Award Number: DAMD17-03-1-0290

TITLE: Novel Therapeutic Approach for Breast Cancer

PRINCIPAL INVESTIGATOR: Devanand Sarkar, Ph.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

REPORT DATE: June 2006

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Limitations of current viral-based cancer gene therapies include lack of cancer-specific targeting and insufficient tumor delivery. To ameliorate these problems I constructed a conditionally replication competent adenovirus (CRCA) manifesting the unique properties of tumor-specific virus replication and production of a cancer-selective cytotoxic cytokine, melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24), which embodies potent bystander antitumor activity. Cancer cell selective tropism was ensured by engineering the expression of the adenoviral E1A protein, necessary for viral replication, under the control of the promoter of progression elevated gene-3 (PEG-3) which functions selectively in diverse cancer cells with minimal activity in normal cells. In the E3 region of this CRCA we introduced the mda-7/IL-24 gene, thereby mediating robust production of this cytokine as a function of adenovirus replication. Infection of this CRCA (designated Ad.PEG-E1A-md-7) in normal mammary epithelial cells and breast cancer cells confirmed cancer cell selective adenoviral replication, mda-7/IL-24 expression, growth inhibition and apoptosis induction. Injecting Ad.PEG-E1A-md-7 into human breast cancer xenografts in athymic nude mice completely eradicated not only the primary tumor but also distant tumors (established on the opposite flank of the animal) thereby implementing a cure. This dual cancer-specific targeting strategy provides an effective approach for treating primary and metastatic breast cancers.
Table of Contents

Cover ..................................................................................................................1
SF 298..................................................................................................................2
Introduction........................................................................................................4
Body....................................................................................................................5
Key Research Accomplishments.......................................................................9
Reportable Outcomes........................................................................................10
Conclusions........................................................................................................11
References.........................................................................................................12
Appendices.......................................................................................................14
Novel Therapeutic Approach for Breast Cancer (DAMD17-03-1-0290)
Devanand Sarkar

Introduction

Despite continuous advancements, the currently available treatment regimens are still inadequate to control the progression of metastatic breast cancer (1, 2) thus mandating the development of additional more effective therapeutic approaches that include gene therapy. Cancer gene therapy typically involves delivery of tumor suppressor, apoptosis-inducing or suicide genes directly into tumor cell (3). Replication incompetent adenoviral (Ad) vectors are frequently used for this purpose because they promote high-level transgene expression (3, 4). However, in most instances, engendering a discernible and significant antitumor response requires administering the Ad multiple times, which can trigger an immune response and viral clearance (5, 6). In these contexts, conditionally replication competent adenoviruses (CRCA) are currently being evaluated because of their effectiveness in killing cancer cells by replication thus requiring fewer administrations (7).

The most important facet of using a CRCA is to ensure cancer cell-specific replication so that the normal cells are spared. To circumvent the problem of tumor-cell specificity we engineered a CRCA in which the expression of adenoviral E1A gene, necessary for replication, is driven by a minimal active region of the promoter of progression elevated gene-3 (PEG-3), which functions selectively in diverse cancer cells with limited activity in normal cells (8, 9). PEG-3 was cloned as an upregulated transcript from a transformation progression rodent cancer model (10, 11) and attractively the activity of its’ promoter (PEG-Prom) was found to be significantly and often markedly higher not only in rodent but also in human cancer cells of diverse origin when compared to normal cells (8, 9, 12). The cancer cell specificity of the PEG-Prom is governed by two transcription factors, AP-1 and PEA-3 (8, 9, 12), which are overexpressed, either singly or in combination, in virtually all types of cancers (13, 14). Employing the PEG-Prom to drive GFP or luciferase via a replication incompetent Ad confirmed prominent cancer cell-specific transgene expression in human prostate and breast cancer cells as well as in malignant glioma cells (8). These observations prompted me to investigate the use of the PEG-Prom to drive expression of the E1A gene, necessary for Ad replication, to create a cancer cell-specific CRCA.

Since cancer cells are genetically and phenotypically complex and frequently harbor multiple abnormalities we reasoned that simply inducing Ad replication in a subset of tumor cells might not be adequate to ensure complete eradication of the disease, especially when compounded by the spread of neoplastic cells to multiple organs. Based on this consideration, I engineered melanoma differentiation associated gene-7 (mda-7)/interleukin (IL)-24 (mda-7/IL-24) to be simultaneously expressed from the E3 region of our CRCA. mda-7/IL-24 possesses the unique property of cancer cell-selective induction of apoptosis without harming normal cells (15, 16). mda-7/IL-24 also has immune modulatory and anti-angiogenic properties as well as potent antitumor bystander effects making it an ideal candidate for cancer gene therapy (15, 17-20). I evaluated the efficacy of the CRCA (Ad.PEG-E1A-mda-7) in vitro and in human breast cancer xenografts in athymic nude mice in vivo. Infection of this CRCA (designated Ad.PEG-E1A-mda-7) in normal mammary epithelial cells and breast cancer cells confirmed cancer cell selective adenoviral replication, mda-7/IL-24 expression, growth inhibition and apoptosis induction. Injecting Ad.PEG-E1A-mda-7 into human breast cancer xenografts in athymic nude mice completely eradicated not only the primary tumor but also distant tumors (established on the opposite flank of the animal) thereby implementing a cure. This dual cancer-specific targeting strategy provides an effective approach for treating breast and other human neoplasms with potential for eradicating both primary tumors and metastatic disease.
Results

PEG-Prom promotes Ad replication and transgene expression selectively in breast cancer cells: To test the dual cancer-specific targeting stratagem and to evaluate the relative effectiveness of Ad.PEG-E1A-mda-7, I created a series of additional Ads, including Ad.CMV-E1A-mda-7, in which viral replication is controlled by the CMV promoter and which also expresses mda-7/IL-24, and Ad.CMV-E1A and Ad.PEG-E1A, in which viral replication is controlled by the CMV promoter or the PEG-Prom, respectively. Additionally, I employed Ad.CMV-mda-7 and Ad.PEG-mda-7, replication-incompetent Ad in which the CMV or the PEG promoter drives mda-7/IL-24 expression, respectively. A replication-incompetent empty Ad, Ad.vec was used as a control. Experiments were performed in four breast cancer cell lines, MCF-7 (wt p53), T47D (mut p53), MDA-MB-157 (p53 null) and MDA-MB-231 (mut p53), and two normal immortal mammary epithelial cell lines, 184A1 and 184AA2. The functionality of these constructs was ascertained following Ad infection by monitoring protein levels of MDA-7/IL-24 and E1A, a marker for adenoviral replication, by Western blot analysis after appropriate viral infection. Western blot analysis detects multiple E1A gene products ranging from 36-50 kDa and multiple glycosylated forms of MDA-7/IL-24 protein ranging from 21-28 kDa. Employment of PEG-Prom, and not CMV promoter, allowed Ad replication (E1A expression) and MDA-7/IL-24 expression only in breast cancer cells but not in normal cells (Fig. 1).

PEG-Prom driven CRCA selectively kills breast cancer cells: Studies were next performed to establish potential selective effects on growth and viability of normal and
breast cancer cells when replication was controlled by the PEG-Prom versus the CMV promoter. In 184A1 and 184AA2 cells, infection with only Ad.CMV-E1A or Ad.CMV-E1A-mda-7, but not with Ad.PEG-E1A, Ad.CMV-mda-7, Ad.PEG-mda-7 or Ad.PEG-E1A-mda-7, induced profound growth inhibition (Fig. 2). In contrast, in all breast cancer cells, Ad.CMV-E1A-mda-7, Ad.PEG-E1A-mda-7, Ad.CMV-E1A and Ad.PEG-E1A infection resulted in significant growth inhibition. Infection with Ad.CMV-mda-7 and Ad.PEG-mda-7 also inhibited growth of the breast cancer cells. These findings indicate that the PEG-Prom allows Ad replication specifically in cancer cells, protecting normal cells from growth inhibition because of Ad replication. The observation that mda-7/IL-24 exerted no direct growth inhibitory effect on normal cells confirms the cancer cell-selectivity of this therapeutic approach.

**PEG-Prom driven CRCA selectively induces apoptosis in breast cancer cells:** To investigate the mechanism of growth inhibition, Annexin V staining assays, which permit differentiation between apoptotic and necrotic cells, were performed (Fig. 3). Infection with only Ad.CMV-E1A and Ad.CMV-E1A-mda-7 elevated the percentage of early apoptotic and late apoptotic (necrotic) 184A1 and 184AA2 cells. However, all of the Ads, except for Ad.vec, resulted in significant apoptosis in the breast cancer cell lines. Infection with the replication competent Ads resulted in predominantly necrosis as evidenced by an increase in late apoptotic cells while infection with Ad.CMV-mda-7 and Ad.PEG-mda-7 resulted in predominantly apoptosis as evidenced by an increase in early apoptotic cells.

**CRCAs eradicate primary and distant tumors:** To expand on the in vitro studies, in vivo assays were performed using nude mice containing established T47D subcutaneous xenografts on both right and left flanks. After palpable tumors of ~75 mm³ developed, in ~4-5 days, seven intratumoral injections with different Ads, 3X per week for the first week and 2X per week for an additional two weeks, were administered to the tumors on the left flank at a dose of 1 x 10⁸ pfu in 100 µl. No injections were given to the right-sided tumors. The experiment was terminated after 6 weeks with injections of Ad.CMV-E1A-mda-7 or Ad.PEG-E1A-mda-7 since tumors on both sides showed regression after only three injections and with seven injections they were completely eradicated (Fig. 4). T47D tumor xenografts grow slowly in the absence of estrogen and in our studies using male nude mice at 6 weeks, the control and Ad.vec injected tumors reached a weight of ~250-300 mg which agrees with a previous report (21). While Ad.CMV-E1A or Ad.PEG-E1A inhibited the growth of tumors on the left flank they had some inhibitory effect on tumors on the right side, which was not statistically significant. Ad.CMV-mda-7 or Ad.PEG-mda-7 eradicated tumors on the left flanks and significantly inhibited tumor growth on the right flanks. The observation that intratumoral injection of Ad.PEG-E1A-mda-7 completely eradicated the primary and distant tumors (comparable to a metastasis) provides confidence that this strategy could prove amenable for successfully treating aggressive cancers.
**PEA-3 and AP-1 confer cancer-cell selective activity of the PEG-Prom:** In the minimum effective element of the PEG-Prom (-118 to +194) there are two important transcription factor-binding sites, one for PEA-3 at −104 and another at +8 for AP-1 (Fig. 5A). These two transcription factors play an essential role in regulating PEG-Prom activity in prostate cancer cell lines and in rodent cell culture systems. Based on this consideration, the potential involvement of PEA-3 and AP-1 in regulating PEG-Prom activity was evaluated in breast cancer cell lines. PEG-Prom activity was ~5-20 fold higher in breast cancer cells than in 184A1 or 184AA2 cells. Mutation in the PEA-3 site reduced promoter activity by ~50% in all cell types. Mutation in the AP-1 site also reduced promoter activity by ~50%, while mutation in both the PEA-3 and AP-1 sites reduced activity by ~75% in all of the cell lines. To further strengthen the role of PEA-3 and AP-1 in regulating PEG-Prom activity, the relative abundance of these proteins was analyzed in breast cancer cell lines and 184A1 and 184AA2 cells. Elevated expression levels of PEA-3 and c-JUN correlated with PEG-Prom activity in the different breast cell lines, with highest activity in MCF-7 and T47D cells that contain the greatest elevation in PEA-3 as well as elevations in c-JUN. These observations provide a mechanistic explanation for the cancer cell-selective activity of the PEG-Prom in breast cancer cells.

Fig. 4. CRCAs eradicate primary and distant tumors. Subcutaneous tumor xenografts from T47D cells were established in athymic nude mice in both right and left flanks and only tumors on the left side were injected with PBS (control) or with the indicated Ad for 3 weeks (total of seven injections). A and C. Measurement of tumor volume. The data represent mean ± S.D. with a minimum of 5 mice in each group. B and D. Measurement of tumor weight at the end of the study. The data represent mean ± S.D. with at least 5 mice in each group. Qualitatively similar results were obtained in an additional study.

Fig. 5. PEA-3 and AP-1 confer cancer-cell selective activity of the PEG-Prom. A. The various cell types were transfected with the indicated plasmids and a β-galactosidase-expression plasmid and luciferase and β-galactosidase activities were measured 48 h post-transfection. The luciferase activity was normalized by β-galactosidase activity. The data represent mean ± S.D. B. The expressions of PEA-3, c-JUN and EF1-α proteins were analyzed by Western blot analyses in the indicated cells.
Training Accomplishments

2. Grantwriter’s Seminars and Workshops, Long Island University, January 6-7, 2006.
3. Training course on Adenovirus amplification and purification, Gene Therapy Center in University of Alabama at Birmingham, Dec 6-9, 2005.
Key Research Accomplishments

5. Document that PEG-Prom-driven CRCA (Ad.PEG-E1A-mdm-7) replicates only in breast cancer cells.
6. Document that PEG-Prom-driven CRCA (Ad.PEG-E1A-mdm-7) expresses MDA-7/IL-24 only in breast cancer cells.
7. Document that PEG-Prom-driven CRCA (Ad.PEG-E1A-mdm-7) inhibits cell viability and induces apoptosis selectively in breast cancer cells but not in normal cells.
8. Demonstrate that intratumoral injection of Ad.PEG-E1A-mdm-7 in established human breast cancer xenografts in nude mice eradicates not only the primary tumors but also distant uninjected tumors established on the opposite flank of the animals.
9. Demonstrate that the transcription factors PEA-3 and c-JUN (a component of AP-1) are overexpressed in breast cancer cells when compared to normal mammary epithelial cells and mediate the cancer-selective function of PEG-Prom.
Reportable Outcome

Publications:


Presentations at Scientific Meetings:


Invited Lecture:

Conclusions

In principle, viral-based gene therapies should be effective options in treating neoplastic diseases. However, in reality, these approaches have not proven as successful as originally intended or envisioned. I have currently addressed these problems and endeavored to produce a viral vector that would obviate major gene therapy hurdles of cancer-specific targeting and poor tumor distribution. To achieve these objectives I reasoned that a dual therapeutic approach resulting in cancer-specific virus replication with simultaneous production of a potent antitumor agent with profound antitumor bystander activity would culminate in an efficacious reagent for treating both primary and distant tumors. I presently describe such a virus, Ad.PEG-E1A-mda-7, that selectively replicates in tumor cells and simultaneously produces the antitumor cytokine mda-7/IL-24. When administered to human breast tumor xenografts established in one flank of a nude mouse potent antitumor activity is evident and remarkably this effect is also manifested in tumors established on the opposite flank of these animals. Moreover, this approach results in a complete eradication of tumors in animals promoting a cure of both primary and distant tumors thus mandating its evaluation in clinical trials for actual translation into the treatment of breast cancer patients.
References:


Dual cancer-specific targeting strategy cures primary and distant breast carcinomas in nude mice


Departments of *Pathology, †Urology, and ‡Neurosurgery, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, New York, NY 10032

Communicated by George J. Todaro, Targeted Growth, Inc., Seattle, WA, August 8, 2005 (received for review May 11, 2005)

Limitations of current viral-based gene therapies for malignant tumors include lack of cancer-specific targeting and insufficient tumor delivery. To ameliorate these problems and develop a truly effective adenovirus gene-based therapy for cancer, we constructed a conditionally replication competent adenovirus (CRCA) manifesting the unique properties of tumor-specific virus replication in combination with production of a cancer-selective cytokotopic cytokine, melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24), which embodies potent bystander antitumor activity. Cancer cell selective tropism was ensured by engineering the expression of the adenoviral E1A protein, necessary for viral replication, under the control of a minimal promoter region of progression elevated gene-3 (PEG-3), which functions selectively in diverse cancer cells with minimal activity in normal cells. In the E3 region of this CRCA, we introduced the mda-7/IL-24 gene, thereby mediating robust production of this cytokine as a function of adenovirus replication. Infection of this CRCA (designated Ad.PEG-E1A-mda-7) in normal mammary epithelial cells and breast cancer cells confirmed cancer cell selective adenoviral replication, mda-7/IL-24 expression, growth inhibition, and apoptosis induction. Injecting Ad.PEG-E1A-mda-7 into human breast cancer xenografts in athymic nude mice completely eradicated not only the primary tumor but also distant tumors (established on the opposite flank of the animal) thereby implementing a cure. This dual cancer-specific targeting strategy provides an effective approach for treating breast and other human neoplasms with the potential for eradicating both primary tumors and metastatic disease. Additionally, these studies support the potential use of mda-7/IL-24 in the therapy of malignant cancers.

