Derivation and Initial Characterization of a Mouse Mammary Tumor Cell Line Carrying the Polyomavirus Middle T Antigen: Utility in the Development of Novel Cancer Therapeutics

Loretta L. Nielsen, Maya Gurnani, Bin Shi, Gaby Terracina, Robert C. Johnson, Jennifer Carroll, J. Michael Mathis, and Gerald Hajian

Tumor Biology [L. L. N., M. B., B. S., G. T.] and Biostatistics [G. H.], Schering-Plough Research Institute, Kenilworth, New Jersey 07033-1300; Safety Evaluation Center, Schering-Plough Research Institute, Lafayette, New Jersey 07848-6032 [R. C. J.]; and Department of Cellular Biology and Anatomy, Louisiana State University Medical Center, Shreveport, Louisiana 71139-3932 [J. C. J. M. M.]

ABSTRACT

Here we describe the derivation of novel cell lines from spontaneous mammary tumors that arose in mouse mammary tumor virus-polyomavirus (MMTV-PyV) Middle T (MidT) transgenic mice. Clonal cell lines from four mixed cell populations were tested for adenovirus transducibility and sensitivity to p53 tumor suppressor gene therapy mediated by SCH58500, a replication-deficient adenovirus that expresses human p53. The MidT2-1 cell line was selected for further characterization in vitro and in vivo. This cell line carried the PyV MidT antigen, had wild-type p53 DNA, and was sensitive to suppression of proliferation by MMAC/PTEN tumor suppressor gene therapy. MidT2-1 cells gave rise to highly aggressive tumors in syngeneic FVB mice in both the mammary fat pad and the peritoneal cavity. The histopathology of MidT2-1 tumors closely resembled the histopathology of the primary transgenic tumors. Tumor growth in vivo was inhibited by p53 gene therapy or by MMAC gene therapy. In addition, combination therapy with a number of anticancer agents had synergistic or additive antitumor activity when used in combination.

INTRODUCTION

MMTV-PyV2 MidT transgenic mice develop aggressive mammary tumors at variable ages in both sexes (1). These tumors metastasize to the lungs with a frequency of >80%. Constitutive activation of c-Src by the PyV MidT antigen is essential for the development of mammary tumors in these mice (2). Activated c-Src in turn results in activation of the PI3K-Akt/PKB signal transduction pathway, which is negatively regulated by the tumor suppressor MMAC/PTEN (3–7). Activated Akt has been implicated in suppression of apoptosis in cells containing ras mutations (8, 9), possibly by suppressing the activation of caspase-9 (10). forkhead transcription factors (11, 12), and/or proapoptotic BAD (13, 14). In one report, Akt-mediated suppression of caspase-9 activity could be partially reversed in vitro by an inhibitor of the FPT enzyme involved in Ras processing (10). One of the effectors of activated Ras is PI3K, which indirectly activates the serine-threonine protein kinase Akt via phosphorylation (15, 16).

p53 is a DNA binding protein that acts as a transcription factor to control the expression of proteins involved in the cell cycle (17, 18). In response to DNA damage, p53 protein accumulates in the cell nucleus, causing cells to undergo cell cycle arrest and DNA repair or apoptosis (19). Functional inactivation of p53 can occur by several mechanisms, including direct genetic mutation, binding to viral oncoproteins or cellular factors (e.g., mdm-2), or alteration of the subcellular localization of the protein (17, 18). Although p53 is not essential for normal development, p53 “knock-out” mice are susceptible to tumors early in life (20). Mutations in p53 have been reported in a majority of clinical cancers, and it has been estimated that p53 function is altered in at least half of all human malignancies (17, 18). SCH58500 (ACN53) is a replication-deficient, recombinant adenovirus that expresses human p53 tumor suppressor (21). In preclinical models, SCH58500 has therapeutic efficacy against a wide range of human tumor types containing nonfunctional p53, and it has enhanced activity in combination with many chemotherapeutic drugs (22–27). SCH58500 cancer therapy is currently undergoing Phase I/II clinical trials.

SCH66336 is a p.o.-active, potent, and selective inhibitor of the FPT enzyme (28, 29). This novel therapeutic agent has activity against a wide variety of human tumor xenografts and also causes regression of tumors in wap-H-ras transgenic mice. Enhanced antitumor activity has been reported in animal models when SCH66336 is combined with various cytotoxic chemotherapeutic agents, including paclitaxel (Taxol), cyclophosphamide, 5-fluorouracil, and vincristine (28, 30). Combination therapy using SCH6636 and SCH58500 had synergistic or additive antiproliferative efficacy in a panel of human tumor cell lines in vitro (31). Greater combined efficacy was also observed in vivo in the DU145 human prostate and the wap-ras/F transgenic mouse cancer models. When the three-drug combination of SCH66336, SCH58500, and paclitaxel was tested, each two-drug interaction displayed such marked synergy that the addition of a third drug to the statistical model only produced additivity. Currently, SCH66336 is undergoing Phase I/II human clinical trials as an anticancer agent.

