Caffeine Increases the Antitumor Effect of Cisplatin in Human Hepatocellular Carcinoma Cells

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Caffeine is thought to increase the antitumor effect of cisplatin or DNA-damaging agents because it is known that caffeine inhibits DNA repair. Caffeine-assisted chemotherapy has been used in the treatment of osteosarcomas. In addition, there are several reports about combination chemotherapy with caffeine for certain malignancies other than osteosarcomas. However, there are no reports that show the utility of combination chemotherapy with caffeine for hepatocellular carcinoma (HCC). We examined the combined effects of caffeine and cisplatin in human HCC cell lines, and screened for a more effective administration method of caffeine in vitro. Human HCC cell lines (HepG2, HLF, HuH-7, and Li-7) were exposed to caffeine (0—0.5 mM) and cisplatin (0—1.2 μg/mL) for 72 h, either alone or in combination. Cell numbers were measured by WST-8 assay, and cell apoptosis was determined by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) binding assay. As a result, caffeine increased the antitumor effect of cisplatin on cell proliferation and cell apoptosis in the HCC cell lines. Moreover, this effect was dependent on the amount of exposure to caffeine. These results suggest that caffeine-assisted chemotherapy is useful for HCC treatment.

Key words caffeine; cisplatin; hepatocellular carcinoma; combination chemotherapy; pharmacodynamics

Hepatocellular carcinoma (HCC) represents an increasing burden of disease worldwide. It is the fifth most common cancer globally and is the third most common cause of cancer-related mortality. Liver injury caused by hepatitis B virus and hepatitis C virus infections or alcohol is a major risk factor for HCC, with the vast majority of cases developing in the presence of underlying cirrhosis. Screening of patients with liver cirrhosis as a population at high risk of developing HCC and the development of imaging modalities such as ultrasonography and computed tomography has improved the early detection of HCC.

Treatment modalities for HCC at an early stage with good hepatic function are fairly well established. These include hepatic resection, percutaneous ethanol injection, transcatheter arterial embolization, microwave coagulation, and radiofrequency ablation. Unfortunately, many patients cannot be treated with these established therapies because a majority of HCC patients present with advanced disease. For these patients, chemotherapy is the only remaining treatment choice. However, there are no standard chemotherapy regimens that have been shown to improve overall survival dramatically.

For HCC patients, a large number of controlled and uncontrolled studies have been performed with most of the major classes of chemotherapy agents given as single or combination therapies. Single or combination regimens including doxorubicin, 5-fluorouracil, capcetabine, cisplatin, gemcitabine, and mitoxantrone have elicited 0 to 27% response rates, 2 to 6 months time to progression or progression-free survival, and 3 to 12 months of overall survival. In recent years, sorafenib has come to be used for HCC treatment. Sorafenib is the first agent to demonstrate a survival advantage over supportive care in HCC. Nevertheless, in a relatively fit group of sorafenib-treated patients (93% Child-Pugh A), median survival was only 10.7 months. Thus, although many chemotherapy regimens have been tried in the effort to control advanced HCC, their effectiveness is unsatisfactory and the prognosis of these patients is extremely poor. Therefore, it is necessary to develop a new chemotherapy for HCC.

Generally, to increase the antitumor effect of anticancer agent, more than two kinds of anticancer agents are often used in combination. However, serious side effects occur more frequently with anticancer agents than with other commonly used medicines. Therefore, there is concern that the enhancement of serious side effects occurs by combination treatment with anticancer agents. If the antitumor effect can be increased by combination therapy with medicines other than anticancer agent, excellent chemotherapy in terms of effectiveness and safety can probably be developed.

