Published in final edited form as:

Ultrasound Med Biol. 2013 December ; 39(12): 2374–2381. doi:10.1016/j.ultrasmedbio.2013.07.017.

ENHANCEMENT OF ADENOVIRUS DELIVERY AFTER ULTRASOUND-STIMULATED THERAPY IN A CANCER MODEL

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Abstract

Improving the efficiency of adenovirus (Ad) delivery to target tissues has the potential to advance the translation of cancer gene therapy. Ultrasound (US)-stimulated therapy uses microbubbles (MBs) exposed to low-intensity US energy to improve localized delivery. We hypothesize that US-stimulated gene therapy can improve Ad infection in a primary prostate tumor through enhanced tumor uptake and retention of the Ad vector. *In vitro* studies were performed to analyze the degree of Ad infectivity after application of US-stimulated gene therapy. A luciferase-based Ad on a ubiquitous cytomegalovirus (CMV) promoter (Ad5/3-CMV-Luc) was used in an animal model of prostate cancer (bilateral tumor growth) to evaluate Ad transduction efficiency after US-stimulated therapy. Bioluminescence imaging was employed for *In vivo* analysis to quantify Ad infection within the tumor. *In vitro* studies revealed no difference in Ad transduction between groups receiving US-stimulated therapy using high, low or sham US intensity exposures at various multiplicities of infection (MOIs) ($p = 0.80$). *In vivo* results indicated that tumors receiving US-stimulated therapy after intra-tumoral injection of Ad5/3-CMV-Luc (1×10^6 plaque-forming units) exhibited a 95.1% enhancement in tumor delivery compared with control tumors receiving sham US ($p = 0.03$). US-stimulated therapy has significant potential to immediately affect Ad-based cancer gene therapy by improving virus bioavailability in target tissues.

Keywords

Cancer; Gene therapy; Microbubble contrast agent; Ultrasound; Adenovirus; Transduction

INTRODUCTION

There is an urgent need to improve delivery of recombinant adenovirus (Ad) to advance cancer gene therapy. Ad vectors have immense potential in cancer therapy because of their ability to infect tumor cells while not injuring healthy tissue. Multiple applications using Ad vectors in cancer have been explored. Therapeutic strategies often involve the triggering of cell death *via* a death-inducing reporter that is specifically driven by a cancer promoter (Choi et al. 2012). Other utilities involve immunotherapeutic approaches aimed at inducing host antitumor immune responses (Lupold and Rodriguez 2005). Direct cancer cell death can be accomplished through delivery of replication oncolytic viruses or non-replicating vectors encoding tumor suppressor genes, suicide genes or anti-angiogenic genes (Kaplan 2005). Tumor cells can be destroyed at both primary and metastatic locations through induction of host antitumor immune responses. Although gene therapy has advanced in the last decade, there are many limitations that prevent routine applications. The effectiveness of gene therapy is directly dependent on successful site-specific delivery (Choi et al. 2013). Limitations of delivery include anti-Ad host immune response, inadequate efficiency of tumor cell transduction and extravasation of the large molecules to their intended site; in addition, the ubiquitous Ad receptor can lead to virus uptake in cell types other than the targeted region (Choi et al. 2012; Fukazawa et al. 2010; Yun 2013). Additional hurdles include the inability to overcome filtration from the liver and the limited infectivity of Ad serotype 5 (Ad5). These limitations lead to necessary advancements in the field of Ad delivery.

