Decreased Replication Ability of E1-Deleted Adenoviruses Correlates with Increased Brain Tumor Malignancy

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Abstract

E1 region replacement adenoviruses are replication defective and are propagated in cells providing adenovirus E1A and E1B proteins. Although they are being developed for antitumor therapies, the proliferative behaviors of these viruses in normal brain tissues or in brain tumors are unknown. To address this, freshly cultured cells from normal human brain and common brain tumors (astrocytomas and meningiomas) were infected using wild-type species C adenoviruses and adenoviruses missing E1A (H5d/312) or E1A plus E1B (H5d/434). Viral DNA replication, late viral protein expression, and production of infectious progeny were characterized. Wild-type adenoviruses grew efficiently in normal brain and brain tumor cells. In comparison, E1-deleted adenovirus DNA replication was delayed and lower in cells derived from normal brain tissues, meningiomas, and low-grade astrocytomas. However, in contrast, E1-deleted adenovirus DNA replication did not occur or was extremely low in cells derived from malignancy grade III and IV astrocytomas. Because wild-type adenoviruses infected and replicated in all cells, the malignancy grade-based differential E1-deleted adenovirus DNA replication was not explained by differential virus uptake. Infectious H5d/312 and H5d/434 production correlated with viral DNA replication. Compared with a 5-day average for wild-type infections, advanced cytopathology was noted ~4 weeks after H5d/312 or H5d/434 infection of meningioma, astrocytoma, and normal brain cells. Cytopathology was not observed after H5d/312 or H5d/434 infection of glioblastoma, anaplastic astrocytoma, and gliosarcoma cells. Because of this tumor grade–based differential growth, the E1-deleted adenoviruses may represent novel tools for studies of brain tumor malignancy. (Cancer Res 2005; 65(19): 8936-43)

Introduction

Astrocytic tumors arise directly at their final grade or may progress over time from lesser to higher grades that are more malignant (1). A study of 715 glioblastomas found that 95% seemed to represent primary (de novo) tumors, whereas the remaining 5% could be traced to tumors that had progressed from lower grades (2). The WHO malignancy grading of astrocytic brain tumors classifies tumors based on several variables where grade I and II tumors include astrocytoma, grade III includes anaplastic astrocytoma, and grade IV includes glioblastoma (3, 4). Unfortunately, the most frequent adult brain tumor is glioblastoma, which is fatal despite surgery, radiotherapy, or chemotherapy. For patients undergoing treatment for glioblastoma, the mean time for survival is under 1 year (2, 5). Whereas there are some very good associative patterns of altered gene expression, DNA loss, mutation, gene amplification, or gene rearrangement, there are no examples of genetic alterations that always occur within a specific astrocytic tumor grade (reviewed in refs. 4, 6). Normal RB (7) and/or p53 (8) cell cycle control pathways may be altered during tumor establishment and/or progression. The recent population-based study by Ohgaki et al. on glioblastoma showed that loss of heterozygosity of 10q was the most prevalent genetic alteration and that it correlated with shorter survival times when associated with epidermal growth factor receptor amplification, TP53 mutation, p16INK4a deletion, or PTEN mutation (2).

The second most common adult brain tumor is meningioma, which arises from meningothelial cells. Meningiomas are usually benign, do not usually invade brain tissue, and are surgically curable when accessible. The meningiomas include a wide range of tumors with various histologies and genetic alterations (4).

Development of new therapies to cure or slow the growth of malignant brain tumors is urgently needed. Much attention has been given to the use of adenoviruses as gene delivery vectors. A major advantage for using adenovirus gene delivery is that adenoviruses enter both proliferating and nonproliferating cells. Clinical and experimental protocols have used replication-defective, replication-conditional, or replication-competent forms of adenovirus vectors (reviewed in refs. 9, 10). Human brain tumor therapies using replication-defective adenovirus vectors were approved partly because animal models proved that adenovirus efficiently delivered genes to brain tissues with no apparent harm to the infected cells (11, 12).

