Contribution of Dual Oxidase 2 (DUOX2) to Hyper-Radiosensitivity in Human Gastric Cancer Cells

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Whole-abdominal radiotherapy (WART) is a primary method for managing gastrointestinal cancers that have disseminated into intra-abdominal tissues. While effective, this approach is limited because of the increased toxicity to normal tissue associated with combined WART and full-dose chemotherapy regimens. Recent studies have demonstrated a survival advantage in a novel treatment paradigm that allows for the safe use of full-dose systemic chemotherapy in combination with low-dose fractionated radiotherapy (LDFRT). Traditionally, radiation doses greater than 120 cGy have been used in radiotherapy because lower doses were thought to be ineffective for tumor therapy. However, we now know that LDFRT can produce hyper-radiosensitivity (HRS), a phenomenon where cells undergo apoptosis at radiation doses as low as 15 cGy, in a number of proliferating cells. The objectives of our current study were to determine whether LDFRT can induce HRS in gastrointestinal cancer cells and to identify biomarkers of chemopotentiality by LDFRT. Our data indicate that three consecutive daily fractions of 15 cGy produced HRS in gastric cancer cells and potentiated a modified regimen of docetaxel, cisplatin and 5'-fluorouracil (mDCF). Colony survival assays indicated that 15 cGy was sufficient to kill 90% of the cells when LDFRT was combined with mDCF whereas a dose almost 10 times higher (135 cGy) was needed to achieve the same rate when using conventional radiotherapy alone. RT² PCR Profiler™ array analysis indicated that this combined regimen upregulated dual oxidase 2 (DUOX2), an enzyme functioning in the production of hydrogen peroxide, without upregulating genes involved in DNA repair. Moreover, downregulation of DUOX2 increased radioresistance at every radiation dose tested. In addition, our data indicate that reactive oxygen species (ROS) increase up to 3.5-fold in cells exposed to LDFRT and mDCF. Furthermore, inhibition of NADPH oxidase abrogated the killing efficiency of this combined regimen. Taken together these data suggest that chemopotentiality by LDFRT in gastric cancer cells may be

due, at least in part, to increased ROS production (DUOX2) without upregulation of the DNA repair machinery. These data thus provide a rationale for further explorations of potential clinical applications of LDFRT, such as in WART, as a chemopotentiality for advanced and metastatic gastric cancers. © 2015 by Radiation Research Society

INTRODUCTION

The treatment of locally advanced and/or metastatic gastrointestinal (GI) tumors is still a challenge despite recent technological and chemotherapeutic advances. Tumors of the GI all present significant challenges when unresectable and/or associated with disseminated intra-abdominal disease. The current standard treatment for these cases involves a combination of 5-fluorouracil-based (5-FU) chemotherapy and localized radiation to the symptomatic primary site. Despite initial responses, the overall long-term outcome for these patients is poor. Disseminated intra-abdominal disease is present in 10–30% of GI cancer cases and is a frequent finding in patients who develop recurrent cancer. Natural history studies have established a 6-month median survival in this group of patients (1). Although GI carcinomas are known to be radiosensitive tumors, it has been a challenge to use full doses of chemotherapy in combination with standard doses of radiation therapy due to the increased toxicity. Whole-abdominal radiotherapy (WART) has been used in cases of GI cancer with disseminated intra-abdominal disease (2). However, the main shortcoming of WART is the inability to combine it with full-dose chemotherapy, which is a significant drawback in the attempt to eradicate disseminated micrometastatic disease. With recent laboratory and clinical data, a novel treatment paradigm allowing the use of full-dose systemic chemotherapy safely in combination with low-dose fractionated radiotherapy (LDFRT) is emerging, where the low-dose radiation sensitizes the tumor to subsequent chemotherapy, resulting in a 90% primary site response rate and 60% nodal site response rate (3). Traditionally, cell survival experiments have suggested that fractionated radiation doses greater than 120 cGy were required to