Since 1989, Tsuchiya et al. have published reports on the use of caffeine-potentiated chemotherapy. Caffeine can safely enhance the cytotoxic effects of anticancer drugs through its DNA repair-inhibiting effect. They demonstrated in several studies that caffeine-potentiated chemotherapy induces a high complete response rate (86%) in patients with osteosarcoma. In addition, they reported an overall 5-year cumulative survival rate of 80.7% for nonmetastatic soft tissue sarcoma patients with a median follow-up period of 52 months. Because the serious side effects such as bone-marrow depression shown in treatment with other anticancer agents did not occur with caffeine, it seems that caffeine-assisted chemotherapy is a safer and more effective form of chemotherapy.

There are several reports about combination chemotherapy with caffeine for certain malignancies other than osteosarcoma, such as pancreatic cancer, stomach cancer, and lung cancer. However, there are no reports that show the utility of combination chemotherapy with caffeine for HCC. Therefore, to develop combination chemotherapy with caffeine for HCC, we examined whether caffeine increases the antitumor effect of cisplatin in HCC cell lines. In addition, we examined the...
both for the indicated time at 37°C. A solution (Sigma-Aldrich, U.S.A.), caffeine (Sigma-Aldrich, U.S.A.), or saline (Mitsubishi, Japan) was obtained from DS Pharma Biomedical (Osaka, Japan). Normal human hepatocyte (ABI 3716) was obtained from DS Pharma Biomedical (Osaka, Japan). HepG2 was maintained in minimal essential medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HLF was maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HuH-7 and Li-7 were maintained in RPMI1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HLF was maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HepG2 was maintained in minimal essential medium (Sigma, Germany) supplemented with 10% fetal bovine serum and 0.1 mm non-essential amino acids (Nacalai Tesque, Inc., Kyoto, Japan). Normal liver hepatocyte was maintained in Serum-Containing Medium Kit (DS Pharma Biomedical Co., Ltd., Osaka, Japan).

**Cell Culture** Human HCC cell lines (HepG2, HuH-7, and Li-7) were obtained from the Riken Bioresource Center Cell Bank (Ibaraki, Japan) and HLF (human liver cancer cell) was obtained from Japanese Collection of Research Bioresource Center Cell Bank (Osaka, Japan). Normal human hepatocyte (ABI 3716) was obtained from DS Pharma Biomedical (Osaka, Japan). This hepatocyte was initiated from normal human liver tissues by centrifugal counter elutriation following dispase digestion of tissue. These cells were maintained at 37°C in a humidified, 5% CO2, 95% air atmosphere and routinely subcultured using 0.25% w/v trypsin-ethylene diamine-tetraacetic acid (EDTA) (Invitrogen, Canada). HuH-7 and Li-7 were maintained in RPMI1640 (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HLF was maintained in Dulbecco’s modified Eagle’s medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum. HepG2 was maintained in minimal essential medium (Sigma, Germany) supplemented with 10% fetal bovine serum and 0.1 mm non-essential amino acids (Nacalai Tesque, Inc., Kyoto, Japan). Normal liver hepatocyte was maintained in Serum-Containing Medium Kit (DS Pharma Biomedical Co., Ltd., Osaka, Japan).

**Cell Proliferation Assay** Cells were seeded in 96-well plates, incubated for 24h at 37°C, and treated with cisplatin (Sigma-Aldrich, U.S.A.), caffeine (Sigma-Aldrich, U.S.A.), or both for the indicated time at 37°C. After the drug treatment, Cell Counting Kit-8 (Dojindo, Tokyo, Japan) was used for the investigation of cell proliferation according to the manufacturer’s instructions. IC50 values for the inhibition of cell proliferation were determined by nonlinear least squares regression (MULTI, Yamaoka et al.265) using the following equation: cell proliferation (1%)=100(1−[(I_{max}·f)·(1+\text{exponent})]), IC_{50}=\text{I}_{50}/(2·\text{I}_{max}−1)^{\text{exponent}}

where I is the initial concentration of cisplatin in the incubation, I_{max} is the maximum concentration of cisplatin, γ is an exponent, and I_{50} is the cisplatin concentration that inhibits cell proliferation by 50% at the concentration in this examination (I_{max}−I_{50}). IC_{50} is the cisplatin concentration that inhibits cell proliferation by 50% of cell viability.

