Immediate recovery from spinal cord injury through molecular repair of nerve membranes with polyethylene glycol

RICHARD B. BORGENS1 AND RIYI SHI

Center for Paralysis Research, Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, Indiana 47907, USA

ABSTRACT A brief application of the hydrophilic polymer polyethylene glycol (PEG) swiftly repairs nerve membrane damage associated with severe spinal cord injury in adult guinea pigs. A 2 min application of PEG to a standardized compression injury to the cord immediately reversed the loss of nerve impulse conduction through the injury in all treated animals while nerve impulse conduction remained absent in all sham-treated guinea pigs. Physiological recovery was associated with a significant recovery of a quantifiable spinal cord dependent behavior in only PEG-treated animals. The application of PEG could be delayed for ~8 h without adversely affecting physiological and behavioral recovery which continued to improve for up to 1 month after PEG treatment.—Borgens, R. B., Shi, R. Immediate recovery from spinal cord injury through molecular repair of nerve membranes with polyethylene glycol. FASEB J. 14, 27–35 (2000)

Key Words: nerve fusion • nerve repair • neurotrauma

The devastating effects of injury to the mammalian spinal cord are not immediate. Severe mechanical injury initiates a delayed destruction of spinal cord tissue, producing a loss in nerve impulse conduction associated with a progressive local dissolution of nerve fibers (axons) (1, 2). This loss of sensory and motor communication across the injury site can produce a permanent paralysis and loss of sensation in regions below the level of the spinal injury. Furthermore, it is clear the most damaging aspect of progressive ‘secondary injury’ (3) of spinal cord parenchyma relative to the loss of behavioral functioning is its effect on white matter. Localized mechanical, biochemical, and anoxic/ischemic injury to white matter may be sufficient to cause the failure of axolemmas to function as a barrier or fence to the unregulated exchange of ions (1). This in turn compromises both the structural integrity of this region of the nerve fiber and its ability to conduct impulses along the cable. For example, elevated intracellular Ca2+ induces depolymerization of microtubules and microfilaments, producing a focal destruction of the cytoskeleton (2, 4, 5).

When K+ rushes down its electrochemical gradient out of the cell, the resultant elevated extracellular concentration contributes to localized conduction block (1, 6).

It would be of particular importance to interrupt this progression of events after acute injury to the spinal cord. This could theoretically be accomplished by immediately repairing or sealing regions of compromised membrane with hydrophilic polymers or surfactants, which could retard or reverse the permeabilization of nerve fiber membranes. This in turn might both rescue these nerve fibers from further degeneration and restore variable levels of physiological and behavioral function.

In recent years the administration of several types of polymers and surfactants to injured cells has been shown to seal or repair their membranes, reversing the progressive permeabilization produced by the insult. For example, application of the molecular surfactant Poloxamine 1107 markedly reduced the leakage of hemoglobin from erythrocytes damaged by radiation (7), whereas Poloxamer 188 facilitated the functional recovery of fibroblasts exposed to potentially fatal heat shock (8). Membrane fusogens such as polyethylene glycol (PEG) are well known to fuse the membranes of numerous cells in culture, producing giant multinucleated ones (9–12). This class of large hydrophilic molecules can likewise reverse the permeabilization of ruptured cell membranes as well as anatomically reconnect their severed processes.

Using a new in vitro isolation and recording chamber (6, 13, 14), we have demonstrated that a local application of an aqueous solution of PEG can functionally and anatomically reconnect completely severed strips of guinea pig spinal cord ventral white matter. This treatment produced axonal fusion associ...
associated with the immediate recovery of compound action potential (CAP) propagation through the original plane of transection (14). In addition, PEG can rapidly restore physiological functioning in 100% of severely crushed spinal cords similarly monitored in isolation (15). Since most clinical spinal cord injuries are compressive/contusive injuries (1), we wanted to know whether the striking in vitro response to PEG could be duplicated in an in vivo spinal cord injury model.

Here we show that in adult guinea pigs possessing severe and standardized spinal cord compression, CAP propagation through the lesion can be restored within minutes after a brief application of PEG. This physiological recovery was also associated with a behavioral recovery. To further explore the possible clinical significance of these data, we also delayed the application of PEG for ~8 h after spinal injury and again observed similar recoveries in the PEG-treated animals, but not in a sham-treated control group.

