A Calpain Inhibitor Enhances the Survival of Schwann Cells *In Vitro* and after Transplantation into the Injured Spinal Cord

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Abstract

Despite the diversity of cells available for transplantation into sites of spinal cord injury (SCI), and the known ability of transplanted cells to integrate into host tissue, functional improvement associated with cellular transplantation has been limited. One factor potentially limiting the efficacy of transplanted cells is poor cell survival. Recently we demonstrated rapid and early death of Schwann cells (SCs) within the first 24 h after transplantation, by both necrosis and apoptosis, which results in fewer than 20% of the cells surviving beyond 1 week. To enhance SC transplant survival, *in vitro* and *in vivo* models to rapidly screen compounds for their ability to promote SC survival are needed. The current study utilized *in vitro* models of apoptosis and necrosis, and based on withdrawal of serum and mitogens and the application of hydrogen peroxide, we screened several inhibitors of apoptosis and necrosis. Of the compounds tested, the calpain inhibitor MDL28170 enhanced SC survival both *in vitro* in response to oxidative stress induced by application of H₂O₂, and *in vivo* following delayed transplantation into the moderately contused spinal cord. The results support the use of calpain inhibitors as a promising new treatment for promoting the survival of transplanted cells. They also suggest that *in vitro* assays for cell survival may be useful for establishing new compounds that can then be tested *in vivo* for their ability to promote transplanted SC survival.

Key words: calpain; caspases; cell death; contusion; spinal cord injury

Introduction

AN INCREASING NUMBER of cell-based therapies are moving towards human use for repair of spinal cord injury (SCI; Chernykh et al., 2007; Dobkin et al., 2006; Knoller et al., 2005; Mackay-Sim et al., 2008; Saberi et al., 2008; Tator, 2006; Yoon et al., 2007). One challenge that may limit their efficacy is the early death of transplanted cells. If these treatments are to reach their full potential, the causes of transplanted cell death need to be determined, and strategies developed to enhance their survival.

Schwann cells (SCs) are a cell type that has been extensively studied for their ability to promote repair following transplantation into the injured spinal cord. Transplanted SCs provide a permissive substrate for axonal growth (Xu et al., 1995, 1997), myelinate axons, and promote functional recovery (Takami et al., 2002) after SCI (Fortun et al., 2009). Despite these benefits, we recently demonstrated that SCs die early

after transplantation, resulting in a reduction in graft size (Hill et al., 2006, 2007; Pearse et al., 2007). Although transplanted SCs die by both necrosis and apoptosis, necrosis predominates (Hill et al., 2007). The causes of transplanted SC death and strategies to prevent it have not yet been assessed.

A number of processes present during collection and implantation of cells could contribute to transplanted cell death and warrant examination, such as detachment of cells from the substratum, removal of serum and mitogens, storage of cells prior to transplantation, and mechanical damage associated with dissociation and injection. At the time of transplantation, SCs are removed from both their trophic support and their substrate, both of which can induce caspase-3 activation and apoptosis in cultured cells, including SCs (Deckwerth and Johnson, 1993; Frisch and Francis, 1994; Koda et al., 2008; Yu et al., 2005). Removal of factors that promote cell survival can induce apoptosis by switching the balance of signaling pathways from survival to death. Replacement of trophic factors

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(Koda et al., 2008) can promote SC survival, and inhibition of apoptotic signaling can promote transplant survival in some paradigms (Chen et al., 2002; Mundt-Petersen et al., 2000; Schierle et al., 1999).

In addition to events in the pre-transplantation interval, factors present in the injury environment that induce host tissue damage could contribute to transplanted cell death and thus should be examined. These include elevated levels of reactive oxygen species, reactive nitrogen species, and nitric oxide (NO; Carrico et al., 2009; Hamada et al., 1996; Liu et al., 1999, 2003; Nakahara et al., 2002; Vaziri et al., 2004; Xiong and Hall, 2007). Blocking factors that induce cell death (for example, formation of reactive oxygen species and lipid peroxidation, or inducible nitric oxide synthase [iNOS] activity) can prevent necrosis and increase cell survival, both *in vitro* and after transplantation into the injured brain (Blasig et al., 2002; Grasbon-Frodl et al., 1996; Matsuda et al., 2005; Nakao et al., 1994). Inhibiting protease activation also may be a useful strategy to promote transplant survival. Calpains, calcium-mediated cysteine proteases, are elevated after injury (Banik et al., 1998; Li et al., 1996; Ray et al., 1999; Wingrave et al., 2003), and calpain inhibitors promote tissue preservation (Corona and Tapia, 2008; Ray et al., 2001; Yu and Geddes, 2007; Yu et al., 2008) and recovery after SCI (Arataki et al., 2005; Colak et al., 2009; Corona and Tapia, 2008; Hung et al., 2005; Yu et al., 2008), making them intriguing candidates to promote transplant survival.

