Adenovirus-mediated Heat-activated Antisense Ku70 Expression Radiosensitizes Tumor Cells in Vitro and in Vivo

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ABSTRACT

Ku70 is one component of a protein complex, Ku70 and Ku80, that functions as a heterodimer to bind DNA double-strand breaks and activates DNA-dependent protein kinase. Our previous study with Ku70−/− and Ku80−/− mice, and cell lines has shown that Ku70- and Ku80-deficiency compromises the ability of cells to repair DNA double-strand breaks, increases radiosensitivity of cells, and enhances radiation-induced apoptosis. In this study, we examined the feasibility of using adenovirus-mediated, heat-activated expression of antisense Ku70 RNA as a gene therapy paradigm to sensitize cells and tumors to ionizing radiation.

First, we performed experiments to test the heat inducibility of heat shock protein (hsp) 70 promoter and the efficiency of adenovirus-mediated gene transfer in rodent and human cells. Replication-defective adenovirus vectors were used to introduce a recombinant DNA construct, containing the enhanced green fluorescent protein (EGFP) under the control of an inducible hsp70 promoter, into exponentially growing cells. At 24 h after infection, cells were exposed to heat treatment, and heat-induced EGFP expression at different times was determined by flow cytometry. Our data clearly show that heat shock at 42°C, 43°C, or 44°C appears to be equally effective in activating the hsp70 promoter-driven EGFP expression (>300-fold) in various tumor cells. Second, we have generated adenovirus vectors containing antisense Ku70 under the control of an inducible hsp70 promoter. Exponentially growing cells were infected with the adenovector, heat shocked 24 h later, and the radiosensitivity determined 12 h after heat shock. Our data show that heat shock induces antisense Ku70 RNA, reduces the endogenous Ku70 level, and significantly increases the radiosensitivity of the cells. Third, we have performed studies to test whether Ku70 protein level can be down-regulated in a solid mouse tumor (FSa-II), and whether this results in enhanced radiosensitivity in vivo, as assessed by in vivo tumor colony formation and by the tumor growth delay. Our data demonstrate that heat-shock-induced expression of antisense Ku70 RNA attenuates Ku70 protein expression in Fsa-II tumors, and significantly sensitizes the Fsa-II tumors to ionizing radiation.

Taken together, our results suggest that adenovirus-mediated, heat-activated antisense Ku70 expression may provide a novel approach to radiosensitize human tumors.

INTRODUCTION

Two distinct processes involving DNA DSBs have been identified in mammalian cells: the repair of DNA damage induced by IR and other insults, and V(D)J recombination during T- and B-cell development. Recent evidence indicates that DNA-PK, activated by DNA-DSB and other insults, and V(D)J recombination during T- and B-cell development, is a central component in both DSB repair and V(D)J recombination. Recent evidence indicates that DNA-PK, activated by DNA-DSB and other insults, and V(D)J recombination during T- and B-cell development, is a central component in both DSB repair and V(D)J recombination. Recent evidence indicates that DNA-PK, activated by DNA-DSB and other insults, and V(D)J recombination during T- and B-cell development, is a central component in both DSB repair and V(D)J recombination. Recent evidence indicates that DNA-PK, activated by DNA-DSB and other insults, and V(D)J recombination during T- and B-cell development, is a central component in both DSB repair and V(D)J recombination.
be purified to high titers; the strains commonly used to construct recombinant viruses are well characterized; and their genome rarely integrates into the host chromosome, thus making them suitable in applications that require efficient and transient expression of vector-borne genes. The recombinant viruses are propagated in special cell lines that express the deleted viral genes in trans, so that viral particles can be made and isolated, but are not themselves capable of replication when infecting nonpermissive cells. Furthermore, the well-characterized adenovirus shuttle vectors and the 293 cells used to produce nonreplicating adenovirus vectors are commercially available.