Ultrasound (US)-stimulated therapy provides a localized technique to enhance agent delivery. Microbubbles (MBs) are clinically used US contrast agents and have proven to be non-immunogenic and non-toxic in nature (Calliada et al. 1998; Cosgrove 2006). In this unique therapy, US-exposed MBs both increase cell membrane permeability and induce localized molecular extravasation (Dijkmans et al. 2004; Ferrara et al. 2007; Lindner et al. 2004; Song et al. 2002; Sorace et al. 2012b). Although there is some evidence disputing the exact duration of this effect, this therapeutic stimulation has been shown to last up to 30 min post-US exposure (Sorace et al. 2013). Depending on the US setup and variance between US parameters used for US therapy, differences in sustained permeability have been found on the order of minutes to hours, and there have even been permanent non-reversible reactions (Böhmer et al. 2010; Caskey et al. 2009; Chin et al. 2009; Stieger et al. 2007; Tamosiunas et al. 2012). US-stimulated therapy has been increasingly popular in pre-clinical models because it is generally non-invasive and has significant potential for translation. US-stimulated therapy has been found to increase delivery of cytotoxic agents to cancer cells and to improve response by greater than 50% compared with drug alone (Casey et al. 2010; Heath et al. 2012; Sorace et al. 2012b). It has also been found that positive effects can be achieved after only a single dose (Sorace et al. 2013). Other applications of this therapy include delivery of drugs through the blood-brain barrier and enhancement of delivery of DNA (Klibanov 2006; McDannold et al. 2012; Sirsi and Borden 2012; Treat et al. 2012). Studies have also indicated that Ad particles can be safely delivered to a localized region by MB packaging to avoid ubiquitous uptake in other cells and liver filtration (Howard et al. 2006; Warram et al. 2012). Improvement of gene delivery through increased extravasation

can occur by displacing endothelial cells and improving pathways for delivery. Thus, US exposure is a good adjuvant treatment for standard gene and drug delivery. Ultrasonic techniques have been explored as an application in gene delivery through heat-sensitive liposomes and heat shock protein promoters. This approach allows for a triggered release after elevating the tissue temperature with US (Frenkel 2008). Another US-based approach to gene delivery is pre-treatment with pulsed high-intensity focused ultrasound, which allows for increased extravasation. In particular, high-intensity focused ultrasound has been found to be a positive treatment in addition to targeted antibody therapy plus adenovirus delivery, resulting in improved tumor regression (Patel et al. 2007). US-stimulated gene delivery using microbubble cavitation and low-intensity fields has recently been explored to improve oncolytic virus transduction and was found to improve tumor uptake analysis without damaging surrounding tissue (Bazan-Peregrino et al. 2013). To the best of our knowledge, applying MBs and low-intensity US-stimulated therapy to enhance a ubiquitous Ad for tumor delivery is a novel application of this US technology.

To establish genetic-based therapeutics as a routine treatment option for cancer patients, delivery barriers must be overcome (de Vrij et al. 2010). In the study described here, US-stimulated therapy was evaluated for its potential to improve Ad vector transduction in an animal model of prostate cancer. Considering the relative tolerability of US-stimulated therapy, positive evaluation of this technique could immediately affect Ad-based therapy trials, leading to improved treatment success and patient survival.

METHODS

Adenovirus preparation

A non-replicative, luciferase reporter-based, serotype 5 Ad on a ubiquitous cytomegalovirus (CMV) promoter (Ad5/3-CMV-Luc) was used to evaluate Ad transduction because of the ease of bioluminescence imaging for evaluation of response. To improve infectivity by eliminating Coxsackie Ad receptor-mediated infection, the Ad5/3-CMV-Luc contains a chimeric infectivity motif that consists of an Ad serotype 3 knob on an Ad serotype 5 fiber (Borovjagin et al. 2005). For the study, particles were amplified in HEK-293 cells and purified using cesium chloride centrifugation gradients. A standard agarose-overlay plaque assay was conducted with HEK-293 cells to determine a viral titer of 1.1×10^{11} plaque-forming units (PFU) per milliliter.

Cell culture

PC3 human prostate cancer cells were purchased from the American Tissue Type Collection (Manassas, VA, USA). The cell line was maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum and 1% L-glutamine. All cells were grown to 80% to 90% confluence before passaging. Cell numbers were determined using a standard hemocytometer, and cell viability was measured by trypan blue dye exclusion.

