Biomaterials 34 (2013) 5907–5914

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of human hair keratin hydrogel on early cellular response to sciatic nerve injury in a rat model

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ARTICLE INFO

Article history:
Received 23 February 2013
Accepted 10 April 2013
Available online 13 May 2013

Keywords:
Keratin
Nerve
Conduit
Schwann cell
Hydrogel

ABSTRACT

Peripheral nerve injuries requiring surgery can be repaired by autograft, the clinical "gold standard", allograft, or nerve conduits. Most published clinical studies show the effectiveness of nerve conduits in small size defects in sensory nerves. Many preclinical studies suggest that peripheral nerve regeneration through conduits can be enhanced and repair lengths increased with the use of a biomaterial filler in the conduit lumen. We have previously shown that a luminal hydrogel filler derived from human hair keratin (HHK) can improve electrophysiological and histological outcomes in mouse, rabbit, and non-human primate nerve injury models, but insight into potential mechanisms has been lacking. Based on the premise that a keratin biomaterial (KOS) hydrogel provides an instantaneous structural matrix within the lumen, the current study compares the cellular behavior elicited by KOS hydrogel to Matrigel (MAT) and saline (SAL) conduit fillers in a 1 cm rat sciatic nerve injury model at early stages of regeneration. While there was little difference in initial cellular influx, the KOS group showed earlier migration of dedifferentiated Schwann cells (SC) from the proximal nerve end compared to the other groups. The KOS group also showed faster SC dedifferentiation and myelin debris clearance, and decreased macrophage infiltration during Wallerian degeneration of the distal nerve tissue.

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1. Introduction

Unlike the central nervous system, the peripheral nervous system (PNS) has the ability to self-repair. Peripheral nerve injuries range in severity from compression injuries, which are capable of spontaneous regeneration, to loss of continuity of the nerve trunk that requires surgical intervention for functional restoration [1–3]. If a tensionless primary nerve repair cannot be performed, the current recommended treatment is a sural nerve three-stranded cable autograft to bridge the defect [4]. Sural nerve autograft is considered the clinical "gold standard" for repair of peripheral nerve defects, although this treatment has several limitations including tissue availability, donor site morbidity, loss of sensation in the tissue innervated by the donor nerve, and the need for an additional surgery [5,6].

Recently, several alternatives to autograft have been approved by the US Food and Drug Administration (FDA) for repair of peripheral nerve defects, including acellular allografts [7] and hollow tubes termed nerve conduits, which are constructed of type I collagen, polyglycolic acid (PGA), or poly-DL-lactide-caprolactone [6,7]. Preclinical studies in a rat sciatic nerve model have shown that type I collagen conduits are superior to PGA conduits and equivalent to autograft in functional recovery and histological outcomes [8,9]. However, the majority of clinical data for these conduits (sold as NeuraGen®, Integra Life Sciences) is limited to repairs of the digital nerve, a small diameter sensory nerve [10,11] and the continuing recommendation for use of nerve conduits is for small gaps (≤3 cm) in sensory nerves only [12–14].

Various preclinical studies have focused on improving peripheral nerve regeneration inside the conduit in order to expand the use of these devices to increased gap lengths and better functional outcomes by using a filler material in the conduit lumen [15]. A nerve conduit filler based on a keratin biomaterial (KOS) hydrogel has been shown to be capable of electrophysiological and histological enhancement following tibial nerve repair in mice and rabbits [16–18] and median nerve repair in non-human primates [Pace et al., unpublished data]. Subcutaneous implant studies of the

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http://dx.doi.org/10.1016/j.biomaterials.2013.04.024

KOS hydrogel in mice revealed that the biomaterial provides a temporary matrix that is degraded by 8 weeks [19]. However, the interaction between the KOS matrix and resident cells needed for nerve regeneration to occur has not been examined.

