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Radiosensitization of human cervical cancer cells by inhibiting ribonucleotide reductase: Enhanced radiation response at low-dose-rates

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Abstract

Purpose—To test whether pharmacologic inhibition of ribonucleotide reductase (RNR) by 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC #663249) enhances radiation sensitivity during low-dose-rate ionizing radiation provided by a novel purpose-built iridium-192 cell irradiator.

Methods and Materials—Cells were exposed to low (11, 23, 37, 67 cGy/hour) dose-rate radiation using a custom-fabricated cell irradiator or to high (330 cGy/min) dose-rate radiation using a conventional cell irradiator. Radiation sensitivity of human cervical (CaSki, C33-a) cancer cells with or without RNR inhibition by 3-AP was evaluated by clonogenic survival and RNR activity assay. Alteration in cell cycle distribution was monitored by flow cytometry.

Results—Increasing radiation sensitivity of both CaSki and C33-a cells was observed with incremental rise in radiation dose-rates. 3-AP treatment led to enhanced radiation sensitivity in both cell lines, eliminating differences in cell cytotoxicity due to radiation dose rate. RNR blockade by 3-AP during low-dose-rate irradiation was associated with low RNR activity and an extended G1-phase cell cycle arrest.

Conclusions—We conclude that RNR inhibition by 3-AP impedes DNA damage repair mechanisms that rely on deoxyribonucleotide production, and thereby increases radiation sensitivity of human cervical cancers to low-dose-rate radiation.

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Keywords

Triapine; cervical cancer; ribonucleotide reductase; radiosensitization

Introduction

After exposure to ionizing radiation (IR), mammalian cells benefit from integrated molecular pathways both that recognize the need for DNA repair and that halt or slow cell cycle progression until the DNA damage has been repaired. The DNA damage response mechanism involves proteins in the base excision repair pathway responsible for the removal of oxidized bases after IR (1). Deoxyribonucleotide triphosphates (dNTPs) used in the repair of DNA damage either can be salvaged from deoxyribonucleosides, drawn from limited intracellular pools which may include oxidized bases (2), or synthesized *de novo* through ribonucleotide reduction (3), which may filter out oxidized bases from dNTP pools (2). The process of repair of IR-mediated DNA damage occurs rapidly in normal and cancer cells, often being essentially complete within three hours (4,5). As such, therapeutics expected to modify repair of radiation-induced DNA damage, and thus enhance radiation sensitivity, should be expected to have their effect during this three-hour period of repair.

The *de novo* synthesis of dNTPs has been suggested to be important in the cellular response to IR-mediated DNA damage (6–8). Formation of new dNTPs depends upon reduction of ribonucleotide diphosphates to deoxyribonucleotide diphosphates, a reaction catalyzed by ribonucleotide reductase (RNR). Mammalian RNR is formed by homodimeric M1 subunits with an active catalytic site, and by either homodimeric M2 or p53R2 (M2b) subunits carrying a free radical critical for catalysis (9,10). Cells restrict RNR activity through S-phase-specific transcription of the M2 gene, binding of dNTP allosteric effectors to the M1 protein, M2 degradation by APC/Cdh1 in late mitosis, p53-dependent p53R2 transcription, and functional inhibition of RNR by p53 protein-protein binding interactions (10–14).

It has been shown that RNR activity increases to supply required dNTPs for DNA damage repair (6,7). Pharmacologic blockade of RNR by the anti-tumor drug 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, NSC #663249) sustains IR-mediated DNA damage, promotes a G1-phase cell cycle arrest, and enhances radiation toxicity in cervical cancer cells (6,7). Because these effects have been demonstrated only in cell culture systems exposed to high radiation dose-rates (i.e., centigray per minute), there is a clear need to study radiobiological phenomena under other clinically relevant radiation dose-rates, in particular those used during brachytherapy (i.e., centigray per hour). We therefore tested the hypothesis that the RNR inhibitor 3-AP enhances low-dose-rate radiation sensitivity through a mechanism of protracted G1-phase arrest due to inactivated RNR. In this study, we also investigate the radiobiological applicability of a purpose-built low-dose-rate cell irradiator for experimental study of human cancers.