The ultimate goal of cancer therapy is a minimally or nontoxic treatment regimen that will ensure complete eradication of both primary and metastatic cancers, thereby producing a cure for this debilitating, life-shortening, and pervasive disease. At present, achieving this objective remains elusive despite improvements in therapies for specific neoplastic conditions. Although potentially effective in early stages of the disease, the standard treatment protocols of surgery, chemotherapy, and radiotherapy, alone or in various combinations, fail short of restraining the disease, which can ultimately progress to metastasis and a failure to respond to therapy (1). These harsh realities highlight the need for alternative modalities of treatment, either alone or in combination, to effectively manage this clinical condition. Newer approaches currently being pursued include gene therapy (targeting specific defects in cancer cells, replacing defective tumor suppressor genes, or promoting cancer-specific viral replication) and immunotherapy (2–5). Conventional treatment failures raise the relevant question of what would constitute an effective cancer therapy. Clearly, a course of treatment that evokes complete destruction of tumor cells with little or no injurious effects toward normal cells represents the definitive objective of cancer gene therapy. In the present work, we describe a dual gene therapy approach using a cancer cell-selective promoter and a cancer cell-specific apoptosis-inducing cytokine gene displaying profound activity toward primary and distant breast tumors, thereby providing a valuable therapeutic for localized and metastatic cancers.

Cancer gene therapy typically involves delivery of tumor suppressor, apoptosis-inducing, or suicide genes directly into tumor cells (2). Replication incompetent adenoviral (Ad) vectors are frequently used for this purpose because they promote high-level transgene expression (6). However, in most instances, engendering a discernible and significant antitumor response requires administering the Ad multiple times, which can trigger an immune response and viral clearance. In these contexts, conditionally replication competent adenoviruses (CRCA) are currently being evaluated because of their effectiveness in killing cancer cells by replication and thus requiring fewer administrations (7, 8). Of equal importance, although antiadenovirus neutralizing Abs significantly attenuate the activity of a replication-incompetent Ad, they have limited or no effect on the activity of a replication-competent Ad (9). This finding argues that administration of a replication-competent Ad in patients with preexisting Ad immunity would still prove efficacious.

The most important facet of using a CRCA is to ensure cancer cell-specific replication, and many unique strategies have been developed to achieve this objective. One approach currently being evaluated clinically uses ONXY-015, a replication-competent Ad that propagates preferentially, although not exclusively, in p53 mutant cells (10). A similar replication-competent Ad, ONXY-411, exploits defects in the Rb pathway, a recurrent alteration in tumors (11). The rationale for using such viruses is that because of intact p53 or Rb pathways, these Ads will not replicate in normal cells, while selectively reproducing and inducing cytolysis in tumor cells containing intrinsic defects in p53 or Rb pathways. A problem confounding this approach relates to the multitude of genetic and phenotypic changes occurring in primary tumors that are commonly exacerbated during tumor progression, thereby restricting the effectiveness of these types of viruses.

To circumvent the problem of tumor-cell specificity, we engineered a CRCA in which replication is driven by a minimal active region of the promoter of progression elevated gene-3 (PEG-3), which functions selectively in diverse cancer cells with limited activity in normal cells (12). PEG-3 was cloned as an up-regulated transcript from a transformation progression rodent cancer model, and attractively, the activity of its promoter (PEG-Prom) was found to be significantly and often markedly higher not only in rodent but also in human cancer cells of diverse origin when compared with normal cells (12–15). The cancer cell specificity of the PEG-Prom is governed by two transcription factors, AP-1 and PEA-3, which are overexpressed, either singly or in combination, in virtually all types of cancers.

Abbreviations: CRCA, conditionally replication competent adenovirus; Ad, adenoviral.

†To whom correspondence should be addressed. E-mail: pbf1@columbia.edu.

© 2005 by The National Academy of Sciences of the USA
Using the PEG-Prom to drive GFP or luciferase by means of a replication-incompetent Ad confirmed prominent cancer cell-specific transgene expression in human prostate and breast cancer cells as well as in malignant glioma cells (12). These observations prompted us to investigate the use of the PEG-Prom to drive expression of the E1A gene, necessary for Ad replication, to create a cancer cell-specific CRCA.

Because cancer cells are genetically and phenotypically complex and frequently harbor multiple abnormalities, we reasoned that simply inducing Ad replication in a subset of tumor cells might not be adequate to ensure complete eradication of the disease, especially when compounded by the spread of neoplastic cells to multiple organs. Based on this consideration, we engineered melanoma differentiation associated gene-7 (mda-7/IL-24) to be simultaneously expressed from the E3 region of our CRCA. mda-7/IL-24 was cloned as a gene transcript upregulated during differentiation of melanoma cells, which possesses cancer cell-selective apoptosis inducing properties without harming normal cells in vitro, in vivo in animal models, and recently in a phase I clinical trial (20–29). Intriguingly, mda-7/IL-24 not only induces apoptosis but also has immune modulatory and antiangiogenic properties as well as potent antitumor bystander effects, making it an ideal candidate for cancer gene therapy (23, 25, 30–34). Owing to these essential qualities as a potential gene therapeutic for cancer, a replication-incompetent Ad expressing mda-7/IL-24 (Ad.mda-7) is currently being evaluated for clinical efficacy in phase II clinical trials (23, 25, 29).

The present studies focus on breast cancer, a common female cancer accounting for 32% of all cancers in women (35). It is estimated that in 2005, a total of 211,240 new cases of invasive breast cancer will be diagnosed in the United States, and the estimated death toll from all forms of breast cancer will be 40,410 (35). Because no consistently successful therapy exists for metastatic breast cancer, we evaluated the effect of Ad.PEG-E1A-mdv-7 in normal and human breast cancer cells by using in vitro cell culture and in vivo in nude mouse tumor models. We reasoned that should cancer-cell-specific efficacy be apparent in this breast cancer model, our dual targeting strategy could be of immense benefit to a significant patient population suffering from primary and metastatic forms of this disease. This possibility has now been validated, indicating that in an experimental animal model complete eradication of both primary and distant breast cancers result after administration of Ad.PEG-E1A-mdv-7. This work provides a solid foundation for developing a potentially effective virus-based therapy for patients with breast cancer, with clear relevance to additional neoplastic diseases.

Materials and Methods

Cell Lines, Culture Conditions, and Viability Assays. MCF-7, T47D, MDA-MB-157, and MDA-MB-231 breast cancer cells and human embryonic kidney (HEK)-293 cells were obtained from American Type Culture Collection and cultured as described in ref. 22. The 184A1 and 184A2A2 cells were kindly provided by Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA) and were cultured in mammary epithelial growth medium containing supplements (MEGM BulletKit, CC-3051; Biowhittaker) and transferrin (5 μg/ml) and insulin (10 mM). Cell viability was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays (26).

Construction of a CRCA. To construct the CRCA (Ad.PEG-E1A-mdv-7), the AdenoQuick cloning system from OD260 (Boise, ID) was used (36). This system uses two shuttle vectors, pE1.2 and pE3.1, in which the transgene cassettes PEG-Prom driving E1A and the CMV promoter driving mda-7/IL-24 were inserted, respectively, before being transferred into a large Ad plasmid (Fig. 1). Ad amplification, purification, titration, and infection were performed as described in ref. 37. Similar strategies were used to generate Ad.CMV-E1A-mdv-7. Ad.CMV-mdv-7 and Ad.PEG-mdv-7 were constructed as described in ref. 12.

Annexin V-Binding Assay. Annexin V binding assays were performed as described in ref. 26.

Preparation of Whole-Cell Lysates and Western Blot Analyses. Preparation of whole-cell lysates and Western blot analyses was performed as described in ref. 24. The primary Abs used were anti-E1A (1:1,000; mouse monoclonal, Upstate Biotechnology, Lake Placid, NY), anti-mdv-7 (1:2,000; rabbit polyclonal), anti-c-JUN (1:1,000; rabbit polyclonal; Santa Cruz Biotechnology), anti-PEA-3 (1:500; mouse monoclonal; Santa Cruz Biotechnology), and anti-EF1α (1:1,000; mouse monoclonal; Upstate Biotechnology).

Human Breast Cancer Xenograft in Athymic Nude Mice. T47D cells (2 × 10⁶) were injected s.c. in 100 μl of PBS in both flanks of male athymic nude mice (NCRnu/nu; 4 wk old; ≈20 g of body weight) (22, 38). After establishment of visible tumors of ≈75 mm³, requiring >4- to 5-d, intratumoral injections of different Ads were given only to the tumors on the left flank at a dose of 1 × 10⁶ plaque-forming units in 100 μl of PBS. No injection was given to the right-sided tumors. The injections were given three times a week for the first week and then twice a week for two more weeks for a total of seven injections. A minimum of five animals was used per experimental point. Tumor volume was measured twice weekly with a caliper and calculated by using the formula π/6 × larger diameter × (smaller diameter)². At the end of the experiment, the animals were killed, and the tumors were removed and weighed.

Transient Transfection and Luciferase Assays. Transient transfection and luciferase assays were performed as described in ref. 12.
In 184A1 and 184AA2 cells, infection with Ad.CMV-E1A generated E1A proteins (Fig. 2). No E1A proteins were detected in MDA-MB-231 cells, infection with all four replication-competent Ads resulted in MDA-7 expression, respectively (12). A replication-incompetent Ad, Ad.vec, was used as a control. Experiments were performed in four breast cancer cell lines, MCF-7 (wt p53), T47D (mut p53), MDA-MB-157 (p53 null), and MDA-MB-231 (mut p53), and two normal immortal mammary epithelial cell lines, 184A1 and 184AA2. The functionality of these constructs was ascertained after Ad infection by monitoring protein levels of MDA-7/IL-24 and E1A, a marker for Ad replication, by Western blot analysis after appropriate viral infection (Fig. 2). Western blot analysis detects multiple E1A gene products ranging from 36 to 50 kDa and multiple glycosylated forms of MDA-7/IL-24 protein ranging from 21 to 28 kDa. Infection of 184A1 or 184AA2 cells with Ad.CMV-E1A or Ad.CMV-E1A-mda-7, but not Ad.PEG-E1A or Ad.PEG-E1A-mda-7, resulted in production of E1A proteins; whereas in breast carcinoma cells, infection with all four replication-competent Ads generated E1A proteins (Fig. 2). No E1A proteins were detected in any cell line after infection with replication-incompetent Ads. In 184A1 and 184AA2 cells, infection with Ad.CMV-E1A-mda-7 and Ad.CMV-mda-7 resulted in MDA-7/IL-24 production (Fig. 3). Western blot analysis after appropriate viral infection (Fig. 2). Western blot analysis detects multiple E1A gene products ranging from 36 to 50 kDa and multiple glycosylated forms of MDA-7/IL-24 protein ranging from 21 to 28 kDa. Infection of 184A1 or 184AA2 cells with Ad.CMV-E1A or Ad.CMV-E1A-mda-7, but not Ad.PEG-E1A or Ad.PEG-E1A-mda-7, resulted in production of E1A proteins; whereas in breast carcinoma cells, infection with all four replication-competent Ads generated E1A proteins (Fig. 2). No E1A proteins were detected in any cell line after infection with replication-incompetent Ads. In 184A1 and 184AA2 cells, infection with Ad.CMV-E1A-mda-7 and Ad.CMV-mda-7 resulted in MDA-7/IL-24 production, whereas infection with Ad.PEG-mda-7 or Ad.PEG-E1A-mda-7 resulted in barely detectable (184A1) or very low (184AA2) levels of MDA-7/IL-24 production (Fig. 2). In breast cancer cells, infection with Ad.CMV-mda-7, Ad.PEG-mda-7, Ad.CMV-E1A-mda-7, or Ad.PEG-E1A-mda-7 generated significant MDA-7/IL-24 production. No MDA-7/IL-24 protein production could be detected in control uninfected cells or after infection with Ad.vec, Ad.CMV-E1A, or Ad.PEG-E1A. In 184A1 and MDA-MB-231 cells, MDA-7/IL-24 protein levels were reduced in comparison with the other cell types studied after infection with Ad.CMV-mda-7 and Ad.CMV-E1A-mda-7. Because a similar reduction in MDA-7/IL-24 protein levels was evident in MDA-MB-231 cells infected with PEG-Prom driven constructs, this reduced expression could reflect a difference in MDA-7/IL-24 transgene expression in these cell types. In total, these findings document that the PEG-Prom facilitates cancer cell-selective replication of Ad and mda-7/IL-24 expression.

Studies were performed next to establish potential selective effects on growth and viability of normal and breast cancer cells when replication was controlled by the PEG-Prom vs. the CMV promoter. In 184A1 and 184AA2 cells, infection with only Ad.CMV-E1A or Ad.CMV-E1A-mda-7, but not with Ad.PEG-E1A, Ad.CMV-mda-7, Ad.PEG-mda-7, or Ad.PEG-E1A-mda-7, induced profound growth inhibition (Fig. 3). In contrast, in all breast cancer cells, Ad.CMV-E1A-mda-7, Ad.PEG-E1A-mda-7, Ad.CMV-E1A, and Ad.PEG-E1A infection resulted in significant growth inhibition. Infection with Ad.CMV-mda-7 and Ad.PEG-mda-7 also inhibited growth of the breast cancer cells. These findings indicate that the PEG-Prom allows Ad replication specifically in cancer cells, protecting normal cells from growth inhibition because of Ad replication. The observation that mda-7/IL-24 exerted no direct growth-inhibitory effect on normal cells confirms the cancer cell-selectivity of this therapeutic approach (23, 28). Of interest, the effect of the various viruses was similar in all four breast cancer cell lines, suggesting that the difference in final levels of MDA-7/IL-24

**Statistical Analysis.** Statistical analysis was performed by using ANOVA, followed by Fisher’s protected least significant difference analysis. *P* < 0.05 was considered significant.

**Results**

To test the dual cancer-specific targeting strategy and to evaluate the relative effectiveness of Ad.PEG-E1A-mda-7, we created a series of additional Ads, including Ad.CMV-E1A-mda-7, in which viral replication is controlled by the CMV promoter and which also expresses mda-7/IL-24, and Ad.CMV-E1A and Ad.PEG-E1A, in which viral replication is controlled by the CMV promoter or the PEG-Prom, respectively. Additionally, we used Ad.CMV-mda-7 and Ad.PEG-mda-7, replication-incompetent Ad in which the CMV or the PEG promoter drives mda-7/IL-24 expression, respectively (12). A replication-incompetent empty Ad, Ad.vec, was used as a control. Experiments were performed in four breast cancer cell lines, MCF-7 (wt p53), T47D (mut p53), MDA-MB-157 (p53 null), and MDA-MB-231 (mut p53), and two normal immortal mammary epithelial cell lines, 184A1 and 184AA2. The functionality of these constructs was ascertained after Ad infection by monitoring protein levels of MDA-7/IL-24 and E1A, a marker for Ad replication, by Western blot analysis after appropriate viral infection (Fig. 2).
To investigate the mechanism of growth inhibition, Annexin V staining assays, which permit differentiation between apoptotic and necrotic cells, were performed (Fig. 4). Infection with only Ad.CMV-E1A and Ad.CMV-E1A-mda-7 elevated the percentage of early apoptotic and late apoptotic (necrotic) 184A1 and 184AA2 cells. However, all of the Ads, except for Ad.vec, resulted in significant apoptosis in the breast cancer cell lines. Infection with the replication competent Ads resulted in predominantly necrosis as evidenced by an increase in late apoptotic cells, whereas infection with Ad.CMV- mdad-7 and Ad.PEG-mdad-7 resulted in predominantly apoptosis as evidenced by an increase in early apoptotic cells. As observed for antiproliferative and antisuivival effects, the various viruses induced similar changes in all four breast cancer cell lines, again, suggesting that the difference in final levels of MDA-7/IL-24 protein also did not impact on apoptosis and necrosis induction in vivo (Fig. 4).

To expand on the in vitro studies, in vivo assays were performed by using nude mice containing established T47D s.c. xenografts on both right and left flanks. After palpable tumors of ~75 mm³ developed, in ~4–5 d, seven intratumoral injections with different Ads, three times per week for the first week and two times per week for an additional 2 wk, were administered to the tumors on the left flank at a dose of 1 × 10⁵ plaque-forming units in 100 μl. No injections were given to the right-sided tumors. The experiment was terminated after 6 wk with injections of Ad.CMV-E1A- mdad-7 or Ad.PEG-E1A- mdad-7 because tumors on both sides showed regression after only three injections, and with seven injections they were completely eradicated (Fig. 5). T47D tumor xenografts grow slowly in the absence of estrogen and in our studies by using male nude mice at 6 wk, the control and Ad.vec injected tumors reached a weight of ~250–300 mg, which agrees with a previous report (39). Although Ad.CMV-E1A or Ad.PEG-E1A inhibited the growth of tumors on the left flank they had some inhibitory effect on tumors on the right side, which was not statistically significant. Ad.CMV- mdad-7 or Ad.PEG-mdad-7 eradicated tumors on the left flanks and significantly inhibited tumor growth on the right flanks. The observation that intratumoral injection of Ad.PEG-E1A-mdad-7 completely eradicated the primary and distant tumors (compa-

rable with a metastasis) provides confidence that this strategy could prove amenable for successfully treating aggressive cancers.

