Preclinical Development

Low-Dose Fractionated Radiation Potentiates the Effects of Cisplatin Independent of the Hyper-Radiation Sensitivity in Human Lung Cancer Cells

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Abstract

In this study, the role of hyper-radiation sensitivity (HRS) in potentiating the effects of cisplatin by low-dose fractionated radiation (LDFRT) was evaluated in four human non–small cell lung cancer cell lines. Presence of HRS and cisplatin enhancement ratio (CER) by LDFRT/2 Gy was assessed using colony-forming and apoptotic assays. Cell-cycle disturbances were studied by flow cytometry. Expression of genes involved in apoptosis was assessed using real-time reverse transcriptase PCR arrays. H-157 cells showed a distinct HRS region, followed by UKY-29 and A549 cells, whereas it was absent in H460 cells, which when lack HRS showed maximum CER with LDFRT (4 × 0.5 Gy) both by clonogenic inhibition and by apoptosis compared with single fraction of 2 Gy whereas the most radioresistant A549 cells had the least CER, with no significant differences between LDFRT or 2 Gy. Interestingly, in H-157 cells, a more pronounced CER was observed with LDFRT when assessed by apoptosis but clonogenic inhibition-CER was higher with 2 Gy than with LDFRT. Excluding H-157 cells, the CER by LDFRT was inversely proportional to radioresistance (determined by $D_{0}$, the dose to reduce survival by 67% from any point on the linear portion of the survival curve or surviving fraction (SF) at 2 Gy (SF2)) of the cells. LDFRT alone or in combination with cisplatin induced larger number of proapoptotic genes than 2 Gy or cisplatin + 2 Gy in cells showing HRS when compared to H460 cells that lack HRS. These findings indicate that chemopotentiation by LDFRT is correlated more with the intrinsic radiation sensitivity of the non–small lung cancer cells than the HRS phenomenon whereas the mode of cell killing is both through apoptosis and clonogenic inhibition. Mol Cancer Ther; 10(2); 292–302. ©2011 AACR.

Introduction

Combined treatment with radiotherapy and chemotherapy has been extensively used in the management of many types of solid malignancies including non–small cell lung cancer (NSCLC; refs. 1, 2). This approach in NSCLC has shown increased response rate, survival, and local control rate (1, 3, 4). cis-Diamminedichloroplatinum (cisplatin; ref. 5) is one of the most widely used chemotherapeutic agents. While several studies have shown cisplatin as a radiosensitizer (6–9), other studies have reported a lack of radiosensitization activity (10, 11) or increased patient survival with high toxicity (8).

Molecular mechanisms of radiosensitization by cisplatin are not completely understood. However, inhibition of repair of radiation-induced DNA damage by cisplatin is thought to be the underlying mechanism (12, 13). Cisplatin has been shown to prevent the translocation of Ku protein along DNA that affects nonhomologous end-joining pathway, thus contributing to cell death (14). There are reports showing that cisplatin treatment can increase DNA repair and enhance survival of cells after radiation exposure (15, 16).

