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Final Narrative Report

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Final Narrative Report 02-3020-SCR-S-O Martin Grumet, Ph.D

## 1. Original Aims of the Project

Following traumatic spinal cord injury there is a short period in which neural cells die and a much longer period during which primarily glial cells continue to die (Liu et al., 1997). Understanding this cytotoxicity is of great importance for the design and testing of new therapeutic modalities to improve recovery following neural injury. While much has been learned about apoptosis and necrosis, little is still known about molecular mechanisms that underlie the death of cells in the spinal cord following injury. Therefore, there is a need for in vitro models to study molecules responsible for cytotoxic activity. In this grant we proposed to analyze the temporal sequence and biochemical nature of the cytotoxic activity using in vitro and in vivo assays.

Our first specific aim was to use in vitro assays to determine quantitatively how much activity is generated and how long it persists following traumatic spinal cord injury using the MASCIS Impactor. We also tested the sensitivity of neural stem cells, which are being evaluated for transplantation to promote nerve regeneration. Parallel studies were performed to analyze the survival of stem cells transplanted into the spinal cord at various times following contusive injury. Our second specific aim was to determine the biochemical nature of the cytotoxic activity.

## 2. Project Progress

To analyze cytotoxic activity in the spinal cord following injury, we developed an in vitro assay to analyze cytotoxic activity in extracts of spinal cords. For the assay we have contused Sprague Dawley rats using the MASCIS Impactor (25 mm wt drop) and then sacrificed the animals 2, 14 or 28 days later and isolated cords without fixation. Segments of the cords (~0.5 cm) were cut from the impact region and homogenized (in 500 $\mu$l buffer for the tissue which is ~ 15 $\mu$l) using a Polytron Homogenizer (Brinkman) in a physiological buffer (PBS containing the Complete protease inhibitor mix from Roche). The extracts were centrifuged in a microfuge for 30 min, 4°C, at 15,000 rpm and the supernatant fraction was collected. Supernatants from contused cords were compared with those isolated form sham controls.

Extracts were then tested for cytotoxic activity in culture in 24 well plates using C6-R glial cells (Friedlander et al., 1998). Equal numbers of cells (~100,000) were plated in each well in 0.5 ml of defined medium (DMEM supplemented with ITS); this medium allows these cells as well as many neural cells to survive with little or no proliferation and facilitates analysis of cytotoxicity without complications of serum such as cell proliferation. The next day, cells are exposed to different concentrations of the extracts in 0.5 ml of defined medium and cultured for 3-4 days. A comparison of extracts from uninjured and post-injured cords showed dramatic differences when the cells were treated with propidium iodide (PI), which labels dead or dying cells, and Hoechst, which labels all nuclei (Fig. 1A). At 4 days following addition of the extracts, cells treated with 6% (by volume) of the normal cord extract did not show PI-labeled dead cells, rather most of the GFP fluorescent cells appeared healthy. We observed many PI-labeled cells in the cultures treated with 2 week contused spinal cord extracts. Also, the strong PI-positive nuclei appear to have less or no GFP fluorescence consistent wit the idea that they loose their cytoplasmic GFP label when the plasma membrane breaks down in dying cells. Thus the total number of cells is obtained from the number of PI-positive cells plus the number of strongly GFP-positive, which are PI-negative. The percent of dead cells is then calculated as the PI-positive/total. Figure 1B shows a representative experiment where there was no cytotoxic activity in controls, low activity at 7 days, maximal activity at 14 days, and low activity at 28 days. This trend was

reproducible in three experiments, however, the baseline and maximal levels of activity varied making it challenging to combine data from different experiments. It appears that maximal cytotoxic activity for these glial cells is with extracts prepared from contused spinal cord 2 weeks following contusion.