The current study was designed to assess if certain conditions hypothesized to contribute to the death of transplanted SCs could be mimicked *in vitro* in adult cultured SCs, and whether these models could be used to rapidly screen compounds for their ability to promote SC survival *in vivo*, following delayed transplantation into the contused spinal cord. We tested whether withdrawal of serum and mitogens was sufficient to induce adult SC apoptosis, and whether application of H₂O₂ was sufficient to induce necrosis in adult cultured SCs. Once models of SC apoptosis and necrosis were established, they were used to screen known inhibitors for their ability to promote SC survival *in vitro*, before testing them for their ability to promote SC survival *in vivo*. The results demonstrate that these models can be effectively used to screen compounds to enhance SC survival, and that treatment of SCs with calpain inhibitors enhances the survival of SCs, both *in vitro* and *in vivo* after transplantation into the contused spinal cord.

Methods

Schwann cell cultures

SCs were extracted from sciatic nerves of female adult Fischer 344 rats (Harlan Sprague-Dawley, Indianapolis, IN), as previously described (Hill et al., 2007), and frozen at passage 2 at -80°C until use. At the time of the experiments, the cells were rinsed with DMEM + 10% heat-inactivated fetal bovine serum (D-10), resuspended in D10 + 3M medium (D-10 + pituitary extract, 20 µg/mL, Biomedical Technologies, Stoughton, MA; forskolin, 2 µM, Sigma-Aldrich, St. Louis, MO; and heregulin, 2.5 nM, Genentech, San Francisco, CA), and plated onto poly-L-lysine-coated (Sigma-Aldrich) culture plates.

For experiments assessing cell survival both *in vitro* and *in vivo*, infection with a lentiviral vector carrying the GFP gene

was used to identify the cells (Hill et al., 2007). For experiments assessing SC death, non-infected SCs were used. For both *in vitro* and *in vivo* experiments, passage 3 cells were used.

Preparation of Schwann cells for *in vitro* assays. Cells (25,000) were plated onto 96-well, white walled, clear-bottom plates (Corning, Corning, NY) and grown for 3 d before all manipulations. All experiments were performed at least three times, and each experiment contained at least four wells per condition.

Preparation of Schwann cells for transplantation. SCs were pretreated with drugs for 1 h prior to collection for transplantation. The drugs were included in all collection media and the transplant medium. 1 × 10⁶ cells resuspended in 5 µL DMEM with the described drug were transplanted (see below).

Apoptosis induction and inhibition

Apoptosis was assayed in adult SCs by using Caspase-Glo 3/7 solution (Promega, Madison WI), per the manufacturer's instructions. Caspase 3/7 activity was measured 3 or 6 h after the cells were treated with D10 + 3M medium, DMEM medium (without serum or mitogens), or 500 µM staurosporine in D10 + 3M. To normalize data across the experiments, the extent of apoptosis induced by serum withdrawal, or staurosporine, was set at 100%, and all results are reported as a percentage of apoptotic cells.

To test whether blocking the caspases would prevent serum withdrawal-induced apoptosis, SCs were pretreated with Ac-YVAD-cmk (YVAD: 125 µM, 250 µM, or 500 µM), or z-VAD.fmk (ZVAD: 25 µM, 50 µM, or 100 µM) for 1 h before serum and mitogen withdrawal. Fresh drug was applied at the time of apoptosis induction, and caspase 3/7 activity was assayed 3 h later.

Necrosis induction and inhibition

The number of dead cells was quantified after application of H₂O₂, with or without the drug treatment, by measuring lactate dehydrogenase (LDH) release using CytoTox-One (Promega) according to the manufacturer's instructions, and read with a microplate reader (excitation wavelength 560 nm; emission wavelength 590 nm). LDH is released from cells with damaged plasmalemmas, characteristic of necrotic cells. To normalize the data across replicate experiments, treatment with 500 µM H₂O₂, which resulted in the death of almost all SCs by 3 h, was set to 100% dead cells, and all results are represented as a percentage of dead cells.

To evaluate SC survival, GFP SC fluorescence was measured. The cells were fixed with 4% paraformaldehyde and rinsed with PBS before assaying with the microplate reader (excitation wavelength 488 nm; emission wavelength 509 nm). In addition to fluorescence readings, the cells were visually examined, and images of each well from three separate experiments were taken. To normalize the data across replicate experiments, treatment with D10 + 3M was set to 100% live cells. All results are represented as a percentage of live cells. In some experiments ATP levels were assayed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega), per the manufacturer's instructions.

