ESTABLISHMENT OF AN IN VIVO MENINGIOMA MODEL WITH HUMAN TELOMERASE REVERSE TRANSCRIPTASE

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OBJECTIVE: The lack of meningioma models has hindered research on the pathogenesis and treatment of this commonly diagnosed primary brain tumor. Animal models of meningioma have been difficult to develop, especially those derived from Grade I tumors, which display very slow growth rates, senesce at early passages, and infrequently survive as explants in vivo. In this study, the authors report the establishment of two benign immortalized meningioma cell lines, Me10T and Me3TSC, that can serve as useful models of human meningioma.

METHODS: Tissue specimens obtained at the time of surgery were cultured in vitro and transduced with human telomerase reverse transcriptase/SV40 large T antigen to establish long-term cell lines. The telomeric activity, growth kinetics, immunophenotype, and karyotyping of the cell lines were investigated. The growth inhibitory effects of the antitumor therapies, hydroxyurea and sodium butyrate, on these cell lines were determined. In addition, immortalized cell lines were implanted subdurally into mice to confirm their ability to form tumors.

RESULTS: Two immortalized benign meningioma cell lines, Me10T and Me3TSC, transduced with catalytic subunit human telomerase reverse transcriptase alone or human telomerase reverse transcriptase and SV40 large T antigen, were established. The meningeal phenotype of the established cell cultures and orthotopic xenografts was confirmed by immunostaining. After subdural injection into athymic nude mice, both cell lines formed identifiable tumors with histological features and immunostaining patterns of human meningioma.

CONCLUSION: The Me3TSC and Me10T cell lines can serve as useful model systems for biological studies and the evaluation of novel therapies on meningioma.

KEY WORDS: Meningioma, Model, Telomerase, Therapeutics

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Meningiomas are mesenchymal tumors that arise from the meningothelial cells of the arachnoid layer that form the external lining of the brain. Meningiomas are the most commonly diagnosed of all primary intracranial neoplasms, constituting 29.2% of all reported cases (10). Although 80 to 90% of meningiomas are benign, these tumors have recurrence rates of up to 20% over 10 years (4, 13). Meningiomas are usually treated by surgical resection, radiation, chemotherapy, or a combination of these treatments because there are few drugs that have proven effective against this tumor type. In addition, the location of the tumor can prevent complete resection or can determine risk of recurrence in itself.

Until now, researchers have developed few reproducible meningioma models. These include the recently established neurofibromatosis Type 2 (NF2) conditional knockout mouse and the IOMM-LEE and F5 malignant meningioma cell lines (17, 22, 27, 53). Other models have used primary cells from benign and malignant meningiomas to establish short-term meningioma xenograft models (21, 31, 35, 36). The primary challenge in developing a reliable meningioma model has been the slow growth rate of meningioma cells, leading to studies based on short-term cell lines and requiring long observation periods for in vivo studies. This is especially true for the modeling of benign meningiomas, which

are not easily established as cell lines or xenografts. Two recent studies have used human telomerase reverse transcriptase (hTERT) to overcome senescence of primary meningioma cell lines (2, 43).

To create a more reliable and biologically relevant meningioma model, we established long-term cell lines from primary benign Grade I meningioma specimens via immortalization with hTERT and SV40 large T antigen. Several studies using primary meningioma cell lines previously identified the specific markers, morphological features, and culture conditions of meningioma cells (1, 16, 34, 38, 39, 44, 47), which we have used to confirm the characteristics of our established cell lines. We also demonstrated the ability of these meningioma cell lines to grow intracranially in nude mice when implanted subdurally, further strengthening the preclinical relevance and usefulness of this model system. This is one of the first studies to report the development of immortalized meningioma cell lines as well as the use of such lines to grow benign human meningioma in vivo.

MATERIALS AND METHODS

Tumor Specimens and Cell Culture

Tissue specimens were obtained from 10 histologically Grade I meningiomas at the time of surgery from the Department of Neurosurgery, Brigham and Women's Hospital, Boston, Massachusetts, under appropriate institutional guidelines. All tissue samples were collected under sterile conditions. Each tumor specimen was minced into 2- to 4-mm^3 pieces and then plated into 75-mm^2 flasks with Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% heat inactivated fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone (Invitrogen, Grand Island, NY) and incubated at 37°C in a humidified atmosphere of 5% CO_2. Media was changed the next day to remove floating cells and every 3 days thereafter. To prevent overgrowth of fibroblasts and other unrelated cell types, regions containing meningioma and epithelial like cells were scraped off and subcultured in this manner for the first two to three passages. Identification of cells was performed daily by observation with a phase-contrast microscope and immunocytochemistry to assure isolation of meningioma cells. As soon as the cell line was established, 0.05% trypsin was used to passage the cells. Viable cells were frozen in liquid nitrogen in 90% FBS/10% dimethyl sulfoxide.

Transduction of Meningioma Cell Lines

To establish long-term meningioma cell lines, four of the 10 primary lines were infected retrovirally with hTERT alone or with hTERT and SV40 large T antigen. All infections were performed using cells under Passage 5 (P5) with pBABE vectors containing hTERT or SV40 large T antigen. Vectors were provided by William Hahn, M.D., Ph.D., Dana Farber Cancer Institute. The 293T packaging cell line was transfected with the vectors using FuGENE6 (Roche, Indianapolis, IN) to produce the retroviral supernatant. Briefly, 1 ml each of retroviral supernatant and Dulbecco's modified Eagle's medium/10%FBS supplemented with 4μl of 4mg/ml polybrene was applied to the meningioma cells and then incubated at 37°C for 6 hours, at which time 6 ml of media was added, followed by overnight incubation at 37°C. Cells infected with hTERT vector were selected for 21 days with increasing concentrations of 0.5, 0.75, 1.00 μg/ml puromycin (Sigma, St. Louis, MO) for 7 days at each concentration. Cells infected with SV40 large T antigen were selected for 21 days with increasing concentrations of 150, 200, 300, and in some cases, up to 400 μg/ml geneticin (Invitrogen, Grand Island, NY).