MATERIALS AND METHODS

Surgery and anesthesia

A total of 51 adult (300 g) guinea pigs were used in two separate experiments. Guinea pigs were anesthetized with an intramuscular injection of 100 mg/kg ketamine HCl and 20 mg/kg xylazine, and the spinal cord was exposed by dorsal laminectomy (16, 17). Subsequently, a constant-displacement 15 s compression of the spinal cord was performed using a modified forceps possessing a detente (18). In this experiment, the lesioning procedure had previously been calibrated to produce an immediate and total loss of CAP conduction through the injury and behavioral functioning of the cutaneous trunci muscle reflex (CTM; see below). For some somatosensory-evoked potential (SSEP) measurements or to sedate animals for behavioral testing and videotaping, guinea pigs were injected with 0.1 cc NaCl 124 mM, KCl 2 mM, KH2PO4 1.24 mM, MgSO4 1.3 mM, CaCl2 1.2 mM, dextrose 10 mM, NaHCO3 26 mM, sodium ascorbate 10 mM, and any excess PEG and/or Krebs’ solution was removed by aspiration. The wounds were closed and animals were kept warm until awaking with heat lamps. Guinea pigs were housed individually and fed ad libitum.

In the first experiment, we attempted to repeat the remarkable complete reversal of functional loss within minutes of severe spinal injury as observed in in vitro trials (14, 15). Thus, PEG was applied within ~15 min of spinal cord compression (experimental n = 14, control n = 11). In the second experiment, PEG application was delayed for ~8 h (experimental n = 11, control n = 11). The former groups were evaluated for ~4 days and the latter for ~1 month after PEG application. In both experiments, documentation of CTM behavior was combined with physiological recording.

An additional four PEG-treated animals were monitored for 1 day after injury, at which time their spinal cord was again exposed at the site of the original injury and crushed again at this location using the same technique as reported above.

Behavioral analysis of the CTM reflex

CTM behavior is observed as a corrugated rippling of back skin in response to light tactile stimulation (Fig. 1B). The behavior is dependent on afferent sensory projections organized as a long tract of axons in each ventral funiculus of the spinal cord, just lateral to the spinothalamic tract (19, 20). The reflex is bilaterally organized as segmental receptive fields, displays little supraspinal control, and usually is permanently lost after severe spinal injury, producing a bilateral region of areflexia below the level of the lesion (17, 19, 20) (Fig. 1A). In such cases, recovery of the CTM reflex in response to tactile or electrical stimulation within the region of areflexia usually is not observed for the life of the animal. The anatomy, physiology, and character of the CTM behavior, both normal and in response to lesioning, have been reported in both rat and guinea pigs (19, 20).

To visualize and quantify the CTM behavior, the shaved back skin of sedated guinea pigs was touched with a monofilament probe, producing contraction of the skin in uninjured or intact receptive fields (Fig. 1B). The boundary between responsive and unresponsive back skin was marked onto the back skin with a marker while the entire study period was videotaped from a platform-mounted camera above. Animals were arranged on a background grid to facilitate the registration of successive video images. Video images were acquired to an Intel Dual Pentium Pro computer. Superimposing of images, the coloring of receptive field boundaries made on the back skin of the animals during CTM testing, and the general management of video images was performed using Adobe Photoshop software. Final plates were constructed with Microsoft PowerPoint software and printed on an Epson Stylus Color 800 printer. Quantitative planimetry of the unit area of receptive fields, or regions of behavioral loss and recovery, was carried out using IP Lab Spectrum software.

Physiological recording of SSEPs

A pair of subdermal electrodes stimulated nerve impulses from the tibial nerve of the hind leg (stimuli trains in sets of 200 at 3 Hz; stimulus amplitude ≤ 3 mA square wave, 200 μs duration). Evoked volleys of CAPs were conducted into the spinal cord, projected to, and recorded from the sensory cortex of the brain. Recording of the nerve impulses at the brain used a pair of subdermal electrodes located above the level of the contralateral cortex, with reference electrodes located in the ipsilateral pinna of the ear. Stimulation, recording, signal averaging, and the computer management of this physiological data used a Nihon Kohden Neuropak 4 stimulator/recorder and PowerMac G3 computer.

