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New Jersey Commission on Spinal Cord Research Final Report

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"Study of activated macrophage transplantation into rat spinal cord contusion model"

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Specific Aims:

Recently, studies have shown that local implantation of macrophages, previously exposed ex vivo to segments of peripheral nerve, led to some axonal regrowth in transected rat spinal cords. To understand the mechanism of this functional recovery, we will transplant eGFP macrophages into the rat spinal cord contusion model.

Aim 1: Determine whether activated macrophages express different levels of cytokines and neurotrophins depending on activation with lipopolysaccharide (LPS) and peripheral nerve (PN). In addition, we will determine the activation state of transplanted macrophage cells in injured spinal cords. The expression level of cytokines and neurotrophins in activated macrophages will be detected by RNase Protection Assay (RPA) and the NGEL gene chip (10,000 rat genes) at different times after activation in tissue culture, as well as after injury and cell transplantation. With a combination of cytokine and neurotrophin probes, the experiments will show whether transplants affect cytokines and neurotrophins levels, as well as other genes. It will help us to understand whether and how transplanted macrophages promote regeneration and repair in injured spinal cords.

Aim 2: Ascertain the fate and activation states of transplanted macrophages? The experiments will establish the distribution and migration pattern of eGFP transplanted macrophages, compared against endogenous macrophages (stained immunohistologically by ED-1). The eGFP transgenic rat expresses the green fluorescent protein (GFP) in all cells and should provide an unambiguous identification of the transplanted cells; we will be validating this by transplanting male eGFP macrophages to female rats and doing in situ hybridization with a custom Y-chromosome probe to confirm that all GFP labeled cells have Y-chromosomes. In addition, the experiments will assess the effects of methylprednisolone, a glucocorticoid steroid that is routinely given in human spinal cord injury, on the number and distribution of endogenous and exogenous macrophages. Using eGFP rats as macrophage cell donors, we will observe the temporal and spatial distribution of transplanted macrophages by confocal microscopy. This will provide direct evidence of transplant survival, migration and distribution in the injured spinal cord.

Aim 3: Establish the effects of activated macrophage transplants on corticospinal tract regeneration and locomotor recovery in rats. The transplants will be done at 1 day and 4 weeks after injury. We will examine the spinal cords to assess corticospinal tract regeneration and remyelination, as well as in situ hybridization to assess long-term activated states of the transplanted macrophages. The morphological data will be correlated with locomotor recovery assessed by weekly BBB scores (Basso-Beattie-Bresnahan). These experiments will indicate whether there are discrepancies in regeneration and recovery associated with early or delayed macrophage transplants.

Overall summary

We have proposed to study the mechanisms of activated macrophages improving spinal cord injury recovery. First, we have assessed the expression of cytokines and neurotrophins in activated macrophages by micro-array and quantitative real time PCR (RT-PCR). Second, we have determined the spatial and temporal distribution of macrophages as well as expression profile of cytokines and neurotrophins in transplanted spinal cords. Third, we have assessed the effect of early and delayed transplants of activated macrophages on long term locomotor recovery and regeneration.

In the past two years, we have made steady progress on this project. A Green Fluorescence Protein (GFP) rat breeding colony was successfully established after mating GFP males with Wild Type (WT) female rats. This is an important step to ensure a constant supply of GFP macrophages for cell culture and animal transplantation. A standardized macrophage extraction and purification protocol was also established after multiple trials. An estimated 3,000,000 cells could be collected from each animal. Purified GFP macrophages remained their morphology and showed ED-1 immuno-reactivity in culture. Different activation methods have been tested under tissue culture conditions and the activated macrophages were collected for micro-array, quantitative RT-PCR and cell transplant studies. Both Lipopolysaccharide (LPS) and peripheral nerve segment (PNS) were used to activate purified macrophage from GFP and WT rats. Microarray and RT-PCR assays were carried out to determine mRNA profile of activated macrophages. Our results showed that many cytokines and related genes were activated after 6 hour LPS activation. Purified GFP macrophages were transplanted into control and injured spinal cords after contusion. The transplanted spinal cords were sectioned and immunohistochemistry staining was carried out. Our preliminary data showed that GFP macrophages could survive several days after transplantation. After 1 week, most of the GFP macrophages were rejected by host tissue. With cyclosporin treatment, most GFP macrophages will survive and become migratory. Our long-term transplant study results have shown that there is no significant difference in locomotor recovery among the groups including control, non-activated and activated macrophage transplant.
Preliminary data, and discussion

GFP rat breeding colony establishment

GFP rat is a transgenic rat with Green Fluorescence Protein gene inserted into rat genome. We have imported 6 GFP male rats from Japan. Weekly mating has been set up between male GFP and WT female rats. Due to the nature of transgenic rats, we have observed several generations of GFP rat litters in terms of litter size, body weight, growth curve, and fertility. We have found no abnormality from the offspring rats. Currently, all macrophage cells are harvested from either GFP rats or their WT littermates. Many transplantation projects have benefited from this GFP colony.