Telomeric Activity Assay

The TRAPeze telomerase detection kit (Intergen, Purchase, NY) was used to measure and compare the level of telomeric activity in the nontransduced and transduced cells. Briefly, 200,000 cells from each cell line were resuspended in 1× CHAPS lysis buffer provided in the TRAPeze Kit. The suspension was incubated on ice for 30 minutes and centrifuged, and lysate was collected. Protein concentration of each lysate was measured using Coomassie protein reagent (Pierce, Rockford, IL). Polymerase chain reaction parameters for each protein lysate consisted of incubation at 30°C for 30 minutes followed by 33 cycles of 94°C for 30 seconds and 39°C for 30 seconds. Polymerase chain reaction products were loaded into a 10% nondenaturing polyacrylamide gel and run in 0.5× Tris borate EDTA at 200 V for 1 hour. Duplicate gels were run for 45 minutes to confirm presence of internal control. For heat-inactivated controls, 5 μl of each sample were incubated at 85°C for 10 minutes. Visualization was performed with SYBR Green stain (Molecular Probes, Inc., Eugene, OR).

Cytogenetics

The Cytogenetics Core Facility at Brigham and Women's Hospital, Boston, Massachusetts, performed a full karyotyping. Metaphase human chromosomes were harvested, gold thioglucose-banded, and analyzed according to standard methods (19). Cytogenic analysis was performed for the original tumor, the nontransduced Me3 and Me10 cells, and the transduced Me3TSC and Me10T cell lines.

Growth Kinetics

The cell doubling time for the transduced cell lines was determined by counting cells using a hemocytometer on a daily basis. On the first day, 1×10^5 cells were plated on six-well tissue culture plates. Cells were trypsinized and counted every 24 hours after plating. Doubling time was calculated from the data obtained at the logarithmic growth phase. The experiment was performed in triplicate.

Immunocytochemistry and Immunohistochemistry

The expression of SV40 large T antigen in Me3TSC cells was confirmed by Western blot analysis (Pab101; Santa Cruz

TABLE 1. Immunostaining materials and methods[a]

Primary antibody (Dako clone)	Positive control IHC	Dilution IHC	Incubation time IHC	Positive control ICC	Dilution ICC	Incubation time ICC	Pretreatment
EMA (E29)	Human breast carcinoma	1:100	1 hr	MCF7	1:100	45 min	No
Cytokeratin (AE1/AE3)	Human breast carcinoma	1:200	O/N 4ºC	MCF7	1:200	45 min	Yes
PR (636)	Human breast carcinoma	1:50	O/N 4ºC	T47D	1:50	45 min	Yes
S-100	Human breast carcinoma	1:2500	O/N 4ºC	N/A	1:1000	45 min	No
Vimentin (V9)	Human breast carcinoma	1:100	1 hr	MCF7	1:500	45 min	No

[a] IHC, immunohistochemistry; ICC, immunocytochemistry; EMA, epithelial membrane antigen; PR, progesterone receptor; N/A, not available.

Biotechnology, Santa Cruz, CA) (data not shown). Cells and corresponding paraffin tissues were tested for the presence of the following known meningioma cell markers: epithelial membrane antigen (EMA), cytokeratin, vimentin, S-100, and progesterone receptor (PR) (Dako Corporation, Carpinteria, CA) (*Table 1*).

Immunocytochemistry

Before staining, cells were plated on collagen-coated cover slips. Cells were fixed with acetic acid in ethanol at −20ºC, washed with 1× phosphate buffered saline, blocked and permeabilized with 3% normal donkey serum and 0.1% Triton-X, and incubated with primary antibody and then with the appropriate fluorescein isothiocyanate secondary antibody (Jackson Lab, Bar Harbor, ME). Cells were visualized with the Nikon T300 Fluorescence microscope and Spot camera (Diagnostic Instruments, Sterling Heights, MI).

Immunohistochemistry

Paraffin slides corresponding to the tissue from which primary cell lines were derived were obtained from the Department of Pathology at Brigham and Women's Hospital. For cytokeratin and PR staining, pretreatment was performed using citrate buffer, pH 6.0, and boiling for 20 minutes before incubation with blocking solution. Tissue was fixed with cold acetone, washed with 1× phosphate buffered saline, and incubated with 1.5% blocking solution. All secondary antibodies were biotinylated (Vector Labs, Burlingame, CA). Pictures were obtained with the Nikon T300 microscope and Spot camera.

Intracranial Tumor Model

Transduced, immortalized primary meningioma cells were implanted subdurally into 12 (six for each cell line) 4-week-old male athymic mice (Charles River, Wilmington, MA). Additional mice were injected with nontransduced cells. A craniotomy was performed 3 mm posterior to coronal suture and 3 mm lateral to midline, and the dura mater was exposed. Using a sharp fine-tip Hamilton syringe (22 gauge), 1×10^6 meningioma cells were injected into the subdural space in a total volume of 5 µl. Sixteen weeks after injection, animals were perfused with 4% paraformaldehyde under deep anesthesia. The brains were removed carefully, placed in sucrose gradient solution, embedded in optimal cutting temperature (Sakura Finetek, Torrance, CA), and stored at −80ºC. Brains were sectioned coronally using a cryostat into 10 µm-thick slices that were mounted on slides and then stained with hematoxylin and eosin according to standard protocol perfusion methods.

Growth Inhibition Assays and Drugs

Exponentially growing Me3, Me10, Me3TSC, and Me10T cells (4×10^3 cells/well) were seeded in triplicate into 96-well culture plates containing 10% FBS in Dulbecco's modified Eagle's medium/Ham's F-12 and were allowed to attach overnight. Cells then were incubated in media containing increasing concentrations of hydroxyurea or sodium butyrate (Sigma, St. Louis, MO). Drug-treated and untreated cells were exposed to an identical dimethyl sulfoxide concentration of 0.04%. A 3-(4,5-dimethylthialzol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) was performed 72 hours after treatment with absorbance of the formazan product measured at 490 nm to determine cell viability expressed as a surviving fraction relative to untreated controls (3). Results for the surviving fraction shown are the mean data from a representative triplicate experiment with error bars for standard deviation.