In this study, we evaluate the potential of a gene-therapy approach to enhance the response of tumors to IR. The hypothesis is that heat-induced overexpression of antisense Ku70 RNA would down-regulate the cellular level of Ku70 and impede the process of DNA repair, thereby enhancing the sensitivity of human tumor cells to IR. We tested this hypothesis in vitro (cell culture) and in vivo (rodent) systems using adenoviral-mediated transfer of an antisense Ku70 under the control of the heat-inducible hsp70 promoter, followed by heat and radiation treatment. Our data demonstrates that heat-induced expression of antisense Ku70 RNA attenuates Ku70 protein level, and significantly sensitizes the FSa-II tumors to both in vitro and in vivo. These results support that adenovirus-mediated, heat-activated antisense Ku70 expression may provide a novel approach to radiosensitize human tumors.

MATERIALS AND METHODS

Cell Culture. For stable transfection experiments, Rat-1 rat fibroblast cells were used. These cells were routinely cultured in DMEM (Life Technologies, Inc.) supplemented with 10% bovine serum and antibiotics (30). For experiments designed to evaluate the heat inducibility of the hsp70 promoter and the efficiency of adenovirus-mediated gene transfer, several human and mouse tumor cell lines were used. The human lines included human squamous cell carcinoma FaDu, prostate carcinoma Du-145, prostate adenocarcinoma PC-3, and glioma U-87MG cells. These cells were purchased from American Type Culture Collection, and routinely cultured in McCoy’s 5A medium containing 15% FCS and antibiotics.

For experiments designed to test whether adenovirus-mediated heat activated antisense Ku70 RNA radiosensitizes tumor cells in vitro and in vivo, mouse tumor FSa-II cells were used. Mouse tumor FSa-II cell lines were derived from the fourth generation tumors of a spontaneous mouse fibrosarcoma FSa-II (31, 32). Routinely, these cells were maintained in McCoy’s 5A modified medium supplemented with 10% fetal bovine serum plus antibiotics, FSa-II cells from the 5th to the 25th passages were used for in vitro experiments.

Heat Shock, Radiation, and Cell Survival. For heat-shock experiments, monolayers of cells were heated in hot water baths in specially designed incubators (33, 34). For γ-ray irradiation, cells were irradiated with a Cs-137 unit (Mark 1 model 68; JL Shephard and Associate, San Fernando, CA) at a dose rate of ~2.5 Gy/min. Survival studies were performed as described previously (33, 34). In brief, cells were trypsinized immediately after irradiation, counted, serially diluted, and plated in 60-mm Petri dishes. After incubation at 37°C for 7–8 days, colonies were fixed, stained, and counted. Surviving fractions were normalized by the plating efficiency. All of the experiments were performed at least three times to ensure that consistent results were obtained.

Antibodies, Preparation of Cell Lysates, and Immunoblots. Protein samples (50 μg) were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The membrane was incubated with antibody as specified, followed by secondary antibody conjugated with horseradish peroxidase. Specific antigen-antibody complexes were detected by enhanced chemiluminescence (Pierce Chemical Co.). The affinity-purified monoclonal antibody 3H10 specific to the Ku70 protein was from Stress Gen. Secondary antibodies were from Boehringer Mannheim.

Preparation of RNA and RT-PCR. Total RNAs were prepared from cultured cell lines and tumor tissues by RNA zol B (TEL-TEST, INC.). Total RNA (2.5 μg) was then heated to 65°C in a mixture with 50 ng of our vector primer 5′-TTCGATTTCTGACGTACGG-3′. After heating, the RNA sample was cooled on ice. Then, 500 μM each of deoxynucleotide triphosphate, 1× reverse transcriptase buffer and 1 μl Moloney murine leukemia virus reverse transcription (NEB) were added to the reaction mixture. The reaction mixture was incubated at 37°C for 1 h then heated to 65°C for 10 min to denature the reverse transcriptase. PCR was performed according to the instructions of the High Fidelity PCR Kit (Boehringer Mannheim). Briefly, 100 ng of cDNA were amplified in a reaction mixture containing 150 ng of vector primer 5′-TTCGATTTCTGACGTACGG-3′ and Ku70 primer 5′-GGTG-GAGTATCGGAAGAGG-3′. The reaction condition was: 94°C for 2 min, 30 cycles of 30 s at 94°C, 30 s at 58°C and 3 min at 72°C, followed by an extension of 7 min at 72°C. PCR products were then separated in a 1% agarose gel, and visualized with ethidium bromide.

