Cancer Biology and Signal Transduction

Longitudinal Time-Dependent Effects of Irradiation on Multidrug Resistance in a Non–Small Lung Cancer Cell Line

Yumiko Kono, Keita Utsunomiya, Shohei Kanno, and Noboru Tanigawa

Abstract

Multidrug resistance (MDR) in cancer is known to decrease the therapeutic efficacy of chemotherapy. The effects of irradiation on MDR in cancer cells remain unclear. Tc-99m methoxyisobutylisonitrile (MIBI) exhibits the same ATP-binding cassette (ABC) transporter kinetics as the chemotherapeutic compound doxorubicin. In this study, we investigated the synergistic effects of chemotherapeutics and irradiation [0 Gy: C (control) group; 3, 6, 9, 12 Gy: I (irradiation) group] in the human non–small lung cancer cell line H1299 exhibiting MDR, on MIBI and doxorubicin ABC transporter kinetics, in vitro and in vivo, respectively. In vitro, inhibition of H1299 cell proliferation by irradiation was found to be irradiation dose dependent. The degree and duration of MDR inhibition in vitro in H1299 were also dose dependent. In the cells of both the C group and 3-Gy I group, no significant difference of MIBI accumulation was observed. In the 6-Gy I group, a higher MIBI accumulation was observed at only 7 days after irradiation relative to the C group. A higher MIBI accumulation in the 9- and 12-Gy I groups with a significant difference from the C group was observed at 4 to 14 days after irradiation. A significant negative correlation between intracellular MIBI accumulation and cell replication was found. In vivo, high accumulation and retention of doxorubicin were observed in irradiated tumors in the H1299 xenograft mice group at 4 to 14 days after 9-Gy irradiation compared with the control mice group. These results provide evidence for a synergistic effect of concurrent chemotherapy and radiotherapy. Mol Cancer Ther; 13(11); 1–7. ©2014 AACR.

Introduction

Multidrug resistance (MDR) in cancer cells is known to decrease the therapeutic efficacy of some types of chemotherapy (1). MDR is due to the overexpression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (Pgp), multidrug resistance–associated protein 1 (MRP1), and others, in the plasma membrane. These overexpressed ABC transporters pump energy-dependent chemotherapeutic agents out from the intracellular volume into the extracellular milieu. MDR cancer cells thus exhibit resistance to chemotherapy resulting in poor outcomes for patients (2, 3).

Concurrent chemoradiotherapy has now been established as a standard treatment based on evidence of improved efficacy in treatment outcomes as compared with only radiation therapy (4–8). However, the response of various tumors to chemoradiotherapy can vary greatly, even for tumors of the same histologic type, for a given treatment regimen. Furthermore, some patients acquire MDR during their first course of chemotherapy, whereas others acquire MDR only during subsequent treatments. In addition, the irradiation protocols that should be used to obtain the greatest beneficial synergy with chemotherapy have not been determined to date.

In chemoradiotherapy, suppression of MDR is the key to increasing the efficacy of many chemotherapeutic agents. However, it should be also noted that with regard to effects of radiation on MDR in cancer cells there are conflicting reports. Some researchers have reported that irradiation inhibits MDR (9, 10), whereas other researchers have reported that irradiation promotes MDR (11). Further research is thus required to elucidate the effects of irradiation on MDR in specific tumor cell types.

Tc-99m hexakis-2-methoxyisobutylisonitrile (MIBI) is a nuclear medicine agent used in diagnostic imaging of the parathyroid and heart. MIBI, as a substrate for Pgp and MRP1, has been shown to have similar pharmacokinetics to chemotherapeutic agents such as doxorubicin (12–15). Therefore, there are a number of clinical research reports assessing the utility of MIBI in predicting the chemotherapeutic response in non–small cell lung cancer (16–23).

The purpose of this study is to investigate how to optimally combine chemotherapy and radiation therapy to achieve the maximum synergistic effect in
chemoradiotherapy. Using doxorubicin in vivo and MIBI in vitro, we investigated the relationship between multiplication and MDR of non–small lung cancer cells and the time-dependent effect of irradiation on MDR in non–small cancer cells.