RESULTS

Cell Culture

Cell cultures were followed by phase contrast microscopy. On seeding of the primary cells, large nuclei and tight cell junctions were observed. Subcultures of Me3, Me5, Me7, and Me10 demonstrated uniform cell patterns, whereas Me1, Me2, Me4, Me6, Me8, and Me9 began to show large vacuoles and cytoplasmic vesiculation leading to early senescence occurring between P4 and P8. The cells became larger and intercellular junctions decreased. These phenomena have been observed previously and are one of the main reasons all meningioma cell studies use cells within the first five to seven passages (38, 47). Transduced cells maintained morphological features similar to those of early passage (P0–P7) nontransduced cells.

Of the 10 specimens plated, two cell lines, Me3 and Me10, remained viable and continued to maintain the same morphological features, allowing cell transformation. After infection, the morphological features for both lines remained the

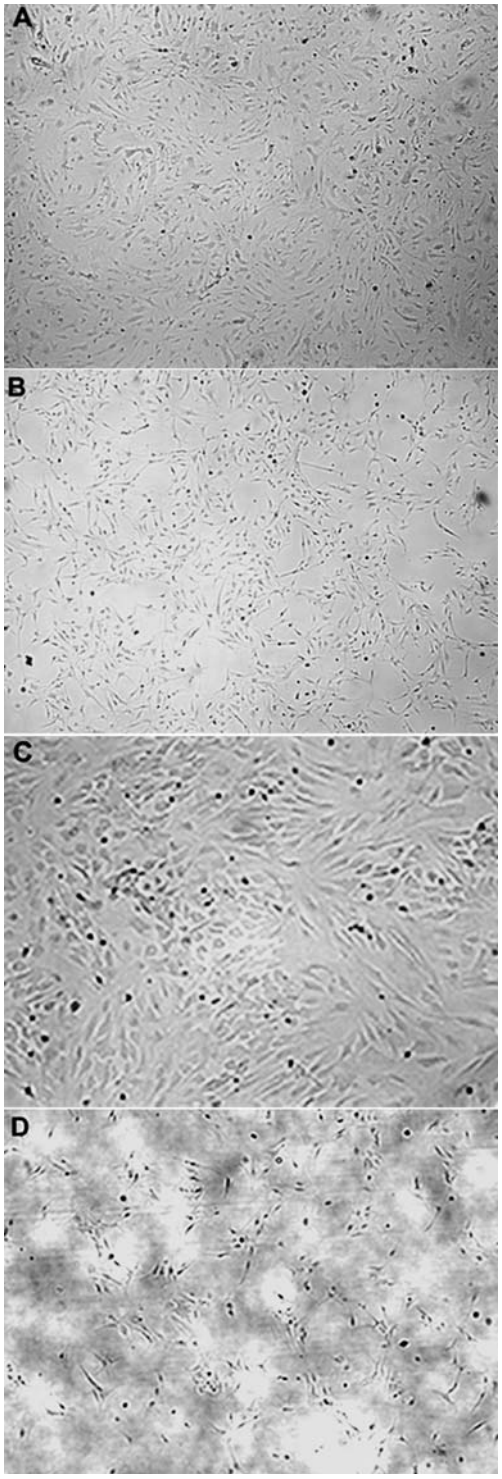

FIGURE 1. *Phase-contrast images of meningioma cells in culture.* A, *Me3 at P3;* B, *Me3TSC at P9;* C, *Me10 at P1; and* D, *Me10T at P2. Original magnification, ×40.*

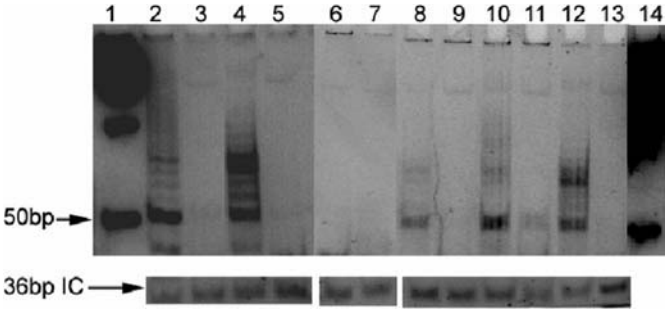

FIGURE 2. *Polyacrylamide gel of telomeric repeat amplification protocol polymerase chain reaction products.* Lanes 1 *and* 14, *50-bp deoxyribonucleic acid ladder;* Lane 2, *Me3TSC P8;* Lane 3, *Me3TSC P8 heat-inactivated negative control (HA);* Lane 4, *TSR8 (+);* Lane 5, *CHAPS (−);* Lane 6, *Me3 P8;* Lane 7, *Me3 P8 HA;* Lane 8, *Me10 P5;* Lane 9, *Me10 P5 HA;* Lane 10, *Me10T P4;* Lane 11, *Me10T P4 HA;* Lane 12, *TSR8 (+);* Lane 13, *CHAPS (−). The Me3TSC and Me10T transformed cell lines show an equal number of repeats as the TSR8-positive control. Positive control, TSR8 (+); negative control, CHAPS buffer (−).*

same as the corresponding nontransduced cells (*Fig. 1*). The cells were cultured successfully from frozen aliquots and were grown to the following passages: Me3, P12; Me3TSC, P28; Me10, P11; and Me10T, P20. Only these cell lines are discussed further.

Transduction with hTERT/SV40

Transduction of Me3 and Me10 cell lines was made using cells at less than P5 to ensure that morphological and cell characteristics of the primary cells would be retained. On immortalization of a subculture of the two lines with hTERT, the observed telomerase activity was much higher than that of the corresponding nontransduced cells. The telomeric repeat amplification protocol assay results for Me3 and Me10 did not show any telomerase repeats, whereas the transduced Me3TSC and Me10T lines showed as many repeats as the control (*Fig. 2*). The heat-inactivated controls for each line did not have any repeats, indicating absence of polymerase chain reaction inhibitors. In addition, the 36-bp internal control was visible for all samples, allowing elimination of false negatives. These results indicate that the lines containing hTERT have higher telomerase activity and thus would proliferate for an extended number of passages. The presence of SV40 was confirmed by Western blot analysis (data not shown).