The dual clinical objectives of the combined modality treatment, improved local control and prevention of distant micrometastases, are constrained by the intrinsic radiation resistance and chemoresistance of solid cancers causing profound patient morbidity. Recently, both in vitro and in vivo studies have shown that low doses of fractionated radiation can be used as enhancers (potentiators) of full-dose chemotherapy and circumvent the...
development of resistance observed with standard clinical doses of radiation and chemotherapy (17–20). The effects of low-dose fractionated irradiation on cell survival have been well studied and the phenomena of hyper-radiation sensitivity (HRS) at doses of less than 0.5 Gy and induced radiation resistance (IRR) at doses of less than 1 Gy are well documented (15, 21). HRS does not induce cellular repair mechanisms often observed at clinically relevant or higher radiation doses and thus provides a plausible explanation as to why there is no induction of radiation resistance with HRS as measured in vitro (21). Furthermore, HRS inducing low dose failed to induce prosurvival transcription factors such as NF-κB and NF-Y that are necessary for increasing the levels of MDR-1 gene (22). Hence, low or no MDR-1 induction in response to low-dose fractionated radiation therapy (LDFRT) will help to enhance the effects of chemotherapy that is often mitigated by MDR-1. Critically, to take advantage of the benefits of HRS in the clinical setting, radiotherapy would have to be extended over 7 to 12 weeks (ultrafractionation) to reach a total dose equivalent to that of standard radiation therapy approaches. Realistically, prolongation of radiation therapy by LDFRT in clinical settings is not possible due to the cost factor and, biologically, there is no clear evidence whether such HRS phenomenon exists in vivo. To avoid such issues, the enhanced cell killing effects of LDFRT in the form of HRS can be exploited by combining it with chemotherapy. The present study investigates the effects of cisplatin in NSCLC cell lines, H-157, H460, A549, and UKY-29, in combination with LDFRT, as well as the conventional dose of radiation. Studies were designed to understand the mechanisms underlying cisplatin-potentiation effects by LDFRT and the role of HRS in determining the cellular response and mode of cell killing. The findings indicate that chemopotentiation by LDFRT is correlated more with the intrinsic radiation sensitivity of the cells than with the HRS phenomenon whereas the cell killing is rendered either by apoptosis or by clonogenic inhibition.

Material and Methods

Cell culture

Four established NSCLC cell lines, H-157, H460, A549, and UKY-29 were used for the present studies. H-157 and H460 cells were provided by Dr. John Yannelli, University of Kentucky, in 2003, and UKY-29 cell line was established using pleural effusion sample from a lung cancer patient at University of Kentucky, Lexington, KY (23). A549 cells were obtained from American Type Culture Collection in 2003. Cell lines were tested and authenticated by RADIL (University of Missouri-Columbia, Columbia, MO), using short tandem repeat markers. The alleles for 9 different short tandem repeat markers were determined for each sample, and the results were compared with the genetic profiles reported by the American Type Culture Collection for each cell line in May 2010. No cross-contamination with other species was observed (Supplementary Table 1). All cell lines were cultured at 37°C and 5% CO2 in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin.

Cell treatments

Cells were treated with cisplatin (Bedford Laboratories) formulated in 0.9% sodium chloride at a stock concentration of 1 mg/mL. A 100-kV industrial X-ray machine (Phillips) was used to irradiate the cells at room temperature. The dose rate with a 2-mm Al plus 1-mm Be filter was ~2.64 Gy/min at a focus surface distance of 10.5 cm.

Treatments were done in the following manner: (i) left untreated; (ii) 0.1- to 6-Gy dose of radiation; (iii) cisplatin (H-157, 0.025–0.5 µg/mL; UKY-29, 0.01–0.5 µg/mL; both H460 and A549, 0.01–0.5 µg/mL). For combined treatments (cisplatin plus radiation), the cells were treated with cisplatin (H-157, 0.04 µg/mL; H460 and UKY-29, 0.25 µg/mL; A549, 0.3 µg/mL) and immediately exposed to radiation, unless specified otherwise, without changing the medium. For multifractionated experiments, cells were exposed to radiation without changing the medium at 0.5 Gy in 4 fractions, with 8-hour time intervals between each fraction. In a separate set of experiments, cells were exposed to a total dose of 6 Gy (2-, 1-, and 0.5-Gy fractions) by using an 8-hour time interval between each fraction.

Colony-forming assay

Clonogenic survival assays were carried out for each treatment group as described previously (17, 18, 24). Briefly, for each treatment, cells were plated in two different cell concentrations in quadruplet sets (total 4 replicates). After overnight plating, cells were treated as explained earlier and left for colony formation. After incubation for 10 or more days, colonies were stained with crystal violet and the colonies containing more than 50 cells were counted. The surviving fraction (SF) was calculated as a ratio between the number of colonies formed and the product of the number of cells plated and the plating efficiency. A plating efficiency of 60% to 80% was obtained depending on the cell line. D0 values (the dose to reduce survival by 67% from any point on the linear portion of the survival curve) were determined using single-hit multitarget model. Cisplatin enhancement ratio (CER) by radiation was calculated using the following formula based on SF:

\[
\text{CER} = \frac{\text{SF} \text{ cisplatin alone}}{\text{SF} \text{ cisplatin + radiation}}
\]