Evoked CAPs are called SSEPs; such measurements were
carried out in every animal prior to spinal cord injury (Fig. 2).

In all animals (at any test period), the failure to record an SSEP after stimulation of the tibial nerve was further confirmed to be due to a lack of conduction through the spinal cord lesion by a control test carried out on the same animal. In this procedure, the medial nerve of the forelimb was stimulated, initiating evoked potentials in a neural circuit unaffected by the crush injury (Fig. 2A–C). To perform this test, recording electrodes were left in place while stimulating electrodes were relocated to stimulate the median nerve, using identical parameters of stimulation.

Statistics

The Mann Whitney two-tailed test was used to compare the means of the data derived from experimental and sham-treated groups. To compare the proportions between groups, Fishers exact test was used. All tests were performed using INSTAT software.

RESULTS

The standardized injury produced a similar loss of CTM functioning in experiments testing the response to the immediate application of PEG and experiments testing the response to the delayed application of PEG. The percent loss of CTM receptive fields (Fig. 1C) was not statistically different between either of the two experiments or between sham-treated and PEG-treated guinea pigs in either experiment ($P>0.4$, Student’s $t$ test, two-tailed).
Only one animal died during the course of this study.

**Behavioral loss and recovery of the CTM reflex**

In both experiments, 19 of the 22 sham-treated animals did not recover CTM functioning (Table 1 and Table 2). During the first experiment, CTM functioning actually worsened by day 4 in two control animals (the region of CTM loss increased by 2% and 15% respectively; Table 1). In contrast, CTM functioning recovered in 10 of 14 PEG-treated animals in the first experiment (~80%; Fig. 1D, Table 1) and in >90% of experimental animals in the second experiment. In all PEG-treated animals, the restored region of CTM competent back skin was observed within the first day after treatment and continued to increase in size with time (Tables 1 and 2). For example, the average unit area of back skin recovering CTM sensitivity nearly doubled from...
cases, this produced a characteristic SSEP for this medial nerve of the forelimb was stimulated. In all procedure carried out on the same animal, where the tibial nerve was further confirmed to be due to a lack failure to record an SSEP after stimulation of the delayed application of PEG. In all animals, the meantime, subsequent measurements were made 4 days after PEG treatment. In the second experi-
ment, subsequent records were taken at 6–8 min,1h,24h, and 4 days after PEG treatment. In the second experiment, subsequent measurements were made 6–8 h, 18–24 h, 3 days, 2 wk, and 1 month after the delayed application of PEG. In all animals, the failure to record an SSEP after stimulation of the tibial nerve was further confirmed to be due to a lack of conduction through the injury by a control procedure carried out on the same animal, where the medial nerve of the forelimb was stimulated. In all cases, this produced a characteristic SSEP for this spinal circuit unaffected by the injury (Fig. 2A–C; Fig. 3).

In this investigation, sham-treated animals never regained the ability to conduct SSEPs through the injury site.

In the first experiment, a detectable SSEP was recorded within a few minutes after PEG application. Quantitative evaluation of 10 of these animal’s electrical records showed that SSEP amplitudes continued to improve, averaging 12% (day 1) to 20% by 1 month after application in the second experiment (Table 2). The increased proportion of animals recovering CTM function and the average increase in the areas of recovered CTM competent back skin in response to PEG were both statistically significant (Tables 1 and 2).

**Physiological measurements of conduction through the spinal cord injury**

Physiological measurements of SSEP conduction were performed in every animal prior to spinal cord injury and within 5–15 min after surgery (Fig. 2B, C; Fig. 3) to provide a basis for later comparison. In the uninjured animal, SSEPs were typically observed to segregate into two peaks: early arriving (latency 20–30 ms) and a later arriving SSEP (35–45 ms; Fig. 2B, C; Fig. 3). In the first experiment, subsequent records were taken at 30 min, 1 h, 24 h, and 4 days after PEG treatment. In the second experiment, subsequent measurements were made 6–8 h, 18–24 h, 3 days, 2 wk, and 1 month after the delayed application of PEG. In all animals, the failure to record an SSEP after stimulation of the tibial nerve was further confirmed to be due to a lack of conduction through the injury by a control procedure carried out on the same animal, where the medial nerve of the forelimb was stimulated. In all cases, this produced a characteristic SSEP for this spinal circuit unaffected by the injury (Fig. 2A–C; Fig. 3).