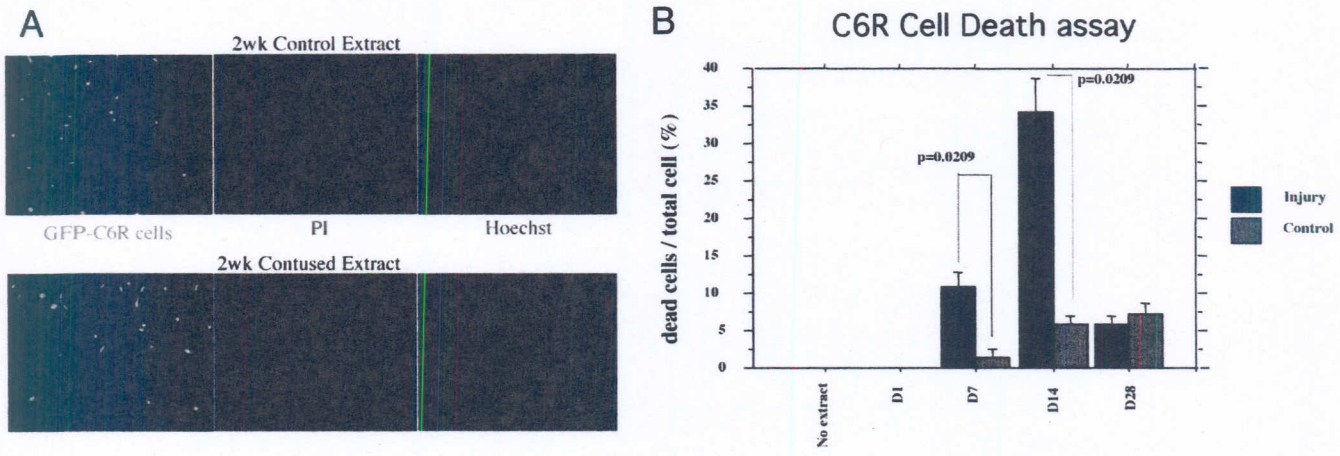



**Figure 1. Cell death assay.** GFP expressing C6-R cells were cultures for 1 day and then extracts of spinal cord were added as indicated (upper panel is control and lower is from contused spinal cord). Four days later the cells were treated with propidium iodide (PI) and Hoechst and were imaged for GFP fluorescence (green), PI (red) and Hoechst (blue). Note the dead cell loose GFP fluorescence and gain red fluorescence. Quantitation in triplicate of the numbers of dead cells in one experiment is shown in panel B, note peak activity in extracts prepared from spinal cords at 14 days (D14) following injury.

Experiments to fractionate the cytotoxic using size exclusion filters indicated that the activity partitioned into both fractions. This underscored the need as noted above, to titrate the extracts in order to determine dose-response relationships and establish specific activities for different fractions. Interestingly, experiments to do this and compare the total activity present even before fractionation by centrifugation, indicated that the majority of the activity is present in the pellet and not in the supernatant. Thus, there may be multiple activities, and the pellet, possibly representing plasma membranes, may represent a rich source of activity. This made fractionation quite complex. Nevertheless, the results indicate peak cytotoxic activity at ~2 weeks after injury which diminished significantly by 4 weeks.

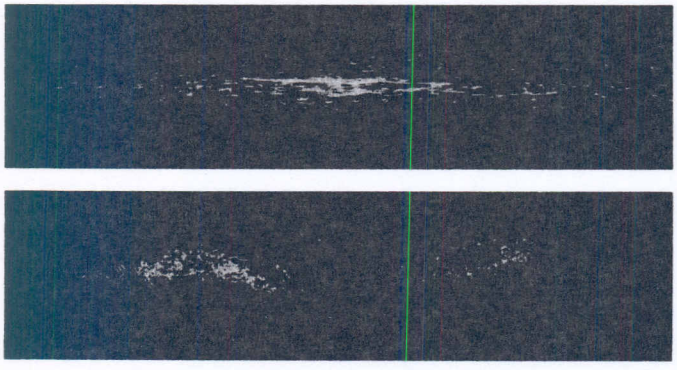
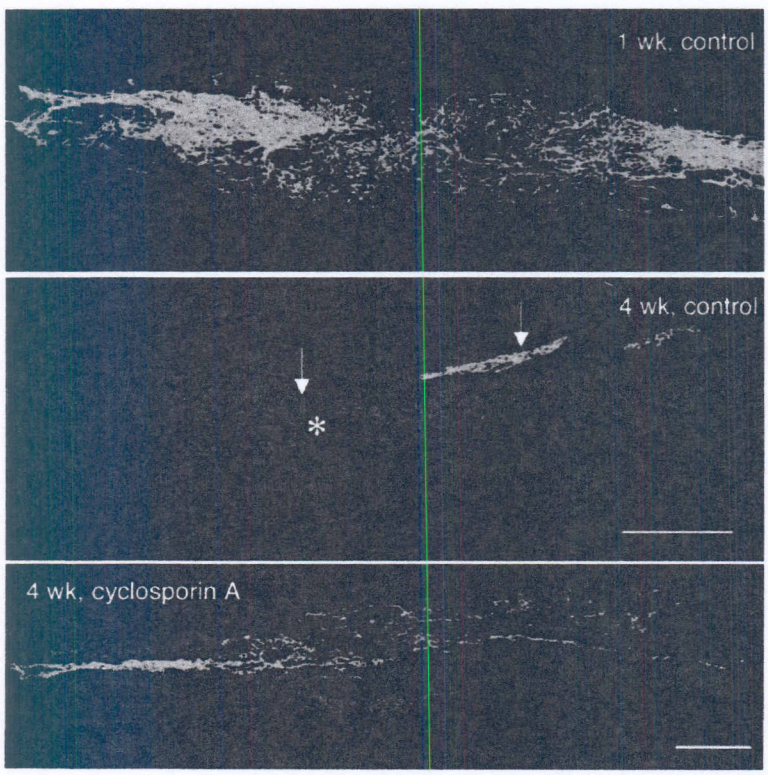