**RESULTS**

**Cytotoxic Effect of Cisplatin in HCC Cell Lines** We examined the cytotoxic effect of cisplatin in four HCC cell lines (Li-7, HLF, HuH-7, and HepG2). Exposure of the tumor cells for 24, 48, or 72h to various concentrations of cisplatin resulted in a concentration-dependent reduction in cell viability that differed between the cell lines (Fig. 1). The mean IC_{50} values of the four HCC cell lines, evaluated by WST-8 assay, are shown in Table 1.

**Apoptosis Assay** Cell apoptosis was detected using the annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (MLB, Japan) as per the manufacturer’s protocol. Briefly, cells were treated with cisplatin, caffeine, or both for 72h. After incubation, cells were trypsinized and suspended in 1 mL of phosphate buffered saline (PBS). In each cell suspension, 2×10^6 cells were centrifuged and re-suspended in 500 μL of 1X binding buffer. In each sample, 5 μL of annexin V-FITC and 5 μL of PI (propidium iodide) were added. The mixture was incubated for 5 min in the dark and immediately analyzed with the use of a FACSCalibur flow cytometer and Cell Quest software (BD Biosciences, San Jose, CA). The annexin V-FITC-positive and PI-negative cells were considered to be early apoptotic cells.

**Statistical Analysis** All results are expressed as the mean±S.D. The significance of differences was determined by two-tailed multiple t-test with Bonferroni correction. Differences were considered significant at p<0.05.

**Table 1. IC_{50} Values of Cisplatin for HCC Cell Lines**

<table>
<thead>
<tr>
<th></th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li-7</td>
<td>N.D.</td>
<td>1.24</td>
<td>0.43</td>
</tr>
<tr>
<td>HLF</td>
<td>2.90</td>
<td>0.71</td>
<td>0.57</td>
</tr>
<tr>
<td>HuH-7</td>
<td>10.33</td>
<td>1.95</td>
<td>1.18</td>
</tr>
<tr>
<td>HepG2</td>
<td>16.06</td>
<td>2.10</td>
<td>0.87</td>
</tr>
</tbody>
</table>

IC_{50} (μg/mL)

**Antiproliferative Effect on HCC Cells by Cisplatin, Caffeine, or Both** To examine whether caffeine can enhance the antiproliferative effect of cisplatin in HCC cell lines, the cells were exposed to caffeine (0.5 mM) and cisplatin...
Effect of Caffeine on Cisplatin-Induced Antiproliferative Effect and Apoptosis Enhancement in Normal Hepatocytes

We examined the effect of caffeine on cell proliferation and apoptosis in normal hepatocytes. Results showed that caffeine exposure inhibited cell proliferation slightly in normal hepatocytes, and caffeine enhanced the antiproliferative effect against normal hepatocytes in combination with cisplatin (Fig. 4A). In contrast, caffeine and cisplatin did not induce apoptosis, and the combination of caffeine and cisplatin did not induce apoptosis either (Fig. 4B).

The Antitumor Effect Was Enhanced by Increasing the Amount of Exposure to Caffeine

To examine whether caffeine’s enhancement of the antiproliferative effect of cisplatin is dependent on the amount of exposure, we exposed HCC cell lines to caffeine while altering the amount of exposure (0—36 mmol·h/L) by prolonging the duration of exposure (0—72 h) or raising the concentration of caffeine (0—0.5 mM) (Fig. 5A). As a result (Fig. 5B), the antiproliferative effect of caffeine was found to increase as the duration of exposure was prolonged or the concentration was raised. In addition, there was a correlation between the antiproliferative effect of cisplatin and the amount of exposure to caffeine. These results suggest that caffeine demonstrated an antiproliferative effect that depended on the amount of exposure to caffeine in all HCC cell lines.