In this investigation, sham-treated animals never regained the ability to conduct SSEPs through the injury site.

In the first experiment, a detectable SSEP was recorded within a few minutes after PEG application. Quantitative evaluation of 10 of these animal’s electrical records showed that SSEP amplitudes continued to improve, averaging 12% of their preinjury level and displaying more typical latencies with time (Fig. 2C, D). Remarkably, within minutes of the spinal injury, the total loss of physiological functioning was reversed in 23 of 25 PEG-treated animals. In the two animals that did not immediately respond to PEG application, SSEP recovery was later observed at the 2 wk time point (Fig. 3, Table 2). In the four animals whose recovered SSEPs were tested by reinjury, the second compression of the spinal cord at the original injury site completely eliminated recovered SSEPs, confirming these were conducted through the lesion.

In summary, all 34 PEG-treated animals recovered SSEP conduction in contrast to the complete failure of all control guinea pigs to conduct evoked potentials through the lesion. Only 3 of 22 sham-treated animals recovered CTM function in both experiments, whereas 20 of 25 PEG-treated animals recov-

<table>
<thead>
<tr>
<th>Table 1. % recovery of CTM, immediate PEG application&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal number</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PEG-treated</td>
</tr>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PEG-treated</td>
</tr>
</tbody>
</table>

<sup>a</sup>The number of animals recovering CTM function is shown before the total number in that group. The increase in the area of back skin regaining sensitivity to tactile stimulation is given as a percent of the total area of CTM behavioral loss. All unit areas in cm<sup>2</sup> were calculated by planimetry from captured video images. <sup>b</sup><i>x</i> = Mean % recovery of the CTM reflex and standard error of the mean. <sup>c</sup>The range of the control data set at 4 days includes the percent increase in the area of CTM loss, which is given as a negative number. <sup>d</sup>Statistical comparison of means not applicable to this data set.

<table>
<thead>
<tr>
<th>Table 2. % Recovery of CTM, delayed PEG application&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal number</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PEG-treated</td>
</tr>
<tr>
<td>Statistic</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>PEG-treated</td>
</tr>
<tr>
<td>Statistic</td>
</tr>
</tbody>
</table>

<sup>e</sup>The number of animals recovering CTM function is shown before the total number in that group. The increase in the area of back skin regaining sensitivity to tactile stimulation is given as a percent of the total area of CTM behavioral loss. All unit areas in cm<sup>2</sup> were calculated by planimetry from captured video images. <sup>f</sup><i>x</i> = Mean % recovery of the CTM reflex and standard error of the mean. <sup>g</sup>NA = Not applicable to this data set. <sup>h</sup>Statistical comparison of means not applicable to this data set. <sup>i</sup>P value: proportion of recovered and unrecovered animals evaluated with Fishers’ exact test, two-tailed.
In animals that had recovered excitability of CTM function, an electrical record obtained from one of the two animals that did not show such an early response is shown at 8 h. Recovered SSEPs measured at 2 wk and 1 month after PEG application are shown for this guinea pig. P1 and P2 as in Fig. 2, SA = stimulus artifact as in Fig. 2.

**DISCUSSION**

This report is the first to show that an immediate and brief application of a hydrophilic fusogen, polyethylene glycol, to the site of a severe compression injury to the adult guinea pig spinal cord results in an immediate recovery of nerve impulse conduction and a progressive recovery of behavioral functioning of the CTM reflex, a quantitative index of white matter integrity (17, 19, 21). An 8 h delay in this application still resulted in a similar recovery of these functions. In sharp contrast, sham-treated animals never recovered the ability to conduct nerve impulses, and the minor occurrence of spontaneous recovery of CTM function was rare compared to the PEG-treated group. We chose to compress the spinal cord since this is more typical of clinical injuries (1, 18).

