[CANCER RESEARCH 58, 2331–2334, June 1, 1998]

Advances in Brief

Suppression of Tumorigenicity of Glioblastoma Cells by Adenovirus-mediated *MMAC1/PTEN* Gene Transfer

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Abstract

Mutated in multiple advanced cancers 1/phosphatase and tensin homologue (*MMAC1/PTEN*) is a novel tumor suppressor gene candidate located on chromosome 10 that is commonly mutated in human glioblastoma multiforme and several other cancer types. To evaluate the function of this gene as a tumor suppressor, we constructed a replication-defective adenovirus (MMCB) for efficient, transient transduction of MMAC1 into tumor cells. Infection of MMAC1-mutated U87MG glioblastoma cells with MMCB resulted in dose-dependent exogenous MMAC1 protein expression as detected by Western blotting of cell lysates. *In vitro* proliferation of U87MG cells was inhibited by MMCB in comparison to several control adenoviruses at equal viral doses, implying a specific effect of MMAC1 expression. Anchorage-independent growth in soft agar was also inhibited by MMCB compared to control adenovirus. Tumorigenicity in nude mice of transiently transduced mass cell cultures was then assessed. MMCB-infected U87MG cells were almost completely nontumorigenic compared to untreated and several control adenovirus-treated cells at equal viral doses. These data support an *in vivo* tumor suppression activity of MMAC1/PTEN and suggest that *in vivo* gene transfer with this recombinant adenoviral vector has a potential use in cancer gene therapy.

Introduction

The investigation of homozygous deletions of human chromosome band 10q23 in brain (1) and breast (2) cancers has recently led to the cloning of a novel gene called *MMAC1*[2] or *PTEN*, respectively. Predicted to encode a protein phosphatase (and also known via this function as TEP1; Ref. 3), *MMAC1/PTEN* was identified as a candidate tumor suppressor gene based on the presence of inactivating mutations (*e.g.*, homozygous deletions or frameshift mutations) in several glioblastoma, breast, and prostate tumors or tumor cell lines (1, 2, 4). Subsequent studies have also demonstrated such mutations in pediatric gliomas, melanomas, and endometrial carcinomas (5–8). Furthermore, germ-line mutations have been found in patients with Cowden disease or Bannayan-Zonana syndrome, autosomal-dominant developmental disorders distinguished in part by predisposition to breast and thyroid malignancies only in the former (9–11). Although not inactivating *a priori*, missense mutations have been observed in conserved residues of the phosphatase motif, in the tensin homology region, or at putative phosphotyrosine residues (5), some of which have been shown to disturb the phosphatase activity of the protein (12).

As with other tumor suppressor genes, a key step in characterizing *MMAC1/PTEN* is to demonstrate an antitumorigenic activity of the wild-type gene in isolated cancer cells. In an initial approach to this goal, Furnari *et al.* (13) transfected wild-type or mutated MMAC1/PTEN expression plasmids into cultured glioma cell lines. Expression of exogenous wild-type MMAC1 inhibited the proliferation *in vitro* of two glioma lines (U87MG and U178) harboring *MMAC1* mutations but did not inhibit a third line (LN229) expressing wild-type endogenous MMAC1; mutant forms of exogenous MMAC1 had no detectable antigrowth effect. In the present study, we sought to evaluate the effects of *MMAC1* gene transfer in glioma cells using an *in vivo* tumorigenicity test. To avoid the potential difficulty of selectively culturing cells expressing growth-inhibitory proteins, we used a replication-deficient recombinant adenovirus at doses sufficient to transiently transduce mass cell cultures without the need for further *in vitro* growth or selection.

Materials and Methods

Cell Lines. The *MMAC1*-mutated glioblastoma cell line U87MG was obtained from the American Type Culture Collection. Cells were maintained in culture medium (DMEM/10% fetal bovine serum/1% L-glutamine) in a humidified atmosphere containing 7% CO_2 at 37°C. 293 embryonic kidney cells were also obtained from the American Type Culture Collection and were grown in culture medium as above.