The radiation enhancement ratio (RER) by cisplatin was calculated as follows:

\[
\text{RER} = \frac{\text{SF} \text{ radiation alone}}{\text{SF} \text{ cisplatin + radiation}}
\]

Quantification of apoptosis

Flow cytometric analysis. Untreated or treated cells as described earlier were incubated in growth media for specific periods of time (from 0 to 48 hours). Adherent and floating cells were harvested by trypsinization. Flow cytometric measurements of cellular DNA content were carried out in ethanol-fixed cells with propidium iodide as
described earlier (25). Propidium iodide-stained cellular DNA was excited at 488-nm using a flow cytometer (FACScalibur; Beckton Dickinson) and the data were acquired using the Cell Quest software (Beckton Dickinson). Apoptotic cells were quantified by gating the sub G0-G1 population.

**TUNEL assay**

The in situ apoptosis detection kit (Roche Diagnostics Corporation), which detects the DNA strand breaks in single cells by terminal transferase-mediated fluorescein-dUTP end labeling (TUNEL), was used as per the instructions provided by the manufacturer. Cells were seeded in chamber slides and exposed to cisplatin alone (IC50 concentrations); 2 Gy, single fraction (2 Gy); combination of cisplatin and 2 Gy, single dose (cisplatin + 2 Gy); and cisplatin plus 4 fractions of 0.5 Gy (cisplatin + LDFRT). After 24 and 48 hours of treatment, cells were stained using TUNEL kit. The stained slides were observed in triple band-pass filter with a Nikon-microphoto epifluorescence microscope. To determine the percentage of cells showing apoptosis, a total of 2,000 cells were counted for each treatment.

Enhancement of cisplatin-induced apoptosis by radiation was calculated using the following formula based on percentage of induction of apoptosis (PIA):

\[
\text{CER} = \frac{\text{PIA}_{\text{cisplatin+radiation}}}{\text{PIA}_{\text{cisplatin alone}}}
\]

**Real-time reverse transcriptase PCR**

Total RNA was extracted from the cells 3 hours after treatment with TRIzol reagent (Life Technologies, Inc.). One microgram of total RNA was reverse transcribed into cDNA with the SuperArray RT2 First Strand Kit (Cat. No. C-03) and instructions provided by the manufacturer. Real-time reverse transcriptase PCR (RT-PCR) was carried out with RT2 profiler PCR array for human apoptosis (SuperArray; Cat. No. APHS-012A) to test the expression levels of 84 genes (Supplementary Table 2) involved in apoptosis with Applied Biosystems 7300 instrument.

**Western blot analysis**

Total protein extracts obtained at 6 hours after treatment of A549 cells were subjected to Western blot analysis as previously described (26). Briefly, membranes were incubated overnight at 4°C with primary antibodies against XIAP (BD Bioscience). The same membrane was stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling) as previously described (27). Densitometric analysis was done using the NIH ImageJ software (http://rsb.info.nih.gov/ij/).

**Statistical methods**

Independent 2-sample Student’s t test was used to test the differences in the means of radiation inactivation estimates and percentage of apoptosis (TUNEL-positive cells) obtained from any given two different treatment groups of cell lines. Because of repeatedly measured SFs at each dose level as well as over different doses, repeated-measures ANOVA for SFs were used with autoregressive variance-covariance matrix. One-way ANOVA to SFs with different fractionating groups were used to determine whether there were differences in mean SFs among different fractionations for a given cell line. Independent 2-sample Student’s t test was used to test whether there are any differences in the mean normalized gene expression level of any given gene between any given two cell lines at a particular treatment regimen. False discovery rate–adjusted P values were calculated to correct for multiple testing. Data management and the statistical analyses were done with SAS version 9.2 for Windows (SAS Institute Inc.). Type I error rate was set to 5% if not stated otherwise due to multiple testing.