In vitro experimentation

Experiment 1. Aliquots (0.1 mL in phosphate-buffered saline [PBS]) of Ad5/3-CMV-Luc were placed in 1.5-mL polypropylene micro-centrifuge tubes at various PFU amounts (0,

3.5, 3.5×10^1 , 3.5×10^2 and 3.5×10^3 infectious virus particles). Groups were then subdivided into low-pressure US, high-pressure US and sham US exposure (control) groups. All groups were evaluated in triplicate. To each tube, 10 μ L of MBs (total of 9.3×10^7 MBs, Definity, Lantheus Medical Imaging, North Billerica, MA, USA) was added immediately before US-stimulated therapy. Groups were exposed to US in a 37 °C water bath with the following acoustic parameters: transmit frequency = 1.0 MHz, peak negative pressures = 0.85 MPa (higher-pressure condition) or 0.1 MPa (low-pressure condition), pulse repetition period = 0.1 s, 10% duty cycle = 10%, duration of US exposure = 5 min. An unfocused, single-element (0.75-in.) immersion transducer (Olympus, Waltham, MA, USA) was placed in series with a signal generator (AFG3002 B, Tektronix, Beaverton, OR, USA) and power amplifier (A075, Electronics and Innovation, Rochester, NY, USA). US intensity measurements were performed in a 37 °C water bath using a hydrophone (Model HGL-0400, ONDA, Sunnyvale, CA) and pre-amplifier setup in series with a digital oscilloscope for voltage signal monitoring and recording. The immersed transducer was manipulated with a precision stepper motor (Velmex, Bloomfield, NY, USA) to locate the spatial peak pressure maximum. Peak negative pressure measurements were determined by converting voltage to pressure measurements using hydrophone calibration data. Tumor and cell samples were placed in the near field.

Adenovirus groups were then added at various multiplicities of infection (MOIs) (0, 0.01, 0.1, 1, and 10) to PC3 cells (3.5×10^2 cells per well in a 24-black-well plate) plated 24 h earlier. Note that an MOI value is defined as the ratio of Ad particles to infectious targets (*i.e.*, number of cells per well). Virus aliquots were allowed to incubate for 1 h and were then removed and replaced with complete medium. After a 24-h incubation period (after Ad vector exposure), luciferin was added and bioluminescence imaging completed. Luciferin (2.5 mg, Caliper Life Sciences, Hopkinton, MA, USA) was diluted into 25 mL of PBS. Medium was removed from the plates and replaced with 1 mL of diluted luciferin (100 μ g) per well to over-saturate the cells. Bioluminescence imaging was performed using the IVIS-100 System (Xenogen, Hopkinton, MA, USA) with an image acquisition time of 300 s (binning of 8 and f/stop of 1) at a fixed stage height. Exposure time was chosen to heighten the signal, while avoiding over-saturation. A region of interest was drawn around each well, and the bioluminescence signal was summarized as total photon counts using equipment software (Living Image 4.2, Xenogen).

Experiment 2. Aliquots (0.1 mL in PBS) of PC3 cells (3.5×10^2) and Ad5/3-CMV-Luc (3.5×10^2 PFU, MOI = 1) in polypropylene micro-centrifuge tubes underwent high-pressure US treatment ($N = 13$) or sham US treatment ($N = 13$) in the presence of MBs in a 37 °C water bath using the same high-pressure US parameters used in experiment 1. After US therapy, cells and Ad were plated in a 24-well plate and then rinsed and replaced with complete medium after 1 h. Twenty-four hours later, plates were imaged for the presence of bioluminescence as described in the previous section.