**Figure 2. Transplantation of GFP-labeled neural stem cells**. Top is control and bottom is contused spinal cord with transplants of neural stem cell line G3.6. Note the survival and spread of the neural stem cells (green) in control spinal cord (red is neurofilament stain). In the bottom panel, cysts develop after injury but cells do not survive in these regions 6 weeks after acute transplant. Cell survival is poor and typically seen rostral and caudal to the injury site; red stain here is for GFAP.

Recent unpublished studies in our laboratory (as well as by others) have revealed that the survival of cells transplanted into contusion sites is very poor immediately following contusion but

improves with increasing times following contusion. We have found, for example, that implantation of C6-R cells, which survive quite well in normal spinal cord (Hormigo et al., 2001), do not survive well in the contused cord when implanted immediately following contusion; their survival improves dramatically when implanted four weeks following contusion, consistent with the reduced cytotoxicity in vitro of extracts prepared 4 weeks following contusion (Fig. 1B). A preliminary report of this work has been published in (Hasegawa and Grumet, 2003). We have analyzed survival of neural stem cells in the spinal cord following contusion. Figure 2 shows horizontal sections of spinal cord that had GFP-labeled neural stem cells injected following spinal cord contusion. Note the cyst formation following contusion with the GFP cells found partially surrounding the cyst and infiltrating into the white matter tracts both rostrally and caudally. However, there is poor survival of the cells in the cyst that develops in the injury site.

Cytotoxic activity in the contused spinal cord may arise from different sources one of which is the immune system. Our standard protocol for cell transplantation includes administration of cyclosporin to the rats following contusion since it has been believed to promote survival. To investigate whether cyclosporin indeed affects cell survival, we performed transplants of GFP labeled cells with and without cyclosporin. At 1 week following transplantation there was little difference but by 4 weeks there were few if any cells detected when cyclosporin was not administered. However, cyclosporin provided significant protection of cell loss at 2 and 4 weeks (Fig. 3). These results indicate the importance of cyclosporin treatment for survival of at least certain transplanted cells. All our experiments with extracts from spinal cords have been treated with cyclosporin, which should presumably limit cell mediated immunity. However, it would not be surprising to find contributions of humoral immunity to cytotoxicity as soluble factors such as cytokines are still generated even with cyclosporin.



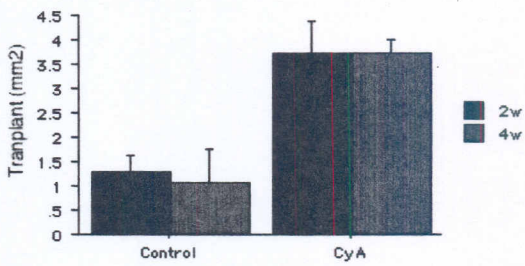




**Figure 3. Cyclosporin protection.** GFP-olfactory ensheathing cells transplanted without (control) or with cyclosporin (CyA). Note on left (bottom panel) the dramatic increase in survival at 4 weeks when treated with cyclosporin in contrast to the poor survival without treatment (middle panel). Cells survive well at 1 week even without treatment (upper panel). *, injury site. Arrows indicate the 2 injection sites. Bars = 1 mm. Graph on top shows Quantitation of the areas ($mm^2$) occupied by the GFP fluorescent cells surviving with or without CyA at 2 and 4 weeks following injury and transplant.

Macrophages infiltrate very soon after injury in contusion and reach a peak level at about 2 wk (not shown), which correlates with the maximal cytotoxic activity detected in the in vitro assay (Fig. 1). Thus, the cytotoxicity may be related to the macrophage response.