RT-PCR Analysis. Total RNA was isolated from U87MG cells (Tri Reagent; Molecular Research Center) per manufacturer's instructions. RNA was reverse transcribed using murine leukemia virus RT (RNA PCR kit, Perkin-Elmer), random hexamer, and other kit reagents, followed by PCR using primers MAC1.6f (5′-CTG CAG AAA GAC TTG AAG GCG TA-3′) and MAC1.6r (5′-GCC CCG ATG TAA TAA ATA TGC AC-3′) matching sequences in MMAC1 exons 2 and 5, respectively. Amplification conditions were 95°C for 1 min, 95°C for 15 min, and 55°C for 30 min for 25 cycles, followed by 72°C for 5 min. Products were cut out from agarose gels, purified (UltraClean, Mo Bio Labs), and directly sequenced using an automated sequencing system (ABI 373A; Perkin-Elmer Corp.).

Viruses. A recombinant Ad containing wild-type p53 (FTCB) was constructed as described previously (14). The genome of this vector has deletions of the E1 and E3 regions and protein IX gene and expresses its transgene under control of the human cytomegalovirus immediate-early promoter/enhancer. The MMAC1/PTEN vector MMCB was constructed in exactly the same manner, except that p53 was replaced with a cDNA encoding full-length MMAC1 (1). The control vector GFCB was constructed to match MMCB except for its transgene, enhanced green fluorescent protein (Clontech). Another matching control vector, ZZCB, was constructed without a transgene. The BGCA control vector expressing *Escherichia coli* LacZ driven by the cytomegalovirus promoter was constructed in a genome with partial E4 deletion in addition to deletions of E1, E3, and protein IX (15) because of packaging size constraints. All of the viruses were grown in 293 cells and purified by DEAE column chromatography as described (16). Virus particle concentrations were determined by Resource Q high-performance liquid chromatography (17), and the primary structure of all of the transgenes was verified by automated sequencing of viral DNA.

Immunodetection of MMAC1 Protein. Cell monolayers were infected for 24 h with GFCB or MMCB at various viral particle numbers/ml in culture

Received 2/16/98; accepted 4/16/98.

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[2] The abbreviations used are: MMAC1, mutated in multiple advanced cancers 1; PTEN, phosphatase and tensin homolog; RT, reverse transcriptase; Ad, adenovirus; FACS, fluorescence-activated cell sorting; pn/ml particle numbers/ml.

medium. Virus-containing solutions were removed at 24 h, and cells were either harvested at this time or refed with growth medium and collected at later time points. Cells were harvested by scraping into cold PBS, centrifuged, and washed once more in cold PBS and then freeze-thawed and resuspended in lysis buffer [50 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0), 150 mM NaCl, 1% NP-40, 5% glycerol, and 0.4 mM EDTA supplemented with 1 mM DTT and 1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim)]. Cell lysates were clarified by centrifugation at 10,000 × *g* for 15 min, and supernatants were normalized for protein content by the Bradford assay (Bio-Rad). Samples were resolved by SDS-PAGE using precast 8% Tris-glycine gels (Novex) and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) for Western blotting. Membranes were blocked with Tris-buffered saline containing 0.05% Tween and 5% skim milk and then blotted with anti-MMAC1 rabbit polyclonal antibody BL74,[3] followed by donkey antirabbit IgG conjugated with horseradish peroxidase (Amersham). MMAC1 was detected by chemiluminescence (enhanced chemiluminescence kit; Pierce Chemical Co.) using Kodak XAR-5 film.

FACS Infectivity Assay. U87MG cells were plated at 2×10^5 cells/well in six-well plates and incubated overnight, and then they were infected with GFCB at concentrations ranging from 1×10^5 to 1×10^9 particles/ml for 24 h. Cells were harvested by trypsinization and assayed by flow cytometry (FACScan; Becton Dickinson) for green fluorescence (525-nm peak detection, filter FL-1). Cells were gated on forward and side scatter, and a cutoff of fluorescence intensity was established such that ~99% of uninfected cells were negative. The percentage of GFCB-infected cells with greater fluorescence than this cutoff was then determined, representing a minimum estimate of the percentage of infected cells.