Construction of Stable Expression Vector and Adenovirus Vector. The plasmids and 293 cells used to produce nonreplicating adenovirus vectors were obtained from Microbitx Inc. (Toronto, Ontario, Canada). To obtain the adenoviral vector containing the heat-shock promoter and human antisense Ku70, mouse hsp70 heat-shock promoter was obtained with Xhol and HindIII digestion from plasmid Nluc (35, 36) and inserted into the pEGFP-1 (Clontech Laboratories, Palo Alto, CA) digested with the Xhol and HindIII. The EGF epitope was replaced with human antisense Ku70 by BamHI and NotI. This expression vector (named pshs70-εKu70) was used for stable transfection of the Rat-1 cell line. The fragment containing the mouse heat-shock promoter and antisense Ku70 was then remove by Xhol and Xbal, and inserted into the adenovirus shuttle plasmid pΔE1s1PA predigested with Xhol and Xbal. Adenovirus shuttle plasmids containing the antisense Ku70 expression cassettes were cotransfected (calcium phosphate) with plasmid pJM-17 into 293 cells (37). Recombination of the expression cassettes from the shuttle plasmids into pJM-17 yielded viral DNA of package-able size that produced adenovirus capable of replicating in 293 cells. Transfected 293 cells were overlaid with agarose to permit isolation of individual virus plaques. Adenoviral vectors expressing the EGFP under the control of the hsp70 promoter were generated as described previously (18).

Virus Clone Selection, Amplification, and Freezing. Virus clones were selected as agarose plugs of individual plaques, and ~10% of the virions from each plaque were used to infect 293 cells in a 60-mm culture dish. The 293 cells were harvested when they were cytopathic, pelleted, suspended in 5 ml of DMEM/F12 containing 5% heat-inactivated horse serum and 10% glycerol, and then frozen at −70°C. The cells were later thawed and virions freed by three freeze-thaw cycles using, alternately, liquid nitrogen and a 37°C water bath. Cell lysates were clarified by centrifugation at 14,000 × g for 10 min at 4°C. After being separated from the pellet, the lysate was stored as aliquots, one of which was later thawed to titrate the virus preparation (37). Each virus sample was tested and selected for use in experiments based on their ability to infect 293 cells and express the antisense Ku70 RNA under the hsp70 promoters.

Selected clones were additionally amplified to produce sufficient viruses for experiments by infecting larger quantities of 293 cells (100 100-mm culture dishes) at an MOI of 0.3. Cells were harvested when ~80% of the culture exhibited cytopathic morphology. After three freeze-thaw cycles, the cell lysates were centrifuged on cesium chloride step gradients at 60,000 × g for 2 h at 20°C (38) to separate viruses from defective particles and empty capsids. Recovered virus bands were dialyzed overnight into PBS (PBS pH 7.4, 0.15 M NaCl, 0.0017 M KH2PO4, and 0.005 M Na2HPO4) Glycerol was then added to 10%, and aliquoted virus suspensions were frozen and stored at −80°C. Again, one aliquot was thawed and used to titrate the virus preparation.

Adenovirus Infection of Experimental Cells. Exponentially growing cells were trypsinized from monolayer cultures, resuspended in DMEM/F12 containing 5% heat-inactivated horse serum (1.5 × 106 cell/ml), pelleted in 15 ml polypropylene centrifuge tubes (5 min at 1200 rpm), and resuspended in the same medium at a density of ~1 × 107 cells/750 μl. The appropriate amount of adenovirus was then added (MOI = 30 or 50), the centrifuge tube was capped tightly and made watertight with a paraffin film, and each tube was mounted horizontally into a rack submerged in a 37°C water bath. The cell suspensions were agitated gently for 2 h by attaching the rack to a wrist-action shaker. Afterward, the cells were diluted into growth medium and then seeded into 35-mm culture dishes such that the cells would be at 80–85% confluency when assayed.