**Results**

**HRS phenomenon was present in H-157, UKY-29, and A549 cells but not in H460 cells**

To investigate the presence of HRS and radiation sensitivity in these cell lines, cells were irradiated at low and high doses of radiation, respectively, and SFs were obtained by colony-forming assay. The presence of HRS in H-157, UKY-29, and A549 cells at low-dose radiation (0.1–1 Gy) was observed as most, moderate, and least prominent phenomenon, respectively (Fig. 1), whereas H460 cells showed no HRS phenomenon (Fig. 1D). These results were confirmed by statistical analysis as explained in the Materials and Methods section with a hypothesis that there is no difference in slope of SF of cells between “high” and “low” doses. Overall, there were statistically significant differences in slope of SF between “high” and “low” doses for cell lines H-157, UKY-29, and A549 but not for cell line H460 (Supplementary Table 3).

To improve the understanding of HRS in a setting of low-dose fractions versus clinically relevant dose fractionation settings, the cells were subjected to 12 fractions of 0.5 Gy or 6 fractions of 1 Gy or 3 fractions of 2 Gy. The decrease in SF was directly proportional to the number of fractions in all the cell lines for all fractionating schemes and the differences were statistically significant (Fig. 2 and Supplementary Table 4).

Overall, all the fractionation schemes were statistically significant and 2 Gy dose per fraction had the highest SF in both H-157 and UKY-29 except at a total dose of 4 and 6 Gy for UKY-29 (Fig. 2A and B and Supplementary Table 4). For UKY-29, the mean estimates (of SF) at a total dose of 4 Gy for 0.5, 1, and 2 Gy dose per fraction were very close to each other and resulted in no statistically significant difference at all possible pairwise comparisons (P values ranges from 0.0331 to 0.5561). However, at a total dose of 6 Gy, the mean differences between all possible pairwise comparisons were statistically significant (P < 0.0003).

Interestingly, in A549 cells that have least HRS phenomenon and H460 cells lacking HRS, significant
differences in clonogenic inhibition were not observed with 0.5- or 1- or 2 Gy fractions (data not shown).

**H-157 cells were most sensitive to cisplatin- and ionizing radiation-induced clonogenic inhibition**

Predictably, continuous incubation of the cells with cisplatin resulted in decreased SF with increasing concentrations (Supplementary Fig 1). The IC$_{50}$ concentrations were 0.04 \(\mu\)g/mL for H-157, 0.25 \(\mu\)g/mL for both UKY-29 and H460, and 0.3 \(\mu\)g/mL for A549 cells, suggesting that H-157 cells were most sensitive to cisplatin treatment and A549 were most resistant. This is similar to the data that were shown earlier, wherein H-157 cells (SF$_2$ = 0.18; $D_0$ = 106 cGy) showed HRS phenomenon and overall greater sensitivity to ionizing radiation along the entire range of radiation doses (0.1–6 Gy) when compared with UKY-29 (SF$_2$ = 0.37; $D_0$ = 149.5 cGy) and A549 (SF$_2$ = 0.66; $D_0$ = 159.4 cGy) cells (Fig. 1A–D). This 6-fold difference in sensitivity to cisplatin together with the presence of the HRS phenomenon may result in an increased chemopotentiation. However, H460 cells that lacked HRS but with a radiation response similar to H-157 cells (SF$_2$ = 0.26; $D_0$ = 110 cGy) showed cisplatin sensitivity similar to UKY-29 cells (Supplementary Fig. 1 and Fig. 1A and D) and maximum CER by LDFRT (Fig. 3).

