Tumor-Derived Cell Culture Model for the Investigation of Meningioma Biology

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Abstract

Meningioma is the most common primary central nervous system tumor. Although mostly nonmalignant, meningioma can cause serious complications by mass effect and vasogenic edema. While surgery and radiation improve outcomes, not all cases can be treated due to eloquent location. Presently no medical treatment is available to slow meningioma growth owing to incomplete understanding of the underlying pathology, which in turn is due to the lack of high-fidelity tissue culture and animal models. We propose a simple and rapid method for the establishment of meningioma tumor-derived primary cultures. These cells can be maintained in culture for a limited time in serum-free media as spheres and form adherent cultures in the presence of 4% fetal calf serum. Many of the tissue samples show expression of the lineage marker PDG2S, which is typically retained in matched cultured cells, suggesting the presence of cells of arachnoid origin. Furthermore, nonarachnoid cells including vascular endothelial cells are also present in the cultures in addition to arachnoid cells, potentially providing a more accurate tumor cell microenvironment, and thus making the model more relevant for meningioma research and high-throughput drug screening.

Key Words: Cultured human meningioma tumor-derived cells, Meningioma model.

INTRODUCTION

Meningioma is the most frequently reported tumor in the Central Brain Tumor Registry of the United States (CBTRUS) with an incidence rate of 8.33 per 100 000 person years, and over 30 000 new cases annually (1). Meningioma is understood to originate from arachnoid cap cells forming extra-axial, intradural tumors at the convexity, falx, skull base, and spinal meninx. Biallelic inactivation of neurofibromatosis 2 (NF2) gene is present in 40%–80% of sporadic meningiomas (2). The second most frequently mutated gene in meningiomas is the TNF receptor associated factor 7 (TRAF7) gene (3) and frequently occurs together with AKT1 E17K, KLF4 K409Q, or PIK3CA mutations (4). Less frequently, SMO L412F and SMO W535L as well as telomerase-reverse transcriptase (TERT) promoter mutations occur in meningiomas. Intriguingly, meningiomas with mutations in NF2 tend to be located in the posterior fossa or lateral convexity, while those of SMO at the olfactory groove, TRAF7 at the skull base, KLF4 at the medial skull base, and AKT1 at the anterior skull base (5, 6). Prostaglandin D2 synthase (PGD2S) is a lineage marker of arachnoid cap cells and expression is frequently retained in meningioma cells (7). The Na-K-2Cl cotransporter and aquaporin 1 were also found to be preferentially expressed in arachnoid granulations and meningiomas (8). Of the 3 histological grades (WHO Grade I–III) and 15 meningioma subtypes included in the current WHO (2016) classification, most cases are slow-growing, well circumscribed, and have a low rate of recurrence (9). The risk of recurrence is estimated by the histological grade, with around 10% recurrence at 5 years for grade I, characterized by low cellularity, the absence of nuclear atypia, lack of sheet-like growth, low mitotic rate, low Ki-67 proliferation index, and lack of brain invasion. Grade II disease has a recurrence rate of 30%–40% at 5 years and identified by brain invasion or a set of other atypical features. Grade III, or anaplastic meningioma has an 80%–100% recurrence rate at 5 years and exhibits malignant behavior with multiple recurrences despite best treatment. Current management of meningioma includes serial imaging for incidental tumor
and maximum safe resection for symptomatic or growing tumor. Radiation therapy (volumetric modulated arc therapy) is offered after surgery for grade II disease, although it is often deferred until radiological progression, depending on the extent of surgery, tumor location, and patient preference. Radiation therapy is strongly recommended for grade III disease. Stereotactic radiotherapy may be applied for inoperable and presumed grade I disease (10–12). Serial imaging is best performed by MRI, except when contraindicated such as patients with severe claustrophobia or MRI-incompatible implanted medical device. Guidelines for the use of gadolinium-containing contrast media are evolving (13), and at our institution contrast is used routinely for grade II and III disease, omitted in pregnancy and severe renal failure, and used in grade I disease on a case-by-case basis depending on patient age, interval stability of tumor, and patient preference. Presently no medical treatment is available to slow tumor growth. This is largely due to the lack of high-fidelity tissue culture and animal models that would facilitate understanding the disease pathology or drug screening. For example, hydroxyurea was shown in a tumor-derived meningioma culture model to effectively inhibit cell growth starting at 0.5 mM or 40 mg/kg/day (14). Subsequently, a clinical trial of 35 patients receiving the dose of 15 mg/kg/day hydroxyurea showed no radiological response (15). Similarly, somatostatin blocked cell proliferation induced by phorbol myristate acetate in a tumor-derived meningioma culture model; however, uninduced cells were not studied (16). A clinical trial of 34 patients with meningioma treated with a long-acting somatostatin analog did not find a change in progression-free survival at 6 months (17). To avoid future failures of translation from disease model to clinical trial, we propose a simple and rapid method for the establishment of meningioma tumor-derived primary cultures for preclinical studies.

