Biomaterials 35 (2014) 7429–7440

Contents lists available at ScienceDirect

Biomaterials

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

The influence of extracellular matrix composition on the differentiation of neuronal subtypes in tissue engineered innervated intestinal smooth muscle sheets

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ARTICLE INFO

Article history:
Received 19 February 2014
Accepted 15 May 2014
Available online 11 June 2014

Keywords:
Neural stem cell
Neural tissue engineering
Extracellular matrix
Adult stem cell
Enteric nervous system

ABSTRACT

Differentiation of enteric neural stem cells into several appropriate neural phenotypes is crucial while considering transplantation as a cellular therapy to treat enteric neuropathies. We describe the formation of tissue engineered innervated sheets, where intestinal smooth muscle and enteric neuronal progenitor cells are brought into close association in extracellular matrix (ECM) based microenvironments. Uniaxial alignment of constituent smooth muscle cells was achieved by substrate microtopography. The smooth muscle component of the tissue engineered sheets maintained a contractile phenotype irrespective of the ECM composition, and generated equivalent contractions in response to potassium chloride stimulation, similar to native intestinal tissue. We provided enteric neuronal progenitor cells with permissive ECM-based compositional and viscoelastic cues to generate excitatory and inhibitory neuronal subtypes. In the presence of the smooth muscle cells, the enteric neuronal progenitor cells differentiated to functionally innervate the smooth muscle. The differentiation of specific neuronal subtypes was influenced by the ECM microenvironment, namely combinations of collagen I, collagen IV, laminin and/or heparan sulfate. The physiology of differentiated neurons within tissue engineered sheets was evaluated. Sheets with composite collagen and laminin had the most similar patterns of Acetylcholine-induced contraction to native intestinal tissue, corresponding to an increased protein expression of choline acetyltransferase. An enriched nitrergic neuronal population, evidenced by an increased expression of neuronal nitric oxide synthase, was obtained in tissue engineered sheets that included collagen IV. These sheets had a significantly increased magnitude of electrical field stimulated relaxation, sensitive maximally to nitric oxide synthase inhibition. Tissue engineered sheets containing laminin and/or heparan sulfate had a balanced expression of contractile and relaxant motor neurons. Our studies demonstrated that neuronal subtype was modulated by varying ECM composition. This observation could be utilized to derive enriched populations of specific enteric neurons in vitro prior to transplantation.

1. Introduction

An uninterrupted enteric nervous system with the preservation of myenteric ganglia is required for intestinal motility and function [1,2]. Motor neurons of the myenteric ganglia pre-dominantly express acetylcholine/tachykinins (excitatory) or nitric oxide/inhibitory peptides/purines (inhibitory) to mediate smooth muscle contraction and relaxation [3,4]. Partial, selective or total loss of neurons is reported in several disorders including, but not limited to, Hirschsprung's disease, achalasia, and inflammation [5–8]. Neural-crest derived enteric neuronal progenitor cells have been isolated from adult mammalian guts, including ganglionic bowel of patients with Hirschsprung's disease [9–12]. These cells have the ability to differentiate into neuronal and glial phenotypes [13–15]. However, there is little information and understanding of microenvironment driven differentiation and limited studies describing subsequent functional behavior of these differentiated neurons in vitro [10,11].

The ECM has been long known to provide permissive and non-permissive environmental cues for migration and differentiation of neuronal progenitor cells [16]. Collagen IV, laminin and heparan

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http://dx.doi.org/10.1016/j.biomaterials.2014.05.037
0142-9612/

sulfate proteoglycans play an important role in guiding the migration of neural-crest derived cells and formation of the enteric nervous system [17–19]. Moreover, myenteric ganglia are surrounded by an ECM composed of type IV Collagen, laminin and a heparan sulfate proteoglycan [20].

Type I fibrillar collagen has favorable biological properties and has been widely used in neural tissue engineering [21,22]. Collagen IV has been demonstrated to promote neurite outgrowth in neuroepithelial progenitor cells and in sympathetic peripheral neurons in vitro [23,24]. Collagen IV aids the colonization of the embryonic gut and modulates selective neurotrophic signaling [20,25]. The IKVAV peptide in laminin interacts with a neurally acquired receptor on post-migratory neural crest stem cells promoting their differentiation [26]. In the gut, heparan sulfate proteoglycan is required for GDNF signaling [27]. Glycosaminoglycan – growth factor interactions additionally stabilize and increase local bioavailability of growth factors, thus inducing neurite outgrowth [28]. The ECM can provide a background upon which cell–cell and cell-matrix signaling can work to regulate phenotypes of differentiating enteric neuronal progenitor cells.

In previous studies using 2D culture substrata, we demonstrated that varying ECM composition of 2D culture substrata influenced neuroglial differentiation of adult enteric neuronal progenitor cells [29]. The number of neurons, neurite lengths and preliminary neuronal network formation were all enhanced in culture substrata that contained collagen IV, laminin and heparan sulfate. The previous studies, however, were carried out on 2D coated glass coverslips. Moreover, we examined neuronal and glial differentiation, without documenting the phenotype or functionality of the differentiated neurons. In the present studies, we utilize tissue engineering as a tool to provide enteric neuronal progenitor cells with 3D viscoelastic ECM microenvironments. Given that the cues for differentiation arising from a contractile phenotype of smooth muscle cells would be constant, we hypothesized that ECM composition could differentially regulate the generation of specific neuronal subsets. The objective of the current undertaking was to determine the presence of differentiated motor neurons obtained and investigate their ability in mediating smooth muscle contraction/relaxation.

