p21WAF1/CIP1 Antisense Therapy Radiosensitizes Human Colon Cancer by Converting Growth Arrest to Apoptosis

Hui Tian, Ellen K. Wittmack, and Timothy J. Jorgensen

Department of Radiation Medicine, Lombardi Cancer Center, Georgetown University Medical Center, Washington, D.C. 20007-2197

ABSTRACT

Substantial evidence suggests that loss of cellular p21WAF1/CIP1 results in increased apoptotic killing by ionizing radiation. We hypothesized that a p21 antisense (AS) oligodeoxynucleotide (ODN) could be used to sensitize cancer cells to radiotherapy. In vitro treatment of colon cancer cells (HCT116/p21−/−) with p21 AS ODN (200 nM) led to inhibition of radiation-induced p21 expression (>95% inhibition, 0–30 Gy), resulting in a loss of G1 arrest and an enhancement of apoptosis to comparable levels and with similar kinetics to HCT116/p21+/− cells (~68% apoptotic cells at 96 h after 10 Gy). In vivo, p21 AS ODN in combination with radiation (i.p. ODN for 6 days at 20 mg/kg/day and 15 Gy) increased apoptosis in s.c. p21+/− tumors in nude mice to levels similar to those of p21−/− tumors (two-fold at 24 h postirradiation) and improved radiocurability of p21−/− tumors to levels comparable to those of p21+/− tumors (p21−/−, two of nine cures versus p21+/−, two of nine cures). Our findings suggest that p21 AS treatment may be a rational approach to improve conventional radiotherapy outcomes.

INTRODUCTION

Resistance to radiotherapy is a common clinical problem for human malignant tumors. For wild-type p53-expressing tumors, radiation-induced cell cycle arrest, as an alternative to apoptosis, may be a major component of the resistance mechanism (1, 2). The p21WAF1/CIP1 gene is transcriptionally activated by p53 and is responsible for the p53-dependent checkpoint that results in G1 arrest after DNA damage (3–5). It has been reported that cells deficient in p21 display a significantly different response to DNA-damaging agents than cells with an intact p21-dependent checkpoint (6). Cancer cells with an intact p21-dependent checkpoint undergo a G1 arrest after DNA damage caused by ionizing radiation or chemotherapeutic drugs, whereas cells with a defective p21 response undergo apoptosis. Detailed analyses have demonstrated that the apoptosis is apparently induced by an uncoupling between mitosis and S phase after DNA damage (1). Instead of undergoing coherent arrest, cells without the p21-dependent checkpoint continue to undergo rounds of DNA synthesis in the absence of mitosis, culminating in apoptosis (1).

In a recent study, Waldman et al. (1) demonstrated that loss of p21 in human colon cancer cells resulted in a tremendous enhancement of radiation-induced apoptosis and was associated with improved radiocurability when the same cells were grown as s.c. tumors in nude mice (2). These findings prompted our interest in targeting p21 as a means of improving cancer radiotherapy. Although there are technical obstacles to long-term inhibition of gene expression in tumors, radiosensitization would presumably only require inhibition for a relatively short period during and after radiation exposure. For this reason, we thought that AS ODNs might provide inhibition of sufficient duration to demonstrate the feasibility of using DNA damage-inducible signals to sensitize irradiated cells to additional treatment. We felt that these studies would provide a useful baseline for future experiments.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Wild-type HCT116 human colon carcinoma cells (HCT116/p21+/+), which express normal p53 and p21, and a derivative in which both p21 alleles had been deleted through homologous recombination (HCT116/p21−/−) were kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins Oncology Center, Baltimore, MD; Ref. 6). The cell lines were maintained in monolayer culture in McCoy’s 5A modified medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Irradiations. All cell and tumor irradiations were performed using a J. L. Shepared Mark I 137Cs irradiator (San Fernando, CA) at 1.5–3.0 Gy/min.