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Generation of Stable Cell Lines Containing Antisense Ku70, and Evaluation of Heat-induced Down-Regulation of Ku70 and Radiosensitization in Vitro. We also constructed expression vectors containing the Ku70 gene in the antisense orientation and under the control of the hsp70 promoter (hsp70/-Ku70). Rodent (e.g., Rat-1) cells were transfected with the constructs and stable cell lines (individually isolated colonies) generated. Using each of these cell lines, the following series of experiments were conducted. First, the dose response and time course of heat-shock induction of antisense Ku70 RNA expression were examined to determine the optimal protocol for subsequent experiments. Cells in monolayers were heated at 43 °C for 30 min, and then incubated at 37 °C for various periods (0, 2, 4, 6, 8, 10, 12, and 24 h). At the end of each incubation period, we determined antisense Ku70 RNA expression (RT-PCR) and Ku70 protein level (Western blot analysis). In subsequent experiments, cell samples were pretreated using the optimum heat-shock and post-treatment time (for the production of maximal antisense Ku70 RNA and the lowest level of Ku70 protein), then irradiated with graded doses of y-ray and their survival evaluated by colony formation assay. The survival curves of control cells were also obtained for comparison.

Verification of the Efficacy of the Adenovirus-mediated Gene Transfer Technique. As a prelude to the use of gene transfer approaches to optimize heat-activated antisense Ku70 expression, we performed in vitro experiments to verify the method of adenovirus-mediated gene transfer using the reporter gene EGFP. Replication-defective adenovirus vectors, containing EGFP and under the control of a hsp70 promoter, were generated in 293 cells with titers ranging from 1.5 to 5 × 10^11 pfu/ml. Exponentially growing human glioma U-87MG, prostate carcinoma Du-145, prostate adenocarcinoma PC-3, and pharyngeal squamous cell carcinoma FaDu cells were infected with the adenovirus vectors (MOI = 50), and then incubated at 37 °C for 24 h. Cells were then heated at 42 °C for 60 min, 43 °C for 30 min, or 44 °C for 30 min, and returned to 37 °C incubation. At different times after heat shock, cells were trypsinized, prepared as single cell suspension, and fixed with formaldehyde (5%). The heat-induced EGFP expression was analyzed by flow cytometry and normalized to that of unheated controls. Experiments were repeated at least twice to ensure reproducibility.

In Vitro Modulation of Ku70 Level via Adenovirus-mediated Gene Transfer, and Evaluation of Heat-induced Ku70 Down-Regulation and Radiosensitization. As described previously, replication-defective adenovirus vectors containing the Ku70 gene in the antisense orientation and under the control of hsp70 promoter were constructed. Recombinant viruses were generated in 293 cells, purified, and titer determined (2.6 to 6 × 10^11 pfu/ml). Exponentially growing FSA-II cells were infected with these adenoviruses, heat shocked at 24 h after infection, and then returned to 37 °C for various times (e.g., 0, 2, 4, 6, 8, 10, 12, and 24 h). At each time point, one group of cells was used to verify the heat-induced Ku70 antisense RNA production and a second group (Ku70 protein level). At 12 h after heat shock (the optimal time for maximum reduction of Ku70 protein), a third group was exposed to 4, 8, and 12 Gy for survival determination by colony formation assay, and compared with that of controls.

In Vivo Modulation of Ku70 Level via Adenovirus-mediated Gene Transfer Technique, and Evaluation of Heat-induced Ku70 Down-Regulation and Radiosensitization. FSA-II, a murine fibrosarcoma that arose spontaneously in a female C3H/He mouse, was used for our study (32). Tumors were grown in the thighs of C3H/He mice by injection of 2 × 10^6 cells in 5 μl. The doubling time in vivo is ~19 days. When the FSA-II tumors had grown to a diameter of 6–8 mm, the adenovirus vectors in a volume of 40 μl were injected intratumorally using a Hamilton microsyringe with a 26-gauge needle at a viral dosage of >1 × 10^10 pfu/tumor. Four injections, 10 μl of viruses per track, were used to improve the distribution of the viruses within the tumor. Glioma and prostate adenocarcinoma tumors were heated to 42.5 °C for 30 min by immersing the tumor-bearing leg in a circulating water bath. At 8, 12, or 24 h after the 42.5 °C heat shock, animals were sacrificed and the tumors excised. Each of the tumors was used for RT-PCR to detect the heat-activated Ku70 antisense expression and the other half for Western blot analysis to determine the level of Ku70 protein.