Methods and Materials

Cell cultures and chemicals

Two cervical cancer cell lines were used: HPV-16 positive, wt-p53 CaSki cells (15) and HPV-naïve, mutated p53 (mut-p53, codon 273 Arg-Cys) C33-a cells (16), obtained from American Type Culture Collection (Rockville, MD). CaSki cells were cultured in RPMI 1640 medium (Grand Island, NY), supplemented with 10% fetal bovine serum, L-glutamine, and 1% penicillin/streptomycin. C33-a cells were propagated in Eagle's minimum essential medium (Grand Island, NY), supplemented with 10% fetal bovine serum, sodium bicarbonate, 1mM sodium pyruvate, and 1% non-essential amino acids.

Chemicals used were purchased from Sigma (St. Louis, MO), unless otherwise specified. 3-AP (NSC #663249) is an investigational agent supplied under a Material Transfer Agreement involving Case Western Reserve University (Cleveland, OH), Vion Pharmaceuticals, Inc. (New Haven, CT) and the National Cancer Institute Cancer Therapy Evaluation Program (Bethesda, MD).

Radiation Treatment

Figure 1 is a schematic illustration of the low-dose-rate irradiator. An incubator (NAPCO 5400, General Laboratory Supply [Pasadena, TX]) capable of maintaining cell cultures at 37°C in a humidified 5% CO₂ atmosphere was outfitted with a 25 cm × 25 cm custom-fabricated acrylic template containing 20 machined parallel grooves 1 cm apart. For our study, the center 14 machined grooves each held a single ribbon of iridium-192 (Ir-192) radioactive seeds ($\Gamma=4.6 \text{ Rcm}^2/\text{mCi/hr}$). Ir-192 ribbons (0.2 cm) contained 14 radioactive seeds, with the individual seeds separated by a 1 cm center-to-center distance. Following the inverse-square law of radiation dosimetry, using a 2 cm thickness for a 60-mm culture plate (Fisher Scientific Inc., Pittsburgh, PA), and a uniform 5 mL of culture media per plate, low-dose-rates of 11, 23, 37, and 67 cGy/hr were achieved when the acrylic template was loaded with 196 1.0 mCi Ir-192 seeds. Film dosimetry verified low-dose-rate at irradiated cell levels. Cells were transferred to and from the irradiator using a removable, sliding acrylic shelf. The shelf provided an Ir-192 source-to-cell distance identical above and below the Ir-192 source acrylic template. Cells were exposed to total radiation doses of 200, 400, 600, 1000, and 2000 cGy at each of four low-dose-rates of irradiation. With the intent of comparing radiation dose-rate effects, cells also underwent high-dose-rate radiation delivered by a conventional Cs-137 γ -irradiator (JL Shepherd Associates, San Fernando, CA) having a dose rate of 330 cGy/min (6).

Clonogenic survival assay and analysis of radiation-drug interactions

Exponentially-growing cells were plated in triplicate on 60-mm dishes to yield 300 cells per dish for 11, 23, 37 cGy/hr irradiation or 600 cells per dish for 67 cGy/hr irradiation. Cells were exposed to 0–2000 cGy of IR delivered at low or high dose-rate with or without 6-hour exposure to 3-AP (1–10 μM) as indicated. Human radiation sensitizing *in vivo* doses of 3-AP provide 6-hour pharmacologic inhibition of RNR, and therefore, a 6-hour exposure of 3-AP was selected for experimental testing (6,7,17). A subset of analyses was conducted at a total dose of 1000 cGy because this dose is one-quarter of the dose of a typical cervical cancer low-dose-rate brachytherapy implant of 4000 cGy (17). To achieve 0–2000 cGy total radiation doses, cells had variable durations of irradiation (e.g., 3 hours for 200 cGy at 67 cGy/hr v. 182 hours for 2000 cGy at 11 cGy/hr). To maintain source-to-cell distances, dose attenuation, and relative cell dosimetry during irradiation, sham 60-mm dishes with culture media were shuffled into and out of the irradiator as needed. Fourteen days after plating, colonies were fixed in 70% ethanol and stained with 0.5% crystal violet. Surviving colonies (>50 cells) were counted. The surviving fraction was normalized by the plating efficiency of non-treated controls.