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overcome the initial DNA repair occurring at sublethal radiation doses. However, recent studies have shown a low-dose hyper-radiosensitivity (HRS) phenomenon in which cells die from hypersensitivity to small single doses (0.5–1 Gy) of radiation (4, 5). When the dose per fraction is reduced to 0.5–1 Gy, the total dose needed to produce comparable tissue damage is reduced. Joiner *et al.* demonstrate that the cell survival curve has a steep hypersensitive slope at <100 cGy, a plateau from 100–120 cGy and a straight-line apoptosis beyond 120 cGy (5). The method of providing multiple fractions of low-dose radiation concurrently maximizes cell death for highly mitotic cells without causing undue toxicity in senescent cells. In fact, Kunos *et al.* have shown that LDFRT was well tolerated and allowed chemopotentialization of docetaxel (DTX) in patients with recurrent epithelial ovarian cancer without adding to the systemic toxicity of docetaxel (6).

Ionizing radiation can induce cell cycle arrest (check-points) to allow DNA repair and prevent the passage of damaged DNA to the next generation of cells. In multifractionated regimen, it is believed that the initial fraction of low-dose radiation can cause arrest of the cells in G₂/M phase, which results in increased cytotoxicity with successive fractions. Krueger *et al.* have shown that inhibiting ChK1 and ChK2, two proteins integral in the G₂/M transition, increased low-dose radiosensitivity (7). Furthermore, low-dose radiation can stimulate DNA repair in normal tissues (8), increase antioxidant capacity and induce immune responses, which collectively produce an effective method of local tumor control (9). Hypoxia and nitric oxide levels also affect the cell's sensitivity to radiation. Reduction of nitric oxide level has been shown to enhance the radiosensitivity of hypoxic non-small cell lung cancer (10). Thus, the identification of cellular pathways that are responsive to low-dose radiation and their contribution to chemopotentialization is significant since it will provide a better measurement of the therapeutic response and help shape the design of future mechanism-based clinical trials for LDFRT as a chemosensitizer in metastatic GI cancer.

MATERIALS AND METHODS

Cell Culture and Treatments

Metastatic gastric carcinoma 5822 (NCI-N87), gastric adenocarcinoma 1739 (AGS) and normal small intestinal epithelial FHs 74 Int were obtained from the American Type Culture Collection (ATCC®, Manassas, VA). The cells were grown as adherent monolayers in their respective recommended media supplemented with 10% fetal bovine serum with 1% pen-strep. Gastric carcinoma 5822 cells were grown in modified essential media (MEM; ATCC), 1739 cells were grown in F-12 Kaighn's media (ATCC) and FH2 74 Int were grown in Hybri-Care media ATCC 46-X™ supplemented with 30 ng/ml epidermal growth factor (EGF). Docetaxel (Taxotere®), 5-fluorouracil and cisplatin [(CDDP) *cis*-diammineplatinum(II) dichloride] were obtained from Sigma-Aldrich®, LLC (St. Louis, MO) in a pure crystalline form and stored in sterile dimethyl sulfoxide (DMSO) (99.9% pure; Sigma-Aldrich) at –20°C. Stock solutions of each drug were diluted to the requirements by successive serial dilution in growth media. The cells

were irradiated with single doses between 0.05 and 1 Gy using a Pantak Seifert X-ray machine (250 kV, 13 mA with a 0.5 mm copper and 1 mm aluminum filter) producing X rays at a dose rate of 0.349 Gy/min (SSD = 82 cm) when using a foam barrier or 2.28 Gy/min (SSD = 32 cm) without a foam barrier. The lower dose rates were used for doses <0.5 Gy. The cells were grown at 37°C, irradiated at room temperature and immediately returned to 37°C as described previously (11).

