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Research Paper

An Elastomeric Polymer Matrix, PEUU-Tac, Delivers Bioactive Tacrolimus Transdurally to the CNS in Rat

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ABSTRACT

Central nervous system (CNS) neurons fail to regrow injured axons, often resulting in permanently lost neurologic function. Tacrolimus is an FDA-approved immunosuppressive drug with known neuroprotective and neuroregenerative properties in the CNS. However, tacrolimus is typically administered systemically and blood levels required to effectively treat CNS injuries can lead to lethal, off-target organ toxicity. Thus, delivering tacrolimus locally to CNS tissues may provide therapeutic control over tacrolimus levels in CNS tissues while minimizing off-target toxicity. Herein we show an electrospun poly(ester urethane) urea and tacrolimus elastomeric matrix (PEUU-Tac) can deliver tacrolimus trans-durally to CNS tissues. In an acute CNS ischemia model in rat, the optic nerve (ON) was clamped for 10s and then PEUU-Tac was used as an ON wrap and sutured around the injury site. Tacrolimus was detected in PEUU-Tac wrapped ONs at 24 h and 14 days, without significant increases in tacrolimus blood levels. Similar to systemically administered tacrolimus, PEUU-Tac locally decreased *glial fibrillary acidic protein* (*GFAP*) at the injury site and increased *growth associated protein-43* (*GAP-43*) expression in ischemic ONs from the globe to the chiasm, consistent with decreased astrogliosis and increased retinal ganglion cell (RGC) axon growth signaling pathways. These initial results suggest PEUU-Tac is a biocompatible elastic matrix that delivers bioactive tacrolimus trans-durally to CNS tissues without significantly increasing tacrolimus blood levels and off-target toxicity.

1. Introduction

In adult mammals, central nervous system (CNS) injury remains a persistent experimental and clinical challenge. Trauma to CNS tissues triggers a pro-inflammatory innate immune response that leads to secondary tissue damage, injury site expansion, and cellular and extracellular matrix (ECM) remodeling (Horn et al., 2008). This default healing response contributes to failed axon regeneration (Peruzzotti-Jametti et al., 2014; Tang et al., 2001) and promotes scar tissue formation (Silver and Miller, 2004), often leading to irreversible CNS neuron death (Russo et al., 2016) and permanently lost neurological function. Thus, combinatorial approaches are needed that can positively modulate the innate immune response to promote functional tissue remodeling over scarring while also providing key neuroprotective and neuroregenerative support to CNS neurons.

Tacrolimus, also called FK506, is a macrolide immunosuppressive drug initially derived from *Streptomyces tsukubaensis* (Prograf ® Astellas, NJ) (Petan et al., 2008) widely used clinically to prevent organ transplant rejection (Starzl et al., 1989) as well as to treat various dermatologic (Madan and Griffiths, 2007) and autoimmune diseases (Chen et al., 2017). Tacrolimus binds to FK506-binding protein (FKBP)

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receptors of the immunophilin family, which comprises at least 15 members in humans. In humans, tacrolimus is thought to prevent organ transplant rejection by suppressing T-cell activation *via* binding to FKBP12 (Xu et al., 2002). In the CNS, FKBPs are widely expressed by numerous cell types, including both neurons and glia, with different cellular populations exhibiting distinct FKBP subtype expression patterns and pharmacokinetics. In the CNS, tacrolimus is hypothesized to modulate the innate immune response by reducing neutrophil and macrophage infiltration and microglial (Zawadzka et al., 2012) and astrocyte activation (Zawadzka and Kaminska, 2005; Szydlowska et al., 2006; Liu et al., 2011), subsequently reducing oxidative stress, secondary damage, and injury site expansion (Fukuta et al., 2015).

Experimentally, tacrolimus can also provide both neuroprotective and neuroregenerative benefits to CNS neurons. Although FKBPs are widely expressed by CNS neurons, tacrolimus appears to modulate neuronal activities by multiple mechanisms, including both FKBP-dependent and FKBP-independent routes (Gold et al., 2005). After optic nerve (ON) ischemia in rats, tacrolimus increases retinal ganglion cell (RGC) survival by suppressing apoptotic signaling (Freeman and Grosskreutz, 2000). After spinal cord injury in rats, tacrolimus increases axon growth and the expression of *growth associated protein-43 (GAP43)*, an axon growth marker (Wang and Gold, 1999; Madsen et al., 1998) and has been reported to improve functional recovery (Voda et al., 2005). In recent studies, tacrolimus has been shown to reduce ischemia reperfusion injury in white matter after cerebral artery occlusion surgery (Fukuta et al., 2015) and to decrease apoptosis in hippocampal neurons (Sharifi et al., 2012). However, most experimental studies have relied on systemic administration to deliver tacrolimus to CNS tissues (Sharifi et al., 2012; Fukuta et al., 2015). Tacrolimus is highly lipophilic and thus accumulates in fatty tissues throughout the body, including the myelin-based white matter in the CNS. However, systemic delivery to CNS tissues is complicated by preferential uptake by other organs, which requires higher systemic doses to effectively increase tacrolimus in CNS tissues. These increases in systemic tacrolimus often lead to often life-threatening toxicity in other tissues and organs.