Most adenovirus vectors used for gene therapy have been designed with deletions in E1A and E1B. E1A is both a positive and a negative transcription regulator of specific viral and cellular genes (13–16). E1A induces transcription of genes involved in adenovirus DNA replication (17–19). The E1B 55-kDa protein in concert with E4 ORF6 proteins is important for viral RNA transport (20). E1B 55-kDa protein interacts with p53 (21), and in cooperation with the protein encoded by E4 ORF6, causes p53 degradation (22, 23). Adenoviruses deficient in E1A or E1A plus E1B have been classified as replication defective or host-range because they replicate efficiently in host cells, such as 293 cells, that synthesize E1A and E1B gene products but replicate poorly or not at all in normal cells which lack adenovirus DNA (24).

Therapy by adenovirus vectors has limitations and risks. Efficacy is limited by the ability of the virus to enter and express, selectively or nonselectively, the desired transgene in tumor cells (25). Because malignant astrocytomas are invasive and migrate throughout the brain parenchyma, invasive cells will escape infection. Only a limited number of viruses can be injected per treatment without causing cytotoxicity or inducing potentially severe immunologic responses. Finally, replication of the vector may cause damage to
normal cells. Understanding and manipulating adenovirus-host cell interactions is of critical importance to successful brain tumor therapy. In the case of astrocytic tumors, this may also depend upon malignancy grade-associated gene expression.

Laboratory-based brain tumor studies are often done using high passage number cell line clones that may no longer resemble the gene expression and infection characteristics of the original tissue. To minimize these differences, tissue explants and low passage number tumor-derived cultures have been used. To our knowledge, there are no reports of adenovirus DNA replication, adenovirus late protein expression, or infectious progeny production in a large sampling of freshly cultured human brain tumor specimens and normal brain cells.

Our studies focused on the ability of adenoviruses to infect and reproduce within newly established cultures of human brain-derived cells. Viruses classified as replication competent and replication defective were evaluated. Whereas the replication-competent adenoviruses were able to produce progeny efficiently, E1-deleted adenoviruses replicated relatively slowly in normal brain and in low malignancy grade brain tumor-derived cells but did not replicate or were even more replication-inhibited in cells from high malignancy grade brain tumors. The temporal delay in the production of progeny virus was linked to slower kinetics of DNA replication. In light of these findings, we suggest that the replication potential of E1-deleted first-generation adenovirus vectors should be accounted for when evaluating the effects of cytotoxic transgenes after infection of normal brain and brain tumor cells. The results also imply that adenoviruses are useful tools for the elucidation of complex cancer cell traits associated with brain tumor progression.

Materials and Methods

Growth of cells. Surgical brain tumor and normal brain tissue specimens were received with consent, were dispersed mechanically in the presence of trypsin/EDTA (Invitrogen Corp., Carlsbad, CA), and cultured using DMEM (Mediatech, Inc., Herndon, VA) containing 20% fetal bovine serum. The cells were subcultured at split ratios of 1:2 or 1:3 and most cells were infected before passage 10. Tumors were classified and graded by a neuropathologist. 293 and A549 cells were grown using DMEM supplemented with 10% FCS and antibiotics (100 units of penicillin and 100 μg streptomycin per mL, Invitrogen). Cells were subcultured at split ratios of 1:2 or 1:3 and most cells were infected before passage 10. Tumors were classified and graded by a neuropathologist. 293 and A549 cells were grown using DMEM supplemented with 10% FCS and antibiotics (100 units of penicillin and 100 μg streptomycin per mL).

Virological methods. Original viral stocks and anti-type 5 adenovirus serum were gifts of C.S.H. Young (Columbia University). Crude lysate preparations of viral stocks were prepared on A549 cells (Ad2, Ad5, Tpx81, and 15.5) or 293 (H5d312 and H5d/434) cells. Titeres were determined by fluorescent focus assay (26) using rabbit anti-Ad5 virion serum. Fluorescent focus forming units were normalized to be equivalent to plaque forming units. Human brain–derived cells were infected using 10, 20, 30, or 90 focus-forming units (ffu) per cell. Wild-type adenovirus yields were determined by plaque assay on A549 cells (27). E1A or E1A plus E1B deletion adenoviruses yields were determined using a fluorescent focus assay on 293 cells.