ODNs. An AS ODN targeted against a sequence in the 3′-untranslated region of human p21 mRNA, as described previously by Poluha et al. (3), was synthesized, fully phosphorothioated, and purified by a proprietary six-step procedure, including two high-performance liquid chromatography steps, cross-flow dialysis, and ultrafiltration by BIOGNOSTIK (Göttingen, Germany). A computer-generated RD sequence ODN of the same length and GC content as the corresponding AS sequence was used as a control. The sequences of the ODNs were as follows: (a) p21 AS sequence (AS), 5′-TGTCATGCGTCTCGGCCGC-3′; and (b) RD control to AS (RD), 5′-CCGCTGAACCAGCGACCA-3′.

Transfection of ODNs. Cells at a density of 5 × 104 cells/ml were plated for overnight incubation. Before the addition of ODNs to exponentially growing cells on tissue culture plates, the cells were replenished with fresh McCoy’s 5A medium with 5% fetal bovine serum. Cells then were treated with ODNs for 4 h in the presence of 5 μg/ml Lipofectin (Life Technologies, Inc., Gaithersburg, MD). After 4 h of incubation at 37°C, ODNs were taken up by more than 80% of the cells, as determined by a fluorescent FITC-labeled RD control ODN. The medium was then removed and replaced with fresh complete medium. The cells were incubated for an additional 20 h. The cells were then exposed to 137Cs γ-irradiation, and a second ODN incubation was performed. Drug treatment was performed 4 h after the second ODN incubation.

Western Blot Analysis. Cell extracts were prepared at various time points after irradiation, and proteins were separated by 15% SDS-PAGE, followed by a Western blot analysis using a mouse monoclonal antibody specific for human p21 (WAF-1, Ab-1; Oncogene Research Product, Cambridge, MA) at a con-
RESULTS

In Vitro Inhibition of Radiation-induced p21 Induction by p21 AS ODN. Treatment of p21+/+ cells with various concentrations of p21 AS ODN, ranging from 50–400 nM, resulted in a concentration-dependent inhibition of radiation-induced p21 expression, whereas the RD control ODN did not inhibit p21 (Fig. 1a). Even at 50 nM, p21 AS ODN prevented p21 from rising above basal levels; at 400 nM, it led to almost a complete block of expression of p21, whereas the RD control ODN, even at 400 nM, produced no inhibition of p21 induction. No change in GAPDH expression was observed (Fig. 1a), and growth rates were determined by measuring three orthogonal diameters of each tumor twice per week, and the tumor volume was estimated by the formula (D1 × D2 × D3)/2. When tumors reached an average volume of approximately 100 mm³, the tumor-bearing animals were randomized into three different treatment arms. ODNs, formulated in normal saline solution at 4 mg/ml, were administered i.p. once daily for 6 consecutive days. Tumors were irradiated on the fourth day of ODN treatment to a dose of 15 Gy. The tumor from one mouse of average tumor size from each group was isolated for Western blot analysis at 24 h postirradiation and isolated for TUNEL assay at 24 h and 120 h postirradiation. Animal studies were done in accordance with a protocol approved by the Georgetown University Animal Care and Use Committee.

Evaluation of the Effects of ODNs in Vivo. HCT116/p21−/− or HCT116/p21+/− cells (5 × 10⁶) suspended in McCoy’s medium were injected s.c. in the sacral region of 6–7-week-old female BALB/c nu/nu (nude) mice. Tumor growth rates were determined by measuring three orthogonal diameters of each tumor twice per week, and the tumor volume was estimated by the formula (D1 × D2 × D3)/2. When tumors reached an average volume of approximately 100 mm³, the tumor-bearing animals were randomized into three different treatment arms. ODNs, formulated in normal saline solution at 4 mg/ml, were administered i.p. once daily for 6 consecutive days. Tumors were irradiated on the fourth day of ODN treatment to a dose of 15 Gy. The tumor from one mouse of average tumor size from each group was isolated for Western blot analysis at 24 h postirradiation and isolated for TUNEL assay at 24 h and 120 h postirradiation. Animal studies were done in accordance with a protocol approved by the Georgetown University Animal Care and Use Committee.
the cationic lipids alone, which were used as a transfection vehicle, did not affect GAPDH protein levels (Fig. 1a, Lane 2). The level of p21 induction was radiation dose dependent, and p21 AS ODN inhibited induction at all radiation doses tested (Fig. 1b).

