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Labeled Schwann Cell Transplantation: Cell Loss, Host Schwann Cell Replacement, and Strategies to Enhance Survival

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KEY WORDS
spinal cord injury; contusion; Schwann cell; survival; immunosuppression; apoptosis; necrosis; rat; transplantation; alkaline phosphatase; PLAP

ABSTRACT
Although transplanted Schwann cells (SCs) can promote axon regeneration and remyelination and improve recovery in models of spinal cord injury, little is known about their survival and how they interact with host tissue. Using labeled SCs from transgenic rats expressing human placental alkaline phosphatase (PLAP), SC survival in a spinal cord contusion lesion was assessed. Few PLAP SCs survived at 2 weeks after acute transplantation. They died early due to necrosis and apoptosis. Delaying transplantation until 7 days after injury improved survival. A second wave of cell death occurred after surviving cells had integrated into the spinal cord. Survival of PLAP SCs was enhanced by immunosuppression with cyclosporin; delayed transplantation in conjunction with immunosuppression resulted in the best survival. In all cases, transplantation of SCs resulted in extensive infiltration of endogenous $p75^+$ cells into the injury site, suggesting that endogenous SCs may play an important role in the repair observed after SC transplantation.

Extensive studies have been undertaken to replace cells after injury and disease in the CNS, including spinal cord injury (SCI), multiple sclerosis, traumatic brain injury, and stroke. Schwann cells (SCs) have been tested because they offer the possibility of autologous transplantation into humans to promote axon regeneration, remyelination, and restoration of conduction (Bunge and Wood, 2005). Recent technical advances now allow us to discriminate host cells from transplanted cells unambiguously by genetic markers (O'Leary and Blakemore, 1997; Kisseberth et al., 1999; Ruitenberg et al., 2002). We initiated study of transplanted SCs to assess their interaction with host cells after SCI. But poor cell survival at 2 weeks led us to examine instead the time course and potential causes of this loss including: downregulation of marker transgene expression, cell death (necrosis, apoptosis, rejection), and effect of the injury or time of transplantation. In the present work, we report our preliminary findings addressing these questions.

SCs were isolated (see Takami et al., 2002) from either adult female wildtype (wt) Fischer 344 or adult female transgenic rats of a Fischer 344 background expressing human placental alkaline phosphatase (PLAP) (a gift from Dr. Mark Noble, University of Rochester; Kisseberth et al., 1999). Cells were grown in DMEM with 10% fetal bovine serum (FBS) and mitogens [forskolin (2 μM), pituitary extract (20 μg/ml), and heregulin (2.5 nM)] until passage 3. The resulting cultures were >95% pure ($p75^+$). Two million SCs in 6 μl of DMEM-F12 were injected into the uninjured (n = 14) or contused (n = 32; 12.5 g-cm with the NYU/MASCIS device) spinal cord of female Fischer 344 rats after a laminectomy at T9/10. Cells were transplanted either acutely (10 min post-contusion) or at 7 days.

Animals for histological analysis were perfused transcardially with heparinized saline followed by 4% paraformaldehyde. The spinal cords were removed, postfixed overnight, cryoprotected in 30% sucrose/phosphate-buffered saline (PBS) for 48 h, frozen on dry ice, and stored at −80°C until 20-μm-thick serial sections were cut on a cryostat. Animals for DNA analysis were decapitated, and the spinal cord containing the injury site and transplant was rapidly removed, frozen, and stored at −80°C.