The taxanes, paclitaxel and docetaxel (Taxotere), inhibit cell replication by enhancing polymerization of tubulin monomers into stabilized microtubule bundles that are unable to reorganize into the proper structures for mitosis (32–34). This results in cell cycle blockage in mitosis and subsequent activation of an apoptotic pathway, which may be p53 independent (35, 36). In human tumor cell lines, paclitaxel has been shown to have synergistic or additive antiproliferative effects when combined with p53 gene therapy mediated by SCH58500 or with the FPT inhibitor SCH66336 (27, 28, 30, 31). Similar results were reported for combination therapy with docetaxel.
and SCH66336 (30). Gemicitabine (Gemzar) is a nucleoside analogue with antiproliferative activity against tumor cells in the S-phase of the cell cycle, primarily by blocking DNA synthesis (37). It is currently approved for the treatment of pancreatic and lung cancers (38, 39).

Here we describe the derivation of novel cell lines from spontaneous mammary tumors in MMTV-PyV MidT transgenic mice. Clonal cell lines from four mixed cell populations were tested for adenovirus transducing and sensitivity to p53 gene therapy using SCH58500. The MidT2-1 cell line was selected for further characterization based on its susceptibility to Ad transduction and sensitivity to p53-mediated suppression of cell proliferation. This cell line carries the PyV MidT antigen, has wild-type p53, and was sensitive to suppression of proliferation by MMC gene therapy. MidT2-1 cells gave rise to highly aggressive tumors in syngeneic FVB mice in both the mammary fat pad and the peritoneal cavity. The histopathology of MidT2-1 tumors closely resembled the histopathology of the primary transgenic tumors. Tumor growth in vivo was inhibited by p53 gene therapy and by MMC gene therapy. In addition, combination therapy with a number of anticancer agents had synergistic or additive efficacy in the MidT2-1 cancer model.

MATERIALS AND METHODS

Transgenic Tumor Cell Derivation. Mammary tumors from female MMTV-PyV MidT transgenic mice (1) were passaged through FVB immuno-competent, syngeneic host mice. Cells from one MidT/TFVB tumor were placed in culture after passage 1 (MidT1 cells) and passage 4 (MidT2 cells). Tumors from two other transgenic mice were similarly passaged through FVB mice to yield MidT3 and MidT4 cells. The mixed cell populations were subsequently cloned. All cells were cultured in 90% DMEM (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum (Life Technologies, Inc.). Cell doubling time for the MidT2-1 cell line was determined in triplicate according to the procedure given by Freshney (40).

Ad Transduction in Vitro. Construction and propagation of SCH58500 (ACN53), MMC Ad, and Escherichia coli β-gal Ad have been described previously (21, 41). The empty Ad vector is identical to SCH58500, but without the p53 transgene. The concentration of total viral particles (PN) in SCH58500 batches was determined by measuring absorption at 260 nm (42). The concentration of total PN in control vector batches was determined by Resource Q Anion Exchange HPLC assay (43). Infectious PN was determined by measuring the concentration of viral hexon protein-positive 293 cells after a 48-h infection period (42). C.I.U. is defined by Musco et al. (44). Ads were administered in phosphate buffer [20 mM NaH₂PO₄ (pH 8.0), 130 mM NaCl, 2 mM MgCl₂, and 2% sucrose].

For Ad transduction of MidT cells in vitro, cells were incubated with β-gal Ad for 48 h, lightly fixed in 0.2% glutaraldehyde, and then assayed for β-gal enzyme activity as follows. The cells were incubated in 1 ml of assay buffer [1.3 mM MgCl₂, 15 mM NaCl, 44 mM HEPES buffer (pH 7.4), 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide (10% final concentration)] for 5–6 h. The number of individual cells scoring positive or negative for β-galactosidase activity was counted in each microscope field. The results from three microscope fields were averaged for each culture dish well, and three wells/treatment group were used for the analysis. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was purchased from Boehringer-Mannheim (Indianapolis, IN). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

