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Enzymatic digestion improves the purity of harvested cerebral microvessels

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ABSTRACT

The harvest of intact cerebral microvessel yields could permit the *in vitro* characterization of mechanisms that underlie numerous vascular-linked central nervous system (CNS) phenomena. Here, we test (1) the effect of mild enzyme digestion on microvessel purity and yield; and then (2) the effect of variable centrifugation and filtration methods on microvessel yields. The brains of female Sprague-Dawley rats (4 weeks-old; $n = 38$) were removed rapidly and homogenized. In Experiments 1 and 2, brain homogenates were incubated in DMEM or a solution of papain (2.5 U/ml), DNAse I (250 U/ml) and dispase II (1 U/ml) in DMEM for 15 min at 37 °C before microvessels were purified using differential (20% Ficoll) and then discontinuous (15/20% Dextran) centrifugation (@3500 × *g*) and collected with glass bead column filtration. Enzymatic digestion decreased microvessel yields (27 vs. 12k/g tissue; $p = 0.053$) but increased microvessel purity by decreasing adherent cells ($p = 0.002$), which included NF-L$^+$ neurons ($p < 0.05$) and GFAP$^+$ astrocytes ($p < 0.001$) and astrocyte endfeet ($p < 0.01$). After one week in culture, >85% of harvested cells morphologically resembled microvessels and expressed the vascular proteins lectin and/or RECA-1. Finally, microvessels yields decreased when discontinuous centrifugation was omitted or nylon mesh filtration was employed. In summary, we found that digesting brain homogenates enzymatically could improve the purity of harvested microvessels that could be cultured for at least a week.

1. Introduction

The endothelium transports oxygen and nutrients to all tissues and organs and modulates their metabolic, secretory, synthetic, and immunologic activity (see Cines et al., 1998). Cerebrovascular integrity is critical for normal central nervous system (CNS) development and lifelong maintenance. For example, age-related changes in cerebrovascular integrity have been implicated in stroke, neurodegenerative diseases and even age-related cognitive decline (see Brown and Thore, 2011). The endothelium also exhibits regional physiological differences that affect the permeability of select factors through the blood brain barrier at circumventricular organs, set up niches that permit or promote the production of neurons postnatally or even render some CNS structures more vulnerable to the effects of ischemia (Palmer et al., 2000; Vogel et al., 2001; Cavaglia et al., 2001). The *in vitro* characterization of microvessels across age and CNS regions may provide insight into the mechanisms behind beneficial and detrimental vascular-linked phenomena.

While published methods for harvesting vascular cell subtypes (i.e. endothelial cells) are relatively abundant, only a handful describes the harvest of intact cerebral microvessels. Early methods simply collect microvessels from mechanically dissociated cerebral tissue with variable pore-sized meshes that discard contaminating cell types in the filtrate (Brendel et al., 1974; Goldstein et al., 1975; Hjelle et al., 1978; Head et al., 1980). Later protocols introduced low- and high-speed density gradient centrifugation to purify microvessels from mechanically dissociated tissue before collecting them on either nylon meshes or on glass beads contained in filtration columns (Harik et al., 1985; Pardridge et al., 1985; Gerhart et al., 1988; Dallaire et al., 1991). Glass bead versus nylon mesh filtration appears to improve microvessel yields and although high-speed versus low-speed gradient centrifugation appears to improve microvessel purity, high-speed centrifugation is also associated with shear stress that alters protein expression and induces DNA damage (Pardridge et al., 1985; Dallaire et al., 1991; Brindley et al., 2011). Combinations of these relatively simple techniques have been employed in studies seeking to characterize pure populations of metabolically active microvessels (Cangiano et al., 1983; Dallaire et al., 1991; Miller et al., 2000; Enerson and Drewes, 2006; Chun et al., 2011), but produced microvessels contaminated with adherent cells in our hands following low-speed centrifugation.

Here we describe a simple combined technique for harvesting relatively pure microvessels. We first incubated cerebral homogenates in a mild enzyme cocktail, typically used to

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dissociate neural progenitor cells from parenchyma and vasculature (Palmer et al., 2000; Peltier et al., 2010) or DMEM and then purified and collected microvessels with low-speed centrifugation and glass bead filtration. Enzymatic digestion produced ~12,000/g of homogenate of microvessels that were relatively devoid of contaminant cells or their fragments. The microvessels appeared healthy and expressed the endothelial markers lectin and RECA-1 for at least one week in culture. Overall our data show that our methodological modification can improve the purity of microvessels harvested from brain homogenate.

2. Materials and methods

2.1. Subjects

The 4 week-old female Sprague-Dawley rats ($n = 38$) from our breeding colony maintained by the University of Florida Animal Care Services used in this study were treated in accordance with relevant University and NIH policies regarding the ethical use of animals for experimentation. These rats were the wild-type offspring of green fluorescent protein-expressing SD-Tg(GFP)2BalRrrrc rats imported from the Rat Resource and Research Center (University of Missouri, MO). They were pair-housed after weaning in shoebox cages located in a specific pathogen free colony room maintained on a 12:12 h light:dark cycle at 23 ± 1 °C and had free access to water and food (Teklad LM-485; Harlan Laboratories).