Materials and Methods

Cell line and tracers

Human non–small lung cancer cells (H1299) transfected with the wtp53 gene (H1299/wtp53), obtained in 2009 from the Division of Oncology, Biomedical Imaging Research Center, Fukui University, Japan, were used in this study. This cell line was cultured in medium containing Geneticin as a selective agent and was thus confirmed to be H1299/wtp53. MDR was demonstrated in the H1299 cells through immunostaining with Pgp and MRP-1 monoclonal antibodies (Fig. 1).

The radiotracer Tc-99m MIBI, a substrate of MDR-related ABC transporters, was used in vitro as a proxy for chemotherapeutic drugs. In vivo, the chemotherapeutic drug doxorubicin was used as a fluorescent tracer.

Cell culture and irradiation

H1299 cells were cultured at 37°C in a conventional humidified 5% CO2 incubator. The cells were grown in DMEM containing 10% (v/v) FBS, streptomycin (50 μg/mL), and Geneticin (G418; 200 μg/mL). The single-cell suspensions, in a canister 105 mm in height and 312 mm in diameter, were γ-ray irradiated (Gammacell 40 Exactor; Nordion International) with a central dose rate of 0.84 Gy/min before cell seeding at room temperature. Five groups, categorized according to radiation dose, were prepared: 0 Gy (control group), 3 Gy (3-Gy group), 6 Gy (6-Gy group), 9 Gy (9-Gy group), and 12 Gy (12-Gy group). After irradiation, the cells were incubated for 1, 4, 7, or 14 days in tissue culture flasks according to the method described above.

Replication study in vitro

At each of 1, 4, 7, and 14 days after γ-ray irradiation, the I groups (3, 6, 9, and 12 Gy) and C group (0 Gy) were trypsinized, centrifuged, and resuspended in 0.5 to 2.0 x 10⁵/225 cm² (0 and 3 Gy: 0.5 x 10⁵/225 cm²; 6 Gy: 1 x 10⁵/225 cm²; 9 and 12 Gy: 2 x 10⁵/225 cm²) flasks in fresh media to exclude the inhibitory effect by confluent. For the H1299 cell line, it was possible to subculture the highest-dose 12-Gy irradiation group in the same manner as the lower-dose groups.

The cell numbers were counted by staining with Trypan blue. For the 14 days incubation group, to prevent the cell cultures in the flasks from becoming confluent, the cells were subcultured into new flasks at 7 days after irradiation. The replication rate (RR) and replication coefficient (RC) at each time point after irradiation, 1, 4, 7, and 14 days, were calculated using the following equations:

\[
RR = \frac{N}{N_0}, \quad RC = \frac{\log_2(N) - \log_2(N_0)}{t - t_0},
\]

Figure 1. Immunostaining of Pgp and MRP-1 in H1299 cell line. Magnification: A, B, C, and D, ×40; A', B', C', and D', ×100. B and B': H1299 expressed Pgp and isotype-matched negative control (A and A'). D and D': H1299 also expressed MRP-1 and isotype-matched negative control (C and C').
where \( N \) is the number of cells after irradiation, \( N_0 \) is the number of cells before irradiation, and \( t - t_0 \) is the number of days under culture.

The replication studies were repeated four times.

**Tc-99m MIBI accumulation study in vitro**

For the MIBI accumulation studies, cells in the C and I groups were trypsinized, after the end of the incubation period, and suspended in 7.0-mL complete medium at a concentration of 1.0 to 2.0 \( \times 10^6 \) cells/mL. The cell suspensions were incubated at 37°C in a stirred water bath. Samples (300 \( \mu \)L) were obtained in duplicate at 1, 15, 30, 45, and 60 minutes after adding 370 kBq MIBI and transferred to 1.5-mL microcentrifuge tubes containing 1-mL ice-cold saline. These samples were then centrifuged at 14,000 rpm for 2 minutes to produce cell pellets, the supernatant was aspirated, and the remaining pellets were carefully washed with 0.5- to 1.0-mL ice-cold saline to remove remaining unbound MIBI. The sample radioactivity was counted using an automatic \( \gamma \) counter (WIZARD 3” 1480; Perkin Elmer, Life Science). The accumulation ratio (CPM\(_{\text{in}}\)/CPM\(_{\text{out}}\)) was calculated as the ratio of radioactivity concentration inside the cell to that found in the supernatant. Using the measurements of radioactivity in the cell pellets and a standard representing the supernatant concentration together with an independent measurement of cell volume, the accumulation ratio equation was calculated as follows (24, 25):