[^{3}H]Thymidine Incorporation Assay. Cells were plated at 5×10^3 cells/well in 96-well microtiter plates (Costar) and incubated overnight. Dilutions of ZZCB, GFCB, FTCB, and MMCB in medium ranging from 1×10^4 to 1×10^9 particles/ml were added in triplicate to the cell monolayers and then incubated for 24 h. Virus-containing solutions were removed at 24 h after infection and replaced with new tissue culture medium for an additional 24 h. Cells were treated with 1 μCi of [^{3}H]thymidine per well 4 h prior to harvesting. Cells were harvested onto glass-fiber filters, and incorporation of [^{3}H]thymidine was determined using liquid scintillation (Top Count; Packard).

Cell Count/Viability Assay. Subconfluent monolayers of U87MG cells were infected in triplicate with MMCB or GFCB Ad at 5×10^6 or 5×10^7 particles/ml for 24 h, after which supernatants were replaced with fresh medium for an additional 48 h. Cells were then harvested by trypsinization, and viable cells were counted by the trypan blue exclusion method using a hemocytometer.

Soft Agar Colony Formation Assay. U87MG cells (1×10^3) infected as described above with 5×10^6 or 5×10^7 particles/ml for 24 h were suspended in tissue culture medium containing 0.35% agar and layered in triplicate onto 0.7% agar in 35-mm tissue culture wells. Cultures were incubated in a humidified atmosphere containing 7% CO_2 at 37°C. Additional 0.35% agar culture medium was added every 5 days. Colony growth was assessed at 14 days postinfection.

Tumorigenicity Assay. U87MG cells were plated at a density of 1×10^7 cells/T225 flask. After overnight incubation, cell monolayers were infected with 5×10^7 or 5×10^8 particles/ml of Ad GFCB, FTCB, BGCA, or MMCB for 24 h. Infected or uninfected cells were harvested by trypsinization, washed in medium, counted in the presence of trypan blue, and injected s.c. (5×10^6 viable cells per flank) into athymic *nu/nu* female mice (Simonsen Laboratories). Mice were scored for tumors at 21 or 30 days; tumor diameters in three dimensions were measured with Vernier calipers, and tumor volumes were calculated as their product.

Results and Discussion

U87MG human glioblastoma cells (18) were chosen for study based on their reported *MMAC1* mutation (1), soft agar colony-forming ability, and s.c. tumorigenicity in nude mice. Consistent with the /gt→/tt mutation of the intron 3 splice donor site (1), RT-PCR products derived from U87MG RNA using primers in exons 2 and 5

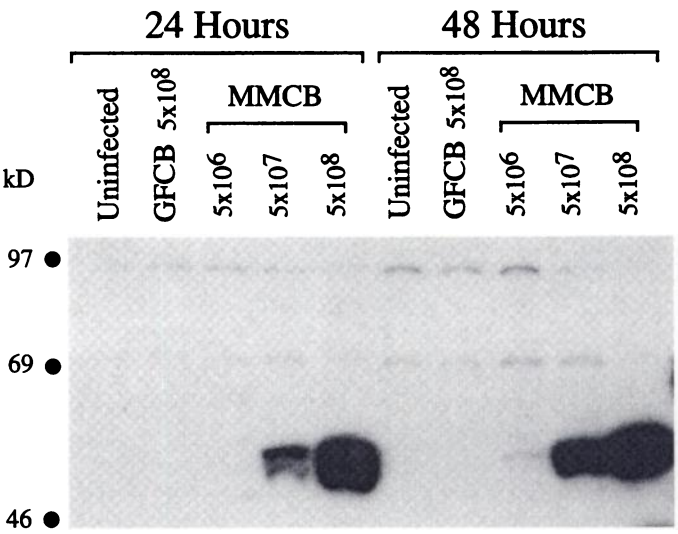

Fig. 1. Exogenous MMAC1 protein expression. U87MG cells were infected with MMCB or GFCB at the indicated concentrations (pn/ml) for 24 h, and then lysates were prepared immediately (24 h) or 24 h later (48 h). Western blotting was performed as described in "Materials and Methods." Protein size markers are shown at left. MMAC1 protein migrated at approximately M_r 55,000 in agreement with Li and Sun (3).

