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Developing an Effective Gene Therapy for Prostate Cancer: New Technologies with Potential to Translate from the Laboratory into the Clinic

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Abstract

Prostate cancer is the second leading cause of cancer-related deaths in men in the U.S. At present, no single or combination therapy has shown efficacy in decreasing disease progression in patients with metastatic disease. A potentially viable approach for treating late-stage prostate cancer is gene therapy. Adenoviruses (Ad) are the most commonly used mode of gene delivery, but progress using this vector has been hampered by concerns over the safety and practicality of viruses including conditionally replicating Ads (CRAds), particularly for intravenous delivery, and the inefficiency of non-viral transfection techniques. Major challenges for effective gene therapy using Ads are the limited infectivity of regular Ad serotype 5 (Ad5) and the inability to specifically deliver the therapeutic directly into diseased tissue without trapping in the liver or elimination by the immune system. The shortcoming in using Ad5 is mostly attributed to a reduction in Coxsackie-adenovirus receptors (CAR) on the surface of cancer cells, which can be mitigated by generating tropism-modified Ads permitting CAR-independent infection of tumor cells. The limitations of systemic gene delivery can now be overcome by using a novel targeted-delivery approach such as ultrasound (US) contrast agents (microbubbles) to deliver effective therapeutic reagents, Ads, or recombinant proteins, combined with ultrasound-targeted microbubble destruction (UTMD), to develop a site-specific therapy in immune competent transgenic mouse models. These unique strategies for enhancing the efficacy of gene therapy provide a direct path to translation from the laboratory into the clinic for developing an effective gene therapy of prostate cancer.

Introduction

Prostate cancer is the most common cancer and the second leading cause of cancer-related deaths in men in the US (Damber and Aus, 2008). It is estimated that 217,730 new cases of prostate cancer will have been diagnosed in 2010 alone. The therapeutic options for patients with prostate cancer include radiotherapy and treatment with cytotoxic chemotherapeutic agents. Despite a palliative benefit, these approaches do not engender a long-term beneficial effect on the overall survival of patients. Late stage prostate cancer patients may benefit from hormone therapy, which removes a primary factor mediating tumor growth, male hormones (androgens) (Di Lorenzo and De Placido, 2006; Sternberg, 2002). Unfortunately, after a few years most patients become non-responsive to this treatment, resulting in uncontrolled disease and patient death. In these contexts, there is a pressing need to develop more effective therapeutic approaches for end-stage prostate cancer patients and genetic therapies represent promising approaches for the treatment of this neoplasm.

The prostate gland is not vital for survival and is accessible by ultrasound, potential therapeutic genes can be injected directly into the primary tumor, tissue-specific promoters exist that can target therapeutic gene expression or viral replication uniquely in the prostate gland, and disease progression can be monitored by measuring prostate-specific antigen (PSA) (Anderson, 1998; Gopalkrishnan *et al.*, 2001; Mabjeesh *et al.*, 2002). Since prostate cancer is commonly a relatively slow-growing disease, it may be necessary to use repeated gene therapy approaches, with single or multiple genes, over the lifespan of the patient. Adenoviruses (Ads) are the most commonly used vehicle for gene therapy approaches because the technology for virus production at high titers is established and Ad structure, genome, and replication cycle are well characterized thus facilitating the engineering of these viruses for therapeutic purposes. Although promising *in vitro* and *in vivo* results have been achieved using Ad vectors, administering unmodified serotype 5 Ads (Ad5) for gene delivery faces a number of clinical limitations. These include down-regulation of Coxsackie-adenovirus (CAR) receptors in cancer cells resulting in failure to transduce the majority of tumor cells by Ad5 (Anderson, 1998; Haviv *et al.*, 2002; Mabjeesh *et al.*, 2002).

An effective systemic gene delivery method is required to ensure safe and targeted delivery as administration of Ad-based gene therapy results in hepatic sequestration of the Ad so that very little reaches the target tumor tissue and clearance of the Ad from the circulation by the immune system. To develop safe and more efficient systemic delivery systems, we are focusing on ultrasound (US) contrast agents (microbubbles) (UTMD: ultrasound-targeted microbubble destruction) to enhance delivery of molecules *in vivo*. This review will summarize unique and novel aspects of effective gene therapy for prostate cancer that offers significant promise for moving basic science studies in the laboratory into the clinic to hopefully develop a *'cure'* for advanced prostate cancer.

