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Primary cell culture of *Echinococcus granulosus* developed from the cystic germinal layer: Biological and functional characterization

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ABSTRACT

Cell cultures of parasitic helminths are an invaluable tool for investigations of basic biological processes, as well as for development of improved chemotherapeutic agents and molecular interactions between host and parasite. We carried out a simple and efficient methodology to isolate *Echinococcus granulosus* germinal cells which were maintained for at least 4 months while cultivated in the presence of reducing agents and hormones. Microscopic analysis of the primary cell culture revealed the presence of cells with similar *Echinococcus* germinal cell morphology and behaviour. Population doubling time was estimated at 48 h, showing a rapid division rate. To discard possible host contamination, the specificity of the primary culture was tested by nested PCR, analyzing *mdh* gene expression and obtaining only one product with the expected size. We also studied the expression of specific *E. granulosus* proteins in primary cell culture. The novel and systematized method described here constitutes a powerful tool for investigations in cystic echinococcosis on biochemical and biological aspects related to the life cycle of the parasite and mechanisms of host–parasite interactions. This method also constitutes a powerful tool for the design of more efficient therapeutic alternatives.

1. Introduction

The cestode *Echinococcus granulosus* (*Eg*) is the causative agent of cystic echinococcosis (CE), an important zoonosis that affects humans and ungulate animals in many countries around the world. In humans, cysts may develop in various anatomical sites following oral ingestion of *Eg* eggs, but most commonly in the liver and lungs (Eckert and Deplazes, 2004). These cysts are composed of three layers. The inner germinal layer produces small immature worms named protoscoleces and is formed by totipotential cells. This layer is supported externally by a non-cellular laminated layer, which is surrounded by a host-produced adventitial layer. The germinal layer contains several cell types including undifferentiated cells with large nuclei and nucleoli, as well as muscle and tegumentary cells. Undifferentiated cells are proliferative and responsible for forming capsules or proliger vesicles which develop protoscoleces asexually by budding (Thompson, 1995).

Traditionally, the treatment of CE depends on total resection of the cyst mass and in many cases is accompanied by chemotherapy. For inoperable cases, chemotherapy with the benzimidazoles albendazole and mebendazole remains the only alternative (Hemphill and Müller, 2009). In patients treated with these drugs, cyst degeneration and in some cases cyst disappearance has been observed. Moreover, the combination of praziquantel and albendazole has been used successfully in the treatment of hydatid disease (Moro and Schantz, 2008). However, in a significant fraction of patients *Eg* metacestodes did not respond to chemotherapy (Pawlowski et al., 2001).

The ability to cultivate parasitic helminths in vitro is a requirement for investigating host–parasite interactions as well as for the identification of targets for parasite chemotherapy (Coustau and Yoshino, 2000). In recent years, very important advances have been introduced for the parasite *Echinococcus multilocularis* (*Em*). Based on an axenic cultivation system, Spiliotis et al. (2008) developed a method for long-term cultivation of *Em* primary cells in the presence of host hepatocytes. In contrast to advances made in

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Em cultivation, little progress in cultivating *Eg* germinal cells has been made recently (Fiori et al., 1988; Furuya, 1991). This has mainly been due to the inability to produce adequate in vitro conditions capable of promoting cell proliferation without contamination with host cells (Spiliotis et al., 2008). Moreover, the possibility of isolating *Echinococcus* cells that are free of host contamination is a prerequisite to establish primary cell lines (Brehm et al., 2006). Cell line creation from the germinative layer represents a suitable in vitro model for the study of the larval stage due to it consisting of proliferating cells that can produce new cysts or differentiate into protoscoleces (Yoneva and Mizinska-Boevska, 2001). In addition, *Eg* cell cultures could be a powerful tool to be used in in vitro experiments to study physiological, biochemical and molecular processes and to establish new chemotherapeutic models.

In the current work, we report the establishment and characterization of a primary cell culture from the germinal layer of *Eg*. Cultured *Eg* germinal cells have the potential to contribute to the understanding of biochemical and biological aspects of the life cycle of the parasite and of mechanisms of host–parasite interactions, and thus may aid the design of more effective therapeutic alternatives.

2. Materials and methods

2.1. Parasite material and cell isolation

Hydatid cysts were collected aseptically from the liver and lungs of infected cattle slaughtered in several abattoirs located in Mar del Plata, Buenos Aires, Argentina. Cyst layers were washed several times with sterile PBS (100 mM NaCl, 15 mM KH_2PO_4, 85 mM Na_2HPO_4; pH 7.2) and antibiotics (100 μg ml^{-1} streptomycin and 60 μg ml^{-1} penicillin G), submerged for 10 min in each step. The germinal layer was separated using a scraper. The pieces produced were treated with 5 vol. of 0.25% trypsin for 30 min at 37 °C with continuous shaking (22 cycles min^{-1}). After 10 min centrifugation (2000*g*, 4 °C), the pellet was resuspended in prewarmed culture medium supplemented with 20% FBS which was added to stop trypsinization.