MATERIALS AND METHODS

Patients

Patients undergoing elective surgery for suspected or previously diagnosed meningioma were eligible for inclusion. Informed consents were obtained per a protocol approved by the Institutional Review Board. Resected surgical specimens were submitted for standard review. In cases where the Pathology Service determined that there was surplus tissue, it was released for research.

Primary Cultures

Tissue samples received were between 100 and 1000 mg, transported in clean containers on ice, and handled with compliance of BL-2 standards. Specimens were washed with cold PBS in a laminar flow hood, cut to small slices with surgical scissors, then further fragmented to pieces approximately 5–10 mm³ with a scalpel. The resulting tissue was washed again with cold PBS and collected by centrifugation at 200 g in a 50 ml conical tube. The supernatant was replaced with 25 ml DMEM (ThermoFisher Scientific, Waltham, MA) and 5 mg freshly added collagenase III powder (StemCell Technologies, Cambridge, MA). Digestion was performed at 37°C for 20 minutes. The cells were again collected by centrifugation at 200 g in a 50 ml conical tube, resuspended in 10 ml of DMEM with a 10-ml then 5-ml pipets, several times each. The suspension was filtered through a 40-µm nylon cell strainer (Corning, 431750, ThermoFisher Scientific). The filter was washed with 5 ml DMEM 3 times, and the filtered cells were collected by centrifugation at 200 g in a 50-ml conical tube. Cells were resuspended in Neurobasal Media with N-2 and B-27 additives, 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco, 21103049, 17502048, 0080085SA, 15140122, ThermoFisher Scientific), plated in 25 cm² tissue culture flasks and incubated at 37°C in humidified air with 5% CO₂. Adherent cultures were obtained by the addition of 4% fetal bovine serum (ThermoFisher Scientific) for 3 days. The culture media was replaced after 3 days, then every 5–7 days. After replacing the media 3 times, penicillin and streptomycin were omitted. Subconfluent cultured cells were detached with 0.25% trypsin-EDTA (25200056, ThermoFisher Scientific), resuspended in Neurobasal Media, with N-2, B-27, and 4% fetal calf serum (FCS) and plated at 10%–30% confluence in 100 mm dishes, 24- or 96-well plates for Western blot, transfection and fluorescent microscopy. Cells were imaged 2 days after transfection.

Cell Imaging

Twenty-five centimeters square tissue culture flasks were imaged with a phase contrast microscope (DMi8, Leica) and images assembled to figures with an image editing software (Photoshop, Adobe). All images have been processed in parallel, using identical settings. The immortalized meningioma cell lines AC007TERT and MN620, kindly provided by Vijaya Ramesh, Massachusetts General Hospital, Boston, MA, were included for comparison (18).