**LDFRT chemopotentiating the effects of cisplatin in all the cell lines**

Cisplatin-potentiating effects of either single fraction of 2 Gy or LDFRT were analyzed by colony-forming assays (Fig. 3A). The results showed that both 2 Gy and LDFRT chemopotentiate the effects of cisplatin in all the cell lines (Fig. 3A). The chemopotentiating effects of LDFRT were maximum in H460 cells with a CER of 52.4 compared with 18.5 for 2 Gy single fraction, followed by UKY-29 cells (Fig. 3B). The differences in CER between 2 Gy and LDFRT were the smallest in A549 cells (1.86 vs. 1.92). Interestingly, only H-157 cells showed enhanced chemopotentiation with single fraction of 2 Gy than LDFRT (7.1 vs. 2.9). To ascertain the role of apoptosis in chemopotentiating effects of LDFRT, induction of apoptosis was determined by sub G$_0$-G$_1$ population assessed by flow cytometric method (Fig. 4). Similar to colony-forming results, both LDFRT and 2 Gy single fraction radiation resulted in CER of more than 1.0 in all the cell lines except A549 (Fig. 3B). H460 cells again showed maximum CER with LDFRT compared with 2 Gy irradiation (8.3 vs. 3.5). While significant differences

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**Figure 1.** Effects of radiation on survival of lung cancer cells assessed by colony-forming assay. Effects of X-ray irradiation (0.1–6 Gy) on survival in H-157 (A), UKY-29 (B), A549 (C), and H460 (D) cells by colony-forming assay. The smoothed lines are determined from fitting exponential regression curves. Insets, SFs at doses from 0.1 to 1 Gy in these 4 cell lines showing the HRS and IRR phenomena. Statistical analysis is shown in Supplementary Table 3.
Potentiation of cisplatin by LDFRT: A transient, robust cell-cycle arrest was observed in the G2-M phase at 24 hours after incubation with cisplatin in UKY-29 cells, whereas this phenomenon was not observed in any other cell lines (Fig. 4A vs. Fig. 4B–D). Thus, the effect of G2-M arrest on chemopotentiation of cisplatin by LDFRT or single fraction of 2 Gy irradiation was assessed by colony-forming assay following incubation of UKY-29 cells for 24 hours with cisplatin and compared with H-157 cells. There were no significant changes in the CER in UKY-29 cells (7.1 vs. 6.4 for 2 Gy and 10.2 vs. 10.2 for LDFRT); however, chemopotentiation of cisplatin by LDFRT and 2 Gy was diminished in H-157 cells (7.1 vs. 5.0 for 2 Gy and 2.9 vs. 1.6 for LDFRT; Supplementary Table 5). The decrease in CER by prior incubation of H-157 cells with cisplatin might be due to the induction of robust repair mechanisms that interfere with lethal apoptotic response mediated by HRS.

Furthermore, the radiation/cisplatin/combination-induced disturbances in the cell-cycle distribution were investigated in all the cell lines (Fig. 4). While both H-157 and H460 cells showed a transient accumulation of cells in the G2-M phase following 2 Gy irradiation, H-157 cells also showed a G2-M block following LDFRT. Neither single fraction of 2 Gy nor LDFRT induced significant changes in the G2-M phase in UKY-29 and A549 cells. LDFRT, however, induced G2-G1 arrest in A549 cells. H-157 cells showed a G2-M phase block following the combined treatment with cisplatin and LDFRT, with no significant changes when cisplatin was combined with 2 Gy (Fig. 4B). On the other hand, H460 cells showed an accumulation in G2-M followed by a block in G0-G1 in response to cisplatin by LDFRT and 2 Gy. Combination of cisplatin with LDFRT resulted in G0-G1 block only. These cell-cycle disturbances might be underlying reasons for increased apoptosis (the percentage of cells undergoing apoptosis is shown for the groups for which induction of apoptosis was significant) in these cells at later time points (Fig. 4B and D). UKY-29 cells showed a consistent G2-M block when cisplatin was combined with 2 Gy, with no significant changes in response to cisplatin + LDFRT treatments (Fig. 4A). In A549 cells, the combination of cisplatin and LDFRT resulted in a transient G0-G1 block (Fig. 4C). In both cell lines, however, hypodiploid peak indicating apoptosis was not observed.