Gene Therapy for Prostate Cancer

Using subtraction hybridization combined with induction of cancer cell terminal differentiation, our laboratory cloned *mda*-7/IL-24 (Jiang *et al.*, 1995), a novel member of the IL-10-related cytokine gene family (Dash *et al.*, 2010a; Gupta *et al.*, 2006; Pestka *et al.*,

2004; Sauane *et al.*, 2003). Subsequent experiments documented that *mda*-7/IL-24 had nearly ubiquitous antitumor properties *in vitro* and *in vivo*, which led to its successful entry into the clinic in an unprecendented 5 years after discovery where acceptable safety and clinical efficacy, when administered by Ad (Ad.*mda*-7; INGN 241), has been demonstrated in Phase I clinical trials in humans with advanced carcinomas and melanomas (Cunningham *et al.*, 2005; Eager *et al.*, 2008; Emdad *et al.*, 2009; Fisher, 2005; Fisher *et al.*, 2003; Fisher *et al.*, 2007; Lebedeva *et al.*, 2007; Lebedeva *et al.*, 2005; Tong *et al.*, 2005). Its mechanism of action involves preferential induction of autophagy and apoptosis in prostate cancer without exerting harmful effects to normal cells (Bhutia *et al.*, 2010; Dash *et al.*, 2010b; 2010c; Gao *et al.*, 2008; Miyahara *et al.*, 2006; Sarkar *et al.*, 2007a; Sauane *et al.*, 2003; Su *et al.*, 2005a; Su *et al.*, 2001a; Su *et al.*, 2006; Yacoub *et al.*, 2008). Additional targets of *mda*-7/IL-24 action have also been investigated supporting its considerable potential as a gene therapeutic for cancer. Forced *mda*-7/IL-24 expression in cancer cells inhibits angiogenesis, stimulates an anti-tumor immune response (Gao *et al.*, 2008; Miyahara *et al.*, 2006), sensitizes cancer cells to radiation-, chemotherapy-, and antibody-induced killing (McKenzie *et al.*, 2004; Su *et al.*, 2005a; Su *et al.*, 2001a; Su *et al.*, 2006), and elicits potent "antitumor *bystander* activity" (Chada *et al.*, 2004; Sauane *et al.*, 2008; Su *et al.*, 2001; Su *et al.*, 2005a). MDA-7/IL-24 protein induces a sustained ER stress response as evidenced by expression of ER stress markers (BiP/GRP78, GADD153, GRP94, and phospho-eIF2α) and production of reactive oxygen species (ROS), indicating that both intracellular and secreted proteins activate similar signaling pathways to induce apoptosis (Sauane *et al.*, 2008).

As prostate cancer requires repeated gene therapy approaches, the use of replication defective Ads to administer therapeutic and cytotoxic genes and conditionally replication competent Ads (CRAds) to selectively induce cytolysis in prostate tumor cells represent feasible treatment options (Anderson, 1998; Mabjeesh *et al.*, 2002). 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 (Su *et al.*, 1997). *PEG*-3 (i) displays elevated expression as a function of oncogenic transformation (by diverse oncogenes) (Park *et al.*, 2001; Su *et al.*, 1997), (ii) induces an aggressive cancer phenotype without promoting transformation when expressed in normal cells (Emdad *et al.*, 2005a; Emdad *et al.*, 2005b; Park *et al.*, 2001; Su *et al.*, 1999; Su *et al.*, 2002; Su *et al.*, 1997), and (iii) is regulated by a gene promoter (PEG-Prom) shown to display elevated expression in both rodent and human tumors (including prostate carcinomas) with negligible expression in normal cells (including human prostate epithelium) (Park *et al.*, 2001; Su *et al.*, 2000; Su *et al.*, 2001b; Su *et al.*, 2005b).

To test the cancer specificity of the PEG-Prom for tumor imaging *in vivo*, we used a firefly luciferase reporter PGL3-PEG-prom-Luc (pPEG-Luc) (Bhang *et al.*, 2011; Su *et al.*, 2005b). After confirmation of the presence of metastatic nodules in the lung by computed tomography (CT) at 4-6 weeks after intravenous administration of the human malignant breast cancer cell line (MDA-MB-231) or melanoma (MeWo), animals (athymic nude mice) received an intravenous dose of pPEG-Luc/PEI polyplex (PolyplusTransfection). Forty-eight hours after plasmid DNA (pDNA) delivery, PEG-Prom-driven gene expression was assessed by bioluminescence imaging (BLI). Quantification of the BLI signal intensity from the

thoracic cavity, which represents Luc expression mainly in lung, showed significantly higher PEG-Prom activity in the model of melanoma or breast cancer metastasis as compared to controls, which did not show a detectable signal (Bhang *et al.*, 2011). Additionally, it was possible to use repeat administrations of pPEG-Luc/PEI in tumor-bearing animals, which permitted us to follow growth and development of new metastatic lesions over time (Bhang *et al.*, 2011).