In the minimum effective element of the PEG-Prom (−118 to +194), there are two important transcription factor-binding sites, one for PEA-3 at −104 and another at +8 for AP-1 (Fig. 6). These two transcription factors play an essential role in regulating PEG-Prom activity in prostate cancer cell lines and in rodent cell culture systems (12, 16, 17). Based on this consideration, the potential involvement of PEA-3 and AP-1 in regulating PEG-Prom activity was evaluated in breast cancer cell lines. PEG-Prom activity was ~5- to 20-fold higher in breast cancer
Discussion

In principle, viral-based gene therapies should be effective options in treating neoplastic diseases. However, in reality, these approaches have not proven as successful as originally intended or envisioned. We have currently addressed these problems and endeavored to produce a viral vector that would obviate major gene therapy hurdles of cancer-specific targeting and poor tumor distribution. To achieve these objectives, we reasoned that a dual therapeutic approach resulting in cancer-specific virus replication with simultaneous production of a potent antitumor agent with profound antitumor bystander activity would culminate in an efficacious reagent for treating both primary and distant tumors. We presently describe such a virus, Ad.PEG-E1A-mda-7, that selectively replicates in tumor cells and simultaneously produces the antitumor cytokine mda-7/IL-24. When administered to human breast tumor xenografts established in one flank of a nude mouse, potent antitumor activity is evident, and remarkably this effect is also manifested in tumors established on the opposite flank of these animals. Moreover, this approach results in a complete eradication of tumors in animals promoting a cure of both primary and distant tumors.

Why are gene therapy approaches using a CRCA not as effective in treating cancer as anticipated? This result may occur because simply having a CRCA that only replicates in cancer cells is not potent enough to treat distant tumors (metastases), and actually achieving a complete destruction of distant tumors might require an additional gene therapy component. We have examined this possibility and developed a bipartite CRCA that also expresses mda-7/IL-24, a cytokine cloned in our laboratory that displays broad-spectrum direct and bystander antitumor activity without affecting normal cells (23, 28, 30, 32). Replication-competent Ads were able to migrate to distant sites in an animal and replicate. However, although administration of a replication-competent Ad alone induced significant growth inhibition in primary tumors, no significant effect was apparent in distant tumors, indicating that at those new tumor sites replication of Ad may not be sufficiently robust to be bioactive. As such, an additional antitumor strategy combined with the replication-competent Ad would be required to provide therapeutic benefit. Administration of Ad.CMV-mda-7 or Ad.PEG-mda-7 completely eradicated the primary tumors and these Ads significantly inhibited the growth of the distant tumors, without eliciting a complete cure of these distant tumors. In contrast, the combination of Ad replication and mda-7/IL-24 expression, resulting from Ad.CMV-E1A-mda-7 and Ad.PEG-E1A-mda-7 induced complete purging of both the primary and distant tumors. The effectiveness of both Ad.CMV-E1A-mda-7 and Ad.PEG-E1A-mda-7 further strengthens the importance of using the PEG-Prom in this therapeutic strategy to protect normal cells from the adverse effects of Ad replication.

Tissue-specific and cancer cell-selective promoters can facilitate conditional Ad replication (40). The human telomerase promoter has been used successfully for this purpose (41). For breast cancer, estrogen and hypoxia-responsive promoters have been used to drive the expression of E1A (42, 43). The advantage of using the PEG-Prom in these cancer contexts is its apparent ubiquitous cancer specificity. PEA-3 and AP-1 transactivation and subsequently PEG-Prom activity are positively regulated by the ras-dependent signaling cascade (18, 19, 44). Because activation of the ras pathway is a frequent event in diverse cancers, including breast cancer, the ability of the PEG-Prom to drive transgene expression in these cancers will be robust and specific (45, 46). Additionally, PEA-3 and AP-1 are frequently overexpressed in a wide spectrum of cancers thereby promoting apparent universal cancer-specific activity of the PEG-Prom. These findings suggest that in addition to successfully treating breast cancer, Ad.PEG-E1A-mda-7 may also provide tangible benefit to an expanded patient population with additional types of cancer.

How does mda-7/IL-24 exert its potent inhibitory effects on distant tumors? Unlike a classical cytokine that acts by binding to its receptors and activating the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway, an intracellular mode of action, especially its localization in the endoplasmic reticulum, has been shown to be a primary mechanism of mda-7/IL-24-induced apoptosis when this gene is administered by Ad infection, plasmid transfection, or by means of a GST-MDA-7 fusion protein (47–49). However, mda-7/IL-24, as a secreted cytokine, also possesses potent bystander antitumor activity, which is exerted by its ability to interact with its cognate IL-20/IL-22 receptors although the signal transduction pathway(s) involved in this bystander activity is unclear (31, 32). Another potential mechanism underlying inhibition of cancer cell growth by mda-7/IL-24 in distant tumors in an in vivo context might be its activation of the immune system. mda-7/IL-24 expression is restricted to melanocytes, and those tissues associated with the immune system such as spleen, thymus, and peripheral blood mononuclear cells and its expression is induced in peripheral blood mononuclear cells upon treatment with phytohemagglutinin or LPS (23, 34, 50, 51). Treatment of peripheral blood mononuclear cells with purified recombinant MDA-7/IL-24 protein, results in the induction of IL-6, IFN-γ, TNF-α, IL-1β, IL-12, and granulocyte/macrophage colony-stimulating factor, all of which are potent immunomodulatory agents (25, 34). These secondary cytokines induced by mda-7/IL-24 might activate antigen-presenting cells to present tumor antigens, thereby triggering an antitumor immune response. Studies in a phase I clinical trial involving intratumoral injection of Ad.mda-7 (INGN 241) suggest that these in vitro effects are recapitulated in the context of patients, supporting the immune modulating properties of this cytokine (25). Although current studies were performed in athymic nude mice that are immunocompromised, these mice still have a spleen and a liver and display potent natural killer cell activity (52). Based on an intact immune system, it is hypothesized that Ad.PEG-E1A-mda-7 would prove even more effective in an immunocompetent animal. In this context, the balance between clearance of Ad by the immune system and the modulation of the immune system by mda-7/IL-24 represent major determinants in the antitumor potency of Ad.PEG-E1A-mda-7 in patients. The robust activity of this Ad suggests a need for only limited administration, which in principle will predispose the activation of the immune system promoting viral clearance. Additionally, the observation that neutralizing anti-Ad Abs do not inhibit replicating Ads indicates that Ad.PEG-E1A-mda-7 might be extremely effectual in inducing complete eradication of primary and metastatic cancers (9).

In summary, although impressive progress continues to be made in the diagnosis and therapy of organ-confined primary cancers, few if any approaches have proven universally suc-
cessful in ameliorating the negative prognosis associated with cancer progression culminating in metastasis. We presently describe an innovative dual cancer-specific targeting therapeutic approach, which obviates two fundamental limitations of current gene therapy strategies, namely confining gene expression uniquely in cancer cells and evoking a potent antitumor bystander effect, which offers promise for potentially promoting a cure for primary and metastatic cancer. Further studies are essential in established animal models of breast cancer that mimic the human disease followed by appropriate clinical trials to effectively translate this approach into a mainstream, viable therapy for primary and metastatic breast and other cancers.

This work is dedicated to Susan Goodman in her struggle to defeat breast cancer. She is an inspiration to us all. This work was supported in part by National Institutes of Health Grants CA035675, CA097318, CA098712, and P01 CA104177; Army Department of Defense Breast Cancer Fellowship Grant DAMD17-03-1-04920; the Samuel Waxman Cancer Research Foundation; and the Chernow Endowment. P.B.F. is the Michael and Stella Chernow Urological Cancer Research Scientist and a Samuel Waxman Cancer Research Foundation Investigator.
Melanoma Differentiation Associated Gene-7 (mda-7)/ Interleukin-24 (IL-24), mda-7/IL-24: Current Perspectives on a Unique Member of the IL-10 Family of Cytokines

Devanand Sarkar¹, Paul Dent² and Paul B. Fisher¹,3,4,*

¹Departments of Pathology, ³Neurosurgery and ⁴Urology, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, 630 West 168th Street, New York, NY 10032, USA; ²Department of Biochemistry, Massey Cancer Center, Medical College of Virginia, Virginia Commonwealth University, 401 College Street, Richmond, VA 23298, USA

Abstract: Developing effective and safe therapies for cancer continues to be a primary objective of both basic researchers and clinicians. However, despite evidence of progress in treating specific tumors, in many instances, especially in the context of metastasis, no effective therapies are available. A focus of our laboratories is to develop improved cancer therapeutics that exploit differences in signaling pathways and gene expression in tumor versus normal cells. An approach, which we have pioneered, is the use of 'differentiation therapy' combined with subtraction hybridization, DISH (differentiation induction subtraction hybridization), to define genes that are relevant to cancer growth control, differentiation and apoptosis. Application of DISH to human melanoma cells permitted the cloning of melanoma differentiation associated (mda) genes that display elevated expression as a function of induction of terminal differentiation and loss of tumorigenic potential in cancer cells. One mda gene, mda-7, has emerged as a potential therapeutic for cancer because of its unique ability to selectively induce apoptosis in cancer cells, without affecting normal cells. Based on structure, location and properties, mda-7, a cytokine belonging to the IL-10 family, is now designated as IL-24. mda-7/IL-24 exhibits multiple levels of anti-cancer effects that include inhibition of angiogenesis, radiosensitization and potent antitumor bystander activities. As a cytokine it also demonstrates immunostimulatory properties. An adenovirus expressing mda-7/IL-24, INGN 241, has entered the clinic and been shown to be safe and display significant activity toward solid tumors, including melanoma, in a Phase I clinical trial. We presently provide a brief overview of mda-7/IL-24.

Keywords: Novel cancer-specific apoptosis-inducing cytokine, signaling pathways, reactive oxygen species, angiogenesis, bystander antitumor activity, radiosensitization, clinical trials.

INTRODUCTION

Interleukin-24 (IL-24), a member of the IL-10 family of cytokines, was first identified and cloned as a transcript selectively upregulated during terminal differentiation of melanoma cells and was given the name melanoma differentiation associated gene (mda)-7 [1-3]. As of July 2005, a search of PubMed using the keywords mda-7 or IL-24 identifies 110 papers out of which 75 were published from 2004 onwards, although the first cloning report of mda-7 was published in 1994 [1]. This sudden surge of studies on mda-7/IL-24 is founded on its unique properties, including cancer-specific apoptosis induction, angiogenesis inhibition, radiosensitization and immune modulation that inspire both cancer biologists as well as immunologists to study the structure and function of this molecule [4-7]. A clear sign of the relevance of this novel cytokines is the fact that in only 10 years since its cloning, mda-7/IL-24 has entered into clinical trials and has shown genuine promise as a gene therapy for cancer in a Phase I trial [8, 9]. As with any novel gene, with extensive studies by diverse groups, different aspects related to the functional activity of mda-7/IL-24 are being revealed, some of which are contrasting, and the present review endeavors to analyze the compendium of studies with an unbiased perspective and to infer logical conclusions based on present data.

mda-7/IL-24: INITIAL CLONING AND CHARACTERIZATION

A hallmark of neoplastic transformation is dedifferentiation and induction of differentiation in cancer cells is one modality of therapy that has been successfully applied for the treatment of cancers, such as leukemia [10-15]. HO-1 human metastatic melanoma cells can be induced to terminally differentiate upon treatment with recombinant human fibroblast interferon (IFN)-β and the protein kinase C activator mezerein (MEZ) [2, 15-20]. Terminally differentiated melanoma cells acquire characteristics of normal melanocytes and enter into an irreversible growth arrest stage. Subtraction hybridization between actively proliferating versus terminally differentiated melanoma cells identified a plethora of genes that are induced during terminal differentiation and these genes were called melanoma differentiation associated (mda) genes [2, 20-23]. Many of the mda genes are primary IFN-β-inducible genes. However one gene product, mda-7, was found to be robustly upregulated only in IFN-β + MEZ-
treated terminally differentiated cells [3]. Expression of mda-7 mRNA was undetectable in untreated HO-1 melanoma cells. However, mda-7 mRNA expression was markedly induced by IFN-β + MEZ treatment in these cells as early as 24 hr and the expression continued to increase during terminal differentiation. Treatment of HO-1 cells with IFN-β + MEZ for 24 hr followed by growth for 72 hr in the absence of inducer resulted in a similar expression level of mda-7 as with continuous treatment, indicating that once the cells were committed to terminal differentiation mda-7 expression continues. Treatment with MEZ alone resulted in a modest but detectable increase in mda-7 mRNA expression. This upregulation involved increased stabilization of the mRNA rather than transcriptional augmentation [24]. Two transcription factor families of proteins, AP-1 and C/EBP, confer the basal transcriptional control of mda-7 gene expression [25].

The mda-7 cDNA is 1718-bp in length with an open reading frame encoding a polypeptide of 206-amino acids with a predicted size of 23.8-kDa [3]. The predicted reading frame is flanked by 5’- and 3’-untranslated sequences of 274-bp and 823-bp, respectively. The 3’-UTR contains three consensus elements (AUUUA) involved in mRNA stability and three polyadenylation signals (AAUAAA). The mda-7 gene is composed of seven exons and six introns and is located in chromosome 1q32 within an interleukin (IL)-10 related gene cluster containing four genes including IL-10, IL-19, IL-20 and mda-7 in linear order spanning 195-kb of genomic DNA (Fig. 1) [26]. Sequence analysis reveals that mda-7 has a 49-amino acid N-terminal hydrophobic signal peptide that allows the molecule to be cleaved and secreted. MDA-7 has three putative glycosylation sites at amino acids 95, 109 and 126 resulting in several molecular sizes of the secreted product. Analysis of the structure reveals that MDA-7 is a member of the four-helix bundle family of cytokine molecules with greatest homology to the IL-10 subfamily, which now includes IL-19, IL-20, IL-22 (IL-TIF) and IL-26 (AK-155) [26-31]. IL-10 and mda-7 share <20% sequence identity, however the presence of an IL-10 signature sequence in mda-7 indicates that it belongs to the IL-10 subfamily. Analysis of the expression profile of mda-7 in different human tissues shows that mda-7 expression is restricted to those tissues associated with the immune system, such as spleen, thymus and peripheral blood leukocytes, suggesting cytokine-like properties of the mda-7 molecule [26]. Based on these observations, mda-7 has been renamed IL-24 by the Human Genome Organization (HUGO) nomenclature system.

mda-7/IL-24 displays at least two distinct biological functions (Fig. 2). At low concentration, mda-7/IL-24 functions predominantly as a cytokine. However, when overexpressed at supra-physiological levels, using a replication-incompetent adenoviral vector, mda-7/IL-24 shows cancer-cell specific growth inhibitory properties without negatively impacting on normal cells.

![Fig. (1). Schematic of the structure of MDA-7/IL-24 protein and its genomic organization.](image-url)

A. Protein structure of MDA-7/IL-24. The numbers indicate amino acid residues. B. Genomic organization of mda-7/IL-24. mda-7/IL-24 is located in chromosome 1q32 within an interleukin (IL)-10 related gene cluster containing four genes including IL-10, IL-19, IL-20 and mda-7/IL-24 in linear order spanning 195-kb of genomic DNA. C. Exon-Intron organization of mda-7/IL-24. The black boxes containing the numbers indicate the exons. The arrow indicates the translation initiation site.
CYTOKINE FUNCTION OF mda-7/IL-24

The IL-10 family of cytokines signal through receptors that are dimers of an R1 type of receptor (with a long cytoplasmic domain) and an R2 type of receptor (with a short cytoplasmic domain) (Fig. 3) [32, 33]. IL-10 signals through IL-10R1 and IL-10R2; IL-20, which is involved in the proliferation of keratinocytes, signals through IL-20R1 and IL-20R2; IL-22, which mediates acute phase response in hepatocytes, signals through IL-22R1 and IL-10R2; and IL-26, the function of which is yet to be elucidated, signals through IL-20R1 and IL-10R2 [29-31, 34, 35]. Different cells were transfected with multiple combinations of IL-10/IL-20 and IL-22 receptors and then treated with conditioned media, containing secreted MDA-7/IL-24, from mda-7/IL-24-overexpressing cells. Employing this approach, it was demonstrated that IL-20R1/IL-20R2 and IL-22R1/IL-20R2 receptor complexes regulate mda-7/IL-24 signal transduction that results in the activation of signal transducers and activators of transcription (STAT), notably STAT-1 and STAT-3 [36, 37].