Enhanced expression of proapoptotic genes was observed in cells showing HRS following LDFRT or combination treatments

We, and others, have earlier shown that a significant amount of apoptosis is induced by LDFRT (17, 18, 29). To understand the mechanisms underlying chemopotentiation of cisplatin by LDFRT, real-time RT-PCR was carried out for 84 genes related to apoptotic pathways in the 4 lung cancer cell lines. Expression of genes following each treatment was compared with untreated samples for each cell line (Supplementary Table 6). Expression of genes in H-157, UKY-29, and A549 cells was also compared with gene expression in H460 cells for the respective treatment.
groups (Fig. 5A–C). Genes were thus classified as upregulated, downregulated, or no change. Although expression of genes changed significantly compared with untreated cells in all of the cells, by setting the threshold as 50-fold differences in expression, only few genes showed modulated expression (Supplementary Table 6). In addition to the genes listed in Supplementary Table 6, several other genes were modulated (<50-fold difference) following various treatments in all the cell lines. Interestingly, in H-157 cells (most prominent HRS and most radiosensitive), proapoptotic genes, CASP5, CASP6, NOD1, CD40, CD40LG, CIDEB, CIDEB, DAPK1, and FASLG, were differentially induced following LDFRT treatments. In UKY-29 cells with moderate HRS, proapoptotic genes CASP10 and RIPK2 were significantly upregulated in LDFRT and cisplatin ± LDFRT groups, with downregulation of FADD in cisplatin ± 2 Gy group. In A549 cells having least HRS, proapoptotic genes CASP8 and DAPK1 were upregulated in LDFRT and cisplatin ± LDFRT groups compared with 2 Gy and cisplatin ± 2 Gy groups. Furthermore, proapoptotic genes BAG-4, CASP10, and NOD1 were downregulated in 2 Gy or cisplatin ± 2 Gy or both the groups. In H460 cells showing no HRS, however, only TNFSF10 was upregulated in LDFRT and cisplatin ± LDFRT groups.

In Figure 5, the percentage of genes with statistically significant up- or downregulated changes in expression is shown when the cells having HRS were compared with H460 cells that did not have HRS. In H-157 cells, LDFRT resulted in upregulation of more proapoptotic genes as compared with H460 cells, followed by cisplatin and cisplatin + 2 Gy treatment groups (Fig. 5A). However, a maximum number of antiapoptotic genes were downregulated in 2 Gy group. In UKY-29 cells, cisplatin, 2 Gy, and cisplatin + 2 Gy groups showed similar upregulation of both pro- and antiapoptotic genes (Fig. 5B). LDFRT groups showed less number of pro- or antiapoptotic genes upregulation. In A549
cells, significant downregulation of antiapoptotic genes was observed in cisplatin, 2 Gy, and cisplatin + 2 Gy groups (Fig. 5C). No significant differences were observed in LDFRT groups. A comprehensive list is provided for the genes that were significantly modulated in H-157, UKY-29, and A549 cells compared with H460 cells in Supplementary Table 7. Because A549 cells were resistant to either LDFRT or 2 Gy irradiation, to

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HRS</th>
<th>Radiosensitivity, SF2/D0</th>
<th>Cisplatin sensitivity, IC50</th>
<th>Colony-forming assay-CER</th>
<th>TUNEL-CER</th>
<th>Flow cytometry-CER</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-157</td>
<td>Most</td>
<td>1 (0.18/106 cGy)</td>
<td>1 (0.04 μg/mL)</td>
<td>2.9</td>
<td>7.1</td>
<td>11.1</td>
<td>6.5</td>
</tr>
<tr>
<td>H460</td>
<td>None</td>
<td>2 (0.26/110 cGy)</td>
<td>2 (0.25 μg/mL)</td>
<td>52.4</td>
<td>18.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UKY-29</td>
<td>Moderate</td>
<td>3 (0.37/149 cGy)</td>
<td>2 (0.25 μg/mL)</td>
<td>10.2</td>
<td>7.1</td>
<td>6.2</td>
<td>1.4</td>
</tr>
<tr>
<td>A549</td>
<td>Less</td>
<td>4 (0.66/159 cGy)</td>
<td>3 (0.3 μg/mL)</td>
<td>1.92</td>
<td>1.86</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Figure 4. Cell-cycle perturbations induced by cisplatin and radiation in lung cancer cells. Flow cytometric measurement of cell-cycle distribution of the cells after incubation with cisplatin (H-157, 0.04 μg/mL; H460 and UKY-29, 0.25 μg/mL; A549, 0.3 μg/mL) followed by immediate irradiation (2 Gy or LDFRT). For LDFRT, 0 hours represents the time after the cells were exposed to the last fraction of 0.5 Gy. A, UKY-29; B, H-157; C, A549; and D, H460.
establish a causal correlation, we studied the expression of XIAP, an inhibitor of apoptosis in these cells. The XIAP was induced in A549 cells following radiation as shown by Western blot analysis, suggesting a causal correlation between the increased expression of XIAP and decreased clonogenic inhibition (Fig. 5D). These results suggest that for certain cells that are radioresistant, the use of specific inhibitors of XIAP may be vital for enhancing cell killing with either LDFRT or 2 Gy.