DNA analysis. Total viral and cellular DNAs were extracted from infected cells, 0.5 μg samples per lane were size-fractionated through 0.8% agarose gels, transferred to Hybond N nylon (Amersham, Piscataway, NJ) membrane filters (26) and hybridized to 32P-labeled DNA probes spanning 0% to 26.5% or 29% to 42% of the adenovirus genome. Autoradiography using Kodak X-Omat AR5 film was done at −90°C with Dupont Cronex Lightning Plus intensifying screens.

Detection of viral proteins within infected human brain–derived cells. Cells (2 x 10⁴) were seeded in 35-mm Falcon tissue culture dishes and infected 24 hours post-plating with 10 ffu per cell of H5d/312 or H5d/434. Cells were incubated for 14 to 18 days, washed thrice with PBS, fixed using 95% methanol, and incubated with rabbit anti-Ad5 virion serum. Cells were washed using PBS and primary antibodies were detected using fluorescein-conjugated goat anti-rabbit IgG.

Results

Wild-type growth patterns of adenovirus in cultured human brain–derived cells. To establish that normal brain–derived and various brain tumor–derived cells were susceptible to productive wild-type adenovirus infections, viral DNA accumulation was documented. Significant levels of viral DNA, indicative of replication, were detected in all wild-type adenovirus–infected cells cultured from normal brain tissues, meningiomas, astrocytomas, anaplastic astrocytomas, glioblastomas, and a gliosarcoma (Fig. 1A). To detect subtle differences in their susceptibility to infection and expression of adenovirus genes important for DNA replication, viral DNA accumulation kinetics were determined using normal brain and gliosarcoma cells cultured from the same patient. The accumulation of viral DNA was followed after coinfection of cells by two wild-type chimeric adenoviruses. The onset of viral DNA synthesis was observed 48 hours after infection and the viral DNA replication kinetics were similar in both the normal brain–derived and the gliosarcoma-derived cell cultures (Fig. 1B).

Cytopathology was noted after infection by wild-type viruses. Wild-type adenoviruses (types 2 and 5) or chimeric adenoviruses with wild-type growth kinetics (Tpx81 and 15.5; refs. 29, 30) were used to infect cells at a multiplicity of infection (MOI) of 10 ffu per cell and the cytopathic effects were recorded daily. Infected cells became swollen (day 3) and detached from the plastic substratum (days 4 and 5). There were no significant differences in the times when cytopathic effects were first observed in infected cells cultured from normal brain tissues and brain tumors (data not shown). There are conflicting reports on the importance of coxsackie and adenovirus receptor levels of malignant brain tumors and susceptibility to adenovirus infection (31, 32). We did not measure coxsackie and adenovirus receptor levels. However, all cell types and cultures displayed overt cytopathology, which signified efficient virus uptake and replication.

The ability of normal astrocyte–derived cells, gliosarcoma–derived cells, glioblastoma–derived cells and meningioma–derived cells to produce infectious progeny was determined. Cells were infected using 10 ffu per cell of Tpx81 and 15.5. Infected cells were cultured for 4 days, viruses were released by freeze-thawing and titers were determined. Virus production by all tested brain-derived cells was significant with a range of 300 to 3,000 infectious viruses produced per cell (Table 1). The greatest amounts of viruses were produced from the normal brain cells and meningioma cells. High passage number U373MG cells also produced efficient yields of wild-type adenovirus (data not shown).

Differential DNA replication of E1-deleted adenoviruses in matched sets of cultured normal brain–derived and tumor–derived cells. Adenovirus deletion mutants, H5d/312 (E1A-deleted; ref. 33) and H5d/434 (contains deletions in E1A and lacks all of E1B; ref. 34), are replication defective and must be grown in cells, such as 293 cells, which provide products of the deleted genes. We determined if cultured human brain cells would support full or limited H5d/312 or H5d/434 DNA replication and determined if replicated genomes were packaged into infectious viruses.

Brain tumor–derived cells were infected by E1A-deleted H5d/312 using an MOI of 10, 30, or 90 ffu per cell. We evaluated the E1A plus...
E1B-deleted H5d/d434 adenovirus using an input of 10 ffu per cell. H5d/d312-infected and H5d/d434-infected cells maintained a confluent monolayer and seemed healthy after 2 weeks of infection. The only change in cellular morphology was slightly swollen nuclei compared with uninfected controls.