The purpose of the current study was to investigate early cell infiltration into the conduit lumen filled with KOS following sciatic nerve injury and repair, to assess the behavior of Schwann cells (SC) in the distal stump during Wallerian degeneration, and identify other cells contributing to nerve regeneration. We hypothesized that KOS provides a permissive matrix that is well tolerated by the PNS cells and tissue, and that it would be instantly available for cellular infiltration without the need to build up the normal physiologic fibrin matrix derived from the proximal and distal stump exudate following nerve transection. To investigate these aspects, a rat sciatic model was used. We postulated that the presence of a hydrogel matrix immediately after injury would result in a greater influx of resident cells, and that the KOS hydrogel in particular would support a larger regenerative cell population. Moreover, we postulated that the presence of KOS would alter SC behavior as these are the cells most responsible for regenerative processes at early stages after injury when the KOS hydrogel would be expected to be intact.

2. Materials and methods

2.1. Preparation of keratin powder and hydrogel

KOS hydrogels were prepared as described previously [17,19]. Briefly, human hair was oxidized with a 2% peracetic acid solution and rinsed with water to remove any residual oxidant. Soluble keratins were extracted into tris(hydroxymethyl)aminomethane (Tris) base and deionized water. The extracted solution was then dialyzed, concentrated, neutralized, lyophilized, and ground into a fine powder. The lyophilized keratin was sterilized via γ-irradiation at a dose of 25 kGy and aseptically reconstituted in phosphate buffered saline (PBS) to form a 15% (w/v) hydrogel.

2.2. Rat sciatic nerve injury pilot study

Eighteen male Sprague Dawley rats (Harlan Laboratories) weighing approximately 250 g were randomized into SAL and KOS groups and underwent unilateral sciatic nerve transection and repair of a 1 cm defect with a NeuraGen® conduit (Integra Life Sciences). All animal procedures were approved by the Wake Forest University Animal Care and Use Committee and conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. Anesthesia was maintained with isoflurane (1.5–2.0 volume %) and all procedures were performed under sterile conditions. The sciatic nerve was transected at the level of the obturator tendon, the proximal stump was placed approximately 1 mm inside one end of a 1 cm × 2 mm ID NeuraGen® conduit, secured using a single 9–0 nylon microsuture (Ethicon), and either a 15% weight/volume (w/v) KOS hydrogel or sterile saline (SAL) was introduced into the conduit lumen. The distal stump was similarly placed into the opposite end of the conduit so that contact was made between the tissue and filler material and sutured, thus leaving a gap of approximately 8 mm between the nerve stumps that was occupied by the filler material. Fascia was closed using 5–0 Vicryl suture (Ethicon) and skin was closed with Michel wound clips (Kent Scientific). Buprenorphine (0.01 mg/kg) was administered after closing for analgesia.

2.3. Rat sciatic nerve injury main study

Bilateral sciatic nerve transections and repairs were performed in 24 male Sprague Dawley rats weighing approximately 250 g (Harlan Laboratories) according to the previously described procedure. The sciatic nerve was transected at the level of the obturator tendon and a 5–6 mm portion of the nerve was removed. The proximal stump was placed approximately 1 mm inside one end of a 1 cm × 2 mm ID bovine collagen I nerve conduit (NeuraGen, Integra Life Sciences), secured using a single 9–0 nylon microsuture (Ethicon) and the lumen filled with either a 15% weight/volume (w/v) KOS hydrogel, sterile SAL, or Matrigel™ ("MAT"; BD Biosciences) ($N = 8$ per group, $N = 4$ animals per time point). The distal stump was similarly placed into the opposite end of the conduit so that contact was made between the tissue and filler material and sutured, thus leaving a gap of approximately 8 mm between the nerve stumps that was occupied by the filler material. Fascia was closed using 5–0 Vicryl suture (Ethicon) and skin was closed with Michel wound clips (Kent Scientific). Buprenorphine (0.01 mg/kg) was administered after closing for analgesia.