Discussion

Our previous in vitro, in vivo, and clinical studies have shown that LDFRT could be used as a chemopotentiator of paclitaxel, carboplatin, and gemcitabine (18–20, 30). However, the question that still remains unanswered is whether the presence of HRS is essential to potentiate the effects of chemotherapeutic drugs by LDFRT. Because there are currently no isogenic in vitro HRS models available that can be used to delineate the mechanisms of chemopotentiation by LDFRT, in the present study, 4 lung cancer cell lines were used that had different degree of HRS to answer this question. It was observed that H-157, UKY-29, and A549 showed HRS whereas H460 cells did not (Fig. 1). Although the reasons for the absence of HRS in some cells are not well understood, it has been suggested that HRS-negative cell lines have a dissociation between ataxia telangiectasia mutated (ATM) activity and early G2-phase checkpoint function, producing an aberrant early G2-phase checkpoint response that evades dose-dependent ATM regulatory control (31). However, H460 cells did not show any G2-phase arrest after LDFRT (Fig. 4D). Therefore, some other mechanisms may be responsible in these cells for failure of exhibiting HRS.

In this study, both radioresistant cells such as UKY-29 and A549 as well as radiosensitive cells such as H-157 did show the presence of HRS. These observations are contrary to the reports that radioresistant cells express increased HRS/IRR (32). In fact, the most pronounced HRS was shown in the most radiosensitive H-157 cells,
sustaining that HRS is not an exclusive phenomenon for radioresistant tumor cells.

The present study findings strongly suggest that LDFRT can chemopotentiate the effects of cisplatin in H460 cells that lack HRS phenomenon as well as in H-157, UKY-29, and A549 cells harboring varying degrees of HRS. Furthermore, a higher CER was observed with LDFRT than single fraction of 2 Gy in all the human lung cancer cell lines studied except A549 (Table 1).

More important, LDFRT potentiated the effects of cisplatin maximally in H460 cells, although they were HRS negative. In addition, a linear relationship was observed between CER and intrinsic radiosensitivity of these cell lines (Fig. 3C). Thus, together these observations show that chemopotentiation of cisplatin by LDFRT may be independent of HRS but depend more on intrinsic radiosensitivity of the cells.

A differential response to different types of fractionated radiation exposures was observed in H-157 and H460 cells. HRS-positive H-157 cells were more sensitive to fractions of 0.5 Gy (a total dose of 6 Gy) than fractions of 1 or 2 Gy, whereas HRS-negative H460 cells showed no differential effect with either of these fractions (Fig. 2A). These findings potentially suggest that low-dose fractionated scheme settings may be advantageous in cells that harbor HRS phenomenon.