Although advanced cytopathology comparable with infection by wild-type adenoviruses was not observed, it was possible that the E1-deleted adenoviruses were produced at lower and less toxic levels by the 14-day time point. To test this idea, we determined if H5d/d312 DNA replication occurred in a matched set of normal (GD88NI) and tumor (GD89NI) cells cultured from a patient with glioblastoma multiforme plus a matched set of normal (GD48NI) and tumor (GD47NI) cells cultured from a patient diagnosed with gliosarcoma. Cells were harvested for DNA extraction after 2 weeks of infection and tested for the presence/levels of viral DNA (Fig. 1C). H5d/d312 DNA was easily detected in the normal brain–derived cells but was undetectable in the tumor-derived cell cultures suggesting that the virus was able to replicate DNA but that detectable DNA replication was limited to normal brain–derived cells.

DNA replication of E1A-deleted adenoviruses is inhibited in anaplastic astrocytoma, glioblastoma, and gliosarcoma cells. To determine if the lack of replication of E1-deleted viruses extended to other brain tumor–derived cells, we evaluated DNA replication in a panel of cells derived from gliosarcoma, astrocytoma, anaplastic astrocytoma, glioblastoma, meningioma, and normal brain. Cells were infected with H5d/d312 at an MOI of 10 ffu per cell and DNA was extracted 14 days later. The presence of adenovirus genomes was evaluated by Southern transfer hybridization. Adenoviral DNAs were present in samples from infected normal brain, meningioma, and astrocytoma, but viral DNA was undetectable or was underrepresented in samples from anaplastic astrocytoma, glioblastoma, and gliosarcoma (Fig. 2). In contrast, all of these cells allowed replication of wild-type adenovirus DNA. The results indicate that E1-deleted adenoviral DNA replication occurred in six different normal brain–derived cell cultures, five meningioma-derived cell cultures, and five low malignancy grade astrocytoma–derived cell cultures but did not occur or was severely decreased (small amounts were detected in one GBM in the panel) in cells derived from two anaplastic astrocytomas, four glioblastoma multiformes, and a gliosarcoma. This survey revealed a strong trend toward an inhibition of E1-deleted adenovirus replication in higher malignancy grade brain tumors.

Two lower signal intensity hybridizing fragments that did not comigrate with H5d/d312-specific DNA fragments were detected in seven of the cell lines. One was the result of partial HindIII digestion of DNAs from cells that allowed replication of E1-deleted adenoviruses. The other was of unknown origin in cells where H5d/d312 DNA replication was not detected. The fragment of unknown origin was not the result of replication-competent adenovirus (RCA) because it was not similar in size to HindIII fragments of the Ad5 genome. This was an important consideration because RCA could arise if E1-deleted viruses acquired E1 region DNA by the process of homologous recombination during growth in 293 cells. RCA would replicate in all brain tumor cells. H5d/d312 and H5d/d434 stocks of viruses did not contain detectable RCA DNA when analyzed by Southern transfer hybridization. Furthermore, RCA were not detected when E1-deleted viral stocks were tested for wild-type growth on A549 cells.

Figure 1. DNA accumulation after infection of cells by wild-type or E1-deleted adenoviruses. A, cells were infected for 72 hours with 10 ffu each of two wild-type chimeric adenoviruses (Tpx81 and 15.5; refs. 29, 30) per cell, total cellular and viral DNAs were extracted, DNAs (0.2 µg per sample) were digested with BamHI and subjected to Southern blot analysis using a 32P-labeled DNA fragment spanning 29% to 42% of the adenovirus genome. Infection-specific cytopathology was observed and the cells contained easily detectable viral DNA. B, a matched culture set of cells was infected and analyzed as in (A) using 0.5 µg DNA per sample. Adenovirus DNA replication was first detected at 48 hours after infection. BamHI recognition sites and hybridization probe used in (A and B) are depicted. Map units are expressed as % adenovirus genome. C-D, cells were infected with H5d/d312 (10 ffu per cell) for 2 weeks, total DNAs were extracted, DNAs (0.5 µg per sample) were digested or run uncut, blotted, and probed using an adenovirus DNA fragment (29-42%). GD88NI tumor = glioblastoma. GD88NI normal brain cells cultured from the patient with the tumor used to establish GD88NI. GD48NI normal brain cells cultured from the patient with the tumor used to establish GD47NI. HindIII and EcoRI recognition sites are depicted. C, ethidium bromide–stained agarose gel. D, Southern blot autoradiogram.
Table 1. Production of infectious wild-type adenovirus from cultured normal brain cells and brain tumor cells

