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Development and characterization of an endothelial cell line from the bulbus arteriosus of walleye, *Sander vitreus*

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ABSTRACT

A cell line has been developed from the bulbus arteriosus (BA) of the walleye (WE), *Sander vitreus* (Mitchill), and is termed WEBA. WEBA produced collagen I, and when held at confluency for days or weeks, spontaneously formed capillary-like tubes. WEBA cells bound fluorescently-labeled *Ulex europaeus* lectin agglutinin I (UEA-1), took up acetylated low density lipoprotein (Ac-LDL), were stained for von Willebrand factor (vWF), and produced nitric oxide (NO). The cytoskeleton consisted at least of α- and β-tubulin, vimentin, and actin, with the actin organized into circumferential bundles. Immunofluorescence staining revealed at least two tight junction proteins, zonula occludens-1 (ZO-1) and claudin 3. Together these results suggest that WEBA is an endothelial cell line. Relatively high doses of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) induced cytochrome P4501A (CYP1A) protein and 7-ethoxyresorufin o-deethylase (EROD) activity in WEBA. As one of the first fish endothelial and BA cell lines, WEBA should be useful in many disciplines in which the teleost cardiovascular system is a focus.

1. Introduction

Studying the teleost cardiovascular system can contribute to understanding the evolution of the vertebrate circulatory system and provide practical information on the diseases and toxicology of both fish and mammals. An example of relevance to medicine is the description of atherosclerosis in salmon and possible insights this provides for the human disease (Farrell et al., 1986). Examples of direct significance to teleost health are the discoveries that several fish viruses have a tropism for the endothelial cells lining the luminal surface of the cardiovascular system (Aamelfot et al., 2013) and that during aquaculture some species develop cardiovascular abnormalities (Pombo et al., 2012). Significant to the health of both mammals and fish is the correlation between exposure to environmental contaminants, such as dioxin-like compounds, and the development of cardiovascular disease (Garrick et al., 2005, 2006; Humblet et al., 2008).

Endothelial cells (EC) are key cellular component of many cardiovascular problems and for mammals are commonly studied in vitro as primary cultures or cell lines. Primary cultures of human umbilical vein endothelial cells (HUVEC) have been used intensively in pathophysiology (Cines et al., 1998). EC lines have been obtained by different immortalization procedures (Ades et al., 1992; Kuruvilla et al., 2007) but also have developed spontaneously (Takahashi et al., 1990). These cell lines retain different subsets of endothelial cell properties and are a valuable research resource (Unger et al., 2002).

By contrast, teleost endothelial cells have been studied in vitro only rarely. Primary cultures were prepared first over a decade ago from the rete mirabilis of eels (*Anguilla* spp.) (Garrick, 2000) and have been used in toxicology and cell biology (Garrick et al., 2005, 2006; Huang et al., 2006). Yet a teleost endothelial cell line has been reported only recently. Polyoma middle T antigen was used to develop an immortal rainbow trout heart cell line (RTH) (Luque et al., 2014). RTH has a cobblestone morphology and underwent phagocytosis but was not examined for other classic markers of endothelial cells such as internalization of acetylated low density lipoprotein (Ac-LDL) and expression of von Willebrand factor (vWF). Possibly another source of fish endothelial cell lines is the bulbus ateriosus (BA). The BA is a regulatory conduit for blood flow from the ventricle to the gills and has been considered a subsidiary chamber of the teleost heart (Grimes et al., 2006). The chamber is lined with endothelial cells surrounded by connective tissues and smooth muscle (Benjamin et al., 1983; Icardo, 2013; Leknes, 2009). The literature contains no formal description of cell lines from this organ (Bols and Lee, 1991), although one (Tmb) from tilapia was noted in passing (Lewis and Marks, 1985) and has recently been used to measure osmotolerance (Gardell et al., 2014).