\[
\text{CPM}_{\text{in}}/\text{CPM}_{\text{out}} = \frac{\text{counts per minute}_\text{pellet} \times \# \text{cells}_\text{pellet}}{\text{counts per minute}_\text{supernatant}}
\]

where \( \text{counts per minute}_\text{pellet} \) is the measured radioactivity rate of the pellet, \( \# \text{cells}_\text{pellet} \) is the number of cells in the pellet, \( \text{counts per minute}_\text{supernatant} \) is the measured radioactivity rate of the supernatant, \( \text{volume}_\text{pellet} = \text{volume}_\text{supernatant} = 300 \mu \text{L} \) and \( \# \text{cells}_\text{pellet} = \text{volume}_\text{pellet} \times \text{density}_\text{pellet} = 300 \mu \text{L} \times (5 \times 10^5 \text{ cells}/\mu \text{L})^\dagger \) (\( \dagger \) is the constant density per 0.1 mL of packed H1299 cells).

The CPM\(_{\text{in}}\)/CPM\(_{\text{out}}\) ratios of the I and C groups were measured at 60 minutes after the addition of MIBI. These MIBI accumulation studies were repeated four times.

**The correlation study**

We analyzed the correlation between cell multiplication and MDR using the RC from the in vitro replication study and the accumulation ratios from the in vivo MIBI accumulation study.

**Doxorubicin fluorescence imaging study in vivo**

This animal study obtained approval from an appropriate animal care committee in conformance with the Guide for the Care and Use of Laboratory Animals [Institute for Laboratory Animal Research (ILAR) 8th Edition, 2010].

Approximately 5.0 million human non–small lung cancer cells, from the same line (H1299), were subcutaneously inoculated into a BALB/c-nu/nu mouse model at the dorsal right shoulder. The tumors were grown until they attained a volume of at least 75 mm\(^3\). Five such mice were prepared as irradiation-free controls and another five were prepared for localized thoracic irradiation. The irradiated mice received a total \( \gamma \)-ray dose of 9 Gy via a lead shield collimated \( ^{137}\text{Cs} \) source (Gammacell 40 Exactor; Nordion International). At 7 days after 9-Gy irradiation, 200 \( \mu \text{g}/100 \mu \text{L} \) of doxorubicin was injected into the tail veins of both the control and irradiated mice. Doxorubicin accumulation (counts/mm\(^3\)) was measured from injection to 1, 60, and 120 minutes after injection using an in vivo fluorescent imaging system (Xenogen-IVIS Lumina; PerkinElmer Inc.). The doxorubicin accumulation ratio, AR\(_{\text{doxorubicin}}\), was defined as the ratio of the irradiated region of interest, ROI\(_{\text{IR}}\), normalized by a nonirradiated region of interest, ROI\(_{\text{NIR}}\), thus

\[
\text{AR}_{\text{doxorubicin}} = \frac{\text{ROI}_{\text{IR}}}{\text{ROI}_{\text{NIR}}}
\]

Specifically, ROI\(_{\text{IR}}\) was defined as a circular region, with a diameter of 6 mm, at the tumor mass of the irradiated site, and ROI\(_{\text{NIR}}\) was similarly defined as a circular region, with a diameter of 18 mm, at the skin of the shielded site. The doxorubicin accumulation ratios of the irradiated groups were compared with the control group.

**Statistical analysis and the correlation study**

The statistical analyses of the results of the in vitro replication and MIBI accumulation studies and the in vivo doxorubicin fluorescent imaging study were all performed using repeated ANOVA. The correlation between the in vitro cell replication and MIBI accumulation studies was determined using the linear Spearman rank correlation coefficient. The threshold for statistical significance for all statistical analyses was set as \( P < 0.05 \).