For the evaluation of the radiation response of the adenovirus-infected tumors, in vivoin vitro colony formation and tumor growth delay assays were carried out. In the in vivoin vitro colony formation assay, treated tumors were excised, minced, and trypsinized. The cell suspensions were filtered, centrifuged, and suspended in McCoy’s 5A modified medium supplemented with 10% fetal bovine serum and antibiotics. Tumor cell concentration was determined by counting the cells using a hemocytometer and then serially diluted, and a known number of cells were plated in 60-mm Petri dishes with 10^5 preirradiated (50 Gy) feeder cells. After 37 °C incubation for 8–10 days, the colonies were fixed, stained, and counted. Surviving fraction is defined as cloning efficiency of cells from treated tumor divided by the cloning efficiency of cells from control tumors. Experiments were repeated three times to ensure reproducibility.

For tumor growth delay end point, each tumor was measured in three orthogonal dimensions (a, b, and c), three times a week until the average diameter exceeded 33 mm. Tumor volume was calculated as ab/c and plotted as a function of time after treatment. Six animals were used for each dose group. Experiments were performed at least twice to ensure the reproducibility of the data.

RESULTS

Construction of Ku70 Antisense Vector and Stable Transfection into Rat-1 Cells. To evaluate the feasibility of using heat-activated expression of antisense Ku70 RNA as a strategy to sensitize tumor cells to IR, we constructed an expression vector (hsp70/-Ku70) containing the Ku70 gene in the antisense orientation under the control of a mouse hsp70 promoter. Using DNA-mediated gene transfer technique, we stably transfected Rat-1 cells with this construct and derived stable cell lines. At 37 °C, the Ku70 level is identical in the parental Rat-1 cells and the stably transfected Rat-1 cells (e.g., Rat-1–7 cells, a cloned cell line that contains the antisense Ku70 under the control of a hsp70 promoter). However, when Rat-1–7 cells were heat shocked at 43 °C for 30 min and then incubated at 37 °C for 0, 4, 8, 12, 24, 36, and 48 h, the antisense Ku70 RNA was significantly induced, with maximum induction between 8 and 12 h (Fig. 1A). In parallel, by 12 h the Ku70 protein level was attenuated to ~15% of the level in control untreated Rat-1–7 cells (Fig. 1B). Concomitantly, the radiosensitivity of the Rat-1–7 cells was increased significantly (Fig. 1C). This increased radiosensitivity was not because of hyperthermic radiosensitization, because the time interval between the two treatments (heat and irradiation) exceeded 10 h (39).

Heat-activated Expression of EGFP from Adenovirus Vector in Human Tumor Cells. To examine the heat inducibility of hsp70 promoter and the efficiency of adenovirus-mediated gene transfer in human tumor cells, replication-defective adenovirus vectors were used to introduce a recombinant DNA construct, containing EGFP under the control of a hsp70 promoter, into human glioma U-87MG, prostate carcinoma Du-145, prostate adenocarcinoma PC-3, and pharyngeal squamous cell carcinoma FaDu cells. At 24 h after infection, cells were heat shocked at 42 °C, 43 °C, or 44 °C, returned to 37 °C incubation for 4, 6, 8, 12, and 24 h, and the heat-induced EGFP expression was determined by flow cytometry and normalized to that of unheated controls (Fig. 2). These data clearly show that the EGFP expression can be induced (>300-fold) and that the hsp70 promoter can be efficiently activated in a variety of human tumor cells. Furthermore, 42 °C is as effective as 43 °C or 44 °C to activate the hsp70 promoter-driven EGFP expression. Similar results were obtained in mouse tumor cells (data not shown).

