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Limited regeneration in long acellular nerve allografts is associated with increased Schwann cell senescence

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ABSTRACT

Repair of large nerve defects with acellular nerve allografts (ANAs) is an appealing alternative to autografting and allotransplantation. ANAs have been shown to be similar to autografts in supporting axonal regeneration across short gaps, but fail in larger defects due to a poorly-understood mechanism. ANAs depend on proliferating Schwann cells (SCs) from host tissue to support axonal regeneration. Populating longer ANAs places a greater proliferative demand on host SCs that may stress host SCs, resulting in senescence. In this study, we investigated axonal regeneration across increasing isograft and ANA lengths. We also evaluated the presence of senescent SCs within both graft types. A sciatic nerve graft model in rats was used to evaluate regeneration across increasing isograft (~autograft) and ANA lengths (20, 40, and 60 mm). Axonal regeneration and functional recovery decreased with increased graft length and the performance of the isograft was superior to ANAs at all lengths. Transgenic Thy1-GFP rats and qRT-PCR demonstrated that failure of the regenerating axonal front in ANAs was associated with increased levels of senescence related markers in the graft (senescence associated β-galactosidase, $p16^{INK4A}$, and IL6). Lastly, electron microscopy (EM) was used to qualitatively assess senescence-associated changes in chromatin of SCs in each graft type. EM demonstrated an increase in the presence of SCs with abnormal chromatin in isografts and ANAs of increasing graft length. These results are the first to suggest that SC senescence plays a role in limited axonal regeneration across nerve grafts of increasing gap lengths.

Introduction

Large complex peripheral nerve injuries necessitate repair with nerve grafts. Nerve autografts are the current standard for clinical nerve reconstruction, but their use is limited by the amount of expendable tissue and donor site morbidity. An alternative approach is to use allografts, but the necessity of systemic immunosuppression limits its application (Brenner et al., 2002; Mackinnon et al., 2001). Recent efforts have concentrated on addressing the immune challenge associated with allografts by removing the immunogenicity of the grafted tissue (Brenner et al., 2005; Fox et al., 2005a, 2005b; Hudson et al., 2004a, 2004b; Ray et al., 2010, 2011; Whitlock et al., 2010a, 2010b). A number of studies have established donor Schwann cells (SCs) as the immunogenic target in nerve allografts (Ansselin and Pollard, 1990; Lassner et al., 1989). In keeping with these findings, various thermal and chemical techniques (Fox et al., 2005a, 2005b; Gulati, 1998; Hudson et al., 2004a, 2004b; Moore et al., 2011a, 2011b; Ray et al., 2011; Sondell et al., 1998; Whitlock et al., 2010a, 2010b) have been devised to create acellularized nerve allografts (ANAs), thereby eliminating the need for immunosuppression.

Axonal regeneration in ANAs has been demonstrated to be similar to that in autografts across short gaps (Moore et al., 2011a, 2011b; Whitlock et al., 2009), but is reduced across longer defects (Whitlock et al., 2009), due to a poorly-understood mechanism. Following nerve repair with ANAs, there is early and progressive migration of SCs from both the proximal and especially the distal nerve stumps (Fornaro et al., 2001; Hayashi et al., 2007; Tseng et al., 2003; Whitlock et al., 2010a, 2010b). Host SCs provide the environment necessary for axonal regeneration in ANAs (Hall, 1986a, 1986b) through synthesis of neurotrophic factors (Bunge, 1993), adhesion molecules (Bixby et al., 1988), and axonal myelination (Bunge, 1993; Levi et al., 1994, 1997) and organization(Fansa et al., 2001). Failure of SCs to provide a positive regenerative environment in ANAs would significantly affect regeneration.

Cells undergo senescence in response to telomere shortening or dysfunction that arises from consecutive cell divisions, DNA damage, oncogenes, and/or other stressors that can cause epi(genomic) dysfunction (Campisi, 2011). The senescent state is characterized by irreversible arrest in proliferation accompanied by altered gene expression and changes in secretory profile (Campisi, 2005, 2011; Collado et al., 2007; Krtolica and Campisi, 2002; Pazolli and Stewart, 2008). Thus the

Abbreviations: SCs, Schwann cells; ANAs, acellularized nerve allografts; SenScs, senescent Schwann cells; GFP, green fluorescent protein; EDL, extensor digitorum longus; DAPI, 4′,6-diamidino-2-phenylindole.

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presence of senescent SCs in nerve grafts would alter the local environment potentially leading to a loss of necessary support required for regenerating axons.

In this study, we investigated the role of SCs in mediating failure of axonal regeneration across long nerve grafts. Specifically, we hypothesized that longer ANAs place a greater proliferative demand on host SCs to fill longer grafts. This demand either exceeds the replication limit of SCs or creates a stressful environment causing them to undergo senescence.

Materials and methods

Animals

Adult male Lewis rats (250 g, Charles River Laboratories, Wilmington, MA) underwent sciatic nerve transection and grafting with a long 60 mm or shorter (20 mm or 40 mm) isografts or ANAs as outlined in Table 1. At the appropriate endpoint, the animals were sacrificed for assessment of grafts for neuroregeneration and senescent SCs (SenScs). An additional cohort of Thy1-Sprague–Dawley transgenic rats (genOway, Lyon, France; Moore et al., 2011a, 2011b), which express green fluorescent protein (GFP) in axons under the control of neuron-specific Thy1 promoter, were used for in vivo visualization of axonal regeneration through long ANAs or isografts.

Surgical procedures and peri-operative care measures were conducted in compliance with the Washington University Institutional Animal Studies Committee and the National Institutes of Health guidelines. All animals were housed in a central animal care facility and provided with food (PicoLab rodent diet 20, Purina Mills Nutrition International, St. Louis, MO) and water ad libitum. They were monitored post-operatively for signs of infection and distress.

