Comparative Analysis of Cell Death Induction by Cisplatin and 5-FU in Human Oral Squamous and Hepatocellular Carcinoma Cell Lines

MASAHIKO OKAMURA[1], JUN SHIMADA[1] and HIROSHI SAKAGAMI[2]

Divisions of *1Oral Maxillofacial Surgery* and *2Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan*

Abstract. *We established the optimal conditions for the induction of cell death by cisplatin (CDDP) and 5-fluorouracil (5-FU) in human oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4) and human hepatocellular carcinoma (HepG2) cell lines. HSC-3 cells were the most sensitive to 48 hours' continuous treatment with CDDP, followed by HepG2, HSC-2 and HSC-4 cells. On the other hand, HSC-4 cells were the most sensitive to 48-hour continuous treatment with 5-FU, followed by HSC-2, HSC-3 and HepG2 cells. CDDP induced internucleosomal DNA fragmentation in HSC-2 and HSC-3 cells, but not in HSC-4 cells, while 5-FU failed to induce internucleosomal DNA fragmentation in all of these cells. The treatment of HSC-2, HSC-3 and HSC-4cells with CDDP for 12 hours (followed by incubation for 36 hours without CDDP) showed comparable magnitude of cytotoxicity and caspase-3 activation with that attained by continuous 48-hour CDDP treatment. On the other hand, the cytotoxicity of 5-FU depended both on the dose and the exposure time. The present study demonstrate that the most effective treatment time is 12 hours for CDDP and much longer for 5-FU in all studied cell lines, underlining the importance of optimizing the treatment time for each chemotherapeutic agent.*

Cisplatin (CDDP) was initially synthesized as a test compound for laboratory use only in 1845. In 1965 Rosenberg *et al.* showed for the first time that the proliferation of *Escherichia coli* was efficiently prevented by CDDP (1). CDDP has shown a relatively wide antitumor spectrum against mouse leukemia (L1210, P388) and animal

tumor cell lines *in vitro*. 5-Fluorouracil (5-FU) was developed in 1957 as a potential anticancer agent by Heidelberger *et al.* (2). At present, CDDP and 5-FU are popular chemotherapeutic agents for the treatment of various malignant tumors, such as head and neck, gastric, esophageal, ovarian and lung cancers. We have previously reported the cell death induction by CDDP and 5-FU using only one human squamous cell carcinoma cell line (HSC-2) (3, 4). It was essential to check the reproducibility of our findings, using different types of oral squamous cell carcinoma cell lines. The aim of the present study was to compare the most effective treatment conditions for three human squamous cell carcinoma (HSC-2, HSC-3, HSC-4) and one human hepatocellular carcinoma (HepG2) cell lines with CDDP and 5-FU. An MTT assay was used to determine the cytotoxic activity. DNA fragmentation and caspase-3, -8 and -9 activities were used as markers for apoptosis.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY, USA); fetal bovine serum (FBS; JRH, Bioscience, Lenexa, KS, USA); 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chem., St. Louis, MO, USA); 5-FU (MW 130.08) (Kyowa, Tokyo, Japan); CDDP (MW 300.05) (Briplatin injection, Bristle Pharmaceutical Co., Tokyo, Japan).

Cell culture. HSC-2, HSC-3, HSC-4 and HepG2 cells (obtained from Riken Cell Bank, Ibaraki, Japan) were cultured at 37°C in DMEM supplemented with 10% heat-inactivated FBS as monolayer culture in the tissue culture dish (Falcon 100x20 mm Style; Becton Dickinson Labware, NJ, USA), under a humidified 5% CO_2 atmosphere. Human promyelocytic leukemic HL-60 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere.

Assay for cytotoxic activity. HSC-2, HSC-3, HSC-4 and HepG2 cells (5×10^3/0.1 mL) were inoculated in 96-microwell plates (Becton Dickinson) and incubated for 24 hours before the treatments. These cells were then treated for 48 hours without (control) or

Correspondence to: Hiroshi Sakagami, Division of Pharmacology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan. Tel: +81 49 285 2758, Fax: +81 49 285 5171, e-mail: sakagami@dent.meikai.ac.jp

with different concentrations of CDDP or 5-FU (1.95 to 62.5 μM), with three replicate wells for each concentration. The relative viable cell number of adherent cells was then determined by the MTT method. In brief, the drug-treated cells were washed once with phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} [PBS(–)], and incubated for 4 hours with 0.2 mg/mL MTT in the culture medium. After removal of the culture medium, cells were lysed with 100 μL of dimethyl sulfoxide (DMSO), and the absorbance at 540 nm of the cell lysate was then measured by a microplate reader (Labsystems Multiscan, Biochromatic Labsystem, Osaka, Japan) attached to an EPSON VP-700 printer. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (3).

Assay for DNA fragmentation. HSC-2, HSC-3 and HSC-4 cells ($1 \times 10^5/3$ mL) were inoculated in 6-well tissue culture plates (Becton Dickinson) and incubated for 24 hours before the treatments. These cells were then treated for 48 hours without (control) or with CDDP (1 to 50 μM) or 5-FU (1.6 to 25 μM). These cells were collected by scraping with a rubber policeman, lysed and digested with RNase A and proteinase K (Boehringer Ingelheim GmbH, Ingelheim, Germany). The DNA was then isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (3). The DNA from apoptotic HL-60 and HSC-2 cells induced by treatment with actinomycin-D (Act-D; Sigma Chem., St. Louis, MO, USA) (1 μg/mL, 6 hours for HL-60 cells; 0.1 μg/mL, 24 hours for HSC-2 cells) were run in parallel as positive controls.

