely to be effective for delivering genes with intracellular mechanisms of action. Finally, although other routes of delivery exist (e.g., nasal instillation),<sup>60</sup> the magnitude of delivery is small.

Another issue is the safety of vectors. Herpes simplex virus vectors can be cytopathic; this can be reduced by using highly purified preparations<sup>61</sup> or helper virus-free systems.<sup>62</sup> Adenoviral vectors can cause considerable inflammation; this can be reduced by careful purification of preparations, encapsulating virus in copolymers to reduce antigenicity, using newer adenoviral vectors that express fewer viral genes, or suppressing host immune responses.<sup>55,63-65</sup> Nonetheless, more progress is needed to ensure greater confidence before these vectors are used clinically.

A major limitation in viral vector systems is that, regardless of means of delivery, few neurons are infected. In some cases, if the gene introduced produces a protein that is secreted, there is the potential for its diffusion over great distances (see reference 31, for example). For proteins whose functions remain intracellular, however, this minimizes the number of cells altered by a gene-transfer intervention. As a result, few of the studies cited reported a decrease in total infarct size, or total number of neurons killed. Instead, end points are derived from studying the small subregion of damage in which there was a high concentration of infected neurons. For example, in one report of protection against ischemic injury, vector was injected at the edge of the infarct, and "protection" consisted of a significant increase in the width of the strip of healthy tissue from the edge of the infarct to the midline of the brain.<sup>26</sup> In other studies (e.g., reference 9), protection consists of increased numbers of transgene-positive surviving neurons on the injured side, relative to the number of positive neurons on the uninjured, contralateral control side. In these studies, the absolute number of neurons spared were too few to alter overall infarct size. (It should be noted that a decrease in the overall size of a lesion or in the total number of dead neurons has been reported in some studies.<sup>30-32</sup>)

A final issue is whether a neuron, if saved from death because of the protective effects of some trans-

gene, actually functions appropriately afterward. Viral vector gene therapy that spared neurons from a chronic degenerative insult (a rodent model of PD) spared function as well.<sup>67,68</sup> To our knowledge, only one study has examined issues of sparing of function following gene-therapy approaches for a necrotic insult. In this study, we established conditions under which overexpression of either the glucose transporter or of Bcl-2 were equally protective, decreasing excitotoxicity to equivalent extents in hippocampal cultures and the hippocampus. We then found that the two routes of protection involved different intermediary steps. Glucose-transporter overexpression constituted an early intervention (saving only up to 1 hour postinsult), maintained neuronal metabolism longer during the insult, and decreased ROS accumulation. In contrast, Bcl-2 overexpression constituted a late intervention (with a time window of saving postinsult of at least 6 hours) and had no sparing effect on metabolism or ROS accumulation. In addition, the glucose-transporter intervention, but not the Bcl-2 intervention, spared function, in that if lesion size was halved with either therapy, only the glucose transporter group was spared spatial maze learning deficits (McLaughlin et al., submitted for publication, 1999).

As gene therapists have considered the cascade of degeneration in the figure, the logical tendency has been to block the "final common pathway" of damage, the point of convergence among various insults. This would offer the advantage of one intervention being efficacious against numerous insults and afford the longest possible therapeutic window of opportunity postinsult. But Bcl-2 overexpression, constituting an intervention nearer the final common pathway than does glucose-transporter overexpression, may be acting too late in the cascade of damage to prevent dysfunction.

Based on this single study, we speculate that gene therapy interventions at early stages of the degenerative cascades of the figure, although perhaps not saving the largest number of neurons, are the most likely to spare function in those neurons that are saved. As such, the point optimizing decreased neuron loss and decreased neuronal dysfunction might come relatively soon after the onset of an insult. This reinforces the notion derived from clinical trials using thrombolytic agents for acute stroke that early intervention (within 3–6 hours) is essential for reperfusion strategies to successfully salvage threatened cerebral tissue.<sup>69-72</sup> If true, this would increase the demands to develop gene-therapy techniques that alter the earliest events following an insult.

**Conclusions.** Progress is needed in three areas. The first is improvement in vector delivery, so that broader areas of tissue can be infected. This is critical if there is to be a transition from using gene transfer to gain insight into the mechanisms of neuron death in artificial microcosms of injured tissue, to actual therapy.

A second area needing improvement is in understanding the viral vectors themselves, as discussed in numerous recent reviews.<sup>55-59</sup> This would include:

- Further improvements in the means of delivery so as to minimize inflammation and cytopathicity
- Improving the duration, strength, and efficiency of expression
- Developing inducible vectors that are turned on and off,<sup>72-74</sup> allowing for greater temporal control of expression, including vectors activated by insults themselves (eliminating the requirement for an exogenous manipulation at the time of the insult)<sup>75</sup>
- Developing vectors in which an array of transgenes can be delivered

Finally, there also must be progress in understanding which genes should be delivered therapeutically; this will emerge from more knowledge about the basic biology of neuron death.

A final point often raised is whether gene-therapy strategies, although elegant, are often superfluous. For example, why overexpress the glucose transporter instead of merely increasing circulating glucose concentrations? There are a number of responses to this issue:

- Gene-transfer approaches often would be complements, rather than substitutes for traditional therapies.
- Insult-inducible gene expression likely is more rapid than the effects of many traditional interventions.
- Gene-transfer approaches allow for altering intracellular levels of proteins, something rarely possible with systemic administration of a protective protein.
- The localized expression of gene-therapy approaches circumvents side effects of diffuse systemic delivery of a protein.

Given the speed with which findings have emerged in this area in the past half decade, one can be cautiously optimistic that gene transfer approaches will continue to be a tool for understanding the biology of neuron death and perhaps even will become an addition to the arsenal of therapeutic responses to necrotic neuronal injury.

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