The lethal dose of $\sim 50\%$ of the cells (LD_{50}) of H_2O_2 for necrosis of cultured SCs was determined by exposing the cells for 3 h at $37^\circ C$ ($6\% CO_2$) to varying concentrations of DMEM-3M containing H_2O_2 ($0-500 \mu M$). After 3 h of exposure the number of live or dead cells was assayed. To test the ability of drugs to inhibit H_2O_2 -mediated necrosis, drugs were applied at the time of, or 1 h before, H_2O_2 application. In all cases $50 \mu M H_2O_2$ was used to induce necrosis, and the number of dead or live cells was measured 3 h later. Necrosis-inhibiting drugs were prepared in D10 + 3M. Cells were treated with the following concentrations of inhibitors to: lipid peroxidase, U83836E ($0.1 \mu M$, $0.3 \mu M$, or $1.0 \mu M$); iNOS, SB203580 ($1.0 \mu M$, $5.0 \mu M$, or $10.0 \mu M$); and calpain, MDL28170 ($50 nM$, $500 nM$, $5 \mu M$, or $50 \mu M$). After 1 h of drug pretreatment, fresh solution of drugs and H_2O_2 was added to the cells for the duration of the experiment. Additional wells received injury alone ($50 \mu M H_2O_2$ in D10 + 3M), or were maintained in D10 + 3M (all live cells). To determine maximal LDH release (all dead cells), $2 \mu L$ of a cell lysis solution, included with CytoTox-One assay, was added to D10 + 3M.

Surgery and transplantation

Eighty adult female Fischer 344 rats ($n = 10/\text{group}$, weight $160-180 g$; Harlan Sprague Dawley) were used in this study. The animals were anesthetized with $1-2\%$ halothane in oxygen. The dura was exposed below thoracic vertebra T9. A spinal cord contusion was induced with the MASCIS/NYU impactor by dropping a $10-g$ weight $12.5 mm$ onto the exposed spinal cord. After injury, the muscles were closed in anatomical layers, and the animals were given $10 mL$ lactated Ringer's solution subcutaneously, followed by buprenorphine ($0.01 mg/kg$) twice a day for the first 2 days, and gentamycin ($5 mg/kg$) for the first 7 days, to prevent infection. The bladders were manually voided twice daily until reflexive urination returned. Food and water were provided *ad libitum*.

Seven days after injury, the animals were re-anesthetized with ketamine ($45-87 mg/kg$) and xylazine ($5-13 mg/kg$), the injury site was re-exposed, and 1×10^6 SCs in $5 \mu L$ DMEM were injected into the injury epicenter as previously described (Hill et al., 2007). In some cases inhibitors were added to the injection medium. The inhibitors tested included U83836E ($0.3 \mu M$); SB203580 ($10 \mu M$); MDL28170 ($50 nM$); Ac-YVAD-cmk ($500 \mu M$); z-VAD.fmk ($25 \mu M$); and a combination of U83836E ($0.3 \mu M$) and YVAD ($500 \mu M$). Following transplantation, the injury site was closed in anatomical layers, and buprenorphine and gentamycin were administered. The animals were housed according to the National Institute of Health and U.S.D.A. guidelines. The Institutional Animal Care and Use Committee of the University of Miami approved all animal procedures.

Tissue processing

The animals were euthanized with a lethal overdose of ketamine and xylazine 10 min or 7 days after transplantation. Forty animals ($n = 5/\text{group}$) were decapitated and the spinal cords were rapidly isolated and flash-frozen for quantification of GFP DNA. The remaining 40 animals ($n = 5/\text{group}$) were perfused with saline (0.9%), followed by 4% paraformaldehyde, and their spinal cords were removed, post-fixed overnight in 4% paraformaldehyde, and cryoprotected with 30% sucrose. Serial sections ($20 \mu m$) encompassing the injury site were cut horizontally on a cryostat. Prior to examination, the

slides were cover-slipped with Vectashield H-1000 (Vector Laboratories, Burlingame, CA) containing Hoechst 33842 ($1:1000$) to visualize nuclei, and sealed with nailpolish.

Quantification of cell survival and transplant volume

Cell survival and transplant volume were quantified using Stereoinvestigator (MicroBrightField, Inc, Williston, VT) as previously described (Hill et al., 2007). Every eighth tissue section was used. Transplant volume was measured at $10\times$ using the Cavalieri function with a $200 \times 200 \mu m$ grid. For quantification of cell numbers the Optical Fractionator function was used (grid: $150 \times 400 \mu m$; sampling box: $50 \times 50 \mu m$ [7 days]; $20 \times 20 \mu m$ [10 min]).

Quantitative real-time polymerase chain reaction

DNA from a $5-mm$ piece of spinal cord tissue centered on the lesion/transplant or cultured cells was collected using the QIAamp DNA Mini Kit (Qiagen Sciences, Valencia, CA), and further purified by alcohol precipitation. Quantitative real-time PCR was performed using $100 ng$ of sample DNA, and primers for GFP DNA (forward primer: $5'GAACGGCATCAAGGTGAACT3'$; reverse primer: $5'TGGGTGCTCAGGTAGTGTT3'$) as previously described (Hill et al., 2007).