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>GD48NI</th>
<th>GD47NI</th>
<th>GD111NI</th>
<th>GD95NI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Derivation</td>
<td>Normal brain</td>
<td>Gliosarcoma</td>
<td>Glioblastoma</td>
<td>Meningioma</td>
</tr>
<tr>
<td>pfu/cell</td>
<td>3,000</td>
<td>1,000</td>
<td>300</td>
<td>3,000</td>
</tr>
</tbody>
</table>

NOTE: $5 \times 10^5$ (GD111NI) or $1 \times 10^6$ (all other tissue cultures) cells per 100-mm plate were infected using 10 ffu each of Tpx81 and 15.5 (29, 30), lysed at day 4 by three cycles of freeze-thawing and viruses were enumerated by plaque assays using A549 cells. GD48NI and GD47NI were derived from the same patient. Abbreviation: pfu, plaque-forming units.

We have cultured specimens from several normal brain and over 150 brain tumor samples from patients undergoing neurosurgical procedures. Most specimens could be established and grown as healthy monolayer cultures with no evidence of adenovirus specific cytopathology. Southern blot analyses of DNAs extracted from uninfected controls did not reveal the presence of intrinsic adenovirus DNAs.

Replication time course of E1-deleted adenoviruses in cultured GD150NI meningioma and A549 cells. Figure 3 depicts adenoviral DNA accumulation after infection of cultured gliosarcoma cells (GD47NI), meningioma cells (GD150NI), and A549 cells with H5dl312 or H5dl434. A549 cells, derived from a human non small cell carcinoma of the lung, are commonly used for wild-type adenovirus stock production. Furthermore, H5dl312 will infect these cells and the E2-encoded 72-kDa DNA binding protein, involved in viral DNA replication, can be detected 22 hours after infection (35). Cells were infected with an MOI of 10 ffu per cell for 2 weeks. DNA samples were digested with *Hind*III and analyzed by Southern transfer hybridization using either an adenovirus DNA probe spanning 29% to 42% or 0% to 26.5% of the adenovirus genome. The 0% to 26.5% probe was chosen because it could detect characteristic fragments that differentiated H5dl312, H5dl434, and 15.5 genomes. Wild-type–sized E1A DNA fragments were not detected in the infected gliosarcoma cells, whereas significant DNA levels were detected in A549 and GD150NI cells.

We next determined if H5dl312 DNA increased over time after infection. A549 cells and GD150NI cells were infected at an MOI of 10 ffu per cell and cells were harvested daily for 6 days. Southern blot analysis indicated increased amounts of viral DNA in A549 cells after day 1 and in GD150NI cells after day 3 (Fig. 4A). Accumulations of E1A-deleted DNAs were gradual compared with the DNA increases seen in wild-type–infected normal brain and gliosarcoma-derived cells depicted in Fig. 1B. The increases in DNA were suggestive of H5dl312 replication in A549 and GD150NI cells.

The amount of virus produced per cell was determined by fluorescent focus assay (Fig. 4B). GD150NI and A549 cells produced infectious viral progeny. Production of infectious virus was not exponential in either situation, and when considered with the results of Fig. 4A, it seems there was a rate-limiting component to viral DNA synthesis. The increases in viral DNA leveled off by day 7 or 10 in these cell lines (Fig. 4C). Furthermore, when compared with the DNA levels obtained after wild-type infection, these mutant adenovirus–infected cells had a much lower viral DNA content. After 2 weeks of infection, GD150NI cells yielded 33 viruses per cell (compare this with yields listed in Table 1). Increases in both viral DNA and infectious viral progeny showed that specific cells allowed H5dl312 growth with low efficiency.