Cytogenetics

The karyotype for the original tumor, Me3 and Me3TSC, is 45,XX,t(1;5)(p?36.1;q?13),del (9)(p13),del(11)(p14);−22. This tumor shows monosomy for chromosome 22, deletions in chromosomes 9 and 11, and translocations between 1 and 5. The karyotype for the original tumor, Me10 and Me10T, is 45, XX,−22, and shows monosomy only of chromosome 22; no other aberrations were observed. Monosomy of chromosome 22 is the most common aberration in meningiomas, occurring in 70% of meningiomas (data not shown).

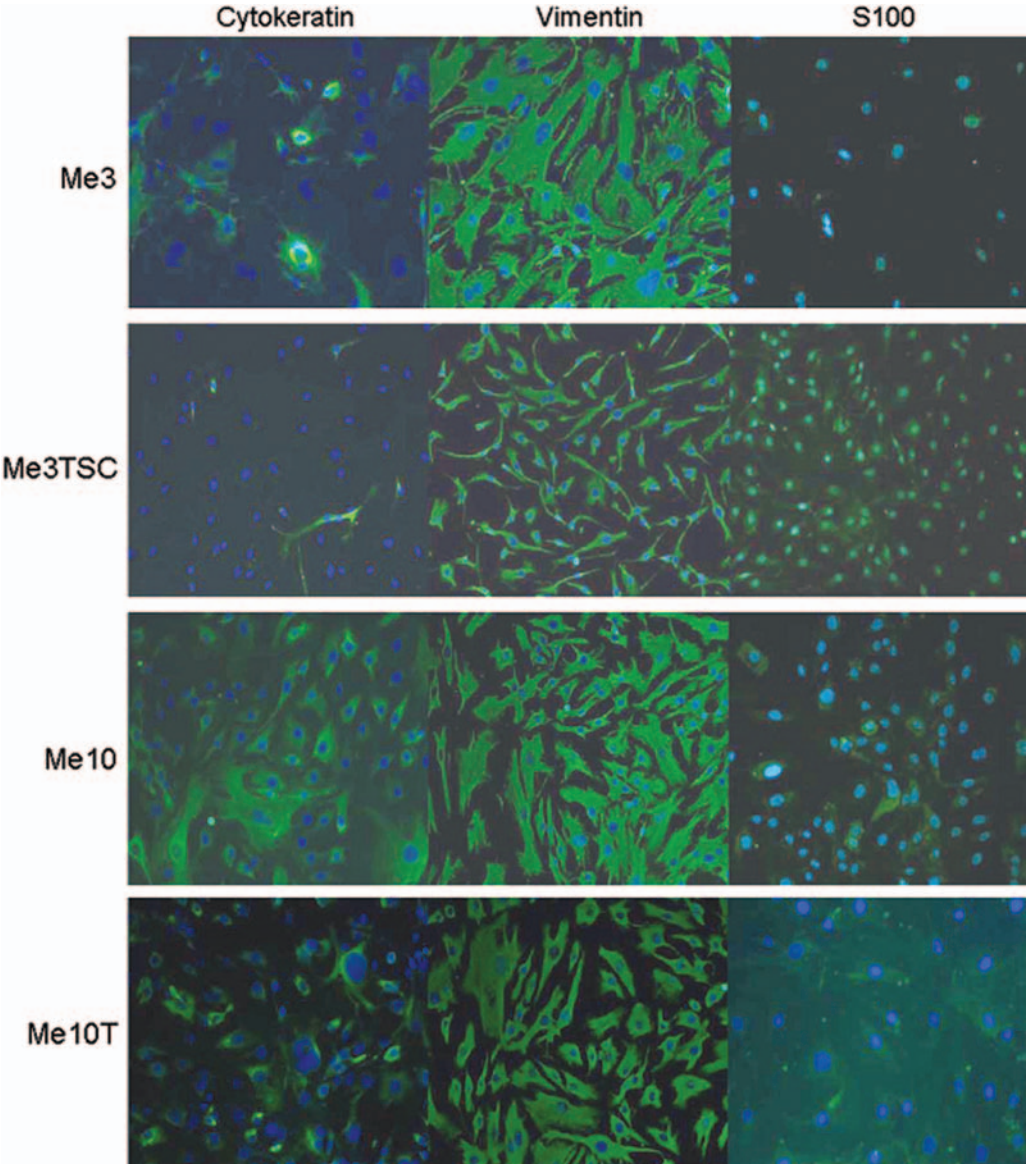

FIGURE 3. *Immunofluorescent staining of meningioma cells with cytokeratin, vimentin, and S100 antibodies. Transduced cell lines maintained expression even at later passages.*

Growth Kinetics

The average doubling time for the nontransduced cells was 14 days for the Me3 cell line and 8 days for the Me10 cell line. In contrast, the average doubling time of the immortalized Me3TSC cells (infected with hTERT and SV40) was 96 hours and for Me10T cells was 144 hours (infected with hTERT only).

Immunocytochemistry and Immunohistochemistry

Cells were passaged at confluence and were stained repeatedly for known meningioma markers. Both cell lines were positive for cytokeratin, vimentin, and S-100, but were negative for EMA and PR (*Fig. 3*). All corresponding paraffin slides from the original tumor were positive for cytokeratin, vimentin, S-100, EMA, and PR. The percentage of nontransduced cells showing positivity decreased with each passage number (data not shown). Electron microscopy analysis on Me3 showed hemidesmosomes and tight cell junctions, both well-documented characteristics of meningioma cells (data not shown) (47).