Two-stage multivariate analyses of variance (MANOVA) statistics analyzed IR-drug interactions at all treatment levels (i.e., combinations) with all levels of every other treatment (18,19). These statistical methods use a balanced complete block factorial design and have been detailed by our group for clonogenic survival assays (18,19). In brief, these methods are a generalization of ANOVA and t-tests appropriate for multiple responses to provide a unified alternative to testing differences among mathematically fitted treatment response vectors one parameter at a time, and then, jointly as a global test for differences between treatment responses. Simply, MANOVA statistics compare overall “curvature” of

different cell survival curves. MANOVA statistics ($\alpha = 0.05$) were computed using statistical software (SPSS 18.0, Chicago, IL).

Flow cytometry for cell cycle analysis

Exponentially-growing cells were plated on 100-mm dishes to yield $0.6\text{--}2.0 \times 10^6$ cells per dish. Cells underwent low-dose-rate irradiation (0–67 cGy/hr) to a dose of 1000 cGy with or without a 6-hour 3-AP (0–10 μM) treatment. A 1000 cGy endpoint was chosen because (a) this dose is one-quarter of the dose of a typical cervical cancer low-dose-rate brachytherapy implant (4000 cGy), (b) substantial G1 cell cycle arrest has been observed between 12 and 18 hours in cells treated with 5 μM 3-AP alone (6), and (c) peak transcriptional activation of RNR M2 occurs 12 to 18 hours after IR (20). Time to reach the 1000 cGy endpoint was 15 hours at 67 cGy/hr and 3 minutes at 330 cGy/min. Flow cytometric analyses were done using a Coulter EPICS XL-MCL flow cytometer (Coulter Co., Miami, FL) using published techniques and analyzed using ModFit LT 3.0 (Verity, Inc., Topsham, ME) (6). Median cell proportions in the G1-phase of the cell cycle were determined using SPSS 18.0.

Ribonucleotide reductase assay

A DNA polymerase extension assay quantified dCTP levels resulting from ribonucleotide reductase activity after IR with or without 3-AP (6,7). The template was 5'-AAA GAA AGA AAG AAA GAA AGG GCG GTG GAG GCG G-3' and the primer was 5'-CCG CCT CCA CCG CC-3' (Integrated DNA Technologies, Coralville, IA). CaSki and C33-a cells were treated as indicated with low-dose-rate radiation and 6-hour 3-AP (5 μM). All cells were harvested at 15 hours for each dose-rate level to associate dCTP level and flow cytometry. Cell extract (5 μL) was added to 45 μL DNA polymerase extension assay reaction mixture: 50mM Tris-HCl (pH 7.5), 10mM MgCl_2 , 5 mM dithiothreitol, 10 μg BSA, 0.25mM template-primer, 0.1 unit Klenow fragment of DNA polymerase, and 1 μM ^3H -dTTP (specific activity 15Ci/mmol, Perkin-Elmer, Waltham, MA). After incubation at 37°C for 30 min, the mixture was spotted onto Whatman DE81 filter paper, dried by infrared lamp, washed 3 times with 5% Na_2PO_4 , rinsed with H_2O and ethanol, and dried again. ^3H -dTTP radioactivity was determined by a liquid scintillation counter, with radioactivity linearly proportional to dCTP concentration (up to 2.0 pmole). Mean and standard deviation are reported (SPSS 18.0).