Transfection

Primary cells were seeded in 24-well plates in Neurobasal Media with N2, B27 and 4% FCS at 50% confluence. The media was changed to 0.4 ml transfection media (OptiMem, 11058021, ThermoFisher Scientific) before transfection. 0.125 nmol of Fluorescein-labeled control siRNA (No. 6201, Cell Signaling Technology, Danvers, MA) diluted in 50 µl transfection media was mixed with 3 µl transfection reagent (Lipofectamine RNAiMAX, 13778150, ThermoFisher Scientific) diluted in 50 µl transfection media, incubated for 5 minutes at room temperature, and added to the cells. The media was changed after 5 hours to neurobasal media without additives or FCS.

Lentivirus Transduction

VSV-G-pseudotyped lentiviral particles were produced by transfecting 293T cells grown in DMEM plus 10% FCS at 10% confluence with 1 ml DNA-calcium phosphate complex in pH 7 HeBS buffer, 50 mM CaCl₂, with 10 µg of the lentiviral plasmid pCDH-CMV-MCS-EF1-copGFP (CD511B-1, System Biosciences, Palo Alto, CA) along with 6.5 µg of pCMVR8.74 and 6.5 µg of pMD2.G (22036, 12259, Addgene, Watertown, MA) in 100-mm dishes. The medium was...
replaced after 12 hours with neurobasal medium without supplements. Lentiviral particles were harvested starting 48 hours after transfection daily for 5 days, and each day 10 ml of fresh neurobasal medium was used as a replacement. Cells and debris were removed from the 50 ml pooled culture supernatant with a 0.45 μm syringe filter (SLHV004SL, MilliporeSigma, Burlington, MA). Primary meningioma cells were transduced with 10 ml of lentiviral vector-containing media in 100-mm dishes. The media was changed to culture media after 4 hours.

Western Blot

Cells cultured in 100-mm dishes were washed with PBS 3 times, collected with cell lifters (07-200-364, ThermoFisher Scientific) followed by centrifugation in 1.7-ml tubes at 500 g, lysed in 2 × RIPA buffer (BP-115X, Boston Bioproducts, Ashland, MA) with protease inhibitors (Complete mini, EDTA-free, Roche, Basel, Switzerland). Thirty micrograms of total protein were loaded per well on a polyacrylamide gel (4%–12% Bis-Tris Plus, ThermoFisher Scientific) and transferred to a 0.45 μm PVDF membrane (88518, ThermoFisher Scientific). After blocking with 5% (wt/vol) fat-free milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), the membranes were incubated overnight with diluted primary antibodies (PGD2S, sc-14825, 1:200, Santa Cruz Biotechnology, Dallas, TX; β-tubulin, Ab7291, 1:10,000, Abcam, Cambridge, UK; epithelial membrane antigen, M061301, 1:100, Agilent, Santa Clara, CA; progesterone receptor, no. 8757, 1:100, CD31 no. 3528, 1:1000, Cell Signaling Technology) at 4°C. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (7074S and 7076S, 1:10,000, Cell Signaling Technology) for 1 hour at room temperature in TBS-T. The blots were developed with ECL (Prosignal Pico, Prometheus Biosciences, San Diego, CA) and imaged using X-ray films at various exposures (Super RX-N, Fuji, Tokyo, Japan) or with a digital camera (iBright CL1000, ThermoFisher Scientific).