In Vivo Experiments

The formation of tumors was observed after subdural implantation of Me3TSC and Me10T cell lines. An open craniectomy technique provided good exposure to the dura mater and a reliable way to perform subdural injections. In addition, using this technique instead of a stereotaxic injection model prevented retrograde flow of tumor cells injected and increased the rate of tumor formation (unpublished data). Mice were killed 16 weeks after tumor injection, and all animals had visible tumors at the injection site. Both macroscopically and microscopically, the tumors showed features of meningioma (*Fig. 4*, *A* and *B*). Grossly, the tumors were well demarcated, firm, nodular masses of tan-white tissue adherent to the dura and leptomeninges on the brain surface. Microscopically, the tumor tissue was densely cellular and was composed of spindled to epithelioid cells with monomorphic round to oval nuclei and moderate amounts of cytoplasm. The tumors showed growth patterns that varied from small nodular meningothelial-like clusters to more fascicular spindled growth. Focal whorls and microcalcifications were present in some tumors. Although nuclei displayed some pleomorphism and had visible nucleoli, other atypical features such as small cells with a high nuclear-to-cytoplasmic ratio, brisk mitotic activity, and sheet-like growth were not observed. One tumor showed focal areas of nuclear condensation and fragmentation consistent with necrosis. Immunohistochemically, the tumors

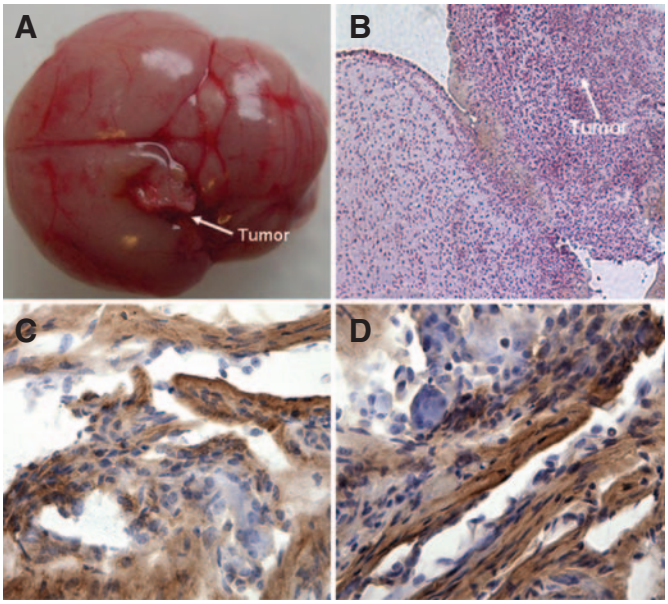

FIGURE 4. A, *photograph showing a grossly visible tumor attached to the cortex in the posterior parietal region of the left hemisphere. The tumor is well demarcated, firm, and white, and conforms macroscopically to meningioma characteristics observed in humans.* B, *photomicrograph showing tumor tissue that is densely cellular and composed of spindled to epithelioid cells with monomorphic round to oval nuclei and moderate amounts of cytoplasm. The tumor volume was 2.03 mm³ (hematoxylin and eosin; original magnification, × 200).* C, *photomicrograph showing that the tumor is weakly positive for EMA; original magnification, × 400.* D, *photomicrograph showing that the tumor is weakly positive for S-100; original magnification, × 400.*

showed weak positive staining for EMA and focal weak staining for S-100 (*Fig. 4*, *C* and *D*).

Growth Inhibition after Drug Therapy

We determined the growth inhibitory effects of hydroxyurea and sodium butyrate in transduced and nontransduced cells by measuring mitochondrial metabolism in an MTS-based cell viability assay. Hydroxyurea previously was shown to inhibit growth in a panel of primary meningioma cell lines (48). After exposure to less than 400 μmol/L hydroxyurea, Me3TSC cells displayed a dose-dependent decrease in viability at either 3 days (not shown) or 6 days with an IC_{50} of 175 μmol/L; the IC_{50} was 300 μmol/L for Me3 untransformed cells (*Fig. 5*). In contrast, Me10 and Me10T cells displayed no significant change in growth when exposed to less than 400 μmol/L hydroxyurea under the same conditions. Sodium butyrate treatment resulted in dose-dependent growth inhibition of Me3, Me3TSC, Me10, and Me10T cells (*Fig. 5*). The observed IC_{50} range (3–7 mmol/L) for sodium butyrate in Me3, Me3TSC, and Me10T cells is consistent with previous observations of growth-inhibitory response in malignant glioma lines (14).

DISCUSSION

Considering the 20% recurrence rate of benign meningiomas and the paucity of effective drugs, a reliable meningioma model is desperately needed, especially one in which potential therapeutics can be tested efficiently in vivo. Our group has established two immortalized meningioma cell lines from Grade I meningiomas and has used these lines successfully to produce tumors in an orthotopic meningioma mouse model. The Me3TSC and Me10T cells have been cultured up to P28 and P20, respectively, more than double the time seen with most primary meningioma cells. These cells also had the ability to form visible meningioma tumors in athymic mice within 112 days of subdural injection. The in vivo meningioma model developed in this study takes into consideration the requirement of specific genetic aberrations for the development of immortalized benign meningioma models. In particular, the Me3TSC cell line, transformed with hTERT and SV40 large T antigen, demonstrates the need for genetic alterations in addition to telomerase activation to produce improved meningioma models.

Meningioma Cell Culture

The need for reliable in vitro meningioma studies has been limited by the need to use early passage cell cultures (16, 21, 24, 31, 36, 39). Meningioma cells usually undergo senescence between P4 and P8. The nontransduced cells in our study began to show changes in morphological features and antigen expression at P2 and senesced by P8. Until now, no single meningioma specific antigen has been identified (47). For this reason, we characterized the cells using an array of previously studied meningioma markers. These include cytokeratin, EMA, PR, S-100, and vimentin. Expression of EMA and PR was present in the original tumor but not the cultured cells. However, strong expression of vimentin and cytokeratin, observed in the meningioma cells at sequential passages and in the corresponding tissues, demonstrated retention of these features from the primary tumor. Loss of expression of EMA and PR and senescence at early passages has been shown previously (8, 9, 26). Interestingly, cells showing loss of EMA expression in culture regained focal EMA positivity in xenograft tumor tissue.

