Effect of Anticancer Drugs and Desferrioxamine in Combination with Radiation on Hepatoma Cell Lines

Won Ho Kim, Chae Yoon Chon, Young Myung Moon, Jin Kyung Kang, In Suh Park and Heung Jai Choi

Several anticancer chemotherapeutic agents (5-fluorouracil, adriamycin and cisplatinum) and desferrioxamine, an iron chelator, were tested with regard to cytotoxicity and to the combined effect on radiation induced cell killing using two human hepatoma cell lines (HepG2 and PLC/PRF/5). Survival fractions were measured by quantitative colorimetric assay (MTT assay) and dose-response curves were plotted. MTT assay could be successfully used in the assessment of radiosensitivity in addition to chemosensitivity, because a good linear relationship between optical densities and cell numbers was observed and cells approached exponential growth for the first 7 days of culture when 5×10^4 or less cells were inoculated per well in our study. Steepness of the final slope (D0), width of the shoulder (D2) and the extrapolation number (n) of radiation survival curves were 106.172 rad, 226.43 rad and 1.25 respectively in HepG2 and 1091.38 rad, 268.42 rad and 1.29 respectively in PLC/PRF/5. After combining anticancer chemotherapeutic agents and desferrioxamine with radiation, the widths of the shoulders were decreased whereas sensitizer enhancement ratios were increased as the concentration of drugs increased in both cell lines. These results suggest that neither anticancer chemotherapeutic agents nor desferrioxamine enhance cell killing induced by radiation alone, but suggested the possibility that they inhibit the repair of radiation damage.

Key Words: Hepatoma, MTT, radiation, desferrioxamine

Hepatocellular carcinoma is one of the most common malignancies and the main cause of cancer death in endemic areas of the hepatitis B virus (Beasley et al. 1981). Surgery is the mainstay of treatment for early cases, but unfortunately, most patients are diagnosed in an advanced stage and/or have liver cirrhosis that makes operation difficult. Thus other treatment modalities including chemotherapy, hepatic artery ligation or embolization and radiation therapy have been used (You et al. 1990). External radiation therapy combined with low dose chemotherapy has been tried for palliation but the results have not been satisfactory (Stillwagon et al. 1991; Habrand et al. 1992).

Desferrioxamine (DFO), a well known iron chelator, has also been reported to have antitumor activity on several cultured human cancer cell lines but has minimal cytotoxicity on normal human cells (Hoffbrand et al. 1976; Lederman et al. 1984; Blatt and Stitely, 1987; Hann et al. 1990). Therefore, it was hypothesized that DFO could be used as a potential anticancer regimen and we reported the synergistic cytotoxic effects of DFO when
combined with 5-fluorouracil (5-FU) but not with adriamycin (ADR) and cisplatinum (DDP) on the human gastric cancer cell line (Kim et al. 1993).

In this study, we carried out MTT assays using two hepatoma cell lines (HepG2 and PLC/PRF/5) in order to investigate the radiobiological characteristics of hepatoma cells and to assess the interactions between radiation and anticancer chemotherapeutic agents as well as desferrioxamine.

MATERIALS AND METHODS

Cell lines and culture conditions

The human hepatoma cell lines used in this study were HepG2 (American Type Culture Collection; ATCC, Rockville, MD, U.S.A.; HB 8065; a hepatoblastoma) and PLC/PRF/5 (ATCC, CRL 8024; a hepatocellular carcinoma). The cells were cultured in a 25 cm² plastic flask (Costar, Cambridge, MA, U.S.A.) with complete media which consisted of Eagle's minimum essential media (JR Scientific & Hazleton Biologic Inc; JRH, Lenexa, KS, U.S.A.) supplemented with 10% heat inactivated fetal calf serum (Commonwealth Serum Laboratories Ltd., Parkville, Australia), 100 IU/ml penicillin (JRH) and 100 μg/ml streptomycin (JRH), at 37°C in a humidified 5% CO₂ incubator.

Drugs

5-Fluorouracil (5-FU; Roche, Seoul, Korea), adriamycin (ADR; II Dong Pharmaceuticals Co., Seoul, Korea), cisplatinum (DDP; Dong A Pharmaceuticals Co., Seoul, Korea) and desferrioxamine (DFO; Ciba Geigy, Basel, Switzerland) were used.