(see "Materials and Methods") were found to be smaller than expected (wild-type size, 317 bp), and the sequence of these products confirmed the absence of exon 3 (data not shown). Although this exon contains 45 bp (15 codons), and an in-frame readthrough product is possible, the missing residues encode a conserved α-helix in the native protein, and their loss ablated growth-inhibitory activity as measured by Furnari *et al.* (13).

Purified recombinant MMAC1-containing Ad (MMCB) was characterized for transgene expression in U87MG cells by Western blotting of cell lysates with a rabbit polyclonal antibody generated against a fusion protein (Fig. 1). Endogenous MMAC1 protein was not detected in uninfected or control virus-infected cells, but it was detected in a dose-dependent fashion in MMCB-infected cells by the end of the 24 h infection period, as well as at 48 h (Fig. 1), 72 h and 96 h (data not shown). This experiment verified the efficient transduction and acute expression of exogenous MMAC1 protein in U87MG glioma cells, as well as validating its detection by Western blotting with antibody BL74. Infectivity of U87MG cells was assessed quantitatively by FACS analysis using GFCB, a recombinant Ad identical to MMCB except for its transgene, which encoded green fluorescent protein (Fig. 2). The expected sigmoidal infectivity curve was obtained, from which it was estimated that 85–90% of cells were infected at a viral dose of 5×10^7 particles/ml for 24 h. Of note is that our dosing parameters are not based on the plaque-forming unit or its derivative, multiplicity of infection, because we have previously shown that adenoviral concentration and infection time are the primary determinants of *in vitro* transduction (19).[4]

In vitro proliferation of MMCB Ad *versus* control Ad-infected U87MG cells was measured by [^{3}H]thymidine uptake over a wide range of viral concentrations (Fig. 3A). U87MG was differentially inhibited by MMCB compared to two control Ads (GFCB and ZZCB) over most viral doses; at high Ad concentrations (1×10^9 particles/ml), a nonspecific inhibitory effect predominated, as has been noted before in some cell lines (20). Inhibition of DNA synthesis by MMCB was comparable to that induced by adenoviral *p53* gene transfer (FTCB; Fig. 3*A*). Growth inhibition was confirmed in a second *in vitro* assay by counting viable cells at 72 h after the start of infection

[3] A. Morimoto, A. E. Berson, G. H. Fujii, P. A. Steck, S. V. Tavtigian, R. Bookstein, and J. B. Bolen, submitted for publication.

[4] D. Giroux, K. Watson, and P. Shabram, manuscript in preparation.

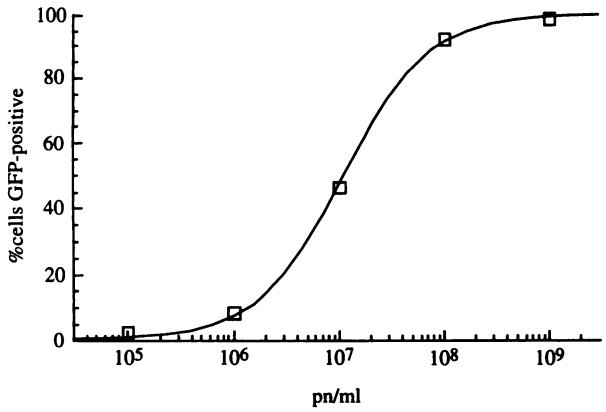

Fig. 2. FACS infectivity assay. U87MG cells were infected with GFCB at the indicated concentrations for 24 h. The fraction of cells expressing green fluorescent protein was quantitated by flow cytometry.

