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The Effect of Poloxamer-188 on Neuronal Cell Recovery from Mechanical Injury

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ABSTRACT

Neuronal injury resulting from mechanical deformation is poorly characterized at the cellular level. The immediate structural consequences of the mechanical loading lead to a variety of inter- and intra-cellular signaling events that interact on multiple time and length scales. Thus, it is often difficult to establish cause-and-effect relationships such that appropriate treatment strategies can be devised. In this report, we showed that treating mechanically injured neuronal cells with an agent that promotes the resealing of disrupted plasma membranes rescues them from death at 24 h post-injury. A new *in vitro* model was developed to allow uniform mechanical loading conditions with precisely controlled magnitude and onset rate of loading. Injury severity increased monotonically with increasing peak shear stress and was strongly dependent on the rate of loading as assessed with the MTT cell viability assay, 24 h post-injury. Mechanical injury produced an immediate disruption of membrane integrity as indicated by a rapid and transient release of LDH. For the most severe injury, cell viability decreased approximately 40% with mechanical trauma compared to sham controls. Treatment of cells with Poloxamer 188 at 15 min post-injury restored long-term viability to control values. These data establish membrane integrity as a novel therapeutic target in the treatment of neuronal injury.

Key words: membrane damage; neuronal cells; Poloxamer; shear stress; traumatic brain injury

INTRODUCTION

Traumatic brain injury (TBI) is one of the leading causes of death and disability in the United States. Each year more than 2 million individuals are affected by TBI (Davis, 2000). The high occurrence and huge cost to the society of TBI (Max et al., 1991; Lehmkuhl et al., 1993; Haffey and Abrams, 1991) has urged the development of various experimental approaches, including both *in vivo* and *in vitro* models, to better understand the mechanisms leading to cell and tissue dysfunction and death.

TBI is multifaceted process. In the aftermath of the initial traumatic event, there is a sequential activation of many cellular and molecular events which can lead to delayed cell damage and death (McIntosh et al., 1998; Graham et al., 1989). This process of secondary degeneration has received a great deal of attention, and many potential therapeutic targets have been identified. However, despite promising *in vitro* and animal studies, clinical trials have been disappointing, especially with respect to long-term recovery from the injury (Faden, 2002; Kermer et al., 1999; Lee et al., 1994; McIntosh et al., 1998; Royo et al., 2003; Verma, 2000).

The immediate structural consequences of the me-

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chanical deformation of neurons during TBI remain poorly characterized, and the mechanisms by which the mechanical stimulus gives rise to both acute biochemical responses and delayed pathology are not known. The loss in cell membrane integrity and plasma membrane modifications, including membrane blebbing and altered permeability, have recently been found to be the major contributors to the development of neuronal damage subsequent to traumatic injury by leading to ionic imbalances and activation of several cellular pathways (Geddes et al., 2003; LaPlaca et al., 1997; LaPlaca and Thibault, 1998; Obrenovitch and Urenjak, 1997; Pettus et al., 1994). In this paper, we describe the development of an *in vitro* model to study the relationship between mechanical loading parameters and injury severity and to elucidate the role of acute membrane damage in subsequent pathology.

To relate cell injury to mechanical trauma, an *in vitro* model in which a precisely controlled uniform stimulus can be applied to a population of cells is required. Most previous *in vitro* models of TBI (Regan and Choi, 1994; Mukhin et al., 1997; Shepard et al., 1991; Murphy and Horrocks, 1993; Lucas and Wolf, 199; Cargill and Thibault, 1996; Ellis et al., 1995; LaPlaca and Thibault, 1997) have some limitations. The most severe limitations of these models are the non-uniformity of the applied mechanical stimuli and the difficulty in controlling strain and strain rate independently.

Previous studies have shown that cell injury depends on both the magnitude and rate of the applied load (Blackman et al., 2000; Cargill et al., 1996; Geddes et al., 2003; LaPlaca and Thibault, 1997). To investigate the mechanism of loading rate dependent responses in cultured cells, we used the controlled cell shearing device (CCSD) described by Blackman et al. (2000), which has been used previously to demonstrate loading-rate dependent injury of the plasma membrane of endothelial cells. This device (Fig. 1) produces a uniform shear stress with precise and independent control of the magnitude and onset rate of the applied stress. The major advantages of this system over existing *in vitro* injury systems to model and study injury is that the magnitude and rate of the mechanical stimulus can be precisely controlled, and the cells can be monitored easily before and after the injury. In addition, the mechanical stimulus is uniformly applied to the entire population of cells.

In the present study, a neuronal cell line, PC2 cells, established by Pittman and colleagues (1993) was used. PC2 cells are a subline derived from pheochromocytoma cell line, PC12 cells, that is highly responsive to nerve growth factor (NGF) (Greene and Tishler, 1976; Levi, 1991), and once differentiated, they resemble sympathetic neurons (Fig. 2B). Controlled impulses of fluid shear stress were applied to these cells using the CCSD. The magnitude and rate dependence of injury severity was assessed in terms of cell viability 24 h post-injury. The effect of mechanical trauma on cell morphology and membrane integrity was also evaluated.

Since initial membrane damage is one of the primary pathophysiological changes occurring in mechanically injured neuronal cells, we used a treatment strategy that

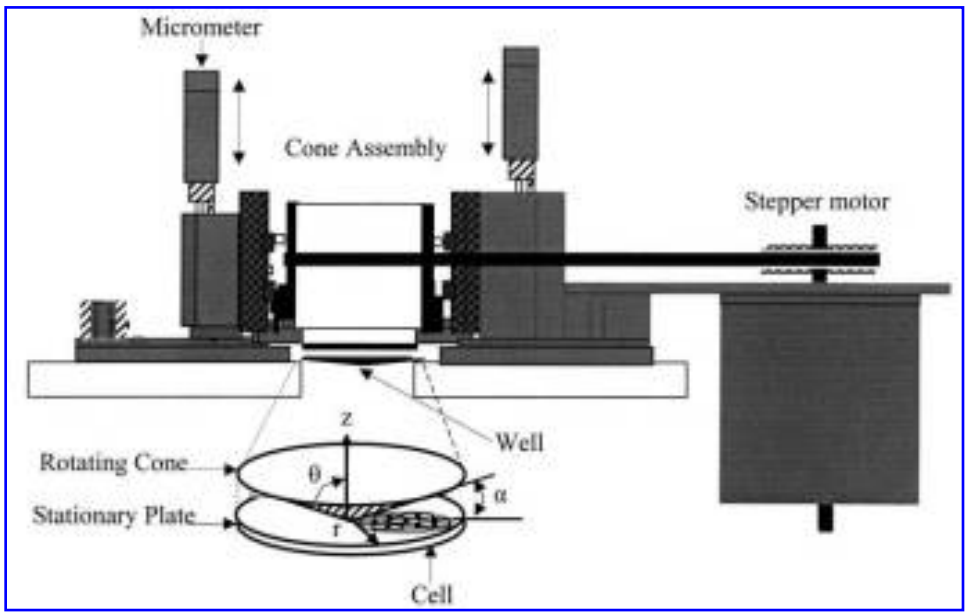

FIG. 1. A schematic representation of the CCSD device (Blackman et al., 2000). It shows the dynamic two-degree of freedom cone assembly, *z*-axis translation via dual micrometers, and rotation via stepper motor.