2.4. Nerve histology

For the pilot study, the animals were anesthetized with isoflurane (1.5–2.0 volume %), euthanized, and the conduit grafts were harvested including 2–3 mm of proximal and distal nerve tissue at 3, 7, and 14 days following surgery. The recovered conduits were placed into O.C.T. compound (Tissue-Tek, Sakura Finetek), flash frozen in liquid nitrogen, and stored at −80 °C. For the main study, the conduits were harvested according to the previously mentioned method at 3 and 7 days after surgery. The nerve grafts were measured and transected at the graft midpoint with a scalpel blade. The distal and proximal sections were placed into O.C.T. compound (Tissue-Tek, Sakura Finetek), flash frozen in liquid nitrogen, and stored at −80 °C. Prior to sectioning, all tissue blocks were equilibrated to −25 °C and either 10 μm longitudinal or transverse serial cryosections were collected with a cryostat (CM1950, Leica Microsystems) and post-fixed in acetone for 15 min at −20 °C. The slides were then stored at −20 °C until staining. For the pilot study, all conduits were sectioned longitudinally and the entire conduit lumen including the distal and proximal nerve ends was labeled. For the main study, all conduits were sectioned transversely and histology from within the conduit lumen was taken from the proximal 2.5 mm of the conduit and is referred to as being from the conduit. Histology performed on the distal stump tissue refers to tissue within the residual nerve stump, immediately adjacent to the gap but still within the conduit opening.

2.5. Immunohistochemistry and immunofluorescence

All stains were performed at room temperature (RT) using either a Dako Universal Slide Staining System or by hand using an optimized staining technique utilizing tris-buffered saline/tween 20 (TBS/T) solutions with or without 0.3% Triton X-100 (Sigma Aldrich) depending on the cellular location of the antigen. The slides were rinsed with TBS/T, blocked in 5% serum corresponding to the species of the secondary antibody for 30 min, incubated in primary antibody for 2 h, rinsed with TBS/T and incubated in secondary antibody for 1 h. Primary antibodies to p75 neurotrophin receptor (NTR) (1:1600, Cell Signaling), neurofilament light chain (NF-L) (1:100, Cell Signaling), neurofilament heavy chain (NF-H) (1:200, Cell Signaling), growth associated protein 43 (GAP43) (1:100, Cell Signaling), and S100 (1:200, Sigma Aldrich) were used to label the tissue. Secondary antibodies including FITC-anti-mouse (rat adsorbed, 1:100, Vector Labs), Alexa Fluor 594 and 488 goat anti-rabbit (1:1000, Life Technologies) were used to visualize the antigens. A FITC-conjugated Lectin from *Lycopersicon esculentum* (tomato) solution (1:150, 1 h, Sigma Aldrich) was used to label macrophages. Myelin was labeled with FluoroMyelin Red (1:300, 5 min, Invitrogen). All stains included a nuclear counterstain with DAPI (1:300, 5 min, Sigma Aldrich) and all slides were mounted with ProLong® Gold Antifade reagent (Life Technologies).

2.6. Conduit cellular infiltration

For the pilot study, 50× magnification monochromatic images were obtained of the entire 1 cm conduit at 3, 7, and 14 days for the DAPI label ($n = 3$ animals per group per time point) with a Zeiss Axio Imager M1 upright microscope and photomerged using Photoshop CS2 software (Adobe) (Fig. 1A & B). The merged images were converted to binary format and total cell numbers were quantified using ImageJ software (NIH). The entire conduit lumen, including the portions of proximal and distal nerve tissue inserted into the conduit, was traced and the area calculated in mm^2. Nuclei/mm^2 was quantified using the analyze particles count function in ImageJ.

2.7. Fluorescent label quantification

Monochromatic 200× and 630× fluorescent images were obtained for each stain using a Leica DM4000 upright microscope (Leica Microsystems) or a Zeiss LSM 510 confocal microscope (Carl Zeiss) with identical capture settings for each group. Three images per animal per time point were obtained and analyzed using ImageJ software. The images were converted to a binary format and either stain density (total pixels/total area) or cell number measurements were determined and averaged for each animal. The tissue section area was traced and measured in μm^2 and stain density was determined by dividing the area of the stain by the total area for each section and expressing the value as a percentage. Macrophage infiltration was quantified in ImageJ by superimposing a grid over the images, manually counting cells that crossed the gridlines in the same pattern across each image, and dividing the cell count by the total area analyzed.

2.8. Growth cone advancement and migrating SC

During surgery the conduits were fastened to the nerves ends via 9–0 epineurial nylon microsuture (Ethicon). The suturing technique placed a 1–2 mm portion of the proximal and distal nerve tissue inside the conduit lumen and the sutures were visible using light microscopy. For the main study, the maximum distance of growth cone advancement from the proximal nerve end was quantified by identifying the growth cones with GAP43/NF-L double immunofluorescence and counting the number of sections between the growth cone and the proximal suture. The total cell

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