To estimate the number of transplanted GFP-positive SCs, samples were normalized to a standard curve generated using cultured GFP-positive SC DNA, mixed with wild-type spinal cord DNA.

Statistical analysis

For all *in vitro* experiments a one-way analysis of variance (ANOVA) was used to determine significance. Dunnett's *post-hoc* analysis was conducted for all necrosis experiments and apoptosis inhibition experiments. Bonferroni *post-hoc* analysis was conducted for experiments concerning apoptosis induction. For *in vivo* experiments, one-way ANOVAs followed by Dunnett's *post-hoc* test were used to compare the number and percentage of surviving SCs, the transplant volume, and the density of cells within the transplant, to determine if drug treatment was effective. Significance was assigned at $p \leq 0.05$. All data are expressed as mean $\pm$ SEM.

Results

Assaying and enhancing Schwann cell survival in vitro

Following implantation into the contused spinal cord, some of the transplanted SCs die via apoptosis (Hill et al., 2007). At the time of preparation for transplantation, the SCs are re-suspended in minimal medium to prevent addition of foreign proteins to the spinal cord. Removal of serum and mitogens from the culture medium is known to induce apoptosis in other cell types, and was examined in SCs to determine if it alone was sufficient to induce SC apoptosis. Removal of serum and mitogens resulted in a significant increase in caspase 3/7 activity in cultured SCs (Fig. 1A: $p \leq 0.005$), equivalent to that observed following application of a known potent inducer of apoptosis, staurosporine (Fig. 1A). In addition to increases in caspase 3/7 activity, condensed cells observed in phase contrast (Fig. 1C and E), and condensed nuclei observed with Hoechst staining (Fig. 1F), following serum and mitogen withdrawal, indicated that increased caspase 3/7

FIG. 1. Serum and mitogen withdrawal induces apoptosis of adult Schwann cells (SCs) in culture, which is prevented by caspase inhibitors. Serum and mitogen withdrawal was sufficient to induce significant apoptosis in adult SCs after 3 h [ANOVA: $F(2,5) = 23.34$] (A). Three hours of exposure to DMEM was used to test caspase inhibitors. Preincubation of SCs with the caspase inhibitors ZVAD or YVAD for 1 h prior to apoptosis induction significantly decreased caspase 3/7 activity 3 h after serum and mitogen withdrawal [ANOVA: $F(7,120) = 98.99$] (B). Images of cells in phase (C, E, G, and I), and nuclear labeling (D, F, H, and J), in D10 + 3M, DMEM, DMEM + ZVAD, and DMEM + YVAD, show condensed cells and nuclei (arrowheads). A reduction in cell number is seen following serum and mitogen withdrawal and partial return following caspase inhibition, as shown by changes in the numbers of healthy nuclei (arrow). Cleavage of a luciferase substrate, measured in relative luminescent units (RLU), was used to detect caspase 3/7 activity, a measure of apoptosis. Luciferase levels in D10 + 3M were used to normalize data across the experiments in A. To assess the effects of inhibitors, luciferase levels following serum and mitogen withdrawal were set to 100% (** $p \leq 0.005$; scale bar = 10 μ m; DMEM, Dulbecco modified Eagle medium; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance).

activity corresponded with apoptosis. Further evidence of SC death was the reduction in both the number of SCs and the number of Hoechst-positive nuclei (Fig. 1C–F) when D10 + 3M was replaced with DMEM. More condensed cells were visible in phase than after Hoechst, which required washing the cells. Similar to removal of both serum and mitogens, removal of either individually was also sufficient to induce the formation of condensed nuclei, but this was not examined further (data not shown).

Next we tested whether blocking caspases upstream of caspase 3/7 could prevent effector caspase activation and subsequent SC apoptosis. Pretreatment of cells with caspase inhibitors (ZVAD or YVAD) prior to serum and mitogen withdrawal resulted in reduced caspase 3/7 activity (Fig. 1B; $p \leq 0.001$), and increased the survival of cultured SCs. Increased SC survival was visible by both phase contrast (Fig. 1E, G, and I), and nuclear detection (Fig. 1F, H, and J).