In vivo experimentation

Animal studies were approved by the Institute of Animal Care and Use Committee at the University of Alabama at Birmingham. PC3 cancer cells (2×10^6 cells per 100 μ L DMEM

without fetal bovine serum) were implanted subcutaneously into the left and right flanks of 5-wk-old nude athymic male mice (Frederick Cancer Research, Hartford, CT, USA) ($N = 24$ animals, $N = 48$ tumors). Tumors were allowed to grow approximately 5 wk to equal tumor size according to caliper measurements (final tumor size = $34.1 \pm 2.8 \text{ mm}^2$). Each animal received a 30- μL (2.8×10^8 MBs) tail vein injection of MBs (Definity) diluted to a final volume of 100 mL with saline. Animals were then submerged in a custom-built 37 °C water bath and remained under isoflurane gas anesthesia for the entire US-stimulated therapy. Two minutes after MB injection, the left flank tumor was exposed to US (at the higher pressure in these experiments, 0.85 MPa). Right flank tumors were not exposed to US. US-stimulated therapy was administered using the previously detailed parameters. This setup allowed exposure of the entire target tumor to US energy while the contralateral tumor was outside the path of US transmission. Immediately after US, the Ad5/3-CMV-uc vector was injected intra-tumorally into both the left and right flank tumors. Animals were divided into three groups and administered different Ad concentrations: 1×10^6 PFU ($N = 12$), $1 \mu 10^7$ PFU ($N = 5$) and 1×10^9 PFU ($N = 7$), which, for the remainder of this article, are referred to as the “low,” “medium” and “high” concentrations of Ad, respectively. Intratumoral doses were administered in a total volume of 50 μL . Animals were imaged for bioluminescence expression before therapy on day 0 (baseline) and again on day 2 (48 h post-therapy) using the following methods. Animals were injected intra-peritoneally with firefly luciferin (2.5 mg), which is catalyzed by luciferase to produce a bioluminescence reporter signal after luciferase gene expression. After a 15-min period allowing for systemic circulation, all animals were oriented so both tumors were visualized and then imaged for bioluminescence expression using the IVIS-100 system (Xenogen Corporation) and established data acquisition protocols. Five animals were imaged simultaneously using a 300-s exposure, f/stop of 1, binning of 8 and fixed stage height. Standardized regions of interest were generated using instrument software to analyze photon counts. Day 0 was used as background signal and was subtracted from the day 2 signals.

Statistical analysis

All experimental data were expressed as means $\pm$ standard errors of the mean and reported as percentage change or bioluminescence count. Analysis of variance was completed to assess differences within *in vitro* data in experiment 1. An unpaired, two-sample *t*-test was used to measure differences in US-stimulated and control cell groups within *in vitro* data in experiment 2. A two-sample paired *t*-test was used to calculate statistical differences between control and US-stimulated tumors within each group. No direct comparisons were made between the various Ad concentration groups. A *p*-value less than 0.05 was considered to indicate statistical significance. Analyses were completed using the SAS statistical software package (Cary, NC, USA).

RESULTS

During *in vitro* experiment 1, various concentrations of Ad particles were subjected to US-stimulated therapy in the absence of cells to determine the effect of US treatment on the infectivity potential of the virus. After US exposure, the Ad was added to plated cells at various MOIs and allowed to incubate. Bioluminescence imaging resulting from successful

Ad infection revealed that there is no significant difference in virus infectivity or vector transduction between exposures to high, low or no (sham) US pressures ($p = 0.80$) (Fig. 1a, c). Only control and high-pressure therapy are illustrated, as exposure to low pressure also results in no qualitative or quantitative differences. The importance of this finding is that it confirms that exposure of the Ad vector to the US intensity levels necessary for inducing membrane permeabilization during US-stimulated therapy has no negative effect on the infectivity potential of the Ad. The various MOIs tested confirm that this observation was consistent across various concentrations of Ad.

