ABSTRACT

Recent developments in the use of small inhibitory RNA molecules (siRNAs) to inhibit specific protein expression have highlighted the potential use of siRNA as a therapeutic agent. The double-strand break signaling/repair proteins ATM, ATR, and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) are attractive targets to confer enhanced radio and chemosensitivity to tumor cells. We have designed and exogenously delivered plasmids encoding siRNAs targeting these critical kinases to human cancer cells to assess the feasibility of this concept as a clinically translatable experimental therapeutic. siRNA led to a ~90% reduction in target protein expression. siRNAs targeting ATM and DNA-PKcs gave rise to a dose-reduction factor of ~1.4 compared with untransfected and control vector-transfected cells at the clinically relevant radiation doses. This was greater than the radiosensitivity achieved using the phosphatidylinositol 3'-kinase inhibitor Wortmannin or DNA-PKcs competitive inhibitor LY294002. A similar increased sensitivity to the alkylating agent methyl methanesulfonate (MMS) was also observed for siRNA-mediated ATR silencing. Together, these data provide strong evidence for the potential use of siRNA as a novel radiation/chemotherapy-sensitizing agent.

INTRODUCTION

RNAi1 was first noted in Caenorhabditis elegans and plants as a novel mechanism of post-transcriptional gene silencing and has since been discovered in many eukaryotes (for reviews, see Refs. 1 and 2). Rapid progress has been made in the use of RNAi and more specifically siRNAs as a means of attenuating the expression of specific proteins both in vitro and in vivo (3–6) enabling any protein target, where the cDNA sequence is known, to be inhibited by these sequence-specific, double-stranded RNA molecules. Highly specialized DNA repair proteins together with upstream sensors and signalers protect mammalian cells from potentially lethal and/or tumorogenic DNA-damaging agents exhibited by cells and animal models developing resistance to DNA damage and repair is highlighted by the extreme sensitivity to DNA-damaging agents exhibited by cells and animal models of DNA-PKcs expression (9–11). Thus, targeted inhibition of these kinases is an attractive approach in the development of potent radiation therapy strategies. To increase the radio- and chemotherapy-mediated cell killing of human tumor cells, we have used a plasmid-based pol III promoter system to deliver and express siRNAs (4) targeted toward ATM, ATR, and DNA-PKcs. Those siRNAs exhibiting the greatest inhibition of target protein expression were used to transfect cells before exposure to DNA-damaging agents and subsequently gave rise to a significant increase in sensitivity to ionizing radiation and alkylating agents compared with empty vector-transfected and untransfected control cells. This work demonstrates the first use of siRNA to augment radiation-mediated killing of human cancer cells and highlights the potential use of siRNA as an adjuvant gene therapy strategy to radiation and chemotherapy. The study presented here provides the necessary basis on which additional developments of siRNA gene therapy vectors can be designed, constructed, and tested in anticipation of clinical translation.

MATERIALS AND METHODS

Cell Culture. DU 145 and PC-3 cells were obtained from the American Type Culture Collection and maintained as adherent monolayer cultures in RPMI culture medium (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Life Technologies). All cultures were grown at 37°C in a humidified atmosphere of 5% carbon dioxide, fed every 5 days with complete medium, and subcultured when confluence was reached.

Plasmids. siRNAs were designed to target the 223–253, 432–462, and 597–627 bp of the ATM mRNA; 134–164, 388–418, and 579–609 bp of the ATR mRNA; and 196–226, 585–616, and 733–763 bp of the DNA-PKcs mRNA sequences with corresponding AUG translation initiation codons of 190, 80, and 58 bp, respectively. The siRNA-encoding complementary single-stranded oligonucleotides, which hybridize to give pSHAG-1 (provided by Dr. Greg Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as described previously (12). Oligonucleotides encoding siRNAs were ligated into pSHAG-1 and pBM-H-compatible overhangs, were designed using the computer program available on the Internet.2 Oligonucleotides encoding siRNAs were ligated into pSHAG-1. The plRE vector (12), which encodes EGFP, was used for cotransfection studies and to assess transfection efficiencies.

