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Lab resource

Cell growth of immortalized arachnoid cells in the presence of fibroblasts and blood products

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ABSTRACT

Object: The pathophysiology of non-obstructive hydrocephalus involves alteration in cerebrospinal fluid (CSF) pathways. The exact mechanism is unknown, but as arachnoid CSF egress is a major route of CSF removal, damage or alteration to the growth of arachnoid cells may influence the rate of CSF absorption. We investigated the effect of soluble factors secreted by fibroblasts and the presence of blood products on arachnoid cell growth.

Methods: An immortalized arachnoid cell line was developed and cells were grown on semipermeable membranes in a culture chamber. Arachnoid cells were plated in Transwells®, with fibroblasts separated from the arachnoid cells. Cell phenotype was analyzed and cell growth rates were determined by manual counts. Similar experiments were conducted with biliverdin, bilirubin, as well as fibroblast challenge. DNA content in the cell cultures was then determined as corroborative data. Cell counts for the additional arachnoid cell lines were calculated at each day and represented the controls.

Results: Cell counts increased with each time point. Arachnoid cells in the three experimental conditions showed a statistically significant decrease in cell counts for each day when compared to the control group. Post hoc analysis showed differences between the control and experimental conditions but no significant difference between groups. The DNA content for each experimental condition was reduced at all time points when compared to the control arachnoid cells, but only became statistically significant at day 7.

Conclusion: Inflammation and hemorrhage are two common conditions associated with the development of hydrocephalus. The arachnoid membrane is exposed to fibroblasts and blood products (bilirubin, biliverdin) in these conditions, and their effect on arachnoid cell growth was studied. We have shown that arachnoid cell growth decreases in the presence of fibroblasts, bilirubin, and biliverdin. Given its intimate relationship with CSF, it is possible that the decreased growth of arachnoid cells may affect absorption and thus contribute to the development of hydrocephalus.

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1. Introduction

Hydrocephalus is a heterogeneous diagnosis often encountered in neurosurgery. It can be congenital in nature, or acquired such as in the setting of neoplasm, infection, hemorrhage, and trauma. The principal concept of hydrocephalus is an abnormal accumulation of cerebrospinal fluid (CSF) either from obstruction or decreased absorption. The pathophysiology of non-obstructive hydrocephalus involves alteration in cerebrospinal fluid pathways potentially at the arachnoid gateway, but the exact mechanism has yet to be elucidated [1–6].

CSF absorption through arachnoid granulations was first described in 1923 by Weed and additional reabsorption via nasal lymphatics, nerve sheaths, spinal subarachnoid spaces, and across ependymal and endothelial brain surfaces has since been identified [7–9]. The arachnoid mater encases the subarachnoid space through which CSF courses and forms the CSF–blood barrier [10,11], This membrane extends from the surface of the cerebrum to the filum terminale and is comprised of arachnoid cells. Arachnoid cells are derived from neuroectoderm and have been shown to secrete cytokines, phagocytize foreign matter, and express antigens/receptors. While there is variation of the cellular phenotypes of arachnoid cells over this large area, it is a potential site for injury, which may contribute to hydrocephalus.

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As arachnoid CSF egress is one of the major routes of CSF removal, damage or alteration to the growth of arachnoid cells may influence the rate of CSF absorption. The object of the present study was to examine substances encountered in disease states associated with hydrocephalus and determine if they influence arachnoid cells. We investigated the effect of soluble factors secreted by fibroblasts and the presence of bilirubin and biliverdin, hemoglobin breakdown products, on arachnoid cell growth.

2. Materials and methods

2.1. Production of retrovirus containing SV40 LgTAg and retroviral transduction

Arachnoid cells were harvested from 21-to 23-day old male Sprague–Dawley rats. At passages 3 or 4, the arachnoid cells were replated into a Biocoat six-well plate (BD Biosciences San Jose, CA). Viral transduction was performed when the cells reached 60–80% confluence. Clarified viral supernatant containing BABE-puro-SV40LT and pBABE-neo-hTERT was applied sequentially to the arachnoid cell primary culture. Target cells were initially at a density of 4×10^4 cells per well of a six-well plate. The media was aspirated and 3 ml virus-containing media was added per well. Polybrene (Millipore) was then added to a final concentration of 4 mg/ml. Cells transduced with pBABE-puro-SV40LT were selected over 14 days with puromycin (Sigma).

We described previously the development and characterization of an immortalized rat arachnoid cell line [12]. In short, retroviral constructs pBABE-neo-hTERT and pBABE-puro-SV40LT, containing hTERT and SV40 LTAg along with the G418 and puromycin resistance genes, were used to transfect the EcoPack2 cells (Clontech Mountain View, CA), which are 293HEK ecotropic feeder cells containing retroviral packaging genes. Cells were seeded 12–18 h prior to use at 5×10^5 cells in 25 cm^2 flasks. 5 mg of pBABE-puro-SV40LT was combined with the Fugene reagent (Roche Indianapolis, IN) and the OptiMem (Invitrogen) serum-free media in 250 ml total reaction volume with 3:1 ratio of Fugene:DNA. This was incubated for 30 min and added to each flask of 293HEK ecotropic feeder cells, with 3 ml of serum-containing media per flask.