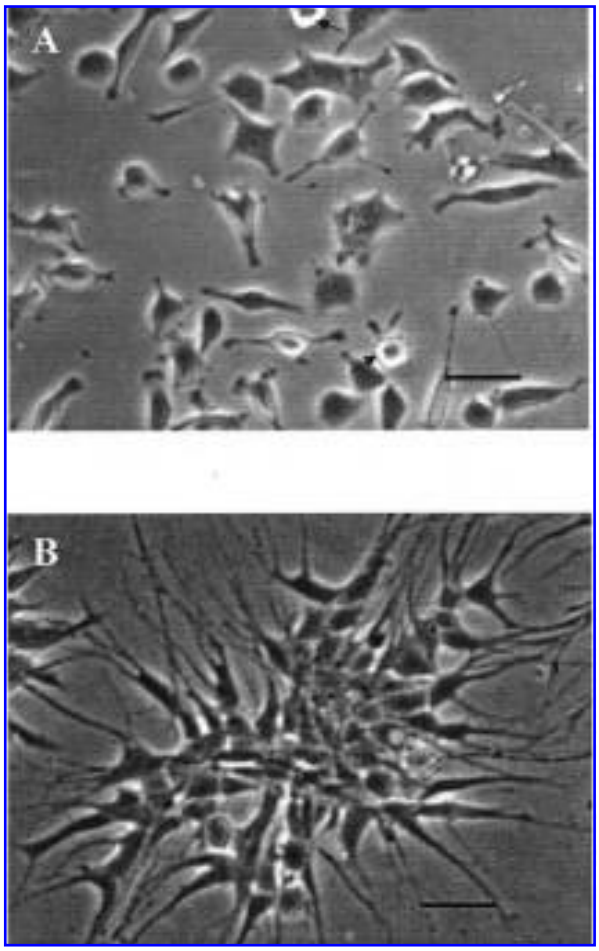

FIG. 2. Phase-contrast images of both undifferentiated PC2 cells with no NGF (**A**) and differentiated PC2 cells with NGF (50 ng/mL) (**B**) seeded on poly-D-Lysine and Matrigel. Undifferentiated cells have extended very short neurites at 7th day of seeding. Arrows, in A, indicates that the cells were still proliferating (cells with round cell bodies) and not differentiated. Differentiated cells (B) have grown in small aggregates, and they have formed small neuronal nets at 7th day of seeding (Pittman et al., 1993; Das et al., 2004), while undifferentiated cells (A) have very small processes and intercellular spaces. Bar = 55 μm.

targeted the recovery of damaged cell membrane after traumatic injury. Poloxamer 188 (P188) is a water-soluble, non-ionic surfactant with tri-block copolymer (Fig. 3). Exposing electroporated cells to this surfactant effectively seals the damaged membranes; thereby, acutely arresting the leakage of intracellular components *in vivo* and *in vitro* (Hanning et al., 2000; Lee et al., 1992, 1994; Merchant et al., 1998; Terry et al., 1999; Borgens et al., 2004). Recently, Marks et al. (2001) reported that P188 is effective in restoring the integrity of injured membranes of electroporated neuronal cells. Since the mechanisms of P188 action are specifically directed at the plasma membrane, the use of this surfactant may provide an alternative approach to treat both acute and delayed post-traumatic injuries in neuronal cells.

FIG. 3. The molecular structure of Poloxamer 188 (P188). It has an amphiphilic, tri-block copolymer structure. This water-soluble surfactant contains a central hydrophobic block of polypropylene oxide (29 PPO) moieties and two peripheral hydrophilic blocks of polyethylene oxide (38 PEO) moieties.

MATERIALS AND METHODS

Cell Culture

PC2 cells (provided by Dr. R. Pittman, Department of Pharmacology, University of Pennsylvania Medical School, PA) were cultured according to the method of Greene and Tishler (1976). Briefly, cells were plated onto both 6-well tissue culture plates (experimental cultures) and Petri dishes (stock culture) in RPMI 1640 (Fisher, Chicago, IL) medium supplemented with heat-inactivated horse serum (10%), fetal bovine serum (5%), penicillin (50 IU/mL) and streptomycin (50 μg/mL), and L-glutamine (2 mM) (Fisher, Chicago, IL). Stock and experimental cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Stock cultures were maintained in petri dishes coated with rat-tail collagen (Biomedical Technologies, Stoughton, MA). For experiments, cells were plated at a density of 2300 cell/cm^2 on thermanox plastic coverslips (Fisher, Atlanta, GA) that were coated with either cell-TAK™ (BD Biosciences, MA) or with 4 μg/cm^2 poly-D-Lysine and 0.20 mg/cm^2 of Matrigel® Basement Membrane Matrix (Becton Dickinson, Bedford, MA). Experimental cells were terminally differentiated by addition of 2.5S NGF (50 ng/mL) (Becton Dickinson, Bedford, MA) in serum-free medium for 7 days. Over the course of 7 days, PC2 cells differentiate into cells with a neural-like morphology (Fig. 2B). However, a small percentage of PC2 cells continued to proliferate even after differentiation with NGF addition as shown by Mills et al. (1997).