A potential role of mda-7/IL-24 as a cytokine and its involvement in the immune system has been highlighted by multiple studies [38-40]. mda-7/IL-24 expression could be induced in peripheral blood mononuclear cells (PBMC) upon treatment with PHA or LPS [38]. In addition, mda-7/IL-24 mRNA was detected by real-time RT-PCR in monocytes and

its expression was upregulated in monocytes by treatment with LPS or in T cells, especially in CD4^+ naive and memory cells, following activation by anti-CD3 mAb [39]. The mouse counterpart of mda-7/IL-24, FISP, was shown to have exclusive expression in Th2 lymphocytes [41]. However, in humans the expression pattern in T cells is different from that in mouse. At earlier time points (6 hr) mda-7/IL-24 expression was downregulated in cells undergoing T1 differentiation (mediating cellular immunity) and slightly upregulated in cells undergoing T2 differentiation (regulating humoral immunity). At 66 hr, the expression increased in cells committed to T1 differentiation [39]. Treatment of Peripheral Blood Mononuclear Cells (PBMC) with MDA-7/IL-24, purified from the conditioned media of a 293 cell line stably expressing mda-7/IL-24, resulted in the induction of IL-6, IFN-γ, TNF-α, IL-1β, IL-12 and GM-CSF [38]. These inductions could be blocked, either completely or partially, by simultaneous administration of IL-10. This blockage by IL-10 might be explained by the observation that IL-10 family members share their cognate receptor subunits and IL-10 shows a 10-fold higher affinity for its receptors than mda-7/IL-24 [39]. It is intriguing to note that while IL-10 and mda-7/IL-24 belong to the same family, functionally they elicit opposite effects. IL-10 is a major suppressor of the immune response and inflammation [34, 42], while mda-7/IL-24 is immunomodulatory. However, treatment with MDA-7/IL-24 does not modulate the proliferative functions.
of PBMC [38]. It was hypothesized that the secondary cytokines induced by MDA-7/IL-24 might activate antigen-presenting cells to present tumor antigens, thereby triggering an antitumor immune response [38]. MDA-7/IL-24 expression could be detected in melanocytes but not in melanomas [3, 43], indicating that loss of mda-7/IL-24 expression in progressing melanoma might contribute to the ineffective immune response to melanoma.

Stimulation of PBMC with PHA or LPS promotes mda-7/IL-24 mRNA and protein generation in CD-3 positive T cells and CD-14 positive macrophages, respectively [40]. The peak induction following these treatment protocols occurred at ~24-h post-treatment and returned to the basal level by 72-hr and the induction was mediated by augmentation of mda-7/IL-24 mRNA stability similar to what transpires during terminal differentiation. However, in a mixed lymphocyte culture system, the induction of mda-7/IL-24 expression persisted even after one week indicating that cytokines released from the lymphocytes induce mda-7/IL-24 expression [40]. Indeed, a number of cytokines, such as IL-1β, IL-2, IL-7, IL-15, TNF-α and GM-CSF, induced mda-7/IL-24 expression [40]. Interestingly, neither type I or type II interferons or IL-4 could induce mda-7/IL-24 expression. IL-2, IL-7 and IL-15 share the common cytokine receptor γc and cytokines binding to γc-containing receptors are involved in T-cell maintenance and homeostasis [44-46] indicating a potential involvement of mda-7/IL-24 in such processes.

ANTITUMOR FUNCTIONS OF mda-7/IL-24: STUDIES PERFORMED IN MELANOMA

Since mda-7/IL-24 is induced during terminal differentiation of human melanoma cells its expression pattern and biological behavior have been extensively examined in this model system [3, 26, 43, 47-51]. Interestingly, while mda-7/IL-24 mRNA expression could not be detected in melanoma cells, mda-7/IL-24 was constitutively expressed in an SV40-transformed human melanocyte cell line (FM516-SV) [3]. Immunohistochemical studies using anti-MDA-7/IL-24 antibody detected cytoplasmic MDA-7/IL-24 protein in normal human epidermal melanocytes, as well as in melanocytes in paraffin-embedded sections of normal skin and nevi [43]. However, MDA-7/IL-24 expression could not be detected in radial growth phase primary melanoma (RGP), vertical growth phase primary melanoma (VGP) or metastatic melanomas [3, 43]. These results suggest that elevated mda-7/IL-24 expression in the context of a melanocyte is still compatible with growth, whereas induction of mda-7/IL-24 expression in human melanoma cells correlates with irreversible growth arrest and terminal cell differentiation. These observations also suggest that mda-7/IL-24 might have a selective yet to be identified physiological function in melanocytes, which may not be compatible with melanoma cell growth.

To evaluate the relationship between mda-7/IL-24 expression and melanoma progression, mda-7/IL-24 and GAPDH levels were determined by comparative RT-PCR in actively growing melanocytes, primary melanomas (RGP and early VGP) and metastatic melanomas [3]. Normal melanocytes express on average 3.7-fold more mda-7/IL-24 than actively growing metastatic melanomas (p<0.001) and 2.2 fold more mda-7/IL-24 than actively growing non-metastatic primary melanomas (p<0.02). The expression of mda-7/IL-24 during melanoma progression was analyzed by ‘Matrigel-assisted’ tumorigenic growth. This approach comprises the co-injection of nontumorigenic or weakly tumorigenic RGP or early VGP primary human melanomas with Matrigel in nude mice [52]. Tumors develop rapidly in Matrigel co-injected animals and sublines can be developed that readily grow as solid tumors in secondary nude mouse re-
Although no significant change in \textit{mda-7/IL-24} expression was evident in an RGP primary melanoma cell line (WM35) before or after Matrigel selection, two independent early VGP primary human melanoma cell lines (WM793 and WM1341B) expressed less \textit{mda-7/IL-24} after Matrigel selection [3]. Immuneactivity for \textit{MDA-7} could be detected in primary melanomas [43, 48]. More intense staining was seen in the thinner tumors, whereas very light or no staining was seen in thick primary tumors [43]. In addition, both the percentage of MDA-7/IL-24-positive cells and the intensity of immunolabeling decreased significantly in the invasive portion of the tumor [48]. Immuneactivity for MDA-7/IL-24 could be detected in only 1 of 15 cases of lymph node metastasis of melanoma [43]. In a separate study, intense MDA-7/IL-24 immunoreactivity was seen in dendritic cells of germinal centers of four of the 20 lymph node metastases examined [48]. However, the relevance of this finding is presently not known. The conclusion reached from these studies is that \textit{mda-7/IL-24} expression is gradually lost during melanoma progression, thereby resulting in a growth advantage in evolving tumor cells. In these contexts, \textit{mda-7/IL-24} could be regarded as a tumor suppressor gene whose loss of expression is involved in melanoma progression.

A splice variant of \textit{mda-7/IL-24} (\textit{mda-7}x) has been identified in normal melanocytes in which exon 2 is spliced directly to exon 4 and exon 4 is spliced to exon 6 [53]. This results in a frame-shift mutation generating a protein of 63-aa residues of which only the first 14-aa residues are identical to \textit{mda-7/IL-24}. It was shown that \textit{mda-7}x physically interacts with \textit{mda-7/IL-24}. However, the functional significance of this observation is unclear since \textit{mda-7}x did not inhibit the growth inhibiting properties of \textit{mda-7/IL-24} in melanoma cells. A gradual decrease in \textit{mda-7}x expression was observed with the progression of the cells from melanocytes to metastatic melanoma. Again, the functional relevance of this observation was not explained or explored.

What happens when \textit{mda-7/IL-24} is overexpressed in melanoma cells? Transient transfection of \textit{mda-7/IL-24} in C8161 human melanoma cells provided the first demonstration of growth inhibitory properties of \textit{mda-7/IL-24} in human melanoma cells [3]. These findings were substantiated by experiments using a replication-incompetent adenovirus expressing \textit{mda-7/IL-24} (Ad.\textit{mda-7}) [43, 49, 50]. Infection with Ad.\textit{mda-7} resulted in significant inhibition of growth in melanoma cells but not in FM516-SV or normal human melanocytes [49, 50]. In a comparative study, Ad.\textit{mda-7} was as potent as Ad.\textit{p53} or Ad.\textit{p21}, a cyclin dependent kinase inhibitor that is a downstream wild-type p53 target gene, in its growth inhibitory effect on melanoma cells [49]. Analysis of programmed cell death (apoptosis) by cell cycle analysis, Annexin V staining and DNA fragmentation analysis revealed an increase in the apoptotic cell population as a function of Ad.\textit{mda-7} infection in melanoma cells, but not in FM516-SV or early passage normal human melanocytes (NHuMEL) [49]. In addition, Ad.\textit{mda-7} infection also resulted in an increase in melanoma cells in the G2/M phase of the cell cycle [43, 49]. The resistance of FM516-SV or NHuMEL to the killing effect of Ad.\textit{mda-7} is an inherent property of these cells rather than an experimental artifact since Ad.\textit{mda-7} effectively infected both cell types resulting in the production of abundant \textit{mda-7/IL-24} mRNA and protein [49]. In addition, infection with Ad.\textit{mda-7} also resulted in the secretion of MDA-7/IL-24 protein in the media by both normal and tumor cells [49]. Studies were performed to ascertain the potential involvement of apoptosis-associated genes in Ad.\textit{mda-7} induction of apoptosis in melanoma cells. Decreases were apparent in the ratio of anti-apoptotic (Bcl-2 and Bcl-xl) proteins to pro-apoptotic (Bax and Bak) proteins suggesting that this change in equilibrium between the two classes of molecules may be a major determinant of induction of apoptosis by Ad.\textit{mda-7} in human melanoma cells [49].

One interesting observation is that there is an inverse relationship between the expression levels of \textit{mda-7/IL-24}, which is downregulated, and inducible nitric oxide synthase (iNOS), which is upregulated, in melanoma. Infection of melanoma cells with Ad.\textit{mda-7} results in profound suppression in iNOS expression [47]. It has been shown that inhibition of iNOS by aminoguanidine (AMG) induces apoptosis in melanoma cells but not in normal melanocytes, underscoring the importance of nitric oxide for melanoma survival [54]. Inhibition of iNOS results in downregulation of bcl-2 and upregulation of bax, caspases and GADD45 among other apoptosis-related molecules. Understanding how Ad.\textit{mda-7} downregulates iNOS will provide additional insights into the apoptosis-inducing function of this molecule.

\textbf{\textit{mda-7 INDUCES GROWTH SUPPRESSION AND APOPTOSIS IN DIVERSE CANCER CELLS}}

Transfection with an \textit{mda-7/IL-24} expression vector documented the wide-ranging antitumor inhibitory properties of \textit{mda-7/IL-24} [55, 56]. \textit{mda-7/IL-24} induced growth suppression in a diverse array of human cancer cell lines, including breast, colon, nasopharyngeal, cervical and prostate carcinoma, melanoma, glioblastoma multiforme, fibrosarcoma and osteosarcoma [4, 55, 56]. In contrast, when \textit{mda-7/IL-24} was transfected into normal cells, including human mammary epithelial cells and skin fibroblasts, only a marginal reduction in growth was observed [55]. These results firmly established that \textit{mda-7/IL-24} functions as a growth-suppressor specifically in the context of cancer cells. The activity of \textit{mda-7/IL-24} was independent of the status of other tumor suppressor genes, such as p53, Rb or p16\textit{ink4a}, indicating that \textit{mda-7/IL-24} is capable of inducing growth-suppression in diverse cancer cell lines irrespective of their tumor-suppressor status [55, 56].

Experiments with Ad.\textit{mda-7} provided further documentation of the growth inhibitory properties of \textit{mda-7/IL-24}. Ad.\textit{mda-7} infection resulted in growth inhibition in several human breast cancer cell lines irrespective of their p53 status (wild type, mutant or null), while sparing normal human mammary epithelial cells (HMEC), immortalized human breast epithelial cells (HBL-100) and human endothelial cells [57, 58]. Analysis of the mechanism underlying the growth-inhibiting properties of Ad.\textit{mda-7} revealed that it induces apoptosis in breast cancer cell lines, as evidenced by DNA fragmentation analysis, flow cytometry and TUNEL assays [58]. Infection with Ad.\textit{mda-7} resulted in an increase in the pro-apoptotic protein Bax in breast cancer cells, but not in HMEC cells, and the ratio between Bax and anti-
apoptotic protein Bcl-2 was significantly higher in breast cancer cells [58]. No changes were apparent in the levels of additional apoptosis-modifying proteins, such as, Bak, Bad, Bag-1 or Bcl-XL, following Ad.mda-7 infection of MCF-7 (wild-type p53) or T47D (mutant p53) breast carcinoma cells. Overexpression of Bcl-2 protected breast cancer cells from Ad.mda-7 mediated apoptosis, indicating that Ad.mda-7 might exert its apoptotic effect by modulating mitochondrial functions. In addition, the cancer growth suppressive effects of Ad.mda-7 were demonstrated in vivo [56, 58]. Infection of MCF-7 cells with Ad.mda-7 prior to injection into nude mice resulted in a statistically significant suppression of tumor development, as defined by tumor volume and tumor weight [58]. Similarly, repeated injection of established human cervical carcinoma (HeLa) xenografts in nude mice with Ad.mda-7 inhibited tumor growth and disease progression [56]. These experimental findings document that Ad.mda-7 directly inhibits human tumor formation and progression in vivo in nude mice.

Additional insights into the mechanism underlying the apoptosis-inducing properties of Ad.mda-7 were obtained by studying non-small cell lung cancer cells [59]. Ad.mda-7 infection induced apoptosis and G2/M cell cycle arrest in both p53 wild type containing lung cancer cells (A549 and H460) and p53 null cells (H1299) while sparing normal human lung fibroblasts (NHFL). Interestingly, Ad.mda-7 infection resulted in a significant increase in the levels of p53 (5.4-fold), Bax (2.7-fold) and Bak (2.5-fold) proteins in A549 and H460 cells as compared to infection with a luciferase expressing adenovirus control. However, this induction was not observed in H1299 or NHFL cells and the expression level of Bcl-2 did not change in any of these cells. In cancer cell lines the induction of apoptosis was associated with activation of the caspase cascade, including cleavage of caspase-9 and caspase-3 and cleavage of PARP, a caspase-substrate. This activation of the caspase cascade occurred earlier (48 hr post-infection) in p53 wild-type cells than in p53 null cells (72 hr post-infection). In NHFL cells activation of the caspase cascade was not observed. Treatment with a pancaspase inhibitor, Z-VAD.FMK, significantly inhibited apoptosis induced by Ad.mda-7 in all the cancer cell lines. Since Ad.mda-7 could kill both p53 wild type and p53 null cells, it was concluded that mda-7/IL-24-mediated tumor cell killing occurred independently of p53, Bax and Bak expression. However, the p53-dependent pathway seen in p53 wild type tumor cells might additionally participate in apoptotic mechanisms thereby amplifying the apoptotic effects of mda-7/IL-24. This hypothesis is supported by the observation that the caspase cascade is activated earlier in p53 wild type cells than in p53 null cells. Additional experiments in DU-145 prostate cancer cells, which are Bax deficient and p53 mutated [60], showed that Ad.mda-7 also induces apoptosis in these cells [56] and activated the caspase cascade [59]. Moreover, no induction of p53 or Bak could be detected in these cells. These observations indicate that in specific cases mda-7/IL-24 can induce apoptosis in a Bax-independent manner. Cleavage of caspase 3 and caspase 9 following Ad.mda-7 infection provides further support that apoptosis is mediated by the mitochondrial (intrinsic) pathway rather than the death receptor (extrinsic) pathway.

In the mitochondrial pathway death signals result in mitochondrial activation, which initiates changes in mitochondrial membrane permeability and subsequent release of pro-apoptotic factors, like cytochrome c (cyto c), apoptosis inducing factor (AIF), second mitochondrial-derived activator of caspase (smac), HtrA2 and endonuclease G [61-68]. Cytosolic cyto c forms an essential part of the apoptosis complex “apoptosome” which is composed of cyto c, Apaf-1 and procaspase 9. Formation of the apoptosome leads to activation of caspase 9, which then processes and activates other caspases, including caspase 3, to orchestrate the biochemical execution of cells [69]. The key regulatory proteins in the mitochondria-mediated apoptotic events are Bcl-2, Bcl-xL, Bax and Bak. Bcl-2 and Bcl-xL appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thus preventing cyto c release, whereas Bax and Bak promote cyto c release from mitochondria [68, 70]. Several studies indicate that Ad.mda-7 can induce apoptosis in specific cancer cells in a Bax-dependent and Bax-independent manner as well as independent of Bak, Bcl-2 and caspase 3 [56, 57, 59]. These observations raise the relevant question as to how the mitochondrial pathway is activated by Ad.mda-7 in diverse cancer cells.

In melanoma cells, infection with Ad.mda-7 induced a maximum change in Bcl-xL expression [49]. Thus, Ad.mda-7-mediated changes in Bcl-xL levels might be important in altering the balance between pro-apoptotic and anti-apoptotic molecules resulting in induction of apoptosis. Ad.mda-7 infection also leads to cleavage of caspase 8 and Bid in lung cancer cells [71]. Activation of caspase 8 occurs through the extrinsic pathway [72]. Activated caspase 8 cleaves Bid and the truncated Bid translocates to mitochondria, induces homo-oligomerization of Bax or Bak resulting in the release of cyto c, thus establishing a cross-talk between the intrinsic and extrinsic pathways [73-76]. In these contexts, it is possible that the secreted MDA-7/IL-24 protein may activate the extrinsic pathway, which in turn activates the intrinsic pathway thereby promoting apoptosis. Further studies need to be performed to answer these questions and shed light on the mechanism of cancer-specific induction of apoptosis by Ad.mda-7.