To identify transplanted cells, tissue was either reacted enzymatically for alkaline phosphatase (AP) activity using NBT/BCIP (nitroblue tetrazolium [NBT] chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) or stained immunohistochemically (see Hill et al; 2004). For enzymatic treatment, tissue was: washed with Tris buffered saline (TBS); heated for 1 h in TBS to 60°C (to denature endogenous AP, leaving only the heat-stable

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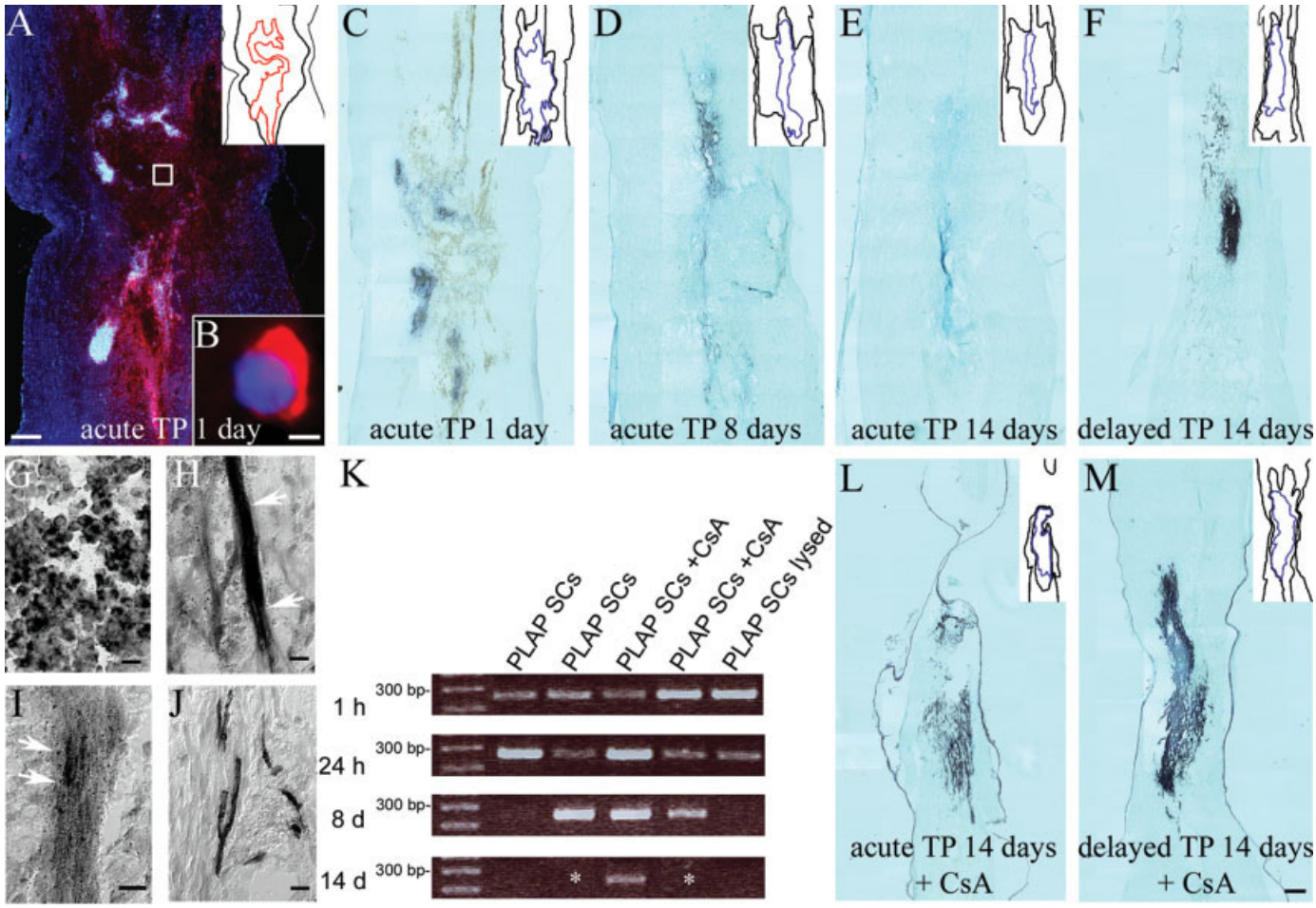