Standardization of macrophage extraction protocol

At the beginning of this project, we have encountered some difficulties of collecting enough macrophage cells from each animal. The results were not consistent and reproducible. In order to perform cell transplantation in a timely fashion, a reliable protocol must be developed before any animal test. First, we tested whether 3% Thioglycollate medium (Tg), which is a commonly used reagent to stimulate macrophage production, would improve the number of cells. Second, we tested different tissue culture dish surfaces in terms of macrophage adhesion. Third, we tested different culture media. 3% Tg did increase macrophage production. But, we were also worried about Tg's inflammatory effects on macrophage. To our surprise, we found plain plastic surface is the best culture surface for macrophage adhesion. After, 48 hours of incubation and wash, almost 99% of the adhesive cells are macrophages. All macrophages retained their morphology and express GFP protein. Most of the purified macrophages express ED-1 under tissue culture condition. A number of improvements were also implemented during the surgery to ensure a strict sterile procedure is followed. The successful rate has been improved to more than 90%. An estimated 3,000,000 cells could be collected from each animal.

Macrophage activation profile from RT-PCR and Microarray

Purified macrophages from both GFP and WT rats were activated with LPS for 6 hours and 24 hours. At the end of activation, cells were harvested and total RNA was collected. In RT-PCR assay, 6 primer sets were selected for their known cytokine activity in macrophages. The RT-PCR results showed that the message levels of the 6 cytokines (IL-1a, IL-1b, IL-6, IL-10 and TNF-a) were significantly elevated after 6 hours of LPS treatment. By 24 hours, all 6 cytokine levels have reached to control level. This result suggests that purified macrophages are activated after 6 hour LPS treatment and prolonged activation by LPS will deactivate macrophages.

Microarray assay was carried out by using the same RNA samples. Both 6 hours and 24 hours LPS treated macrophages were compared with non-treated ones. Data analysis was carried out by Gene Spring software. We used 2 fold changes as a cut off point. In summary, after 6 hours LPS incubation, 138 genes were up regulated, and 55 genes were down regulated. At 24 hours, 123 genes were up regulated, and 49 genes were down regulated at least 2 folds respectively. These results provided an overall macrophage mRNA profile under LPS activation. There is a number of cytokines elevated including IL-1a, IL-1b, IL-6, IL10, and TNF-a, which are consistent with RT-PCR data. From these preliminary results, we believe that 6 hour LPS activation is a good starting point for macrophage activation. It will be interesting to know whether the transplanted macrophages remain active and secret cytokines after transplantation. We have already carried out transplantation of 6 hour LPS-activated macrophages into injured spinal cords.

Macrophage transplantation survival needs immunosuppressant treatment

Purified GFP macrophage cells were transplanted into non-injured and injured spinal cords after contusion. We injected GFP macrophages at proximal and distal to the impact site. After 24 hours, 48 hours, 72 hours, 7 days, 14 days and 28 days, rats are perfused

and spinal cords are sectioned. GFP macrophages were visualized with Zeiss confocal microscope. In the non-injured spinal cord, very few cells survived after 1-week transplantation. This result suggests that the transplanted cells might be rejected by host tissue. In order to study long-term effect of transplantation macrophages, we decided to use cyclosporin A (10 mg/kg daily SC) for long-term experiments. In the injury transplant, the data showed that the transplanted macrophages remained alive after 72 hours. They are not restricted to the injection site but rather migratory. After 1 week, very few GFP macrophages were visible. Combined with non-injured data, we suggest that most of the GFP macrophages were rejected from the spinal cord after 1 week. With daily cyclosporin treatment, the transplanted macrophages remain healthy and widely distributed inside spinal cord.

Locomotor recovery after macrophage transplant

We have carried out several long-term survival studies to compare early (1 day) and delayed (4-week) transplants of optimally activated (control, LPS and peripheral nerve) macrophages and their effects on regeneration and locomotor recovery in the rats. Purified GFP macrophages were activated and transplanted into contused spinal cord at different time points. All animals were kept alive for more than 8 weeks and score weekly for locomotor recovery after cell transplantation. 2 weeks before sacrifice, BDA were injected into cortex to label corticospinal tract. All animals were perfused with 4% PFA and spinal cords were sectioned for histological study. We have compared locomotor recovery (BBB score) in control, non-activated macrophage and activated macrophage transplantation in contused spinal cords. Unfortunately, our data suggested there was no significant difference in term of BBB scores among different groups. Histological studies have show there were few survival GFP cells near the inject site and these cells remained ED-1 positive. There was no significant difference among the BDA labeled corticospinal tract either. These experiments suggested that activated macrophage has no beneficial effect in acute and chronic contusion models.

Our data suggests that it is possible to use GFP marker to visualize transplanted cells, macrophages will be rejected without cyclosporine treatment, transplanted macrophage remain activated inside spinal cord, and macrophage transplant has little effect on locomotor recovery and regeneration in contusion model. In order to carry out clinical trial, more animal data are needed to prove activated macrophage trasplanattion beneficial effect on spinal cord injury.