Although apoptosis occurs following SC transplantation, six times more cells die by necrosis (Hill et al., 2007). Because of the

large necrotic component, we sought to identify factors present after SCI that could induce SC necrosis *in vitro*, and that may contribute to necrotic SC death *in vivo*. Oxidative stress, among other factors, is implicated in cell death both after SCI and *in vitro*. To determine whether oxidative stress produced by application of H_2O_2 is sufficient to induce SC necrosis, varying concentrations of H_2O_2 were applied to cultured SC monolayers. Treatment of cells with H_2O_2 (0–500 μ M) for 3 h resulted in a concentration-dependent decrease in SC survival, as determined by cytological examination and GFP fluorescence (Fig. 2C), and LDH release (Fig. 2A). To determine the LD_{50} , cultures were assayed for GFP fluorescence relative to untreated cultures, and for LDH release relative to conditions in which no cell survival was observed. The LD_{50} was 50 μ M (Fig. 2A and C). Although H_2O_2 can also be used to induce apoptosis *in vitro*, measurements of ATP showed a $96.5\% \pm 1.1\%$ decrease in ATP levels compared to untreated cells, indicating that the H_2O_2 concentrations used induced necrotic, not apoptotic, cell death (Leist et al., 1997; Saito et al., 2006).

FIG. 2. H₂O₂-induced death of adult cultured Schwann cells (SCs) is concentration-dependent and is reduced following inhibition of lipid peroxidation and calpain activity. H₂O₂ application *in vitro* induces SC death, as measured by LDH release (A), and reduces the number of surviving adult SCs, as measured by GFP fluorescence (C), in a concentration-dependent manner when assessed after 3 h of exposure. To determine the LD₅₀ of H₂O₂, data were normalized to 500 μM H₂O₂ for cell death (A), and 0 μM H₂O₂ (D10 + 3M) for all live cells (C), and is presented as percent survival. The LD₅₀ of H₂O₂, determined to be ~ 50 μM H₂O₂, was subsequently used to test inhibitors. Data are normalized to 100% cell death following treatment with the cell lysis solution included in the CytoTox-One kit for maximal LDH release. Application of the lipid peroxidase inhibitor U83836E significantly reduced the percentage of dead cells (B), when cultures were treated with it either at the time of H₂O₂ application, or 1 h before. [ANOVA: $F(21,238) = 37.9$]. Pretreatment of cultures with the lipid peroxidase inhibitor U83836E, or the calpain inhibitor MDL28170, for 1 h prior to application of H₂O₂, or application of the lipid peroxidase inhibitor U83836E at the time of H₂O₂ application, significantly enhanced GFP fluorescence after 3 h over H₂O₂ application alone [D; ANOVA: $F(21,242) = 23.2$]. Inhibition of iNOS did not prevent H₂O₂-induced cell death. Data were normalized so that D10-3M is 100% live cells (* $p \leq 0.05$, ** $p \leq 0.005$; GFP, green fluorescence protein; ANOVA, analysis of variance; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LD₅₀, lethal dose of ~ 50% of cells).

Having established that 50 μM H₂O₂ was sufficient to induce the death of ~ 50% of SCs *in vitro*, we tested whether administration of pharmacological inhibitors either 1 h prior to or at the time of injury could prevent SC death *in vitro*. Wells treated with the lipid peroxidase inhibitor U83836E or the calpain inhibitor MDL28170 contained more intact GFP-positive cells than wells treated with 50 μM H₂O₂. Representative images of GFP-positive SCs in individual wells for each treatment condition are shown in Figure 3A–H. Quantification of cell survival by measuring GFP fluorescence determined that pretreatment of SCs with a calpain inhibitor or lipid peroxidase inhibitor significantly enhanced SC survival 3 h after H₂O₂ administration (Fig. 2D; MDL28170: $p \leq 0.001$ for 50 nM, 500 nM, and 5 μM; U83836E: $p \leq 0.001$ for 0.1 μM, 0.3 μM, and 1.0 μM). In addition to the enhanced SC

survival detected following pretreatment, the lipid peroxidase inhibitor promoted SC survival (i.e., elevated GFP fluorescence) when administered at the time of injury (U83836E: $p \leq 0.05$ for 0.1 μM and 0.3 μM). Both pretreatment and treatment at the time of H₂O₂ administration resulted in a reduction in LDH release following application of the lipid peroxidase inhibitor (Fig. 2B; U83836E pretreatment: $p \leq 0.05$ for 0.1 μM, 0.3 μM, 1 μM; treatment at the time of injury: $p \leq 0.05$ for 0.3 μM). Unlike the lipid peroxidase inhibitor, the calpain inhibitor did not enhance SC survival when given at the time of injury (Fig. 2B), indicating that a delay is required for protease inhibition to promote SC survival. Protease inhibition by pretreatment of SCs with the calpain inhibitor MDL28170 at concentrations that enhanced SC survival did not result in a reduction in LDH release, suggesting that the

FIG. 3. More GFP-positive Schwann cells (SCs) survive H_2O_2 -induced injury *in vitro* following application of inhibitors of lipid peroxidation and calpain activity. Representative cultures from each condition are shown following application of inhibitors before (A–D), or at the time (E–H) of H_2O_2 -induced injury. SCs were bipolar and covered the dishes when maintained in D10 + 3M (D). Addition of H_2O_2 decreased the number of GFP-positive SCs after 3 h (H). Treatment of SCs before injury with the lipid peroxidase inhibitor (A), and the calpain inhibitor (C), or at the time of injury with the lipid peroxidase inhibitor (E), resulted in improved SC survival compared to H_2O_2 -treated cells (H; scale bar = 100 μ m; GFP, green fluorescence protein; iNOS, inducible nitric oxide synthase).

calpain inhibitor was not able to prevent cell membrane breakdown and subsequent LDH release.