Screening for a More Effective Method of Caffeine Administration

For altering the dose of caffeine, there are two methods: prolonging the duration of exposure or raising the concentration. However, it is not clear which method is more effective for the administration of caffeine. Therefore, we examined the effect of caffeine on cell proliferation by prolonging the duration of exposure (0—72 h) or raising the concentration (0—1 mM) under conditions in which the amount of exposure was kept constant (24 mmol·h/L) (Fig. 6A). Results (Fig. 6B) showed that the antiproliferative effect of caffeine in combination with cisplatin was equivalent between the two methods in all HCC cell lines.

DISCUSSION

Caffeine-assisted chemotherapy has been used in the treatment of osteosarcomas. Although the chemotherapy is not currently performed globally, it was shown to have good clinical performance. Caffeine has been reported to affect cell cycle function, induce programmed cell death or apoptosis, and perturb key regulatory proteins, including the tumor suppressor protein, p53.27,28 There are several reports about combination chemotherapy with caffeine for certain malignancies other than osteosarcomas.29—25 However, there are no reports that show the utility of combination chemotherapy with caffeine for HCC. Therefore, we examined the combined effects of caffeine and cisplatin against HCC, which has a very poor prognosis.

The concentrations of cisplatin used in this study (0.4—1.2 μg/mL) reflect the clinical concentration because these cisplatin concentrations are near the blood concentration after 72 h (about 1 μg/mL) when cisplatin is administered intravenously to humans at 35 mg/m².30 Cisplatin remains in the systemic circulation for a long time because of its long biological half-life. Therefore, we experimented by applying cisplatin exposure for 72 h at a level near the clinical concentration.
In this study, we showed that caffeine increased cisplatin-induced cell death or apoptosis, and enhanced the antiproliferative effect in combination with cisplatin in three HCC cell lines (Li-7, HLF, HuH-7) (Figs. 2, 3). These results suggest that caffeine enhances the antitumor effect against HCCs in combination with cisplatin.

In response to cisplatin-induced DNA damage, eukaryotic cells activate ataxia telangiectasia and Rad-3-related (ATR)
kinase through Chk1 to arrest the cell cycle and initiate DNA repair.\(^{31,32}\) In contrast, caffeine has been reported to inhibit ATR kinase (IC\(_{50}\): 1.1 mM).\(^{33}\) These results suggest that caffeine enhances cisplatin's antitumor effect through prevention of DNA repair function by inhibition of ATR kinase caused by cisplatin exposure. Therefore, inhibition of ATR activity may be one of the mechanisms by which caffeine regulates sensitivity to cisplatin.

We showed that caffeine inhibited cell proliferation slightly in HCC cell lines without causing apoptosis alone (Figs. 2, 3). Hashimoto et al.\(^{34}\) have reported that lower levels of caffeine (0.25—1 mM) suppressed cell cycle progression at the G0/G1 phase without causing apoptosis. Because caffeine has also been reported to inhibit phosphatidyl inositol-3 (PI-3) kinase activity directly (IC\(_{50}\): 0.075—1 mM),\(^{33,35}\) they suggested that the inhibitory effects of caffeine on cell growth signaling through Akt/GSK-3β may result from the direct inhibition of PI-3 kinase,\(^{34}\) which is upstream of Akt/GSK-3β. Because we used caffeine at a low concentration (0.1—1 mM) in this study, the inhibitory effects of caffeine alone on the cell growth were probably caused by the inhibition of PI3-kinase.

We showed that caffeine could not increase cisplatin-induced apoptosis or cell death in HepG2. Because inhibition of ATR kinase activity may be one of the mechanisms by which caffeine regulates sensitivity to cisplatin.

