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

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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

RNAi 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 tumorigenic lesions resulting from DNA damage (for recent reviews, see Refs. 7 and 8). Pivotal to these repair pathways are the DNA damage sensors ATM and ATR and DNA-PKcs. The essential role these proteins play and 8). Pivotal to these repair pathways are the DNA damage sensors

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3 The abbreviations used are: RNAi, RNA interference; siRNA, small inhibitory RNA; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; MMS, methyl methanesulfonate; EGFP, encoding the green fluorescent protein; FACS, fluorescence-activated cell sorter; DRF, dose-reduction factor; PI3K, phosphatidylinositol 3-kinase.

4 Internet address: http://katahdin.cshl.org/9331/RNAi.

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 3 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 Hairpin- and BamHI-compatible overhangs, were designed using the computer program available on the Internet.2 Oligonucleotides encoding siRNAs were ligated into pSHAG-1 (provided by Dr. Greg Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) as described previously (12). Name designation of the resulting plasmids was pATM-1, -2, or -3; pATR-1, -2, or -3; and pDNA-PKcs-1, -2, or -3 based on the target protein and region of mRNA downstream from the AUG codon (1 being the closest to the AUG codon). The pREV vector (12), which encodes EGFP, was used for cotransfection studies and to assess transfection efficiencies.

Transfection of Cells. A total of 2 × 10^6 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 pREV-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 × 10^6 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 (≥10^5 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. At the appropriate time after transfection, cells were trypsinized and diluted to the appropriate cell density into 100-mm culture dishes to give 50–100 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...
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 \( \frac{1}{100} \) of 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

\[
S \exp(\frac{-\alpha D}{D^2})
\]

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 untransfected populations. 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-PKcs, and \( \beta \)-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 \( \mu \)g 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-PKcs were reprobed for \( \beta \)-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 Versa-Doc gel documentation system (Bio-Rad).

**RESULTS**

siRNA-mediated Down-Regulation of ATM, ATR, and DNA-PKcs. 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 (4). 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 \( \beta \)-actin expression. We found that 48 h post-transfection was the time at which greatest down-regulation (\( \leq 90\% \) in transfected cells) was observed (Fig. 1). By 96 h post-transfection, target protein levels had risen back to levels comparable
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). In addition, transfection of cells with siRNA plasmids that effectively reduced ATR protein levels also failed to enhance radiosensitivity (DRF = 0.98, 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 cultures showed similar results to those observed in DU 145 and PC-3 cells transfected with siRNA encoding the targeted regions. Furthermore, cotransfection of cells with siRNA plasmids that effectively reduced ATR protein levels also failed to enhance radiosensitivity (DRF = 0.98, 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.
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 irradiation (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). DU 145 cells were transfected with either an EGFP-expressing plasmid alone (Untransfected) or together with pSHAG-1 and pATM-2 (A–B) or pDNA-PK-1 (D–E) and FACS-sorted 48-h post-transfection to enrich the transfected population. FACS-sorted cells were immediately seeded for clonogenic survival assays (B and E), and the remaining cells were used to obtain protein extracts for Western analyses (A and D). Target protein expression is plotted as the normalized expression in EGFP +ve-sorted cells compared with EGFP −ve-sorted cells. C and F, clonogenic survival curves for DU 145 cells respectively treated with DMSO or high, nontoxic concentrations of the PI3k inhibitor Wortmannin or specific competitive DNA-PKcs inhibitor LY294002 1 h before and 24 h after irradiation.
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 $\approx 90\%$ 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 $\approx 1.15$ and 1.4, with a $\approx 1.5–1.8$- 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 $\approx 30\%$ of the cells lacking ATR.

As previous work has demonstrated that such selected fibroblasts clones lacking ATR were most 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 $\approx 50\%$ 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 killing caused by DNA-PKcs and ATM siRNA appears modest (average DRFs of $\approx 1.15$; 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-separated cells had a $\approx 80\%$ 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 $\approx 3$-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 P33i3k 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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