**SIGNALING PATHWAYS MODIFIED BY AD.mda-7**

Although the ultimate end-point of Ad.mda-7 infection is the induction of apoptosis, in different cell types it induces this effect by activating different signal transduction pathways. As such, any conclusion inferred on Ad.mda-7-induced apoptosis will be dependent on the cell type employed for the study. One very interesting observation is that although mda-7/IL-24 is a secreted cytokine known to activate JAK-STAT pathway, apoptosis induced by Ad.mda-7 occurs in a JAK-STAT-independent manner [77]. Treatment with tyrosine kinase inhibitors (Genistein and AG18) and JAK-selective inhibitor (AG490) did not inhibit Ad.mda-7-induced apoptosis and Ad.mda-7 could effectively induce apoptosis in multiple mutant cell lines defective in JAK-STAT signaling. In addition, no correlation was observed between patterns of expressions of mda-7/IL-24 receptor mRNA and susceptibility to Ad.mda-7 in different cell lines. An adenovirus expressing mda-7/IL-24 without the signal peptide (Ad.Sp mda-7), that generated intracellular mda-
7/IL-24 without being secreted, showed equal effectiveness as Ad.mda-7 in inducing apoptosis in prostate cancer cells without affecting normal immortal prostate epithelial cells [78]. Following Ad.mda-7 infection, inhibition of mda-7/IL-24 secretion by non-cytotoxic doses of tunicamycin or brefeldin A demonstrated that the intracellular protein was sufficient to induce apoptosis [79]. Subcellular localization identified the intracellular mda-7/IL-24 to be located in endoplasmic reticulum (ER) indicating that mda-7/IL-24 might induce ER stress culminating in apoptosis [78, 80]. Induction of proteins associated with the stress response (unfolded protein response or UPR) such as BiP, GADD34, PP2A, XBP1 and caspase 7 and 12 was observed upon Ad.mda-7 infection [79] and mda-7/IL-24 genetically engineered to be targeted to ER, but not to the nucleus, could effectively induce apoptosis [79].

**mda-7/IL-24 AND p38 MAPK**

Pretreatment with inhibitors of diverse signaling pathways followed by Ad.mda-7 infection revealed that inhibition of p38 MAPK pathway by its specific inhibitor SB203580, abrogates the apoptosis-inducing effect of Ad.mda-7 [50, 81]. Activation of p38 MAPK is directly related to the induction of apoptosis in several model systems. In FO-1 melanoma cells, but not in FM516-SV immortalized normal melanocytes, Ad.mda-7 infection resulted in phosphorylation of p38 MAPK and its downstream target HSP27 [50]. This observation indicates that the phosphorylation of p38 MAPK might be a critical event in the cancer-selective apoptosis-inducing property of Ad.mda-7. Several studies have shown that apoptosis triggered by p38 MAPK activation is mediated by the induction of growth arrest and DNA damage inducible (GADD) family of genes that include GADD34, GADD45α, GADD45β, GADD45γ and GADD153. Overexpression of each GADD gene causes growth inhibition and/or apoptosis and combined overexpression of the GADD genes leads to synergistic or cooperative antiproliferative effects [82]. Ad.mda-7 infection resulted in marked induction of GADD153, GADD45α and GADD34 and a modest induction of GADD45γ in melanoma cell lines in a time- and dose-dependent manner, but not in normal melanocytes [50]. This induction correlated with the production of apoptosis in these cell lines. Inhibition of the p38 MAPK pathway, both with SB203580 or by an adenovirus expressing a dominant negative p38 MAPK, abrogated the induction of the GADD family of genes by Ad.mda-7 and also protected melanoma cells from apoptosis. In addition, inhibition of the GADD family of genes by an antisense approach rescued cells from Ad.mda-7-mediated cell death. SB203580 treatment also protected prostate, breast and lung cancer cells from Ad.mda-7-mediated cell death and activation of p38 MAPK following Ad.mda-7 infection has been observed in ovarian cancer cells [77, 81, 83]. These findings indicate that activation of the p38 MAPK pathway followed by induction of the GADD family of genes plays a crucial role in the cancer-selective apoptosis-inducing effect of Ad.mda-7.

A prominent effect of Ad.mda-7 infection of melanoma cells is downregulation of the anti-apoptotic protein BCL-2 [49]. Previous studies demonstrate that GADD153 functions by downregulating the bcl-2 promoter [84]. Based on these considerations, it was hypothesized that inhibition of the p38 MAPK pathway, resulting in inhibition of GADD153 induction, should prevent Ad.mda-7-mediated BCL-2 downregulation. Indeed, treatment with SB203580 prevented the downregulation of BCL-2 by Ad.mda-7, strengthening the conclusion that the p38 MAPK and GADD family of genes are essential for the killing effects of Ad.mda-7 in melanoma cells [50]. The induction of the GADD family of genes following Ad.mda-7 infection is not restricted to melanoma cells, since this induction is also observed in Ad.mda-7-infected glioblastoma multiforme [85], ovarian cancer [86] and prostate cancer cells and in pancreatic cancer cells infected with Ad.mda-7 in combination with anti-sense K-ras (unpublished data).

**mda-7/IL-24 AND PKR**

Ad.mda-7 induces apoptosis in lung cancer cells via upregulation of double-stranded RNA-dependent protein kinase (PKR) [71]. PKR is recognized as a mediator of antiviral and antitumor responses in target cells. Two dsRNA-binding domains reside in the NH2 terminus, and interaction with dsRNA or other activators modifies the conformation of PKR allowing it to undergo autophosphorylation and activation [87-89]. Once activated, PKR is able to phosphorylate a variety of target substrates, the most well characterized being eIF-2α, which can lead to the inhibition of protein synthesis, growth suppression and induction of apoptosis [90-92]. Infection with Ad.mda-7 resulted in phosphorylation of PKR and also its downstream targets eIF-2α, Tyk2, Stat1, Stat3 and p38MAPK [71]. Ad.mda-7 infection increased PKR protein but not PKR mRNA suggesting a regulation at a post-translational level [93]. Ad.mda-7 infection also produced apoptosis that was associated with activation of caspase 3, 8 and 9 and cleavage of Bid and PARP [71]. The activation of PKR appeared to be upstream of caspase activation because pretreatment with caspase inhibitors failed to block PKR phosphorylation. However, treatment with a specific serine/threonine kinase inhibitor, 2-amino purine (2-AP), blocked Ad.mda-7-mediated apoptosis-induction and also activation of PKR and eIF-2α. An involvement of PKR in Ad.mda-7-mediated apoptosis was confirmed in PKR null (-/-) mouse embryonic fibroblasts (MEF). Infection with Ad.mda-7 induced apoptosis in PKR wild type MEFs but not in PKR null (-/-) MEFs emphasizing the importance of PKR in Ad.mda-7-mediated cell death. Interestingly, a physical interaction between PKR and mda-7/IL-24 was demonstrated by co-immunoprecipitation assays using anti-PKR or anti-mda-7/IL-24 antibody [93]. Immunoprecipitation using anti-phospho-threonine or anti-phospho-serine, but not with anti-phospho-tyrosine, antibody demonstrated that in the presence of mda-7/IL-24 both PKR and mda-7/IL-24 become phosphorylated in A549 lung cancer cells. Additionally, studies performed in PKR+/+ and PKR-/- MEFs also revealed that only in PKR+/+ MEFs, which are susceptible to Ad.mda-7 mediated apoptosis, mda-7/IL-24 becomes phosphorylated indicating that the potential interaction between PKR and mda-7/IL-24 results in PKR auto-phosphorylation and phosphorylation of mda-7/IL-24. The importance of PKR interaction and phosphorylation of mda-7/IL-24 has not been demonstrated in other cell types apart from lung cancer cells [81, 93]. Therefore, the generalization of these findings in
Ad.mda-7-induced apoptosis remains to be determined. One important observation is that p38 MAPK lies downstream of PKR indicating that activation of p38 MAPK might be a critical event for Ad.mda-7 to exert apoptosis.

**mda-7/IL-24 and Reactive Oxygen Species (ROS)**

Reactive oxygen species (including singlet oxygen and hydrogen peroxide as well as free radicals such as superoxide anion and hydroxyl radicals) regulate apoptosis and cell proliferation in response to a variety of stimuli [94]. Ad.mda-7 infection produced a 3- to 5-fold increase in ROS production in prostate cancer cells but not in normal immortal human prostate epithelial cells [95]. A decrease in mitochondrial membrane potential (ΔΨm; occurring at 6–8 hr post-infection) preceded ROS production (occurring at 10–20 hr post-infection), which coincided with increased Annexin V binding, an indicator of early apoptosis, suggesting that ROS production might be a basic event mediating Ad.mda-7-induced apoptosis. Indeed, treatment with non-cytotoxic doses of anti-oxidants such as NAC or Tiron protected prostate cancer cells from Ad.mda-7-induced apoptosis, while non-cytotoxic doses of arsenic trioxide or NSC656240 (a dithiophene), known inducers of ROS production, potentiated Ad.mda-7-induced apoptosis. Treatment with cyclosporine A (CsA) or bongkrekic acid (BA) that inhibit the mitochondrial permeability transition (MPT) inhibited the decrease in ΔΨm and protected cells from Ad.mda-7-induced apoptosis while PK11195, an inducer of MPT, potentiated these events. Treatment with NAC or z-VAD.fmk, a pan-caspase inhibitor, blocked Ad.mda-7-induced apoptosis, but did not inhibit the decrease in ΔΨm indicating that Ad.mda-7 facilitates mitochondrial changes prior to ROS generation in a caspase-independent manner [95]. Bcl-2 and Bcl-xL, anti-apoptotic proteins that are involved in regulation of mitochondrial function and protection from apoptosis, differentially protect prostate cancer cells from apoptosis induced by Ad.mda-7 [96]. Overexpression of Bcl-xL in DU-145 and PC-3 cells and Bcl-2 in LNCaP cells, but not *vice versa*, provided protection from apoptosis and inhibit the decrease in ΔΨm and generation of ROS following Ad.mda-7 infection [96]. Studies performed in lung cancer cells showed that Ad.mda-7 infection resulted in cytochrome c release followed by induction of apoptosis without changes in ΔΨm [97]. This study probably missed the window of ΔΨm changes since it was measured 48 hr after Ad.mda-7 infection.

The importance of ROS generation in apoptosis induced by Ad.mda-7 was confirmed in pancreatic cancer cells [98]. Pancreatic cancer cells are inherently resistant to Ad.mda-7-induced apoptosis [99]. However, non-cytotoxic doses of ROS inducers such as As2O3, dithiophene (NSC656240) or retinamide (HPR) rendered these cells susceptible to Ad.mda-7-induced apoptosis [98]. Treatment of pancreatic cancer cells stably expressing mda-7/IL-24 mRNA with these agents induced apoptosis at concentrations not affecting growth of the parental cells. These agents also augmented Ad.mda-7-induced ROS production and helped overcome the translational block of mda-7/IL-24 expression in mutant and wild type K-ras-expressing pancreatic cancer cells.

Renal cell carcinoma (RCC) cells are resistant to Ad.mda-7-induced apoptosis because they lack coxsackie virus and adenovirus receptor (CAR) that facilitates adenoviral infection [100]. However, plasmid-mediated transfection of mda-7/IL-24 or treatment with mda-7/IL-24 fused with GST (GST-MDA-7) significantly inhibited growth of RCC cells [100]. A synergism was observed between GST-MDA-7 and As2O3 in inducing RCC cell killing, which could be inhibited by NAC. Combination of GST-MDA-7 and As2O3 induced apoptosis and activated p38 MAPK and JNK1/2 in RCC cells. All these studies in multiple cell types indicate that ROS generation and p38 MAPK activation might be central events in mediating Ad.mda-7-induced apoptosis. The molecular mechanisms by which ER-localized mda-7/IL-24 promotes these strategic events are future challenges that will help unravel the complexities of mda-7/IL-24 action.

**mda-7/IL-24 and β-catenin and PI3K pathway**

Microarray studies revealed that various members of the β-catenin and PI3K pathways were differentially regulated upon Ad.mda-7 infection in lung cancer cells [81]. Ad.mda-7 infection, but not Ad.p53 infection, resulted in nuclear/cytoplasmic to plasma membrane translocation of β-catenin without altering its steady state level in breast and lung cancer cells, but not in human umbilical vein endothelial cells (HUVEC). This translocation occurred within 24 hr of infection and before the induction of apoptosis assessed by Annexin V staining. A correlate to these findings was that Ad.mda-7 infection inhibited β-catenin mediated transcriptional activation employing the TopFlash/FopFlash system that includes the TCF/LEF promoter. E-cadherin expression was increased following Ad.mda-7 infection, which decreased cell migration and increased cell adhesion. Two negative regulators of β-catenin, the tumor suppressors APC and GSK-3β, were upregulated by Ad.mda-7 infection. However, no physical interaction of mda-7/IL-24 with β-catenin, APC or GSK-3β was observed. In these contexts, the mechanism behind these potential gene changes and mda-7/IL-24 action remains to be determined.

Ad.mda-7 infection in breast and lung cancer cells, but not in HUVEC, downregulates proto-oncogenes of the PI3K pathway, such as PI3K (p85), FAK, ILK-1 and PLCγ, and upregulates the PI3K inhibitor PTEN [81]. These studies demonstrate cancer-specific gene expression changes evoked by Ad.mda-7 in specific cancer cells and provide indirect evidences of inhibition of the β-catenin and PI3K pathways in mediating Ad.mda-7-induced apoptosis. Further stringent genetic studies employing siRNA or overexpression approaches need to be performed to confirm these intriguing but preliminary observations.

Contrasting results have been documented regarding susceptibility of pancreatic cancer cells to Ad.mda-7 infection. While our studies identified pancreatic cancer cells to be resistant to Ad.mda-7, another report showed that Ad.mda-7 infection could elicit apoptosis in these cells [101]. This difference may reflect variations in the viral titers and/or vector constructs employed by the two groups. Upregulation of APC, GSK-3β and PTEN and downregulation of β-catenin were observed in pancreatic cancer cells upon high titers.
Ad.mda-7 infection suggesting a potential involvement of inhibition of the β-catenin and PI3K pathways in mediating Ad.mda-7-induced apoptosis [101]. Unfortunately, additional experiments were not performed to validate these observations.

**mda-7/IL-24 AND FAS-FASL**

Studies using a single ovarian carcinoma cell line MDAH2774 revealed a novel signal transduction pathway of Ad.mda-7-mediated killing [83]. While activation of PKR, p38 MAPK and JNK was observed 48 hr after Ad.mda-7 infection in MDAH2774 cells, but not in normal ovarian epithelial cells, activation (phosphorylation) of c-Jun and ATF2 could be observed at 24 hr post-infection. c-Jun and ATF2 are involved in activation of AP-1 and NF-κB pathways and Ad.mda-7 infection augmented AP-1 and NF-κB activity [83]. It was observed that Fas and FasL have AP-1 and NF-κB binding sites in their promoters. These molecules were induced following Ad.mda-7 infection and the Fas promoter was activated by Ad.mda-7 infection. A dominant negative 1xB inhibited Fas induction by Ad.mda-7. The activation of Fas-Fasl signaling pathway by Ad.mda-7 infection was documented by down-regulation of FLICE-like inhibitory protein (FLIP), cleavage of Bid, cytochrome c release from the mitochondria and activation of caspase-8. Apoptosis induced by Ad.mda-7 in MDAH2774 cells could be inhibited by a dominant negative FADD, siRNA for Fas or FasL neutralizing antibody. These findings suggest that activation of NF-κB resulting in activation of Fas-Fasl signaling cascade mediates apoptosis in MDAH2774 cells following Ad.mda-7 infection [83]. However, a separate study using 293 cells stably overexpressing mda-7/IL-24 showed that mda-7/IL-24 alone did not induce NF-κB activation, but it augmented TNF-α-induced NF-κB activation and downstream gene expression and protected the cells from TNF-α-induced apoptosis [102]. These studies were performed in a single clone of 293-mda-7 cells and these findings might therefore represent clonal bias rather than a general phenomenon.

A separate study using four different ovarian cancer cells, OV-4, SKOV3, HEY and SKOV3.ip1,1 revealed different levels of susceptibility of these cells towards Ad.mda-7 infection [103]. While 20 and 23% of OV-4 and SKOV3 cells were apoptotic, only 10% and 12% of HEY and SKOV3.ip1 cells were apoptotic following Ad.mda-7 infection. Apparently, there is some inherent resistance of ovarian cancer cells to Ad.mda-7-induced apoptosis, which could be overcome by retargeting Ad.mda-7 to CD40 or EGF receptors. However, this study did not analyze the signaling pathway of Ad.mda-7-mediated apoptosis, thereby precluding validation of the findings of Gopalan et al. Our recent findings using the same four ovarian cancer cells indicate the importance of p38 MAPK activation and GADD gene induction in Ad.mda-7-mediated apoptosis induction [86].