**Disabling of Radiation-induced G₁ Arrest by p21 AS ODN.** p21-mediated G₁ arrest after DNA damage is the hallmark of wild-type p53-expressing cells (6). For this reason, we sought to determine whether p21 AS ODN could inhibit radiation-induced G₁ arrest. This was accomplished by treating cells with p21 AS ODN in combination with radiation and then analyzing by flow cytometry for reduced transit from G₁ to S phase (i.e., G₁ arrest), as measured by a depletion of the S-phase fraction. This assay was repeated in duplicate for each treatment group and cell line. Representative data are shown (Fig. 2). At 24 h postirradiation, untreated p21⁺/⁺ cells displayed a decreased portion of cells in S phase after irradiation (8.9% versus 38.1% for unirradiated cells), consistent with G₁ arrest. This was accompanied by an increased fraction of cells in G₂, suggesting that both radiation-induced G₁ and G₂ arrest were functional in these cells (Fig. 2, a and b). In p21⁻/⁻ cells, however, irradiation did not alter the S-phase fraction (47.3% for unirradiated cells versus 49.5% for irradiated cells; Fig. 2, a and b), which is consistent with previous reports of defective G₁ arrest in this cell line (1, 6). Additionally, there were fewer cells in G₂, consistent with a recent report that p21 also plays a role in G₂ arrest (8). When p21⁺/⁺ cells were treated with p21 AS ODN-treated cells and irradiated, a relatively high fraction of cells (30.0%) were in S phase after irradiation compared with cells treated with RD control ODN (13.2%), suggesting that G₁ arrest had been inhibited by AS treatment. Although inhibition was not complete (unirradiated AS ODN-treated cells showed as high as 41.6% of cells in S phase), this is probably due to the fact that not all of the cells were transfected by the ODN, which is consistent with FITC-labeled ODN uptake experiments that showed 80% transfection efficiency (see transfection methods, above).

**Enhancement of Radiation-induced Apoptosis by p21-antisense-ODN in Vitro.** Exposure of p21⁺/⁺ cells to 10 Gy of radiation led to only 13.8% of cells undergoing apoptosis at 96 h postirradiation, whereas 58.7% of p21⁻/⁻ cells underwent apoptosis (Fig. 3b). A total of 200 nm p21 AS ODN increased apoptosis in the irradiated p21⁺/⁺ cells to the same levels (66.3%) and with the same kinetics as the p21⁻/⁻ cells, but RD ODN did not (12.6%). p21 AS ODN itself caused little apoptosis in unirradiated cells (12.5%). Thus, p21 AS ODN successfully converted a radioresistant phenotype to a more sensitive one in terms of apoptosis (Fig. 3a and b), which is considered a major mechanism by which cells are killed by radiation.

**p21-antisense ODN Increased Radiocurability in p21⁻/⁻ Tumors.** Because of the encouraging data in vitro, we tested the in vivo efficacy of p21 AS ODNs using a nude mouse xenograft model, in which the p21⁺/⁺ cells were injected s.c. in nude mice to produce tumors that were then treated with p21 AS ODNs in combination with radiation.

