Regeneration and Scar Tissue Formation: Developing Novel Approaches for Analyzing Macrophage-Mediated Regeneration

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Introduction

The ultimate goal for optimal healing after traumatic injury or surgery is the complete regeneration of damaged tissue. While some animals such as zebrafish, salamanders, and certain species of rodents have the ability to successfully regenerate after complex tissue injury, most mammals have poor regenerative ability and instead form scar tissue.

The African Spiny mouse (*Acomys cahirinus*) and common lab mouse (*Mus musculus)* are commonly used as a comparative mammalian model for studying the mechanisms of regeneration due to their effective regenerative and scar-forming abilities shown after ear punctures, respectively[1,2].

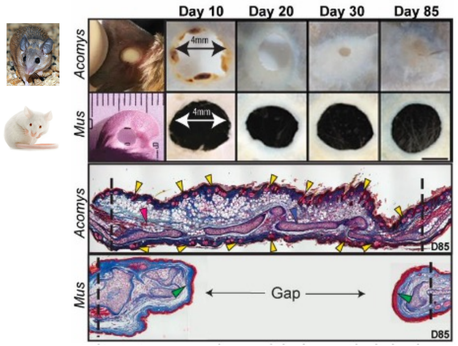

Figure 1. A comparative model of musculoskeletal tissue regeneration and scar formation a) Following a 4mm biopsy punch through the external ear pinnae, *Mus* never replaces lost tissue whereas *Acomys* regenerates missing structures including hair follicles (yellow arrow), elastic cartilage (blue arrow), muscle (red arrow), adipose tissue, nerve, and vascular supply.

Previous studies have determined that macrophages play a key role in both guiding complex tissue regeneration in these highly regenerative animals and promoting scar tissue formation in humans and other mammals[3].

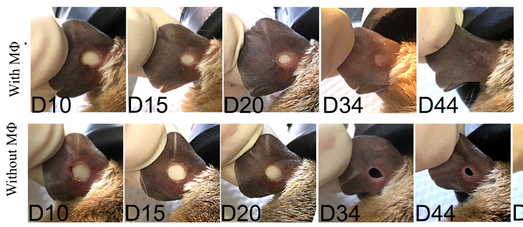

Figure 2. Regeneration of the ear punch is inhibited when macrophages are depleted from the tissue. Macrophages were depleted in *Acomys* ears using Clodronate-liposomes (bottom panel) and ear closure was compared to PBS-liposome controls (top panel). Without macrophages, regeneration is stalled for up to two weeks until macrophages return to the tissue[3].

Macrophages, the orchestrators of inflammation, are abundant after almost every injury and secrete products that influence the actions of all cell types, from keratinocytes to fibroblasts to endothelial cells, which makes them an important cell population to study as a signaling center for regeneration. Regenerative macrophages have been found to secrete higher amounts of proteins such as PDGF-AA, LTF, and IL-1α compared to scar-forming macrophages (Simkin, unpublished), which is being further studied to determine the potential role of these proteins in the promotion of regeneration. PDGF-AA, an important regulator of cell growth, proliferation, and angiogenesis, has been shown to play a prominent role in promoting regenerative phenotypes in vitro.

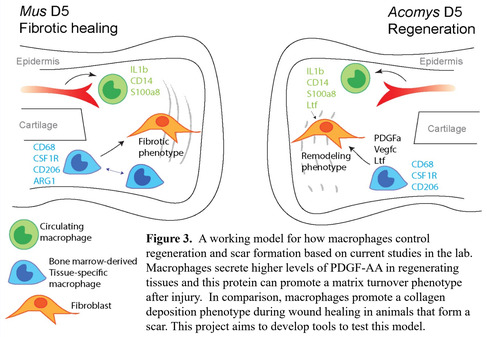

Figure 3. A working model for how macrophages control regeneration and scar formation based on current studies in the lab. Macrophages secrete higher levels of PDGF-AA in regenerating tissues and this protein can promote a matrix turnover phenotype after injury. In comparison, macrophages promote a collagen deposition phenotype during wound healing in animals that form a scar. This project aims to develop tools to test this model.

