The cancer growth suppressor gene mda-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice

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ABSTRACT  A differentiation induction subtraction hybridization strategy is being used to identify and clone genes involved in growth control and terminal differentiation in human cancer cells. This scheme identified melanoma differentiation-associated gene-7 (mda-7), whose expression is upregulated as a consequence of terminal differentiation in human melanoma cells. Forced expression of mda-7 is growth inhibitory toward diverse human tumor cells. The present studies elucidate the mechanism by which mda-7 selectively suppresses the growth of human breast cancer cells and the consequence of ectopic expression of mda-7 on human breast tumor formation in vivo in nude mice. Infection of wild-type, mutant, and null p53 human breast cancer cells with a recombinant type 5 adenovirus expressing mda-7, Ad.mda-7 S, inhibited growth and induced programmed cell death (apoptosis). Induction of apoptosis correlated with an increase in BAX protein, an established inducer of programmed cell death, and an increase in the ratio of BAX to BCL-2, an established inhibitor of apoptosis. Infection of breast carcinoma cells with Ad.mda-7 S before injection into nude mice inhibited tumor development. In contrast, ectopic expression of mda-7 did not significantly alter cell cycle kinetics, growth rate, or survival in normal human mammary epithelial cells. These data suggest that mda-7 induces its selective anticancer properties in human breast carcinoma cells by promoting apoptosis that occurs independent of p53 status. On the basis of its selective anticancer inhibitory activity and its direct antitumor effects, mda-7 may represent a new class of cancer suppressor genes that could prove useful for the targeted therapy of human cancer.

Abnormalities in cellular differentiation are common occurrences during cancer development and progression (1, 2). Correction of these defects resulting in the reversion of tumor cells to a more-normal differentiated phenotype represents a potentially useful therapeutic strategy (1, 2). Although the mechanism underlying cancer growth suppression and terminal differentiation is unknown, it is hypothesized that these changes result from the activation of genes negatively regulating cell growth and the suppression of genes promoting the cancer phenotype (1, 2). Induction of terminal differentiation can occur with and without the initiation of programmed cell death (2). Identification of the genes mediating these phenomena should provide mechanistic insights into these processes and also may elucidate potentially novel and selective targets for cancer therapy.

Induction of terminal differentiation combined with the molecular approach of subtraction hybridization, differentiation induction subtraction hybridization, is permitting the identification of critical gene changes associated with and controlling induction of terminal differentiation in human melanoma cells (1, 3, 4–6). The combination of recombinant human fibroblast interferon (interferon b) and the antileukemic compound mezerein elicits an irreversible loss of proliferation and induces terminal differentiation in human melanoma cells (7, 8). Several melanoma differentiation-associated (mda) genes have been isolated that either correlate with or directly influence human melanoma cell growth and differentiation (1, 3, 4–6). These include the cyclin-dependent kinase inhibitor p21, identified as mda-6, waf-1, cip-1, sdi-1 (4, 5, 9), and several novel genes, including mda-7 and mda-9 (10, 11). Partial screening of a human melanoma temporally spaced differentiation inducer-treated cDNA (differentiation induction subtraction hybridization) library identified the mda-7 cDNA consisting of 1,718 bp that encode a novel protein of 206 amino acids with a predicted Mr of 23,800 (1, 3, 11). Induction of growth arrest and terminal differentiation in human melanoma cells results in elevated expression of mda-7 (1, 3). Moreover, the level of mda-7 expression inversely correlates with human melanoma progression, with highest levels found in actively proliferating normal melanocytes and lowest levels in metastatic melanoma (3). Ectopic expression of a transfected mda-7 gene in H0–1 human melanoma cells suppresses growth without inducing terminal differentiation, suggesting that this gene is involved in growth control and indirectly contributes to the terminal differentiation process (3). Additionally, ectopic expression of a transfected mda-7 gene induces growth suppression and a reduction in colony formation in cancer cell lines of diverse origin with multiple genetic defects (11). In contrast, no overt biological response is engendered in normal human epithelial or fibroblast cells by ectopic over-expression of mda-7 (11, 12).

The present studies investigate the mechanism by which mda-7 selectively inhibits the proliferation of breast cancer cells and not normal mammary epithelial cells. Evidence is presented documenting a strong correlation between ectopic expression of mda-7 and induction of apoptosis in breast cancers and also may elucidate potentially novel and selective targets for cancer therapy.

Abbreviations: pfu, plaque-forming unit; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; HMEC, human mammary epithelial cells; HMC, high molecular weight.