Likely the most widely stocked and important commercial and recreational freshwater fish species in North America is *Sander vitreus* (Mitchill) or walleye (WE) (Fenton et al., 1996). WE aquaculture has also begun (Summerfelt, 2005). Thus there is a need for cell lines to

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study at the cellular level the physiology and diseases of this species. However, the few WE cell lines developed in the past appear to have been lost. Recently, the caudal fin of *S. vitreus* was explored as a cell line source, and a fibroblast cell line (WE-cfin11f) (Vo et al., in press) and an epithelial-like one (WE-cfin11e) (Curtis et al., 2013) were described.

In this report the development and characterization of an endothelial cell line (WEBA) from the bulbus arteriosus (BA) of walleye (WE) is described. WEBA has several endothelial properties, including UEA-1 binding, Ac-LDL uptake, nitric oxide (NO) production, and vWF expression, and responds to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) with the induction of cytochrome P4501A (CYP1A).

2. Materials and methods

2.1. Tissue culture supplies and fish

The basal medium Leibovitz's L-15 and fetal bovine serum were from Hyclone (ThermoFisher Scientific, Burlington, ON). Penicillin and streptomycin (P/S) for supplementing L-15 and dimethyl sulfoxide (DMSO) for cryopreservation were purchased from Sigma-Aldrich (St Louis, MO). Other tissue culture supplies were purchased through VWR International (Mississagua, ON) and included trypsin (Lonza) for subcultivation or passaging, 12.5 cm^2, 25 cm^2 and 75 cm^2 flasks (BD Falcon), 4-chamber slides (BD Falcon) and slide flasks (Nunc). TryplE, a trypsin derivative, was used initially for establishing primary cultures and was purchased from Invitrogen. The fish were juvenile walleye of the Bay of Quinte Stock and obtained and handled as described previously (Vo et al., in press).

2.2. Initiation of BA primary cultures and development of WEBA cell line

In the laminar flow hood, a whole bulbus arteriosus (BA) was removed from the heart of the fish and placed in a sterile plastic Petri dish containing Ca^{2+} and Mg^{2+}-free Dulbecco's Phosphate Buffered Saline solution (DPBS, Cellgro) with 200 U/mL penicillin, 200 μg/mL streptomycin, and 0.5 μg/mL amphotericin B. The BA was cut into smaller pieces that were then rinsed with DPBS with antibiotics/antimycotics twice prior to being transferred into 25 cm^2 culture flasks for initiation of primary cultures. BA primary cultures were initially kept at 20 °C in L-15 medium with 10% fetal bovine serum (FBS, Hyclone), 200 U/mL penicillin, and 200 μg/mL streptomycin. Media change was done daily during the first three days and then every three–five days. The first passage was performed on the BA primary cultures with TryplE after approximately a month in cultures. Subsequent subculturing successfully resulted in the continuous propagation of cobblestone-shaped cells that eventually gave rise to the WEBA cell line. May-Grunwald Giemsa staining was performed to examine the organization and morphology of the cells. All chemicals were purchased from Sigma Aldrich, unless otherwise specified.

To examine the effect of FBS on the cell proliferation, cell number in cultures with different FBS concentrations was monitored with a Coulter counter (Vo et al., in press). Walleye serum would have been an interesting medium supplement but was unavailable in sufficient volume and quality to test. Approximately 8×10^4 cells in triplicates were seeded per well in 6-well plates. The next day 3 well cultures were used to establish day 0 cell count. Cultures were then changed to L-15 with 2%, 5%, 15% or 20% FBS and incubated at 26 °C during the course of the experiment. Cell counts were done on days 2, 4 and 7.

2.3. Maintenance of other fish cell cultures

Rainbow trout liver epithelial (RTL-W1) cell line (Lee et al., 1993) was maintained in L-15 with 5% FBS. Walleye fin fibroblastic (WE-cfin11f) (Vo et al., in press) and epithelial (WE-cfin11e) (Curtis et al., 2013) cell lines were established from caudal fins of juvenile walleye and grown in L-15 with 10% and 5% FBS, respectively. Rainbow trout and walleye cells were subcultured by trypsin on a weekly basis and kept at 20 °C and 26 °C for routine maintenance, respectively.