Transfection of Cells. A total of 2 × 10⁵ cells was seeded into each well of a six-well tissue culture plate (Falcon). The next day (when the cells were 70–80% confluent), the culture medium was aspirated, and the cell monolayer was washed with prewarmed sterile PBS. Cells were transfected with the appropriate construct using LipofectaminePlus reagent (Life Technologies) according to the manufacturer’s protocol. Green fluorescence of pRE-transfected cells was quantified at each time point by FACS analysis and used to ascertain transfection efficiencies for cells transiently transfected with siRNA-encoding plasmids.

FACS Analysis. For each sample, 1 × 10⁶ cells were analyzed on a LSR flow cytometer (BD Biosciences, San José, CA) with an excitation wavelength of 488 nm and FITC collection wavelength using a band-pass filter at 530 ± 15 nm. Dead cells were gated out of the samples by forward and side scatter. The level of EGFP fluorescence in live cells was determined using the Becton Dickinson CellQuest program. FACS sorting (≥10⁶ fluorescence on a four-log scale) was carried out at the same excitation/emission wavelengths using a BD FACS Vantage SE (BD Biosciences).

Clonogenic Survival.

Clonogenic Survival. At the appropriate time after transfection, cells were trypsinized and diluted to the appropriate cell density into 100-mm culture dishes to give ≥50 colonies/dish after irradiation and then irradiated at 0.78 Gy/min to the desired dose using a Gammacell 40 ¹³⁷cesium irradiator (Atomic Energy, Ottawa, Canada). For MMS clonogenic assays, at the appropriate time

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after transfection, cells were treated for 1 h with MMS solubilized in DMSO and diluted in serum-free media (SFM) or an equivalent percentage of DMSO/SFM as a control, washed twice in PBS, and trypsinized and plated as explained above. Ten days after radiation or drug treatment, colonies comprising 50 cells were counted after staining with 50% Crystal Violet (Sigma-Aldridge). Cell survival was plotted as a function of dose and fitted using the linear quadratic model $S = \exp(-\alpha D - \beta D^2)$, where $S$ is the cell survival, $D$ is the dose of radiation, and $\alpha$ and $\beta$ are constants. DRFs, the factor by which the dose of radiation or drug can be reduced in the presence of the sensitizing agent to achieve the same level of cell killing in the absence of the sensitizing agent, were calculated as the dose required to give 10% cell survival (90% clonogenic cell killing) from fitted clonogenic survival curves for pSHAG-1-transfected and siRNA-transfected cells. DRFs were calculated at 80 and 30% clonogenic cell killing for cells respectively treated with LY294002 and MMS, because the fitted survival curves did not reach 10% cell survival.

**Immunoblots.** Whole cell extracts were separated on 4–15% acrylamide gels (Bio-Rad, Hercules, CA) using standard SDS-PAGE techniques. Antibodies for ATM, ATR, DNA-PKc, and β-actin were obtained from Dr. Michael Kastan (St. Jude Children’s Research Hospital, Memphis, TN), Oncogene Research Products (San Diego, CA), and Sigma Biochemicals (St. Louis, MO), respectively. A total of 2–20 μg of protein extracted from each transfected cell population was loaded onto each gel, electrophoresed at 100 V for 3 h at 4°C, and then transferred overnight at 50 mA, 4°C, onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were probed with primary and secondary antibodies at optimized concentrations, and protein expression was visualized using an enhanced chemiluminescence kit (Amersham-Pharmacia). Membranes probed for ATM, ATR, and DNA-PKc were reb-probed for β-actin to normalize for loading and/or quantification errors and to allow comparisons of target protein expression to be made between transfected and untransfected populations. Protein expression was quantified using a Vespa-Doc gel documentation system (Bio-Rad).