Considering the cancer-specific expression property of the PEG-Prom, we constructed a bipartite CRAd [called a Cancer Terminator Virus (*CTV*)] in which the expression of E1A and E1B genes of Ad, necessary for replication, is regulated by the PEG-Prom (**Figure 1**) (Sarkar *et al.*, 2005). This novel biCRAd (*CTV*) also expressed *mda*-7/IL-24 in the E3 region (Ad.PEG-E1A-*mda*-7). To test our hypothesis of cancer-specific activity and therapeutic effectiveness of the Ad.PEG-E1A-*mda*-7, experiments were done in three prostate cancer cell lines, androgen-nonresponsive DU-145 and PC-3 cells, and androgen-responsive LNCaP cells and their Ad.*mda*-7-resistant variants (i.e., DU-145-Bcl-2, DU-145-Bcl-x_L, PC3-Bcl-2 and PC-3-Bcl-x_L, DU-145, and PC3 that stably expresses Bcl-2 and Bcl-x_L). As a control, P69 normal prostate epithelial cells immortalized by SV40 T/t antigen were used (Sarkar *et al.*, 2007b). From Western blot analysis it was evident that infection of normal immortal human P69 prostate epithelial cells with Ad.CMV-E1A (CRAd where E1A is driven under CMV promoter control) or Ad.CMV-E1A-*mda*-7 (bipartite CRAd where both E1A and *mda*-7/IL-24 are driven by the CMV promoter) but not with Ad.PEG-E1A (CRAd where E1A is driven under CMV promoter control) or Ad.PEG-E1A-*mda*-7 (*CTV*) resulted in production of E1A proteins; whereas in prostate cancer cells, infection with all four replication-competent Ads generated E1A proteins. In P69 cells, infection with Ad.CMV-E1A-*mda*-7 or Ad.CMV-*mda*-7 resulted in MDA-7/IL-24 protein production, whereas infection with Ad.PEG-*mda*-7 or Ad.PEG-E1A-*mda*-7 (*CTV*) resulted in barely detectable levels of MDA-7/IL-24 protein production. In prostate cancer cells, infection with Ad.CMV-*mda*-7, Ad.PEG-*mda*-7, Ad.CMV-E1A-*mda*-7, or Ad.PEG-E1A-*mda*-7 (*CTV*) generated significant MDA-7/IL-24 production. No MDA-7/IL-24 protein production could be detected in uninfected control cells or following infection with Ad.vec, Ad.CMV-E1A, or Ad.PEG-E1A (Sarkar *et al.*, 2007b). These findings document that the PEG-Prom facilitates cancer cell-selective replication of Ads and concomitant *mda*-7/IL-24 expression. The effects of the engineered Ads on cell viability and apoptosis were evaluated in the various prostate cell lines. In P69 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 (*CTV*), induced profound growth inhibition (Sarkar *et al.*, 2007b). In contrast, in all prostate cancer cells, both parental and *mda*-7/IL-24-resistant, Ad.CMV-E1A-*mda*-7, Ad.PEG-E1A-*mda*-7 (*CTV*), Ad.CMV-E1A, and Ad.PEG-E1A infection resulted in significant growth inhibition, indicating potential therapeutic applications of the *CTV* in prostate cancer patients frequently showing Bcl-2 and Bcl-x_L over-expression. Replication of Ad.PEG-E1A-*mda*-7 results in robust amounts of *mda*-7/IL-24 production resulting in a potent antitumor immune response. Moreover, *in vivo* assays in established melanoma, breast cancer, and therapy-resistant prostate cancer xenografts in athymic nude (immunocompromized) mice showed that injection of Ad.PEG-E1A-*mda*-7 completely eradicated not only the primary tumors but also distant tumors (Sarkar *et al.*, 2007b; Sarkar *et al.*, 2008; Sarkar *et al.*, 2005).

Construction of tropism modified Ads for enhanced therapeutic efficacy for prostate cancer cells

The most frequently used serotype of Ad for gene therapy has been recombinant forms of the type 5 Ad (Ad.5). Ad.5 utilizes CAR for infective entry into cells (Glasgow *et al.*, 2004). In many tumor types, for example malignant glioma, ovarian cancer, renal cancer, and prostate cancer, and particularly in primary tumor specimens, it has been noted that the expression of CAR is reduced or absent in tumor cells compared to surrounding non-tumor tissue. Reduced CAR expression precludes efficient transduction of cancer cells by Ad.5 (Paul *et al.*, 2008; Tsuruta *et al.*, 2007). This finding may in part explain why gene therapy approaches using Ad.5 have not been as successful as the studies performed *in vitro* using established cell lines.