Cell Viability and Survival

Cell viability was measured with a WST-1 assay kit (Roche Applied Science, Indianapolis, IN) as recommended by the manufacturer. The 5822 cells were plated at 25,000 cells/well in 200 µl of media, the 1739 cells were plated at 5,000 cells/well in 200 µl of media in quadruplicates in 96-well plates one day before irradiation and treatment. Each plate was irradiated at one of the following doses every 24 h for three consecutive days: 0, 0.05, 0.1, 0.15, 0.25, 0.5 and 1 Gy. After irradiation the cells were exposed to a chemotherapy regimen based on a planned phase II study of LDFRT for patients with peritoneal carcinomatosis from gastric or gastroesophageal junction primary adenocarcinomas. The chemotherapy doses were adjusted to produce 50% survival in a cell-based assay. On day 1 the cells were irradiated and treated with 5-FU (3.8 µM) and DTX (0.5 mM). On day 2 the cells were irradiated and treated with 5-FU (3.8 µM). On day 3 the cells were irradiated and treated with 5-FU (3.8 µM) and CDDP (1.6 µM). On day 4 the cells were either analyzed for viability (WST-1), RNA profiling or allowed to grow further to assess survival on day 10. For cell survival assay, the cells were treated as described above except that 300 cells were plated in 6-well plates and colonies (≥50 cells) were fixed, stained and counted ten days later (12). Because only low-dose radiation was given, we used the same number of cells in triplicate and irradiated the cells after they were attached. Radiation exposure was performed in 6-well plates. All six wells on each plate were irradiated at the same time for each dose of radiation. The colonies (60–90) were manually counted. Plating efficiency was 20–30%. Relative survival is expressed as a percentage of surviving colonies in reference to the mean plating efficiency of three sham-irradiated control plates. Survival was also evaluated in the presence of the NAD(P)H oxidase inhibitor diphenylene iodonium (DPI, 50 nM) (Sigma-Aldrich). To determine the effect of radiation on a modified regimen of docetaxel, cisplatin and 5'-fluorouracil (mDCF) and the effect of mDCF on radiation we calculated enhancement ratios as follows.

Radiation enhancement ratio for mDCF (mDCF ER) based on surviving fraction (SF):

$$\text{mDCF ER} = \frac{\text{SF}_{\text{mDCF alone}}}{\text{SF}_{\text{mDCF+radiation}}}; \quad (1)$$

and radiation enhancement ratio (RER) by mDCF:

$$\text{RER} = \frac{\text{SF}_{\text{radiation alone}}}{\text{SF}_{\text{mDCF+radiation}}}. \quad (2)$$

A ratio >1 is considered enhancement of either chemopotentialization by radiation (mDCF ER) or radiosensitization by mDCF (RER).

The contribution of radiation exposure to the induction of genes exposed to drugs was calculated as follows:

$$\text{Fold RER} = \frac{\text{Fold induction}_{\text{mDCF+radiation}}}{\text{Fold induction}_{\text{mDCF alone}}}. \quad (3)$$

A ratio >1 indicates that exposure to radiation contributes to further increase of gene expression induced by chemotherapy.

Identification of Biomarkers and Cellular Pathways

To determine which genes or cellular pathways are implicated in HRS, RNA was extracted 24 h after cells were exposed to three

FIG. 1. Gastric cancer cells treatment plan. The gastric cells were exposed to different radiation doses for three consecutive days (day 1–3). On day 1 the cells were irradiated and treated with 5-fluorouracil (5-FU; 3.8 μ M) and docetaxel (DTX; 0.5 mM). On day 2 the cells were irradiated and treated with 5-FU (3.8 μ M). On day 3 the cells were irradiated and treated with 5-FU (3.8 μ M) and cisplatin (CDDP; 1.6 μ M). On day 4 the cells were either analyzed for viability (WST-1), RNA profiling or allowed to grow further to assess survival on day 10.

consecutive days of radiation (0.15 Gy) and chemotherapy as described above. The RNA was harvested according to protocol using RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed and analyzed using the 7300 Real-Time PCR System (Applied Biosystems®, Carlsbad, CA) in the following RT² PCR Profiler™ Arrays (QIAGEN): Human Cancer PathwayFinder™, Human Hypoxia Signaling Pathway, Human Nitric Oxide Signaling Pathway and Human Drug Metabolism. Controls were treated similarly with vehicle alone (DMSO) and fold induction was calculated as a ratio of treated samples over untreated samples. Validation of dual oxidase 2 (DUOX2) induction was verified by RT-PCR performed on RNA extracted from cells treated as described above and by Western blots. For Western blots, protein were extracted with RIPA buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and (30 μ g) were loaded on 8% SDS-PAGE, transferred on PVDF membrane and hybridized to DUOX2 rabbit polyclonal antibody (Pierce™, Waltham, MA). Downregulation of DUOX2 was performed by transfection of DUOX2 shRNA-GFP plasmid (OriGENE, Rockville, MD) with FuGENE® HD (Madison, WI) in 5822 cells and transfection efficiency was monitored by fluorescence. Cell viability was measured as described above.