PyV MidT Transgene PCR. The presence of the PyV MidT oncogene in MidT2-1 cells was confirmed by PCR. DNA from MidT2-1 and an unrelated mouse mammary cell line from CI5513 was isolated and then cycled for 25 cycles consisting of 10 s at 94°C, 10 s at the appropriate primer annealing temperature, and then stored at −20°C. Exons 5–8 of the p53 gene were isolated from MidT2-1 DNA by the PCR (46) using oligonucleotide primers published previously (47). Exons 5–8 were isolated individually, as well as a 1.6-kb product spanning exons 5–8. PCR reactions were performed in 20-µl volumes containing 1 mM of each primer, 200 µM each of dATP, dGTP, dCTP, and dTTP with 1.5 mM MgCl₂, 1 unit of Taq polymerase, and template DNA in Taq buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.01% gelatin]. PCR was performed with initial denaturation at 94°C for 2 min and then cycled for 25 cycles consisting of 10 s at 94°C, 10 s at the appropriate annealing temperature, and then stored at −20°C. The PCR fragments were analyzed by agarose gel electrophoresis and directly subcloned into the pCR 2.1 plasmid vector (Invitrogen).

Sequence of the p53 coding exons 5–8 was performed on PCR products using internal T7 forward and M13 reverse as well as intron-specific oligonucleotides (46). Direct PCR sequencing reactions were performed using a PRISM Dye Primer Cycle sequencing kit using Taq polymerase on an automated 373A DNA Sequencer (ABI). Sequencing reactions were performed as recommended by the kit manufacturer. Sequencing of each exon was performed on four independent PCR reactions.

p53 Protein Detection. MidT cell lysates were prepared and assayed in a p53 mutant-selective ELISA (Onconec Sciences, Cambridge, MA) according to the manufacturer’s instructions. For p53 immunohistochemistry, five-µm, formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated. The sections were then postfixed in cold ethanol/acetic acid (2:1) for 10 min and washed in PBS for 2 × 5 min. Antigen retrieval was performed by microwaving the slides for 10 min in 10 mM sodium citrate (pH 6.0). After cooling to room temperature and one wash in PBS for 5 min, endogenous peroxidase activity was blocked with 1.5% aqueous hydrogen peroxide for 10 min. After two 5-min washes in PBS, the slides were blocked with the protein blocking reagent from the ABC/DAK staining system (Santa Cruz Biotechnology, Santa Cruz, CA) and processed according to the manufacturer’s instructions. The primary antibody was the mouse monoclonal, anti-p53 PAB1801 (Lab Vision) diluted 1:100 in ABC antibody diluent. The incubation time for primary antibody was 60 min. For negative controls, primary antibody was substituted with blocking reagent. The secondary antibody was the biotinylated goat antimouse antibody included in the ABC kit. The slides were counterstained with hematoxylin (1:4; Sigma) for 3 min, washed in water for 5 min, 95% ethanol 3 × for 30 s each wash, 100% ethanol two times for 1 min each wash, and Clear Rite (Richard-Allen, Kalamazoo, MI) two times for 1 min each wash. They were coverslipped using Permount (Fisher Scientific, Pittsburgh, PA).

Akt Western Blots. Cells were lysed in SDS-sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycine, 50 mM DTT, and 0.1% bromophenol blue] and Western blotted according to the manufacturer’s directions in the PhosphoPlus Akt-Ser473 Antibody kit (New England BioLabs). Briefly, 20 µl of each sample were loaded onto an SDS-PAGE gel (NuPAGE 4–12% Bis-Tris Gel; Novex) and run at 240 milliamps for 1.5 h. Then the proteins were electrotransferred from the gel to nitrocellulose membrane (Novex). The nitrocellulose membrane was blocked in SuperBlock (Pierce) at room temperature for 1 h and then incubated with primary antibodies (1:1000), rabbit polyclonal anti-phospho-AKT (Ser-473), or rabbit polyclonal anti-AKT at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h and then incubated with primary antibodies (1:1000), rabbit polyclonal anti-phospho-AKT (Ser-473), or rabbit polyclonal anti-AKT at 4°C overnight.

Downloaded from cancerres.aacrjournals.org on August 14, 2017. © 2000 American Association for Cancer Research.
Drug Interaction Studies in Vivo. SCH66356 was synthesized by Schering-Plough, and its structure has been published (28, 29). SCH66366 (100 μM) in DMSO was diluted with culture media for in vitro studies. Paclitaxel (Taxol) was purchased from Calbiochem. Docetaxel (Taxotere; Rhone-Poulenc Rorer) and gemcitabine (Gemzar; Eli Lilly) were purchased from Drug Fair (Westfield, NJ). Paclitaxel and docetaxel were dissolved in absolute ethanol to 10 mg/ml and then diluted in culture media immediately before use. Gemcitabine was dissolved in DMSO.