CAR is expressed in established cell lines at a higher level than that observed in primary tumors in patients. An approach to circumventing the low efficiency of Ad.5 infection of tumor cells involves “tropism modification” in which the virus capsid proteins that normally associate with CAR are modified, permitting CAR-independent infectivity of tumor cells. To achieve enhanced infectivity, the infective viral capsid “knob” has been modified to bind to surface integrin proteins whose expression is enhanced upon transformation (RGD/DRGD modification) (Glasgow *et al.*, 2004; Paul *et al.*, 2008; Tsuruta *et al.*, 2007). Additionally, insertion into the knob of multiple lysine residues (pK_7/PK) which will increase viral interaction with cells by electrostatic effects, or by including portions of type 3 Ad in the viral capsid knob (Ad.5/3), has been engineered (Tsuruta *et al.*, 2007).

Experiments were performed to directly compare the activity of Ad.5/3 chimeric viruses (expressing luciferase or *mda*-7/IL-24) vs. Ad.5 viruses (expressing luciferase or *mda*-7/IL-24) in the context of prostate carcinoma cells that contain reduced levels of CAR on their surface (**Figure 2**, Figure 2A). For this purpose, we chose PC-3 cells, which have a reduced level of CAR in comparison with DU-145 or LNCaP prostate carcinoma cells (Figure 2B). As a first test of comparative transduction efficiency we constructed Ads that express luciferase and evaluated relative expression as a function of infection of PC-3 cells. When PC-3 cells were infected with Ad.5/3-Luc, the level of luciferase activity with lower p.f.u. was significantly greater than that observed with Ad.5-Luc (Figure 2C). On the other hand, the level of luciferase activities in DU-145 with Ad.5/3-Luc and Ad.5-Luc was comparable. Studies were next performed using two Ads genetically engineered to express *mda*-7/IL-24, including Ad.*mda*-7 (*mda*-7/IL-24 cloned into an Ad.5 virus) and Ad.5/3-*mda*-7 (*mda*-7/IL-24 cloned into an Ad.5/3 virus) (Dash *et al.*, 2010b). Infection of PC-3 cells with Ad.5-*mda*-7 was significantly less effective in reducing cell proliferation and viability than Ad.5/3-*mda*-7. This differential effect correlated with a reduced level of MDA-7/IL-24 protein being produced in PC-3 cells infected with Ad.*mda*-7 vs. Ad.5/3-*mda*-7 (Figure 2D). Correlating with the *in vitro* data, Ad.5/3-*mda*-7 also showed profound enhanced antitumor activity (Dash *et al.*, 2010b) as compared to Ad.5-*mda*-7 in PC-3 xenograft models using nude mice. These findings provide definitive evidence for enhanced therapeutic efficacy of the Ad.5/3-*mda*-7 virus vs. Ad.5-*mda*-7 in prostate cancer cells with reduced CAR. Further results provide definitive evidence for enhanced therapeutic efficacy of the Ad.5/3-*mda*-7

virus vs. Ad.5-*mda*-7 in ovarian carcinoma, malignant glioma, and renal carcinomas with reduced CAR (Hamed *et al.*, 2010; Park *et al.*, 2010; Yacoub *et al.* 2010).

Microbubble-assisted gene therapy

The quest for novel, safe, and more efficient systemic gene delivery systems has recently highlighted ultrasound (US) contrast agents (microbubbles) as a potential candidate for enhancing delivery of molecules to target tissue (Goldberg *et al.*, 1994; Larina *et al.*, 2005; Lawrie *et al.*, 2000; Ng and Liu, 2002). Currently used US contrast agents (microbubbles) contain high-molecular weight gases with less solubility and diffusivity, which improves microbubble persistence and allows passage through the microcirculation. Microbubbles can be injected in peripheral veins, as more robust bubbles can re-circulate through the systemic circulation numerous times, surviving for several minutes within the bloodstream (Goldberg *et al.*, 1994). The ideal microbubble diameter is likely 2.5 to 4 μm. This is small enough to prevent entrapment within the pulmonary capillary bed (ranging from 5 to 8 μm in diameter), but big enough to entrap and protect viral vectors such as Ad from the environment. The gas-filled microspheres effectively lower the energy threshold for non-thermal cavitation. Ultrasound-targeted microbubble destruction (UTMD) enables focal release of entrapped materials as well as the creation of small shock waves that increase cellular permeability (Pitt *et al.*, 2004). In addition, the microbubbles protect the viruses from rapid degradation by the immune system, thus allowing for intravenous injection rather than direct target organ delivery by catheter-based approaches or operative bed injection (Howard *et al.*, 2006). This is particularly important in cancer gene therapy of potentially inaccessible tumors because the microbubbles may also limit the amount of inflammatory response to the viruses and may allow repeated injections.