Production of Reactive Oxygen Species

Intracellular production of reactive oxygen species (ROS) was measured by the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes®, Eugene, OR). Gastric carcinoma 5822 and 1739 cells were seeded as 0.5×10^6 and treated as described above (Fig. 1). On day 4 the cells were harvested, collected and gently washed with $1 \times$ phosphate buffered saline. The cells were suspended in Hanks' balanced salt solution (HBSS) buffer and incubated with 10 μ M H2DCFDA or 0.5 μ M DCF for 20 min or 5 min, respectively, at 37°C. Propidium iodide was added in the last 5 min and the cells were stored on ice before data acquisition. Cells were analyzed using a FACScan™ (Becton-Dickinson, San Jose, CA). Ten thousand events were analyzed per sample. The mean intensity of fluorescence was analyzed by CellQuest™ software (Largo, FL).

Statistical Analysis

Statistical analysis was performed on relative (%) viability and survival of cells exposed to radiation and chemotherapy over cells exposed to radiation. Calculations were performed using the Student's *t* test. Probability values <0.05 are considered significant.

RESULTS

Chemopotentiality by LDFRT in Gastric Cancer Cells

Current first-line chemotherapy for metastatic gastric cancer commonly consists of docetaxel, cisplatin and 5-fluorouracil. However, the toxicity with this regimen is significant and alternative more tolerable approaches are needed. Recently, a less toxic and comparably effective modified DCF regimen was reported in a phase II trial (13). In an effort to determine whether LDFRT could potentiate mDCF we treated two gastric cancer cell lines, as shown in Fig. 1. The cells were exposed to different radiation doses for three consecutive days in the presence or absence of the following chemotherapy regimen; 5-FU and docetaxel on day 1, 5-FU on day 2 and 5-FU and cisplatin on day 3. To evaluate the effect of radiation on mDCF we selected the chemotherapy doses at the IC₅₀. Cell viability (Fig. 2A–B) was measured on day 4 and clonogenic survival (Fig. 2C–D) was evaluated on day 10. The data shown in Fig. 2A–B indicate that in both the metastatic gastric carcinoma 5822 and gastric adenocarcinoma 1739 cells, HRS was induced with radiation alone at 0.15 Gy (white bars). A statistically significant reduction of cell viability was observed with 0.15 Gy irradiation compared to sham irradiation (0 Gy) in both cell lines followed by a typical increase in radioresistance up to 1 Gy. Similar response was observed with mDCF (black bars) in the presence of 0.15 Gy resulting in more cells killed with the combined modality compared to mDCF alone (0 Gy). Clonogenic survival assays (Fig. 2C–D) mirrored the cell viability data and indicate that LDFRT potentiated mDCF compared to mDCF alone (0 Gy) at every radiation dose. For a better appreciation of chemopotentiality by irradiation, the data are expressed as a percentage of untreated cells (0 Gy). Figure 2C indicate that in 5822 gastric cells, 0.15 Gy was sufficient to kill 90% of the cells when LDFRT was combined with mDCF whereas a dose of almost ten times higher (1.35 Gy) was needed to

FIG. 2. Panels A and B: Cell viability assay (WST-1) performed on the indicated gastric cell lines after exposure to the indicated radiation doses for three consecutive days (white bars) or radiation plus chemotherapy as indicated in Fig. 1 (black bars). The data are expressed as a percentage of untreated (0 Gy) cells. Bars indicate standard deviation of triplicate. Panels C and D: Clonogenic survival assays performed on the indicated gastric cell lines. The cells were treated identical to those in panels A and B except that the cells were allowed to grow until day 10, fixed and stained. Relative survival is expressed as a percentage of untreated cells (0 Gy) or cells treated with mDCF alone (insets). * $P < 0.05$; ** $P < 0.005$.

achieve the same rate when using conventional radiotherapy alone. In fact, the mDCF enhancement ratio by irradiation in the 5822 cells was >1 at every radiation dose (Table 1), indicating that radiation enhanced the killing efficiency of mDCF. Data expressed as a percentage of cells treated with mDCF alone (0 Gy, inset) also indicate chemopotentialization by irradiation, especially at higher radiation doses. Chemopotentialization by irradiation was also observed in the 1739 cells (Fig. 2D, Table 1). Both cell lines also had a radiation enhancement ratio by mDCF higher than 1 for all radiation doses tested (Table 1). These data indicate that LDFRT can chemopotentialize mDCF in gastric cancer cells and that mDCF can sensitize gastric cancer cells to LDFRT.