Based on these current in vitro studies, we hypothesize that macrophage produced PDGF-AA can promote collagen degradation after injury and improve regenerative capacity in a scar-forming animal. In order to test this hypothesis in vivo, we aim to create a macrophage that secretes high levels of PDGF-AA and can be introduced to a scar-forming injury.

The purpose of this study was to develop a novel approach through which macrophage-mediated regeneration can be further investigated. The method of focus was Polyhedrin Delivery Systems (PODS, Cell Guidance Systems), which are stable nanoscale (200 nm-5 µL) co-crystals built from the polyhedrin protein that encapsulate growth factors in a protein shell, protecting and preserving their function until sustained release[4].

Methods

Cell Culture
RAW 264.7 cells, an immortalized macrophage-like cell line derived from Balb/c mice, were cultured using standard cell culture techniques. The cell culture media consisted of 500 mL Dulbecco's Modified Eagle Medium, with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin, and 0.1% Gentamycin.

PODS
Macrophages from cell culture were seeded in a 96-well plate at a density of 3000 cells/well. PODS crystals were reconstituted at 200 million co-crystals/mL in dH2O. PDGF-AA PODS were added to wells at a concentration of 1 POD/cell, 5 PODS/cell, and 10 PODS/cell. Empty PODS containing no cargo protein were added as a negative control, and RAW 264.7 cells with no PODS and PDGF-AA PODS with no cells were also added.

Imaging & Microscopy
96-well plate was imaged using NIS Elements software after a 24-hour period. RAW 264.7 cells were seeded at a density of 250,000 cells in Lab-Tek II chamber slides with Green Fluorescent Protein (GFP) PODS at 5 PODS/cell and imaged using confocal microscopy and 3D imaging (DAPI and FITC lens). PDGF-AA and GFP PODS were plated with macrophages (5 PODS/cell) in 35 mm glass bottom dishes and imaged over a 16-hour time period using an Olympus VivaView FL live cell imaging incubator microscope.

ELISA Analysis
Medium from the 96-well plate conditioned by the loaded macrophages was collected and analyzed for the presence of PDGF-AA by Duoset Human PDGF-AA enzyme-linked immunosorbent assay (ELISA) using general Elisa protocol. A microplate reader was used to obtain results from the assay and a standard curve was generated using Microsoft Excel and Prism software.

Results

Macrophages successfully phagocytize PODS

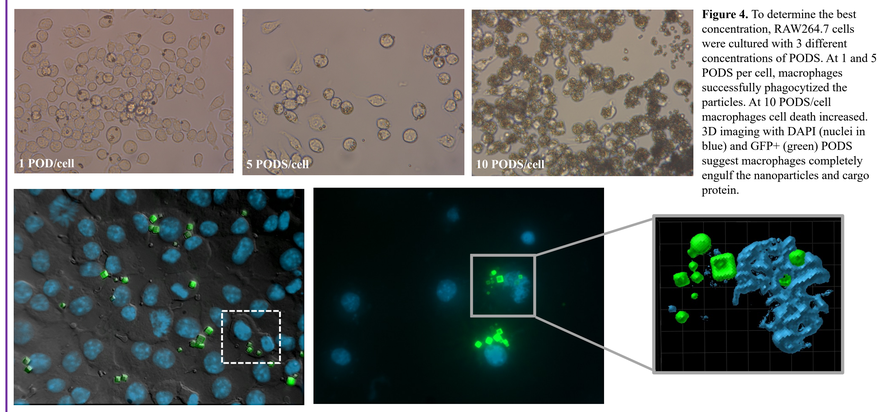

Figure 4. To determine the best concentration, RAW264.7 cells were cultured with 3 different concentrations of PODS. At 1 and 5 PODS per cell, macrophages successfully phagocytized the particles. At 10 PODS/cell macrophages cell death increased. 3D imaging with DAPI (nuclei in blue) and GFP+ (green) PODS suggest macrophages completely engulf the nanoparticles and cargo protein.