**mda-7/IL-24 AND PANCREATIC CANCER**

An interesting model to study the signaling mechanism of Ad.mda-7-mediated apoptosis is pancreatic cancer. Pancreatic cancer is a complex disease in which multiple subsets of genes undergo genetic change, either activation or inactivation, during tumor development and progression [104, 105]. A frequent genetic alteration in pancreatic cancer involves activation of the K-ras oncogene (85-95% cases). Infection with Ad.mda-7 alone, at doses effective in inducing apoptosis in the vast majority of tumor cells, does not kill pancreatic cancer cells either with mutK-ras or wrtK-ras [99]. Pancreatic cancer cells (both mutK-ras and wrtK-ras) have abundant CAR and upon Ad.mda-7 infection mda-7/IL-24 mRNA can be detected. However, this mRNA is not translated into protein and therefore the pancreatic cancer cells are resistant to apoptosis-induction by Ad.mda-7. In contrast, MDA-7 protein can be detected in the cell lysates of mutK-ras pancreatic cancer cells following treatment with the combination of Ad.mda-7 and antisense (AS) K-ras phosphorothioate oligodeoxynucleotides (PS ODN) [99]. This combinatorial treatment also results in significant growth inhibition and apoptosis only in mutK-ras pancreatic cancer cells. Similarly, a combination of Ad.mda-7 with MAPK inhibitors or siRNA for MEK1 or MEK2 and a bipartite adenovirus expressing mda-7 and K-ras antisense induce apoptosis in mutK-ras pancreatic cancer cells (unpublished data).

Possible insights into the mechanism underlying this synergy come from a recent study by Rajasekhar et al. documenting that Ras and Akt signaling pathways exert profound affects in defining which subsets of mRNAs associate with polysomes and become translated into protein [106]. Moreover, a surprising observation was that blocking Ras or Akt signaling only modestly affected mRNA levels of genes when monitoring total cellular RNA, whereas the levels of polysome associated mRNAs were greatly altered following Ad.mda-7 infection [99]. It was documented by down-regulation of FLICE-like inhibitory protein (FLIP), cleavage of Bid, cytochrome c release from the mitochondria and activation of caspase-8. Apoptosis induced by Ad.mda-7 in MDAH2774 cells could be inhibited by a dominant negative FADD, siRNA for Fas or FasL neutralizing antibody. These findings suggest that activation of NF-κB resulting in activation of Fas-Fasl signaling cascade mediates apoptosis in MDAH2774 cells following Ad.mda-7 infection [83]. However, a separate study using 293 cells stably overexpressing mda-7/IL-24 showed that mda-7/IL-24 alone did not induce NF-κB activation, but it augmented TNF-α-induced NF-κB activation and downstream gene expression and protected the cells from TNF-α-induced apoptosis [102]. These studies were performed in a single clone of 293-mda-7 cells and these findings might therefore represent clonal bias rather than a general phenomenon.

In addition to its direct effect on cancer cells resulting in apoptosis, Ad.mda-7 also decreases tumor growth by inhibiting angiogenesis. Although Ad.mda-7 does not have any deleterious effects on the growth of normal human vascular endothelial cells (HUVEC), infection with Ad.mda-7 inhibits differentiation and tube formation by HUVEC in vitro [107]. Ad.mda-7 infection also downregulates the expression of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β mRNA, known modulators of angiogenesis, in human lung cancer cells. Subcutaneous tumor xenografts in nude mice injected with Ad.mda-7 had low levels of CD31 staining, which is a marker highly expressed by neovascular endothelial cells [107]. Similar findings were observed using MDA-7/IL-24 protein affinity purified from culture supernatant of HEK-293 cells stably expressing mda-7/IL-24 [108]. MDA-7/IL-24 inhibited HUVEC differentiation and migration, but not proliferation. These effects appeared to be directly mediated by MDA-7/IL-24 and were independent of IFN-γ or IP-10. Addition of blocking antibody to IL-22R1 protected HUVEC from the inhibitory activity of MDA-7/IL-24 on tube formation indicating that MDA-7/IL-24 exerted its effects via interaction with its receptors resulting in STAT3 activation. However, the re-
requirement of STAT3 activation for inhibition of tube forma-
tion was not confirmed by direct assays, such as using siRNA targeting of STAT3. In an interesting set of experi-
ments, xenografts of A549 human lung cancer cells were estab-
lished in nude mice in the lower right flank and after the estab-
ishment of the tumors either parental 293 cells or 293- mda-7 cells were implanted in the upper right flank [108]. A significant reduction in tumor growth was observed only with 293-mda-7 implantation and these tumors showed low levels of CD31 staining. Since A549 cells do not contain mda-7/IL-24 receptors [109], it can be inferred that MDA-
7/IL-24 systemically inhibited tumor growth by inhibiting angiogenesis rather than affecting the tumor cells directly. These results confirm that Ad.mda-7 can induce cancer cell growth inhibition by multiple pathways, including induction of apoptosis and suppression of angiogenesis, further docu-
menting the potential of mda-7/IL-24 for cancer gene ther-
apy.

Bystander Activities of mda-7/IL-24

Infection with Ad.mda-7 induces apoptosis predominantly employing an intra-cellular mode of killing most likely by eliciting ER stress [78, 79]. However, current studies are describing the receptor-dependent apoptosis-inducing properties of secreted MDA-7/IL-24 protein – the ‘bystander effect’. The first evidence of a ‘bystander effect’ came from our studies in pancreatic cancer cells [99]. Transf-
fection of pancreatic cancer cells with a K-ras antisense ex-
pression vector followed by infection with Ad.mda-7 in vitro resulted in a dramatic reduction in cloning ability in vitro and a complete loss of tumorigenic potential following in-
fec tion of combination-treated cells into athymic nude mice. Since this combination treatment resulted in an estimated maximum of ~8% of cells receiving both molecules, that is, 100% delivery of mda-7/IL-24 with Ad.mda-7, a maximum ~3-4% transfection of cells with the K-ras antisense gene and potentiation of transfection following adenovirus infec-
tion (hence ~8% transfection efficiency), whereas no tumors developed, we evoked the hypothesis that production and secretion of MDA-7/IL-24 protein (and/or potentially other gene products) by cells receiving both agents (presumptive 8% of the treated population) resulted in a potent ‘bystander antitumor effect’ in the adjacent tumor cells. Similar indirect evidence of a ‘bystander effect’ of MDA-7/IL-24 was also observed in non-small cell lung carcinoma cells H1299 [57]. Upon Ad.mda-7 infection, confocal microscopy revealed that the majority of cells expressing MDA-7/IL-24 were undergoing apoptosis. However, a fraction of cells that did not express MDA-7/IL-24 also showed apoptotic changes indicating that these cells became apoptotic either due to unde-
tectable levels of intracellular MDA-7/IL-24 or because of a ‘bystander effect’ produced by MDA-7/IL-24 expressing cells.

The indirect evidences of the bystander properties of MDA-7/IL-24 have now been confirmed by using purified proteins or conditioned media from Ad.mda-7-infected cells [51, 109]. Normal immortalized human cells, such as mel-
ocytes (FM-516-SV), astrocytes (PHFA-IM) or prostate epithelial cells (P69), were infected with Ad.mda-7 and then overlaid with soft agar containing prostate cancer cells [109]. While the normal cells were unharmed upon Ad.mda-7 in-
fection, the secreted MDA-7/IL-24 expressed from these cells significantly inhibited the anchorage-independent growth of the prostate cancer cells. Cocultivation experi-
ments, in which different normal cells, infected with Ad.mda-7, were mixed with non-infected HeLa-GFP cells, revealed apoptosis induction in GFP-positive cells. However, similar experiments using Ad.SP mda-7, that expresses non-
secreted MDA-7/IL-24, did not induce apoptosis in HeLa-
GFP cells indicating that it is the secreted MDA-7/IL-24 that is responsible for eliciting the bystander effects. Co-
cultivation of Ad.mda-7-infected P69 cells with DU-145, BxPC-3 or A549 cells inhibited the invasion ability of DU-
145 and BxPC-3 cells, but not of A549 cells that lack mda-
7/IL-24 receptors indicating that the bystander activity of mda-7/IL-24 is mediated via its cognate receptors. Condi-
tioned media from Ad.mda-7-infected PHFA-IM cells activ-
ted STAT3 in DU-145 and BxPC-3 cells, but not in A549 cells [109]. Purified MDA-7/IL-24 protein activated STAT3 in pancreatic cancer cells, but not in A549 cells, and induced apoptosis in pancreatic cancer cells and melanoma cells, but not in A549 cells [51]. Further confirmation of the receptor-
dependence of this effect was obtained by using anti-MDA-
7/IL-24, anti-IL-20R1 and anti-IL22R1 antibodies all of which inhibited MDA-7/IL-24-induced apoptosis in pancre-
atic cancer cells [101]. Support for a direct biological effect of mda-7/IL-24 protein in pancreatic cancer cells comes from the recent observation that treatment with purified GST-MDA-7 fusion protein induces apoptosis in normally resistant pancreatic cancer cells [80].

An interesting recent study demonstrates the bystander activity of mouse mda-7/IL-24 (mIL-24) [110]. Tumors were established either subcutaneously or in the spleen of mice using mouse hepatoma ML-1 cells that resulted in metastasis in the liver. Plasmids expressing mouse mIL-24 were deli-
vered to the quadriceps muscle of these mice via electropora-
tion. In the subcutaneous model, ML-1 cells formed tumors on the backs of animals 2 months after injection, at which point mIL-24 was administered. On day 140, after injection of mIL-24, 60% of the mIL-24-injected animals and 0% of the control animals survived. In the metastatic hepatoma model, mIL-24 was injected 10 days after establishment of the ML-1 tumor. On day 50, 90% of the mIL-24-treated mice and 40% of the control mice survived. mIL-24 inhibited angiogenesis in the tumors as revealed by CD31 staining. In-
terestingly, while mIL-24 treatment induced a 50% reduction in the liver tumor mass in comparison to the control liver, it did not have significant effect on tumor growth in the spleen, which is the organ the tumor cells were originally inoculated. It is not clear why mIL-24 could inhibit the metastatic tumor growth in the liver but not the primary tumor established in spleen. This differential effect might reflect a concentration-
dependent gradient of mIL-24 required for biological activity, in which a defined threshold dose is required to inhibit growth, and this level is achieved in the liver, but not in the spleen.

Our recent studies in animal models provide further proof of the ‘bystander activity’ of mda-7/IL-24 (Fig. 4) [111]. Subcutaneous xenografts from the human breast carcinoma cell line T47D were established on both the right and left flanks of athymic nude mice and only the tumors on the left side were injected with Ad.mda-7. Of therapeutic relevance,
shown to activate JNK1/2/3 signaling, potentially [112]. Ionizing radiation, in a variety of cell types, has been noted mediating the radiosensitization properties of Ad. mda-7. Activation of the JNK pathway plays an important role [115-117], which is mediated by suppression of angiogenesis [114]. The radiosensitization properties of primary nude mouse tumor-maintained human glioblastoma tumors [112, 113]. Recent studies have extended this observation to treatment of rodent and human malignant glioma cell lines [112].

RADIOTHERAPY IS A STANDARD ADJUVANT THERAPY FOR CANCER TREATMENT

Radiotherapy is a standard adjuvant therapy for cancer treatment. Regrettably, cancers acquire radio-resistance with progression. Fortunately, one unique aspect of mda-7/IL-24 is an ability to overcome this resistance, thereby sensitizing tumor cells to radiation. Ad.mda-7 infection greatly enhanced growth inhibition and apoptosis induction in malignant human glioma cell lines exposed to radiation, irrespective of p53 status and concurrent with upregulation of the GADD genes [85]. Heterogeneity of p53 status is common in the clinical settings of malignant glioma, where tumor cells with a wild type and a mutant p53 genotype can be found within the same cancer, making this observation particularly relevant from a gene therapy viewpoint. Work by Yacoub et al. has extended these findings to show a greater than additive efficacy of combined Ad.mda-7 and radiotherapy in treatment of rodent and human malignant glioma cell lines [112, 113]. Recent studies have extended this observation to primary nude mouse tumor-maintained human glioblastoma multiforme cells [114]. The radiosensitization properties of Ad.mda-7 have also been described for lung cancer cells [115-117], which is mediated by suppression of angiogenesis. Activation of the JNK pathway plays an important role in mediating the radiosensitization properties of Ad.mda-7 [112]. Ionizing radiation, in a variety of cell types, has been shown to activate JNK1/2/3 signaling, potentially via the generation of ceramide [118]. In some cell types, e.g., lung carcinoma cells, Ad.mda-7, as a single agent, has been shown to cause JNK1/2 activation [81, 115, 119], whereas in other cell types, e.g., glioma, breast and prostate carcinoma, Ad.mda-7 and purified MDA-7/IL-24 protein showed little or no discernable effect in activating this pathway [77, 100, 112]. In glioma, breast and prostate carcinoma cells, however, radiation and Ad.mda-7 induces prolonged activation of the JNK1/2/3 pathway [85, 112-115, 120]. Inhibition of JNK1/2/3 signaling using the relatively specific inhibitor SP600125 or by a dominant negative JNK abolished the radiosensitization properties of Ad.mda-7 and GST-MDA-7 in these cell types.

The ability of radiation to enhance the efficacy of mda-7/IL-24 to induce growth suppression and apoptosis in a cancer-specific manner represents a significant means of increasing the therapeutic index of mda-7/IL-24. Recent studies indicate that radiation can be combined with Ad.mda-7 to induce apoptosis in both parental prostate cancer cells and prostate cancer clones displaying inherent resistance to mda-7/IL-24 because of overexpression of Bcl-2 or Bcl-xL [120]. These observations provide further impetus for the combinatorial use of radiation and mda-7/IL-24 to target cancer cells for death, including those cancer cells that may display resistance to either agent used alone.

CLINICAL TRIALS USING AD.mda-7

The extensive provocative observations relative to the pleiotropic cancer-specific effects of mda-7/IL-24 in in vitro systems and animal models, paved the way for initiation of Phase I clinical trials using Ad.mda-7, designated INGN 241, in twenty-eight patients with multiple solid tumors, such as melanoma, squamous cell carcinoma of head and neck. Ad.mda-7 not only inhibited the growth of the left sided tumors that were injected but also significantly inhibited the right-sided non-injected tumors. These studies suggest that in a clinical setting mda-7/IL-24 may be equally effective in inhibiting primary and metastatic tumors by its direct growth inhibitory properties and bystander properties.

Fig. (4). Conditionally replication competent adenoviruses (CRCA) expressing mda-7/IL-24 inhibits both primary and distant tumors. Subcutaneous tumor xenografts from T47D breast cancer cells were established in athymic nude mice in both right and left flanks and only tumors on the left side were injected with PBS (control) or with the indicated Ad for 3 weeks (total of seven injections). Measurement of tumor volume. The data represent mean ± S.D. with a minimum of 5 mice in each group. Ad.CMV-E1A-mda-7 and Ad.PEG-E1A-mda-7: viral replication is controlled by the CMV promoter and the PEG-Prom, respectively and also expresses mda-7/IL-24; Ad.CMV-E1A and Ad.PEG-E1A: viral replication is controlled by the CMV promoter or the PEG-Prom, respectively. Ad.CMV-mda-7 and Ad.PEG-mda-7: replication-incompetent Ad in which the CMV or the PEG promoter drives mda-7/IL-24 expression, respectively; Ad.vec: replication-incompetent empty Ad.
neck, lymphoma, hepatoma and carcinomas of breast, colon, lung, adrenal gland, bladder, parotid, lip, kidney and penis, by intratumoral injections [4, 5, 8, 9]. The patients were stratified into eight cohorts according to viral dose (2 × 10^10 to 2 × 10^12 viral particles), time of post-treatment biopsy (24 h to 30 days) and treatment mode (single dose, divided dose or multiple injections [8, 9]. The first 3 cohorts of patients received 2 × 10^10, 2 × 10^11 and 2 × 10^12 viral particles (vp), respectively and biopsy was taken 24 h post injection [8]. The fourth and fifth cohort of patients received 2 × 10^12 vp and biopsy was taken 48 and 96 h post-injection, respectively. The sixth cohort received 2 × 10^12 vp in divided doses and biopsy was taken 48 h post-injection. The seventh cohort received 2 × 10^12 vp and biopsy was taken on day 30 post-injection and the eighth cohort received multiple doses (2 × 10^12 vp twice weekly for three weeks) an biopsy was taken on day 30 post-injection. In all the patients, including those in cohort 8, the adverse events were mild and only one patient in cohort 8 showed grade 3 serious adverse event (SAE) involving fatigue and was removed from the study [9].

Common toxicities related to INGN 241 included injection site pain and fever (<grade 2). Fever generally occurred within 24 h of injection and was observed more frequently at higher doses. All fevers resolved within 48 h of injection. No other significant short-term or long-term toxic effects were identified.

INGN 241 vector DNA and mRNA copies were detected in all injected lesions. Expression was highest near the injection site and decreased toward the perimeter of the tumor [9]. Protein expression of MDA-7/IL-24 determined by immunostaining parallel DNA expression [8]. Apoptosis evaluated by TUNEL assay closely correlated with the expression pattern of the MDA-7/IL-24 protein [8, 9]. These results convincingly demonstrate correlation of delivered DNA with expressed mRNA/protein and the intended functional effect (apoptosis).