DUOX2 Can Mediate HRS in Gastric Cancer Cells

In an effort to delineate the molecular mechanisms underlying chemopotentialization by LDFRT in gastric cancer cells we performed gene profiling arrays of cellular pathways known to be involved in cellular sensitivity to chemotherapy and radiation. Previous studies have shown that nitric oxide can sensitize mammalian cells to low-dose radiation (14). We thus analyzed this cellular pathway first.

We treated gastric cancer cells with radiation alone, mDCF alone or a combination of the two modalities as shown in Fig. 1. We exposed the cells to 0.15 Gy dose of radiation because this was the lowest dose that produced HRS in both cell lines (Fig. 2A–B). Our data (Fig. 3A) indicate that the DUOX2 gene was by far the most upregulated in response to the combined treatments in 5822 cells. Exposure to either three consecutive daily doses of 0.15 Gy or mDCF alone was also sufficient to upregulate DUOX2 in 5822 cells (Fig. 3B). In addition, upregulation of DUOX2 mRNA and protein (Fig. 3C–D) was observed in the 1739 cells as well, after exposure to either the combined regimen or each modality separately. However, DUOX2 mRNA was not upregulated by the combined regimen (Fig. 3C) or by each individual modality (data not shown) in the normal epithelial intestinal FHsInt74 cells. To verify the functional significance of DUOX2 upregulation we downregulated DUOX2 in 5822 cells and measured cell viability in response to three consecutive daily exposures to low-dose radiation. The data in Fig. 4A indicate that downregulation of DUOX2 (Fig. 4C) significantly increased radioresistance at 0.15 Gy exposure and at every radiation dose tested (data not shown). The radioresistance enhancement ratio calcu-

TABLE 1
Enhancement Ratios for 5822 and 1739 Gastric Cancer Cells

5822			1739		
Dose (Gy)	mDCF ER	RER by mDCF	Dose (Gy)	mDCF ER	RER by mDCF
0	1	3.2	0	1	2.6
0.05	1.7	4.6	0.05	1.3	3.0
0.1	1.9	2.3	0.1	1	1.9
0.15	2.8	4.4	0.15	1.6	2.5
0.25	2	3.1	0.25	1.9	2.9
0.5	5.8	9.4	0.5	4.1	4.6
1	7.5	6.1	1	5.9	2.5
1.5	40.5	5.6	1.5	ND	ND
2	202.3	11	2	93.5	1.7

Notes. mDCF enhancement ratio (mDCF ER) by radiation and radiation enhancement ratio (RER) by mDCF were calculated as described in the Materials and Methods section. A ratio >1 indicates enhancement. ND = not determined.

lated by shDUOX2 cell viability over scrambled shRNA cells is 2.4, which indicates enhancement (Fig. 4B). Moreover, the effect was even more pronounced in 1739 cells stably transfected with shDUOX2 where cell viability

was more than 40% higher than the control (scrambled) when exposed to the combined regimen (Fig. 4D). On the other hand, overexpression of DUOX2 increased cell sensitivity to the combined regimen (data not shown). These data indicate that DUOX2 levels can in fact modulate gastric cancer cell sensitivity to LDFRT.