Targeting ligands on the surface of microbubbles permit the selective accumulation of these particles in the areas of interest, such as up-regulated levels of receptor/prognostic marker molecules on vascular endothelium or tumor cells. Decorated microbubbles coupled (covalently or non-covalently) with small targeting ligands/peptides have been designed to achieve maximum tissue-specific accumulation for enhanced US-based imaging (Weller *et al.*, 2005). Studies involving Phase I to III clinical trials have demonstrated that US contrast agent (microbubbles) are safe and well tolerated even at higher doses (Bhatia and Senior, 2008).

Proof-of-principle for site-specific delivery of Ad.PEG-E1A-mda-7 (CTV) in vivo

To confirm the ability of microbubble (Targestar-P) to deliver viruses efficiently and specifically, we performed a pilot study in which tumor xenografts were established in both flanks of athymic nude mice by injecting each site with 2×10^6 DU-145 human prostate carcinoma cells (**Figure 3**). The DU-145 tumor-bearing nude mice (n = 5) were then injected in their tail vein with 100 mL of Targestar-P contrast agent that was reconstituted with Ad-GFP or water as control as previously reported, and a portable SonoSite Micro-Maxx ultrasound platform (SonoSite, Inc., Bothell, WA) equipped with an L25 linear array transducer set at 0.7 Mechanical Index (MI), 1.8 MPa for 10 minutes, was used to sonoporate only the tumor implanted on the right side (Figure 3A). Tissues from different sites were then harvested and snap frozen. Figure 3B shows specific delivery to the right

tumor as evidenced by expression of the green fluorescence protein (GFP) in an immunoblot in which total protein extracts were run on a 10% SDS-PAGE gel. As a GFP control, GST-GFP fusion protein was loaded (Figure 3B). Protein gel loading was normalized using β-actin as a control. These results support the application of microtubules for systemic delivery of viruses. We next explored the systemic delivery of the antitumor gene *mda-7*/IL-24 by microbubbles. For this proof-of-principle study, we used DU-145-Bcl-x_L (DU-145 ectopically express Bcl-X_L), an Ad.*mda*-7-resistant variant of DU-145 human prostate cancer cells (Greco *et al.*, 2010). The therapeutic arm of this work included the *CTV* (Ad.PEG-E1A-*mda*-7) (Sarkar *et al.*, 2006; Sarkar *et al.*, 2002; Sarkar *et al.*, 2008; Sarkar *et al.*, 2005). DU-Bcl-x_L tumor xenografts were established on both flanks of nude mice by injecting 2×10^6 cells in each side of the animal. Treatment was initiated when the tumor reached a size of 250-350 mm^3. Four injections of the various Ad/microbubble complexes into the tail vein once per week (total of 4 weeks) were administered followed by US for 10 minutes on the tumor on the right side only. No treatment was performed on the tumor xenografted on the left flank. Animals receiving the Ad-GFP-microbubble complexes plus US treatment showed no statistically significant effect on the growth of DU-145-Bcl-x_L tumors. *CTV*/Microbubble elicited a sustained growth inhibition of the therapy resistant DU-Bcl-x_L tumor xenografts in both primary and distant tumors (Greco *et al.*, 2010). A Western blotting analysis of total protein extracts from the harvested tumors showed expression of MDA-7/IL-24 protein in both the tumor samples implanted on the right and left flank validating the "*bystander*" effects of MDA-7/IL-24 previously reported (Sauane *et al.*, 2008; Su *et al.*, 2005a). The amplified expression of MDA-7/IL-24 in the non-injected left tumor may also reflect secondary viral infection by the CRAd (Sarkar *et al.*, 2007b). Control tumors treated with Ad-GFP-microbubble complexes were mostly TUNEL negative. Treatments with the *CTV*-microbubble complexes plus US disrupted the tumor cyto-architecture, which correlated with an increase in the number of TUNEL positive tumor cells in both sonoporated right and untreated left tumors. Control tumors treated with Ad-GFP-microbubble complexes were TUNEL negative. B-mode ultrasound-scan of DU-Bcl-x_L tumors showed dramatic volume reductions in the tumors after 2 and 4 weeks of treatments with *CTV*-microbubble complexes and US leading to the eradication of the tumor xenograft. Additionally, no tumor regrowth in the primary or distant sites was evident in *CTV*-microbubble complex and US-treated DU-Bcl-x_L animals after an additional three weeks post-treatment (Greco *et al.*, 2010). To investigate if the tumor would recur after a longer period of time following the last treatment, three out of ten animals initially treated with *CTV*-microbubble complexes were not sacrificed at the endpoint of the study and were maintained for an additional 3 months. The mice were then sacrificed and dissected to look for potential tumor recurrence and/or eventual tumor spread. We did not observe any local tumor recurrence or distant metastasis in the lungs or liver in these mice that were treated with *CTV*-microbubble complexes.