It is likely that this severe constant displacement injury produced axotomy in an unknown proportion of nerve fibers. However, it is unlikely that the ability of PEG to fuse the proximal and distal segments of axons (14) was responsible for the recovery of physiological and behavioral function reported here. We have shown that reconnection of transected axons by PEG requires precise alignment and precise pressure applied to the carefully abutted proximal and distal segments (14). This was not possible or attempted in this study. Rather, we think the interaction of PEG with damaged axolemmas of crushed spinal cords led to an immediate sealing of breaches in these membranes, producing a reversal of permeabilization.

**Molecular repair of cell membranes**

Historically, PEG has been used to fuse many individual cells *in vitro* into one large multinucleated cell (9–12, 22). This technique was an early advancement allowing the transfer of genetic material between cells and the production of giant cells, such as neurons, to facilitate electrophysiological study of their membranes. Hence, the name ‘fusogen’ is sometimes applied to this class of water-soluble polymers that are able to produce membrane union and the intermingling of cytoplasm. PEG-mediated membrane fusion is still an important model for endogenous biomembrane and vesicular fusion though its mode of action is unclear (22, 23). It is believed that PEG induces a dehydration of closely approximate membranes, allowing their structural components to resolve into each other (22). After rehydration, complex molecular morphologies of the lamellae apparently spontaneously reassemble based in part on the polar forces associated with the aqueous phase of the membrane. Some have argued that the PEG-mediated fusion process must involve first rupture and then rescaling of the adjacent bilayers (24, 25) whereas others have opined that membrane rupture is not required for this process (26).

Our preliminary *in vitro* evaluation of the anatomy of axonal repair after mechanical compression (13) has revealed that a 2 min application of PEG produced sealing of membrane lesions at the site of a standardized compression. Sealing was indicated by the exclusion of horseradish peroxidase uptake by injured fibers in the PEG-treated group compared to sham-treated spinal cords (to be reported elsewhere). Such immediate repair of membrane breaches sufficient to inhibit the uptake of large molecular weight dyes should also arrest or reduce permeabilization allowing the nonspecific flux of ions across it. We think it is this ‘sealing’ behavior of PEG that both restores excitability and reverses anatomical dissolution of the nerve fiber. It may be that PEG functions as a detergent or sealant in a manner hypothesized for surfactants such as the poloxamers, polaxomers, and dextrans (27, 28). These agents may cover or be absorbed into regions of membrane damage where their hydrophobic tails extend into the adjacent aqueous domains of the membrane, somehow binding, closing, or covering discontinuities in the bilayer (29). Surfactant-mediated membrane sealing has
been shown to prevent myonecrosis and calcein leakage from damaged skeletal muscle cells after electrical trauma (8, 27, 28), facilitate recovery from lethal heat shock in fibroblasts (29), and reduce tissue damage in testicular ischemia-reperfusion injury (30). Even though the interaction of surfactants and large hydrophilic molecules like PEG with cell membranes may or may not share certain biophysical mechanisms of action, it is likely that their biological/medical importance is this shared ability to seal compromised cell membranes. Exploitation of this ability by using both types of agents offers a new way of thinking about the treatment of cell and tissue trauma.

**Trauma to nervous tissue as a function of trauma to the single cell**

It is well known that the anatomical consequences of compressive/contusive injuries are more severe days rather than hours after injury. This fact has led to the concept of 'secondary injury' and a proliferation of theories of how such delayed and progressive destruction of spinal cord tissue may occur (1, 3, 31). This pathophysiology is complex and incorporates biochemical alterations (such as free radical-mediated damage, endogenous neurotoxicity, and derangement of the ionic extracellular environments inside and outside cells), progressive ischemic injury, and cell-mediated damage—for example, 'bystander' damage caused by infiltration of healthy parenchyma by enormous numbers of macrophages (32, 33). Although these distinctions are important in understanding spinal cord injury, it may be more convenient and instructive to consider their consequences as end points in a final common pathway that begins with permeabilization of the membranes (2) of neurons principally, but not exclusively. For example, disruption of the axolemma may be so severe as to lead to axotomy, but less severe or 'nondisruptive' injury to the nerve fiber may also do so (4, 5). In both scenarios, unregulated entry of Ca$^{2+}$ into the cytosol appears to be the seminal event in the progressive dissolution of the axon. Some axons may survive intact but fail to conduct action potentials across the region of damage (31). Conduction block may result from focal demyelination, accompanied by a breakdown in the ion exchange/ion exclusion properties of normal membrane. Axotomy or conduction block both lead to a break in the conduction pathway and functional deficits. It is at this level where the action of fusogens and membrane sealants swiftly restrict or reduce ion leakage into or out of injured cells helping to restore ionic equilibrium, membrane excitability, and ultimately preserving crucial anatomies.