Tumors were treated with i.p. injections of ODNs once daily for 6 consecutive days. On the fourth day of treatment, the tumors were irradiated once with 15 Gy. p21 AS and RD ODN at 5 mg/kg/day were compared with or without irradiation for p21⁺/⁺-derived tumors. p21⁻/⁻-derived tumors were irradiated as a positive control. Of the nine radiation-treated animals with p21⁻/⁻ tumors, all showed growth delay, and two were cured of their tumors. None of the animals with p21⁺/⁺ tumors (35 tumor-bearing animals among five treatment arms) showed ODN-mediated growth delay or cure (data not shown), yet neither was there any apparent toxicity caused by ODN treatment. For this reason, we conducted a second experiment in which we escalated the p21 AS ODN dose from 0 to 20 mg/kg/day (Fig. 4a–d). Again, we did not see growth delay or toxicity associated with p21 AS ODN treatment, but we did see cures: 1 of 8, 2 of 7, and 2 of 8 animals for 5, 10, and 20 mg/kg/day, respectively (Fig. 4, b–d). These cure rates were similar to what we had seen for the p21⁻/⁻ tumors (Fig. 4e) and consistent with the cure rate (5 of 13 animals) for 15 Gy that was reported by Waldman et al. for p21⁻/⁻ tumors (2).

**Inhibition of Radiation-mediated p21 Induction and Enhancement of Radiation-induced Apoptosis by p21 AS ODN in Vivo.** Western blot analysis of tumor samples 24 h postirradiation showed that p21 protein was very highly induced in samples of p21⁺/⁺ tumors (Fig. 5a, Lane 1 versus Lane 2). An inhibition of p21 induction was found in p21 AS ODN-treated tumors (Fig. 5a, Lane 2 versus Lane 3). This was accompanied by a near doubling of the apoptotic index from 5.1% to 9.0% at 24 h postirradiation (Fig. 5c). In comparison, p21⁻/⁻ tumors treated with radiation had an apoptotic index of 9.5% at 24 h postirradiation, suggesting that p21 AS ODN administration in vivo achieved almost maximal potentiation of radiation-induced apoptosis at this time point (Fig. 5c, hatched bars). Similar patterns of radiation-induced apoptosis were also seen at 120 h postirradiation (Fig. 5c). Thus, p21 AS ODN induced a p21⁻/⁻ apoptotic radiation phenotype in tumors derived from p21⁺/⁺ cells.

**DISCUSSION**

Many malignant tumors display resistance to radiotherapy. Thus, there is great interest in determining the regulatory events involved in this radioresistance, so that they might be targeted for an improved radiotherapeutic effect. Resistance to cancer radiotherapy can occur in...
many ways. One protective mechanism in wild-type p53-expressing tumors is thought to involve radiation-induced cell cycle arrest, which allows the cells to repair damage before cell division proceeds (2, 9).

Apoptosis, or programmed cell death, is an important lethal response of cells to ionizing radiation that has been studied extensively in recent years (10–14). Consequently, much new information about its underlying mechanisms has emerged. This provides opportunities to explore the potential of using molecular mediators of apoptotic processes as targets to enhance radiotherapeutic toxicity to tumors.

In this regard, we now know that many apoptotic responses are p53 mediated (13–15). However, the dual role of p53 in both cell cycle arrest (a protective response; Refs. 2 and 6) and apoptosis (a lethal response; Refs. 2, 16, and 17) remains to be sorted out (18–20), and it is unclear how modulation might affect radiation resistance for any particular tumor type.

p21 is an inhibitor of cyclin-dependent kinase that is transcriptionally activated by p53 (4, 5, 21). Activation of p21 is thought to be essential for G1 arrest in cells, and it has been shown that mutation of p21 in tumors both abrogates radiation-induced G1 arrest and enhances radiation-induced apoptosis, presumably as a consequence of cell cycle progression under a sustained damage burden (1, 6, 22). Several lines of evidence suggest that p21 protects cells against DNA damage-induced, p53-mediated apoptosis (22–25).

We chose AS ODNs as a practical means to target p21 for downregulation both in vitro and in vivo. We attempted to show that AS inhibition of p21 could inhibit radiation-induced p21 protein expression and G1 arrest, resulting in enhanced apoptosis in a human colon cancer cell line, HCT116. We chose this cell line because of the availability of a matched cell line that was null in p21 (6), thereby affording a positive isogenetic control for tumor cell radioresponses in the absence of p21 expression. Additionally, the cell cycle profile of these cell lines had been previously well characterized (1, 2, 6).