Few studies have followed the expression of epithelial and mesenchymal antigens in primary meningioma cells (20, 38, 39). These studies show that cells taken from benign meningiomas express vimentin and cytokeratin. A study by Ng and Wong (38) showed EMA expression in 94% of stained meningioma cells and tissue, keratins in 50% of stained meningioma cells and tissue, and vimentin in 98% of stained meningioma cells and tissue. Although all cell lines in our study were positive for cytokeratin and vimentin, the percentage of positive cells for each line varied. Furthermore, the percentage of positive cells decreased with the passage number for the nontransduced cells, but remained approximately the same for transduced, immortalized cells, confirming retention of epithelial-like characteristics.

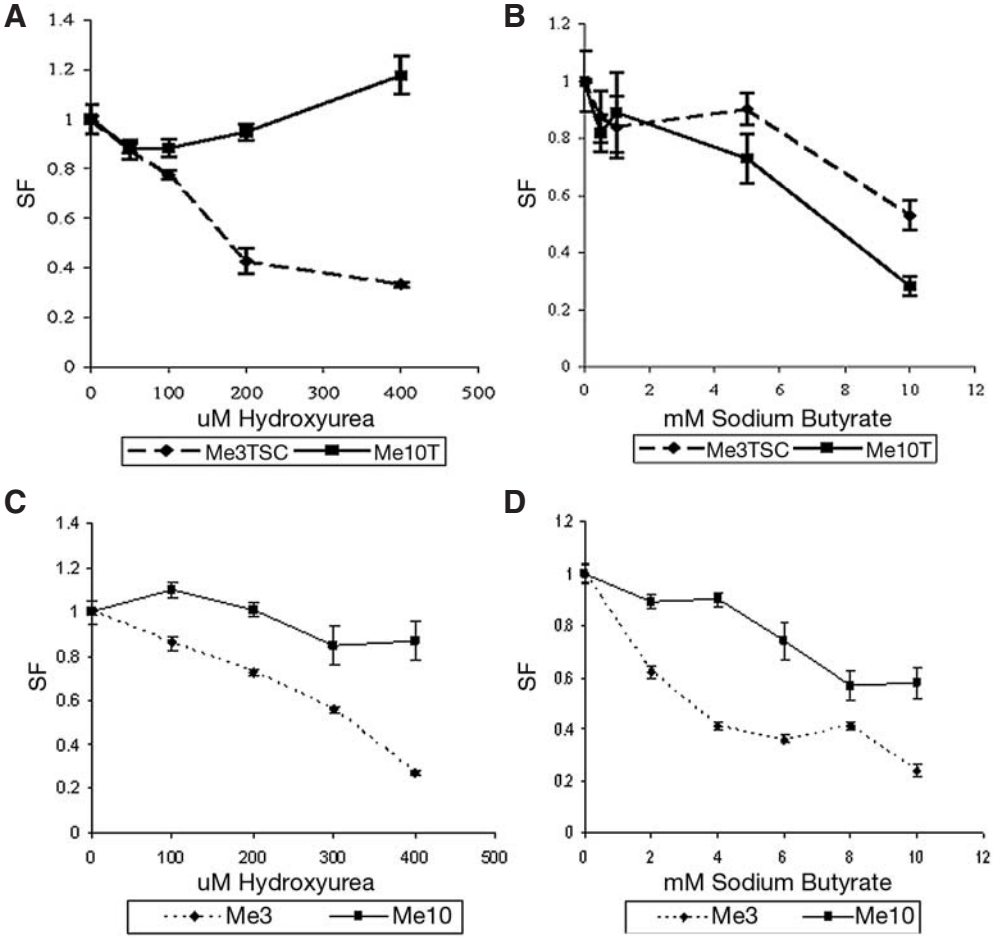

FIGURE 5. *Line graphs showing growth inhibition of Me3, Me10, Me3TSC, and Me10T cell lines. The surviving fraction (*SF*) was estimated using an MTS-based cell viability assay. Data are presented as the mean + standard deviation of a triplicate experiment.* A *and* C, *effects of hydroxyurea on meningioma cells in vitro. Exponentially growing Me3, Me10, Me3TSC, or Me10T cells were exposed to 0 to 400 μM hydroxyurea for 6 days with media and hydroxyurea added at Days 0 and 3.* B *and* D, *effects of sodium butyrate on meningioma cells in vitro. Exponentially growing Me3, Me10, Me3TSC, or Me10T cells were exposed to 0 to 10 mmol/L sodium butyrate for 6 days with media and sodium butyrate added at Days 0 and 3.*

Despite loss of EMA expression in the cells, strong expression of cytokeratin is consistent with previous findings, which demonstrate that meningiomas express keratin filaments in addition to vimentin in vitro. These results also confirm the absence of fibroblast cells because fibroblasts do not express keratins in vivo or in vitro. We also found expression of S-100 in the primary tumor tissue and in lower concentration in the cells, even at early passages. S-100 is a specific neuronal marker found in 18 to 100% of meningiomas. S-100 protein has been found in arachnoid granulations and reconfirms the lineage of the meningioma cells (1, 44, 51). Expression of vimentin, cytokeratin, and S-100 emphasizes the complex mesenchymal and epithelial features of meningiomas.

Cell Immortalization with hTERT/SV40

Although the absence of EMA and PR may indicate their function in tumor cell proliferation, telomeric activity is also a significant factor in determining the life span of a tumor cell and can be used to extend the life span of normal or benign cells. The life span of many cells lines has been extended by the introduction of telomerase (5). The function of hTERT in tumorigenesis has been well characterized. The successful immortalization of normal human cells with hTERT was shown in the early 1980s and has since been proven to be a way to bypass senescence. Ectopic expression of hTERT alone does not make a normal cell tumorigenic. The reactivation of telomerase is merely one of the first steps in rendering the cell more susceptible to oncogenic mutagenesis (18, 50).