For 3- and 4-day assays, MidT2-1 cells were plated at a density of 1–1.5 × 10^5 cells/well on a 96-well plate and cultured for 4 h at 37°C and 5% CO2. For 7-day assays, 250 MidT2-1 cells were seeded into culture wells of 96-well plates and allowed to attach for 3 h under the same culture conditions. For MMC Ad and paclitaxel, MMC Ad, paclitaxel, or the appropriate vehicle was added to each well, and cell culture was continued for 3 days. For MMC Ad and SCH58500, MMC Ad, SCH58500, or the appropriate vehicle was added to each well, and cell culture was continued for 3 days. For MMC Ad and SCH66366, tumor cells were incubated with SCH66366 or vehicle for 7 days. MMC Ad was added to some cells for the last 3 days. For paclitaxel/docetaxel and SCH58500, paclitaxel/docetaxel, SCH58500, or the appropriate vehicle was added to each well, and cell culture was continued for 3 days. For one assay, SCH58500 was added 24 h after paclitaxel. For SCH58500 and gemcitabine in assay 1, SCH58500, gemcitabine, or the appropriate vehicles were added to each well, and cell culture was continued for 3 days. In assay 2, gemcitabine or Ad buffer was added to each well. Twenty-four h later, SCH58500 was added to some wells. Cell culture was continued for a total of 4 days. For SCH66336 and SCH58500, tumor cells were incubated with SCH66336 or vehicle for 7 days. SCH58500 was added to some cells for the last 3 days. For SCH66336 and paclitaxel/docetaxel, the cells were incubated with paclitaxel/docetaxel or vehicle for 4 h, washed, and then SCH66336 or vehicle was added, and the incubation continued for 7 days. For SCH66336 and gemcitabine, the cells were incubated with SCH66336 for 7 days. Gemcitabine was added to some cells for the last 4 days.

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole bromide assay (48). Briefly, 25 μl of 5 mg/ml 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyloxazole bromide were added to each well and allowed to incubate for 3–4 h at 37°C and 5% CO2. Then, 100 μl of 10% SDS detergent were added to each well, and the incubation was continued overnight. Fluorescence in each well was quantitated using a Molecular Devices microtiter plate reader. Cell proliferation data from drug interaction studies were analyzed using the Thin Plate Spline methodology of O'Connell and Wolfinger (49).

Histology. Tissue samples were fixed in 10% buffered formalin, processed overnight in a Miles VIP tissue processor, and then embedded in paraffin. Five-μm tissue sections were cut with a Leitz microtome, and then the sections were stained with a routine Harris H&E stain (50).

Ad Treatment in Vivo. FVB mice were from the SPRI colony. Breeding stock for the SPRI colony of MMTV-PyV MidT transgenic mice (1) were a generous gift from Dr. William Muller (McMaster University, Hamilton, Ontario, Canada). All mice were maintained in a virus antigen-free-hairless facility. All animal procedures were performed in accordance with the rules set forth in the NIH Guide for the Care and Use of Laboratory Animals and approved by the SPRI Animal Care and Use Committee.

MMAC Gene Therapy. MidT2-1 transgenic mouse mammary tumor cells (1 × 10^7) were injected into the mammary fat pad of each female FVB mouse. Tumors were allow to establish for 3 weeks and then randomized into three treatment groups on day 0. Group 1 was dosed with 0.1 ml of vehicle, group 2 with empty Ad vector, and group 3 with MMC Ad, intratumorally once a day on days 0–4 and 7–11. There were 10 mice/group. Mice in the vehicle control group were humanely sacrificed on day 16 because of high tumor burden.

p53 Gene Therapy. In study 1, 5000 MidT2-1 transgenic mouse mammary tumor cells were injected into the peritoneal cavity of each female FVB mouse. Mice were given i.p. therapy on days 8–11, 14–17, 21–24, and 28–30. The Ad dose was 1 × 10^9 Plaques Infective/animal (3.6 × 10^7 C.I.U. SCH58500 and 1.8 × 10^7 C.I.U. empty Ad). In study 2, 5000 MidT2-1 transgenic mouse mammary tumor cells were injected into the peritoneal cavity of each female FVB mouse. The Ad dose was 1 × 10^9 Plaques Infective/animal (3.6 × 10^7 C.I.U. SCH58500 and 1.8 × 10^7 C.I.U. empty Ad). The cisplatin dose was 0.1 mg/kg/day, and the paclitaxel dose was 0.25 mg/kg/day. Mice were given i.p. therapy on days 7–11, 14–18, and 21–25. Data from survival studies were analyzed using the Kaplan-Meier statistical method, and log-rank probabilities were generated using StatView 5.0 software (SAS Institute, Cary, NC).