Fig. 1. Placental alkaline phosphatase (PLAP) staining over time after acute transplantation, DNA evidence for cell death, and enhancement of cell survival after delayed transplantation and/or immunosuppression. After acute transplantation, PLAP-labeled cells decrease with time (**A–E**). At 24 h, few host or grafted cells were present within the lesion center (**A,C**). A single PLAP$^+$ cell is shown at higher magnification in **B. A,B:** PLAP immunohistochemistry, red; nuclei counterstained with Hoechst, blue). At this time, grafted cells remained spherical, lacked processes, and were mostly present in clumps around the lesion margin, as shown by increased blue nuclear staining (**A**), and PLAP enzymatic staining (**C**). One clump is shown at higher magnification in **G**. By 8 days, surviving cells had integrated into the spinal cord (**D**) and elongated groups of Schwann cells (SCs) evenly stained for PLAP were present (**H**); by 14 days, however, few PLAP-labeled SCs remained (**E,I**). Survival of grafted SCs was enhanced by delaying transplantation (**F**). In contrast to the punctate staining of cells surviving 14 days after acute transplantation (**I**), individual bipolar cells were healthy appearing, densely stained and scattered throughout the lesion after delayed transplantation (**F,J**). Immunosuppression further augmented survival after both acute (**L**) and delayed (**M**) transplantation. PCR of spinal cord DNA containing grafted PLAP cells produced a 248-bp product (**K**). PLAP DNA PCR products were run on a 1% agarose gel from fresh frozen spinal cords of individual animals, no samples were run in lanes with * at 14 days. PLAP DNA was detected in all PLAP SC transplants (intact or lysed) at 1 and 24 h. At 8 days, PLAP DNA was detected in the spinal cord of 1 of 2 nonimmunosuppressed animals and in both immunosuppressed animals, but not in the spinal cords of animals receiving lysed PLAP cells. At 14 days, PLAP DNA was detected only in samples from animals receiving immunosuppression [both fresh frozen (shown), and fixed]. **C–F,L,M:** PLAP enzymatic staining (purple-black). **J–M:** Nomarski images, PLAP enzymatic staining (black). **A,C–F,L,M** upper right corners: diagrams of injury sites (black) and regions of transplant (**A:** red; **C–F,L,M:** blue). Scale bars = 100 μm in **A,C–F,L,M**; 10 μm in **G–J**; 2.5 μm in **B**.

PLAP enzyme active); preincubated in AP buffer (pH 9.5); and reacted with NBT (0.35 mg/ml; Roche, Indianapolis, IN)/BCIP (0.225 mg/ml; Roche) for 1.5 h with lavamisol (5 mM), to block any residual endogenouos AP activity. The resulting precipitate ranged in color from purple to black (Figs. 1C–F,L,M and 2A–F). Some tissue sections were counterstained with methyl green, which produced aquamarine nuclei (Fig. 2A–C,E,F). Slides were air-dried and mounted with Vectamount (Vector Laboratories, Burlingame, CA). For double label immunostaining, tissue was washed with PBS; blocked with 5% normal goat serum and 0.1% Tween-20 in PBS; incubated with primary antibodies overnight at 4°C (PLAP mouse monoclonal IgG2a, 1:200, Sigma, St. Louis, MO; p75, IgG1 supernatant; from IgG192 hybridoma cells provided by Eugene Johnson); washed; and incubated with secondary antibodies for 2 h at room temperature (goat-anti-mouse IgG2a-594, 1:200; goat-anti-mouse IgG1-488, 1:200) (Molecular Probes, Eugene, OR). Slides were examined in a Zeiss Axophot microscope (obtaining color images with an Optronics DEI-750 CE digital camera; Fig. 2), or an Olympus 1X70 inverted microscope with or without Nomarski optics (obtaining black-and-white images with a Cooke SensiCam QE high-performance digital camera; Figs. 1 and 3).