For *in vitro* experiment 2, Ad particles were subjected to US-stimulated therapy in the presence of PC3 cells (MOI = 1) to determine the effects of US treatment on cellular response to infection. The control group contained Ad5/3-CMV-Luc, cancer cells and MBs without US treatment. Figure 1b and d illustrate that there was no difference in bioluminescence expression after Ad infection between the therapy group ($5.4 \times 10^5 \pm 1.9 \times 10^4$ counts) and the control group ($5.3 \times 10^5 \pm 2.0 \times 10^4$ counts) ($p = 0.92$). These results suggest that there was no cellular internalization of the Ad. US-stimulated therapy had neither negative nor positive effects when applied to a combination of MBs, cells and Ad.

Ultrasound-stimulated gene therapy effects on Ad5/3-CMV-Luc transduction in an *in vivo* model of prostate cancer were also analyzed. Bilateral flank tumors were to provide *in situ* control tumors that did not were not exposed to US. After US-stimulated therapy of treatment tumors, Ad5/3-CMV-Luc was immediately administered to the three animal groups *via* an intra-tumoral injection using low, moderate and high concentrations of Ad. Forty-eight hours after treatment, the low concentration therapy group had a $95.1 \pm 35.1\%$ increase in bioluminescence expression compared with the control group ($p = 0.03$). At the moderate concentration, the therapy group exhibited a $12.1 \pm 6.4\%$ increase compared with the control, trending toward significance ($p = 0.06$). Finally, at the high concentration, the therapy group exhibited no difference ($10.1 \pm 22.8\%$ increase) compared with the control group ($p = 0.09$) (Fig. 2a). The average photon/second tumor expressions for the different concentrations are (therapy) and 157.1 (control) for low, 629.1 (therapy) and 546.8 (control) for moderate and 212,877.9 (therapy) and 154,899 (control) for high. Qualitative analysis revealed the visual differences in overall bioluminescence expression between treated and control tumors receiving low, moderate and high Ad concentrations, at baseline and 48 h. Figure 2b comprises representative bioluminescence images illustrating enhancement of infectivity at 48 h in the tumors subjected to US-stimulated therapy, as compared with contralateral control tumors and baseline images. Further analysis of the low Ad concentration group revealed that at baseline, there were no statistical significant differences between control tumors and tumors subjected to US therapy ($p = 0.27$). Individual analysis of the animals in the group administered the low concentration of Ad indicates that in 75% of the animals infectivity increased, and in 17% there was relatively no change (which is defined as less than 20% change as compared with the control); one animal had a negative response (Fig. 3).

DISCUSSION

Enhanced tissue-specific delivery of Ad-based vectors has the potential to significantly improve current cancer gene therapy protocols. The strategies investigated here represent original advances in delivery of Ad to intended regions to improve virus transduction within the target tumor tissue. The *in vitro* studies confirmed that Ad infectivity was not affected by either the high or low US pressure condition (frequency = 1.0 MHz). Although in the approach used in this study the virus was injected after US-stimulated therapy, evaluation of the direct effects of US-stimulated therapy on Ad particles was necessary for future translation and investigations. We can thus be confident that in future studies, administration of multi-dose or intra-tumoral injections before therapy will not alter the gene therapy vector during exposure to the US energy levels detailed in this article. Another limitation of the *in vitro* studies is that there is a difference in the US intensity that reaches the cells *in vitro* and *in vivo* because of the polypropylene tubes that house the cells in suspension. This was also calculated with hydrophone measurements, and adjustments *in vitro* have to be made accordingly to equalize the pressures received. Differences in intensity have been found to decrease by 15%–50% when cells in suspension versus monolayers are analyzed (Sorace et al. 2012a). It is difficult to directly compare *in vitro* and *in vivo* conditions in any study; therefore, assumptions are made.