Transplantation of Schwann cells following pretreatment with necrosis and/or apoptosis inhibitors

Having established *in vitro* that inhibition of apoptosis by application of caspase inhibitors, and inhibition of necrosis by application of lipid peroxidase or calpain inhibitors, could enhance the survival of SCs, we tested whether pharmacological pretreatment of transplanted SCs improved their survival 7 days following transplantation into the spinal cord injured 7 days previously. SCs were pretreated with inhibitors of caspases (YVAD or ZVAD), lipid peroxidase (U83836E), iNOS (SB203580), calpain (MDL28170), or based on the *in vitro* results, the combination of U83836E and YVAD.

Transplanted GFP-positive SCs were detected within the injured spinal cord in all cases (Fig. 4A–P). Pretreatment of SCs with the lipid peroxidase inhibitor, the iNOS inhibitor, or the caspase inhibitor (ZVAD), did not enhance transplant survival. Pretreatment of the cells with the calpain inhibitor MDL28170, however, significantly increased both the total number and the percentage of surviving SCs ($p \leq 0.02$; Fig. 4Q), doubling transplant survival (medium: $15 \pm 0.7\%$; calpain inhibitor: $28 \pm 5.5\%$). Pretreatment of the SCs with caspase inhibitors resulted in a slight but non-significant increase in the percentage of surviving SCs (YVAD: $22 \pm 4.0\%$; YVAD + U83836E: $21 \pm 3.3\%$). Upon gross morphological examination, grafts pretreated with the calpain inhibitor and one of the caspase inhibitors (YVAD) appeared larger than untreated transplants at 7 days (Fig. 4E, F, and H); however, no significant differences were detected in transplant volume between the groups (Fig. 4S). Similarly, MDL28170 showed a trend toward more GFP DNA as detected by real-time PCR, but high variability between samples (Fig. 4R) meant that significant differences were not detectable. The effect of sustained inhibition after *in vivo* application of the inhibitors was not tested.

Discussion

In this study we identified factors that contribute to the death of SCs transplanted into the injured spinal cord. We also tested inhibitors of specific factors implicated in inducing or signaling cell death for their ability to promote SC survival. We demonstrated that SCs are susceptible to apoptosis induced by serum and mitogen withdrawal, and to necrosis induced by oxidative stress. Importantly, these results show that blocking calpain activity enhances the survival of SCs *in vitro*, and that a single pretreatment is sufficient to enhance the survival of transplanted SCs 7 days after transplantation into the contused spinal cord.

Mechanisms of cell death

The mechanism by which cells die is a continuum between apoptosis and necrosis (Zeiss, 2003). Mild cellular damage leads to apoptosis, for which ATP levels remain sufficient to activate protein synthesis and intracellular signaling, including initiator and effector caspases, allowing for a more controlled cell death. More extreme cellular damage leads to necrosis, during which ion homeostasis is lost (including $[Ca^{++}]_i$), ATP is depleted, and ultimately cells lyse and release damaging cell contents including calpains (calcium-dependent cysteine proteases), acid hydrolases, hydrogen peroxide, and hydrogen ions (van Cruchten and Van Den, 2002). Transplanted SCs die by both apoptosis and necrosis (Hill et al., 2007). The causes of the cellular damage leading to transplanted SC death and how to prevent it remain to be elucidated.

Death of transplanted cells in other models

Poor survival of transplanted cells is not restricted to SCs transplanted into the injured spinal cord. Transplanted cell death has been reported across a variety of cell types and injury models, with cell survival ranging from 1–35%. In the

FIG. 4. Pretreatment of Schwann cells (SCs) with a calpain inhibitor increases SC survival 7 days after transplantation. Grafts of each of the treatment conditions (A–H), with enlargements of individual cells within the grafts (I–P), are shown here. The insets in A–H outline the injury and transplant sites for each condition. Surviving GFP-positive SCs were quantified stereologically (Q), or by real-time PCR for GFP DNA (R). Pretreatment of SCs with the calpain inhibitor resulted in a significant increase in the percentage of surviving SCs [ANOVA: $F(7,31) = 3.422$; Q]. Inhibiting caspase activity with YVAD showed a trend toward increased cell survival. No significant differences were observed in transplant volume (S). Dots on the grafts in Q, R, and S represent values of individual animals within each group ($*p \leq 0.05$; scale bar = 10 μm ; ANOVA, analysis of variance; GFP, green fluorescence protein; PCR, polymerase chain reaction).

