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Research report

An acellular cerebellar biological scaffold: Preparation, characterization, biocompatibility and effects on neural stem cells

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ABSTRACT

Biomaterial and regenerative medical research has diversified and developed rapidly. A biological scaffold consisting of an extracellular matrix (ECM) functions not only as a supportive material but also as a regulator of cellular functions. Although decellularized scaffolds have been widely applied for the repair of non-central nervous system (CNS) tissues, their efficacy in the CNS has not been extensively investigated. In this report, we describe a dynamic decellularization protocol that combined intracardial perfusion and a series of treatments to effectively remove the cellular components from the cerebellum, which is a unique and relatively simple CNS structure. The resulting cerebellar scaffold retained neurosupportive proteins and growth factors and, when tested with neural stem cells (NSCs) in vitro, was found to be cytocompatible and to stimulate the proliferation and migration of these cells. NSCs that were cultured in vitro on the scaffold differentiated into neurons and astrocytes, as indicated by their expression of βIII-tubulin and glial fibrillary acidic protein (GFAP). Through subcutaneous and intracranial implantation experiments, this preliminary study demonstrated the in vivo biocompatibility of the cerebellar scaffold and indicated its potential for future applications. Thus, our study demonstrated that the cerebellar ECM scaffold provided tissue-specific advantages for regenerative medical applications.

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1. Introduction

Over the past decade, the field of regenerative medicine has emerged through the integration of materials science, tissue engineering, and stem-cell, molecular, and developmental biology, the goal of which is to create functional and biocompatible tissues or organs for use in repair or replacement procedures in patients based on clinically relevant approaches (Badylak et al., 2011b). One of the most important advancements in this field has been the production of biological scaffolds containing functional and structural extracellular matrix (ECM) components, which are logical and ideal scaffolds for organ and tissue regeneration (Barkan et al., 2010; Nelson and Bissell, 2006; Vorotnikova et al., 2010).

All tissues and organs consist of cells and the associated ECM, which is a product of structural and functional molecules secreted by the resident cells and constitutes a unique, tissue-specific three-dimensional environment (Calve et al., 2010; Ott et al., 2008). The native ECM dynamically interacts with the resident cells in response to variations in the microenvironment and has been shown to play an important role in the attachment, proliferation, migration, and differentiation of cells (Chastain et al., 2006; Koochekpour et al., 1995; Simon-Assmann et al., 1995; Williams et al., 2008; Yang et al., 2011). It is highly desirable to preserve the native composition and ultrastructure of the ECM during the process of tissue and organ decellularization (Ott et al., 2010; Petersen et al., 2010).

To date, acellular scaffolds have been widely applied in non-central nervous system (CNS) tissues and have been used to successfully repair or replace skin, cardiac valves, blood vessels, the bladder, the urethra, the small bowel, skeletal muscles (Badylak et al., 2011a; Bolland et al., 2007; Butler et al., 2005; Conconi et al., 2004; O'Connor et al., 2002; Palminteri et al., 2007; Parnigotto et al., 2000; Quarti et al., 2011), and even more complex organs, such as the lung and heart (Ott et al., 2008, 2010). However, due to the

Abbreviations: ECM, extracellular matrix; CNS, central nervous system; NSCs, neural stem cells; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; H&E, hematoxylin and eosin; SDS, sodium deoxycholate solution; GAGs, glycosoaminoglycans; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; BDNF, brain derived neurotrophic factor; NGF, nerve growth factor; SEM, scanning electron microscopy.

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specific characteristics of the CNS and its complicated response to injury, until now, few decellularized ECM scaffolds have been tested for the repair of CNS tissue (DeQuach et al., 2011; Guo et al., 2010; Medberry et al., 2013; Ribatti et al., 2003).

The cerebellum, one of the most thoroughly studied areas of the CNS, functions in the control of smooth and skillful movements, and it is involved in higher cognitive and emotional functions (Sillitoe and Joyner, 2007; ten Donkelaar et al., 2003). The unique and relatively simple architecture of the three-layered cerebellar cortex and the well-defined afferent and efferent fiber connections have made the cerebellum a favored research area and a good candidate for studying the tissue engineering and developmental biology of the CNS (Erceg et al., 2011; Hoshino, 2012; Ito, 1999).

The goals of the present study were to develop a method to decellularize cerebellar tissue, to characterize the resulting scaffold with respect to its composition and its in vitro effects on neural stem cells (NSCs), and to evaluate its in vivo biocompatibility. Comparing with previously described decellularization studies (DeQuach et al., 2011; Guo et al., 2010; Medberry et al., 2013; Ribatti et al., 2003), our protocol was able to provide a cerebellar ECM scaffold that maintained the original microstructure of the cerebellum and indicated its potential for future applications.