Microbubble Encapsulated Ads Display Reduced Immunogenicity

Although Ads are the most commonly used vector for gene therapy, major disadvantages of this vehicle are its ability to be sequestered in the liver and to elicit robust innate immune and inflammatory responses when injected systemically (Koizumi *et al.*, 2007).

Improvement of therapeutic index of Ad-based gene therapy requires a method to shield viruses from exposure to the immune system. We hypothesized that microbubble (MB) encapsulated Ads (at a dose of 1×10^{12} p.f.u.) or recombinant protein (10 nM) will not be exposed to the immune system following systemic injection. As predicted, microbubble-encapsulated Ad.vec (non-replicative Ad which do not express any transgene) treated with complement (Greco *et al.*, 2010) did not elicit an innate immune response (i.e., activation of IL-6, TNF-α and IFN-γ) at 12 hours following intravenous tail vein injection into the C57B6 mice, whereas the Ad.vec alone or microbubble-encapsulated Ad.vec without complement treatment were immunogenic (**Figure 4**). We also observed a similar inflammatory cytokine profile after 24 hours of intravenous injection into mice (data not shown). Interestingly, neither the His-MDA-7 (Histidine tagged recombinant MDA-7/IL-24) nor the microbubble-encapsulated His-MDA-7 induced an immune response following intravenous injection into immunocompetent C57B6 mice. These results suggest that the microbubble can protect the viruses from exposure to the immune system and His-MDA-7 may not be immunogenic (Figure 4).

Conclusion

Therapy of cancer using Ads has been restricted for a number of reasons, particularly when utilizing systemic administration routes. These include: limitations in tumor transduction efficiency that are frequently mediated by a reduction in the number of CAR that regulate Ad entry into cancer cells (Paul *et al.*, 2008; Tsuruta *et al.*, 2007); sequestering of Ads in the liver limiting virus delivery to disseminated tumors (Koizumi *et al.*, 2007); neutralization of viruses by the immune system (Koizumi *et al.*, 2007); and absence of broad-spectrum anti-tumor agents capable of selectively killing cancer cells and provoking elimination of disseminated metastatic tumors through potent "*bystander*"anti-tumor activity (Fisher, 2005; Sarkar *et al.*, 2007b; Sarkar *et al.*, 2008; Sarkar *et al.*, 2005). We have attempted to overcome these barriers to achieve effective systemic therapy of cancer using a number of innovative approaches. We have modified the infectivity tropism of Ad by producing chimeric viruses containing regions of both Ad type 5 and Ad type 3, Ad.5/3, which allow CAR-independent transduction of tumor cells. Ad.5/3 shows superiority in transducing genes in a CAR-independent manner in prostate cancer and is effective in cells with both low and high CAR receptors (Dash *et al.*, 2010b; Hamed *et al.*, 2010; Park *et al.*, 2010; Yacoub *et al.*, 2010). To prevent trapping of Ads in the liver and elimination of viruses by the immune system, we have developed a novel approach in which Ads, both replication incompetent and conditionally replication competent, are incorporated in a perfluorocarbon microbubble that is treated with complement (to inactivate and mask viruses on the surface of the microbubble from the immune system) and then administered systemically and released in the tumor microenvironment through ultrasound, i.e., the UTMD approach (Greco *et al.*, 2010). Early phase clinical studies suggest that *mda*-7/IL-24 may be an effective agent for gene therapy of primary and metastatic cancers (Cunningham *et al.*, 2005; Tong *et al.*, 2005). This novel IL-10-family member cytokine selectively kills cancer cells without harming normal cells, displays potent systemic "*bystander*" antitumor effects, inhibits tumor angiogenesis, stimulates the immune system resulting in long term antitumor effects, and potentiates the therapeutic activity of currently used modalities of therapy,

including radiation, chemotherapy, and monoclonal antibodies (Emdad *et al.*, 2007; Gupta *et al.*, 2008; Lebedeva *et al.*, 2007; Su *et al.*, 2006; Dash *et al.*, 2010b). We have now generated Ad.5/3-*CTV* (tropism modified CTV), which we intend to evaluate this virus for delivery into the prostate of immunocompetent prostate cancer transgenic mice (Hi-Myc) by the UTMD technique. Successful completion of our proposed studies using tropism-modified viruses, including the *CTV*, and the UTMD technology will provide a direct path for translation into the clinic for potentially improving the therapy for advanced prostate cancer and other difficult to treat neoplastic diseases.