MTT assay

The cells were harvested from exponential phase maintenance cultures using trypsin-ethylenediamine tetraacetic acid (trypsin-EDTA; JRH) treatment of monolayer cultures. After washing three times with complete media, single cell suspensions were prepared and cells were counted using a hemocytometer by staining with trypan blue and appropriate dilutions were made. Various numbers (5 x 10⁴ to 5 x 10⁶) of cells were inoculated in each well of 96-well microtiter plates (Costar) in a total volume of 200 μl/well and cultured for 18 hours at 37°C in a humidified 5% CO₂ incubator for the adherence of cells. After irradiation and/or adding drugs, the plates were subsequently cultured for various periods (0 to 11 days) to determine an adequate assay condition.

To perform MTT assay, the culture medium was removed from the wells ensuring that the monolayer of cells were not disturbed. Then 50 μl of MTT solution (2 g/ml; Sigma Chemical Co., St Louis, MO, U.S.A.) were added to each well and incubated for 4 hours. The plates were then centrifuged at 450 x g for 5 minutes and the remaining MTT solution was removed cautiously. Then 150 μl of 100% dimethyl sulfoxide (DMSO; Sigma Chemical Co.) were added to each well and the plates were shaken for 10 minutes on a plate shaker to dissolve the formazan crystal. Absorbance (optical density; OD) reading on each well were performed at 570 nm (single wavelength) using a multiwell spectrophotometer (ELISA processor II; Behringerwerke, Marburg, W. Germany). Cell free wells for absorbance readings contained no cells but they did contain media.

All experiments were performed at least twice in eight duplicated wells.

Irradiation procedures and assessment of radiation response

Irradiations were performed using a ⁶⁰Co source in a 6000 Ci telecobalt irradiation room at a dose rate of 142 rad/min.

Radiation survival curves for mammalian cells are usually presented in the form shown in Fig. 1, with dose plotted on a linear scale and the surviving fraction on a logarithmic scale. The survival curve may be described in terms of a steepness of the initial slope (D₁) due to single-event killing, a steepness of the final slope (Dₙ) due to multiple event killing, and some quantity (either n or D₁) to represent the size or width of the shoulder. The quantities D₁ and Dₙ are the doses required to reduce the fraction of surviving cells to 37% of its previous value. As illustrated, D₁ is the dose required to reduce the fraction of surviving cells to 0.37 on the initial straight portion of the survival curve.
Calculation of survival fraction

The survival fraction was calculated from the following equation:

\[ \text{Survival fraction} = \frac{\text{mean OD in test wells} - \text{mean OD in cell free wells}}{\text{mean OD in control wells} - \text{mean OD in cell free wells}} \]

Assessment of effect of drugs in combination with radiation

The effect of drugs in combination with radiation was assessed by the sensitization enhancement ratio calculated from the following equation:

\[ \text{Sensitizer enhancement ratio} = \frac{D_0 \text{ without drug}}{D_0 \text{ with drug}} \]

RESULTS

Relationship between optical density and cell number

For each cell line, cells suspended in 200 \( \mu l \) of complete media were inoculated in each well of microtiter plates in serial dilutions from 10^6 to 5 \times 10^6 cells/well and cultured for 18 hours at 37°C in a humidified 5% CO\(_2\) incubator for the adherence of cells and then MTT assays were performed. Fig. 2 shows a strong linear relationship between optical densities and cell numbers in representative experiments in each cell line using a linear regression (R^2: 0.991 in HepG2 and 0.997 in PLC/PRF/5).

Time course of growth of each cell line

For each cell line, cells suspended in 200 \( \mu l \) of complete media were inoculated in each well of microtiter plates in serial dilutions from 5 \times 10^6 to 5 \times 10^5 cells/well and cultured for 18 hours at 37°C in a humidified 5% CO\(_2\) incubator for the adherence of cells and then MTT assays were performed daily or every other day until the 9th day in HepG2 and until the 11th day in PLC/PRF/5 during the subsequent culture. Fig. 3 shows the representative experiments relating the cell number per well and the duration of culture in each cell line. For the first 7 days, cells approached exponential growth when 5 \times 10^5 or
less cells were inoculated per well. Therefore, a concentration of $5 \times 10^5$ cells/well and a culture duration of 7 days were selected for further experiments.

**Fig. 2.** Relationship between the numbers of hepatoma cells and optical densities in the MTT assay. Various numbers of hepatoma cells were inoculated in each well of microtiter plate and then incubated for 4 hours after adding MTT. Optical densities at 570 nm were measured by multiwell spectrophotometer.