Adenovirus-mediated Heat-activated Antisense Ku70 Expression Radiosensitizes FSA-II Tumor Cells in Vitro. To examine the effect of adenovirus-mediated, heat-activated antisense Ku70 expression on the radiosensitivity of FSA-II tumor cells, we successfully constructed an adenovirus shuttle plasmid containing the heat-inducible antisense Ku70 and produced the recombinant adenovirus in high titer (~6 × 10^11 pfu/ml). Exponentially growing FSA-II tumor cells were infected with these replication-defective adenovirus vectors (MOI = 30), and 24 h later heated at 43 °C for 30 min (or sham treated) and then returned to 37 °C incubation for 12 h. The heat-
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Fig. 1. Ku70 antisense RNA and Ku70 protein expression in transfected Rat-1→ cells. Rat-1→ (Rat-1 cells stably transfected with human Ku70 gene in the antisense orientation under the control of a hsp70 promoter), were subjected to heat shock at 43°C for 30 min, then incubated at 37°C for 0, 4, 8, 12, 24, 36, and 48 h. Total RNA and protein were prepared at each time point. A, Ku70 antisense (αKu70) expression was determined by RT-PCR. C, control unheated Rat-1→ cells; WT, parental Rat-1→ cells; M, DNA marker. B, Ku70 protein was determined by Western blot analysis and presented as percentage of Ku70 in control cells. β-Actin was used as internal control. No HS, control Rat-1→ cells without heat shock; HS, Rat-1→ cells heat shocked and followed by 12 h incubation at 37°C. C, Rat-1→ cells were heat shocked at 43°C for 30 min, returned to 37°C for 12 h, then subjected to graded doses of γ-ray and survival determined. The survival for Rat-1→ cells after a 43°C, 30 min treatment is ~100%; bars, ±SD.

Adenovirus-mediated Heat-activated Antisense Ku70 Expression Radiosensitizes FSa-II Tumors in Vivo. To modulate Ku70 protein level in vivo, the recombinant adenovirus vectors (>1×10⁹ pfu in 40 μl) were introduced into 6–8-mm diameter FSa-II tumors by four intratumoral injections. At 24 h after infection, the animals were anesthetized, and the tumor-bearing legs heated at 42.5°C for 30 min. Twelve h later, the animals were sacrificed and the tumors excised. As shown in Fig. 4, the 42.5°C heat shock induced antisense Ku70 RNA expression in FSa-II tumors (Fig. 4A) and significantly attenuated Ku70 protein (Fig. 4B).

We then examined whether the down-regulation of Ku70 level would sensitize these tumors to IR. At the optimal time after heat shock, i.e., 12 h (for maximum reduction of Ku70 level), the radiation response of the adenovirus-infected, heat-shocked tumors was assessed. For in vivoin vitro colony formation assay, treated tumors were irradiated with graded doses of γ-ray. These tumors were then excised, tumor cell suspension prepared, and cell number counted using a hemocytometer. One half of the tumor cells were used for the determination of Ku70 level and the other half for survival determination (colony formation assay). As shown in Fig. 4C, the 42.5°C heat shock, which induced antisense Ku70 RNA expression and attenuated Ku70 protein level, significantly sensitized the FSa-II tumors to IR.

For tumor growth delay, 12 h after heat shock, the tumors were irradiated with 30 Gy of γ-ray. Subsequently, each tumor was measured in three orthogonal dimensions (a, b, and c), three times a week until an average diameter exceeded 13 mm. In Fig. 4D the measured tumor volume relative to that at the time of irradiation is plotted as a function of days after irradiation. These data clearly showed that the 42.5°C heat shock, which induced antisense Ku70 RNA expression and attenuated Ku70 protein, prolonged the tumor growth delay significantly.