Internalization of the Ad vector, which ultimately leads to tissue infection, is triggered by interaction of the viral penton with epithelial integrins. From there it is processed to the nucleus and is eventually expressed (Lupold and Rodriguez 2005). The Ad requires receptor-mediated internalization; therefore, intra cellular delivery through membrane permeabilization could decrease expression (Greber et al. 1993). *in vitro* experiments indicated that increased Ad transduction did not occur and therefore was not due to increased infection by altering the mechanisms of the adenoviral process or internalization. This is consistent with previous studies analyzing gene transfer with US-stimulated therapy (Miller and Quddus 2000; Price et al. 1998). Bioluminescence signal measurements between *in vitro* experiments indicated the precision of infection at a MOI of 1. US alone has the capacity to increase the temperature of the surrounding medium, which could potentially alter infection rate; however, there was no change in water bath temperature as monitored throughout each US treatment session. No differences were found in Ad infectivity *in vitro* when US-stimulated therapy was applied directly to cells. Previous experiments had indicated no decreases in cell viability under similar US-stimulated conditions (Sorace et al. 2012b). The MOI of 1 was chosen for this experiment so as not to over-saturate the cells to more accurately evaluate and quantify interactions between cells and Ad. Considering that receptor-mediated internalization is required for successful virus infection, US-induced internalization would not lead to reporter transduction. Therefore, observation of a decrease in bioluminescence signal within the US group would indicate that the viral particles were sequestered inside the cell and not available for traditional infection. Because no such observation was made, it was concluded that US-stimulated internalization did not occur. It is proposed that the large size of the Ad vector compared with drug molecules decreases its ability to be internalized through a membrane permeabilization effect of US-stimulated therapy.

Detailed *in vivo* studies evaluated enhancement of Ad delivery after US-stimulated therapy in an animal model of prostate cancer. US-stimulated therapy is effective because when induced with lower-intensity US parameters, MBs mechanically oscillate and interact with endothelial cells. This massaging mechanism allows for displacement of cells and breaking of gap junctions, allowing further permeability and extravasation into the tumor (Frenkel 2008). The greatest enhancement of Ad transduction was observed at the lowest vector dose, whereas little or no change was observed at the highest doses. At the highest dose, Ad availability at the cancer cell level was not a boundary for transduction because of the high concentration (tissue saturation) of the Ad vector. Considering that the purpose of the study was to highlight enhancement of transduction by US-stimulated therapy, the lowest dose provided the greatest potential for improvement. Although 75% of the animals studied had a positive outcome when administered a low concentration of Ad in combination with US-stimulated therapy, one animal had a decrease in Ad tumor delivery. The negative response in this animal could possibly be the result of poor intra-tumoral injection. An additional source of variation within the group could be the variability between tumor vascularity and differences in necrotic regions within the tumor. Nevertheless, the use of this promising US technology would improve the bioavailability of low Ad vector doses and allow a lower dose to achieve the same therapeutic effect as a high dose. This outcome could help reduce toxic effects in patients, which currently hinder widespread use of gene therapy techniques in the clinic. As opposed to intra-venous injections, intra-tumoral injections can offset the limitations of adenoviral gene delivery such as the anti-Ad host immune response and the ubiquitous Ad receptor leading to adenoviral uptake in all cell types.

Several studies detailed in the literature have investigated enhancement of gene transfection efficiency with US-stimulated delivery in various tissue types. Specifically, with plasmid DNA integrated into a MB shell, once the injected agents reached the target tumor tissue, high-intensity US energy destroys the MB and triggers localized payload (DNA) delivery (Sirsi et al. 2012). Using bioluminescence imaging techniques, this group was able to determine a significantly higher region of expression within the tumor than in normal tissue. Research analyzing the longitudinal effects of antiangiogenic gene therapy on hepatocellular carcinoma revealed a significant decrease in micro-vessel density and increase in apoptosis using US-stimulated therapy with plasmid compared with plasmid alone (Nie et al. 2008). Various other studies have investigated US-stimulated delivery of genetic material in the heart (Bekeredjian et al. 2005; Shohet et al. 2000; Tsunoda et al. 2005), pancreas (Chen et al. 2006), skeletal muscle (Wang et al. 2005; Zhang et al. 2006), kidney (Koike et al. 2005), central nervous system (Shimamura et al. 2005) and solid tumors (Nie et al. 2008; Wang et al. 2009; Warram et al. 2012).