RESULTS

Derivation and Morphology. Mammary tumors from female MMTV-PyV MidT transgenic mice were passaged through FVB immunocompetent host mice and dispersed into cell culture, and the mixed cell populations were subsequently cloned. Fig. 1 shows in vitro data for representative clones from four MidT mixed cell populations. We derived 3 MidT1 clonal cell lines, 3 MidT2 clonal cell lines, 6 MidT3 clonal cell lines, and 5 MidT4 clonal cell lines. Only MidT2 clones were easily transduced by Ad (Fig. 1A) and sensitive to killing by p53 gene therapy (ED50, 46 C.I.U. SCH58500/cell; Fig. 1B) but not by control Ad vectors. Therefore, MidT2 mixed cell populations or MidT2 clone 1 (MidT2-1) cells were used for in vivo exper-
iments. MidT2-1 cells had a cell doubling time of 13.3 ± 0.7 h in 10% serum (± SE).

The morphology of MidT2-1 cells is shown in Fig. 2A. Note the moderate nuclear:cytoplasmic ratio characteristic of epithelial cells and the resemblance of MidT2-1 cells to cells in tumors from MMTV-PyV MidT transgenic mice (Fig. 2, B–D). Pathological analyses were performed on well-established, primary mammary fat pad tumors and lung metastases from 10 female MMTV-PyV MidT transgenic mice. The primary tumors were typically well demarcated, multilobulated, and often had variable to conspicuous central necrosis. Cells were

either densely compact in a multilobular pattern or were arranged in long, ribbon-like columns of columnar-type cells, forming the periphery of a lumen often containing pink, homogeneous, sometime vacuolated proteinaceous material. In addition to containing deeply eosinophilic secretory (proteinaceous) material, these gland-like structures are often large, irregular, and commonly coalesced to form very prominent lakes of secretory material. The cells were oval with a moderate amount of eosinophilic cytoplasm and generally indistinct cell borders. Nuclei were oval with stippled chromatin and commonly had a single prominent nucleolus. Mitoses were common. Cells occasionally resembled squamous or sebaceous-type cells, often in conjunction with production of keratin-like material (“keratin pearls”). Multiple, conspicuous metastases were present in the lungs of all transgenic mice examined. Tumor colonies were most frequently located subpleurally, and the metastases generally recapitulated the parent tumor. For example, there were areas of squamous cell differentiation (“keratin pearls”) in several metastases.

Pathological analyses were also performed on well-established, MidT2 mammary fat pad tumors and lungs from 10 female FVB mice. The tumors were typically well demarcated, multilobulated, and often with variable to conspicuous central necrosis (Fig. 2, E–G). Tumor cells were densely arranged into variably sized lobules separated by thin strands of connective tissue and vessels. Occasionally, cells lined small, variably sized, gland-like structures. In contrast to the transgenic tumors, lumens in MidT2 tumors were oval and lacked secretory material. The tumor cells were (as above) oval with a moderate amount of eosinophilic cytoplasm and had generally indistinct cell borders. Nuclei were oval with stippled chromatin, commonly had a single prominent nucleolus, and a moderate nuclear:cytoplasmic ratio. Mitoses were common. Lung tumors were generally smaller and fewer in number than in the parent transgenics. This was not surprising, given the higher tumor burden in the transgenics at the time of tissue harvest and the more aggressive growth rate of the MidT2 mammary fat pad tumors, which allows less time for lung colonization before mouse death from the primary tumor. However, the morphology of the MidT2 lung metastases that were present closely resembled the morphology of the transgenic mouse lung metastases.

MidT2 i.p. tumors from nine female FVB mice were also analyzed. Tumors were distributed throughout the peritoneal cavities as both small and large nodules (Fig. 2H). They were commonly found on the peritoneal surface of abdominal organs but were also found invading the surfaces of the diaphragm, liver, pancreas, and intestines. In one mouse, there was a mild capsular (surface) invasion of the kidney with minimal compression of the parenchyma. Some tumors nodules were densely cellular and separated into smaller, variably sized oval lobules by generally thin layers of connective tissue. Central necrosis and multiple mitoses were common.