LDFRT Increases ROS

Because DUOX2 is a member of the NAD(P)H oxidase family that can produce reactive oxygen species, including superoxide and hydrogen peroxide (15), we then evaluated the effect of LDFRT and mDCF on ROS levels. The cells were labeled with the oxidation sensitive probe H2DCFDA that can be oxidized to its fluorescent product DCF by hydroperoxides and other prooxidants. The data shown in Fig. 5A–B indicate that indeed each modality can increase ROS levels in both cell lines and that the combined regimen produced the highest levels, up to 3.5 times more, compared to untreated cells (Fig. 5B). To control for possible changes in probe uptake, ester cleavage or efflux of the probe, the cells were also labeled with H2DCFDA oxidation-insensitive analogue DCF. As shown in Fig. 5D, no changes in

FIG. 3. Panel A: RNA profiling performed on the nitric oxide pathway. The metastatic gastric carcinoma 5822 cells were treated with 0.15 Gy radiation combined with chemotherapy for three consecutive days as indicated in Fig. 1. On day 4 the cells were harvested and the RNA analyzed. Panels B and C: Real-time PCR (RT-PCR) was performed on RNA extracted from 5822, 1739 or FHsInt74 cells to measure dual oxidase 2 (DUOX2) levels after the same treatment as shown in panel A (X ray + drug) or radiation alone (X ray) or chemotherapy alone (drug). Panel D: Western blot analysis of DUOX2 levels in 1739 cells after exposure to 0.15 Gy for three consecutive days (X ray), chemotherapy (drug) or radiation plus chemotherapy (X ray + drug). Fold induction was measured by densitometry normalized to actin and untreated sample.

FIG. 4. Panel A: Downregulation of DUOX2 confers resistance to radiation in 5822 cells. DUOX2 was transiently downregulated by shRNA (shDUOX2) and cell viability (WST-1) was assessed after three consecutive days of irradiation at the indicated dose. Control was performed with scrambled RNA (SC). Data are expressed as a percentage of untreated cells (0 Gy). * $P < 0.05$. Panel B: Radioresistance enhancement ratio (RRER) conferred by DUOX2 downregulation. The ratio was calculated by dividing the percentage of cell viability obtained with shDUOX2 by the percentage of cell viability obtained with control scrambled RNA (SC). Panel C: Western blot of DUOX2 downregulation in 5822 cells stably transfected with DUOX2 shRNA (shDUOX2) or control scrambled RNA. GAPDH is used as a loading control. Panel D: 1739 cells were stably transfected with either shDUOX2 or control scrambled RNA and cell viability was determined as in panel A after exposure to combined regimen of LDFRT and mDCF. ** $P < 0.005$.

fluorescence levels were observed when the cells treated with the dual modalities were labeled with the oxidation-insensitive probe (Fig. 5D). Moreover, when the cells were treated with the oxidation-sensitive probe in the presence of the NAD(P)H oxidase inhibitor DPI, the levels of the fluorescent product decreased significantly with all treatments (Fig. 5C). Furthermore, treating the cells with DPI abrogated the cell-killing efficiency of LDFRT combined with mDCF (Fig. 5E). These data thus support a primary role for DUOX2 in producing ROS and mediating LDFRT-induced HRS.

Amiloride Binding Protein 1 and Lactoperoxidase, but Not the Conventional DNA Damage Response, Are Upregulated by LDFRT Combined with mDCF

In an effort to better understand the molecular events underlying HRS, we next evaluated the effect of chemopotentiation by LDFRT on the expression of genes associated with the cancer pathways, the hypoxia pathways and the drug metabolizing pathways. The data shown in Table 2 indicate that several genes of the cancer pathways were affected by the combined regimen. In general, genes

receiving treatment alone were affected similarly to genes receiving the combined regimen (data not shown). Several genes upregulated by the combined treatment in both gastric cell lines [e.g., Integrins (ITGA1) and GzmA] are either involved in the production of or responding to ROS (16, 17). This is consistent with the induction of DUOX2 (Fig. 3), an enzyme that generates hydrogen peroxide. Genes usually associated with the classical DNA damage response such as ATM, TP53, RB1 and BRAC1 were either downregulated or not affected by the combined regimen in both gastric cell lines and in normal epithelial intestinal cells FHSInt74 (Table 2). In the cancer pathway, only a few genes, including CDKN1A/p21, APAF1 and ANGPT2, were upregulated to higher levels by the combined treatment compared to each individual modality (Table 2). Some of these genes are likely to contribute to chemopotentiation by LDFRT by promoting apoptosis. For example, p21 can increase ROS levels and induce cell death in certain cancer cells (18, 19), the production of hydrogen peroxide by DUOX2 could promote an interaction between caspase-9 and apoptotic protease-activating