We found that p21 AS ODNs were highly effective in inhibiting radiation-induced p21 expression in vitro, and that inhibition reduced G1 arrest in a population of cycling cells to a level consistent with the ODN transfection efficiency of those cells. We also saw a maximal increase in apoptosis among those cells when compared to cells that completely lacked p21 expression (i.e., HCT116/p21−/−).

We extended our in vitro findings to the same cell lines in vivo by growing them as s.c. tumors in nude mice and measuring both p21 expression and apoptosis in irradiated tumors. Waldman et al. (2) had contended that the improved radiocurability of p21−/− tumors was due to the enhanced apoptosis that they saw in vitro (1), but they had never actually measured the apoptotic responses in tumors themselves, and others have questioned that enhanced apoptosis was involved (26).

Western blots of tumor extracts showed that p21 AS ODN was effective in reducing p21 levels in irradiated tumors, although not as strongly as in vitro. It is not clear whether this decreased efficiency was due to an overall reduced uptake of ODNs throughout the tumor or whether only a subfraction of cells took up ODNs. We speculate that the latter may be the case because, despite a low overall inhibition of p21, an increase in apoptotic cells was seen. The enhancement of tumor apoptosis by p21 AS ODN was relatively high at 24 h postirradiation (experimental day 9). Because ODN treatment was terminated at experimental day 6, there may have been some recovery of p21 function by the later apoptotic time point. Being that p21−/− tumors had significantly greater apoptosis 120 h postirradiation, a longer treatment with p21 AS ODN might have more closely mimicked the p21−/− tumor response and produced a greater tumor growth delay. Nevertheless, an improvement in radiocurability was seen. Although it is unclear how enhanced cure is achieved without enhanced growth

Fig. 4. The effect of p21 AS ODN on growth of irradiated s.c. tumors in nude mice. Tumors were treated with 15 Gy in combination with p21 AS ODN at 0, 5, 10, and 20 mg/kg/day according to the protocol described in “Materials and Methods.” Individual tumors volumes were measured and plotted as shown, with each animal represented by a different symbol. The relative volumes of cured tumors (i.e., no measurable tumor) were included as 0.001 for the purposes of plotting on logarithmic scale. The cure rate (CR), defined as the number of animals without detectable tumor at the end of observation period (>11 weeks) relative to the total number of animals in the treatment group, is shown at the bottom left corner of each panel. Response of individual p21−/− tumors is shown for comparison. P = 0.22 for the effect of p21 AS ODN on cure rate, by single-tailed x2 test.
were isolated and sectioned at 24 h (15 mg/kg/day). As depicted is 24 h postirradiation (15 Gy) treated in combination with p21 AS ODN (20 μg/kg/day) as a counterstain to identify total nuclei (left panel), in which apoptotic cells appear bright. DAPI staining of protein lysates from tumor samples taken 24 h postirradiation. p21 AS ODN (20 μg/kg/day) at 20 mg/kg/day are compared with normal saline (NS), with and without irradiation (15 Gy) in HCT116/p21+/- and HCT116/p21-/- tumors. a, representative micrograph (×20) of apoptotic cells detected by TUNEL assay in vivo, quantitation of apoptosis detected by TUNEL assay in vivo, c, quantitation of apoptosis detected by TUNEL assay in vitro. The tumors were isolated and sectioned at 24 h and 120 h postirradiation (15 Gy). Samples at 24 h post irradiation were analyzed by Western blot analysis of protein lysates from tumor samples taken 24 h postirradiation. p21 AS ODN (20 μg/kg/day) is used to damage cells (data not shown). Others have shown that p21-deficient cells are also sensitive to camptothecin (2), etoposide (2), and cisplatin (27). Ruan et al. (28) have shown that p21 AS-expressing adenovirus can sensitize human glioma cells to cisplatin.