Since the destruction of spinal cord white matter is dynamic, delayed, progressive, and produced by different mechanisms of action, it is not surprising that a delay in PEG treatment still produced recoveries in physiological and behavioral function. The contribution to any functional and physiological deficit arising from mechanical compression of nervous tissue should be viewed as a balance between the irreversible loss of critical anatomies and endogenous mechanisms of repair such as spontaneous axonal sealing (34). It is likely that PEG may facilitate several of these repair processes and at different times after injury.

The immediate recovery of excitability implicates a sealing function of PEG, but it is not a perfect seal. We have reported that the amplitudes of CAPs restored by PEG treatment were nearly doubled by subsequent application of the fast potassium channel blocker 4-aminopyridine (15). Thus PEG repaired membranes probably remain somewhat leaky to potassium, which pushes their resting potential toward that of K$^+$ and limits CAP amplitudes. Recently we have developed a method to apply PEG to clinical cases of spinal injury in dogs (see below). We have noted that during the 2 min application of PEG to the exposed spinal cord, the minor seepage of blood from capillaries too small to be cauterized during the laminectomy procedure was immediately stopped (J. P. Toombs and R. B. Borgens, unpublished observations). We believe it possible that PEG may seal or fuse very small breaches in endothelium and that if this occurs within the contused cord, it may also contribute to a reduction in the progressive vascular insult accompanying acute severe compression.

**The CTM behavioral model and central nervous system (CNS) injury**

We do not use walking or other indirect measures of posture and limb movement in spinal rodents (35, 36) as a means to study the loss and recovery of behavior (37). This is due in part to the prevalence of locally controlled and generated patterned stepping in small animal models of spinal cord injury, the largely unknown character of the anatomical basis subserving these complicated behaviors, and the large numbers of animals required to credibly demonstrate differences between control and experimental treatments (37). We used a defined sensorimotor behavior, the CTM reflex, as a behavioral index of spinal cord white matter integrity (17, 19, 21, 37). In transected spinal cords, the CTM never spontaneously recovers for the life of the animal, whereas in severe compression injured cords spontaneous recovery is infrequent, as shown here. Moreover, propagation of nerve impulses through identified CTM spinal tracts within the ventral funiculus of...
the cord must be reestablished to the identified pools of motor neurons in order to restore CTM functioning. Finally, regions of CTM loss do not recover by collateral cutaneous sprouting since peripheral cutaneous innervation is undisturbed by the spinal injury (17, 19, 37).

This report provides clear evidence of a behavioral recovery dependent on an identified neural circuit within the damaged mammalian CNS in response to this experimental treatment. Together with our previous reports (13, 14), it proposes molecular repair and fusion of nerve membranes as a novel treatment of severe trauma to both peripheral nervous system and CNS tissue. Our continuing experiments will determine the critical window of time for PEG application, test PEG derivatives [such as PEG/4-aminopyridine (14) and PEG-complexed free radical scavengers] designed to improve PEG-mediated repair, and move clinical tests of PEG application to naturally produced cases of paraplegia in dogs (38, 39).

This work was supported by Center funds from the Center for Paralysis Research, Purdue University, and a generous gift from Mrs. Helen Skinner. We thank Debra Bohnert and Loren Moriarity for expert technical assistance and Carie Brackenbury for manuscript preparation.

REFERENCES


Received for publication May 14, 1999.
Revised for publication August 12, 1999.