**RESULTS**

siRNA-mediated Down-Regulation of ATM, ATR, and DNA-PKc. For each protein target, we designed siRNA complimentary to three different regions of the corresponding mRNA at increasing distance from the AUG translation initiation codon. Each siRNA was synthesized as complimentary oligonucleotides and cloned in the pSHAG-1 vector. The resulting constructs were then screened for their ability to down-regulate target protein expression. The human prostate cancer line DU 145 was transfected with each pSHAG-1/siRNA construct; protein extracts were obtained from 24–96 h post-transfection, and Western blot analyses were performed for target protein and normalized to β-actin expression. We found that 48 h post-transfection was the time at which greatest down-regulation (≤90% in transfected cells) was observed (Fig. 1). By 96 h post-transfection, target protein levels had risen back to levels comparable

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**Fig. 1. siRNA-mediated down-regulation of ATM, ATR, and DNA-PKc proteins in DU 145 cells after 48-h transfection with siRNA-encoding plasmids.** A, C, and E, Western blots for ATM, ATR, and DNA-PKc, respectively. Membranes were probed with antibodies for target protein, and expression levels were normalized for loading by probing for β-actin. Protein expression for ATM, ATR, and DNA-PKc was quantified using a Bio-Rad Versa-Doc imager and Quantity One analysis software and expressed as a percentage compared with that calculated in untransfected cells (B, D, and F, respectively). Given that the respective transfection efficiencies were calculated as ~45, 40, and 50, ≤90% inhibition of target protein are evident in the transfected population.
with empty vector-transfected and untransfected cells (data not shown). We found that for both ATR and DNA-PKcs, the greatest down-regulation was seen with the siRNA targeting the region closest to the translation initiation sequence (pATR-1 and pDNA-PK-1, respectively), whereas the two regions further downstream in the mRNA sequence gave the highest amount of protein inhibition for ATM (pATM-2 and pATM-3).

**siRNA Silencing of Repair Proteins Renders Human Prostate Tumor Cells Sensitive to DNA-damaging Agents.** Previous studies have demonstrated that loss of function of ATM, ATR, or DNA-PKcs results in increased cellular sensitivity to DNA-damaging agents. To ascertain if siRNA-mediated attenuation of expression of ATM, ATR, and DNA-PKcs results in a subsequent sensitizing effect to such modalities, we transiently transfected DU 145 and PC-3 human prostate cancer cells with the ATM and DNA-PKcs-targeted, siRNA-encoding plasmids that were shown to give the greatest inhibition of target protein expression. At the appropriate time post-transfection where protein levels were shown to be the lowest, we treated the resulting heterogeneously transfected cultures with ionizing radiation. Cellular sensitivity was ascertained by clonogenic survival assays (Fig. 2). siRNA-mediated inhibition of these DNA repair proteins conferred an increased sensitivity to ionizing radiation in siRNA-transfected cell populations compared with untransfected or pSHAG-transfected cells. This increased radiosensitivity corresponded to DRFs of 1.1 and 1.21 for DNA-PKcs silencing in DU 145 and PC-3 cells, respectively, and 1.16 and 1.14 for ATM silencing in DU 145 and PC-3 cells, respectively, with an increase in sensitivity of 1.5–1.8-fold noted at 6 Gy. In addition, DU 145 cells transfected with ATR-targeted, siRNA-encoding plasmids exhibited an increased sensitivity (DRF = 1.38) to the alkylating agent MMS (Fig. 3). In a similar set of experiments, the expression of nontargeted siRNA in these cells failed to result in any evident radiation sensitization (DRF = 0.99, data not shown). Together, these data suggest that transfection and subsequent expression of plasmid-based siRNA does not, itself, result in an altered radiation response phenotype but actually requires specific targeting to produce such phenotypic alteration.