FIG. 5. Panels A and B: Detection of cellular reactive oxygen species. The indicated gastric cells were treated as indicated and exposed to the oxidation sensitive and cell permeant reagent 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA). After diffusion into the cell, H2DCFDA is deacetylated by cellular esterases to a nonfluorescent compound, which is later oxidized by ROS into 2',7'-dichlorofluorescein (DCF). Intensity of DCF fluorescence is detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 and 529 nm, respectively. Representative results of three experiments are shown. Panel C: Same as panel A, except that the cells were treated in the absence or presence of the NAD(P)H oxidase inhibitor DPI and results of three independent experiments are expressed as mean fluorescence intensity (MFI) of the respective controls. Panel D: Same as panel A, except that the cells were incubated with the oxidation-insensitive DCF probe. Results of two independent experiments are expressed as mean fluorescence intensity (MFI) of control. Panel E: Survival assay in the presence of DPI. The cells were treated with 150 nM DPI and exposed to the indicated treatments. Survival was performed as stated early in Fig. 2 and data are expressed as a percentage of their respective controls. * $P < 0.05$.

factor 1 (Apaf-1) (20) and upregulation of ANGPT2 can activate caspase-3 (21).

The combined regimen also upregulated genes activated by ROS in the hypoxia pathway. CASP1 (22), HIF1A (23), COL1A1 (24) and MT3 were all upregulated by the combined treatments (data not shown). In the drug metabolism array, only two genes, lactoperoxidase (LPO) and amiloride binding protein 1 (ABP1) were upregulated to higher levels by the combined treatments than by each individual modality (Fig. 6). In fact the fold radiation enrichment ratios were 3.56 and 1.65 for LPO and ABP1, respectively (Fig. 6D). LPO uses hydrogen peroxide as a substrate and can contribute to oxidative stress, and ABP1 also known as diamine oxidase, is a biomarker for 5-FU GI toxicity (25). Taken together these data indicate that

chemopotentialiation by LDFRT can be mediated, at least in part, by upregulation of DUOX2 and genes associated with ROS production without upregulation of classical DNA repair genes.

DISCUSSION

The goals of our study were to determine whether LDFRT can induce HRS in gastrointestinal cancer cells and to identify biomarkers of chemopotentialiation by LDFRT. Chemopotentialiation by LDFRT could allow the safe use of full-dose systemic chemotherapy in combination with whole-abdominal radiotherapy to manage gastrointestinal cancers that have disseminated into intra-abdominal tissues. In this study, our first aim was to determine whether two

TABLE 2
Example of Cancer Pathway Gene Fold Induction Relative to Control after Combined Treatments in 5822, 1739 Gastric Cancer Cells and FHsInt74 Normal Epithelial Intestinal Cells

Gene name	Fold relative to control		Fold RER $\frac{\text{mDCF}+\text{XR}}{\text{mDCF}}$	Gene name	Fold relative to control		
	mDCF + X ray				mDCF + X ray		
	5822 cells	1739 cells			5822 cells	1739 cells	FHsInt74 cells
ITGA1	960	1.41	0.07	ATM	Down	1.31	0.95
ITGB3	1.10	2.11	0.05	TP53	Down	0.75	0.72
ITGB5	6.79	1.31	0.39	BRCA1	Down	1.24	0.02
MTA1	241.6	1.13	0.97	RB1	Down	1.33	0.11
MTA2	52	2.53	0.68	E2F1	Down	1.74	ND
ANGPT2	14.75	1.16	1.49	TNFRSF10B	Down	1.52	ND
APAF1	6.26	2.59	2.43	TNFRSF1A	Down	0.78	
CDC25A	4.65	2.45	0.86	CASP8	Down	1.09	ND
CDKN1A/p21	25.2	2.94	5.26	BCL2	Down	0.6	ND
CDK2	2.89	1.46	1.18	PDGFB	Down	1.05	ND
GZMA	5.1	4.74	0.32	PDGFA	Down	0.92	ND
HTATIP2	8.74	1.42	0.74	TGFB1	Down	1.74	ND
TIMP1	18.5	1.07	1.33	MMP2	Down	0.93	ND
FRG2	26.46	2.48	1.16	MYC	Down	1.66	ND
NME4	33.53	1.12	1.11	JUN	Down	1.13	ND

Notes. Genes involved in DNA repair pathways were either not induced (1739, FHsInt74 cells) or downregulated (5822 cells). Fold radiation enhancement ratio (RER) was calculated by dividing the fold induction observed with the combined regimen (mDCF + X ray) by the fold induction observed with chemotherapy alone (mDCF). ND = Not determined.