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In this study, we showed that caffeine and cisplatin did not increase apoptosis of normal hepatocytes either alone or in

Fig. 5. (A) The Two Methods of Increasing the Amount of Exposure and (B) The Antitumor Effect Was Increased by Increasing the Amount of Exposure to Caffeine

\(\bullet\) Under conditions in which the duration of exposure was kept constant (72 h), caffeine was used at a concentration of 0.33 mM, 0.5 mM, or 1 mM. (C) Under conditions in which the concentration was kept constant (0.5 mM caffeine), the duration of exposure was prolonged for 24 h, 48 h, or 72 h. The medium was replaced with new medium corresponding to the well in both methods at intervals of 24 h. In addition, HCC cells were exposed to cisplatin for 72 h at a concentration based on each HCC cell's IC\(_{50}\) for cisplatin. Data represent the mean±S.D. of three separate experiments, each performed in duplicate.
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combination (Fig. 4B). On the other hand, caffeine exposure inhibited cell proliferation slightly and increased the anti-proliferative effect against normal hepatocytes in combination with cisplatin (Fig. 4A). Jha et al. \(^{36}\) examined caffeine’s effect on G2-phase delays in three human normal cell lines (GM2149, GM4626, AG1522) and three human tumor cell lines (HeLa, MCF7, OVGI) after exposure to gamma rays. All six cell lines displayed similar G2-phase delays after radiation. However, the gamma-ray-induced G2-phase delays were eliminated by caffeine in the tumor cell lines but were unaffected in all of the normal cell lines. These results suggest that caffeine may preferentially target tumor cells while having little effect on normal cells. Furthermore, caffeine is already used for osteosarcoma treatment and a report has shown that there were no critical side effects against the liver at a concentration of about 0.4 mM.\(^{37}\) Therefore, we consider that caffeine-assisted chemotherapy is probably a safe treatment method for HCC patients.

As shown in Figs. 2 and 3, our results suggest that the combination of caffeine and cisplatin is useful for HCC treatment. However, there is no evidence for the most effective administration method of caffeine. Therefore, we examined whether caffeine’s enhancement of the antiproliferative effect of cisplatin is dependent on the duration, the maximum concentration, or the amount of exposure.

In the experiment with increases in the amount of exposure to caffeine to HCC cell lines (Fig. 5A), the antiproliferative effect increased according to increases in the amount of exposure to caffeine (Fig. 5B). This result suggests that the antitumor effect of caffeine depends on the amount of exposure in the range used in this study. Moreover, to screen for the most effective method of caffeine administration, we examined the effect of caffeine on cell proliferation by prolonging the duration or raising the concentration under conditions in which the amount of exposure was kept constant (Fig. 6A). In this experiment, the antiproliferative effect depended on not only the maximum drug concentration and the duration of exposure, but also the amount of exposure to caffeine (Fig. 6B). These results suggest that administration that increases the amount of exposure to caffeine is more effective in HCC patients.

Caffeine is thought to increase the risk of side effects such as insomnia at increased blood concentration levels,\(^{38,39}\) and these effects in adults may be observed at serum concentrations above 30 \(\mu g/mL (0.154 \text{ mM})\).\(^{40}\) On the other hand, the serum concentration of caffeine required for fatal poisoning has been reported as about 80 to 700 \(\mu g/mL (0.412 \text{ to } 3.60 \text{ mM})\).\(^{40}\) In patients treated with caffeine-assisted chemotherapy, although a toxic reaction occurred in most patients, the toxic events were well tolerated and fatal side effects such as serious hepatic toxicity and serious kidney toxicity did not occur at caffeine concentrations near 0.4 mM (about 80 \(\mu g/mL\)).\(^{37}\) Therefore, 0.333 mM caffeine in this study would be tolerable in humans.

To reduce the side effects of caffeine, it must be administered at a lower blood concentration level. Because the antiproliferative effect of caffeine depends on the amount of exposure to it, it seems preferable to administer caffeine for a long time at a low concentration for safer and more effective treatment. To establish safer treatment, further investigations of caffeine at concentrations below 0.333 mM are necessary.

In conclusion, combination chemotherapy with caffeine and cisplatin is useful for HCC treatment; the most effective and safest method of administration of caffeine is administering it for a long time at a low blood concentration level (about 0.4 mM serum concentration of caffeine or less).