DISCUSSION

It has now been firmly established that DNA-PK plays important roles in vital cellular processes that involve DNA end joining, especially DNA DSB repair and V(D)J recombination. Recently, using Ku80−/−, Ku70−/−, and DNA-PKcs−/− model systems, we and others have clearly shown that deficiency in any of the three subunits of DNA-PK leads to decreased DNA DSB repair and increased radiosensitivity, both in vitro and in vivo (12, 40, 41). In particular, absence of Ku70 or Ku80 led to a ~2.5-fold increase in the slope of the radiation survival curve. These findings motivated us to design methods to down-regulate the Ku70 level in tumors, which may eventually be translatable to radiotherapy of human cancers and improved clinical outcome.

The use of hyperthermia as an adjuvant in cancer radiotherapy, based on the potentiation of radiation effect by heat treatment, has been the subject of clinical investigation for some time (23, 42, 43).
Thus far, the potential of such synergism has yet to be realized in the clinic because of our inability to deliver sufficient cytotoxic heat dose in a spatially and temporally controlled manner. This is especially true for deep-seated tumors, for which improvement is needed in the equipment used for planning, implementing, and monitoring hyperthermic treatments. Alternatively, improvement may be possible through better understanding of the biology of heat shock in combination with radiation. In this regard, basic science investigation has unraveled important mechanistic knowledge of the heat shock phenomenon, including transcription control, protein trafficking, chaperon function, and the role of hsps in cancer immunotherapy (19).

Relative to the above discussion, our study is of significance in recognizing that the heat shock promoter is a powerful inducer of transcription with potential utility in gene therapy approaches. Also of relevance is that the heat dose, both in terms of the absolute temperatures and the spatial uniformity, for activating the hsp70 transcriptional control may be significantly less stringent than that required for cell inactivation, thus lessening the technical requirement of heat delivery. As is clearly shown in Fig. 2, heat shock at 42°C, 43°C, or 44°C appears to be equally effective in activating the hsp70 promoter-driven EGFP expression. Furthermore, hyperthermia has been used to modify local tumor environment to increase liposomal drug delivery to tumors (44, 45). At temperatures of 41°C-43°C, hyperthermia has been shown to increase blood flow (46) and oxygenation (47). Hyperthermia has also been shown to increase the permeability of tumor vessels to antibodies (48–51). In pilot studies, using replication-defective adenovirus that constitutively expresses EGFP from the cytomegalovirus promoter, we found that a 41°C or 42.5°C heat shock can enhance the virus delivery significantly (data not shown). If this observation can be confirmed in other tumor systems, it would imply that mild heat treatment is an effective method to improve the spatial distribution of the virus delivery to tumors.

Although the use of constitutive promoters may be an alternative approach, the use of heat-shock induction may have several advantages. The application of hyperthermia provides additional geometrical selectivity in the induction of antisense Ku70. Furthermore, as described in the previous paragraph, there is evidence indicating mild

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Fig. 3. Attenuation of the Ku70 protein in FSa-II tumor cells by adenovirus-mediated heat-activated antisense Ku70 expression and their effect on radiation response in vitro. FSa-II tumor cells were infected with adenoviral vectors (MOI = 30) containing the human Ku70 antisense under the control of mouse hsp70 promoter. After virus infection (24 h), cells were exposed to 43°C for 30 min, and returned to 37°C incubation for 0–48 h. Total RNA and protein were prepared at each time point. A, Ku70 antisense expression determined by RT-PCR. C, control cells, no virus, no heat shock. Ku70 antisense (αKu70) was indicated on the left margin, B, the percentage of Ku70 protein expression in infected cells measured at 12 h after heat shock. Ku70 protein expression was determined by Western blot analysis, and β-actin used for internal control. The values shown were normalized to that of the uninfected controls (–/–). –/–: cells with no virus infection and no heat shock; –/+ : cells with no virus infection but heat shocked; +/+ : cells with virus infection but not heat shocked; +/– : cells with viral infection and heat-shocked, the Ku70 protein expression was determined at 12 h after heat shock. C, γ-ray survival curves: [] cells were infected with virus, 24 h after infection cells were heated at 43°C for 30 min, returned to 37°C for 12 h, and then exposed to graded doses of γ-ray. A, infected with virus but not heat-shocked. ■, no virus, no heat shock. ●, no virus, but heat-shocked and irradiated 12 h afterward. The survival for FSa-II cells after a 43°C, 30 min treatment is ~100%; bars, ±SD.