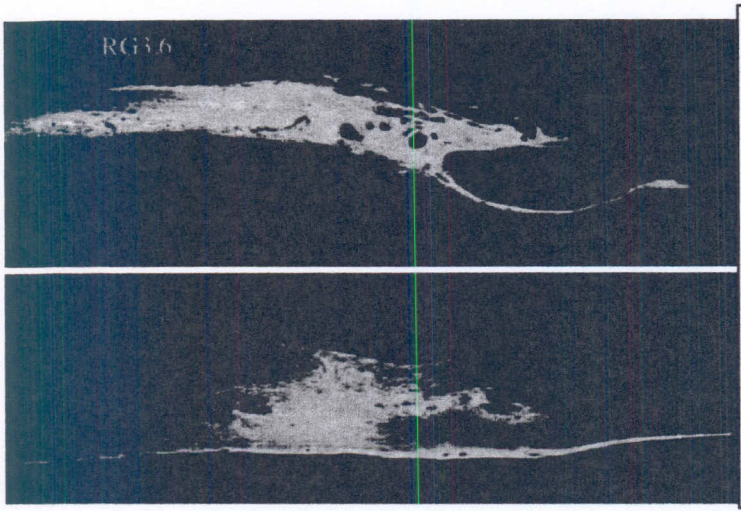



**Figure 4. Transplantation of GFP-labeled neural stem cells 4 weeks after injury.** Transplants of GFP-labeled neural stem cell clone G3.6. Note the extensive survival and spread of the neural stem cells (green) (red is neurofilament stain) by comparison to the poor survival of GFP+ cells observed in the injury site implanted immediately following injury (compare with lower panel in Fig. 2). Interestingly, transplants at 4 weeks were usually restricted to certain regions that could be rostral as shown in the two examples here or caudal, but some cells can cross the injury site (e.g., around the cyst in top panel).

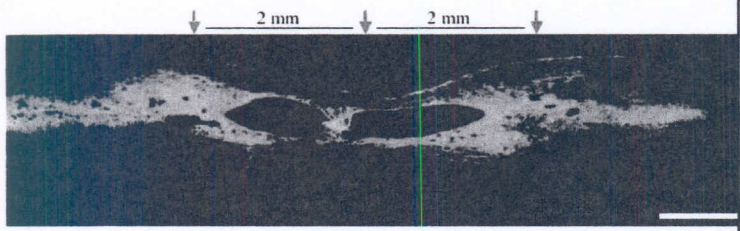



**Figure 5. Acute Transplantation of G3.6 cells rostral and caudal to the injury site.** We discovered that the 3-point delivery protocol (indicated by arrows) resulted in significant cell survival 6 weeks later. This resulted in greater preservation or growth of neurofilaments (Fig. 6).