Short and colleagues (33) showed the influence of cell-cycle phases on the HRS phenomenon. Cells synchronized in G2-M showed a significantly more pronounced HRS than synchronized cells in G1 or S. These findings present an interesting explanation of the underlying mechanisms of chemopotentiation by LDFRT in the studies from our laboratory using paclitaxel or docetaxel (18, 19). In the present study, incubation of cells with cisplatin for 24 hours resulted in a transient accumulation in the G2-M phase only in UKY-29 cells (Fig. 4A). However, in these cells, CER was not enhanced following either LDFRT or 2 Gy treatment compared to when cisplatin was added immediately prior to irradiation (Supplementary Table 5). This is in contrast to the earlier reports wherein LDFRT could enhance the chemopotentiation effects of paclitaxel that arrest cells in the G2-M phase (18, 19). One explanation could be that the G2-M phase arrest induced by cisplatin is similar to the classical G2-M phase block induced by radiation due to the accumulation of damaged G1- and S-phase cells in the G2-M phase. Cells arrested in the G2-M phase by this mechanism do not show HRS, as only a recently described second G2-M checkpoint (34) is thought to be involved in induction of HRS (28).

Furthermore, in H-157 cells, the chemopotentiation effects were reduced when cells were incubated with cisplatin 24 hours prior to LDFRT, although no G2-M block was observed (Fig. 4B and Supplementary Table 5). Similar results were obtained by Marples and Skov (35) in CHO cells, as cisplatin increased resistance to subsequent low-dose radiation exposures by abolishing HRS. Because H-157 cells showed HRS, it is likely that a DNA-damaging agent such as cisplatin can trigger the repair mechanisms so that HRS is lost and IRR is induced when cells are exposed to subsequent doses of radiation (32, 36). Alternatively, both cisplatin and radiation may target the same subpopulation of the cells. Pretreatment, thus, eliminates the sensitive subpopulation so that the remaining cell populations are more resistant to subsequent treatments (36).

Our previous studies in head and neck tumor cell lines and mice xenografts showed increased apoptosis rather than clonogenic death in LDFRT-mediated chemopotentiation (18, 19), suggesting that preferred mode of killing in chemopotentiation by LDFRT is apoptosis. This is supported by the TUNEL assay results of the present study wherein it was found that chemoenhancement ratio was significantly higher for apoptosis than for clonogenic inhibition in H-157 cells. A relationship between apoptosis and the prevalence of low-dose HRS has been shown previously (29). Hence, HRS-negative H460 cells showed increased clonogenic inhibition than apoptosis when compared with HRS-positive H-157 cells. However, UKY-29 and A549 cells harboring HRS did not show significant induction of apoptosis following LDFRT, thus supporting the previous observations that apoptosis may be indicative of HRS but is not a prerequisite for HRS (37, 38). Furthermore, in H460 cells, significant differences in the expression of genes related to apoptosis were not observed with any of the treatments when compared with the untreated group (Supplementary Table 6). For other lung cancer cells that harbor HRS, significant changes in pro- and antiapoptotic gene expression were observed as expected. Several proapoptotic genes such as BAG3, NOD1, CASP5, and CASP6 were induced in H-157 cells, followed by UKY-29 and A549 cells. The expression of apoptotic genes thus significantly correlated with the biological response as well as with the HRS status of the cells.

Of importance, the expression of several proapoptotic genes was significantly higher in untreated HRS-positive A549, UKY-29, and H-157 cells than in HRS-negative H460 cells (data not shown). Following various treatments, a limited number of genes showed significant enhanced expression in these three HRS-positive cells compared with HRS-negative H460 cells (Supplementary Table 7). Significant among them were induction of FASLG in H-157 cells by LDFRT, CASP1 in UKY-29 cells and induction of CIDEB by LDFRT, and downregulation of NOD1 by 2 Gy in A549 cells.

In summary, the present findings show that LDFRT chemopotentiates the effects of cisplatin in human lung cancer cell lines irrespective of HRS status and mode of cell killing could be either apoptosis or clonogenic inhibition. This indicates that this regimen may benefit all cases of NSCLC. However, further studies to elucidate the molecular mechanisms of chemopotentiation of cisplatin by LDFRT are needed to fully exploit this approach as a novel therapeutic modality in the clinic for the treatment of lung cancer.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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References

Stewart DJ, Molepo JM, Eapen L, Montpetit VA, Goel R, Wong PT, Hazuka MB, Crowley JJ, Bunn PA Jr, O'Schaake-Koning C, Van Den Bogaert W, Dalesio O, Festen J, Hoo...
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