2. Materials and methods

2.1. Preparation of the cerebellar biological scaffold

All of the surgical and experimental procedures were reviewed and approved by the Animal and Ethics Review Committee. On the basis of our repeated experimental investigation, the protocol parameters were determined, which was suitable for decellularizing mouse cerebellum. The cerebella of 18- to 20-g C57BL/6 mice were harvested under aseptic conditions. The mice were anesthetized using chloral hydrate and were transcardially perfused with a 1.0% sodium deoxycholate solution (SDS) for 5 min (at 20 ml per min). The cerebella were removed and immediately placed in phosphate-buffered saline (PBS) at 4 °C. To maintain sterility, all of the subsequent steps were performed in a laminar flow hood. All of the non-CNS tissue was separated and removed. The following series of agitated bath treatments at 25 °C were used for decellularization: 1.0% SDS (60 min, 100 rpm), deionized water (10 min, 60 rpm), 0.02% trypsin/0.05% EDTA (30 min, 60 rpm), deionized water (10 min, 60 rpm), 1.0% Triton X-100 (60 min, 100 rpm), 1.0 M sucrose (15 min, 60 rpm), and deionized water (30 min, 60 rpm). The decellularized cerebella were immersed in antibiotic-containing PBS (100 U/ml penicillin G, 100 U/ml streptomycin, and amphotericin B) for 72 h. The acellular cerebellar biological scaffolds were stored in PBS until used (Fig. 1).

Unless otherwise noted, all of the chemicals were purchased from Sigma, and all solutions were either filter-sterilized or autoclaved before use.

2.2. Characterization of the cerebellar ECM components

2.2.1. Characterization of the residual DNA in the cerebellar ECM

To qualitatively assess the residual DNA in the ECM scaffold, the following procedure was conducted: the scaffold was fixed using 4% neutral buffered formaldehyde and then embedded in paraffin, sectioned, and stained using 4′,6-diamidino-2-phenylindole (DAPI) or hematoxylin and eosin (H&E). After digesting the comminuted ECM scaffold using 0.1 mg/ml of a proteinase K solution, the content and lengths of the residual DNA were quantitatively investigated. The proteins were digested until no white precipitate was found at the interface that formed following repeated phenol/chloroform extractions and centrifugations (10,000 × *g*), and the extract was then mixed with 100% ethanol and 3 M sodium acetate. After centrifugation, the DNA pellet was rinsed using 70% ethanol, centrifuged, and dried. The double-stranded DNA was quantified using PicoGreen (Invitrogen), according to the manufacturer's instructions. Gel electrophoresis of the DNA extracts on a 1.0% agarose gel containing ethidium bromide (2 h at 60 V) and imaging using ultraviolet transillumination revealed the base-pair length of the DNA that remained in the cerebellar ECM scaffold. The decellularization efficacy was evaluated according to the following criteria: (1) the absence of visible nuclei in H&E- and DAPI-stained sections; (2) <50 ng dsDNA per mg of lyophilized ECM (dry weight); and (3) no DNA fragments exceeding 200 bp in length (Arenas-Herrera et al., 2013; Crapo et al., 2011).

2.2.2. Protein constituents of the cerebellar ECM

The procedure for examining the protein content was described previously (Arenas-Herrera et al., 2013; Crapo et al., 2012). Briefly, unstained sections of native cerebella and the cerebellar scaffold were deparaffinized, rehydrated, and stained for collagens using Masson's trichrome stain, stained for myelin using the Luxol fast blue stain, and stained for glycosoaminoglycans (GAGs) using the Alcian blue/PAS stain. They were then blocked using 2% normal goat serum; incubated serially with primary antibody, H_2O_2, a peroxidase-conjugated secondary antibody, and diaminobenzidine; counterstained using H&E; and dehydrated using ethylene–xylene.

A sandwich enzyme-linked immunosorbent assay was performed according to the manufacturer's instructions (Chemicon, USA) to determine the contents of brain-derived neurotrophic factor (BDNF) and nerve-growth factor (NGF) (Kauer-Sant'Anna et al., 2007). Briefly, samples were homogenized in PBS containing 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′*N*′-tetraacetic acid (EGTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The wells of 96-well flat-bottom plates were coated with samples that had been diluted 1:2 in sample diluent or, to prepare standard curves for quantification, with 7.8–500 pg of BDNF or NGF, and they were then incubated for 24 h. After coating, the sample diluent was used to wash the plates four times. After being diluted 1:1000 in the sample diluent, a monoclonal anti-BDNF rabbit antibody or a polyclonal anti-NGF rabbit antibody was applied for 3 h at room temperature. Then, the samples were incubated with 1:1000 diluted peroxidase-conjugated anti-rabbit antibody for 1 h at room temperature after washing. After the sequential addition of the streptavidin enzyme, the substrate solution, and the stop solution, the levels of BDNF or NGF were determined based on the absorbance at 450 nm. A direct relationship between the BDNF or NGF concentration and the optical density (OD) was demonstrated using the standard curves. Using bovine serum albumin as a standard, the amount of total protein present was determined using Lowry's method.

2.3. In vitro characterization of the cerebellar ECM

Human NSC lines were used to evaluate the in vitro effect of the decellularized cerebellar ECM scaffold on their proliferation, migration, and differentiation. The derivation of the human NSC lines is described in Sun et al. (2009). Established human NSC lines are cultured on laminin (10 mg/L)-coated dishes (Iwaki) in expansion medium comprised of RHB-A medium (Stem Cell Sciences Ltd., UK), recombinant mouse EGF (10 ng/ml, Peprotech), and recombinant human FGF-2 (10 ng/ml, Peprotech). The expansion medium was changed every 2 days.

We used a urinary bladder ECM scaffold as a reference material to evaluate whether the cerebellar ECM scaffold offered tissue-specific advantages in vitro. Urinary bladder matrix was prepared as previously described (Reing et al., 2009). Briefly, connective tissues were removed from the serosal surface of the bladder.

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