Clinically, tacrolimus is typically administered systemically either orally or by injection (Varghese et al., 2014; Yamazoe et al., 2014). The chemical properties of tacrolimus require careful consideration with regard to systemic administration. The method of administration, frequency, and dosage, must be carefully monitored to maintain effective therapeutic tissue levels without inducing toxicity in off-target tissues. Improperly regulated systemic levels can lead to a number of life-threatening side effects, including, but not limited to, diabetogenicity, nephrotoxicity, neurotoxicity, and oncogenicity (Randhawa et al., 1997; Starzl et al., 1989). Moreover, tacrolimus administration is further complicated by the number and distribution of FKBP binding proteins. Tacrolimus acts on at least 15 FKBP binding receptors in humans differentially expressed by cellular populations in different tissues and organs. Therefore, tacrolimus administration must be carefully optimized to maintain effective concentrations within the tissues of interest to maximize efficacy while minimizing toxic side-effects both to targeted and to non-targeted cellular populations and tissues. Thus, strategies are being developed to deliver tacrolimus locally. Local delivery approaches include hydrogels (Gabriel et al., 2016), inhalants (Deuse et al., 2010), and micelle (Lapteva et al., 2014) or polymer encapsulation technologies (Tajdaran et al., 2015). However, these technologies are limited for many CNS applications due to ineffective and/or inconsistent tacrolimus delivery to specific CNS tissues, incompatible mechanical properties, and the inability to remain intact and/or localized to the injury site.

To deliver tacrolimus locally and controllably to CNS tissues, this study developed a biodegradable and elastic matrix using poly(ester urethane) urea (PEUU) (Guan et al., 2002). PEUU polymers have desirable mechanical characteristics, including high elasticity and strength, good cell-adhesive properties, and controllable biodegradation, all of which can be tuned to match the tissue of interest. By modifying a previously developed PEUU electrospinning platform (Stankus et al., 2004), tacrolimus and PEUU were successfully blended into PEUU polymer matrices. This method allowed precise control over both the PEUU and tacrolimus concentrations as well as control over the size and the thickness of the PEUU-Tac matrices. PEUU-Tac material properties and release kinetics were analyzed *in vitro*. The dose-dependent effects of tacrolimus on primary RGC viability, toxicity, and differentiation were analyzed *in vitro*. Finally, PEUU-Tac matrices were used as ON wraps to analyze local, trans-dural tacrolimus delivery both to CNS tissues and to the blood in an acute ON ischemia model in rat.

2. Materials and Methods

2.1. Animals

Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed and maintained according to the guidelines set forth by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) and the DOD Animal Care and Use Review Office (ACURO). All procedures complied with the American Association for the Accreditation of Laboratory Animal Care (AALAC).

2.2. Retinal Ganglion Cells

Primary RGCs were isolated from female and male postnatal day three (P3) Sprague-Dawley rat pups, purified by immunopanning, and cultured in NB-SATO media as described (Barres et al., 1988). RGCs were seeded ($5 \times 10^3/cm^2$) in cell culture plates coated with poly-D-lysine (70 kDa, 10 μg/ml; Sigma-Aldrich Corp., St. Louis, MO, USA) and laminin (2 μg/ml, Sigma-Aldrich Corp.). Tacrolimus (Invitrogen) was diluted in 100% EtOH to make a 20 mM stock. The tacrolimus stock was then diluted in NB-SATO as specified and the RGC cultures maintained at 37 °C in 10% CO_2 for 3 days *in vitro* (DIV).

2.3. RGC Viability

Viability was analyzed after 3 DIV using a calcein and propidium iodide based live/dead kit per manufacturer's instructions (Life Technologies, R37601). For analysis, the first five non-overlapping fields of view per well, moving from the left well edge, were imaged at 20× using standard epi-fluorescence fluorescein and rhodamine filter sets (Zeiss, Axio Observer). Experimentally blinded individuals analyzed live and dead cells using ImageJ (National Institutes of Health, Bethesda, MD, USA). Data represent triplicates from four experimental repeats, totaling at least 27 fields of view and at least 300 neurons per group as previously described (Faust et al., 2017). Significance between groups was determined by one-way analysis of variance (ANOVA) as noted in Section 2.13.

2.4. RGC Neurite Growth

After 3 DIV, RGCs were fixed with 4% paraformaldehyde (Alfa Aesar; 30525-89-4) in PBS, washed with PBS (2×), and permeabilized with 0.2% triton X-100 in PBS for 15 min. After blocking for 1 h (1% BSA in PBS, Fisher Scientific), the RGCs were incubated with anti-β III tubulin (1:300, TUJ-1, Millipore, RRID: AB 570918) at 4 °C overnight, washed in PBS (3×), incubated with a FITC-rabbit anti-chicken IgY H + L secondary (1:150, #31501, Thermo Scientific) for 3 h, washed with PBS (3×), counterstained with the nuclear marker DAPI (1:3000, Invitrogen, D1306) for 20 min at room temperature, and washed in PBS (2×, 5 min each). The RGCs were imaged randomly as described above and neurite growth measured by blinded individuals as described (Steketee et al., 2011) using the ImageJ plugin, NeuronJ (National Institutes of Health, Bethesda, MD, USA). The first ten non-contacting RGCs encountered, moving right from the left edge of the well, were analyzed from