**Effect of 5-FU, ADR, DDP and DFO on survival of each cell line**

For each cell line, $5 \times 10^5$ cells suspended in 180 $\mu$l of complete media were inoculated in each well of microtiter plates and cultured for 18 hours at 37°C in a humidified 5% CO$_2$ incubator for the adherence of cells. Then 20 $\mu$l of various concentrations of 5-FU, ADR, DDP, and DFO diluted with phosphate-buffered saline (PBS) were added and subsequently cultured for 7 days. MTT assays were performed and the dose-response curves were plotted after calculating the survival fraction (Fig. 4). Fifty percent growth inhibiting concentrations (IC$_{50}$) were calculated from the log equation of dose-response curve. IC$_{50}$ of 5-FU, ADR, DDP and DFO were 0.208 $\mu$g/ml, 0.054 $\mu$g/ml, 1.074 $\mu$g/ml and 5.777 $\mu$g/ml respectively in HepG2, and 0.725 $\mu$g/ml, 0.050 $\mu$g/ml, 0.902 $\mu$g/ml and 10.052 $\mu$g/ml respectively in PLC/PRF/5 (Table 1).

**Effect of radiation on survival of each cell line**

For each cell line, $5 \times 10^5$ cells suspended in 200 $\mu$l of complete media were inoculated in

**Fig. 3.** Time course of growth of hepatoma cells. Various numbers of hepatoma cells were inoculated in each well of microtiter plate and cultured at 37°C in a 5% CO$_2$ incubator. MTT assays were performed every or everyother until 11th day of culture.
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**Fig. 4.** Dose-response curves of 5-fluorouracil, adriamycin, cisplatinum, and desferrioxamine in hepatoma cells. For each assay, $5 \times 10^6$ hepatoma cells were inoculated in each well of microtiter plates and cultured for 18 hours at 37°C in a humidified 5% CO$_2$ incubator for the adherence of cells. Then various concentrations of each drug diluted with phosphate-buffered saline were added and cultured subsequently for 7 days. MTT assays were performed and drug dose-response curves were plotted after calculating survival fraction. The results were analysed as percent of survival against the final concentration of each drug. Each data depicts the mean percent of survival±SD of 3 different experiments, each consisting of 8 wells.

<table>
<thead>
<tr>
<th>Drugs(μg/ml)</th>
<th>HepG2</th>
<th>PLC/PRF/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Fluorouracil</td>
<td>0.208</td>
<td>0.725</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.054</td>
<td>0.050</td>
</tr>
<tr>
<td>Cisplatinum</td>
<td>1.074</td>
<td>0.902</td>
</tr>
<tr>
<td>Desferrioxamine</td>
<td>5.777</td>
<td>10.502</td>
</tr>
</tbody>
</table>

Table 1. Concentration of anticancer chemotherapeutic agents and desferrioxamine required to reduce growth of hepatoma cells to 50%(IC$_{50}$) of control

Each well of microtiter plates and cultured for 18 hours at 37°C in a humidified 5% CO$_2$ incubator for the adherence of cells. Then plates were irradiated with various doses (0 to 1,000 rad) and subsequently cultured for 7 days. MTT assays were performed and radiation dose-response curves were plotted after calculating the survival fraction (Fig. 5). Steepness of the final slope (D$_{90}$), width of the shoulder (quasithreshold dose; D$_{10}$) and the extrapolation number ($n$) were calculated from the extrapolation of the log-linear plot of the...
Fig. 5. Radiation dose-response curves in hepatoma cells. For each assay, $5 \times 10^6$ hepatoma cells were inoculated in each well of microtiter plate and cultured for 18 hours at 37°C in a humidified 5% CO$_2$ incubator for the adherence of cells. Then plates were irradiated with various doses and cultured subsequently for 7 days. MTT assays were performed and radiation dose-response curves were plotted after calculating survival fraction. The results were analysed as percent of survival against the dose of radiation. Each data depicts the mean percent of survival $\pm$ SD of 3 different experiments, each consisting of 8 wells.