We utilize a previously described tissue engineered model of intestinal longitudinal smooth muscle sheets, where uniaxial alignment of a smooth muscle monolayer was facilitated by the use of substrate microtopography [30]. We innervated these tissue engineered sheets using enteric neuronal progenitor cells embedded with hydrogels of varying ECM composition. Differentiated neuronal composition (cholinergic, nitrergic, peptidergic) within tissue engineered sheets was evaluated. Functional neuronal physiology mediating smooth muscle contraction/relaxation was also evaluated in the tissue engineered sheets using real-time force generation measurements.

2. Materials and methods

2.1. Materials

All tissue culture reagents (including media, supplements, and natural mouse laminin) were purchased from Invitrogen (Carlsbad, CA). Rat tail type I collagen and mouse collagen type IV were purchased from BD Biosciences (Bedford, MA). Heparan sulfate was purchased from Celsus (Cincinnati, OH). Growth factors were purchased from Stemgent (Cambridge, MA). All primary antibodies were purchased from Abcam (Cambridge, MA), unless specified otherwise.

2.2. Isolation and primary culture from adult rabbit GI tissues

Enteric neuronal progenitor cells were isolated from jejunal biopsies of adult New Zealand White rabbits using a collagenase/dispase digestion technique and cultured in neuronal growth media, as described previously [29]. Cells aggregated to form enteric neurospheres. Longitudinal smooth muscle cells were isolated from the adult rabbit sigmoid colon as described previously [30]. Isolated longitudinal smooth muscle cells were expanded in culture until confluency.

2.3. Composition and characterization of ECM hydrogels

ECM hydrogels were made with the following components:

i) Collagen I gels (800–1600 μg/ml);
ii) Collagen I (800 μg/ml) and Collagen IV (200 μg/ml) gels composite gels;
iii) Collagen I and Collagen IV with laminin (5–10 μg/ml);
iv) Collagen I and Collagen IV with laminin and heparan sulfate (10–20 μg/ml).

Other components of the gel included: 1% fetal calf serum, 0.1× antibiotics in Dulbecco's modified Eagle's medium. 0.1 N Sodium hydroxide was used to adjust pH to ~7.4 for gelation.

2.3.1. Rheological characterization of ECM hydrogels

Oscillatory rheometry (ATS RheoSystems) was used to measure viscoelastic moduli of ECM gels. 20 mm parallel base plates were used to perform a stress sweep of the sample at 1 Hz. ECM gels were allowed to gel in situ between the parallel plates at 37 °C. The viscoelastic modulus was obtained from a linear region of the stress–strain curve, at strains lower than 10%, within the sensitivity ranges for torque and strain of the rheometer. 3–5 individually manufactured ECM gels were measured to determine an average viscoelastic modulus. Compositions that resulted in a matrix viscoelasticity within the range of 150–300 Pa were utilized for further experimentation, so as not to let stiffness be a variable in influencing neuroglial differentiation.

2.3.2. Characterization of ultrastructure of ECM hydrogels

Sample preparation of ECM hydrogels for scanning electron microscopy was adapted from Stuart et al. [31]. Gels were dehydrated through graded ethanol (10%–100%). Hydrogels were dried at critical point using carbon dioxide exchange. The resulting dehydrated ECM discs were mounted onto metallic stubs with conducting carbon tape, sputter coated with gold, and visualized using an AMRAY 1910 Field Emission Scanning Electron Microscope. Constant working distance and magnification were maintained to image all samples. NIH Image J was used to measure and compare fiber diameters. Porosity was determined using Image J from micrographs obtained from at least three-independent samples of dehydrated ECM gels.

2.4. Tissue engineering innervated intestinal smooth muscle sheets

The tissue engineering process was adapted from Raghavan et al. [30]. Briefly, 500,000 longitudinal smooth muscle cells were aligned uniaxially for 4 days on 35 mm diameter circular Sylgard molds containing wavy microtopographies. Enteric neurospheres were treated with Accutase to obtain single cell suspensions. 200,000 cells were resuspended in the appropriate ECM solution and overlaid on the aligned smooth muscle monolayer. Upon gelation, neuronal differentiation medium (neurobasal-A) was added, supplemented with B27 and 1% fetal bovine serum. Differentiation medium was exchanged every second day. Enteric neuronal progenitor cells were allowed to differentiate within the hydrogel for a period of 10 days. Smooth muscle cells compacted the ECM hydrogel over the next 10 days, forming ~1 cm long innervated smooth muscle sheets, anchored between silk sutures. Phase microscopy was used to image neuronal differentiation at the edge of the tissue engineered sheets.

2.5. Biochemical characterization of neuroglial composition in tissue engineered sheets

At day 10, tissue engineered sheets were harvested in radioimmunoprecipitation buffer to isolate protein. Protein concentration was estimated spectrophotometrically using the Bradford assay. 20 μg of protein from each sample was resolved electrophoretically and transferred to polyvinylidene difluoride membranes. Membranes were blotted with antibodies for neuronal βIII Tubulin, neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), and Smoothelin. β-Actin was used to confirm equal loading. HRP-conjugated secondary antibodies were used to visualize proteins using enhanced chemiluminescence.

2.6. Immunohistochemical characterization of neuron composition in tissue engineered sheets

Tissue engineered sheets were fixed in 4% formaldehyde and washed extensively in glycine buffer. Immunohistochemical staining was performed following previously established protocols utilized for staining differentiated neurons within bioengineered tissues [32]. Sheets were blocked with 10% horse serum and permeabilized in 0.15% Triton-X for 45 min. Permeabilized sheets were incubated with primary antibodies directed against Vasoactive Intestinal Peptide (VIP), ChAT and nNOS for 60 min at room temperature. Following antibody incubation, sheets were washed three times with phosphate buffered saline, pH 7.4. Tissue engineered sheets were incubated with appropriate fluorophore conjugated secondary antibodies for 45 min, washed in phosphate buffered saline and imaged using an