**Results**

**Replication study in vitro**

Irradiation suppressed the cell RR in all the I groups relative to the C group. This RR suppression was found to be dose dependent, over the entire time interval of observation from 1 to 14 days. Although the RR decreased after irradiation, the cells continued to multiply in all I groups with the exception of the 12-Gy (high dose) I group (Fig. 2). In Table 1, the RC of all the I groups is compared with that of the C group. In 1 day after irradiation, no statistically significant difference for the RC was found relative to the C group. Besides, for all I groups,
a significant RC dose-dependent decline was found from 4 to 14 days after irradiation.

**Tc-99m MIBI accumulation study in vitro**

Intracellular MIBI accumulation was observed, for both the C group and all I groups, after MIBI addition. The CPM\textsubscript{in}/CPM\textsubscript{out} ratio attained a plateau at about 30 minutes after the addition of MIBI (45- and 60-minute data were within ± SE) in all groups. A dose-dependent increase in the CPM\textsubscript{in}/CPM\textsubscript{out} ratio, indicating increased intracellular MIBI uptake, was observed from 4 to 14 days after irradiation in all the I groups (Fig. 3A). The graph consisting of 60-minute data of all periods (Fig. 3B) shows the time-dependent effect of inhibition of MDR by irradiation. Using this result, the CPM\textsubscript{in}/CPM\textsubscript{out} ratio at 60 minutes after addition of MIBI was measured from 4 to 14 days for both the C and I groups to determine the longitudinal time effect of irradiation on MDR (Fig. 3B). For both the C group and 3-Gy I group, no statistically significant difference in MIBI accumulation was observed from 1 to 14 days after irradiation. For the 9- and 12-Gy I groups, a statistically significant higher CPM\textsubscript{in}/CPM\textsubscript{out} ratio was observed from 4 to 14 days after irradiation compared with the C group. For the 6-Gy I group, a significantly higher CPM\textsubscript{in}/CPM\textsubscript{out} ratio was observed at the 7th day. However, unlike the 9-Gy I group, there was no significant difference at the 14th day. Suppression of MDR was thus found to be irradiation dose dependent.

**Correlation study in vitro**

The Spearman rank correlation coefficient analysis found a statistically significant negative correlation between CPM\textsubscript{in}/CPM\textsubscript{out} and RC (Fig. 4).

**Doxorubicin fluorescent imaging in vivo**

In both the C group and 9-Gy I group, whole mouse body fluorescence increased, after doxorubicin injection, for the first 10 minutes. After 10 minutes, whole body fluorescence slowly decreased until the measurement endpoint at 120 minutes after injection. At 1 minute after injection, a similar accumulation of doxorubicin was observed in both the C group and the 9-Gy I group. On the other hand, at 60 and 120 minutes, a large accumulation of doxorubicin was observed at the irradiated site of the 9-Gy I group. A statistically significant higher AR\textsubscript{doxorubicin} was found in the 9-Gy I group at 60 and 120 minutes after injection relative to the C group (Figs. 5 and 6).

**Discussion**

The in vitro cell replication study demonstrated the non–small lung cancer cell line H1299/wtp53 sensitivity to γ-ray irradiation. Dose-dependent inhibition of cell replication was observed in all the groups subject to γ-irradiation. The largest inhibition was observed in 7 days after irradiation. The most probable cause is DNA damage. Furthermore, it was found that this cancer cell line was resilient to single-dose irradiation, even at the 12-Gy high dose, as demonstrated by the recovery of replication capacity of the irradiated cells after the 14 days.

<table>
<thead>
<tr>
<th>RC</th>
<th>1 day</th>
<th>4 days</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0 Gy)</td>
<td>−0.06 (0.50)</td>
<td>0.87 (0.06)</td>
<td>0.65 (0.03)</td>
<td>0.83 (0.01)</td>
</tr>
<tr>
<td>3 Gy</td>
<td>−0.06 (0.15)</td>
<td>0.75 (0.04)a</td>
<td>0.54 (0.04)a</td>
<td>0.76 (0.01)a</td>
</tr>
<tr>
<td>6 Gy</td>
<td>−0.27 (0.33)</td>
<td>0.56 (0.05)a</td>
<td>0.45 (0.10)a</td>
<td>0.55 (0.03)a</td>
</tr>
<tr>
<td>9 Gy</td>
<td>−0.26 (0.47)</td>
<td>0.29 (0.08)a</td>
<td>0.19 (0.05)a</td>
<td>0.16 (0.03)a</td>
</tr>
<tr>
<td>12 Gy</td>
<td>−0.29 (0.22)</td>
<td>0.19 (0.05)a</td>
<td>−0.09 (0.04)a</td>
<td>−0.09 (0.10)a</td>
</tr>
</tbody>
</table>

NOTE: Significance testing was done using the pairwise Mann–Whitney U test. Significant difference between control (0 Gy) and other irradiated groups.

