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Low Dose Rate Radiosensitization of Hepatocellular Carcinoma *In Vitro* and in Patients[1]

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Abstract

Transarterial radioembolization (TARE) with ^{90}Y microspheres delivers low dose rate radiation (LDR) to intrahepatic tumors. In the current study, we examined clonogenic survival, DNA damage, and cell cycle distribution in hepatocellular carcinoma (HCC) cell lines treated with LDR in combination with varying doses and schedules of 5-fluorouracil (5-FU), gemcitabine, and sorafenib. Radiosensitization was seen with 1 to 3 μM 5-FU (enhancement ratio 2.2–13.9) and 30 to 100 nM gemcitabine (enhancement ratio 1.9–2.9) administered 24 hours before LDR (0.26 Gy/h to 4.2 Gy). Sorafenib radiosensitized only at high concentrations (3–10 μM) when administered after LDR. For a given radiation dose, greater enhancement was seen with LDR compared to standard dose rate therapy. Summarizing our clinical experience with low dose rate radiosensitization, 13 patients (5 with HCC, 8 with liver metastases) were treated a total of 16 times with TARE and concurrent gemcitabine. Six partial responses and one complete response were observed with a median time to local failure of 7.1 months for all patients and 9.9 months for patients with HCC. In summary, HCC is sensitized to LDR with clinically achievable concentrations of gemcitabine and 5-FU *in vitro.* Encouraging responses were seen in a small cohort of patients treated with TARE and concurrent gemcitabine. Future studies are needed to validate the safety and efficacy of this approach.

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Introduction

Hepatocellular carcinoma (HCC) is a leading cause of cancer-related deaths worldwide [1]. Transarterial radioembolization (TARE) with yttrium-90 (^{90}Y) microspheres is one of the many treatment options available for patients with unresectable HCC. Because tumors in the liver derive most of their blood supply from the hepatic artery versus the portal vein [2], this therapy preferentially targets the tumor and spares uninvolved liver parenchyma. Prior reports have shown that TARE with ^{90}Y microspheres is associated with a 42% partial response rate [3,4] and longer progression-free survival than chemoembolization [5].

Concurrent chemoradiotherapy has proven to be more efficacious than radiation alone in the majority of gastrointestinal malignancies. A drug which preferentially sensitizes HCC to the cytotoxic effects of low dose rate radiation (LDR) produced by ^{90}Y microspheres would potentially improve the efficacy of this therapy. Candidate drugs for radiosensitization include gemcitabine and 5-fluorouracil (5-FU) in addition to agents with known efficacy in HCC such as sorafenib. Gemcitabine and 5-FU are used routinely in combination with external beam radiation therapy for several intra-abdominal malignancies including pancreatic and gastric cancer [6–8]. Sorafenib was shown in a preclinical study to be an effective radiosensitizer in HCC when given after radiation therapy but not when given before treatment [9].

In the current study, we evaluated the potential of gemcitabine, 5-FU, and sorafenib to radiosensitize HCC to ^{90}Y microspheres. Because the mean dose rate achieved during an administration of ^{90}Y microspheres is

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0.05 to 0.5 Gy per hour, we used a novel *in vitro* LDR model system that could deliver a dose rate in this range. We assessed clonogenic survival, DNA damage repair, and cell cycle distribution in HCC cells *in vitro*. Additionally, we report our early clinical experience of combining TARE with gemcitabine in patients with primary liver cancer and liver metastases.

Materials and Methods

Cell Culture and Drug Treatment

Human HCC cell lines (Hep3B, HepG2) were maintained in F-12 or RPMI media supplemented with 10% fetal bovine serum and penicillin/streptomycin. Experiments involving 5-FU were carried out in dialyzed serum with leucovorin. Gemcitabine (Eli Lilly, Indianapolis, IN), 5-FU/leucovorin (Sigma-Aldrich, St. Louis, MO), and sorafenib (University of Michigan Pharmacy, Ann Arbor, MI) were tested in combination with LDR. Drugs were diluted in PBS to appropriate concentrations which were selected to correspond to clinically achievable levels.

Radiation Techniques

LDR was delivered using a custom-built LDR device consisting of an array of cesium-137 sources. This array is shielded by interlocking 6-cm–thick pieces of Cerrobend and resides inside a cell culture incubator at 37°C. Dose homogeneity determined by film was within ± 5%. Cells were irradiated at a dose rate of 0.07, 0.10, or 0.26 Gy/h for 16 hours to a total dose of 1.1, 1.6, or 4.2 Gy. Standard dose rate radiation (SDR) was delivered using a Philips RT250 orthovoltage unit (Kimtron Medical, Oxford, CT) at a dose rate of approximately 2 Gy per minute to a total dose of 2 to 4 Gy. Dosimetry was carried out using an ionization chamber connected to an electrometer system directly traceable to a National Institute of Standards and Technology calibration.