Molecular Characterization. The presence of the PyV MidT transgene in MidT2-1 cells was confirmed. As shown in Fig. 3, a

Fig. 3. PCR analysis confirms the presence of the PyV MidT transgene in MidT2-1 cells. Lane 1, R/F6–10 wap-H-ras transgenic mouse mammary tumor cell line. Lane 2, MidT2-1 MMTV-PyV MidT transgenic mouse mammary tumor cell line. Lane 3, molecular weight marker.

MID T SYNGENEIC MOUSE TUMOR MODEL
adenovirus that expresses human p53 tumor suppressor. SCH66336 is a farnesyl protein transferase inhibitor that targets the processing of Ras and other farnesylated proteins. SCH58500 Paclitaxel Paclitaxel 24 h before SCH58500, 3-day assay Synergy (P = 0.0099).

The p53 status of MidT2-1 cells was determined at both the DNA and protein levels. DNA for p53 exons 5–8 was cloned by PCR and sequenced. All exons had wild-type p53 DNA sequence. Syngeneic MidT2-1 tumor or cultured cell homogenates had 2.0 ± 0.4 pg p53/µg of total protein (n = 8). For comparison, p53mut MDA-MB-468 human breast tumor cells had 381.4 pg of p53/µg of total protein in the same assay, strongly suggesting that MidT2 cells express wild-type p53 protein. p53 immunohistochemistry on tumor sections failed to detect overexpression of p53, confirming wild-type p53 status for these tumors (data not shown). These observations are consistent with the report of wild-type p53 status in primary mammary tumor cells from MMTV-PyV MidT transgenic mice (51).

MidT2-1 cells were expected to have constitutive phosphorylation of Akt because of constitutive stimulation of upstream Src activity by the transgenic PyV MidT antigen in these cells. The MMAC/PTEN tumor suppressor gene modulates Akt phosphorylation by affecting P13K activation (3–5, 7). As shown in Fig. 4, MidT2-1 cells had high levels of phosphorylated Akt. MMAC gene therapy suppressed Akt phosphorylation by 47% and also had a profound, dose-dependent inhibitory effect on cell proliferation. In contrast, treatment with empty Ad vector or SCH58500 had no effect on the levels of phosphorylated Akt in MidT2-1 cells, although p53 gene therapy did inhibit cell proliferation very effectively.

The efficacy of MMAC gene therapy was next tested in vivo. FVB mice with MidT2-1 mammary fat pad tumors were dosed with intratumoral, recombinant Ads (Fig. 5). Adenovirus vector had no effect on tumor growth for the first 11 days of dosing. However, by day 14, tumors treated with Ad vector were significantly smaller than tumors treated with vehicle (P ≤ 0.03 for days 14–16). In severe combined immunodeficient mice, this vector-specific growth suppression is mediated by natural killer cells (52). MMAC gene therapy had a significant growth inhibitory effect on MidT2-1 mammary fat pad tumors compared with either vehicle or Ad vector (P ≤ 0.0099). Compared with vehicle treatment, MMAC Ad inhibited tumor volume by 47% on day 3, with inhibition peaking at 86% by day 16. Tumors treated with MMAC Ad were an average of 61% smaller than tumors treated with Ad vector from days 3 to 23.

### Drug Interaction Studies

**p53 Ad** gene therapy significantly improved the survival of FVB mice bearing i.p. MidT2-1 tumors (Fig. 7A; P = 0.0026). The 50% survival (ED50) values for each treatment combination were given in Table 1. MMAC Ad and p53 Ad demonstrated a synergistic interaction between the two recombinant adenoviruses (P = 0.00001). B, isobole graph for SCH58500 and paclitaxel demonstrating synergy (P = 0.0496). C, isobole graph for SCH58500 and paclitaxel when MidT2-1 cells were incubated with paclitaxel 24 h before the addition of SCH58500. The data demonstrate synergy (P = 0.0002). Multiplicity of infection (m.o.i.), C. I. U./cell.