Immortalized arachnoid cells were incubated at 37 °C in humidified atmosphere of 95% air and 5% CO_2 in a culture media containing Eagle's essential medium with 10% fetal bovine serum (FBS) (Remel Inc., Lenexa, KS), nonessential amino acids (Sigma–Aldrich, Saint Louis, MO), glutamine (BioWhittaker Inc., Walkersville, MD), streptomycin, and penicillin (Gibco Invitrogen Corp., Grand Island, NY) at 10 μl/cc each; medium was changed twice per week.

2.2. Immunostaining

Cells were grown on four-well poly-D-lysine culture slides (BD Biocoat) and fixed with 4% formaldehyde in phosphate buffered saline (PBS). The cells were permeabilized with 0.5% Triton X-100 in PBS for 5–7 min and washed 3× with a washing solution containing 10% FBS and 0.2% Tween-20 in PBS before application of primary antibodies. Double-staining was carried out sequentially as needed with species-specific primary antibodies. In general, cells were incubated with primary antibodies for 1 h, washed with the washing solution and incubated with secondary antibodies for 1 h. Finally, cells were washed with washing solution and nuclei were stained with Hoechst dye (Sigma) for 15 min. Slides were air-dried and water-soluble fluorescence mounting solution (Dako) and cover slips were applied. Fluorescence images were captured with a Nikon Eclipse E600 microscope with Diagnostic Instruments Spot Insight CCD camera. Images were post-processed with Adobe Photoshop CS3. The following antibodies and dilutions were used for immunostaining: rabbit anti-vimentin antisera (Abcam, Cat. #8545) at 1:1, rabbit polyclonal anti-cytokeratin (Dako, Cat. #Z0622) at 1:100, mouse monoclonal anti-cytokeratin18 (Abcam, Cat. #668) at 1:20, mouse monoclonal anti-desmoplakin (Abcam, Cat. #16434) at 1:10. The following secondary antibodies were used, all at 1:500 dilution: Alexa Fluor 488-conjugated anti-rabbit or anti-mouse IgG (Invitrogen), Alexa Fluor 555-conjugated anti-rabbit.

2.3. Arachnoid cell growth with fibroblasts

The Transwell™ system (Corning Inc. Corning, NY) with polyester membranes (0.33 cm^2 per well and pore size of 3.0 μm) was used to determine if fibroblast soluble factors affected growth rate. The Transwells were plated with primary rat brain fibroblasts (Cell Biologics, Chicago, IL) in the apical chamber and arachnoid cells in the basal compartment. Arachnoid cells in each basal compartment were seeded at 4×10^4, and growth rates were counted using a hemocytometer. For each day (ie days 3, 5, 7), the arachnoid cells were removed from the basal compartment using 0.05% trypsin-0.02% EDTA (Gibco Invitrogen Corp. Grand Island, NY) diluted 1:3 in PBS and counted.

2.4. Arachnoid cell growth with Bilirubin, fibroblasts with bilirubin, biliverdin, and fibroblasts with biliverdin

The paradigm was performed separately in the additional four experimental conditions. The perturbing substrate was added to the apical compartment and the arachnoid cells plated in the basal compartment. A concentration of 1.71 mM of bilirubin was used both with and without fibroblasts. Biliverdin at a concentration of 0.01 mM was used for the third and fourth experimental conditions.

2.5. DNA content

To verify the proliferative activity of our arachnoid cells, DNA content in cell cultures was determined using the DNeasy Blood and Tissue Kit (Qiagen Sciences; Maryland). The cells were mechanically disrupted and then lysed using the lysis buffer supplied in the kit. The proteins and polysaccharides are salt-precipitated and the cell debris and precipitates were removed by centrifugation. Binding buffer and ethanol were added to clear the lysate and to promote DNA binding to the DNeasy membrane. Following centrifugation and washing, the DNA was eluted in low-salt buffer. Absorbance scanning was used to determine content and purity at the 260 nm wavelength, according to the manufacturers' instructions.

2.6. Statistical analysis

Analysis of variance was used to analyze cell counts and DNA content were performed using SPSS statistical software (version 20.0; SPSS Inc. Chicago, IL). Post hoc analysis was completed for those tests which were statistically significant ($p < 0.05$).

3. Results

3.1. Immortalized rat arachnoid cell line

We have previously demonstrated the development of an arachnoid cell line with characteristics of the arachnoid cell phenotype [12–15]. The cells in the current experiments show morphology identical to primary rat arachnoid cells prior to and after confluence. They have stable expression of cytokeratin, vimentin,