2.2. In vitro culture of cells

The culturing procedure was based on that developed for *E. multilocularis* primary culture (Spiliotis et al., 2008) with a number of modifications. Cells were plated on 12-well plates with a density of approximately 10^5 cells/cm^2 and cultivated in 2 ml/well of 199 medium (Gibco BRL) at 37 °C. The culture medium was supplemented with 10% FBS, 10% hydatid fluid, reducing agents (5×10^{-5} M 2-mercaptoethanol and 100 μM L-cysteine), 2 mM L-glutamine (Bio-Rad, USA), 4 mg ml^{-1} glucose (Sigma, USA), 1 mM sodium pyruvate (Sigma, USA), 0.6 U ml^{-1} insulin (Eli Lilly and Co., USA) and antibiotics (penicillin, streptomycin and gentamicin 100 μg ml^{-1}). The final medium pH was 7.5. Cells were cultivated for at least 4 months, reaching total confluence. The culture medium was changed weekly. The number of viable cells was determined by trypan blue exclusion analysis (Redondo et al., 2007). Cell cultures were observed and photographed periodically using an inverted microscope.

2.3. Reverse-transcription (RT)-PCR experiments

Different RNA extractions were performed from *Eg* primary culture cells (2–3 weeks of cultivation), protoscoleces and host tissues as documented above. Total RNA was isolated from different samples using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions (Cumino et al., 2009). For RT-PCR analysis, total RNA treated with DNase (RQ1 RNase-free DNase; Promega) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo-dT. PCR amplifications were performed using primers for the *Eg* malate dehydrogenase gene (*Eg mdh*, Accession No. AAC28239), specifically detecting *Eg* cells by nested PCR. The outer PCR was performed using the primer pair *mdh*Fwe, 5′-GACTCGGGATGACCTCTTTTCGATGAATGCAT-3′ as forward primer and *mdh*Rv 5′-GAGTACAATGTATAGACTAGTAGGC GGCTGCAAA-3′ as reverse primer, and was predicted to amplify a 706 bp segment. The second PCR (nested PCR) was performed using primers *mdh*Fwi 5′-GGAATTGGTGGCTCGAATACAAAACGCA-3′ and the same reverse primer as in the first reaction, and was predicted to amplify a 359 bp DNA fragment. In addition, specific primers were designed for *Bos taurus* β-tubulin-encoding gene (*Bt tub*) to discard possible contamination with host cells (*tub*Fw 5′-GCTCAACGTGCAGAACAAGAACAGCA-3′ and *tub*Rv 5′-CCTCCTCGA ACTCGCCCTCTTCCT-3′). As a positive control for the *Bt tub* gene, cDNA was produced from bovine liver. Additionally, as a positive control for the *Eg mdh* gene, cDNA was produced from protoscoleces. PCRs were run on a PCR Sprint thermal cycler (Thermo Electron Corporation) for 20 cycles of 94 °C (2 min), 49 °C for *Eg mdh* or 52 °C for *Bt tub* (1 min) amplifications, and 72 °C (1 min), plus a single elongation step at 72 °C for 10 min. The second PCR was carried out similarly to the outer PCR except that 30 cycles were performed, with an annealing step at 53 °C for 30 s. RNA quality control and PCR products were separated on a 1% agarose gel, stained with cyber safe (Invitrogen, USA) and visualized under UV light. Parallel reactions were performed without reverse transcriptase to monitor possible genomic DNA contamination in the reactions.

2.4. Immunoblot analysis

Protein extractions were performed on primary cell lysate and protoscolece lysate which resulted from ultrasonication (into ice bath for 120 s) in lysis buffer (250 mM sucrose, 50 mM Tris–HCl, 6 mM β-mercaptoethanol and 1.5 mM EDTA at pH 7.5). The homogenates were centrifuged at 80,000*g* for 10 min. Proteins were quantified using a Bradford assay and were loaded at the same concentration for SDS–PAGE. Immunodetections were performed as previously reported (Cumino et al., 2009). Fifty micrograms of parasite protein per well were separated on 12% polyacrylamide gels and electrotransferred to nitrocellulose membranes (Bio-Rad, USA). Polypeptides were revealed with rabbit polyclonal antibodies against *Eg*AT1 fusion protein corresponding to an *Eg* amino acid transporter (Camicia et al., 2008) and protoscolece total proteins, both diluted at 1:300. A goat anti-rabbit IgG alkaline-phosphatase conjugate (A 8025; Sigma) was used as secondary antibody and bands were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.5. Electron microscopy

For scanning electron microscopy (SEM), cells cultivated for 3–4 weeks were adhered to plastic slides and fixed with 2.5% v/v glutaraldehyde in 0.1% v/v sodium cacodylate buffer for 2 h at 4 °C, and were washed several times in cacodylate buffer. The specimens were then dehydrated by sequential incubations in increasing concentrations of ethanol (50–100%) and finally immersed in hexamethyl-disilazane for 18 h. They were sputter-coated with gold and inspected on a JEOL JSM-6460 LV scanning electron microscope at 15 kV.