#### Table 1. Analysis of in vitro drug interactions in MidT2-1 transgenic mouse mammary tumor cells

<table>
<thead>
<tr>
<th>Drug 1</th>
<th>Drug 2</th>
<th>Protocol</th>
<th>Isobole analysis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMAC Ad&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Paclitaxel</td>
<td>Simultaneous drugs, 3-day assay</td>
<td>Synergy (P = 0.0496)</td>
</tr>
<tr>
<td>MMAC Ad</td>
<td>SCH58500</td>
<td>Simultaneous drugs, 3-day assay</td>
<td>Synergy (P = 0.0001)</td>
</tr>
<tr>
<td>SCH58500</td>
<td>Paclitaxel</td>
<td>Simultaneous drugs, 3-day assay</td>
<td>Synergy (P = 0.0002)</td>
</tr>
<tr>
<td>SCH58500</td>
<td>Paclitaxel</td>
<td>Paclitaxel 24 h before SCH58500, 3-day assay</td>
<td>Synergy (P = 0.0001)</td>
</tr>
<tr>
<td>SCH58500</td>
<td>Docetaxel</td>
<td>Simultaneous drugs, 3-day assay</td>
<td>Synergy (P = 0.0025)</td>
</tr>
<tr>
<td>SCH58500</td>
<td>Gemicitabine</td>
<td>Gemicitabine 24 h before SCH58500, 4-day assay</td>
<td>Additivity (P = 0.7308)</td>
</tr>
<tr>
<td>SCH58500</td>
<td>Gemicitabine</td>
<td>Simultaneous drugs, 3-day assay</td>
<td>Additivity (P = 0.0206)</td>
</tr>
<tr>
<td>SCH66336</td>
<td>Paclitaxel</td>
<td>SCH66336 for last 3 days, 7-day assay</td>
<td>Additivity (P = 0.0206)</td>
</tr>
<tr>
<td>SCH66336</td>
<td>Docetaxel</td>
<td>SCH66336 for last 4 days, 7-day assay</td>
<td>Additivity (P = 0.0994)</td>
</tr>
<tr>
<td>SCH66336</td>
<td>Gemicitabine</td>
<td>SCH66336 for last 4 days, 7-day assay</td>
<td>Additivity (P = 0.0994)</td>
</tr>
</tbody>
</table>

<sup>a</sup>P for synergy or antagonism. <sup>b</sup>MMAC Ad is a replication-deficient adenovirus that expresses human MMAC/PTEN tumor suppressor. SCH58500 is a replication-deficient adenovirus that expresses human p53 tumor suppressor. SCH66336 is a farnesyl protein transferase inhibitor that targets the processing of Ras and other farnesylated proteins.
Because of the widespread use of paclitaxel and platinum drug chemotherapy for ovarian/peritoneal cancers, combination therapy with paclitaxel/cisplatin was tested in the i.p. MidT model (Fig. 7, B and C). Treatment significantly increased mouse survival ($P = 0.0001$). Chemotherapy improved survival ($P = 0.0370$), and this survival benefit was further augmented by $p53$ gene therapy ($P = 0.0032$). When the study was halted on day 134, eight mice in the chemotherapy plus SCH58500 group were still alive, as compared with one mouse in the chemotherapy-alone group. The 50% survival (ED$_{50}$) values for each treatment group were day 50 for vehicle, day 48 for empty Ad, and day 87 for SCH58500.

**DISCUSSION**

In this report, we describe the development and initial characterization of a novel cancer model derived from mammary tumors in MMTV-PyV MidT transgenic mice (1). It is known that the PyV MidT antigen hyperstimulates c-Src tyrosine kinase but is not able to overcome $p53$-dependent growth arrest and does not induce $p53$-dependent apoptosis (53). Therefore, the MidT antigen was not expected to interfere with the antiproliferative activity of $p53$ gene therapy in MidT cells. Clonal cell lines from four mixed cell populations were tested for adenovirus transducibility and sensitivity to $p53$ tumor suppressor gene therapy mediated by SCH58500, a replication-deficient adenovirus that expresses human $p53$. The MidT2-1 cell line was selected for further characterization. This cell line carries the PyV MidT antigen, has wild-type $p53$, is easily transduced by Ad, is sensitive to suppression of proliferation by SCH58500, and is sensitive to suppression of proliferation by MMAC/PTEN tumor suppressor gene therapy. MidT2-1 cells give rise to highly aggressive tumors in syngeneic FVB mice in both the mammary fat pad and the peritoneal cavity. The histopathology of MidT2-1 tumors closely resembles the histopathology of the primary transgenic tumors. Tumor growth in vivo was inhibited by $p53$ gene therapy or by MMAC gene therapy.

Development of mammary tumors in MMTV-PyV MidT transgenic mice requires constitutive activation of c-Src by the polyomavirus MidT antigen (2). Activated c-Src in turn results in stimulation of the PI3K-Akt/PKB signal transduction pathway, which is negatively regulated by the tumor suppressor MMAC (3–7). Ad-mediated MMAC gene therapy has been shown to suppress Akt phosphorylation and cell proliferation in a number of glioblastoma and prostate tumor cell lines, with no effect on total Akt levels (41, 54). We observed similar results in MidT2-1 cells in vitro, as well as inhibition of MidT2-1 mammary fat pad tumor growth in vivo.