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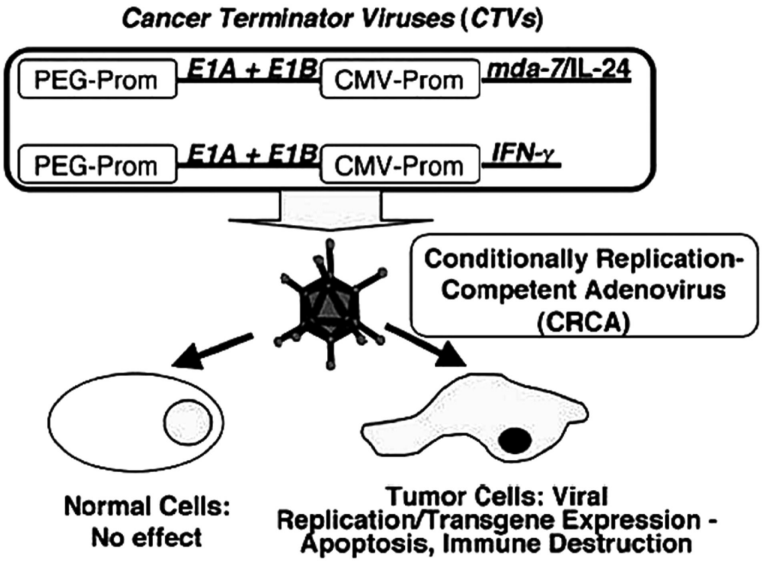

Figure 1.

Schematic representation of cancer terminator viruses (CTVs). In the CTVs the PEG-Prom drives the expression of E1A and E1B genes thus ensuring cancer-specific replication while the CMV-Prom regulates the expression of either *mda*-7/IL-24 or IFNγ in the E3 region of the Ad. These conditionally replication competent adenoviruses (CRCA) do not harm normal cells but induce oncolysis by Ad replication and diverse tumor-suppressor effects of the expressed transgene. (Reproduced with permission of the publisher, from Sarkar et al., 2005).

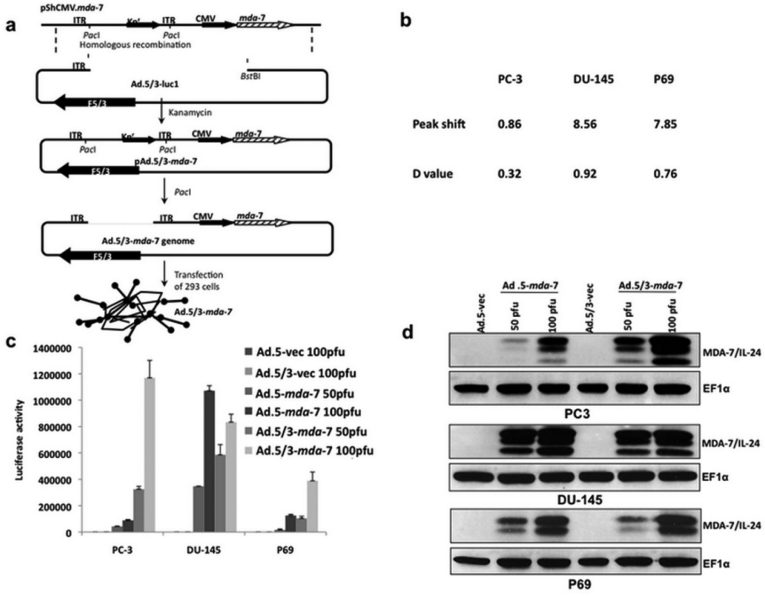

Figure 2.

Tropism-modified adenovirus (Ad.5/3) shows enhanced infectivity in PC-3 low Coxsackie-adenovirus receptor (CAR) prostate cancer cells. (a) Schematic representation showing the construction of tropism-modified Ad for delivery of *mda*-7/IL-24. (b) Expression of CARs on the surface of DU-145 and PC-3 prostate carcinoma cells and P69 SV40 immortalized normal prostate epithelial cells. (c) P69, DU-145 and PC-3 cells were infected with the indicated p.f.u. per cell of Ad.5-Luc or Ad.5/3-Luc; and luciferase activity was determined 48 hours later. (d) P69, DU-145 and PC-3 cells were infected with the indicated p.f.u. per cell of Ad for 48 hours and total proteins were isolated. The expressions of MDA-7/IL-24 and EF-1a (as a loading control) proteins were analyzed by Western blotting analyses. (Reproduced with permission of the publisher, from Dash et al., 2010a).