Results

Enhanced radiosensitization of cervical cancer cells to low-dose-rate irradiation by 3-AP

Radiation dose-response curves are presented for CaSki cells (Fig. 2A,B) and C33-a cells (Fig. 2D,E). Raising the dose-rate from 11 cGy per hour to 67 cGy per hour increased cell kill (Fig. 2A, D; $P < 0.004$ each). Combination dose-response curves of 3-AP + IR cytotoxicity are presented in Figures 1B, E. The addition of 3-AP increased cell kill across the 11, 23, 37, and 67 cGy per hour dose-rates such that no significant differences in cell kill were apparent among the four dose rates at each of three clinically-relevant 3-AP drug levels (Fig. 2B $P=0.22$, Fig. 2E; $P=0.28$). Comparing two clinically-relevant dose rates (i.e., >300 cGy/min for conventional external beam radiation and 67 cGy/hr for low-dose-rate brachytherapy) with and without 3-AP (5 μM), a higher cell kill was observed in the presence of 3-AP after the low-dose-rate irradiation than after high-dose-rate irradiation (Fig. 2C, F). Taken together, these findings support the hypothesis that pharmacologic blockade of RNR, when cells demand dNTPs for DNA repair, sensitizes cells to IR and increases cell death.

3-AP inhibits ribonucleotide reductase during low-dose-rate irradiation

To discover whether 3-AP hampered activity of RNR during conditions of continuous low-dose rate irradiation, cells had no treatment (0 cGy/hr) or were exposed to 5 μ M 3-AP alone (0 cGy/hr) or IR (11, 23, 37, and 67 cGy/hr for 15 hours) with or without 5 μ M 3-AP (Fig. 3A,B). The effect of 3-AP on RNR activity was investigated through analysis of intracellular dCTP levels as a marker for RNR activity. A rise in dCTP level in irradiated cells is an indicator of increased RNR activity. Low-dose-rate irradiation did induce increased RNR activity by elevating intracellular dCTP levels in CaSki and C33-a cells, relative to untreated cells ($P < 0.01$ each). 3-AP treatment alone substantially reduced dCTP levels in both cell lines, when compared to untreated cells ($P < 0.01$ each). Under continuous low-dose-rate irradiation conditions, 3-AP blocked RNR such that a rise in dCTP levels after IR was not observed (IR alone v. IR + 3-AP, $P < 0.01$ each).

Increased G1-phase cell cycle arrest in cervical cancer cells after blockade of ribonucleotide reductase

To explore RNR inhibition and cell cycle alteration after low and high-dose-rate irradiation, CaSki and C33-a cells treated with IR and/or 3-AP were analyzed for DNA content by flow cytometry (Fig. 4). Sixteen hours after the start of irradiation, both cervical cancer cell lines showed a G1 cell cycle block with 5 μ M or 10 μ M 3-AP treatment (Fig. 4A,B). At each 3-AP dose, a substantial fraction of cells was in the G1-phase of the cell cycle after 16 hours of continuous low-dose-rate IR. In contrast, when cells were exposed to high-dose-rate irradiation for 3 minutes to deliver 1000 cGy, 16 hours later most cells were arrested in the G2/M-phase of the cell cycle if 1 μ M 3-AP had been present, but were in a G1-phase arrest if exposed to 5 μ M or 10 μ M 3-AP. Furthermore, when exposed to the clinically-relevant combination of low-dose-rate radiation and 3-AP treatment, cells of both cervical cancer lines showed an increased fraction of cells that were blocked in G1-phase and an increased sub-G1 population suggestive of enhanced cell cytotoxicity from the combination treatment (Fig. 4A,B).

Discussion

Cells exposed to IR sustain DNA damage in the form of base alterations, single-strand breaks, and double-strand breaks. At least one mechanism to supply essential dNTPs for DNA repair is the synthesis of new dNTPs by RNR. In response to IR-mediated DNA damage, RNR increases its catalytic activity to increase available pools of dNTPs (6,7). Pharmacologic blockade of RNR by anticancer drugs such as 3-AP reduce RNR catalytic activity, perpetuate DNA damage, and increase IR-mediated cell cytotoxicity (6). Our findings are consistent with a mechanism by which pharmacologic blockade of RNR by 3-AP enhances IR-mediated cell cytotoxicity.