The biologically significant, but modest, degree of radiosensitization observed after transfection of ATM and DNA-PKcs siRNA-encoding plasmids (Fig. 2) is a function of the heterogeneous populations resulting from transient transfection where the transfection efficiencies are ~25–40%. To enrich the transfected population and demonstrate a more representative clonogenic survival of the transfected/siRNA-expressing cells, we cotransfected DU 145 cells with plasmids encoding siRNA-targeting ATM or DNA-PKcs together with a plasmid EGFP and FACS-sorted, EGFP-expressing cells 48-h post-transfection. Western blot analyses of protein extracts from FACS-sorted cells confirmed that siRNA silencing of repair proteins resulted in decreased protein expression compared to controls. These data suggest that siRNA-mediated attenuation of repair proteins can sensitize cells to DNA-damaging agents, providing a potential therapeutic strategy for the treatment of prostate cancer.
sorted cells transfected with siRNA-encoding plasmids confirmed that EGFP-expressing, FACS-sorted cells had vastly reduced target protein expression compared with those deemed untransfected (attributable to a lack of EGFP expression) (Fig. 4, A and D, respectively). Clonogenic survival assays demonstrated that the FACS-enriched ATM and DNA-PKcs siRNA-transfected cells exhibited a substantial increased sensitivity to ionizing radiation (respective DRFs of 1.46 and 1.36, with an increase in sensitivity of ~3-fold noted at 6 Gy) compared with cells transfected with just the EGFP-encoding plasmid or those transfected with pSHAG-1 (Fig. 4, B and E, respectively).

To highlight and further characterize the amount of radiosensitization caused by ATM and DNA-PKcs siRNA, we ascertained the increase in radiosensitivity caused by the PI3k inhibitor Wortmannin, which inhibits ATM, ATR, and DNA-PKcs activity, and the specific DNA-PKcs competitive inhibitor LY294002. DU 145 cells were treated with either DMSO, 10 μM Wortmannin, or 10 μM LY294002 1 h before and 24 h after exposure to ionizing radiation, and cellular radiosensitivity was determined by clonogenic survival assays (Fig. 4, C and F). Treatment with Wortmannin and LY294002 led to an increased radiosensitivity (respective DRFs of 1.4 and 1.1) that was less than that caused by ATM or DNA-PKcs siRNA (DRFs of 1.46 and 1.36; Fig. 4, B and E), thus demonstrating the efficacy of siRNA targeting specific DNA repair factors.

**DISCUSSION**

Proteins involved in the detection, signaling, and repair of DNA damage after exposure to cytotoxic agents are attractive targets when considering exogenous modulation of DNA repair capacity as a means to increase cellular sensitivity to ionizing radiation and/or chemotherapeutic agents. As such, controlled targeted inhibition of the DNA damage signaling/repair factors ATM, ATR, and DNA-PKcs combined with localized conformal radiotherapy or systemic delivery of chemotherapeutic drugs would make an attractive adjuvant gene therapy approach for many solid tumors.

In an attempt to design and develop radiation- and chemo-sensitizing gene therapy strategies, we have assessed the ability of exogenously delivered plasmid-based siRNA to target the protein kinases ATM, ATR, and DNA-PKcs and increase the cellular sensitivity of human prostate cancer cells to DNA-damaging agents. The radiation resistant prostate cancer cells DU 145 and PC-3 were transiently transfected with plasmids encoding siRNA and treated with commonly used classes of cancer therapeutics, namely ionizing radiation and alkylating agents (MMS). Cell survival was determined by clonogenic survival assays. Cells transfected with siRNA-encoding plasmids were rendered sensitive to ionizing radiation (via targeting of ATM and DNA-PKcs) and the alkylating agent MMS (ATR). Thus,
we report the first use of siRNA as a novel approach to modulating cellular responses of human cancer cells to DNA-damaging agents.

Initial screening of siRNA for their effectiveness showed that all three target proteins were down-regulated by $\leq 90\%$ from 24- to 72-h post-transfection (Fig. 1), with protein levels being comparable with the levels seen in untransfected and pSHAG-1-transfected cells at 96 h (data not shown). These findings are consistent with previous data reporting the half-lives of these proteins to be in the region of 24–48 h (13, 14). These findings may also result from loss of expression of the transgene as cells divide or, perhaps, by other unknown cell defense mechanisms.