\(^aP < 0.05.\) Values are expressed as mean (SD); \(n = 4.\)
The effect of \( \gamma \)-irradiation on MDR in H1299/wtp53 was investigated using Tc-99m MIBI. MIBI, with similar pharmacokinetics to chemotherapeutic drugs such as doxorubicin, is known to be a substrate of the over-expressed ABC transporters responsible for MDR in cancer cells. Therefore, suppression of MDR via irradiation can be effectively investigated using the change in intracellular uptake of MIBI. Furthermore, based on this pharmacokinetic property, a number of clinical studies have reported that MIBI imaging may be used to obtain an early estimate of the therapeutic response to chemotherapy by assessing the cancer MDR (16–23). In this study, MIBI was used to investigate the response of MDR in H1299/wtp53 in vitro. The increase in intracellular MIBI retention that was observed from the 4 to 14 days after irradiation is evidence for the suppression of MDR in H1299/wtp53 due to \( \gamma \)-irradiation. Furthermore, the amount and duration of MDR suppression were found to be \( \gamma \)-irradiation dose dependent, a finding that we suggest may be important for clinical chemoradiotherapy. Finally, these in vitro results are further supported by the in vivo result, obtained via fluorescent imaging in a mouse model, that MDR to the uptake of the chemotherapeutic agent doxorubicin is suppressed by \( \gamma \)-irradiation. This result can be considered as additional evidence for the in vivo suppression of MDR by \( \gamma \)-irradiation in the non–small lung cancer cell line H1299/wtp53.

The molecular basis of the effect of irradiation on MDR is not yet clear. Xie and colleagues (9) have reported that fractionated irradiation-induced radioresistant esophageal cancer cells are more sensitive to certain types of chemotherapeutic drugs, including doxorubicin. Our study extends prior reports of MDR suppression by irradiation. Conversely, it has also been reported that irradiated cancer cells can develop resistance to chemotherapy. Overexpression of ABC transporters associated with MDR has been observed in human cancer cell lines after irradiation (26, 27). Bottke and colleagues (11) have reported that irradiation can induce functionally relevant MDR gene and protein expression. Thus, further research is clearly required to elucidate the molecular basis of the response of different cancer cell lines to radiotherapy with regard to MDR.

This study focused on MDR in cancer cells over the short-time interval of 14 days immediately after \( \gamma \)-irradiation. MDR suppression due to \( \gamma \)-irradiation in the non–small lung cancer cell replication was also reduced. This set of results provides additional experimental evidence in support of the synergistic efficacy of chemoradiotherapy as reported by clinical studies (4–8).
MIBI across the cell membrane for influx is passive transport by diffusion and for efflux is active transport by the ABC transporters which are overexpressed in cancer cells resulting in MDR. The current hypothesis is that γ-irradiation disrupts, in some manner, the cellular active transport process. However, to date, to the best of our knowledge, no studies have been published with regard to this hypothesis. In this article, we did not investigate how the underlying molecular mechanisms are affected by γ-irradiation. However, based on the negative correlation between cancer cell replication and MIBI accumulation, we suggest that γ-irradiation may cause MDR-related protein damage or suppression of the active transporter’s metabolic function.

Conclusion

MDR in the non–small lung cancer cell line H1299/ wtP53 was suppressed after γ-irradiation. The amount and duration of the suppression depended on the γ-irradiation dose. A statistically significant negative correlation was found between cell replication and MDR suppression after γ-irradiation in this cell line.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: Y. Kono, K. Utsunomiya

Development of methodology: Y. Kono, K. Utsunomiya

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Kono, K. Utsunomiya

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Kono, K. Utsunomiya

Writing, review, and/or revision of the manuscript: Y. Kono, K. Utsunomiya

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Kono, K. Utsunomiya, S. Kanno

Study supervision: K. Utsunomiya, N. Tanigawa

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References


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