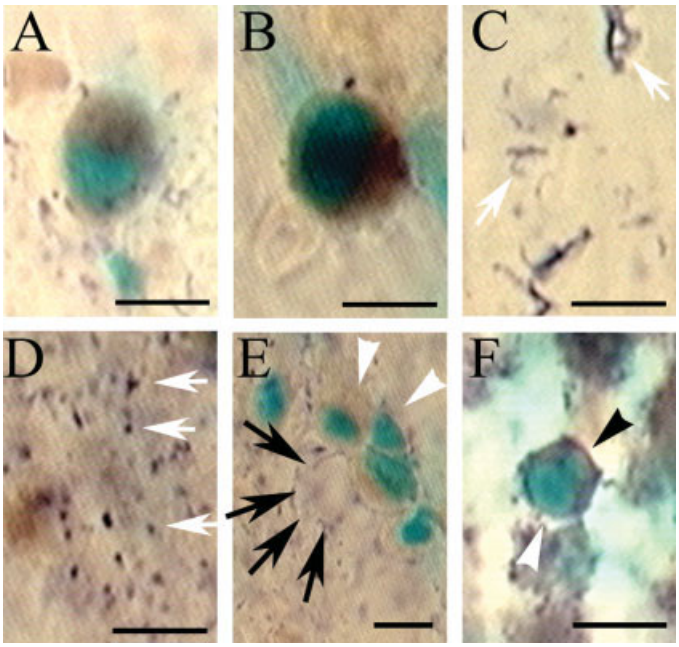

Fig. 2. Morphology of cells and histological evidence of cell death early after transplantation. Cells varied in enzymatic placental alkaline phosphatase (PLAP) staining intensity from pale (**A**) to intense (**B**). Evidence for necrotic cell death included noncellular strands (arrows, **C**) and punctate spots (arrows, **D**) of PLAP cell membrane remnants within and around the injury site and circular "ghost" profiles without nuclei (arrows in **E**) next to faintly stained PLAP cells (arrowheads in **E**). Apoptotic PLAP cells (shrunken with pyknotic, methyl green-stained nuclei; arrowheads) were present (**F**). **A–F:** PLAP enzymatic staining (purple-black), nuclei counterstained with methyl green (aquamarine; see arrowheads). Scale bars = 5 μm in **A–F**.

To detect PLAP DNA, DNA was extracted from either a 10-mm block of spinal cord containing the transplant or 2×10^6 cultured PLAP cells (Qiagen DNA kit, Valencia, CA), according to the manufacturer's instructions. Spectrophotometry showed that DNA could be extracted from fresh-frozen or fixed material. Total DNA from each sample was quantified and 215 ng was amplified using primers to the SV-40 poly A region of the inserted PLAP transgene [Kisseberth et al., 1999; 50 ng left primer, CTGATGAATGGGAGCAGTGGTGGAATG; 50 ng right primer, CAGTGCAGCTTTTTCCTTTGTGGTGTA; 0.2× SyBr green (Molecular Probes, Eugene, OR); 1.25 U *Taq*; 5 mM $MgCl_2$; and 0.2 mM dNTPs, in buffer (Invitrogen, Carlsbad, CA)]. A dilution series of DNA collected from cultured cells, equivalent to 1, 5, 10, 50, 100, 500, 1,000, 5,000, 10,000, and 50,000 cells was run to estimate the lowest number of PLAP cells detectable. DNA was amplified using a Rotogene 3000 PCR machine (Corbett Research, Sydney, Australia; DNA was denatured at 95°C, 10 min; followed by 40 cycles of 95°C, 20 s; 68°C, 20 s; 72°C, 20 s; 82°C, 20 s) and end point gels were run to detect the presence or absence of PLAP DNA. Duplicate samples from each cord were run in duplicate experiments.

To understand the loss of PLAP label, spinal cords were initially examined histologically after transplantation into uninjured or acutely contused spinal cord (for animal details, see Table 1). As in a study transplanting only 40,000 SCs into uninjured spinal cord (Iwashita et al., 2000), there were fewer labeled cells with increasing time post-transplantation. At three and 24 h, the main area of the transplant showed strong PLAP staining localized to spherical cells that lacked processes. At 8 days, $PLAP^+$ cells had integrated into the spinal cord; long bipolar cells were in parallel arrays among host cells. By 14 days, $PLAP^+$ cells were no longer found.