An orthotopic MidT tumor model similar to ours has been reported by another group (51). A cell line established from MMTV-PyV MidT transgenic tumors (MT1A2) expressed wild-type murine $p53$ but not mutant $p53$ protein. Overexpression of human $p53$ introduced via an Ad vector suppressed DNA synthesis and cell proliferation and induced apoptosis in a dose-dependent manner. This supports the theory that tumor cells from MMTV-PyV MidT mice express wild-type $p53$ but have a defect in the $p53$ signal transduction pathway. Primary tumor cells from MMTV-PyV MidT transgenic mice (1) were harvested, disaggregated, and injected into the mammary fat pads of syngeneic FVB hosts. Subsequently, intratumoral therapy with recombinant Ads expressing interleukin 2 or $p53$ demonstrated greater efficacy in combination (51). Mice rechallenged with fresh tumor cells 2–3 months later failed to develop cancer, suggesting antitumor
immunity. MTV-PyV MiDT transgensics in the FVB background strain developed CTLS specific for the MiDT tumor antigen after treatment with both Ads. Combination therapy with a number of anticancer agents had synergistic or additive efficacy in MiDT-2 cells in vitro. In particular, MMAC gene therapy synergized with SCH58500 or paclitaxel. Synergistic interactions between MMAC and p53 gene therapies have not been reported previously for any tumor cell line. Similarly, interaction studies have not been reported previously for paclitaxel combined with MMAC gene therapy nor for gemcitabine combined with SCH58500 or SCHR6336. In the i.p. MiDT-2 tumor model in vivo, p53 gene therapy was effective by itself and also enhanced the survival benefits of paclitaxel/cisplatin chemotherapy.

When exploring interactions between chemotherapy and SCH58500, it is tempting to assume the chemotherapy drug is interacting only with wild-type p53 protein. However, the chemotherapy may also be interacting with the Ad vector used to deliver p53. In Nielsen et al. (27), synergistic interactions between SCH58500 and paclitaxel were demonstrated in a panel of human tumor cell lines in vitro. These drug interaction effects were mediated, at least in part, by the unexpected ability of low nmol concentrations of paclitaxel to enhance Ad transduction of tumor cells in vitro. In other words, more tumor cells were infected with SCH58500 and exposed to high levels of wild-type p53 protein when paclitaxel “sensitized” them to transduction by recombinant Ad. This effect appears to be mediated by increased numbers of CAR1 receptors on the cell membrane after paclitaxel treatment. Grace et al. (23) reported that coadministration of paclitaxel with SCH58500 had no effect on the depth of Ad penetration into SK-OV-3 peritoneal xenografts in severe combined immunodeficient mice. However, combination therapy did cause an elevation in the total number of tumor cells undergoing apoptosis as compared with single drug therapy. The novel murine tumor cell line described in this report (MiDT2-1) also responded to SCH58500 and paclitaxel combination therapy with synergistic or additive reductions in cell proliferation in vitro, depending on the dosing regime.

The use of combination treatment has become a mainstay in cancer treatment. In this study, we used a novel transgenic mouse tumor cell line to suggest new combinations that might be explored in clinical cancer care. These include gene therapy using the tumor suppressors MMAC and p53 and chemotherapy using farnesyl transferase inhibitors, the microtubule-stabilizing taxanes, and the DNA synthesis inhibitors gemcitabine and cisplatin. The precise biological mechanisms by which these therapies induce their antitumor effects are not fully elucidated. However, the work presented here suggests that many of these therapeutic approaches have synergistic antitumor activity when used in combination.

ACKNOWLEDGMENTS

Thanks to Dr. Ming Liu for his diligence in procuring clinical grade drugs for use in these studies and to Janet Dell for technical assistance. Thanks also to Drs. Robert Bookstein and Chandra Kumar for helpful scientific discussions.

REFERENCES


20. Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Butel, J. S., and Bradley, A. Mice deficient for p53 are developmentally normal but susce...


Derivation and Initial Characterization of a Mouse Mammary Tumor Cell Line Carrying the Polyomavirus Middle T Antigen: Utility in the Development of Novel Cancer Therapeutics

Loretta L. Nielsen, Maya Gurnani, Bin Shi, et al.

Cancer Res 2000;60:7066-7074.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/24/7066

Cited articles
This article cites 48 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/24/7066.full#ref-list-1

Citing articles
This article has been cited by 5 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/60/24/7066.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.