It is clear that the dose rate at which cells are exposed to radiation impacts the DNA damage response (Fig. 2). Under conditions of low-dose-rate radiation, cells are subject to a constant low rate bombardment with IR over extended time periods. To optimally fix repeatedly damaged DNA bases and strand breaks, a cell must have a constant supply of DNA precursors for DNA repair enzymes and polymerases. The *de novo* pathway through RNR is likely one operative mechanism supplying requisite dNTPs in the cell. At very low-dose-rates (11 cGy/hr and 23 cGy/hr), cells balance the pace of DNA damage with DNA repair, maintaining viability. At a clinically-relevant dose rate (67cGy/hr), DNA damage accumulates at a sufficient rate to increase cell cytotoxicity.

The iron-chelating RNR inhibitor 3-AP interferes with the diferric iron-stabilized free radical critical for catalytic function of RNR small subunits (21). Through this effect, 3-AP

potently disrupts RNR and impedes DNA repair (6). Under conditions of low-dose-rate radiation where sustained dNTP demands are relatively high, inhibition of RNR renders the pathway of ribonucleotide reduction for new dNTPs ineffective. Our results show that this effect substantially enhances the radiation sensitivity of cells. This pronounced radiosensitization effectively eliminated the well-known radioresistance of cells that is associated with low radiation dose-rates. Comparing clinically-relevant high (330 cGy/min) and low (67 cGy/hr) radiation dose rates, 3-AP treatment had a greater effect at 200cGy. 3-AP drug exposure for six hours during repetitive DNA damage induced by low-dose-rate radiation substantially lowered cell survival, suggesting that impaired repair of DNA damage promoted cell kill. These observations suggest that the repeated demand for dNTPs that cannot be supplied by an inactive RNR in the presence of 3-AP alters the ability of the cell to respond to radiation damage and increases radiation sensitivity, promoting cell death. However, cells respond to irradiation by extracting and replenishing dNTPs from intracellular pools which may include oxidized bases (2) or synthesized *de novo* through ribonucleotide reduction (3) which may filter out oxidized bases from dNTP pools (2), facilitating repair of DNA. In this study, cell RNR was inactivated by 3-AP for only the first six hours of irradiation, while irradiation may have continued well beyond this therapeutic window. In this scenario, perhaps DNA repair occurred in the absence of RNR inactivation by 3-AP but still under bombardment of low-dose radiation. Whether repeated 3-AP exposure over long-lasting low-dose radiation times would enhance cytotoxicity has not been tested.

A G1-phase cell cycle checkpoint arrest as a result of inhibited RNR small subunits follows 3-AP treatment under low or high dose-rate radiation conditions (Fig. 4). To begin to understand the importance of this observation, we first studied the dose-rate effect on cell cycle progression after IR. High-dose-rate IR results in a predominant G2/M-phase cell cycle checkpoint arrest, so that unrepaired DNA damage inflicted during the previous G1- or S-phases is not passed to daughter cells in mitosis. The G2/M-phase checkpoint restriction follows inhibition of cyclinB/CDK1 kinase (22,23). Low-dose-rate IR results in a more biphasic distribution, with cells accumulating both at the G1/S-phase and G2/M-phase cell cycle checkpoints to allow DNA repair prior to DNA replication or mitosis, respectively (i.e., low-dose-rate G1-phase 29% v. high-dose-rate G1-phase 4%, Fig. 3). The dominant G1 checkpoint response is mediated by the ATM(ATR)/CHK2(CHK1)-p53/p21 pathway, which is capable of inducing sustained and sometimes even permanent G1-phase arrest (24). However, cervical cancer cells have abrogated G1 checkpoint responses due to (a) HPV-16 E6 protein, facilitating rapid p53 turnover and low intracellular levels of p53 (25–28), or (b) mutated p53, which is unable to transcriptionally activate p21CIP1/WAF1, an inhibitor of cyclin-dependent kinases (24), which silences the G₁/S-promoting cyclin E/Cdk2 kinase. In our study, increasing 3-AP concentration during low- or high-dose-rate IR resulted in a higher proportion of cells arrested in G1-phase of the cell cycle, a phenomenon likely independent of p53 and perhaps related to 3-AP-targeted inhibition of RNR impeding progression to S-phase (6,7). As such, our findings are consistent with a mechanism of disrupted RNR function by 3-AP resulting in slowed or halted G1-phase transition.