A loss of $PLAP^+$ SCs with time also followed transplantation into the acutely contused spinal cord (Fig. 1). At 24 h, the injury site contained few nuclei (both host and transplanted cells), although transplanted cells were present in small clumps around the injury margin (Fig. 1A, C,G). By 8 days, transplanted cells, bipolar and thus SC-like, were integrated among host cells within and adjacent to the lesion (Fig. 1D,H). By 14 days, however, only a few cells remained (Fig. 1E,I). This temporal profile of PLAP staining suggests that most cells die early after injury, but that some cells survive the first week and integrate into the spinal cord only to be lost in a second wave of cell death occurring between 8 and 14 days.

To prove that disappearance of PLAP protein expression was not due to transgene downregulation, PCR was performed to detect PLAP DNA at 1 or 24 h after transplantation into uninjured spinal cord and 1 h, 24 h, 8 days, or 14 days after transplantation into the acutely contused spinal cord (for animal details, see Table 2). PLAP DNA was detectable in all animals up to 24 h after transplantation of intact or lysed cells into either the uninjured or acutely injured spinal cord. PLAP DNA was detectable beyond 1 week in only 25% of animals receiving intact cells, and in no animals receiving lysed cells (Table 3). PLAP DNA from transplanted intact cells was detectable at 1 h, 24 h, and 8 days, but not at 14 days in the acutely contused cord (Fig. 1K). The loss of PLAP SC DNA proves that the loss of PLAP staining observed histologically is not due to decreased transgene expression but to SC death after transplantation into the uninjured or acutely contused spinal cord. Although some $PLAP^+$ cells were observed histologically at 14 days, we were unable to detect any PLAP DNA by PCR. Using a standard curve, we established that 100 cells needed to be present within the sample to detect PLAP DNA, corresponding to 10,000 cells within a 10-mm spinal cord segment. Thus, under the conditions used, histological staining was better able to detect a small number of cells in a large volume of tissue.

SC transplants were examined for morphological evidence of necrosis and apoptosis after injection into the uninjured or acutely contused spinal cord (Fig. 2). As early as 3 h, necrotic SCs were observed in the uninjured spinal cord, whereas apoptotic SCs were observed in both the uninjured and acutely contused spinal cord by 24 h. At early time points, the cells remained spherical and lacked processes (Fig. 2A,B). Within and around the transplant there were intact cells with punctate membrane staining, noncellular strands of PLAP staining suggestive of lysed PLAP cell membranes (Fig. 2C,D), and ghost cells, regions the size of cells enclosed by a faintly PLAP stained border but without a nucleus (Fig. 2E), all