Injury Protocol

PC 2 cells cultured on coverslips were subjected to shear stress by using the controlled cell shearing device (CCSD) (Blackman et al., 2000) on the 7th day after plating. Prior to experimentation, the cells were rinsed with

1 × Eagle's Balanced Salt Solution (EBSS) (Life Technologies, Inc., Rockville, MD) two times to remove any unattached cells. A mechanical loading profile was developed with the goal of producing cellular injury with similar characteristics to that observed in the *in vivo* studies of Pettus, et al. (1994); specifically, the increased membrane permeability to macromolecules found in moderate to severe injury. Based on the previously demonstrated loading-rate dependence of cell injury, we varied loading rate over a wide range. This was achieved by rapidly increasing the applied shear stress from zero to variable peak value. In preliminary studies, using the trapezoidal shear stress profile used previously to injure endothelial cells (Blackman et al., 2000), we found that high and sustained levels of shear stress caused significant detachment of cells during loading. Therefore, following the initial rapid increase in shear stress to its peak value, it was immediately reduced to a lower steady value (Fig. 4). The rationale for maintaining the lower steady level of shear stress following the initial impulse comes from electroporation studies of Zhelev and Needham (1993), which showed that membrane tension could stabilize membrane pores. Again, the goal of this model was to produce the membrane permeability change observed *in vivo*. Following the injury, cells were incubated at 37°C for 24 h. The two types of controls were used at each time point: untouched controls, which were kept in incubator after their medium was changed under cell culture hood, and sham (uninjured cells) controls, which were handled as injured ones except for the application of the mechanical loading. Based on the results obtained from magnitude and rate dependence studies, the combination of 100 dyn/cm^2 peak shear stress and 20 dyn/cm^2 of steady shear stress for 200 msec was used for all studies with P188 treatment.

Assessment of Cell Viability

Trypan-Blue Staining (Sigma, St. Louis, MO) was used to determine the number of dead cells compared to live cells in both injured and control samples as previously described (Colowick and Kaplan, 1963). After 24-h incubation following the injury, dead cells were identified due to dye intake.

Additionally, the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Sigma) was used to quantify cell viability. MTT dye is reduced to form a dark blue formazan dye by dehydrogenases present in only metabolically active cell mitochondria. The assay was performed as described before by Mosmann et al. (1983) with some modifications. Briefly, at 24 h post-injury, cells were incubated with 1 mg/mL of MTT at 37°C for 3 h. Then, 1 mL of extraction buffer (20% SDS, 50% DMF (Sigma) was added into each well to dissolve formazan crystals as described by Hansen et al. (1989). After the overnight incubation with extraction buffer at 37°C, the cell solutions were used to measure absorbance at a test wavelength of 570 nm and a reference wavelength (background) of 630 nm.

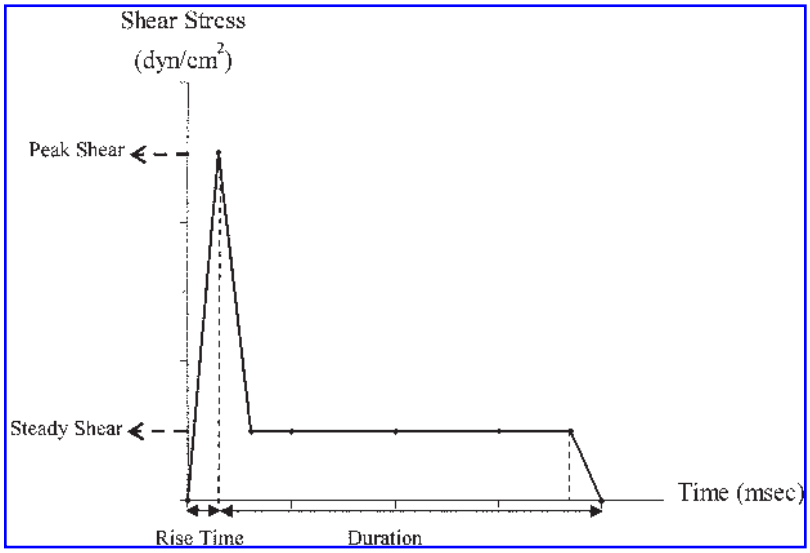

FIG. 4. Schematic representation of shear stress profile used as a mechanical input to injure the cells. A rapid increase to the peak shear stress (15-msec rise time) was followed by a lower, steady shear stress which was applied for 185 msec. Total loading duration was 200 msec.

Assessment of Membrane Integrity

Lactate dehydrogenase (LDH) release into extracellular space was assayed as a measure of membrane integrity, using commercially available CytoTox-ONE™ kit (Promega, Madison, WI) as described by manufacturer. The extracellular medium was sampled (100 μL) prior to mechanical stimulus, right after the mechanical stimulus, within 15 min, 1 h, 3 h, 6 h, and 24 h post-stimulus. For each time point, sampling of extracellular bath was made three times and transferred into 96-well tissue culture plates (Fisher, Chicago, IL). At each time point, control cells were lysed to get the total amount of LDH present. Sham controls were sampled in the same way as injured cells. Following the incubation with the reagent, each reaction was stopped with an addition of 50 μL of Stop Solution provided with the kit to prevent the further generation of fluorescent product. Values were normalized to volume, and the LDH release rate was calculated according to the difference in cumulative LDH release between time points divided by the time interval. LDH release was assessed using a CytoFluorometer with an excitation wavelength of 570 nm and an emission wavelength of 590 nm.

Application of Poloxamer-188 (P188) and Dose Dependency Analysis

To test dose dependency of the P188 induced neuroprotection, three different concentrations of P188 were applied to mechanically injured cells: low (30 μM), medium (100 μM), and high (1000 μM). The applied doses of P188 were all below the critical micelle concentration (CMC = 6.3 mM at 37°C). Above their CMC, surfactants self aggregate to form micelles causing the (active) surfactant monomer concentration to remain constant independent of the total surfactant concentration (Kabanov et al., 1995). First, cells were mechanically loaded, and P188 was added into the cell medium within 10–15 min following the injury, gently agitating the medium to evenly distribute the applied surfactant. Sham controls (with and without P188 treatment) and untreated injured cells were treated identically including the gentle agitation. Cells were incubated for 24 h before assessing viability with the MTT assay as described above.

Assessment of Cell Death Mechanism

To test the occurrence of apoptosis in injured cells at 24 h, PC2 cells were stained with fluorescently conjugated Annexin-V (Vybrant™ Apoptosis kit, Molecular Probes, Eugene, OR) according to manufacturer's instructions. Briefly, cells were washed with ice-cold 1 × EBSS and fixed with 4% paraformaldehyde. After cells were labeled with Annexin-V, they were mounted and observed with Fluorescent microscope. Annexin-V binds to phosphatidylserine which translocates to the outer leaflet of the plasma membrane in cells undergoing apoptosis.

Statistical Analysis

The data were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's test to determine significance of data between groups. A p value of <0.05 was considered as significant.

RESULTS

Injury Dose Dependence and Cell Viability following the Shear Injury

Our previous studies showed that maintaining the high peak velocity for too long (more than 30 msec) resulted in widespread cell detachment from the substratum during the mechanical loading (data not shown). Also, the use of different cell attachment proteins (collagen, cell-TAK™, and poly-D-Lysine with Matrigel®) for cell attachment to substratum demonstrated that the proper attachment of the cells was a critical factor to create high mechanical injury on the cells with fluid shear stress. In the initial experiments, cell-TAK™ was used to attach the cells to surface. Using this attachment factor, the most severe injury without significant detachment of cells resulted in approximately 20% loss of viability at 24 h. Then, we started to use poly-D-Lysine and Matrigel,® which were previously proved to be not only effective for strong attachment of PC12 cells to the surface but also to be effective for cell differentiation (Keshmirian et al., 1999). This surface treatment allowed us to achieve higher injury severity without significant detachment of cells.