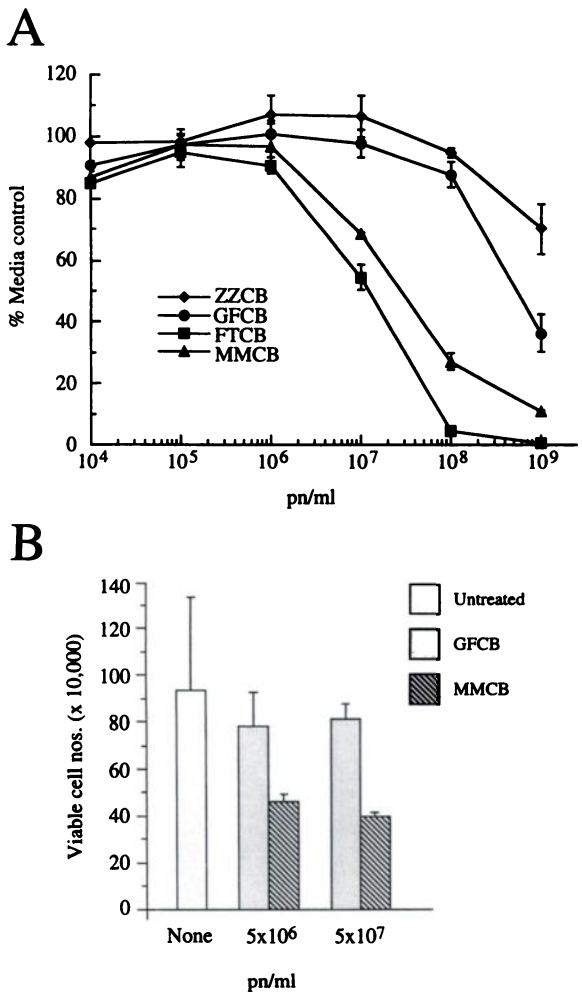

Fig. 3. Inhibition of *in vitro* proliferation by MMCB. *A*, [^{3}H]thymidine uptake. U87MG cells were infected with MMCB or control Ads at the indicated concentrations for 24 h. Results are plotted as percentages of medium-treated control (mean ± SD). *B*, viable cell count assay. U87MG cells were infected with MMCB or GFCB as in *A*. Mean cell numbers at 72 h after infection are plotted; *bars*, SD (three replicates).

(Fig. 3*B*). MMCB reduced cell numbers at this time point by about 50% compared to GFCB at equal doses. This inhibition was comparable in magnitude to that observed by Furnari *et al.* (13) using transient plasmid transfection. MMCB- and GFCB-infected cultures had similar viability rates at 72 h, and morphological evidence of cell death, such as cell blebbing or nuclear fragmentation, was not seen with MMCB treatment (data not shown).

Effects of MMAC1 on anchorage-independent growth were assessed by colony formation in soft agar following transduction by MMCB *versus* GFCB or FTCB. The latter was included to validate the assay with an established tumor suppressor gene. At a dose of 5×10^6 particles/ml for 24 h, colony formation with MMCB or FTCB was inhibited by approximately 50% compared to the GFCB control, whereas a >95% inhibition (relative to GFCB) could be achieved at 5×10^7/ml of either MMCB or FTCB (Fig. 4). Therefore, a dose-dependent, gene-specific effect of MMAC1 was evident in this *in vitro* assay.

Two tumorigenicity assays were performed with 5×10^6 MMCB-infected U87MG cells per injection compared to the same number of cells infected by three different control Ads, GFCB, FTCB, and BGCA (Table 1). Differences between experiments 1 and 2 included the use of two dose levels *versus* one and termination at 21 *versus* 30 days, respectively. MMCB-infected U87 cells were completely nontumorigenic at 21 or 30 days with the exception of three very small tumors (~10 mm^3) at the lower dose level in experiment 1. Tumors formed in all 39 mice injected with uninfected or control Ad-infected cells. Reporter gene-containing control Ads GFCB and BGCA had some activity in reducing average tumor size compared to buffer-treated cells, a nonspecific "adenoviral effect" previously noted by us