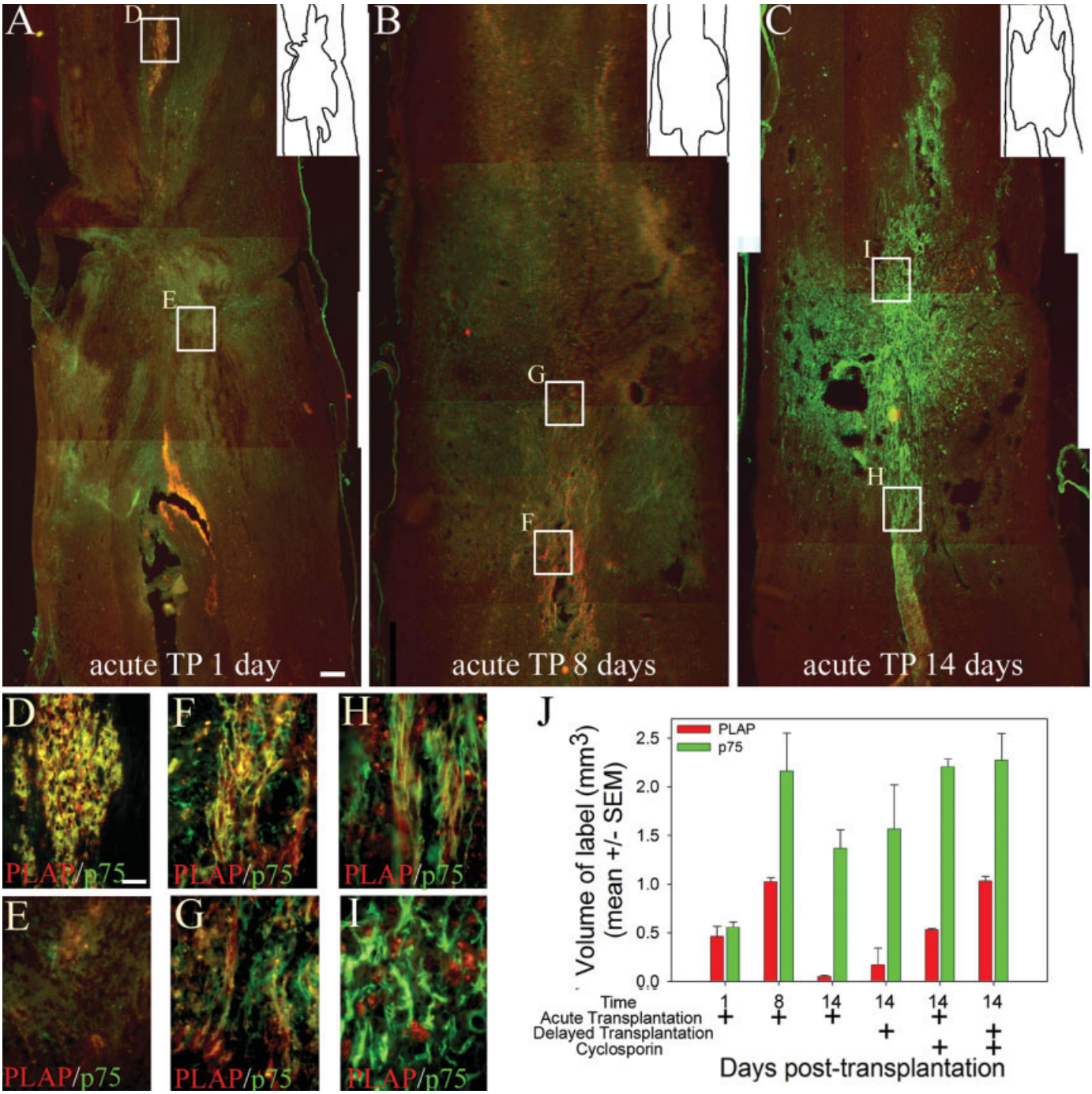

Fig. 3. Endogenous SCs invade the spinal cord concurrent with the death of transplanted Schwann cells (SCs). p75 staining (green) increased, while placental alkaline phosphatase (PLAP) staining (red) decreased during the first 14 days after transplantation of PLAP SCs into the acutely contused spinal cord (**A–C,J**). Initially p75 was only expressed in PLAP SCs (**A,D**), and not at the injury epicenter (**A,E**). At 8 days, PLAP SCs continued to express p75 (**B,F**), but regions were found with little PLAP staining accompanying p75 staining (**B,G**). By 14 days, when only a few PLAP SCs were present (**C,H**), p75 was located throughout the lesion epicenter (**C,I**). **J:** Volume of PLAP and p75 staining quantified by outlining the lesion site (excluding the roots) in every eighth tissue section and marking positive staining with the Cavalieri method, using Stereoinvestigator. Early after transplantation, p75 and PLAP volumes were similar. Within the first week, p75 staining volume increased becoming greater than that of PLAP by 14 days, regardless of the extent of cell survival (**J**). The PLAP volume measurements highlight the second wave of cell death between 8 and 14 days (**J**); the first wave occurred before 1 day. The increased PLAP volume between 1 and 8 days (**J**) corresponded with the change in cell morphology from densely packed, round cells at 1 day (**D**) to dispersed bipolar cells at 8 days (**F**). Outlines of injury sites, **A–C**. Scale bars = 100 μm in **A–C**; 25 μm in **D–I**.

characteristics of necrosis. Individual cells exhibited varying staining intensities (Fig. 2A,B). Some cells contained condensed fragmented nuclei (Fig. 2F; arrowheads) indicative of apoptosis. Further studies are under way to quantify the relative contributions of necrosis and apoptosis using immunohistochemical markers.