CNS, transplant survival has been quantified following implantation of neural progenitor cells into the injured spinal cord (Marsala et al., 2004), and brain (Bakshi et al., 2005), and implantation of dopaminergic neurons into models of Parkinson's disease (Barker et al., 1996; Emgard et al., 1999; Marsala et al., 2004). Outside of the CNS, transplant survival has been quantified following implantation of cardiomyocytes into the heart (Reinecke and Murry, 2002), and pancreatic islet cells into the liver (Pawelec et al., 2008).

Causes of transplanted cell death

Examination of strategies to enhance transplant survival in other transplantation models have demonstrated that the survival of transplanted cells is affected by cell preparation, the immediate post-graft interval, and the chronic graft site (Pileggi et al., 2006; Sortwell, 2003). In the current study we examined the contributions of cell preparation and factors present in the immediate post-graft interval on the survival of SCs.

Preparation of SCs for transplantation involves detachment of cells from their substratum, resuspension in a trophic fac-

tor-free medium, storage on ice during transport, and injection through a small cannula. Previously, we demonstrated that storage of cells prior to transplantation and passage of cells through the injection cannula minimally affects SC survival (Hill et al., 2007). Both trophic factor withdrawal and substrate detachment have been implicated in SC death in other models (Deckwerth and Johnson, 1993; Koda et al., 2008). In the current study, removal of serum and mitogens, without substrate detachment, was sufficient to induce SC apoptosis. This suggests that processing of cells for transplantation induces mild cellular damage, and some of the transplanted cells may already be susceptible to death at the time of transplantation.

Although some transplanted SCs die by apoptosis, most die by necrosis (Hill et al., 2007), indicating the presence of factors that induce more extreme cellular damage. Many biochemical reactions are activated after SCI that can induce substantial cellular damage. Among the factors that have been implicated in tissue damage and cell death after SCI are oxidative stress and release of lytic enzymes such as proteases (Banik et al., 1998; Ellis et al., 2005; Liu et al., 1999, 2003; Xiong

et al., 2007; Xiong and Hall, 2009). Oxidative cell death can be modeled *in vitro* by application of H_2O_2 . Application of H_2O_2 to SCs in culture was sufficient to deplete ATP and induce necrotic SC death (Leist et al., 1997; Saito et al., 2006), implicating oxidative stress as a potential cause of transplanted SC necrosis.

Enhancing Schwann cell survival

A number of strategies have proved efficacious at enhancing transplant survival in other cell types. Most of these studies have focused on enhancing the survival of dopaminergic neurons in Parkinson's models (Sortwell, 2003). Successful strategies have included pretreatment of cells with caspase and iNOS inhibitors, or application of antioxidants (Brundin et al., 2000; Karlsson et al., 2002; Love et al., 2002; Mundt-Petersen et al., 2000; Nakao et al., 1994; Othberg et al., 1997; Schierle et al., 1999; Zawada et al., 2001). To date, there have been no reports of treatments to enhance the survival of transplanted SCs. To explore whether these same strategies are effective at preventing transplanted SC death, we tested several inhibitors of apoptosis and necrosis.

Caspase inhibition

Caspase inhibition has been extensively used to enhance cell survival when apoptosis is present (Bilsland et al., 2002; Gottron et al., 1997; Hansson et al., 2000; Mundt-Petersen et al., 2000; Schierle et al., 1999). In the current study, application of the caspase inhibitors ZVAD and YVAD, at concentrations found effective in other cell types, successfully prevented SC apoptosis and enhanced SC survival following serum and mitogen withdrawal. Despite decreased caspase 3/7 activity *in vitro*, and the presence of caspase-3-positive SCs within the first 24 h after transplantation (Hill et al., 2007), pretreatment of SCs with caspase inhibitors prior to transplantation did not enhance SC survival. Pretreatment of SCs with caspase inhibitors may be sufficient to reduce apoptosis induced by serum and mitogen withdrawal; however, the additional toxic factors present within the injured spinal cord would likely overwhelm this inhibition. Supplementation of the transplant medium with trophic factors, and prevention of anoikis, may help to overcome the induction of apoptosis, resulting in healthier cells at the time of transplantation. However, alone they may not be sufficient to prevent the substantial cellular damage that occurs following implantation of the cells into the injured spinal cord. Strategies that focus on reducing cellular damage at the time of transplantation may be more beneficial.