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cancer cells. This process is characterized by an up-regulation of the proapoptotic effector Bax and an increase in the BAX/BCL-2 protein ratio (12, 13). A direct effect of mda-7 “gene therapy” on the growth of human tumor xenografts in nude mice also is demonstrated. On the basis of the selective breast cancer growth inhibitory properties of mda-7 and its apparent ability to distinguish and spare normal cells from growth inhibition and apoptosis, the mda-7 cDNA represents a potentially effective antigrowth agent for breast cancer gene therapy.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Growth and β-Galactosidase Assays. MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453, and T47D human breast carcinoma cell lines were obtained from the American Type Culture Collection and were cultured as recommended. Normal human breast epithelial cells included immortal HBL-100 (American Type Culture Collection) and early passage mammary epithelial cells [human mammary epithelial cells (HMEC), passage nos. 9–12] (Clonetics, San Diego). HMEC cells were grown in serum-free defined medium supplied by Clonetics. To study the effect of mda-7 on monolayer colony formation or cell growth, cells were infected with 100 plaque-forming units (pfu)/cell of Ad.mda-7 S, Ad.Vec, or Ad.β-gal, and colony formation (3 to 4 weeks) or cell growth (daily over a 14-day period, with a medium change at days 4, 7, and 10) was determined (3, 11). To evaluate the effect of mda-7 coexpression with Bcl-2 or Ad E1B, MCF-7 or T47D cells were transfected with 10 μg of an mda-7 gene cloned in a PMAM-neo vector (3), alone or in combination with 10 μg of a Bel-2 (pSFFV-Bcl-2) (14) or an Ad E1B (pCMV.E1B) (15) expression vector by using the lipofectin method (11). In situ β-galactosidase assays were performed by using standard protocols (16).

Construction and Assaying of Recombinant Adenoviruses. The recombinant replication-defective Ad.mda-7 S was created in two steps, as described for Ad.mda-7 antisense (11). Production of infectious virus in 293 cells, analysis of recombinant virus genomes to confirm the recombinant structure, plaque purification, and titration of virus were performed as described (17).

Cell Cycle Analysis. Fluorescence-activated cell sorter analysis was performed as described (18). The percentage of cells in the various phases of the cell cycle was estimated manually by gating the G1/G0, S, and G2/M regions of the histograms. The percentage of cells to the left of the G1/G0 region (the A0 region), representing apoptotic cells containing less than a diploid content of DNA, also was estimated by gating the appropriate region of the histograms.

DNA Extraction, Fragmentation Assay, and Terminal Deoxyribonucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay. DNA was extracted, and fragmentation assays were performed as described (18) 2 and 4 days after infection of cells with 100 pfu/cell of Ad.mda-7 S or Ad.Vec. A modified TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) method (19) was used to evaluate apoptosis in cells treated for the fragmentation assay.

Immunohistochemistry, Immunoprecipitation, and Western Blotting. These assays were performed as described (3, 11, 20–22). However, immunoreactivity in Western blotting assays was detected by enhanced chemiluminescence (ECL) (Amer sham).

Tumor Studies. MCF-7 cells were infected with 100 pfu/cell of Ad.mda-7 S or Ad.Vec, were incubated at 37°C for 96 hr, were resuspended at 2.5 × 10⁶ cells/ml in PBS, and were mixed 1:1 with Matrigel (Collaborative Research), and 400 μl of this suspension (1 × 10⁶ per animal) was injected s.c. into nude mice (Taconic Farms) (16, 23). Four weeks after injection, animals were killed, and the tumors were removed, were snap frozen in liquid nitrogen, and were weighed. Data are presented as tumor weight. In addition, a tumor volume ratio was calculated. This is an index of tumor progression over the course of a study (23).

RESULTS

mda-7 Selectively Induces Apoptosis in Human Breast Cancer Cells with Different p53 Genotypes. To define a potential mechanism by which mda-7 induces its selective effect on cancer versus normal cells and to define potential therapeutic applications for mda-7, we constructed replication-defective adenoviruses expressing mda-7 (Ad.mda-7 S) or, as a control, the β-galactosidase (Ad.β-gal) gene. Infection of human breast cancer cells, including MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-453, and T47D with 100 pfu/cell of Ad.mda-7 S reduced growth and colony formation in comparison with untreated cells or cultures infected with a recombinant Ad lacking the mda-7 gene Ad.CMV null (Ad.Vec) (Figs. 1 and 2 and data not shown). Infection of the same cell types with 100 pfu/cell of Ad.β-gal resulted in β-galactosidase expression in the majority of treated cells and no significant change in growth properties (Figs. 1 and 2 and data not shown). Because the different breast cancer cell lines contain either wild-type p53 (MCF-7), mutant p53 (MDA-MB-231, MDA-MB-453, and T47D), or null p53 (MDA-MB-157), these results document that the growth-inhibitory activity of mda-7 occurs independently of the mode of action of this extensively studied tumor suppressor gene that is frequently altered in human cancers.