Various shear stress magnitudes and loading rates were tested to optimize mechanical injury of cells. The severity of cell injury for different loading rates was quantified using trypan blue cell viability assays with cells cultured on cell-TAK. Using various rise times while maintaining same peak shear stress (30 dyn/cm^2) produced a range of onset rates. The decrease in viability was strongly dependent on the loading rate (Fig. 5). The most severe injury was a 19.2 ± 3.02% loss of viability produced at an onset rate of 1000 dyn/cm^2 · sec. Higher onset rates and magnitudes of peak shear stress caused significance detachment of cells from the cell-TAK substrate.

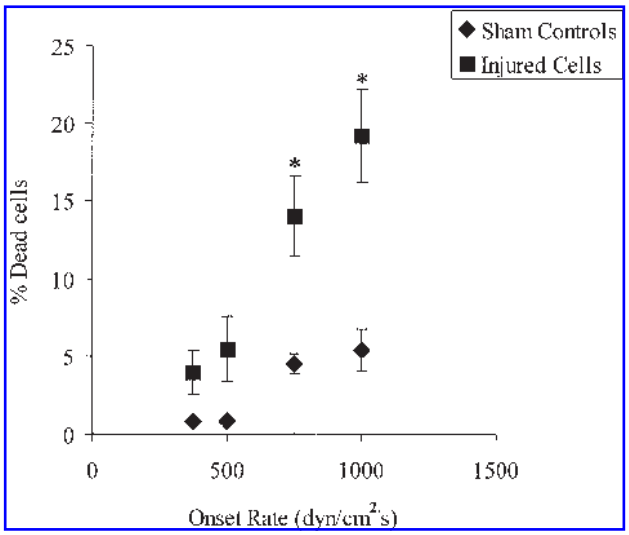

FIG. 5. Loading rate dependence of neuronal cell injury as assessed by the trypan blue assay performed at 24 h after the injury. Cells seeded on Cell-TAK™ were loaded with the same shear stress magnitude (30 dyn/cm^2) and duration time (200 msec). The onset rate of shear stress strongly influenced the magnitude of injury. The two larger onset rates (1000 and 750 dyn/cm^2 · sec) produced significantly higher death rates than either the sham controls or the cells loaded with at the two lower onset rates (500 and 375 dyn/cm^2 · sec; *$p < 0.05$). For 1000 dyn/cm^2 · sec, n = 10 (injury) and n = 5 (sham control); for 750 dyn/cm^2 · sec, n = 8 (injury) and n = 2 (sham control); for 500 and 375 dyn/cm^2 · sec, n = 2 (injury and sham control). Data analysis was performed with one-way ANOVA. Each point represents mean ± SD.

The dose dependence of loading conditions was determined with MTT assay (Fig. 6). Increasing peak shear stresses caused greater loss in cell viability. Using poly-D-Lysine and Matrigel® as the cell attachment substrate allowed the application of higher peak shear stress (100 dyn/cm^2) without significant cell detachment. The data demonstrated that the maximal injury severity was up to 40% ± 6% with these conditions (Fig. 6). This injury severity with these loading magnitudes and rates were significantly reproducible ($p < 0.001$).

Change in Membrane Integrity following Injury

LDH release to extracellular space serves as a simple measure of cell damage, specifically membrane integrity. LDH is an enzyme which is found in the cell cytoplasm, and its high molecular weight prevents it from crossing intact cell membranes. To characterize the effect of mechanical input on acute membrane damage, LDH release rate was measured at various time points starting from right before the mechanical loading and ~5 min, 15 min, 1 h, 3 h, 6 h, and 24 h post-injury in sham controls (three cultures) and injured cells (four cultures). Acute release of LDH was more than five times higher in injured cells at 5 min post-injury ($p < 0.05$) and returned to baseline values by 1 h (Fig. 7). The rate of LDH release was not significantly different from sham controls at later time points. The cell populations lysed at the end of 24 h post-injury showed that total amount of released LDH at 5 min post-injury was approximately 10% of maximum LDH content for each cell population.

Changes in Cell Morphology following Injury

Mechanically injured cells showed distinct morphological alterations within the first 5 min post-injury (Fig. 8). Most of cells remained attached after injury (Fig. 8B). At 5 min post-injury, many cells exhibited reduced phase contrast indicating a loss of intracellular material (Fig. 8B). Occasionally, poorly attached cell processes were broken by the application of shear stress. By 24 h, some cells started to exhibit beaded plasma membrane in both cell body and axonal processes, and cell shrinkage was apparent (Fig. 8D). Some cells became loosely attached to substratum or completely detached from the substratum by 24 h (detected by visual examination under light microscope). Control cell cultures did not show these morphological changes (Fig. 8A,C). A significant portion of injured cells at 24 h exhibited positive Annexin-V staining compared to sham (uninjured) controls (Fig. 9).

Neuroprotective Effect of P188 in PC2 Neuronal Cells following the Injury

To assess the neuroprotective effect of P188 on neuronal survival following the mechanical injury, PC2 neuronal cells were mechanically loaded with CCSD device as described in Materials and Methods. Then, three different concentrations (30, 100, and 1000 μM) of P188 were applied to both sham controls and injured cells 10–15 min after the mechanical loading. Treatment with P188 restored cell viability in a dose-dependent manner. Viability of injured cells at 24 h as assessed by the MTT assay showed that the full recovery was observed with the application of 100 μM P188, which was well below the CMC, and was not improved further with the application of a higher concentrations of P188 (Fig. 10). The survival rate of the injured cells (91.5 ± 9.8%) treated with 100 μM of P188 was not statistically different from the uninjured sham controls (99.5 ± 2.7%). The results showed that application of 100 μM P188 significantly ($p < 0.001$) improved the survival of injured cells compared to the untreated injured cells (58.5 ± 13.4%).