TABLE 1. Proportion (and Numbers) of Transplanted Rats With PLAP SCs Detectable by Histology

	Time post-transplantation			
Group	3 h	24 h	8 days	14 days
Uninjured spinal cord	1/1	2/2	2/2	0/1
Contused spinal cord				
Acute transplantation	—	2/2	2/2	3/3[a]
Acute transplantation + cyclosporin	—	—	—	3/3[a]
Delayed transplantation	—	—	—	2/2
Delayed transplantation + cyclosporin	—	—	—	2/2

[a]One animal from each of these groups was also included in the DNA analysis. Numerator is the number of rats with detectable PLAP staining. Denominator is the total number of rats in the group.

*TABLE 2. Number of Rats Used to Detect Transplanted PLAP SCs by PCR**

		Time post-transplantation			
Group	Schwann cells	1 h	24 h	8 days	14 days
Uninjured spinal cord	Wildtype	1	1	—	—
	PLAP	2	2	—	—
	Lysed PLAP	1	1	—	—
Contused spinal cord					
Acute transplant	PLAP	2	2	2	2[a]
	Lysed PLAP	1	1	1	1
Acute transplant + cyclosporin	PLAP	2	2	2	2[a]

*PCR was performed on DNA collected from 10 mm of freshly frozen tissue centered on the transplant.
[a]Spinal cord from one animal from each group was perfused with 4% paraformaldehyde and the DNA was then isolated from every fourth tissue section.

TABLE 3. Percentage (and Numbers) of Transplanted Rats With PLAP DNA Detectable by PCR

	Time post-transplantation	
Schwann cells	≤24 h	≥8 days
Wildtype	0% (0/2)	—
Lysed PLAP	100% (4/4)	0% (0/2)
PLAP	100% (8/8)	25% (1/4)
PLAP + cyclosporin	100% (4/4)	100% (4/4)

Each cell preparation includes all transplants performed (injured and uninjured). Time post-transplantation is divided into early, ≤ 24 hours (1 hour and 24 hour animals), and late, ≥ 8 days (8 and 14 day animals). Numerator is the number of rats with detectable DNA. Denominator is the total number of rats in the group.

The acute loss of labeled SCs within the injured spinal cord, occurring at a time when host neurons and glia are undergoing cell death (Grossman et al., 2001), is likely mediated by the injury environment. To test the role of this acute injury environment, we delayed SC transplantation for 7 days after contusion and examined cell survival 14 days later. Previous experiments with unlabeled SCs suggested that they survive best when transplanted either immediately or after a delay of at least 1 week (Martin et al., 1996). Delaying transplantation for 7 days improved survival of PLAP SCs (Fig. 1F,J), supporting a role of the acute injury environment in the loss of injected cells.

Despite improved survival with delayed transplantation, only a small region of PLAP staining was observed at 14 days (Fig. 1F), suggesting that other factors must influence SC survival. For example, the second wave of SC death occurring after SC integration into the spinal cord tissue could result from transplant rejection. Previously, long-term survival of PLAP precursor cells was observed after transplantation into the acutely injured spinal cord; the use of different rat strains necessitated immunosuppression in these experiments (Hill et al., 2004). In the current studies, allogenic transplants were prepared using Fischer rats as donors and recipients. Genetic similarity, generally, obviates immunosuppression under these conditions. It has been demonstrated recently, however, that expression of foreign proteins within CNS cells transduced in vivo can elicit immune-mediated rejection, which is prevented by immunosuppression (Doi et al., 2004). This suggests that foreign proteins expressed within the CNS can be appropriately presented to host immune cells and recognized as non-self, resulting in immune-mediated rejection.