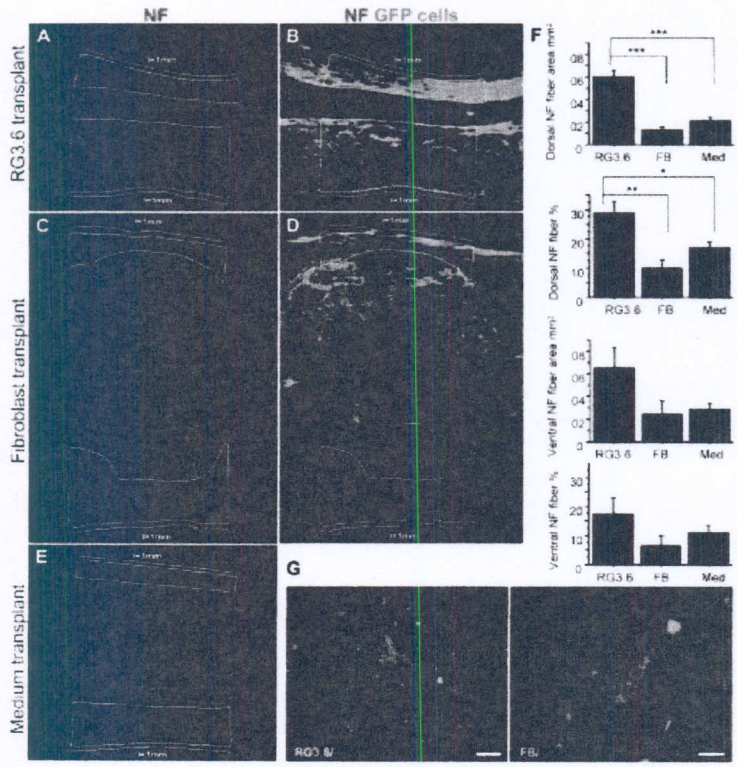



**Figure 6. Acute Transplantation of G3.6 results in increased staining for neurofilaments (NF).** Using the 3-point delivery protocol shown in Fig. 5, we observed increased organization and preservation or growth of neurofilaments. Sections stained for NF are shown for rats transplanted with RG3.6 (A and B), fibroblasts (C and D) and medium (E) 6 weeks after spinal cord injury. B and D are merged images. Spared dorsal and ventral fiber tracts were outlined (A-E) within 1 mm from the dorsal and ventral surfaces of the spinal cord in para-sagittal sections and then measured using Zeiss confocal LSM software (F). Percentages of dorsal and ventral NF+ areas were normalized to the outlined areas (F). Data shown are means ± SEM with 3 rats per group. (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Panel G shows that RG3.6 cells (left) promote growth of neurites whereas fibroblasts did not (right).

Our experiments revealed two approaches to circumvent the problem of cytotoxicity in vivo. Given that the peak cytotoxicity in vitro was at 2 weeks and diminished dramatically by 4 weeks, we tested for cells survival and found poor survival in vivo acutely when transplanted into the injury site (Fig. 2, lower panel) and much better survival when transplanted at 4 weeks. Implantation into the 4 week-old injury site provides information on cell behavior during the chronic phase following contusive injury. At the time of transplant (4 weeks following injury), cysts have formed that are surrounded by a dense matrix containing chondroitin sulfate proteoglycans among other molecules that may be inhibitory. Thus it is not surprising that in these situations the migration of the cells is quite variable (e.g. Fig. 4). However, survival was quite good. Thus, delayed transplantation may be a viable approach but may require additional treatments to neutralize or remove inhibitory molecules such as chondroitin sulfate proteoglycans. This is an area that we are continuing to pursue.

The second approach to circumvent the problem of cytotoxicity in vivo is to implant the cells acutely in regions that are adjacent to the site of injury. Our results indicate that cysts typically form in a rostrocaudal region of ~1-2 mm. Preliminary results using single injections at the site of injury or rostral or caudal indicated good cell survival 1-2 mm away from the injury site. Therefore, we developed a method of implanting cells immediately following contusion ~1-2 mm rostral and caudal to the center of the contusion site that can easily be defined at the time of injury (Fig 5). The results show good cell survival 6 weeks later with cells extending far beyond the site of injection into adjacent fairly normal cord as well as bridging across the injury site (Fig 5). Measurements of the areas including the GFP cells that were positive for neurofilaments in the dorsal and ventral fiber tracts indicated that more neurofilaments were present with RG3.6 transplants than with fibroblasts or media controls (Fig 6). These results suggest that the RG3.6 either preserved regions rich in NF by associating with those cells and possibly protecting them or inducing regrowth of axons. Both of these may also occur and future studies will investigate these issues. Preliminary studies indicate in culture that RG3.6 cells can promote neurite outgrowth by contrast to fibroblasts which do not (Fig. 6G), providing support for the idea that radial glia interact with axons and may promote axon growth as described above.

**3. Project Challenges**

A problem was encountered that much of the cytotoxic activity may be associated with an insoluble fraction that previously was discarded and never analyzed. This made biochemical characterization very challenging and we decided to focus more on the cell transplantation studies that revealed two approaches to promoting the survival of transplanted cells into contused spinal cords.

**4. Implications for future research**

One limitation of cell transplantation acutely following spinal cord contusion is the poor survival of cells in the injury site. Our results suggest two approaches to promoting the survival of transplanted cells into contused spinal cords. One is to transplant acutely adjacent to the injury site where the cells can survive, migrate and may have multiple affects including tissue preservation and possibly axonal regrowth. The second approach indicates that a suitable delay, which in the rat contusion model was 4 weeks, allows transplanted cells to survive in the injury site but issues with their migration remain to be resolved.

**5. Plans to continue research**

As noted above, we plan to continue with both acute and chronic transplantation and we are planning to submit a grant to the NIH to support this work. We have already received funding from the

NJCSCR to pursue alternative approaches to generate radial glia for transplantation that do not rely on the introduction of the v-myc oncogene that was used to generate the RG3.6 cells.

## 6. Publications

Hasegawa, K. and Grumet, M. Trauma induces tumorigenesis of cells implanted into rat spinal cord. J. Neurosurgery 98:1065-1071 (2003).

Hasegawa, K., Chang, Y.-W., Li. H., Berlin, Y. Ikeda, O., Kane-Goldsmith, and Grumet, M. Embryonic radial glia bridge spinal cord lesions and promote functional recovery following spinal cord injury (submitted)