2.4. Cell line authentication by DNA barcoding

The same procedure was followed as previously described for the walleye fin fibroblast cell line WE-cfin11f (Vo et al., in press). DNA barcoding was based on amplification of cytochrome c oxidase subunit 1 (COX1) gene using a PCR primer cocktail designed for teleosts (Cooper et al., 2007; Ivanova et al., 2007) as previously described for fish cell lines in particular (Sansom et al., 2013). A 655 bp region of the walleye COX1 gene was sequenced. DNA sequence was compared and matched to the species identification in both the Barcode of Life Data (BOLD) (Ratnasingham and Hebert, 2007) (http://www.barcodinglife.org) as well as the NCBI BLAST databases (http://www.ncbi.nlm.nih.gov/BLAST). The WEBA samples were run along with samples from the walleye fin fibroblast WE-cfin11f cell line.

2.4.1. General fluorescence microscopy procedures

Regardless of the cellular component being targeted for visualization, the procedures for fluorescence microscopy had some common steps as recently described by Gignac et al. (2014). Microscopy was done on cultures in Nunc slide flasks. Prior to fixation, cultures were rinsed several times in phosphate buffered saline (PBS). With a few exceptions, fixation was 3% paraformaldehyde (PFA) for 20 min at 4 °C. This was followed with 0.1% Triton X-100 in PBS for 10 min to permeabilize cells. For collagen I, vimentin and zonula occludens-1 (ZO-1), fixation was ice-cold absolute methanol for 20 min at 4 °C. This was followed by a quick wash in PBS to rehydrate cells. Prior to the application of primary antibodies, cells were rinsed in PBS and incubated for 1 h in a blocking buffer (BB): 10% goat serum, 3% bovine serum albumin and 0.1% Triton X-100 in PBS. Details of the primary antibodies, their dilution in BB, and their application conditions are described in the sections below for particular cellular components. After the primary antibody incubation, cultures were washed with PBS three times with rocking and subsequently incubated with Alexa Flour 488®-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (1:1000 in PBS) for 1 h. Cultures were then washed five times with PBS, allowed to dry, and mounted in Fluoroshield medium containing DAPI. Fluorescence images were taken with a Zeiss LSM 510 laser-scanning microscope and confocal images were acquired and analyzed using a ZEN lite 2011 software.

2.5. Fluorescence microscopy of collagen type I in WEBA cultures

WEBA cells were seeded into Nunc slide flasks at approximately 5×10^5 cells per flask and allowed to grow at 26 °C for up to 2 days prior to fixation and staining. An affinity purified rabbit polyclonal antibody against salmon collagen I (Cedarlane, Burlington, ON) was used at 1:400 dilution for 1 h.

2.6. Phagocytosis, Ac-LDL uptake, and UEA-binding

The internalization of polystyrene beads by fish cells in monolayer cultures was monitored with a Nikon inverted phase contrast microscope. Polysciences Inc. (Warrington, PA) was the source of polystyrene carboxylated modified beads (2.0 μm ± 0.1 μm in diameter, 2.5% solid in water). Each confluent culture in a 25 cm^2 flask received 4 mL of 1:1000 beads in L-15 with 10% FBS and was incubated at room temperature. After overnight incubations, monolayers were washed vigorously with PBS and observed and photographed.

The binding of fluorescently-labeled *Ulex europaeus* agglutinin I (UEA-1) to WEBA cells was examined. After 5×10^5 cells were seeded in Nunc slide flasks for 2 days at 26 °C, cells were washed twice with PBS, fixed in 2% PFA for 20 min, washed quickly once with PBS and