We demonstrate that siRNA-mediated inhibition in the expression of these target proteins confers an increased sensitivity to therapeutically relevant DNA-damaging agents (Figs. 2 and 3). For ATM and DNA-PKcs, siRNA-mediated attenuation of protein expression was manifested as an increased radiosensitivity for both heterogeneously transfected cells and FACs-enriched cells (respective DRFs of $\sim 1.15$ and $1.4$, with a $\sim 1.5$–$1.8$- and 3-fold increase in radiosensitivity at 6 Gy in heterogeneously and transfected cells, respectively; Figs. 2 and 4). In addition, we show that siRNA-mediated inhibition of ATR expression results in an increased sensitivity to the alkylating agent MMS (Fig. 3). As mentioned previously, we did not see an increased sensitivity to ionizing radiation in either DU 145 or PC-3 cells after transfection of ATR siRNA (data not shown), unlike the slight increased radiosensitivity reported for fibroblasts deficient in ATR (15, 16). The most likely explanation for this difference is that both Chiby et al. and Wright et al. used selected cell populations of which all were ATR deficient, whereas in the transient transfections experiments we describe, there were only $\sim 30\%$ of the cells lacking ATR.

As previous work has demonstrated that such selected fibroblasts clones lacking ATR were more sensitive to the alkylating agent MMS than any other DNA-damaging agents tested (16), we treated DU 145 cells with MMS after transient transfection of ATR siRNA-encoding plasmids. Although DU 145 cells are somewhat resistant to alkylating agents because of a mutation in the mismatch repair protein hMLH1 (17, 18), we show that transfection of only $\sim 50\%$ of cells with an ATR siRNA-encoding plasmid gave rise to an overall decreased cell survival (DRF of 1.38) after treatment with MMS (Fig. 3).

Although the observed increased radiation-mediated cell kill caused by DNA-PKcs and ATM siRNA appears modest (average DRFs of $\sim 1.15$; Fig. 2), it has to be emphasized that the survival curves shown represent a population of cells where only a minority (25–40%) are transfected and, thus, also have depleted levels of each target protein. To address this problem, we cotransfected DU 145 cells with the siRNA-encoding plasmid and a plasmid encoding EGFP, which we used to enrich the transfected population via FACs sorting. Western blot analysis showed that the FACs-sorted cells had a $\sim 80\%$ reduction in both ATM and DNA-PKcs expression (Fig. 4, A and D), which resulted in biologically significant DRFs of 1.46 and 1.36, with a 3-fold increased sensitivity noted at 6 Gy (Ref. 19; Fig. 4, B and E). Furthermore, the radiosensitivity exhibited by the enriched cells was greater than that seen in cells treated with the PI3K inhibitor Wortmannin or specific DNA-PKcs competitive inhibitor LY294002 (respective DRFs of 1.4 and 1.1; Fig. 4, C and F). The use of LY294002 before and after exposure to radiation highlights the increase in radiosensitivity that can be achieved by inhibition of DNA-PKcs activity in DU 145 cells. Moreover, this approach also results in a slightly greater radiosensitization than that seen in normal human fibroblasts transfected with double-stranded siRNA targeting DNA-PKcs (20). Finally, enrichment of ATM siRNA-transfected DU 145 cells gave an increased radiosensitivity that is comparable with previous work using antisense approaches in the similarly radiation-resistant prostate cancer cells PC-3 and glioblastoma cells U-87 (14, 21).

The work presented here demonstrates the first reported use of siRNA as a novel tool for modulating killing of human cancer cells by DNA-damaging agents, including radiation. The inherent specificity of this approach provides a powerful method of target protein down-regulation that can be incorporated into several existing viral and nonviral vector delivery platforms. We are currently studying several such systems and anticipate that the improved delivery of our siRNAs achieved by using one of these systems will aid future studies based on our initial results presented here.

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REFERENCES


Enhanced Radiation and Chemotherapy-mediated Cell Killing of Human Cancer Cells by Small Inhibitory RNA Silencing of DNA Repair Factors