The function of telomerase in brain tumor progression continues to be studied. The percentage of meningiomas containing telomerase activity increases with histological grade. Telomerase activity is found in up to 21% of benign meningiomas and in 100% of malignant meningiomas. However, Cabuy and de Ridder (7) demonstrated that the absence of telomerase activity in most benign meningiomas indicates that it is not necessary for meningioma development. Therefore, it is reasonable to presume that telomerase functions primarily to extend the life span of the primary cells. This study also used the telomeric repeat amplification protocol assay to measure telomerase activity, unlike other studies that used hTERT messenger ribonucleic acid quantity to measure telomeric activity indirectly. Furthermore, Simon et al. (49) noted that telomeric activity has been observed in nonneoplastic conditions and in 0 to 28% of benign neoplasms.

The role of telomerase in meningioma progression is thought to occur at later stages and not at the initial development of meningioma from the arachnoid cap cells but the

mechanism of progression is still not understood completely. Ectopic expression of the catalytic subunit hTERT in benign meningioma cells does not induce malignant progression because it is likely that the telomeric activity is being reactivated and not increased. Even with an increase in activity, induction of progression would have to occur with specific anomalies in chromosomes and molecular pathways (6, 7, 15, 49, 50).

Immortalization of the Me3TSC cell line established in this study specifically involved transduction with hTERT as well as SV40 large T antigen. Transduction with SV40 leads to disruption of the p53 and pRb pathways. Baia et al. (2) recently reported that immortalization of a Grade I meningioma-derived cell line, such as our Me3TSC line, with telomerase behaved like the parental cell line but did not grow beyond population doubling 20. They overcame the senescence of meningioma cells with human papillomavirus oncogene E6/E7 and hTERT to demonstrate that additional genetic changes are required for Grade I meningiomas to grow in culture. In our study, the doubling time of the cells transduced with both SV40 large T antigen and hTERT was shorter than the cells transduced with hTERT only. The histopathological examination of the in vivo tumors also revealed that the cell line transduced with both SV40 large T antigen and hTERT have some atypical changes when compared with the cells transduced with hTERT only. These findings confirm the need for loss of p53 and pRb functionality to produce transformed, immortalized cells. Although loss of p53 enhances genomic instability, this is indicative of the genetic alterations observed in meningioma progression. Our specific use of the catalytic subunit hTERT and SV40 large T antigen can, therefore, be interpreted as a successful method of extending the life span of benign meningioma cells.

Cytogenetics

Analysis of characteristic genetic aberrations in meningiomas has been used to distinguish between those involved in initial development as opposed to alterations involved in malignant progression. Most of the genetic aberrations observed in the karyotypes obtained from cytogenetic analysis for the transitional Me3TSC and meningothelial Me10T transduced and nontransduced cell lines are consistent with previously shown genetic aberrations. Benign meningiomas often have losses on 1p, 6q, 10q, 14q, and 18q (11, 26, 28, 42). Both cell lines studied are from female patients and have monosomy of chromosome 22, which is found in up to 70% of meningiomas. Chromosome 22 contains the NF2 gene, producer of the protein merlin that leads to cytoskeletal reorganization and thus tumor formation.

The Me3 cell line contains a translocation between 1p36.1 and 5q13. Mutations and deletions on 1p have been reported previously. However, translocations between chromosomes 1 and 5 have not been observed before. Rearrangements on chromosomes 5 and 11 were reported in Cerda-Nicolas et al. (11) and Lopez-Gines et al. (29). In addition, losses on 9p have been found only in 5% of benign meningiomas. Still, Me3 has a deletion of 9p13, whereas losses in higher grades were found at p14, p15, and p16, locations associated with blocking degradation of p53 and transcriptional control of pRB, respectively. The only, yet critical, characteristic alteration in Me10 is monosomy of chromosome 22.

Cytogenetic analysis in this study was performed to confirm consistency between morphological features, immunostaining, and primary tissue. The anomalies found in Me3 correlate with previously reported meningioma genetic studies. Monosomy of chromosome 22 in Me10 also is especially crucial in noting the cell type because loss of NF2 is extremely common in meningiomas (26).

In Vitro and In Vivo Models

Recent recognition of the potentially invasive and complex behavior of meningiomas, regardless of grade and subtype, has instigated several attempts to develop in vitro and in vivo models for this vastly common brain tumor (17, 21, 24, 31, 35, 36, 52). The ease of studying the primary tissue as opposed to slow-growing cell lines has rendered the development of few mouse models. These in vitro models used primary tumor tissue, either as blocks or as cell suspensions, to develop meningioma xenograft models. Most studies involving meningioma Grades I and II have used short-term cell lines, usually within the first 8 passages (34, 40, 41). We have developed two immortalized cell lines, Me3TSC and Me10T, that can be used continually for in vitro and in vivo studies.

Other existing meningioma models are of the malignant phenotype. Two malignant meningioma cell lines, F5 and IOMM-LEE, have been established (27, 55). The IOMM-LEE cells were established from an intraosseous tumor and resemble hemangiopericytomas by electron microscopy; therefore, it is unclear whether or not these cells are of meningothelial origin. F5 cells have also been used in conducting magnetic resonance imaging studies to monitor the rapid growth of malignant meningiomas in vivo (53).

Long observation periods are one major obstacle in existing in vivo meningioma models. The recently reported meningioma animal studies, including those using NF2 mouse models, require long observation periods ranging from 9 to 11 months, with intracranial tumors present only in 30% of the animals (22). Studies using well-established benign meningioma cell lines, such as those made in this study, can aid in elucidating the factors involved in the slow progression of meningiomas and can lead to better in vivo models. We successfully used our immortalized cell lines to establish an improved in vivo meningioma model. Our orthotopic meningioma model produces visible tumors at 112 days that are histologically similar to human meningioma. These results are comparable with those of Püttmann et al. (43), who showed that hTERT-mediated immortalization of benign meningioma cells can produce tumors after 107 days in athymic mice. Our model also confirms that additional disruption of the p53 and pRb pathways produces transformed cells that more closely resemble the genetic characteristics present in meningioma progression.