To demonstrate the ability of P188 to facilitate resealing of mechanically injured membranes, its effect on LDH release was measured (Fig. 11). For this experiment,

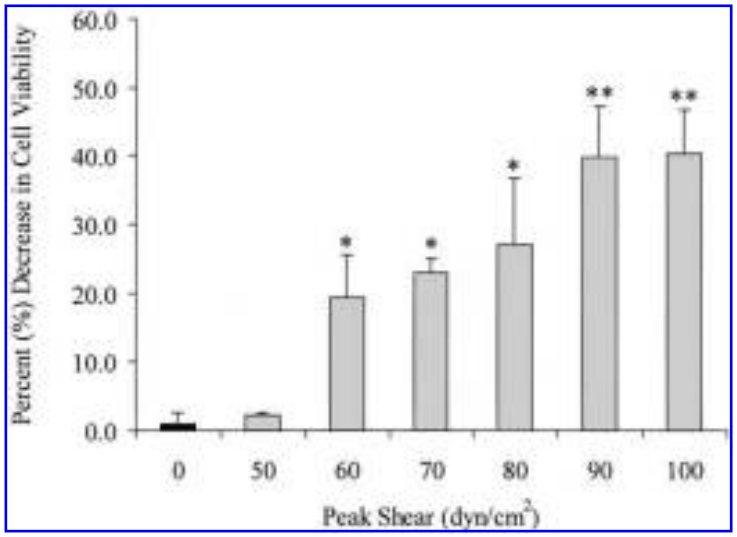

FIG. 6. Dose dependent change in cell viability (%) in injured PC2 cells seeded on poly-D-Lysine and Matrigel® Matrix. The cell viability was quantified with MTT assay. Injury protocol included various peak shear stresses applied with 15-msec rise time and 20 dyn/cm^2 steady plateau shear stress. The maximum injury created on PC2 cells was significantly higher at 100 dyn/cm^2 peak shear stress (n = 6). Because the rise time was constant, the onset rate of shear stress (peak stress/rise time) changed with peak shear stress, ranging from 3333 to 6667 dyn/cm^2 · sec. For 50 and 60 dyn/cm^2, $n = 3$; for 70 dyn/cm^2, $n = 5$; for 80 dyn/cm^2, $n = 6$; for 90 dyn/cm^2, $n = 3$; and for 100 dyn/cm^2, $n = 6$. There were significant differences ($*p < 0.05$ and $**p < 0.001$) between injured cells and sham controls ($n = 9$). Each bar represents mean ± SD. The multiple comparisons of means were performed with Tukey's test following one-way ANOVA.

100 μM P188 was applied to cells at 10 min post-injury in order to determine its effect at the 15-min time point when the release rate in injured cells was still significantly greater than the controls (Fig. 7). The peak LDH release rate at 5 min was similar to the untreated injury experiment (Fig. 7). In contrast, P188 treatment at 10 min resulted in a release rate at 15 min that was not significantly different from controls (Fig. 11). In untreated injured cells, the release rate at 15 min was approximately three times the controls (Fig. 7). As with untreated cells,

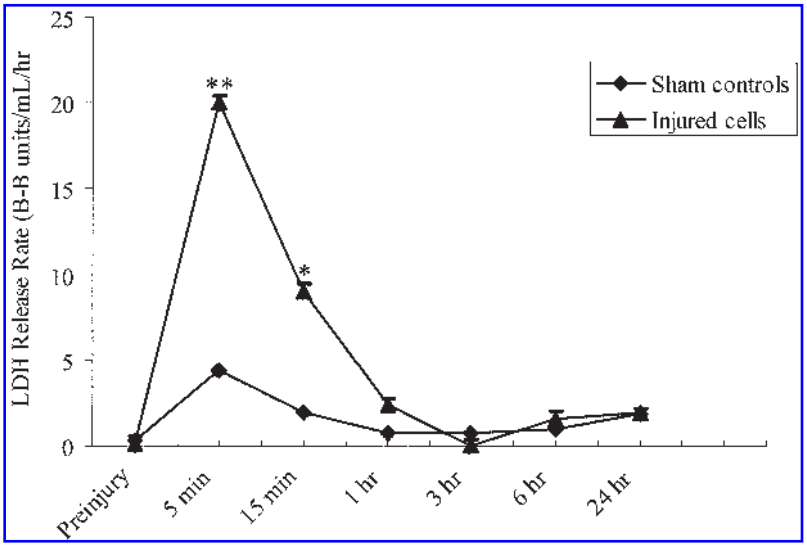

FIG. 7. LDH response of injured PC2 cells seeded on poly-D-Lysine and Matrigel®. LDH release rate (Berger-Broida (B-B) units/mL/h) for injury control ($n = 3$) and injured ($n = 4$) were taken right before the injury (preinjury), 5 min, 15 min and 1, 3, 6, and 24 h after the injury. Bars represent mean ± SD ($*p < 0.05$ and $**p < 0.001$).