In the first six cohorts the injected lesions were excised between 24 and 96 h after injection, therefore no conclusion about clinical activity could be drawn [9]. No patients in cohort 7 (injected tumor excised on day 30) demonstrated evidence of response. However, of the patients receiving multiple injections (cohort 8), two of the seven patients achieved clinically significant responses (>50% shrinkage of injected lesion) to INGN 241. One patient with refractory metastatic melanoma had a 2 × 2 cm supraclavicular nodule injected. Within the first week a partial response was achieved. Local regional erythema and warmth developed within 24 h of injection, but regression was not observed until day 5. As the erythema resolved regression continued to the point at which no palpable disease was identified. However, evidence of systemic activity was not observed. After the first week a second lesion was injected on the same patient. The lesion was on the dorsum of the right hand. Maximum diameter of the second lesion was also 2 cm and within the first week again complete regression was demonstrated. This time the site of injection was resected and microscopically a marked inflammatory lymphoplasmacytic infiltrate was observed throughout the residual nodule. There was extensive necrosis and no viable tumor cells. A third lesion located in the anterior right thigh in the same patient was injected. However, the third lesion despite undergoing a minor response did not fulfill criteria for a partial response. This patient remained alive with continued detectable disease at uninjected sites 491 days after initial injection. No further treatment was administered. A less dramatic response was seen in another patient with squamous cell carcinoma of the penis in which one of several skin nodules was injected. The injected nodule had a maximum diameter of 3 cm. It underwent a significant central necrotic response satisfying criteria for a partial response. However, other regionally located nodules did not show evidence of response and within several months following the initial course the perimeter of the injected lesion and several uninjected lesions progressed [9].

The immunostimulatory activity of mda-7/IL-24 was demonstrated in the clinical trials [8]. Transient increases in circulating cytokines, such as IL-6, IL-10 and TNF-α, were observed with INGN 241 injection. Significantly higher elevations of IL-6 and TNF-α were observed in patients receiving repeat treatment who demonstrated evidence of activity. The majority of patients also showed a marked increase in CD3^+ and CD8^+ T cells at day 15 following injection, suggesting that INGN 241 may be associated with a TH1 response. At the molecular level, β-catenin expression was reduced in six of nine patients and iNOS expression was reduced in four of nine patients tested. In conclusion, these initial clinical studies reveal that INGN 241 (Ad.mda-7) is well tolerated when administered via intratumoral injection and repeat dosing with 2 × 10^{12} viral particles per injection could be utilized in subsequent clinical investigations via intratumoral injection.

**NEW DEVELOPMENTS IN mda-7/IL-24 DELIVERY APPROACHES**

The overwhelming evidences regarding the potent antitumor functions of mda-7/IL-24 are inspiring endeavors to develop approaches that further augment its cancer-specific effects. Nanoparticle-mediated delivery of mda-7/IL-24 expression plasmid by the intravenous route significantly inhibited lung metastasis generated from human lung cancer cells in nude mice, indicating that systemic administration of mda-7/IL-24 effectively inhibits metastatic tumor growth [121]. Infectivity enhanced adenoviruses expressing mda-7/IL-24 have been developed that overcome the resistance of several ovarian cancer cells to Ad.mda-7 [103]. Oncolytic Ads, based on ONYX-015, having deletions in the E1B gene thus replicating in p53-deficient cells, have been engineered to express mda-7/IL-24 that show stronger antitumor activity than that induced by ONYX-015 or Ad.mda-7 alone in colorectal carcinoma cells [122]. We have developed an oncolytic Ad in which viral replication is controlled by the cancer-selective promoter of the PEG-3 gene (PEG-Prom) [111]. Unlike ONYX-015 that depends upon the p53-status of the cancer cells, this approach ensures Ad replication in any cancer cell irrespective of its tumor suppressor status owing to the activity of the PEG-Prom only in cancer cells and not in normal cells [125]. mda-7/IL-24 was simul-
Ad.PEG-E1A-mda-7 completely eradicated both primary tumors and distant non-injected tumors. The summation of these findings suggest that mda-7/IL-24 is a highly effective therapy for multiple cancers and it will, therefore, be imperative that more stringent clinical trials be performed to translate these encouraging observations into an FDA-approved gene therapeutic for diverse cancers.

**mda-7-LIKE MOLECULES IN OTHER SPECIES**

Southern blot analysis of DNAs from different species using an mda-7/IL-24 cDNA probe identified homologous sequences in the genomic DNAs of yeast, monkey, cow, dog and cat suggesting that mda-7/IL-24 is an evolutionary conserved gene [3]. Three subsequent studies have identified mda-7/IL-24-like molecules in rat and mouse [41, 126, 127]. C49a was identified by differential display PCR performed between wound edge RNA isolated from rat excisional skin wounds and non-wounded controls [126]. The protein product, consisting of 183-amino acid residues, has 58.7% identity to the complete MDA-7/IL-24 protein and 69% identity (82% positives) in a conserved 492-bp coding segment. Wounded rat dermal cells expressed markedly elevated levels of c49a/C49A before and during the proliferative phase of repair, suggesting a role in proliferation rather than growth inhibition. An additional mda-7/IL-24-like molecule named mob-5 was also isolated by differential display PCR between rat embryo fibroblast cells Rat1 and Rat1:IRas cells containing an inducible oncogenic Ha-ras [127]. In Rat1 cells, mob-5 expression was induced by oncogenic Ha-ras and Ki-ras. MOB-5 protein is essentially identical to the C49A protein except for two amino acid mismatches. MOB-5 is a secreted protein and since its expression is induced as a function of induction of the oncogenic ras pathway it was proposed that mob-5 is involved in positive regulation of cell growth. Both in the human and rat genomes the IL-10 cluster contains IL-24/mob-5, IL-20, IL-19 and IL-10 in a sequential order [128]. A recent report has shown that mob-5 can bind to human mda-7/IL-24 receptor complexes and enhance STAT DNA-binding [128]. The murine pro-B cell line Ba/F3 requires IL-3 for survival and undergoes apoptosis upon IL-3 withdrawal. Both mob-5 and mda-7/IL-24 could substitute for IL-3 and facilitate the growth of Ba/F3 cells in a receptor-dependent manner. Overexpression of h-ras augmented mob-5 induced STAT DNA-binding and knocking out activated k-ras from human colon carcinoma cells down-regulated IL-20R1, IL-20R2 and IL-22R receptors, indicating a regulation of expression of mob-5 and mda-7/IL-24 and the receptor complex by ras. These findings contrast with the cancer-specific apoptosis-inducing properties of mda-7/IL-24 confirmed in multiple laboratories [4-6, 129]. In the rodent system, overexpression of oncogenic ras also induces irreversible growth arrest resulting in senescence [130]. In these contexts, the identification of mob-5 downstream of ras might be a part of the ras-mediated senescence-induction pathway.

RET/PTC oncoproteins are a family of fusion proteins derived from chromosomal rearrangements involving the tyrosine kinase domain of the c-RET proto-oncogene and are detected early in the development of a variety of differentiated thyroid carcinomas [131]. Microarray analysis between PC CL3 rat thyroid cells and PC CL3 cells overexpressing RET/PTC3 (RP3) identified c49a/mob-5 (rat mda-7/IL-24) to be activated by RP3 [132]. Transgenic mice expressing human RP3 in the thyroid gland develop thyroid hyperplasia and solid papillary carcinomas. These RP3-expressing mice but not wild-type mice showed expression of rat mda-7/IL-24 in the thyroid gland. Neutralization of rat mda-7/IL-24 by an antibody against rat mda-7/IL-24 inhibited the proliferation of RP3-expressing rat thyroid cells. RP3\(^{53,7}\) mice develop advanced thyroid malignancies containing regions of both differentiated and undifferentiated carcinoma. In differentiated regions of tumors, which expressed high amounts of RP3 protein, specific staining for rat mda-7/IL-24 was detected. In undifferentiated regions of tumors expressing reduced or no RP3 protein, the presence of rat mda-7/IL-24 was reduced. These findings indicate that rat mda-7/IL-24 might be required for the survival of differentiated thyroid cells, while with progression and dedifferentiation its expression is lost. All these studies in rodent systems, indicate that with physiological levels, rat mda-7/IL-24 (c49a/mob-5) might be required for the survival and maintenance of newly developed differentiated cells (which correlates with its induction during terminal differentiation) as observed during wound healing or induction by oncogenes. Additionally, rat mda-7/IL-24 might be required for the survival of cells of the immune system that will facilitate the activation of an anti-tumor immune response.

The mouse mda-7/IL-24 like molecule, termed FISP (IL-4-induced secreted protein), was identified in type 2 T helper lymphocytes (Th2) by representational difference analysis between Th2 cells and Th1 cells [41]. It encodes a secreted protein of 220 amino acids, which displays 93% identity with MOB-5/C49A and 69% identity with MDA-7. FISP expression is induced during Th2 differentiation and its expression is promoted by TCR signaling involving protein kinase C activation and STAT-6 dependent IL-4R signaling. There are several common properties between mda-7/IL-24 and FISP, including expression of both molecules predominantly in the immune system. FISP mRNA expression could not be detected in any other tissues apart from Th2 lymphocytes. Both mda-7/IL-24 and FISP are induced during the process of differentiation and their expression is promoted by a combinatorial treatment with a cytokine and a protein kinase C activator. Although the function of FISP is not known it appears as if this gene is more closely related functionally to mda-7/IL-24 than c49a/mob-5. A very recent report has documented that mouse IL-24 (mIL-24) inhibits growth of mouse hepatoma cells [110] providing direct confirmation of growth inhibitory properties of IL-24 in different species further supporting a closer functional relationship between mda-7/IL-24 and FISP than and c49a/mob-5 (rat mda-7/IL-24).

**CONCLUSION AND FUTURE DIRECTIONS**

The information gleaned during the last decade on the functions of mda-7/IL-24 have primarily employed overexpression strategies or administration of conditioned media obtained from cells secreting MDA-7/IL-24. Although these studies using ‘supraphysiological’ levels of mda-7/IL-24 have provided invaluable insights into specific functional aspects of this interesting cytokine, the physiological role(s) of mda-7/IL-24 remain to be defined. A major hindrance in
achieving this goal is the technical difficulties encountered in generating large quantities of recombinant MDA-7/IL-24 protein, since this cytokine is extremely labile/unstable in its pure form. We have observed that adding a GST-tag to the molecule stabilizes it and like soluble untagged MDA-7/IL-24 protein GST-MDA-7 protein is effective in inducing apoptosis selectively in a wide variety of transformed cells. However, to address the physiological role(s) of MDA-7/IL-24 it will be necessary to produce pure untagged MDA-7/IL-24 protein. Additionally, to obtain structural information and to perform structure-function analyses it will be important to produce sufficient quantities of untagged MDA-7/IL-24 protein to obtain its crystal structure. It will also be mandatory to ascertain the physiological levels of MDA-7/IL-24 in serum. What are the pathological/immunological conditions that increase MDA-7/IL-24 levels? What are the consequences of this upregulation? These questions require immediate answers especially considering the recent successful achievement of a next hurdle without engendering toxicity while exhibiting even greater clinical efficacy following enhanced delivery, this unique cytokine generating large quantities of recombinant MDA-7/IL-24 it will be necessary to produce pure untagged MDA-7/IL-24 protein. Additionally, to obtain structural information and to perform structure-function analyses it will be important to produce sufficient quantities of untagged MDA-7/IL-24 protein to obtain its crystal structure. It will also be mandatory to ascertain the physiological levels of MDA-7/IL-24 in serum.

ACKNOWLEDGEMENTS

The present studies were supported in part by National Institutes of Health grants CA56757, CA097318, CA098712 and P01 CA104177 to PBF; National Institutes of Health grants DK52825, CA88906, CA72955, CA108520 and P01 CA104177, to PD; Department of Defense grant DAMD17-03-1-0262 to PD; Department of Defense Army Postdoctoral Fellowships DAMD17-03-1-0317; the Ludwig Foundation for Pancreatic Cancer Research to PBF; the Samuel Waxman Cancer Research Foundation to PBF; the Chernow Enowment to PBF. PBF is the Michael and Stella Chernow Urological Cancer Research Scientist and a SWCRF Investigator. PD is a Universal Leaf Professor in Cell Signaling.

REFERENCES

Received: January 06, 2006  Accepted: March 16, 2006
Unique conditionally replication competent bipartite adenoviruses - cancer terminator viruses (CTV): efficacious reagents for cancer gene therapy

Devanand Sarkar\textsuperscript{1}, Zao-zhong Su, and Paul B. Fisher\textsuperscript{1}

Departments of Pathology, Urology and Neurosurgery, Herbert Irving Comprehensive Cancer Center, Columbia University Medical Center, College of Physicians and Surgeons, New York, NY 10032

\textsuperscript{1}Corresponding authors: Departments of Pathology and Urology, College of Physicians & Surgeons, Columbia University Medical Center, 630 W 168\textsuperscript{th} St., BB-1501, New York, NY 10032.
Phone: 212-305-3642; Fax: 212-305-8177; e-mail: ds2039@columbia.edu and pbf1@columbia.edu
Abstract

The frequent resistance of aggressive cancers to currently available therapies, such as radiotherapy and chemotherapy, mandates development of targeted, non-toxic and more efficacious treatment protocols. Conditionally replication competent adenoviruses (CRCAs) that induce oncolysis by cancer-specific replication are currently being evaluated in clinical trials. However, a single modality approach may not be sufficient to completely eradicate cancer in a patient, because most cancers arise from abnormalities in multiple genetic and signal transduction pathways. The promoter region of rodent progression elevated gene-3 (PEG-3), cloned and characterized in our laboratory, embodies the unique property of increased activity in a broad range of tumor cells, both rodent and human, when compared to normal counterparts. Bipartite adenoviruses were engineered to express the E1A gene, necessary for viral replication, under control of the PEG-3 promoter (PEG-Prom) and simultaneously express a second transgene in the E3 region that encodes an apoptosis-inducing and immunomodulatory cytokine, either immune interferon (IFN-γ) or melanoma differentiation associated gene-7/interleukin-24 (mda-7/IL-24). These conditionally replication competent bipartite adenoviruses, referred to as cancer terminator viruses (CTVs), facilitated cancer-selective adenovirus replication, robust transgene expression and apoptosis induction with complete eradication of both primary and distant (metastatic) human cancers xenotransplanted in athymic nude mice. These findings suggest that CTVs might prove efficacious for the therapy of primary and advanced neoplastic diseases.
Introduction

Cancer gene therapy classically involves delivery of tumor suppressor, apoptosis-inducing or suicide genes directly into tumor cells.\(^1\,^2\) Replication incompetent adenoviral (Ad) vectors are frequently used for this application because they promote high-level transgene expression.\(^2\) However, in most instances, achieving a discernible and significant antitumor response mandates administering the Ad multiple times, which can provoke an immune response that mediates viral clearance.\(^2\) In these contexts, conditionally replication competent adenoviruses (CRCAs) are currently being evaluated because of their effectiveness in killing cancer cells by replication, thus requiring fewer administrations.\(^3\,^4\) Ads embody several attributes that support their utility as oncolytic agents including a lytic replication cycle, high stability, efficient genome transfer and low pathogenicity.\(^5\,^6\) Moreover, the technology for Ad production at high titers is well established and Ad structure, genome and replication cycle are well characterized accordingly facilitating the engineering of these viruses for therapeutic purposes.\(^5\,^6\) Of equal import, while anti-Ad neutralizing antibodies significantly attenuate the activity of a replication-incompetent Ad, they have limited or no effect on the activity of a replication-competent Ad.\(^7\) This argues that administration of a replication-competent Ad in patients with preexisting Ad immunity would still prove effective. Additionally, the availability of animal models, such as Syrian hamsters and cotton rats, supporting Ad replication also allows stringent pre-clinical characterization of these Ads prior to their testing in patients in clinical trials.\(^8\,^9\)

**CRCA: different strategies to achieve cancer-selective replication:**
The most important aspect of using a CRCA is to ensure cancer cell-specific replication and a number of unique strategies have been designed to achieve this objective. Ad early proteins, E1A and E1B, facilitate cell transformation by their capacity to induce cell cycle progression through the S-phase and provide protection from apoptosis consequently allowing viral replication to occur. E1A proteins bind to the retinoblastoma (Rb) family of proteins, thereby releasing the E2F transcription factors that regulate expression of genes involved in cell proliferation and chromatin synthesis. Binding of E1A to Rb activates p53, but the E1B 55K protein binds to p53 and targets it for degradation. The E1B 19K protein is a homologue of the anti-apoptotic protein Bcl-2. An adenovirus deficient in E1B 55K was generated (ONYX-015 or dl1520) based on the hypothesis that it would replicate in p53 mutant cells, a common feature in cancer cells, but not in p53 wild-type (normal) cells. Recent data, however, demonstrate that it is the loss of E1B 55K-mediated late viral RNA export, rather than p53-inactivation that restricts ONYX-015 replication in primary cells. Cancer cells that support replication of ONYX-015 provide the RNA export function of the E1B 55K protein. An alternate approach exploiting the Rb pathway is to remove the Rb-binding CR2 region of E1A (dl922/947) thereby allowing Ad replication in cancer cells deficient in the Rb pathway. A number of additional modifications in dl922/947 have been generated such as ONYX-150, replacing the E1A promoter with the E2F-1 promoter, ONYX-410, replacing the E4 promoter with the E2F-1 promoter and ONYX-411, replacing both the E1A and E4 promoters with the E2F-1 promoter. Ad-ΔE1B19/55, deleted for sequences encoding E1B 19K and 55K proteins, has also been generated and evaluated for cancer-selective activity.