FIG. 6. Panels A–C: RNA profiling performed on drug metabolism PCR array. The metastatic gastric carcinoma 5822 cells were treated as stated early in Fig. 1A or chemotherapy alone (Fig. 1B, drugs) or radiation alone (Fig. 1C, X ray) and the RNA analyzed as stated in Fig. 3D. Only two genes, LPO and ABP1 (black boxes) were upregulated to higher degrees with the combined regimen than with each individual treatment. Panel D: The fold radiation enhancement ratio (RER) was calculated by dividing the fold induction obtained with radiation plus chemotherapy (X ray + drug) over the fold induction obtained with chemotherapy alone.

gastric cancer cell lines were positive for HRS. HRS was originally thought to be a prerequisite for chemopotentialization by LDFRT, but in a recently reported study this concept was challenged when intrinsic radiation sensitivity in non-small lung cancer cells was shown to correlate more than HRS with chemopotentialization by LDFRT. Our data are in good agreement with these recent findings. In fact, HRS was observed at radiation doses as low as 0.15 Gy in both 5822 and 1739 cells but chemopotentialization by LDFRT was more pronounced in the 5822 cells, the most radiosensitive of the two cell lines (Fig. 2 and Table 1). The mechanisms underlying HRS are still not completely understood but are associated with a failure to arrest in early G₂ and increased apoptosis (26, 27). Nonetheless, it has been suggested that mechanisms other than a failure to arrest in early G₂ can lead to HRS since some cells lacking the capacity to arrest in G₂ after LDFRT are also negative for HRS (28). In an effort to identify biomarkers for HRS and improve our current understanding of chemopotentialization by LDFRT we analyzed different cellular pathways after exposure to a combined regimen of mDCF and LDFRT. Our data indicate that in the nitric oxide pathway DUOX2 is the gene that by far was the most upregulated in response to the combined treatments of mDCF and LDFRT (Fig. 3). Moreover, downregulation of DUOX2 increased radioresistance (Fig. 4). DUOX2 is an enzyme involved in the generation of H₂O₂ and ROS. Interestingly, DUOX2 is expressed in a number of radiosensitive tissues such as the thyroid, airway epithelial cells, salivary gland and gastrointestinal tract. By increasing the levels of ROS in these tissues, DUOX2 is likely to enhance the killing efficiency of LDFRT and activate ROS-responsive genes. Our data indicate that, indeed, a limited number of genes regulated by ROS are upregulated to higher levels by the combined regimen than by either treatment alone. These genes may represent a signature of chemopotentialization by LDFRT. Among these genes, CASP1 is a likely candidate for chemopotentialization by LDFRT since it requires ROS for its activation and has also been reported to be upregulated by LDFRT in lung cancer cells (28). The increased production of ROS by the combined modalities and the inhibition of the killing efficiency of the combined regimen by a NAD(P)H oxidase inhibitor (Fig. 5) strongly support this possibility. In addition, our data indicate that the combined regimen of mDCF and LDFRT failed to activate the classical DNA damage response (Table 2) suggesting that DNA repair would be impeded while DNA damage would be increased by ROS production. This is consistent with earlier studies indicating that exposure to low-dose radiation is sufficient to increase DNA damage, but does not activate DNA repair (29, 30). Chemopotentialization by LDFRT thus appears to be mediated, at least in part, through DUOX2 activation and failure to activate the DNA damage response in gastric cancer cells.

The study presented here supports a rationale for further clinical explorations of potential applications of LDFRT,

such as in WART, as chemopotentializers for advanced and metastatic gastric cancers.

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