2.2. Brain extraction and tissue preparation

The rats were CO_2-asphyxiated and decapitated. Their brains were extracted aseptically and rapidly placed into ice-cold phosphate-buffered saline (PBS) containing 300× penicillin–streptomycin–amphotericin (PSA; MediaTech, VA). The meninges, superficial large blood vessels and choroid plexi were removed with sterile forceps on a Zeiss Stemi DV4 stereo microscope (Zeiss MicroImaging, NY). The weighed brains were homogenized in ice-cold DMEM (5 ml) using a Potter-Elvehjem Tissue Grinder (approx. 0.005-in. clearance; 10 up-and-down strokes). In Experiment 1, tested the effect of digesting homogenates in an enzyme cocktail versus DMEM prior to purification by centrifugation and collection by glass bead filtration on microvessel yield and purity. ($n = 4$ brains per condition; 3 replications). In Experiment 2, we conducted more precise confocal analyses of purity on microvessels harvested from enzymatically digested versus control homogenates ($n = 5$ brains per condition). In Experiment 3, we tested the effect of manipulating gradient centrifugation and filtration methods on microvessel yields by passing the enzymatically digested homogenate of 4 pooled brains through (1) 20% Ficoll centrifugation, 15/20% Dextran centrifugation and then glass bead filtration, (2) 20% Ficoll centrifugation, 15/20% Dextran centrifugation and then nylon mesh filtration or (3) 20% Ficoll centrifugation and then glass bead filtration.

2.3. Enzymatic digestion

Brain homogenates were pelleted using a Sorvall Legend T centrifuge (Thermo Scientific, NC; 3500 × g for 10 min) and then suspended in 10 ml of DMEM or a cocktail containing papain (2.5 U/ml), dispase II (1 U/ml) and DNAse I (250 U/ml; all from Worthington Biochemicals, NJ) in DMEM before being incubated at 37 °C for 15 min. The enzymes were inactivated with 10 ml of 10% defined fetal bovine serum (FBS; Hyclone) before the cells were re-pelleted by centrifugation (3500 × g) for 10 min.

2.4. Microvessel purification by differential and density gradient fractionation

Pelleted cells were suspended in a solution of 20% Ficoll 400 (Fisher Scientific, Fairlawn, NJ) in DMEM and then centrifuged (3500 × g) for 20 min (with the brake off). The microvessel-containing pellet was suspended in 9 ml of 15% Dextran 500 (Fisher Scientific) layered onto 3 ml of 20% Dextran 500 to form a discontinuous gradient, which was centrifuged (3500 × g) for 20 min (with the brake off). The microvessel-enriched pellet was prepared for filtration. In Experiment 3, we omitted the dextran discontinuous gradient centrifugation step that potentially contains large microvessels (Dallaire et al., 1991).

2.5. Glass bead filtration

Pelleted microvessels were suspended in 25 ml of DMEM and passed slowly through a fritted drying column (Supelco Analytical, MO) containing a 3-cm layer of glass beads (400–600 μm; Sigma–Aldrich, MO). The emptied column was then washed 3× with DMEM to filter non-adherent debris and small cells through gaps between the glass beads. The microvessel-bound glass beads were removed from the column and agitated gently in 50 ml of DMEM to detach the microvessels. The microvessel-containing suspension was decanted and microvessels pelleted by centrifugation (3500 × g) for 10 min before plating.

2.6. Mesh filtration

Pelleted microvessels were suspended in 25 ml of DMEM and then collected by passing the solution through a 40 μm Nylon mesh (Fisher Scientific). The filtrate was washed 3× with DMEM to remove smaller cells and debris. The microvessel retentate was collected in DMEM and then pelleted by centrifugation (3500 × g) for 10 min before being plated. Yields obtained from glass bead versus nylon mesh filtration were compared because mesh filtration has been applied extensively to microvessel isolation (Brendel et al., 1974; Hjelle et al., 1978; Yousif et al., 2007).

2.7. Cell culture

Multiwell chamber slides and 6 cm plastic culture dishes were coated with 0.3 mg of rat tail collagen (BD Biosciences)/ml acetone dried on their surface. Pelleted microvessels were suspended in DMEM containing 20 ng/ml endothelial cell growth supplement (ECGS; BD Biosciences, MA), 100× PSA and 15% heat-inactivated FBS and plated (Hatley et al., 2003). In Experiment 1, 50% of the media was replaced 48 h and after one week, the microvessel cultures were fixed in 4% paraformaldehyde for 10 min for immunohistochemical processing. In Experiment 2, the microvessel cultures were fixed in 4% paraformaldehyde 1 h after plating for immunohistochemical processing.

2.8. Immunohistochemistry

Cells were rinsed repeatedly in tris-buffered saline (TBS; pH 7.4) before and between steps. The cells were blocked in a 3% Normal Donkey Serum and 0.1% Triton-X solution for 20 min and then incubated overnight at 4 °C in a primary antibody cocktail (all 1:500; see below). The following day, the cells were incubated in the appropriate fluorophore-conjugated streptavidin and maximally adsorbed secondary antibodies (all 1:500; Jackson Immunoresearch, PA) for 4 h at RT and then 4′,6-diamidino-2-phenylindole (DAPI; 1:10,000;