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Fig. 4. Heat-induced antisense Ku70 RNA and Ku70 protein expression in FSa-II tumors, and their effect on radiation response in vivo. Recombinant adenovirus vectors (≥1 × 10⁹ pfu in 40 μl) were introduced into FSa-II tumors (6–8 mm in diameter) by four intratumoral injections. At 24 h after infection, the animals were anesthetized and the tumor-bearing legs heated at 42.5°C for 30 min. Twelve h later, the antisense Ku70 RNA expression, the Ku70 protein level, and the radiation response of FSa-II tumors were determined. A, antisense Ku70 RNA (αKu70) by RT-PCR; B, Ku70 protein expression by Western blot analysis. +/+ : with viral infection and heat shock; –/+ : no viral infection but heat shocked; –/– : control, no viral infection and no heat shock. Radiation response as determined by (C) in vivo/vivo colony formation and (D) tumor growth-delay assay respectively. In C, ● control, no virus, no heat shock; ○ adeno-virus-infected, heat-shocked and irradiated 12 h after heat shock. In D, ○ control, no virus, no heat shock and no irradiation; □ adeno-virus-infected only, no heat shock and no irradiation; ▼ no virus, no heat shock, 30 Gy alone; ▽ no virus, heat-shocked, and irradiated with 30 Gy at 12 h after heat-shock; ■ adeno-virus-infected, heat-shocked and irradiated with 30 Gy at 12 h after heat shock.
hyperthermia may lead to improved viral vector distribution, and the heat-shock promoter is one of the strongest inducer of gene expression.

In this study, we have provided the proof of principle of using adenovirus-mediated, heat-induced activation of antisense Ku70 as a strategy for radiosensitization in vitro and in vivo. First, we have constructed an expression vector containing the Ku70 gene in the antisense orientation under the control of a mouse hsp70 promoter, and have shown that heat-induced activation of antisense Ku70 reduces the cellular level of Ku70 and leads to radiosensitization in stably transfected Rat-1 cells (Fig. 1). Second, we have adopted the use of adenovirus for the delivery of the hsp70 promoter-driven antisense Ku70 expression cassette into rodent tumor cells and have provided data that radiosensitization is achieved (Fig. 3). Third, we have demonstrated that in FSA-II tumors, adenoviral-mediated heat-activated antisense Ku70 RNA attenuates Ku70 protein expression, and significantly sensitizes FSA-II tumors to IR as determined by in vivoin vitro survival and by tumor growth delay. Control experiments indicate that the heat dose, applied by itself (without the adenoviral delivery) 12 h before irradiation, did not lead to radiosensitization. Similarly, the injection of the adenoviral vector by itself (without applying heat) also did not result in radiosensitization. Thus, it is the combined adenoviral and heat application that caused radiosensitization, a result of heat-induced activation of the antisense Ku70 RNA, leading to down-regulation of Ku70 protein level and compromising DNA DSB repair.

We hope that these studies will lead to additional preclinical and clinical trials involving disease sites that can benefit from radiosensitization. Advances in conformal and intensity-modulated radiation therapy have provided a powerful tool in the treatment of cancer by locally irradiating the tumors with minimal damage to healthy tissues. However, a number of tumors, such as glioblastomas, are resistant to radiation. The work presented here is a preclinical feasibility study to increase the sensitivity of radioresistant cells to IR. Translation to a clinical protocol would require much additional thought and consideration. However, it is conceivable that intratumoral injection of the viral vector would be guided by computed tomography images, similar to what is commonly practiced in brachytherapy. Application of hyperthermia would be by radiofrequency antennae along the same needle tracks used for intratumoral injection. If tumor cells in general and radiation-resistant tumor cells in particular could be sensitized by down-regulating the cellular level of Ku70, new approaches could be developed as an adjuvant to radiation therapy.

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