To test whether survival of allogenic PLAP SCs could be enhanced by immunosuppression, 10mg/kg cyclosporin was administered intraperitoneally daily (see Hill et al., 2004), after either acute or delayed transplantation. Fourteen days later, all rats receiving immunosuppression (n = 6) exhibited surviving PLAP cells (Fig. 1K,L,M). Improved survival with immunosuppression was confirmed with PCR after acute transplantation; PLAP DNA was detectable in all of animals at 1 h, 24 h, 8 days, and 14 days post-transplantation (Fig. 1K; Table 3), although the estimated number of surviving cells detected both histologically and by PCR at 14 days was low (<50,000 cells). Using immunosuppression in conjunction with delayed transplantation further enhanced PLAP SC survival 14 days post-transplantation (Fig. 1M). The data suggest that immunosuppression is required for survival of SCs from transgenic PLAP rats, even though both host animals and grafted cells are from inbred rats of a Fischer background. These results suggest that a potential immune response to genetic markers used to track transplanted cells must be considered.

A surprising finding of these studies was that, in both the uninjured and contused spinal cord, the volume of p75 staining increased with increasing time post transplantation despite the death of labeled cells (Fig. 3). p75 has been used routinely to identify transplanted SCs, despite substantial evidence that host SCs invade the injured spinal cord (reviewed in Bunge and Wood, 2005). Within the injury, p75 was initially co-localized with $PLAP^+$ SCs. At 8 days the volume of PLAP staining increased as the PLAP cells integrated into the lesion site; at this time, however, only 50% of the p75 staining colocalized with PLAP staining (Fig. 3J). By 14 days, most p75 staining within the lesion did not correspond with PLAP staining. Thus, a substantial number of endogenous SCs invade SC transplants in which few transplanted cells survive. The time course of loss of PLAP staining and the increase in the volume of p75 staining suggests that the death of transplanted SCs and the infiltration of host SCs occur simultaneously. The infiltration of endogenous SCs may play, therefore, an important role in recovery after transplantation into spinal cord injuries.

Our preliminary experiments reported here reveal that the loss of cellular label is due to the death of

transplanted cells rather than transgene downregulation, with apoptosis, necrosis and rejection playing a role. Although there was a loss of labeled cells with time, both delaying transplantation and immunosuppression improved PLAP SC survival. This additive effect indicates multiple causes of cell death after PLAP cell transplantation. Early results from a more comprehensive experiment currently underway using lentivirally transduced SCs expressing green fluorescent protein intracellularly indicate an early loss of cells (via necrosis and apoptosis) after delayed transplantation as well. Because the cell death observed with PLAP SCs may be a general phenomenon of transplanted SCs, not specific to PLAP cells, additional strategies may be needed to improve acute survival of injected cells.

Concurrent with transplanted SC death, host SCs invaded the transplant site in greater numbers than after injury alone. Recently SCs also have been shown to invade partial spinal cord lesions containing labeled olfactory ensheathing glia (Ramer et al., 2004) and bone marrow stromal cells (Lu et al., 2005). In addition, we have observed invasion of endogenous $p75^+$ SCs into Matrigel and fibrin cables containing transplanted lysed SCs (with and without surrounding polymer channels, respectively) placed into completely transected spinal cord (unpublished data). Together, these observations raise a number of important questions. Do dying SCs release factors that induce endogenous SC migration? If so, what are they? To what extent do endogenous SCs account for the remyelination, regeneration and improvement in conduction observed in previous studies? Given the propensity for host SC migration into transplants of lysed SCs and the loss SCs after transplantation, the possibility exists that the effects observed in studies with unlabeled SCs or SCs labeled with dyes may have been due to host SC invasion. Further experiments are needed to assess the relative contributions of host and grafted cells to repair after SCI. Understanding cell survival is particularly important for experiments that use transduced cells to express growth promoting molecules as a means of improving neurological repair.

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