Immunofluorescence and Immunohistochemistry

Paraffin blocks were sectioned at 5 μm, deparaffinized and rehydrated. Slides were boiled in 10 mM sodium citrate buffer, pH 6.0 and kept at 99°C for 10 minutes, then immersed in purified water for 5 minutes at room temperature. Endogenous peroxidase activity was blocked in 3% H2O2 in methanol for 15 minutes at room temperature. Sections were washed 2 times for 10 minutes each with 0.4% Triton X-100 in PBS plus 1% FCS, then blocked with 0.4% Triton X-100 in PBS plus 5% FCS for 30 minutes at room temperature. Primary antibodies (PGD2S, sc-14825, 1:100, Santa Cruz Biotechnology, epithelial membrane antigen, M061301, 1:100, Agilent, progesterone receptor, no. 8757, 1:100, CD31 no. 3528, 1:100, Cell Signaling Technology, Ki-67, GA626, 1:100, Agilent) were added in 0.4% Triton X-100 in PBS plus 1% FCS on the sections and incubated at room temperature for 2 hours, washed 2 times with 0.4% Triton X-100 in PBS plus 1% FCS. Label-conjugated secondary antibodies (7074S, 7076S, 1:1000, Cell Signaling Technology, Alexa Fluor Plus 647, A32733, Alexa Fluor Plus 488, A32723, 1:1000, ThermoFisher Scientific) were added in 0.4% Triton X-100 in PBS plus 1% FCS and incubated at room temperature for 2 hours. Sections were washed 2 times with 0.4% Triton X-100 in PBS plus 1% FCS for 10 minutes each. Slides for immunofluorescence were mounted in a DAPI-containing medium (Vectashield Plus, H-2000, Vector Laboratories, Burlingame, CA). Slides for immunohistochemistry were developed with a peroxidase kit (Vectastain Elite, PK-7200, Vector Laboratories), counterstained with hematoxylin, dehydrated with ethanol and xylene then mounted.

Image Analysis

Raw microscopy images were color separated (DAPI, Ki-67, and PGD2S) using the open-source software (Python CziFile). The DAPI and Ki-67 channels were then analyzed using image analysis software running a cell nucleus detection pipeline (CellProfiler). The PGD2S channel was profiled in a fixed border radius around each detected nucleus for both the Ki-67 and DAPI channels, using open-source software (NumPy, OpenCV). The average value for each nucleus in both categories was collected into the 2 sample categories for comparison.

RESULTS

Human meningioma-derived cell cultures were established successfully in 10 instances out of 14 surgical samples, although the yield of viable cells varied. The 4 failures were due to delays in handling the tumor specimen resulting in non-viability before cell dissociation. Initial doubling time showed no correlation with tumor grade or Ki-67 proliferation index (Table). Cells grew as tumor spheres in serum-free media and did not exhibit loss of viability after 14 days. The time to reach confluency was increasing after each passage and eventually the cells failed to reach confluence. Therefore, transfections were carried out at passage 2–3. Adherent cultures were efficiently transfected with oligonucleotides (e.g. siRNAs), and did not exhibit loss of viability after 5 hours of exposure to a liposomal transfection reagent (Fig. 1B). Cells were transduced with green fluorescent protein (GFP)-expressing lentivirus with high efficiency with no apparent toxicity (Fig. 1C). The lineage marker PGD2S is expressed by most cultures, although to a variable degree, as shown by Western blot. CD31 immunoblot suggested the presence of vascular endothelial cells (Fig. 1D).

PGD2S expression was similar in each tumor specimen when tested by immunofluorescence versus Western blot (Figs. 1D and 2A). Tumor-derived cell cultures showed similar PGD2S expression to corresponding tumor tissues (Fig. 1D). Epithelial Membrane Antigen expression showed a weak correlation between cultured meningioma cells versus tumor samples (Figs. 1D and 3A). Of note, progesterone receptor expression was not detected in cultured meningioma.
cells, although a subset of tumor samples showed expression both by Western blot and immunofluorescence (Figs. 1D and 3B). Hematoxylin and eosin staining of tumor tissue sections is shown for morphological reference (Fig. 3C). Based on the double-staining for PGD2S and Ki-67 proliferative marker, the samples can be divided into 3 groups, one with high PGD2S expression and low Ki-67 index (MEN014 and MEN015), the second with low PGD2S expression and high Ki-67 index (MEN016, MEN017, MEN023, and MEN024), and the third with medium PGD2S expression and moderate Ki-67 index (MEN018, MEN020, MEN023, and MEN026) (Fig. 2A). Of note, the Ki-67 index observed by immunofluorescence and the formal Ki-67 index reported by pathology did not show close correlation suggesting large regional heterogeneity, or selective growth of a subpopulation of tumor cells in vitro. Tumor tissue showed variable vascularity by CD31 immunohistochemistry, with similar CD31 expression in cultured tumor-derived cells by Western blot, suggesting that meningioma tumor-derived cultures are intermixed with vascular endothelial cells proportionally to tumor vascularity (Fig. 2B). Distribution analysis of PGD2S expression showed a subpopulation of Ki-67-negative cells with high PGD2S expression. This subpopulation was absent in Ki-67-positive cells, suggesting that there is cell heterogeneity within the tumor, with actively proliferating cells exhibiting a less differentiated phenotype (Fig. 2C).