Advanced cytopathology can occur 3 to 4 weeks after infection of permissive cells using H5dl312 or H5dl434. We were able to follow the course of infection in normal brain–derived, brain tumor–derived, and A549 cells by infecting them at an MOI of 10 ffu per cell and culturing them with medium changes and/or subculturing as needed. Cells were infected with H5dl312 or
H5dl/434 and followed over a period of 42 days. GD47NI and GD39NI from a gliosarcoma and a glioblastoma, respectively, seemed healthy and displayed no overt cytopathology throughout the infection period. DNA was extracted from these cells at day 42 or 31 after infection. The amount of adenovirus DNA in GD47NI was below the level of detection, whereas very faint bands, compared with those of replication-positive cells, could be detected in the DNA extracts of GD39NI (Fig. 5A). DNA was extracted from three different normal brain–derived cultures and a meningioma cell culture after advanced cytopathology was evident and swollen cells began detaching from the substratum. Large amounts of viral DNA were present in these cells (Fig. 5A). Similar results were obtained using H5dl/312 (data not shown).

Adenovirus-encoded late proteins were detected in permissive cells after E1-deleted adenovirus infection (Fig. 5B). Two representative examples of E1-deleted adenovirus permissive and nonpermissive cells are shown. Staining of GD47NI cells revealed that only 1% of H5dl/312-infected cells expressed adenovirus-encoded late proteins. Furthermore, cytopathology was not detected after long-term culture. In contrast, most GD96NI meningioma cells infected by H5dl/434 expressed adenovirus late proteins. Cytopathology was evident in the brightest cells.


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**Replication of H5dl/312 in commonly used high passage brain tumor–derived cell lines.** Rat C6 glioma cells and the cells of human brain tumor–derived lines: T98G, U373MG, U138MG, A172, and U87MG were infected with H5dl/312 at a multiplicity of 10. DNA was extracted 2 weeks after infection, and the presence of adenoviral DNA was determined by Southern transfer hybridization (Fig. 5C). In contrast to low passage number cells, three of the five human cell lines allowed E1-deleted adenovirus DNA to replicate to easily detectable levels. In addition, U138MG cells allowed replication to a much lower but still detectable level. Viral DNA from infected T98G cell extracts was below the level of detection. The cells containing higher amounts of adenoviral DNA also displayed infection-specific cytopathology when observed for longer periods. Rat C6 glioma cells did not allow DNA replication to detectable levels. Wild-type adenovirus 2 or 5 readily infects rat cells but the infections are semipermissive, resulting in low amounts of progeny viruses (36). This species-specific decrease in replication may be the reason that H5dl/312 DNA was not detected after 2 weeks. C6 glioma cells are used to model brain tumors and are efficiently infected with adenovirus vectors (37). Replication of H5dl/312 in specific high passage number cell lines derived from high malignancy grade human brain tumors implies either that the block to the replication to E1-deleted adenoviruses was never present or that it was lost over time in culture.

**Discussion**

Replicative behavior is an important aspect of adenovirus-associated therapy of brain tumors. It can affect therapeutic efficacy and safety. Replication would cause cell death and release progeny virions to initiate secondary rounds of infection. Selective replication in tumor cells would be beneficial and would increase the therapeutic index of the vector. Replication and killing of normal cells would be unwanted but might be tolerated depending on the rate of viral replication, host immune responses, and the benefit as an antitumor agent. To date, the most commonly used adenovirus vectors replace the E1A and E1B gene regions with transgene expression cassettes. Although E1-deleted adenoviruses are replication defective, they display wild-type growth kinetics at high multiplicities within HeLa cells (38). E1-deleted adenovirus vectors expressing therapeutic genes have been and are being evaluated for the treatment of brain tumors (39).