**Table 2. Parameters of radiation survival curves of hepatoma cell lines**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Hepatoma cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HepG2</td>
</tr>
<tr>
<td>$D_0$ (rad)</td>
<td>1061.72$\pm$ 68.95</td>
</tr>
<tr>
<td>$D_1$ (rad)</td>
<td>226.43$\pm$ 1.25</td>
</tr>
<tr>
<td>$\nu$</td>
<td>1.25$\pm$ 0.02</td>
</tr>
</tbody>
</table>

$D_0$: steepness of final slope of radiation survival curve
$D_1$: width of shoulder of radiation survival curve
$\nu$: extrapolation number of radiation survival curve

For each cell line, $5 \times 10^6$ cells suspended in 180 µl of complete media were inoculated in each well of microtiter plates and cultured for 18 hours at 37°C in a humidified 5% CO$_2$ incubator for the adherence of cells. Then the plates were irradiated with various doses (0 to 1,000 rad) and immediately 20 µl of various concentrations of 5-FU, ADR, DDP, and DFO diluted with PBS were added. MTT assays were performed after 7 days of subsequent culture. Radiation dose-response curves combined with various dose of 5-FU, ADR, DDP and DFO were plotted (Fig. 5 for HepG2; Fig. 6 for PLC/PRF/5). $D_0$ was decreased, whereas sensitizer enhancement ratios were increased as the concentration of drugs were increased in both cell lines (Fig. 6, 7, 8).

**DISCUSSION**

Tissue culture offers the opportunity to examine the temporal aspect of combination treatments without the complexities of *in vivo* pharmacokinetics (Beltelsen et al. 1984; Finlay and Baguley, 1984). Information of this type is important since not only can a drug's pharmacokinetics be varied in man by altering the schedule of administration, but the radiation fractionation can be adapted to op-
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Fig. 6. Effect of 5-fluorouracil, Adriamycin, Cisplatinum and Desferrioxamine in combination with Radiatoin in HepG2. For each assay, 5 x 10^6 cells were inoculated in each well of microtiter plate and cultured for 18 hours at 37°C in a humidified 5% CO2 incubator for the adherence of cells. Then plates were irradiated with various doses and immediately various concentrations of 5-fluorouracil, Adriamycin, Cisplatinum and Desferrioxamine were added to each well. After subsequent culture for 7 days, MTT assays were performed and dose-response curves were plotted. The results were analyzed as percent of survival. Each data depicts the mean percent of survival of 3 different experiments, each consisting of 8 wells.

timized pharmacokinetic schedules (Hamberger and Salmon, 1977).

Radiation effects on the survival of cell lines have been generally assessed with clonogenic assay following the original description of the technique (Puck and Marcus, 1955). However, limitations include the time taken for colonies to form and the inability to measure survival in cells which do not grow as colonies, thus alternative assays have been sought over the years. Among them, a quantitative colorimetric assay using a tetrazolium salt and usually referred to as the "MTT assay", quantitates living but not
Fig. 7. Effect of 5-fluorouracil, adriamycin, cisplatinum and desferrioxamine in combination with radiation in PLC/PRF/5. For each assay, $5 \times 10^6$ cells were inoculated in each well of microtiter plate and cultured for 18 hours at $37^\circ C$ in a humidified 5% CO$_2$ incubator for the adherence of cells. Then plates were irradiated with various doses and immediately various concentrations of 5-fluorouracil, adriamycin, cisplatinum and desferrioxamine were added to each well. After subsequent culture for 7 days, MTT assays were performed and dose-response curves were plotted. The results were analysed as percent of survival. Each data depicts the mean percent of survival of 3 different experiments, each consisting of 8 wells.

dead cells and has the advantage of being rapid, precise and semi-automatable (Mosmann, 1983). This assay measures the ability of viable cells to reduce MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), a yellow tetrazolium salt, to a purple formazane precipitate; a process which requires active mitochondrial enzyme, succinate dehydrogenase, of viable cells (Wasserman and Twentyman, 1988). The MTT assay is used as the standard determinant of drug responsiveness in the National Cancer Institute’s anticancer drug screening programme. In addition, this assay has been used for the
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![Graphs showing sensitization enhancement ratios](image)

**Fig. 8. Sensitizer enhancement ratios by 5-fluorouracil, adriamycin, cisplatinum and desferrioxamine in hepatoma cell lines.** Sensitizer enhancement ratio was calculated from dividing steepness of final slope ($D_a$) of radiation survival curve combined with drug by that without drug.

evaluation of the radiation response of cultured tumor cells (Stratford et al. 1989; Price and McMillan, 1990) and showed good agreement with clonogenic assay (Carmichael et al. 1987; Wasserman and Twentyman, 1988). In our study, cell numbers were accurately estimated by the MTT assay and cells approached exponential growth for the first 7 days of culture when $5 \times 10^6$ cells were inoculated per well and longer when lesser cells were inoculated. Therefore, we thought that the MTT assay could be successfully used in the assessment of radiation response as well as the drug response of hepatoma cells. $IC_\infty$ of 5-FU, ADR, DDP and DFO in both hepatoma cell lines were much lower than in the gastric cancer cell line previously reported (Kim et al. 1993). However, direct comparison of $IC_\infty$ is difficult due to differences in cell type and in the duration of culture after adding drugs.