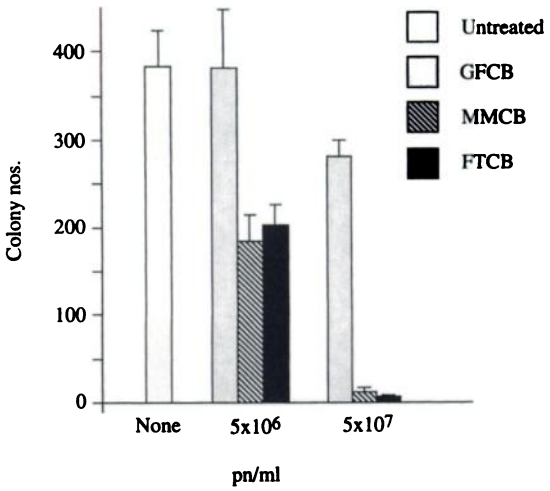

Fig. 4. Soft-agar colony formation. U87MG cells were infected with MMCB, GFCB, or FTCB at the indicated concentrations for 24 h. Mean colony numbers are plotted; *bars*, SD (three replicates).

Table 1 *Tumor formation in nude mice injected with U87MG cells infected ex vivo with MMCB and control Ads*[a]

Vector (No. of particles/ml)	No. of mice with tumors/ No. of mice injected	Mean tumor volume (mm^3) ± SD
Experiment 1, 21 days		
None	6/6	443 ± 55
BGCA (5×10^7)	6/6	375 ± 61
BGCA (5×10^8)	6/6	290 ± 25
FTCB (5×10^7)	6/6	68 ± 14
MMCB (5×10^7)	3/6	10 ± 6
MMCB (5×10^8)	0/6	
Experiment 2, 30 days		
None	5/5	1461 ± 743
BGCA @ (5×10^7)	5/5	435 ± 79
GFCB @ (5×10^7)	5/5	696 ± 312
MMCB @ (5×10^7)	0/5	

[a] Cells were infected for 24 h and then washed and prepared for injection. Mice were scored for tumor formation after the indicated number of days. BGCA, LacZ-expressing Ad; FTCB, p53-expressing Ad; GFCB, green fluorescent protein-expressing Ad; MMCB, MMAC1/PTEN-expressing Ad.

(14, 20). The p53-containing Ad had a more dramatic effect on average tumor size (~68 mm^3), yet tumors still formed in six of six mice. These results are consistent with the growth-inhibitory effects of *p53* Ad gene transfer in U87MG cells reported elsewhere, although these cells contain *p53* alleles with the wild-type sequence (21, 22). In any case, these data indicate a gene-specific tumor suppression activity of MMCB in U87MG cells at moderate viral doses.

Using a recombinant adenoviral gene transfer system, we have shown an *in vitro* growth inhibition activity of MMAC1/PTEN in U87MG cells in agreement with Furnari *et al.* (13). However, the inherent limitations of *in vitro* growth assays and their uncertain relevance to *in vivo* tumorigenicity are well recognized. The use of a recombinant Ad was helpful in circumventing the technical difficulty of studying tumor cells stably expressing potentially growth-inhibiting proteins such as MMAC1. For example, we have repeatedly failed to isolate MMAC1-expressing clones of U87MG cells transfected with either constitutive or inducible expression plasmids (data not shown). Technical drawbacks of recombinant Ads for these studies include variation in infectivity of various cultured tumor cells (20) and a variable nonspecific inhibitory effect of Ad, for which several control procedures are indicated. Nonetheless, a specific tumor suppression activity of wild-type MMAC1 was clearly detected in the *in vivo* tumorigenicity assay. Biochemically characterized mutants of MMAC1, including those defective for its phosphatase activity (12), have been investigated separately using a retroviral transduction system.[3] Our data support a role for *MMAC1/PTEN* inactivation in glioblastoma tumorigenesis and further suggest that *MMAC1* transfer *in vivo* may be considered as a potential cancer therapy approach.

Acknowledgments

We thank Dan Maneval, Beth Hutchins, Murali Ramachandra, David Teng, and Peter Steck for discussions; Wendy Hancock, Elaine Hurney, and Erlinda Quijano for cell culture; Suganto Sutjipto and Doug Cornell for virus production; Debbie Muilwijk for animal care; and Donal MacGrogan, Uyen-Chi Dang, Alina Levy, and Whei-Mei Huang for molecular biology support.

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