**Figure 3.** Analysis of viral DNAs in gliosarcoma–derived cells, meningioma–derived cells, and A549 cells after 2 weeks of infection by E1A-deleted (H5dl/312) or E1A plus E1B-deleted (H5dl/434) adenoviruses. DNAs (0.5 µg per sample) were digested with HindIII. A, blot filter probed with a 32P-labeled adenovirus DNA fragment (29-42%). B, blot filter was probed a second time using an adenovirus DNA fragment (0-26.5%) without stripping of signal depicted in (A). C, HindIII restriction endonuclease site maps of H5dl/312 and H5dl/434. H5dl/312 contains a deletion spanning 448 to 1,349 bp. The H5dl/434 deletion coordinates are 2.6% to 8.7%. HindIII fragments of 15.5 that hybridize to 0% to 26.5% Ads5 probe are identical to those of Ads. HindIII fragments of 15.5 that hybridize to the 29% of 42% probe differ from Ads by an additional site at 41.0% resulting in the four fragments depicted (5.32, 3.28, 2.08, and 1.32 kbp).
The replication profiles of E1-deleted adenoviruses within human brain or human brain tumor cells have not been described. We designed experiments to test the hypothesis that because brain tumors often lose functional pRB, which results in transcriptionally active E2Fs, E2F1-dependent transcription of adenovirus E2 would occur and E1-deleted adenoviruses would replicate much better in brain tumor cells compared with normal astrocytes or normal meningotheial cells. It had been shown that replacement of E1 by an expression cassette containing an E2F1-responsive promoter resulted in higher transgene expression in rat C6 glioma cells compared with normal rat cells (40). Furthermore, adenovirus vectors, where the endogenous E1A promoter was replaced by E2F1-responsive elements, replicated better in tumor cells compared with normal cells (41). In contrast to prediction, we found that E1A-deleted or E1A plus E1B-deleted adenoviruses were able to replicate, albeit with delayed kinetics compared with wild-type adenoviruses, in low passage number cell cultures of normal brain and low malignancy grade tumors such as meningioma and astrocytoma grade I or II. Surprisingly, they lost the ability to replicate or replicated poorly in cells derived from higher malignancy grade brain tumors.

Progression of a low-grade tumor to a higher-grade tumor or direct establishment of a high malignancy tumor leads to an E1-deleted adenovirus DNA replication deficiency. Because efficient adenoviral DNA replication depends on increased transcription of the adenovirus E2 promoter after a wild-type infection, a simple hypothesis is that transcription profiles are altered in malignant brain tumor cells and adenovirus genes involved in DNA replication are repressed or not expressed.

The host-range phenotype of H5 dl312 has been defined as a differential plaquing ability on 293, HEK, and HeLa cells (42). Although plaquing efficiency was below \(10^{-6}\) on HeLa and HEK cells, infection of these cells at high multiplicities (80 or 800 plaque-forming units per cell) resulted in yields of virus similar to those of wild-type 5 at 72 hours after infection (42). In contrast, a small increase in viral yield was observed after infecting HeLa cells using an MOI of 8 plaque-forming units/cell. In the absence of E1A proteins, the rate of expression of other early adenovirus genes and the major late promoter was extremely low (17). The multiplicity-dependent replication of H5dl312 in HeLa cells was likely the result of more genomes per cell allowing more transcription to produce enough protein to move the replicative cycle forward. However, we did not detect levels of viral replication similar to those seen in normal astrocytes after infection of newly cultured anaplastic astrocytoma and glioblastoma cells using an MOI of 90 ffu per cell. At this MOI, replication of H5dl312 in normal brain cells and permissive brain tumor cells was still temporally delayed compared with replication of wild-type virus at an MOI of 10 ffu per cell.

In contrast to low passage number cell cultures, E1-deleted adenovirus grew in high passage number glioblastoma-derived cell lines with ample DNA replication and hexon expression. T98G cells were somewhat refractory to infection (data not shown), possibly because they express very low levels of coxsackie and adenovirus receptor (32). Low passage number cell strains differed from high passage number cell lines raising a possibility that cell culture selects for cells that also happen to allow replication of E1-deleted adenoviruses.

The first viral transcription detected after infection of human cells by wild-type adenoviruses is from E1A (43). The proteins encoded by E1A interact with a number of cellular proteins that are involved in regulation of gene expression (reviewed in refs. 44, 45). The modification of host cell transcriptional control results in conditions favorable for viral replication by altering the transcription of viral and cellular genes.