The combination of partly effective treatment modalities into improved cancer treatment regimens remains an attractive challenge. Of particular promise is the combination of anticancer chemotherapeutic agents with radiation therapy (Byfield et al. 1982). Thus agents which modify tumor cell radiation response have been investigated in an attempt to improve therapeutic outcome. The potential for such a combination involving 5-FU was demonstrated early by Heidelberger et al. (1958), who noted that doses of radiation therapy which were inhibitory but not curative for rodent tumors were made curative by combining radiation therapy with 5-FU. In addition, subsequent in vitro studies showed that 5-FU could quantitatively enhance cell killing by radiation in a synergistic manner as measured by an increase in the steepness of the radiation survival curve (Bagshaw, 1961; Berry, 1966).

The mechanisms by which 5-FU influences the cellular response to radiation is poorly understood. The enhancement of radiation responsiveness was confirmed in vivo using the mouse leukemia AKR system by Vietti et al. (1971). They hypothesized that 5-FU possibly acted by inhibiting the repair of sublethal radiation damage, because the most striking reduction in cell survival was obtained when the drug was administered immediately following radiation. This concept, that 5-FU inhibits or delays the repair of sublethal radiation damage measured by the reduction in the width of the shoulder ($D_s$) of the radiation survival curve (Shipley et al. 1971), was further supported by in vitro studies (Nakajima et al. 1979). In the clinical field, some investigators reported that 5-FU significantly enhanced the effect of radiation and prolonged survival in patients (Gollin et al.
1972; Arnott, 1975), whereas others observed no benefit from the combination therapy (Carr et al. 1972). These conflicting results may be attributed to the differences in the combination schedule, the type of cancer, the clinical stages at the time of treatment, and the difficulty in evaluating the response objectively (Nakajima et al. 1979; Vokes et al. 1989).

ADR, as an inhibitor of mitochondrial and tumor cell respiration, causes reduced oxygen consumption by the cells in the outer layers of the tumor and leads to improved oxygenation and radiosensitivity of the centrally located hypoxic cells in addition to inhibiting enzymatic repair of radiation induced breaks in DNA (Durand, 1976). Thus this drug could also be regarded as a radiosensitizer (Byfield et al. 1977). Enhancements of the cytotoxic effect of DDP in combination with radiation have also been reported (Kyriazis et al. 1983; Choi et al. 1988). In our study, D, was decreased but sensitizer enhancement ratios, which mean changes of D, by drugs, were increased as the concentrations of 5-FU, ADR and DDP increased. These results suggest that 5-FU, ADR and DDP are not good candidates for a radiosensitizer though the possibility of inhibiting recovery from radiation damage by these drugs could not be excluded: Iron is essential for the growth of all cells including tumor cells because ribonucleotide reductase, a rate limiting enzyme for DNA synthesis, requires the continual presence of oxygen and iron (Reichard and Ehrenberg, 1983). Furthermore, it was reported that iron depletion causes tumor cell death but shows little cytotoxic effect on normal human diploid cells in vitro. Therefore, it has been reported that DFO, which is a hydroxylamine produced by *Streptomyces pilosus* and has been widely used as a therapeutic iron-chelating agent in iron overload (Mondell et al. 1982), could also be used as an anticancer agent (Hoffbrand et al. 1976; Lederman et al. 1984; Blatt and Stitely, 1987; Estrov et al. 1987; Hann et al. 1990), especially if combined with other anticancer chemotherapeutic agents (Kim et al. 1993).

The effect of DFO in combination with radiation has not been reported yet. However, it could be hypothesized that DFO might block the repair of radiation injury because it inhibits ribonucleotide reductase, a key enzyme regulating the deoxyribonucleotide pool balance (O'Dwyer 1987). In contrast, DFO might act as a radioprotector, because it chelates metal based compounds, such as Rh, Pt, Co, Cu and Fe, which have been shown to have a radiosensitizing effect (Stratford, 1992). Our results suggested that DFO might inhibit the repair of radiation damage but not enhance radiation induced cytotoxicity in hepatoma cells because D, was decreased but sensitizer enhancement ratios were increased as the concentration of DFO increased.

In conclusion, 5-FU, ADR, DDP as well as DFO did not enhance cell killing induced by radiation alone, but they might inhibit the repair of radiation damage.

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