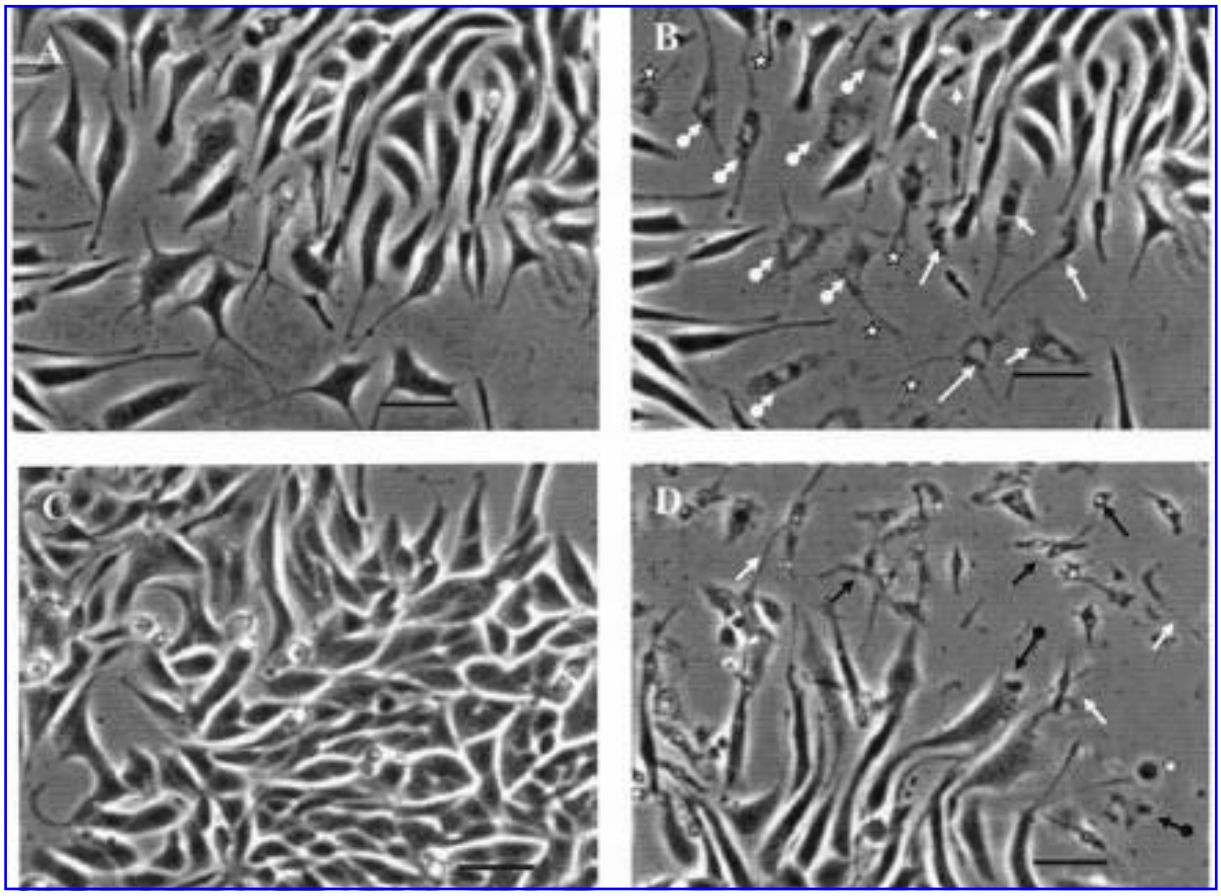

FIG. 8. Phase contrast images of PC2 cells right before injury (**A**), 5 min post-injury (**B**), sham control 24 h post-injury (**C**) and injured cells 24 h post-injury (**D**). Phase images in A and B represent the same population of cells right before and 5 min after the injury. Cells were seeded on poly-D-Lysine and Matrigel. Normal cells were heterogeneous in shape (mostly with spindle or flat cell body). Observations at 5 min post-injury (B) showed that severely injured cells displayed swellings and more granular cytoplasmic appearance (circle ended arrows); some cells showed decreased size in cell body (white arrows), and some axonal processes were observed as broken (white stars). At 24 h post-injury (D), beaded cell membranes (circle ended arrows), beaded or broken axonal processes (white arrows), detached cells (white star) were observed in injured cultures. Control cells (A and C) did not show any of these alterations at these specific time points. Bar = 55 μm.

in P188 treated cells, there was no significant difference between injured and control cells at time points from 1 h to 24 h post-injury.

DISCUSSION

We have developed a model that allowed us to reproducibly create a severe injury in a population of cultured neuronal cells. The loading parameters used in this study were developed based on our understanding of the magnitude and rate dependence of cell injury. Previous studies have used a variety of techniques for applying mechanical loads to cells including simple elongation of axons (Galbraith et al., 1993), biaxial stretch of two-dimensional culture substrates (Cargill and Thibault, 1996) and fluid shear stress (LaPlaca et al., 1997, Blackman et al., 2000). A common feature of these studies was the finding that the injury severity depended strongly on the rate at what the mechanical load was applied. The configuration of our model—fluid shear stress applied to cells cultured on a rigid substrate—is not intended to mimic explicitly the *in vivo*, three-dimensional tissue deformation that occurs during TBI. Rather, the mechanical loading profile was developed to replicate the cellular injury characteristics observed *in vivo*, specifically the mechanical disruption of the plasma membrane (Pettus et al., 1994).

The mechanical trauma produced by the dynamic application of fluid shear stress resulted in a rapid and tran-

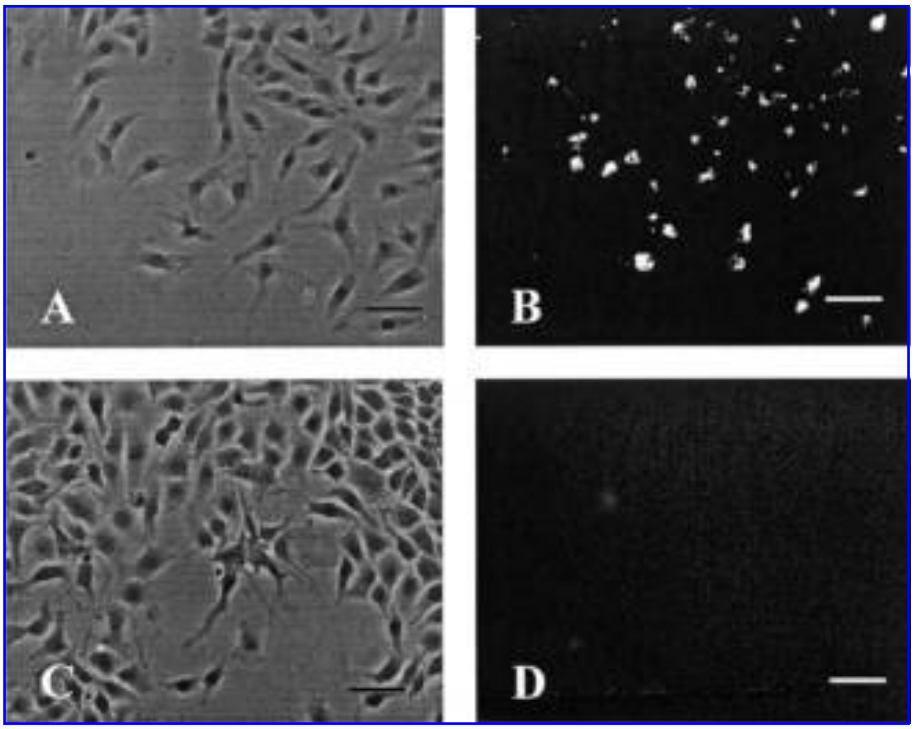

FIG. 9. Annexin-V staining of injured cells. At 24 h post-injury, cells were stained with fluorescently labeled Annexin-V (**A**, phase contrast; **B**, fluorescence). Annexin-V labeled a significant portion of the injured cells indicating the translocation of phosphatidylserine to the outer leaflet of the plasma membrane that occurs in cells undergoing apoptosis. Sham (uninjured) controls displayed very little Annexin-V binding (**C**, phase contrast; **D**, fluorescence). Bar = 55 μm.

sient increase in membrane permeability to macromolecules (LDH) indicating acute membrane damage. The acute and transient nature of the membrane damage in response to applied mechanical forces was consistent with both *in vitro* (Geddes et al., 2003) and *in vivo* (Pettus et al., 2004) injury models. The severity of the injury as assessed by cell viability at 24 h was dependent on both the magnitude of stress and the rate of application consistent with previous work on cell injury. Thus, we have produced the physical finding characterizing neuronal injury *in vivo* (i.e., membrane damage). Furthermore, the loading-rate dependence of the response suggests that the physical mechanism by which we produced the injury is similar to other models as well as the *in vivo* condition.