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Figure 4. Growth of H5dl312 in A549 and GD150NI cells. A and C, time course of H5dl312 viral DNA accumulation in A549 and GD150NI cells. Cells were infected using 10 ffu per cell and DNAs were collected daily. DNAs (0.5 µg each) were digested with HindIII and analyzed by Southern transfer hybridization using a 32P-labeled 29% to 42% adenovirus DNA probe. B, analysis of viral yield by fluorescent focus assay. Cells were harvested at various times after infection, pelleted, resuspended in infection fluid, viruses were released by three cycles of freeze-thawing, and viral titers were determined using a fluorescent focus assay. Viral yields per cell were calculated.
Viral DNA replication is dependent on expression of E2A, which is controlled by cellular and viral gene products (reviewed in ref. 44). For example, cellular E2F1 was identified as a transcriptional activator of adenovirus E2 (46) and is released from pRB by E1A. The host cell restricted DNA replication defects of H5d1312 and H5dl434 could be a direct result of not being able to release E2Fs from pRB. In addition, the adenovirus E4 ORF6/7 protein cooperates with free E2F to enhance transcription from the E2A promoter (47). It is possible that transcription of E4ORF6/7 was absent or severely diminished in low passage number anaplastic astrocytomas and glioblastomas.

Besides E2Fs, E1A products interact with cellular proteins, which affect viral replication. A recent report has shown that murine Sur2 is needed for efficient replication of mouse adenovirus type 1 in mouse embryo fibroblasts (48). Sur2 is a subunit of the transcription mediator complex, which bridges transcription regulators to RNA polymerase II. Human Sur2 has been shown to interact with Ad5 E1A (49). However, human cell lines lacking Sur2 are not available for testing E1-deleted adenoviral replication.

Because of its role in cell cycle control, loss of E2F regulation is thought to also play a role in brain tumor establishment. Most astrocytic tumors have a defective p16INK4A/pRB/cyclinD/CDK4 pathway (50). Protein levels of p16INK4A or pRB were reduced or the genes encoding these proteins were deleted or not expressed in most gliomas. In addition, the levels of cyclin D, cyclin E, and cdk4 were increased, which would be predicted to phosphorylate RB and release active E2F transcription factors. If inactivation of RB by deletion, underexpression, or phosphorylation caused an increase in the transcriptionally active E2F family members (E2F1, E2F2, and E2F3), E1-deleted adenovirus DNA replication would be expected to be greater in cells derived from glioblastoma. However, the amounts of E2F1 protein were reported to be variable and often lower in brain tumor cells (51). There was no correlation of E2F1 protein level with histopathologic grade. Although the functional activity of E2F1 was not examined, these data suggest that changes in E2F1 levels are not solely responsible for E1-deleted adenovirus replication differences.

Perhaps the best correlation with the E1-deleted adenovirus block to replication and host cell modifications is the association of tumor malignancy grade with total loss of chromosome 10 or loss of heterozygosity at 10p14-pter, 10q23-24, or 10q25-pter. Several potential tumor suppressors are located in these regions. An important tumor suppressor located on 10q25.3-q26.1 is "deleted in malignant brain tumors-1" (DMBT-1). DMBT-1 is reported to be present in normal brain cells, meningiomas, and low-grade astrocytomas but is lost (homozygous deletion), mutated, or downregulated in at least 40% of anaplastic astrocytomas and at least 80% of glioblastomas (references in ref. 2). Expression of DMBT-1 was tested in a subset of the high passage number cells used in our studies. DMBT-1 RNA was detected by reverse transcription-PCR in U-87MG cells (52) but was not detected in U138MG cells (52). U373MG cells contained an intact DMBT-1 gene with two missense mutations (53). A549 cells expressed DMBT-1 with two missense mutations that are found in normal populations (54). A172 (55, 56) and T98G (56) have translocations in this region, but the status of DMBT-1 is not known. It is possible that E1-deleted adenoviruses rely on DMBT-1 or the expression of another gene in the 10q25 region for expression of viral proteins involved in replication.

Differential replication of E1-deleted vectors in human brain-derived and brain tumor-derived cells has implications for gene therapy and the study of brain tumor biology. For gene therapy, the results underscore the importance in defining adenovirus replication and toxicity in conjunction with the effects of cytotoxic
transgenes. Persistence of transgene expression may also be affected by slowly replicating adenoviruses, depending on the permissivity of the host cell. In the context of brain tumor biology, the current database of knowledge concerning the control of adenovirus gene expression may allow us to use it as a biological tool to uncover the molecular mechanisms controlling expression of malignancy traits associated with medically important higher-grade astrocytic tumors.

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3. Acknowledgments

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