Use of the In Vitro Meningioma Model for Screening Therapeutics

The meningioma cell lines described may serve as a useful model system for the evaluation of conventional and targeted therapies. As an initial screen of therapeutic compounds in this model, we evaluated the growth inhibitory effects of the antitumor compounds hydroxyurea and sodium butyrate. Hydroxyurea is a free radical quencher, which decreases the level of cellular deoxyribonucleotides through inhibition of the cellular enzyme ribonucleoside diphosphate reductase (25). Hydroxyurea has been used in the treatment of recurrent, unresectable meningiomas for which radiation treatment is not an option. The clinical efficacy of this treatment remains controversial. However, several reported series suggest that treatment may result in disease stabilization in a significant subset of patients (12, 33). Hydroxyurea treatment showed mixed efficacy with no inhibitory effect on the growth of Me10T and Me10 cells with doses as high as 400 μmol/L, whereas in Me3TSC and Me3, a 50% survival fraction was observed at 175 and 300 μmol/L, respectively. The relatively high IC_{50} and mixed efficacy mirrors clinical experience, where reported clinical efficacy has been marginal at best (30, 37). Because hydroxyurea is well tolerated systemically, it may be possible to improve on its efficacy using combination therapies, which may be evaluated in our model.

Histone deacetylase (HDAC) inhibitors are a novel class of anticancer drugs that modulate gene expression by inhibiting the chromatin-modifying effects of the histone deacetylases (52). HDAC inhibitors can induce growth arrest, differentiation, apoptosis, or a combination thereof in a wide variety of tumor types (32). Sodium butyrate is a short-chain fatty acid HDAC inhibitor that has been shown to inhibit tumor growth and to promote differentiation in multiple tumor types (14).

Currently, histone deacetylase inhibitors such as NVP-LAQ824, suberoylanilide hydroxamic acid, and Pivanex (Titan Pharmaceuticals, San Francisco, CA) are undergoing clinical trials as monotherapy or combined therapies for solid tumors and hematological malignancies (23, 45, 46, 54). The growth inhibition observed with sodium butyrate treatment suggests that HDAC inhibition may be a viable approach to meningioma therapy. Further studies are underway to determine the efficacy of this and other targeted therapies in our model system.

In conclusion, we have established two immortalized meningioma cell lines, Me3TSC and Me10T, via transduction with hTERT and SV40. We have demonstrated this transformation in our in vivo model and confirmed the need for specific genetic alterations in the production of a successful meningioma model. Our model can be used to test potential therapeutics for meningiomas efficiently.

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COMMENTS

The creation of immortalized meningioma cell lines for in vitro and in vivo studies is a major achievement. Although any manipulated cell line must be used with caution when compared with non-manipulated tumor cultures, stability and reproducibility with meningioma cultures offers a great chance to further test potential therapy. I hope that the laboratory will now show how these lines can be further investigated to test novel treatment options.

Joseph M. Piepmeier
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The authors are to be congratulated for achieving something that has thus far eluded most translational scientists interested in meningioma biology and therapeutics. They have developed a very successful in vivo model system recapitulating the genotype and phenotype of a Grade I meningioma after the immortalization of two meningioma cell lines. They achieved this in a very clever and novel fashion by using a telomerase construct along with the SV40 T antigen. The SV40 antigen is a logical addition to this strategy because of the known effects that it has on p53 and retinoblastoma function. Thus, this novel strategy produced an immortalized cell line which recapitulated a Grade I meningioma after a subdural injection in athymic animals. More importantly, the model system had similar cytogenetic features as the original tumor and could be effectively studied with antitherapeutics. In this case, hydroxyurea, which has a modest track record as an antimeningioma therapy, was used in addition to a histone deacetylase inhibitor. It would have been very interesting to determine the therapeutic efficacy of radiation therapy in vivo, which was not done in this study. Hopefully, the authors will proceed along this pathway in the future. Notwithstanding, this is a very important study that has provided a very useful model system for low-grade meningiomas that will

enable investigators to test new and potentially successful therapeutic agents, which has not been possible to date.

Mitchel S. Berger
San Francisco, California

The authors have successfully immortalized two cell lines and established an in vivo meningioma model that produces visible extracerebral tumors 112 days after intracranial injection. These are histologically similar to human meningiomas. The authors have used human telomerase reverse transcriptase-mediation immortalization of benign meningioma cells in producing their cell lines. They have also confirmed that the additional disruption of p53 and retinoblastoma pathways produces transformed cells that resemble the genetic characteristics present in meningiomas that have progressed.

Although both in vitro and in vivo rodent models have significant limitations in assessing the true effectiveness of therapies, this model would certainly help in the evaluation of novel therapies for meningiomas. However, the usefulness will depend on the reliability of the development of tumors after intracranial injection and whether or not the tumors will grow large enough for imaging using high-strength magnetic resonance imaging. Nevertheless, the authors are to be congratulated for this excellent work.

Andrew H. Kaye
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The authors describe their development of an animal model of intracranial meningiomas using immortalized cell lines generated by transduction of low-passage meningioma explants with either human telomerase reverse transcriptase alone or in combination with the SV40 large T antigen. These transformed cell lines were characterized for cytogenetic complement, growth characteristics, telomerase and typical meningioma marker expression, and drug sensitivity. Subsequently, the cell lines were used for xenograft transplantation into the subdural space of nude mice to create an intracranial meningioma model.

Given the current lack of reliable animal models of meningiomas, any progress in this field is useful. There are, however, several features of this model that limit its ultimate utility. The physiological relevance of the generated meningioma cell lines is unclear, given the genetic changes required to transform primary meningioma explants. Subsequent conclusions regarding drug sensitivity, growth characteristics, marker expression, etc. (when related back to the primary meningioma cells) may not be translatable in the clinical setting. The prolonged time interval required to demonstrate even microscopic tumor growth in xenografted animals would be a hindrance to performing controlled, large-scale experiments exploring new therapeutic options for meningiomas. More studies with additional animals to document the temporal growth pattern and verify the consistency of tumor growth would be desirable before pursuing therapeutic studies. Overall, however, given the lack of in vivo meningioma alternatives for preclinical testing, this model can be useful if its limitations are addressed in future studies.

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