Clonogenic Survival Assay

After radiation was complete, cells were suspended and counted then plated at set densities based on the dose of radiation received. Cells were incubated until visible colonies were present. Colonies were fixed with methanol/acetic acid (7:1) and stained with crystal violet. The number of colonies containing ≥ 50 cells was determined. Enhancement ratios were calculated by dividing the surviving fraction without drug by the surviving fraction with drug for each dose of radiation with an adjustment for plating efficiency. Experiments were performed in at least triplicate, and the mean and standard error were calculated.

Cell Cycle Distribution

Cell cycle distribution was determined using propidium iodide (PI, 0.018 mg/ml) staining and flow cytometry. Cells were fixed in 70% ethanol at the appropriate time points then incubated with PI before quantification using flow cytometry. Trout erythrocytes were used as the internal standard. Data were analyzed using FlowJo (Tree Star, Ashland, OR). Single-cell populations were gated, and histograms were modeled using the Watson method. A fixed distance between the G1 and G2 peaks was used for each cell line based on untreated controls.

γH2AX Detection

Cells were fixed with 70% ethanol after treatment at the appropriate time points. Fixed cells were incubated with anti-γH2AX mouse antibody (Millipore, Billerica, MA) at a concentration of 1:500 overnight followed by fluorescein isothiocyanate–labeled anti-mouse secondary antibody (Sigma-Aldrich) for 2 hours. Cells were then counted with flow cytometry. Trout erythrocytes were used as the internal standard. FlowJo software was used to quantify the percentage of cells staining positive for γH2AX.

Transarterial Radioembolization and Gemcitabine in Patients

Thirteen patients with primary liver cancer or liver metastases were treated with a single dose of gemcitabine (200–400 mg/m^2) 1 day before TARE with TheraSpheres (Nordion, Ottawa, Canada). Radioembolization dose was defined as the dose to the entire lobar volume. Response was determined based on the Response Evaluation Criteria in Solid Tumors (RECIST). Survival endpoints were calculated from the start of treatment. Local failure was defined as progression in the region of the liver targeted with TARE. Patient were typically seen 1, 3, and 6 months after treatment with follow-up imaging obtained 2 to 3 months after treatment then every 4 to 6 months or as clinically indicated. Data were retrospectively collected and analyzed under an Institutional Review Board–approved protocol.

Statistical Analysis

The mean and standard error were calculated using Microsoft Excel Software (Seattle, WA). For *in vitro* studies, a Student's *t* test was used to compare treatment groups. A *P* value of ≤ .05 was considered statistically significant. Experiments were performed in at least triplicate to ensure reproducibility. The Kaplan-Meier method was used to determine overall survival, local progression-free survival, and time to local failure for all patients treated. Median survival was calculated with JMP software (version 10; SAS, Cary, NC).

Results

Low Dose Rate Radiosensitization

To test our hypothesis that systemic therapy enhances the cytotoxic effect of LDR, we first determined the optimal schedule and concentration of each agent. Clonogenic survival assays with HCC cell lines were performed using gemcitabine, 5-FU/leucovorin, and sorafenib at different dosing schedules. Schedules were chosen based on our experience using these agents with external beam radiation therapy. For gemcitabine, cells were treated for 2 hours either 1 day before or just before LDR. Both schedules resulted in effective radiosensitization at a cytotoxic concentration of gemcitabine (100 nM); however, at noncytotoxic concentrations (10–30 nM), treatment 24 hours before LDR was required for optimal radiosensitization (Figure 1*A*). Similar to our findings with gemcitabine, treatment with 5-FU resulted in greater radiosensitization if started 24 hours before LDR compared to treatment just before LDR (Figure 1*B*). This schedule provided greater enhancement ratios at cytotoxic (3–10 μM) and noncytotoxic concentrations (1 μM) of 5-FU.

Prior reports demonstrate that sorafenib radiosensitizes if administered after radiation but has protective effects if given before [9]. Using this information, we treated cells with sorafenib at the start of or immediately after LDR. Sorafenib was not an effective radiosensitizer at noncytotoxic concentrations (0.3–1 μM) with either dosing schedule. However, at a cytotoxic concentration (10 μM), radiosensitization was observed with both schedules (Figure 1*C*).

Using the optimal dosing schedules determined from the prior experiment, we next tested the effect of changing the radiation dose