Keratin Biomaterials Activate Regenerative Cells and Promote Peripheral Nerve Regeneration at Early and Late Stages in a Mouse Model

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Statement of Purpose: Nerve defects are a common result of peripheral nerve injury and present a significant challenge surgically. Clinically, the gold standard for nerve repair is implantation of an autograft. Nerve conduits provide another option to this problem but are currently limited to smaller nerve defects. It has been shown that the insertion of tissue engineered scaffolds into an empty conduit can support regeneration. However, no neuroinductive biomaterial capable of stimulating nerve growth has been identified. In a recent study, we demonstrated the ability for a keratin conduit filler to accelerate functional peripheral nerve regeneration in a mouse model at early time points, and that this activity was at least partly due to the activation of Schwann cells (SC).[1] Gross and histological investigation showed increased vascularization of nerves that regenerated trough a keratin-based gel. We hypothesize that this increase in vascularity is due keratin's ability to mediate endothelial cells (EC) behavior in addition to that of SCs. The goals of this study were to 1) Investigate the effect of keratin on SC and EC activity *in vitro* and 2) Assess long-term functional regeneration through a keratin scaffold in a mouse model.

Methods: Human hair was obtained from a local barber shop and cut into small length fibers. Keratin fractions were prepared by treating hair fibers with peracetic acid, followed by extraction with aqueous tris base and deionized (DI) water. The extracts were combined and dialyzed against DI water. The dialyzate was neutralized, concentrated, lyophilized, and the resulting keratin solid ground into a fine powder. Hydrogels were prepared by re-hydrating the keratin powder. Micro-architecture of the self-assembled gels was observed by scanning electron microscopy (SEM) of lyophilized samples. Proliferation of SC and EC in the presence of keratin was assessed using an *in vitro* MTS assay. Migration in response to keratin biomaterials was investigated using a modified Boyden chamber. Cell adhesion to keratins was assessed using a parallel flow chamber system. Real time PCR was used to determine changes in gene expression upon exposure to keratin. To determine the effects of keratin on nerve regeneration *in vivo*, a tibial nerve axotomy model was used. The tibial nerve of adult mice was transected and a 4 mm defect was generated under a microscope. Each animal underwent repair using either 1) empty silicone conduit, 2) keratin filled conduit or 3) autograft. Six weeks, 3 and 6 months following injury and repair, electrophysiology testing was conducted to assess functional recovery. Nerve tissues were sectioned and stained with toluidine blue. Histomorphometric analysis was performed to assess nerve area, axon number, axon size and vascularization.

Results: Examination of lyophilized hydrogels by SEM showed a fibrous and highly porous architecture. Schwann cell biocompatibility upon exposure to keratin containing solutions was excellent. Keratin was not cytotoxic and increased SC and EC proliferation at concentrations ranging from 0.1µg/mL to 1 mg/mL over serum containing media control. Migration of SC and ECs in response to keratin was significantly enhanced at several concentrations. SC and EC adhesion on keratin coatings was higher than on uncoated and comparable to fibronectin. Exposure to keratin resulted in upregulation of several Schwann cell specific genes, primarily S100β and CD104. Six weeks following nerve transaction and repair, regeneration was apparent in empty, keratin-hydrogel filled conduits and autograft. Only 50% of animals in the empty groups showed complete regeneration across the defect, versus 100% in both keratin and autograft groups. By 3 and 6 months, nerve tissue was visible within the conduit in all groups. Nerves that regenerated through the keratin hydrogel had lower conduction delays and greater amplitudes than nerves that regenerated through empty conduits. At early time points, functional recovery was superior not only to empty conduits, but also to autografts. Histological analysis of toluidine blue cross sections confirmed an increase in axon number and size in nerves that regenerated through the keratin hydrogel. In addition, regenerated nerves showed an increase in the number and size of newly formed blood vessels over both empty and autograft controls at 6 weeks. At 3 and 6 month time points, differences between treatment groups were reduced but still significant for several functional and histological outcome measures.

Conclusions: This data suggests that human hair keratins activate Schwann and endothelial cells *in vitro* and may induce the recruitment of these regenerative cells *in vivo.* The insertion of keratin hydrogel into a nerve guidance conduit demonstrated a high degree of regeneration in a mouse model as confirmed by functional testing and histological analysis.

References: 1. Sierpinski P, Garrett J, Ma, J, Apel PJ, Klorig D, Smith T, Koman, LA, Atala A and Van Dyke ME. Biomaterials 29: 118-128 (2008).