**DISCUSSION**

The molecular events leading to meningioma formation is poorly understood and no medical treatment is available due to the lack of appropriate disease models. Cell lines derived from human tumor can be easily cultured and tested, however, these are either modified with oncogene overexpression or established from grade III meningiomas, and had acquired multiple genetic changes during many passages, and unlikely to represent a high-fidelity model (20). Although the meningioma mouse xenograft flank model was shown to be useful, tumor cells with complex karyotypes grew preferentially better than those with fewer chromosomal abnormalities (21). Intracranial injection of meningioma-derived cells resulted in asymptomatic but microscopically identifiable tumor growth after a period of 3 months (22). Tumor-derived meningioma cells were successfully cultured and studied in comparison to matched cultured arachnoid cells from the same patient (23). A PGD2S promoter-driven Cre recombinase combined with one flox-flanked and one null Nf2 allele resulted in the development of meningiomas in 6 of 16 mice over 15 months. Additional genetic changes with p16 and p53 homozygous knockouts reduced the time to tumor development (24).

In this study, we describe a primary culture model for meningioma using grade I and II tumor samples. This model is simple to implement and is appropriate for cell-based assays, such as drug screening, oligonucleotide transfection, viral transduction, and standard molecular biology procedures. The cell morphology, growth properties, and lineage marker PGD2S expression varied between individual cultures, however, there is a correlation between levels found in tumor tissue and cultured tumor cells. Therefore, loss of lineage marker expression probably occurs during tumor development in vivo, rather than during culture. It is important to note that the cultures are not homogenous, as shown by the presence of the CD31 vascular endothelial marker. It is advised that experiments are performed at low passage number. Attempts to immortalize cultured tumor-derived cells by ectopic expression of TERT were unsuccessful. Although these cells were not tested for xenograft tumor formation in immunodeficient mice, it is likely that tumor growth would be too slow to be practically useful. Despite these drawbacks, this model may facilitate the understanding of meningioma biology and may be appropriate for preclinical trials of meningioma therapeutics.

**Limitations and Explanations**

In this work, we set out to develop a meningioma model with the possibility to modulate cell functions via transfection or viral transduction, suitable for high-throughput drug screening. These goals can be achieved by cultured cells, but not with achieved specimens, direct analysis of fresh tumor tissue
or xenotransplants. Cell lines are not believed to be a good representation of the disease due to transformation-related changes and genomic instability. For each future application of the primary culture model, it is essential that several independent cultures are used and each is characterized for the genetic alterations relevant for the study. Meningioma-derived cultured cells are not expected to show genomic instability due to intact DNA repair pathways and rapid senescence. It is a limitation of the model that each culture is unique and requires characterization by the user. While exome sequencing of the tumor tissue or cultured meningioma-derived tumor cells is beyond the scope of the current work, array comparative genomic hybridization of tumor tissue was done in select cases of the cohort for clinical indications (Table).
FIGURE 2. (A) Immunofluorescence analysis of tumor tissue sections for the lineage-specific marker PGD2S (magenta) and Ki-67 (green). (B) Immunoperoxidase analysis of tumor tissue sections for the vascular-endothelial marker CD31. (C) Distribution of PGD2S expression as quantified by immunofluorescence (x-axis) analyzed in Ki-67-negative cells (left panel) and Ki-67-positive cells (right panel) in MEN020.
FIGURE 3. (A) Immunoperoxidase analysis of tumor tissue sections for epithelial membrane antigen. (B) Immunoperoxidase analysis of tumor tissue sections for progesterone receptor. (C) Hematoxylin and eosin staining of tumor tissue sections.
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REFERENCES