In our initial studies, we used two different cell adhesion factors (collagen and Cell-TAK™) to achieve cell attachment. These studies showed that loosely attached cells were easily detached with increasing shear stress and onset rates and prevented the application of more severe loading conditions. Especially, cells seeded on collagen easily came off from the surface even with the application of lower shear stresses (20 dyn/cm^2) (data not shown). Cell-TAK™ provided better attachment of PC2 cells and allowed up to 20% injury on the cells detected by trypan blue staining. Cells seeded on combination of poly-D-Lysine and Matrigel® adhesive molecules further increased the strength of the cell attachment to the substratum. The combination of these adhesive molecules allowed the application of higher loading levels and produced an injury up to 46% on the cells, as detected with the MTT assay. These studies demonstrated that the proper attachment of the cells to the substratum is an essential factor to mechanically injure the cells.

The response of the cells to external stimuli may be affected by the mechanical properties of cells, which may vary according to cell type, culture conditions, and cell–cell interactions. Because of this, the levels of mechanical loading used in this study to injure neuronal cells should not be interpreted as absolute without reference to the specific conditions of the experiment. Rather, the appropriate point for comparison should be the level of damage to the cell membrane produced by the mechanical loading.

In this study, PC2 cells were used because they resemble primary neurons upon terminal differentiation with the application of NGF. These cells have been well characterized and have become a model for many studies related to identification and characterization of neuronal signaling pathways (Heneka et al., 1998), to study cell death

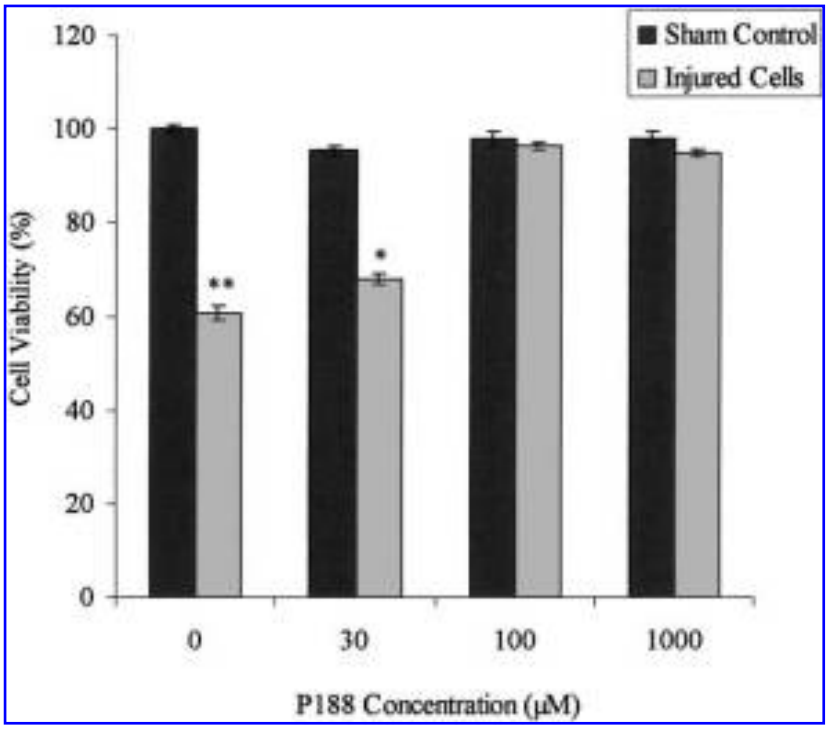

FIG. 10. Change in cell viability of PC2 cells seeded on poly-D-Lysine and Matrigel with P188 treatment. Dose-dependence of P188 induced neuroprotection at three different concentrations—30, 100, and 1000 μM—was measured with MTT assay. Cell viabilities for sham control ($n = 10$), injured ($n = 9$), shams with P188 ($n = 6$ for each concentration), and injured with P188 ($n = 9$ for each concentration) were examined 24 h post-injury. The cell viability was fully restored by P188 at 100 μM and 1000 μM (no significant difference with uninjured sham controls). Bars represent mean. $*p < 0.05$ and $**p < 0.001$ compared to sham controls. The multiple comparisons of means were performed with Tukey's test following the one-way ANOVA.

(Dubreuil et al., 2003; Kitazawa et al., 2004; Ulloth et al., 2003; Yang et al., 2004), especially apoptosis, in neuronal cells and cytoskeletal structure and function (Kobayashi and Mundel, 1998; Nakayama et al., 2001; Okabe and Hirokawa, 1988; Tahir et al., 1992). Although no other types of cells were examined in the present study, cell viability studies and onset rate dependency of traumatic injury produced on cells with CCSD were similar to previous studies conducted with human derived neuronal cells (Ellis et al., 1995; LaPlaca et al., 1997; LaPlaca and Thibault, 1998). This suggests that the response at high strain levels may be very similar in different neuronal cells.

The release of LDH to extracellular space following injury was used to characterize the membrane damage due to mechanical loading. The large transient extracellular release of LDH was consistent with the LDH release data previously reported by LaPlaca in mechanically loaded cells (LaPlaca and Thibault, 1997). The immediate release of LDH (at 5 min post-injury) demonstrated that the membrane breakdown was due to direct mechanical damage rather than the result of secondary degeneration or loss of cell viability. A subpopulation of cells had altered morphology immediately after injury. The reduction of phase contrast in some cells suggests the loss of cytoplasmic material. Since the detected LDH release was less than 10% of the total LDH in the culture and the percentage of cells displaying the injured morphology acutely was somewhat greater than 10%, it is unlikely that the acute release is due to the complete lysing of this subpopulation of the cells. Thus, it is more likely that a larger population of cells was injured acutely and exhibited some degree of spontaneous recovery.