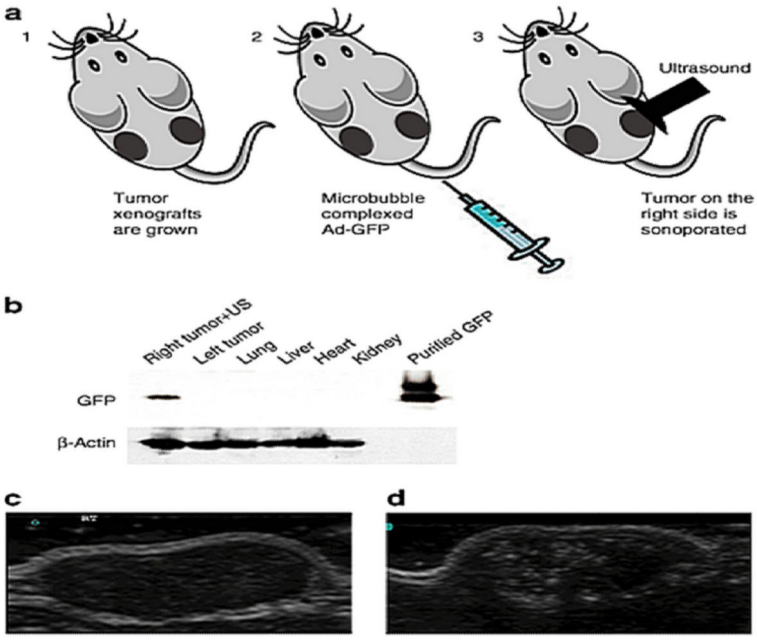

Figure 3.

Microbubble-assisted gene delivery. (a) Schematic representation of the microbubble delivery of Ad-GFP complexes and ultrasound (US) release in a tumor target site of the mouse. (b) Western blotting analysis of Ad-GFP/microbubble–transduced DU-145 tumor xenografts. Immunoblot showing the expression levels of green fluorescent protein (GFP) in DU-145 cells following ultrasound-targeted microbubble/Ad transduction of GFP at 96 hours. Only the tumor on the right flank was sonoporated for 10 min resulting in the delivery and expression of GFP. The left tumor, heart, lung, liver, and kidney were negative for GFP expression. GST–GFP was used as a positive control. Protein gel loading was normalized using β-actin as a control. (c) Ultrasound imaging and US contrast enhancement of in vivo transduced DU-145 tumor xenografts. B-mode US imaging of a tumor before MB contrast agent injection. (d) B-mode US imaging of the same tumor depicted in c following injection of microbubbles/Ad-GFP complexes. MBs cavitation within the targeted tumor dramatically enhances the tumor image within the US field of view. Ad, adenovirus; MB, microbubbles. (Reproduced with permission of the publisher, from Greco et al., 2010.)

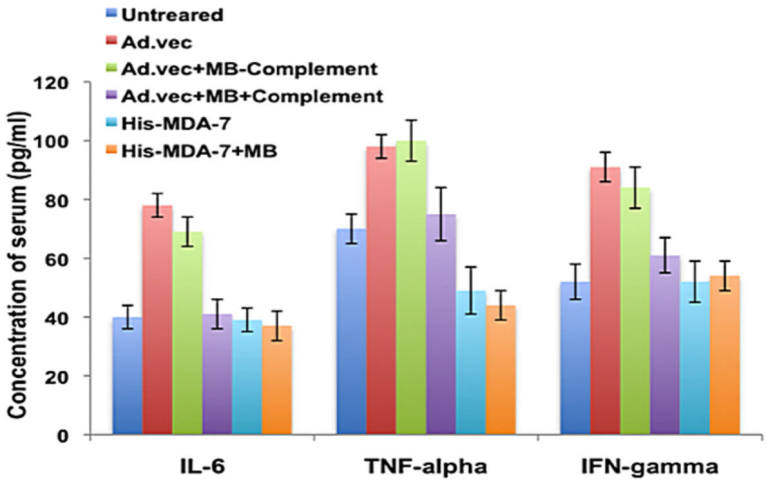

Figure 4.

Microbubble Encapsulated Ads Display Reduced Immunogenicity. The indicated vectors were injected systemically (i.v.) into the tail veins of C57B6 mice (n = 5). Complement (Sigma) was added to one set of the microbubble/Ad complex, whereas another microbubble/Ad complex was not treated with complement. 12 hours after the injections serum was collected from the mice. The indicated cytokines were tested using the Bio-Plex mouse cytokine 23-plexpanel kit with mouse serum samples as described by Bio-Rad Laboratories.