Live Cell Imaging

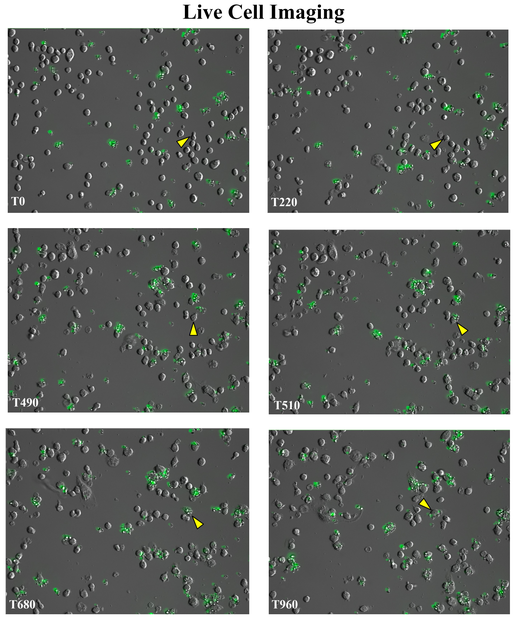

Figure 5. RAW 264.7 cells were cultured with 5 PODS per cell and imaged every 10 minutes over the course of 16 hours. Macrophages successfully phagocytized PODS, however distribution was uneven amongst cells. Live cell imaging revealed "hogging" of PODS by single macrophages. Live cell imaging also suggests proliferation was not inhibited by PODS. Time is indicated in minutes.

Macrophages secrete higher levels of PDGF-AA after treatment with PODS

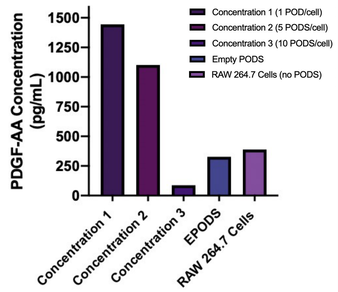

Figure 6. RAW 264.7 cells were cultured with different concentrations of PODS for 72 hours. Conditioned media was then collected from the cells and assayed for levels of PDGF-AA.

Discussion

- Live cell imaging revealed that most PDGF-AA were phagocytized by RAW 264.7 cells over a 16-hour period
- Confocal microscopy and 3D imaging indicated that PODS are readily, consistently, and efficiently taken up by professional phagocytes. The ELISA indicated that following phagocytosis, there is sustained release of a cytokine encapsulated into PODS crystals, which could be detected in a reliable and dose-dependent manner in macrophage culture media.
- An analysis of macrophage behavior indicated that macrophages effectively phagocytize PODS cargo protein at a concentration of 5 PODS/cell, as higher concentrations led to partial cell death
- RAW 264.7 cells were found to secrete at a biologically active level (1 ng/mL) after incubation with PDGF-AA PODS
- Incubating RAW 264.7 cells with PDGF-AA PODS tripled the typical amount of platelet-derived growth factor produced by the macrophages
- The uptake of PODS crystals did not appear to be cytotoxic at low levels or fundamentally alter the characteristics of the RAW 264.7 cells, such as mobility or chemotaxis.

Conclusion & Future Directions

Overall, the preliminary results obtained from this study suggest that the PODS crystals are a promising vector for modifying the cytokine secretion profile of phagocytic cells. This sustained growth factor technology can potentially be utilized to target macrophages for therapeutic purposes and in Trojan horse drug delivery strategies to treat cancer and other diseases. Future research efforts will be aimed at applying the Polyhedrin Delivery System to mouse models to observe the difference in immortalized vs. primary cell responses to PODS. The in vitro PDGF-AA PODS-loaded macrophages will also be introduced into the in vivo scar-forming model to test the potential role of PDGF-AA on regeneration. The findings of these studies will contribute significantly to future efforts in developing a targeted approach to manipulating macrophages to control healing outcomes and advancing regenerative medicine.

References

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