The effectiveness of ONYX-015 has been evaluated in multiple clinical trials either alone or in combination with chemotherapy. Intratumoral injection of ONYX-015 in combination with cisplatin or 5-fluorouracil resulted in a 63% overall response rate, with 27% of patients showing full clinical response in a phase II trial in head and neck cancer. ONYX-015 has been used for intratumoral...
injection in unresectable pancreatic cancer, intraperitoneal injection in recurrent/refractory ovarian cancer, hepatic arterial infusion in liver cancer, intracerebral injection in malignant glioma and as mouth wash in premalignant oral dysplasia. Intravenous administration of ONYX-015 has been shown to be safe and well tolerated. However, the overall objective clinical responses fall drastically short of the anticipated outcome based on preclinical studies indicating the need for further modifications to achieve the desired endpoint of tumor elimination with enhanced patient survival.

Transcriptional targeting, in which tumor or tissue-specific promoters and enhancers regulate the expression of the E1A and E1B genes, is another approach ensuring cancer-specific Ad replication. The most frequent promoter used for this purpose is the human telomerase (hTERT) promoter, since telomerase activity is frequently higher in cancer cells than in normal cells. The fact that the core of solid tumors has a hypoxic environment has led to the use of hypoxia response elements in regulating E1A expression. Promoters responding to hypoxia and estrogen have been used for targeting breast cancer. Tissue-specific promoters, such as prostate-specific antigen (PSA) promoter or probasin promoter or a combination of both for prostate cancers, α-feto-protein promoters for liver cancer and MUC1 promoter for breast cancers, have been used to drive E1A expression. A PSA-promoter-driven oncolytic Ad (CV706) has been evaluated in a Phase I clinical trial by intraprostatic injection following radiotherapy and has demonstrated safety and viral dose-dependent reduction in PSA level. However, no objective clinical response in terms of tumor regression was observed. Ad[I/PPT-E1A], where E1A gene expression is controlled by a recombinant regulatory sequence PPT, comprising of a PSA enhancer, a PSMA enhancer and a T-cell receptor gamma-chain alternate reading frame protein promoter, induces prostate-specific viral replication and cytolysis and inhibits growth of prostate cancer xenografts in nude mice that are independent of androgen level. Survivin, often overexpressed in brain tumors, promoter has been used to drive E1A in malignant gliomas. β-catenin-responsive promoters
have been used to drive the Ad early promoter for cancers with an activated wnt-signaling pathway, such as colorectal and liver cancers. The E1A promoter has been replaced by the promoter/enhancer of the melanocyte and melanoma-specific tyrosinase gene and mutations have been engineered in E1A gene to prevent the mutant protein to interact with and inactivate pRb and p300 thus precluding viral replication in normal cells (AdTyrΔ2Δ24). In a melanoma cell/keratinocyte coculture assay AdTyrΔ2Δ24 showed melanoma cell-specific Ad replication and in in vitro assays it killed melanoma cells, but not non-melanoma cancer cells. AdTyrΔ2Δ24 has been further improved by incorporating the tyrosinase promoter to drive the E4 gene (Ad2Xtyr) and the efficacy of this approach was demonstrated in in vitro organotypic raft cultures. In a similar approach, the Cyclooxegnase-2 (Cox-2) promoter has been used to drive E1A as well as E4 expression.

A selectivity strategy to ensure cancer-specific Ad replication has been developed based on deletions of adenovirus Virus-Associated (VA) RNAs (dl331). The VA RNAs are necessary for virus replication because they inactivate the RNA-dependent protein kinase protein kinase R (PKR), a kinase that otherwise would block protein translation in response to infection. However, downstream effectors of Ras can also inactivate PKR, and therefore, the need for VA RNA genes should be bypassed in cells with an active Ras pathway. dl331 has been shown to inhibit in vivo growth of xenografts developed from pancreatic cancer cells containing mutated Ras but not wild-type Ras. However, VA-RNA lacking Ad has recently been shown to effectively replicate both in the presence and absence of activating Ras indicating the involvement of additional mechanism ensuring Ad replication.

Although oncolytic adenoviruses have been found to be safe in clinical trials, as a single agent they elicit very limited clinical responses and it is only in combination with radio- or chemotherapy that they show appreciable effects. In these contexts, arming these viruses with additional agents offers potential to augment their oncolytic properties. Ad.TKRC, an E1B 55K-deleted Ad (ONYX-015) in
which Herpes simplex virus thymidine kinase (HSV-Tk) was connected to E1A by an internal ribosome entry site, showed increased growth inhibition of colon cancer xenografts in nude mice in the presence of the pro-drug ganciclovir. In CRAd-NTR(PS1217H6), the bacterial nitroreductase gene was inserted at the site of E1B 55K deletion and it showed increased efficacy in the presence of the pro-drug CD1954. A phase I clinical trial in prostate cancer has been performed with intraprostatic injection of Ad5-CD/TKrep that delivers a cytosine deaminase/herpes simplex virus-1 thymidine kinase fusion gene in an ONYX-015 backbone. Simultaneous treatment with 5-fluorocytosine and the ganciclovir prodrug resulted in ≥25% decrease in serum prostate-specific antigen in 7 of 16 (44%) patients, and 3 of 16 (19%) patients demonstrated a ≥50% decrease in serum prostate-specific antigen. These clinical responses were further augmented when this viral approach was combined with radiotherapy.

In addition to suicide genes, immunomodulatory and apoptosis-inducing genes have also been incorporated in a replicating Ad backbone. Examples of immunomodulatory genes inducing antitumor immunity include granulocyte-macrophage colony stimulating factor (GM-CSF) and the co-factor B7-1, the former inserted in E1B 55K and the latter inserted in E3 region of an ONYX-015 backbone (YKL-GB). In a syngeneic mouse melanoma model YKL-GB demonstrated antitumor activity and conferred long-lasting immunity against a tumor re-challenge. A similar GM-CSF expressing oncolytic Ad (CG0070) has been evaluated in bladder cancer. Oncolytic Ad, in which replication is driven by the hTERT promoter, has been engineered to express interferon-γ (IFN-γ) (CNHK300-hIFN-gamma or CNHK300-mIFN-gamma) and has been found to induce regression of liver cancer xenografts in both immunocompetent and immunodeficient animals. Ad.HS4.AFP.E1a/TRAIL, in which an HS4 insulator containing α-fetoprotein promoter driven E1A expression is coupled with TNF-related apoptosis-inducing ligand (TRAIL) expression, showed enhanced oncolysis of hepatocellular carcinoma. Systemic administration of E1A-deleted Ad expressing antisense checkpoint kinase 1
(chk1), a crucial checkpoint modulator, with a low dose of cisplatin cured 80% of orthotopic mouse hepatic carcinomas that were resistant to cisplatin.\textsuperscript{52}

\textit{mda-7/IL-24: A Novel Member of the IL-10 Cytokine Gene Family with Cancer-Selective Apoptosis-Inducing Activity:}

Subtraction hybridization between terminally differentiated/irreversibly growth arrested and actively growing melanoma cells permitted the identification and cloning of a spectrum of genes displaying elevated expression as a function of terminal differentiation and cancer reversion in melanoma cells.\textsuperscript{53-57} One of these genes, melanoma differentiation associated gene-7 (\textit{mda-7}), displays elevated expression in normal melanocytes and a step-wise decrease in expression during the process of melanoma development and progression from nevus, to radial growth phase melanoma, to vertical growth phase melanoma to metastatic melanoma.\textsuperscript{56} These studies suggest that \textit{mda-7} may function as a negative regulator of melanoma cell growth and progression. Structural and sequence homology and functional conservation indicates that \textit{mda-7} belongs to the IL-10 family of cytokines that include IL-19, IL-20, IL-22, \textit{mda-7} and IL-26 and has therefore been re-designated IL-24.\textsuperscript{58,61} Chromosome 1q31-32 contains a gene cluster comprising IL-10, IL-19, IL-20 and \textit{mda-7}/IL-24. IL-10 family cytokines are secreted $\alpha$-helical proteins. Northern blot analysis revealed \textit{mda-7}/IL-24 expression in human tissues associated with the immune system such as spleen, thymus, peripheral blood leukocytes and normal melanocytes.\textsuperscript{58} When expressed at supraphysiological levels, by means of an adenoviral (Ad) expression system, \textit{mda-7}/IL-24 induces growth suppression and programmed cell death (apoptosis) in a broad spectrum of human cancers, including melanomas, malignant gliomas, osteosarcomas and carcinomas of most tissue origins.\textsuperscript{62-64} In contrast, \textit{mda-7}/IL-24 has a negligible effect on growth and does not induce
apoptosis in normal human melanocyte, astrocyte, epithelial, endothelial or skin fibroblast cells. Ad.mda-7 has also shown antitumor and recently antiangiogenic activity in human tumor nude mouse xenograft models, including those involving human breast, cervical, lung and pancreatic carcinomas. Ad.mda-7 also stimulates the immune system to generate secondary cytokines, such as TNF-α, IFN-γ and IL-1 that might evoke an antitumor immune response. Additionally, Ad.mda-7 synergizes with radiation to augment radiation-induced cell death. A phase I/II trial evaluating Ad.mda-7 activity by intratumoral injection in patients with advanced carcinomas and melanomas was performed and the results indicate that mda-7/IL-24 is safe and could induce as much as 70% apoptosis in tumors following a single injection of recombinant virus and multiple injections resulted in clinical responses. An increase in circulating IL-6, IL-10 and TNF-α and CD3+ and CD8+ T cells were observed in Ad.mda-7-injected patients confirming the immunostimulatory properties of the cytokine. These exciting results provide direct support for using mda-7/IL-24 in developing effective gene-based therapies for cancer.

**PEG-3 Promoter: Novel Cancer-Specific Promoter:**

Using subtraction hybridization we cloned a novel rodent gene, progression elevated gene-3 (PEG-3), in the context of tumor progression in transformed rat embryo cells. PEG-3: (i) displays elevated expression as a function of oncogenic transformation (by diverse oncogenes), during cancer progression and after DNA damage (γ irradiation); and (ii) induces an aggressive cancer phenotype and enhanced angiogenesis and genomic instability when ectopically expressed in rodent and human tumors, without promoting transformation when expressed in normal cells. Additionally, the PEG-3 gene promoter (PEG-Prom) has been isolated and shown to display elevated expression in both rodent and human tumors, with negligible expression in normal cells.
adenovirus vectors expressing a transgene (such as GFP or luciferase) under the control of the PEG-Prom documented high GFP or luciferase expression in cancer cells, with little to no expression in normal cells. These observations strongly suggested that the PEG-prom might be an essential tool to facilitate cancer cell-specific gene expression and Ad replication.

**CRCA and CTV: Our Experience:**

To facilitate cancer-specific replication we employed the PEG-Prom to regulate expression of the E1A gene of Ad, necessary for replication. To enhance the therapeutic potential of these viruses we also engineered these Ads to produce either mda-7/IL-24 or IFN-γ (Ad.PEG-E1A-mda-7 or Ad.PEG-E1A-IFN-γ), and termed these viruses ‘cancer terminator viruses’ (CTVs) (Fig. 1). Ad.PEG-E1A-mda-7 was evaluated in a breast cancer model while Ad.PEG-E1A-IFN-γ was evaluated in a pancreatic cancer model. Both of these CTVs permitted Ad replication, transgene expression and induction of cell death (apoptosis and necrosis) only in cancer cells but not in their normal counterparts. In animal models, human breast and pancreatic cancer xenografts were established in both the left and right flanks of athymic nude mice. Intratumoral injections of Ad.PEG-E1A-mda-7 or Ad.PEG-E1A-IFN-γ were given only to tumors on the left side. Very interestingly, this treatment protocol resulted in complete eradication of not only the injected left-sided tumors but also the un-injected right-sided tumors. However, a replicating Ad alone, without any transgene expression, eradicated only left-sided tumors and had little to no effect on the right-sided tumors. These findings indicate that the secreted proteins, such as mda-7/IL-24 or IFN-γ, were released into the circulation and they exerted their antitumor effects either by direct inhibition of growth (or induction of apoptosis) or by indirect-effects such as stimulation of antitumor immunity or inhibition of angiogenesis. Indeed, the Ad.PEG-E1A-IFN-γ CTV was shown
to exert a potent NK-cell-mediated antitumor immune response. These provocative findings provide compelling evidence that our novel approach and reagents will not only kill primary tumors but may also impact on distant metastases. Current studies are in progress to stringently evaluate these and additional CTVs in transgenic immunocompetent animal models, which if proven successful would pave the way for evaluation of safety and ultimately determination of therapeutic benefit in clinical trials.

Conclusion

Developing safe and effective gene-based therapies for cancer represent major endeavors of both academic and commercial laboratories. Although we are still not close to achieving the objective of producing the perfect cancer gene therapeutic, genetically modified conditional replication competent adenoviruses (CRCA) are demonstrating some efficacy in inhibiting tumor growth. Multiple approaches are being evaluated to increase stringent cancer-specific replication and various structural permutations and combinations with diverse agents with potent cancer-inhibitory and destroying actions are being tested. Our approach, the ‘cancer terminator virus’ (CTV), is appealing because (i) the employment of the PEG-Prom to drive Ad replication ensures stringent control of Ad replication only in cancer cells without any toxicity to normal cells; (ii) the high PEG-Prom activity in cancer cells of diverse origin suggests that our approach might be applicable to any cancer indication; (iii) the cancer-specific direct apoptosis induction by mda-7/IL-24 will profoundly augment oncolysis by Ad replication; (iv) the indirect tumor-suppressor functions of secreted mda-7/IL-24, such as inhibition of angiogenesis, stimulation of the immune system and potent ‘bystander antitumor effect’ will help eradicate not only the primary tumors but also distant metastasis; and (v) in principle, based on the robust cell killing effect of Ad.PEG-E1A-mda-7, cancer eradication may be achieved with few, even one, injection of the Ad
thus overcoming the limitation of viral clearance by an intact immune system. Additionally, engineering modifications in Ad tropism to enhance infectivity would significantly increase the efficacy of Ad.PEG-E1A-\textit{mda-7}. We anticipate that our proposed approach will evoke a complete eradication of primary and distant tumors, with minimal toxicity to normal organs, leading to a possible “cure” in cancer patients.

**Acknowledgements**

The present studies were supported in part by NIH grants R01 CA35675, R01 CA97318, R01 CA98712, and P01 CA104177, Army DOD grant DAMD17-03-1-0290, the Lustgarten Foundation for Pancreatic Cancer Research, the Samuel Waxman Cancer Research Foundation and the Chernow Endowment. PBF is the Michael and Stella Chernow Urological Cancer Research Scientist and a SWCRF Investigator. The contributions to our studies by Ms. Eun Sun Park and Mr. Nichollaq Vozhilla are greatly appreciated.
Reference:


34. Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of
calydon virus 764, an attenuated replication competent adenovirus for prostate

DC, Chen Y, Henderson DR, Carducci MA, Nelson WG, Simons JW. A phase I
trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for
the treatment of locally recurrent prostate cancer following radiation therapy.

conditionally replicating adenovirus for hormone-dependent and hormone-

37. Van Houdt WJ, Haviv YS, Lu B, Wang M, Rivera AA, Ulasov IV, Lamfers ML,
Rein D, Lesniak MS, Siegal GP, Dirven CM, Curiel DT, Zhu ZB. The human
survivin promoter: a novel transcriptional targeting strategy for treatment of

38. Fuerer C, Iggo R. Adenoviruses with Tcf binding sites in multiple early promoters
show enhanced selectivity for tumour cells with constitutive activation of the wnt

adenoviruses targeted to melanoma: specific viral replication and cytolysis by
expression of E1A mutants from the tyrosinase enhancer/promoter. Cancer Res


64. Sarkar D, Dent P, Fisher PB. Melanoma Differentiation Associated Gene-7 (mda-7)/ Interleukin-24 (IL-24), mda-7/IL-24: Current Perspectives on a Unique
Member of the IL-10 Family of Cytokines. Anti-inflammatory Anti-allergy Agents Med Chem 2006, in press.


81. Su Z, Shi Y, Fisher PB. Cooperation between AP1 and PEA3 sites within the progression elevated gene-3 (PEG-3) promoter regulate basal and differential


Figure legend

Figure 1. Schematic representation of Cancer terminator viruses (CTVs). In CTVs PEG-Prom drives the expression of E1A and E1B genes thus ensuring cancer specific replication while CMV-Prom regulates the expression of either mda-7/IL-24 or IFN-γ in the E3 region of the adenovirus. These conditionally replication competent adenoviruses (CRCA) do not harm normal cells but induce oncolysis by adenoviral replication and diverse tumor-suppressor effects of the expressed transgene.