Fig. 5. The effect of p21 AS ODN on radiation-induced p21 induction and apoptosis in vivo as shown by Western blot and TUNEL staining. a, Western blot analysis of protein lysates from tumor samples taken 24 h postirradiation: p21 AS ODN (AS) at 20 mg/kg/day is compared with normal saline (NS), with and without irradiation (15 Gy) in HCT116/p21+/- and HCT116/p21-/- tumors. b, representative micrograph (×20) of apoptotic cells detected by TUNEL assay (left panel), in which apoptotic cells appear bright. DAPI was used as a counterstain to identify total nuclei (right panel). The p21+/- tumor sample depicted is 24 h post irradiation (15 Gy) treated in combination with p21 AS ODN (20 mg/kg/day). c, quantitation of apoptosis detected by TUNEL assay in vivo. The tumors were isolated and sectioned at 24 h and 120 h post irradiation (15 Gy). Samples at 24 h post irradiation correspond to those used in Western blot analysis shown in Fig. 5a. The percentages of apoptotic cells from p21+/- tumor and p21-/- tumor produced by various treatments are based on the counting of TUNEL-positive cells relative to total DAPI-stained nuclei from the images.

delay, it is important to note that Waldman et al. (2) likewise showed significant improvement in radiocurability of p21-/- tumors at 7.5 Gy relative to p21+/- tumors at 15 Gy, although the p21+/- tumors showed a greater growth delay. Thus, growth delay may not be a good predictor of radiocurability in this xenograft model.

The χ² P for the cure rates (0.22) falls short of statistical significance. Nevertheless, when tumor results showing p21 inhibition, enhanced apoptosis, and increased radiocurability are taken together, they strongly suggest that AS effects comparable to those seen in vitro are occurring in vivo as well. With an improved ODN/radiation dose regimen and better ODN uptake, these AS effects might very well be exploited for clinical benefit.

This study differs from many other ODN inhibition studies in that the target protein is being tremendously induced by the lethal stimulus. In this regard, targeting of p21 might seem to be a formidable task for an ODN-based strategy, yet our results clearly show that near total inhibition of p21 can be achieved at ODN concentrations comparable to those used for inhibiting constitutively expressed proteins. We interpret this to be a demonstration that high-level inducibility of a protein is not necessarily a disqualification for an AS ODN therapeutic approach.

With regard to the reduced efficiency of p21 inhibition in tumors, it is important to note that in vitro, a pre- and postirradiation incubation with p21 AS ODN with lipid-based transfection was required for maximal ODN uptake and p21 inhibition. In vivo, we were able to simulate the duration of exposure by treating for 6 consecutive days, with radiation delivered midway. It is unclear whether the lipid-free i.p. injections that we used were capable of achieving a comparable level of ODN uptake to that required for p21 inhibition in vivo.

Although this work has used radiation and a colon cancer carcinoma cell line as a model system, we do not believe that this approach is limited to radiation or colon cancer. We have been able to achieve similar inhibition of p21 induction and enhanced radiation-induced apoptosis with the same in vitro transfection protocol in human prostate cancer LNCaP cells, and we have been able to demonstrate similar inhibition of G1/G0 block when Adriamycin, rather than radiation, is used to damage cells (data not shown). Others have shown that p21-deficient cells are also sensitive to camptothecin (2), etoposide (2), and cisplatin (27). Ruan et al. (28) have shown that p21 AS-expressing adenovirus can sensitize human glioma cells to cisplatin.

There is some debate about the relationship between radiation-induced apoptosis and radiocurability of tumors because in vitro clonogenic survival can be uncoupled from apoptotic responses (2, 26). Nevertheless, it has been clearly demonstrated in the HCT116/p21-/- xenograft model that enhanced apoptosis is associated with tumor cure after radiation treatment (1), and, at least for this model system, a high apoptotic index is a good prognostic indicator. Although others have recently reported that p21 AS ODNs can inhibit radiation-induced growth arrest in squamous cell carcinoma cells (29), we believe our study to be the first to show that p21 AS can convert growth arrest into apoptosis as a strategy to enhance cancer radiotherapy.

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