In contrast to malignant breast tumor cells, infection of immortal normal human breast epithelial cells, HBL-100, with 100 pfu/cell of Ad.mda-7 S resulted in a similar kinetics of growth and cloning efficiency in liquid medium as found after infection with Ad.Vec or Ad.β-gal (Fig. 1). Unaltered growth kinetics was also evident in early passage normal HMEC infected with the three viruses (Fig. 1). These results extend previous observations by using the less efficient approach of DNA transfection, indicating that ectopic expression of mda-7 selectively inhibits the growth of breast cancer cells in vitro (11).

A consistent observation with many tumor cells infected with Ad.mda-7 S is a change in cellular and nuclear morphology suggestive of programmed cell death. Fluorescence-activated cell sorter analyses of DNA content were performed to determine the effects of mda-7 on apoptosis-associated DNA degradation and to explore whether alterations in cell cycle progression occur. In all of the breast cancer cell lines, a hypodiploid (A0) peak appeared or increased after infection with Ad.mda-7 S relative to infection with Ad.Vec (data not shown). This putative apoptotic response was not evident after infection of normal human breast cells, HBL-100 or HMEC, with 100 pfu/cell of Ad.mda-7 S (Fig. 2A, and data not shown). When MCF-7 and T47D cells were analyzed for MDA-7 protein by using indirect immunofluorescence with MDA-7-specific mAbs 2 days after infection with 100 pfu/cell of Ad.mda-7 S, intense nuclear staining was visible (Fig. 4A and data not shown). In contrast, none of the parameters indicative of apoptosis occurred in HMEC or HBL-100 cells after infection with 100 pfu/cell of Ad.mda-7 S. The absence of an effect in normal breast cells did not result from a failure to infect these cell types and express genes controlled by β-galacto-
sidase staining after infection with Ad. β-gal) or to produce the MDA-7 protein (Figs. 1 and 5). Immunoprecipitation analysis of [35S]methionine-labeled cell lysates from MCF-7, T47D, HBL-100, and HMEC cells with MDA-7 mAb indicated the presence of equivalent amounts of both the predicted MDA-7 protein (∼23.8 kDa) and a high molecular weight (HMC) interacting protein (∼90–110) in MCF-7, T47D, and HBL-100 cells and reduced levels of both proteins in HMEC cells (Fig. 5). These findings establish that mda-7 can induce apoptosis differentially in breast carcinomas but not in normal breast epithelial cells. Moreover, this selective apoptotic-inducing effect is not a direct consequence of differential levels of the MDA-7 protein or the HMC interacting protein in breast cancer versus normal breast epithelial cells.

**Induction of Apoptosis in Human Breast Cancer Cell Lines Correlates with an Elevation in BAX Levels.** Programmed cell death reflects a balance between signaling events and molecules that either promote or inhibit apoptosis (12, 13, 24, 25). Current data support the hypothesis that the ratio of death antagonists to agonists determines whether a cell will respond to apoptotic signals. Proteins such as Bcl-2, Bcl-XL, Mcl-1, Bcl-w, and Ad E1B (19 and 55 kDa) protect cells from specific programs of apoptosis whereas BAX, Bad, Bak, and Bcl-Xs proteins stimulate apoptosis in specific target cells (12, 13, 24, 25). We, therefore, determined by Western blotting if induction of mda-7-induced apoptosis in human breast carcinoma cells altered the expression of specific proteins associated with

**Fig. 1.** Effect of Ad. Vec, Ad. β-gal, and Ad.mda-7 S on the growth of normal breast and breast cancer cells. The indicated cell types were infected with 100 pfu/cell of Ad. Vec, Ad. β-gal, or Ad.mda-7 S, and cell growth was determined over a 14-day period. Triplicate samples varied by ≤10%. Similar results (±15%) were obtained in two additional replicate studies. In the lower panels, β-galactosidase activity was assayed in HMEC (A), HBL-100 (B), T47D (C), and MCF-7 (D) cells 48 hr after infection with the Ad. β-gal.

**Fig. 2.** Effect of Ad. mda-7 S on growth and BAX protein levels in human breast cancer cells. The experimental growth protocol was as described in the legend to Fig. 1. The breast carcinoma cell lines analyzed include MDA-MB-157 (A), MDA-MB-231 (B), and MDA-MB-453 (C). D provides immunoblot analyses of BAX expression 2 days after infection of the indicated breast cancer cell line with 100 pfu/cell of Ad. Vec or Ad.mda-7 S. Coomassie blue staining of gels indicated equal protein loading.