Despite the apparent recovery of membrane integrity as indicated by LDH release, a large portion of the cells proceeded to die by 24 h as detected by MTT assay. Since there was no significant LDH release detected at these later time points, the loss of viability at 24 h post-injury most likely indicates apoptosis rather than necrosis. Annexin-V labeling of injured cells supports this conclusion. Although Annexin-V can also bind to phoshpatidylserine in the inner leaflet in cells in the late stages of necrosis after the membrane has become permeable, the lack of significant LDH release at 24 h post-injury suggests that the membranes are still intact at this time point. Finally, morphological features of the injured cells at this time point included membrane blebbing and cell

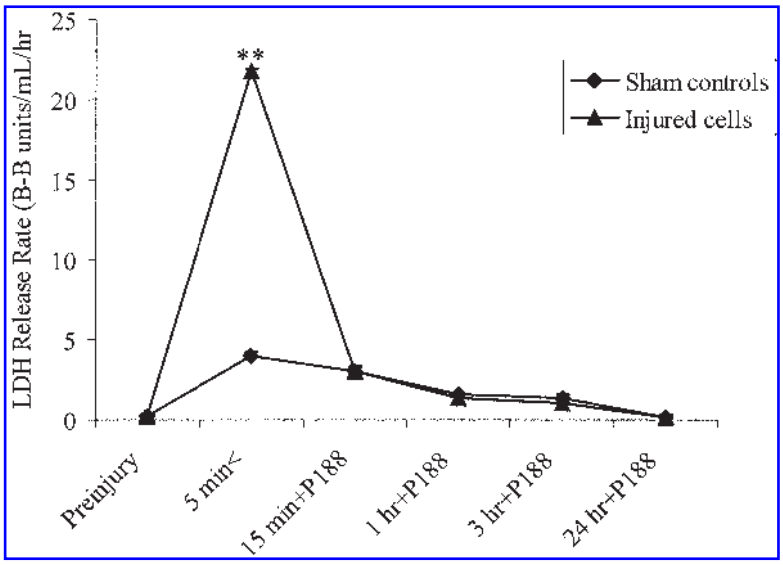

FIG. 11. LDH response of injured PC2 cells treated with P188. Cells were seeded on poly-D-Lysine and Matrigel®. 100 μM P188 was added into the culture medium of injured cells and sham controls at 10 min post-injury time. LDH release rate (Berger-Broida [B-B] units/mL/h) for injury control ($n = 2$) and injured ($n = 3$) were taken right before the injury (pre-injury), 5 min, 15 min and 1, 3 and 24 h after the injury. At 5 min, LDH release in injured cells was significantly greater than in sham controls. Following P188 addition at 10 min, release rates at 15 min and after were not significantly different from controls. Bars represent mean ± SD (**$p < 0.001$).

body shrinkage consistent with the previously described features of apoptosis (Allen et al., 1997).

The heterogeneity of cellular responses in terms of both acute and long-term morphological changes may arise from several sources. Even though the nominal wall shear stress produced by the cone-and-plate device is uniform across the culture surface, the stresses acting on any individual cell will depend on its surface topography and the spatial organization of the cells in the field (Barbee et al., 1995). Differences in cell–cell and cell-substrate interactions can also affect structural and mechanical properties of the cells. LaPlaca et al. (1997) reported that isolated neurons experienced greater deformation and higher calcium transients in response to dynamic shear stress compared to cells located in aggregates. This finding seems to be consistent with the acute and long-term morphological indication of injury in this model (Fig. 9), though we did not evaluate this quantitatively. Finally, variations in the expression of the signaling molecules involved in the response to injury can yield heterogeneity in susceptibility to the pathological processes initiated by the trauma.

We have demonstrated that treatment of injured cells with P188 significantly improved cell viability following traumatic injury *in vitro*. P188 has been previously shown to promote the resealing of disrupted plasma membranes in a variety of *in vitro* experimental models (Lee et al., 1992; Marks et al., 2001; Maskarinec et al., 2002; Merchant et al., 1998; Pandanilam et al., 1994). Our LDH analysis with P188-treated cells also suggests that P188 rapidly promotes membrane resealing and cell recovery after mechanical injury. The timing of the application of P188 after injury is critically important with regard to the potential clinical application of this treatment strategy. For this initial demonstration, we felt that treatment immediately after the injury would have given an overly optimistic view of its potential, not to mention the virtual impossibility of actually delivering any treatment so soon after a real instance of TBI. Although our data and that from other cell culture models (Geddes et al., 2003) suggest a relatively transient change in permeability with membranes resealing spontaneously in less than 1 h, other studies suggest a somewhat longer period of membrane disruption (2–4 h; Ellis et al., 1995). Recently, the study conducted by Borgens et al. (2004) in adult guinea pig spinal cord injured with compression showed that subcutaneous injection of P188 after 6 h post-injury provided a significant recovery of spinal cord function. Based on our LDH release data, we considered a 15-min interval after the injury to be a good test for its ability to rescue injured neurons in this model. However, with the recognition that the permeability changes *in vivo* are more persistent than in our model, the 15-min time point in this model may similarly correspond to a longer, clinically-relevant window of opportunity for TBI.

In addition to the timing of treatment, the clinical po-

tential of P188 will also depend on having an effective mode of delivery. Long-term toxicity studies in animals and man reveal it to be safe even for intravenous use (Edlich et al., 1973). The surfactant is not biodegradable and it is rapidly excreted in the urine unmetabolized (Schmolka, 1977). It has been previously approved by Food and Drug Administration (FDA) as skin wound cleanser for use in humans in 1978. In an *in vivo* model of electrical injury in which skeletal muscle was electroporated, P188 delivered intravenously was able to restore membrane integrity (Lee et al., 1992). In a spinal cord compression injury model, P188 administered subcutaneously promoted restoration of cord function (Borgens et al., 2004). As with all therapeutic agents targeted to the brain, adequate delivery to the site of injury will have to be achieved including crossing of the blood brain barrier if injected intravenously.

The putative mechanism of action of P188 is the promotion of resealing membrane pores created by trauma. The loss of membrane integrity triggers many downstream events resulting in delayed cellular damage (Lee et al., 1992; Marks et al., 2001). Some mildly injured cells may make adaptive responses to recover from the injury while severely injured cells may be unable to recover from the initial injury and proceed to necrosis. Cells that acutely recover membrane integrity may still enter apoptosis later due to inter- or intra-cellular signaling events initiated by the initial traumatic injury. The ability of P188 to speed the restoration of membrane integrity after injury may limit the uncontrolled release of cytotoxic molecules that mediate the long term cell death response. Alternatively, insertion of P188 into the plasma membrane of injured cells may help to protect the membrane from oxidative damage as demonstrated in a model of excitotoxic cell death (Marks et al., 2001).

Although the maintenance of plasma membrane integrity is generally accepted to be a necessary condition for cell viability, the partial or transient loss of membrane integrity is seldom considered as a causative factor in cellular pathology. More often membrane damage is considered either as an all-or-none acute response to some injurious stimulus causing cell lysis and immediate death or as the final common event in necrotic cell death. We have shown that mechanical injury of neuronal cells does not result in the immediate irreversible cell lysis and death of the cells, but rather it produces membrane damage that, if left untreated, leads to cell death over the ensuing 24 h. Treatment with an agent which promotes resealing of membrane defects was able to rescue cells from death. This suggests that initial mechanically-induced plasma membrane damage is a critical feature of traumatic injury and its sequelae. Furthermore, our data establish cell membrane integrity as a potential therapeutic target in the treatment of neuronal trauma.

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