Enhanced radiation sensitivity was expected and observed in cells treated with IR and 3-AP, due to the inhibition of RNR by 3-AP. This effect was most pronounced under conditions of low-dose-rate IR when demands for dNTPs are high resulting from repetitive DNA damage, but unable to be supplied by inactive RNR in the presence of 3-AP. 3-AP has been shown to be well tolerated when co-administered to patients along with cisplatin and radiation for the treatment of gynecological malignancies (17). Therapeutic radiation strategies incorporating inhibitors of RNR hold promise for treatment of human cancers, perhaps most especially for cervical cancer, the second leading cause of worldwide female cancer-related mortality.

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Figure 1.

A schematic diagram of an Ir-192 low-dose-rate irradiator is provided. The irradiator accommodates three 60mm cell culture plates placed at each of five different levels above the acrylic template according to the desired pre-determined radiation dose rates. The irradiator allows one 100-mm culture plate at each of five different levels below the acrylic template according to the desired pre-determined radiation dose rates. Iridium-192 sources are loaded into 14 machined grooves within a central shelf as indicated. See text for details.

Figure 2.

3-AP increases cell radiation sensitivity to low-dose-rate irradiation. CaSki (A) and C33-a (D) cells were treated with low-dose-rate IR (0 to 2000 cGy). A significant dose-rate effect of radiation-related cell cytotoxicity was observed ($P < 0.004$). CaSki (B) and C33-a (E) cells were treated to a total dose of 1000 cGy at each of four low dose rates. Escalating concentrations of 3-AP (1–10 μM) were included in the culture medium for the initial 6 hours of irradiation. No substantial dose-rate effect was seen for CaSki ($P=0.22$) or C33-a ($P=0.28$) cells. CaSki (C) and C33-a (F) cells were treated with a clinically-relevant concentration of 3-AP (5 μM) and either high (330 cGy/min) or low (67 cGy/hr) dose-rate radiation. 3-AP added to IR (200 or 400 cGy total dose) increased cell kill under both dose-rate conditions. Error bars are $\pm$ SD for triplicate data.

Figure 3.

3-AP inhibits ribonucleotide reductase under continuous low-dose-rate irradiation. CaSki (A) and C33-a (B) cells were treated with low-dose-rate IR (0–67 cGy/hr) and/or 3-AP (5 μM). Cells were harvested at 15 hours. Compared to untreated cells, low-dose-rate irradiation was associated with an increase in dCTP level ($P < 0.01$, each). 3-AP alone substantially reduced dCTP levels ($P < 0.01$, each). Compared to the corresponding low-dose-rate irradiation condition, IR plus 3-AP was associated with lower dCTP levels ($P < 0.01$, each). Error bars are $\pm$ SD for triplicate data.

Figure 4.

3-AP increases the proportion of cells in the G1-phase of the cell cycle during irradiation. CaSki (A) and C33-a (B) cells were treated with 1000 cGy radiation either at 67 or 330 cGy/hr with or without 6-hour 3-AP (1–10 μ M). Cells were harvested ~16 hours after the start of irradiation. The median proportions of G1-phase cells are reported for each histogram. Gray histogram boxes depict G1-phase and G2/M-phase cells as indicated. White histogram boxes depict S-phase cells. Cells treated with radiation alone showed a G2/M-phase cell cycle arrest, while cells treated with radiation and 3-AP showed a greater proportion of G1-phase cell cycle arrest. For comparison, 3-AP alone arrests CaSki (46%) and C33-a (41%) cells at the G1-phase of the cell cycle at 18 hours after the start of 3-AP treatment (6).