**Fig. 3.** Induction of nucleosomal DNA degradation in human breast cancer cells, but not in normal breast epithelial cells, infected with Ad.mda-7 S. The indicated cell types were infected with 100 pfu/cell of Ad. Vec or Ad.mda-7 S and were analyzed for nucleosomal DNA degradation 4 days after infection.
significantly higher in breast cancer cells (Fig. 6B) of BAX to BCL-2 protein ratios revealed BAX
by DMB-453) or null p53 (MDA-MB-157) (Fig. 2) cell lines containing mutant p53 (MDA-MB-231 and MDA-
of 18–21 kDa (25). Up-regulation of BAX after infection with Ad.
protein) cells after infection with Ad.
both MCF-7 (p53 wild-type protein) and T47D (p53 mutant protein) cells after infection with Ad.mda-7 S but not with Ad.Vec (Fig. 6A). Ad.mda-7 S infection of MCF-7 and T47D cells also resulted in elevated levels of processed BAX protein of 18–21 kDa (25). Up-regulation of BAX after infection with Ad.mda-7 S was also evident in additional breast carcinoma cell lines containing mutant p53 (MDA-MB-231 and MDA-MB-453) or null p53 (MDA-MB-157) (Fig. 2D). Comparison of BAX to BCL-2 protein ratios revealed BAX/BCL-2 to be significantly higher in breast cancer cells (Fig. 6B and data not shown), implicating BAX as a potential component in mda-7-induced programmed cell death. Ectopic expression of mda-7 in HBL-100 cells variably modified BAX expression, which is generally quantitatively less than that observed in breast cancer cells. Moreover, the ratio of BAX/BCL-2 was consistently lower in HBL-100 cells after infection with Ad.mda-7 S than in the breast cancer cells. In contrast, in early passage HMEC, representing the best approximation of normal breast epithelial cells, Ad.mda-7 S failed to induce the BAX protein. No consistent changes were seen in other apoptosis-modifying proteins, including BAK, BAD, BAG-1, and BCL-X, after infection of breast carcinoma or normal breast epithelial cells with Ad.mda-7 S (Fig. 6B and data not shown). On the basis of these observations, it appears that BAX may be a crucial regulator of apoptosis induced selectively in breast cancer versus normal mammary epithelial cells after ectopic overexpression of mda-7-.
Overexpression of Bcl-2 and Ad E1B proteins protects cells from apoptosis induced by diverse stimuli (13, 24, 25). This effect may be mediated by the formation of a stable complex between BAX and BCL-2 or BAX and Ad E1B proteins by heterodimerization, thereby nullifying the apoptotic-inducing effect of BAX (26–29). Because high levels of the antiapoptosis proteins BCL-2 or Ad E1B can counteract the proapoptotic signaling of BAX, studies were performed to determine whether overexpression of Bcl-2 or Ad E1B would protect breast carcinoma cells from mda-7-induced growth suppression and apoptosis. Cotransfection of MCF-7 and T47D cells with a pMAMneo-mda-7 expression construct (permitting controlled expression of mda-7 by dexamethasone and containing a neomycin resistance gene permitting colony selection in G418) (3) and a pSFFV-bcl-2 expression construct (express-
Ectopic Expression of mdm-7 in Human Breast Carcinoma Cells Inhibits Tumor Development in Nude Mice. On the basis of in vitro studies indicating selective growth-inhibitory and apoptosis-inducing effects of mdm-7 when overexpressed in cancer cells, we investigated the effect of ectopic expression of mdm-7 on tumor formation by breast cancer cells in vivo in nude mice. The breast carcinoma cell line MCF-7 was infected with 100 pfu/cell of either Ad.mdm-7 S or Ad.Vec 96 hr before implantation in nude mice. In the control Ad.Vec-infected cells, tumors developed in all groups within 7 days and grew progressively during the course of the experiment (4 weeks). In contrast, the group of animals injected with tumor cells infected with Ad.mdm-7 S exhibited a statistically significant (P < 0.01) suppression of tumor development, as defined by tumor volume and tumor weight (Fig. 8 and data not shown). Moreover, Ad.mdm-7 S also inhibited the growth of MCF-7 tumors initiated in nude mice (100–150 mm³) before a 3-week Ad.mdm-7 S therapy protocol (three weekly intratumoral injections of 1 × 10⁸ pfu/injection in 100 μl over four sites) (data not shown). These experimental findings document that Ad.mdm-7 S can inhibit tumor formation and progression directly in vivo.