Phase I and II trials to evaluate Ad vector delivery in humans are underway. Ad vectors are being explored because of their high transduction efficiency compared with a retrovirus or lentivirus. Phase II clinical studies on Ad-based prostate-specific antigen (PSA) vaccine are also being conducted. The PSA vaccine has been deemed tolerable with minimal toxic effects compared with more conventional anti-cancer drugs, and the investigators hope that the Ad vector will produce immunity to the PSA and destroy cancer cells producing PSA (NCT00583024) (Department of Defense 2007 [cited from 2013]). Along with the vaccines

that are being studied, there is also a phase I trial using an Ad5.SSTR/TK.RGD gene therapy vector, which is an infectivity-enhanced Ad that expresses a therapeutic thymidine kinase suicide gene and a somatostatin receptor (SSTR) for imaging patients with recurrent gynecologic cancer. When used in combination with a chemotherapeutic drug (ganciclovir), this novel Ad vector has been found to induce cancer cell apoptosis (Kim et al. 2012). This particular study used an Ad to image gene transfer and monitor therapeutic response and represents one of the first studies of its kind to prove tolerability and efficacy in humans. Notwithstanding, these authors noted that further refinements in enhancing Ad vector infectivity are needed. Incorporation of US-stimulated gene therapy may help overcome this problem.

The ability to protect Ad vectors from systemic clearance and liver retention while enhancing their bioavailability within the tumor is an important advancement in gene delivery to target tumors. Previous research has indicated that US-stimulated therapy can enhance delivery of both drugs and plasmids for cancer treatment. To the best of our knowledge, this article represents the first study using US-stimulated therapy to improve delivery of Ad to the target tumor. Our result illustrating that Ad infection can be considerably enhanced after a single session of US-stimulated therapy is a significant finding in the field of cancer gene therapy and warrants further investigation.

Acknowledgments

The authors thank Anton Borovjagin, School of Dentistry at the University of Alabama at Birmingham (UAB), for the generous donation of the Ad vector used in this article. This research project was supported in part by Grant \PC111230 from the Prostate Cancer Research Program of the Department of Defense and a pilot award from the Department of Radiology at UAB.

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Fig. 1.

Representative bioluminescence images of plated cancer cells incubated with adenovirus (Ad) vectors after exposure to high-pressure ultrasound (US)-stimulated therapy or sham (control) US (a). Detailed analysis of the bioluminescence signal (counts) representing Ad infection efficiency after the Ad vector was exposed to acoustic conditions akin to those used during US-stimulated therapy (c). Sham or US exposure at low or high acoustic pressures did not produce any differential effects on or alterations in Ad infectivity potential. US-stimulated therapy of cancer cell cultures incubated with an Ad vector resulted in no significant differences in bioluminescence images (b) or Ad infectivity rates (d), which again indicates US had no negative effects on Ad vector infectivity or the transduction pathway. Illustrated here are means $\pm$ standard errors of the means. MOI = multiplicity of infection.

Fig. 2.

Ultrasound (US)-stimulated therapy improves delivery of the adenovirus (Ad) vector to target flank tumors, especially at low Ad concentrations (a). Representative bioluminescence images at baseline and 48 h after US-stimulated therapy for various Ad concentrations denoted as low, moderate and high (b). US-stimulated therapy produced an increase in bioluminescence signal measurements (*via* increased Ad infection) over contralateral control tumors at various Ad concentrations. Illustrated here are means $\pm$ standard errors of the means.

Fig. 3.

Analysis of individual animals in the low adenovirus (Ad) concentration group, which exhibited the greatest enhancement after ultrasound (US)-stimulated therapy. Of the animals investigated, 75% of the tumors produced increased bioluminescence signals compared with contralateral tumors. These findings were attributed to improved Ad retention and infectivity in the target tumor receiving US-stimulated therapy.