Lipid peroxidase inhibition

Application of antioxidants and lipid peroxidase inhibitors are among the most successful strategies implemented for enhancing the survival of transplanted cells in other models (Brundin et al., 2000; Karlsson et al., 1999, 2002; Love et al., 2002; Nakao et al., 1994; Othberg et al., 1997). A number of lipid peroxidase inhibitors have been developed, including methylprednisolone and lazaroids (e.g., tirilazad mesylate and U83836E; Hall and Springer, 2004; Villa and Gorini, 1997). U83836E, a second-generation lazaroid with enhanced lipid peroxyl scavenging, is neuroprotective (Giese et al., 1995; Kabadere et al., 2004; Vignes and Hugon, 2006), and enhances

transplant survival of dopaminergic neurons (Karlsson et al., 1999, 2002; Nakao et al., 1994). Application of U83836E to SC cultures decreased SC necrosis and increased SC survival in response to H_2O_2 -induced injury. Pretreatment of the cells with U83836E prior to transplantation, however, was not sufficient to improve SC survival within the contused spinal cord. The failure of U83836E to enhance SC survival under conditions that are effective for dopaminergic neuron survival highlights the difficulty in translating successful strategies between different paradigms. Part of the lack of effect may reflect differences in the number of cells initially transplanted. In our study, substantially more cells were present (U83836E: $62,899 \pm 7952$; untreated: $71,885 \pm 3440$) at 7 days after transplantation than in previous studies (Karlsson et al., 1999; Nakao et al., 1994), which may result in higher levels of lipid peroxidation and free radical production.

iNOS inhibition

iNOS inhibition is effective at promoting dopaminergic neuron survival (Zawada et al., 2001). Peroxynitrite, which is produced by the interaction of nitric oxide (NO) and superoxide ions ($O_2^{\cdot-}$), is implicated in tissue damage after SCI (Xiong et al., 2007; Xiong and Hall, 2009). Reducing levels of NO by inhibition of inducible nitric oxide synthase (iNOS), attenuates the loss of neurons (Wang et al., 2005; Xu et al., 2006), and p38 signaling (Stirling et al., 2008), seen after SCI. Inhibition of iNOS via application of SB203580 was not effective at enhancing SC survival *in vitro* after H_2O_2 administration, or *in vivo* following transplantation. Whether a single application of iNOS inhibitor failed to reduce peroxynitrate production, or whether iNOS is not involved in the death of transplanted SCs, remains to be resolved.

Calpain inhibition

In addition to the role of oxidative stress in tissue damage, uncontrolled release of proteases (including calpains and cathepsins) can exacerbate tissue damage after SCI. The role of calpains in the controlled degradation of intracellular proteins is emerging as an important component of necrotic cell death, and calpain-specific cleavage products can be used to detect necrosis of transplanted cells, including SCs (Emgard et al., 2003; Hill et al., 2007). After SCI, inhibition of calpains enhances tissue preservation and function (Arataki et al., 2005; Colak et al., 2009; Corona and Tapia, 2008; Hung et al., 2005; Ray et al., 2000, 2001; Yu and Geddes, 2007; Yu et al., 2008; Zhang et al., 2003). MDL28170 was developed as a irreversible inhibitor of both isoforms of calpain. It reduces both calpain activity (Zhang et al., 2003), and the lysosomal cysteine protease cathepsin B, albeit at a lower affinity. MDL28170 significantly enhanced SC survival *in vitro*, without reducing LDH release. Because calpains are involved in the breakdown of intracellular proteins, inhibiting calpain may prevent the breakdown of cells whose membranes are disrupted, allowing LDH release. MDL28170 pretreatment also enhanced SC survival *in vivo*; it doubled the number of SCs present 7 days following transplantation into the contused spinal cord. Of all the strategies tested, inhibition of cysteine proteases was the most successful.

In sum, in this study we developed an *in vitro* method of replicating aspects of necrosis and apoptosis seen in a rat model of SCI. We demonstrated that elevated levels of H_2O_2

and withdrawal of serum and mitogens, two conditions present during the transplantation of SCs into the injured spinal cord, are sufficient to induce the death of cultured SCs. Furthermore, the *in vitro* protocol we established permits the quick assessment of drugs for their ability to promote SC survival prior to *in vivo* testing, thus saving time and resources for *in vivo* testing only of the most promising drugs. Most importantly, we demonstrated for the first time that targeting calpains, proteases involved in cellular breakdown and remodeling, is a beneficial strategy for promoting cell transplant survival.

Acknowledgments

We would like to acknowledge and thank the Miami Project Animal Surgery and Care Core, Yelena Pressman, Dr. Beata Frydel, Jenissia Velloz-Jeanty, and Dianna Willis for their assistance with animal surgery and care, Schwann cell production, microscopy, word processing, and figure formatting, respectively.

This research was sponsored by The State of Florida, National Institute of Neurological Disorders and Stroke grants 009923 and 038665, the Miami Project to Cure Paralysis, and the Helen Wilshire Walsh fund. C.E.H. and M.B.B. are members of the Christopher and Dana Reeve Foundation International Research Consortium. M.B.B. is the Christine E. Lynn Distinguished Professor of Neuroscience.

Author Disclosure Statement

No competing financial interests exist.

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