FIG. 8. Effect of mdm-7 on the growth of MCF-7 cells in nude mice. MCF-7 cells were uninfected (CONTROL) or infected with 100 pfu/cell of Ad.Vec or Ad.mdm-7 S and were incubated for 4 days at 37°C. Cells were removed with trypsin, were mixed with Matrigel (1:1), and were injected into nude mice (10⁶ cells/animal). Results are shown as tumor weight in grams ± SD. Statistical significance was determined with a Student's t test using the computer program SIGMA STAT (Jandel, San Rafael, CA), and a P value of 0.05 is indicated by an asterisk. Two additional tumorigenicity studies have been performed with qualitatively similar results (data not shown).

DISCUSSION

In the present studies, we describe the antitumor activity and apoptosis-promoting properties of a cancer growth suppressor gene, mdm-7. On the basis of the previously confirmed genetic defects in the human tumor cell lines analyzed, including mutations in p53 and/or RB, it is evident that the biological activity of mdm-7 does not depend on the action of these tumor suppressor genes (11). The capacity of a cancer suppressor gene, such as mdm-7, to efficiently inhibit the growth of wild-type, mutant, and null p53 breast carcinoma cells, as well as cancer cells with additional defects (11), supports the intriguing possibility, confirmed by in vivo studies, that mdm-7 may prove efficacious in the gene-based therapy of human breast and other cancers. Moreover, because elevated expression of mdm-7 in normal cells does not elicit a deleterious effect, a problem often encountered when using conventional gene therapy approaches should be avoided.

The mechanism by which mdm-7 differentially inhibits growth and induces apoptosis in breast cancer versus normal mammary epithelial cells remains to be determined. Preliminary studies using mAbs specific for mdm-7 demonstrate an association of this protein with chromatin in cells undergoing mitosis (data not shown). Moreover, in human melanoma cells, induction of terminal differentiation by treatment with interferon β plus mezerein results in translocation of mdm-7 from the cytoplasm into the nucleus of differentiated cells. These two observations suggest that mdm-7 may be associated with chromatin remodeling, which is apparent during both mitosis and differentiation. On the basis of primary protein sequence analysis, mdm-7 does not appear to possess any nuclear localization signals, suggesting that the MDA-7 protein requires an association with a cytosolic chaperone to translocate into the nucleus (3, 11). In this context, identification of proteins that interact with mdm-7 (such as the HMC) (3, 11) and that facilitate migration into the nucleus may provide insights into the mechanism by which mdm-7 selectively suppresses malignant but not normal mammary epithelial cell growth.

The Bcl-2 gene family members are important genetic elements in maintaining homeostasis between cell survival and death. The present study demonstrates that mdm-7-induced
apoptosis in breast cancer cells is associated with the up-regulation of the proapoptotic protein BAX whereas levels of BCL-2, BCL-X1, BAK, BAD, and BAG-1 were unchanged. The observation that the cell death signal induced by MDA-7 can be counteracted by overexpression of Bel-2 or its viral homologue adenovirus E1B (12, 27, 28) is consistent with a prominent role for MDA-7-induced up-regulation of BAX in the apoptotic mechanism used by this tumor suppressor. In this regard, recent studies suggest that BAX also may function as a tumor suppressor (29–31).

Although BAX expression is regulated positively by wild-type p53 (27), the ability of mda-7 to induce BAX is clearly p53-independent, suggesting that alternative pathways can be involved in BAX up-regulation after ectopic overexpression of mda-7. At present, it is unknown whether mda-7 directly or indirectly induces BAX expression in cancer cells. However, given the lack of similarity of mda-7 to any known transcription factors, we suspect that it at least requires other cofactors. The lack of a significant biological effect of mda-7 in normal cells may involve a failure of MDA-7 protein to accumulate in the nucleus and induce the appropriate gene expression changes, such as Bax, that are necessary to inhibit cell growth and/or induce apoptosis or a combination of these effects. These possibilities are amenable to experimental testing. Defining the molecular basis of action of mda-7 is merited and should provide important insights into the role of this cancer growth suppressor gene in tumor development and progression. This information also should assist in exploiting mda-7 for cancer therapy.

Ectopic expression of mda-7 by using a recombinant adenovirus delivery approach induces growth suppression and apoptosis in human breast cancer cell lines in vitro and inhibits human breast tumor growth in vivo in nude mice. In contrast, normal breast epithelial cells do not exhibit analogous changes in cell growth or survival after ectopic overexpression of mda-7. These findings indicate that mda-7 represents a class of cancer-specific growth-arresting and apoptosis-inducing genes that may prove efficacious for the targeted therapy of breast cancer.

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