Experimental design

Lewis and Thy1-GFP Sprague–Dawley transgenic rats were randomized to undergo sciatic nerve transection and repair with isografts or ANAs. Lewis and Thy-1 GFP rats received isografts from Lewis and Sprague–Dawley donors, respectively. Isografts for each specific group were used as positive control for both short- and long-term studies. Lewis (*RT-1*^{1} MHC) and Sprague–Dawley (*RT-1*^{b} MHC) rat strains are known to be completely MHC incompatible and thus were used as allograft donors to one another. Sciatic nerve allografts harvested from donor rats were chemically processed and decellularized using a series of detergents as described by Hudson et al. (2004a, 2004b) and Moore et al. (2011a, 2011b). Two end time points of short term (10 weeks) and long term (20 weeks) were designated for the study. For the short term (10 weeks), 20, 40, and 60 mm nerve grafts were engrafted. For the long term (20 weeks), 40 and 60 mm grafts were implanted. Following harvest, nerves were analyzed for axonal regeneration and reinnervation using histomorphometry, in vivo imaging, and extensor digitorum longus (EDL) muscle weight and electrically evoked force measurements. Select nerves from the short term endpoint engrafted with 60 mm ANAs and isografts were assessed for presence of SenSCs: Immunohistochemistry and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were used to measure markers of SCs (S100) and cellular senescence (β-galactosidase, p16^{INK4A}, p53, and IL-6); Electron microscopy was used to examine nuclei for reorganization of heterochromatin associated with senescence (Table 1).

Table 1
Groups, time points and animal numbers.

	Graft length	10 week endpoint	20 week endpoint	Analysis
Isograft	20 mm	n = 7	–	Histomorphometry
	40 mm	n = 6	n = 14	
	60 mm	n = 6	n = 6	
	20 mm	n = 2	–	In vivo imaging (Thy1-GFP rats)
	40 mm	n = 2	–	
	60 mm	n = 3	–	
	20 mm	n = 6	–	EDL muscle force/mass
	40 mm	n = 6	n = 3	measurement
	60 mm	n = 6	n = 6	
	60 mm	n = 9	–	Histology
	60 mm	n = 4	–	qRT-PCR
	60 mm	n = 1	–	EM
Allograft	20 mm	n = 7	–	Histomorphometry
	40 mm	n = 6	n = 10	
	60 mm	n = 8	n = 6	
	20 mm	n = 2	–	In vivo imaging (Thy1-GFP rats)
	40 mm	n = 2	–	
	60 mm	n = 3	–	
	20 mm	n = 6	–	EDL muscle force/mass
	40 mm	n = 6	n = 3	measurement
	60 mm	n = 6	n = 6	
	60 mm	n = 9	–	Histology
	60 mm	n = 4	–	qRT-PCR
	60 mm	n = 1	–	EM

Surgical procedures

Surgical procedures were performed under aseptic conditions and with the aid of an operating microscope (JEDMED/KAPS, St. Louis, MO) as described previously (Moore et al., 2011a, 2011b). The animals were anesthetized with subcutaneous delivery of Ketamine (75 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and dexmedetomidine (0.5 mg/kg, Pfizer Animal Health, Exton, PA). Donor nerves were harvested as described previously (Moore et al., 2011a, 2011b). The dissection was extended proximally and distally to allow harvest of 32–35 mm of nerve, which was later trimmed to 30 mm or 20 mm lengths as necessary. Sciatic nerves were transferred to aseptic tubes to undergo acellular processing (Hudson et al., 2004a, 2004b) or immediately used as fresh nerve isografts. Donor animals were then euthanized. We employed a novel long 60 mm rodent nerve graft model by coapting two 30 mm acellularized or fresh sciatic nerves using a minimum of a single 9–0 nylon epineurial suture and fibrin sealant (Baxter Healthcare Corp., Deerfield, IL; Whitlock et al., 2010a, 2010b). For 40 mm grafts, the same model was applied using coaptation of two 20 mm nerves.

Recipient rats underwent exposure of the right sciatic nerve. The recipient nerve was transected at 5 mm proximal to the sciatic trifurcation. The defect was reconstructed with an isograft or ANA (20, 40, or 60 mm) and secured to the proximal and distal nerve stumps using a minimum of a single 9–0 nylon epineurial suture and fibrin sealant (Moore et al., 2011a, 2011b; Whitlock et al., 2010a, 2010b). For 20 mm graft, the grafted nerves were settled with "S" style at original sciatic nerve bed. For 40 and 60 mm nerve grafts, the grafted nerves were shaped like a loop and inserted into an under-skin "pocket" around the femur (Fig. 1A). Thy1-GFP rats underwent intra-operative imaging of the newly-implanted nerve graft, for the purposes of later comparison of axonal regeneration, prior to closure of incision.

At the 10- and 20-week endpoints, the animals were sacrificed for analysis of the graft for axonal regeneration and SC senescence. Thy1-GFP rats underwent in vivo imaging of the exposed nerve prior to harvest. The sciatic nerve was harvested en bloc ~5 mm proximal and ~5 mm distal to the interposed graft. The nerves were stored in 3% glutaraldehyde or 4% paraformaldehyde for histomorphometry and electron microscopy (EM) analysis or immunohistochemical analysis, respectively. Samples for qRT-PCR analysis were stored in RNAlater solution (QIAGEN, Valencia, CA) and stored at −80 °C prior to extraction. The EDL muscle was harvested from both the experimental and the contralateral sides and weighed to evaluate percent muscle recovery following denervation.