Assay for caspase activation. HSC-2, HSC-3 and HSC-4 cells ($5 \times 10^5/10$ mL) were inoculated to a tissue culture-dish (Falcon) and incubated for 24 hours before the treatments. These cells were treated for 48 hours without (control) or with CDDP (1, 5, 10 μM) or 5-FU (1, 10 μM), followed by washing twice with PBS(–) and then lysed with lysis solution (MBL, Nagoya, Japan). After the samples were stood for 10 minutes on ice and centrifuged for 10 minutes at 10,000 xg, the supernatant was collected. Lysate (100 μL, equivalent to 150 μg protein) was mixed with 100 μL of 2 x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA) (MBL, Nagoya, Japan). After incubation for 4 hours at 37°C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a microplate reader. Comparison of the absorbance of *p*NA generated by the lysate of treated cells with that of a control cell lysate allows the determination of the relative caspase activity (expressed as % of control), according to the manufacturer's instructions (MBL).

The time-course of apoptosis induction. HSC-2, HSC-3 and HSC-4 cells were treated with CDDP (0 to 62.5 μM) for 3, 6, 12 or 48 hours, washed once and then incubated without CDDP for another 45, 42, 36 or 0 hours. HSC-2, HSC-3 and HSC-4 cells were treated with 5-FU (0 to 62.5 μM) for 12, 24 or 48 hours, washed once and then incubated without 5-FU for another 36, 24 or 0 hours. These cells were subjected to MTT, DNA fragmentation and caspase activity assays as described above.

Statistical treatment. Student's *t*-test was used to assess the statistical significance between the results from the two groups.
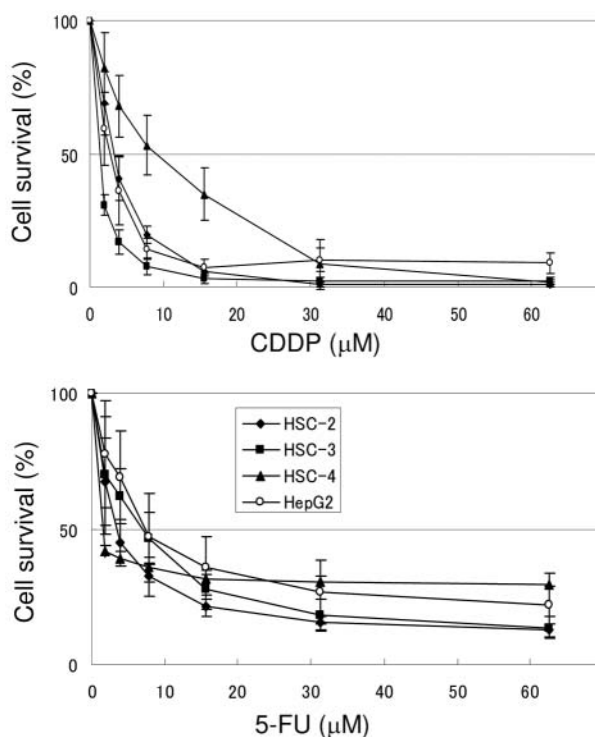

Figure 1. *Cytotoxic activity of CDDP and 5-FU against HSC-2, HSC-3, HSC-4 and HepG2 cells. Near confluent cells were incubated for 48 hours without (control) or with the indicated concentrations (μM) of CDDP or 5-FU and the relative viable cell number (% of control) was determined by the MTT method. Each value represents the mean ±S.D. from 3 independent experiments.*

Results

Induction of cytotoxic activity. HSC-3 cells were the most sensitive to 48-hour continuous treatment with CDDP ($CC_{50}=1.4\pm0.1$ μM), followed by HepG2 (2.8 ± 1.1 μM), HSC-2 (3.2 ± 0.9 μM) and HSC-4 cells (9.3 ± 4.4 μM). On the other hand, HSC-4 cells were the most sensitive to continuous treatment for 48 hours with 5-FU ($CC_{50}=1.7\pm0.1$ μM), followed by HSC-2 (3.4 ± 2.0 μM), HSC-3 (6.9 ± 2.7 μM) and HepG2 (8.7 ± 5.6 μM) (Figure 1).

Induction of DNA fragmentation. Treatment with CDDP (1 to 50 μM) for 48 hours induced internucleosomal DNA fragmentation in HSC-2 and HSC-3 cells, but not in HSC-4 cells. In contrast, treatment of 5-FU (1.6 to 25 μM) for 48 hours did not induce internucleosomal DNA fragmentation in any of these cell lines (Figure 2).

Caspase activation. Treatment of HSC-3 cells with CDDP (1, 5 μM) for 48 hours resulted in a 2.5- to 3.5-fold increase in the caspase-3 activity. CDDP (1, 10 μM) activated the caspase-3 to lesser extents in HSC-2 (1.7- to 2.2-fold) and

CDDP 48 hours
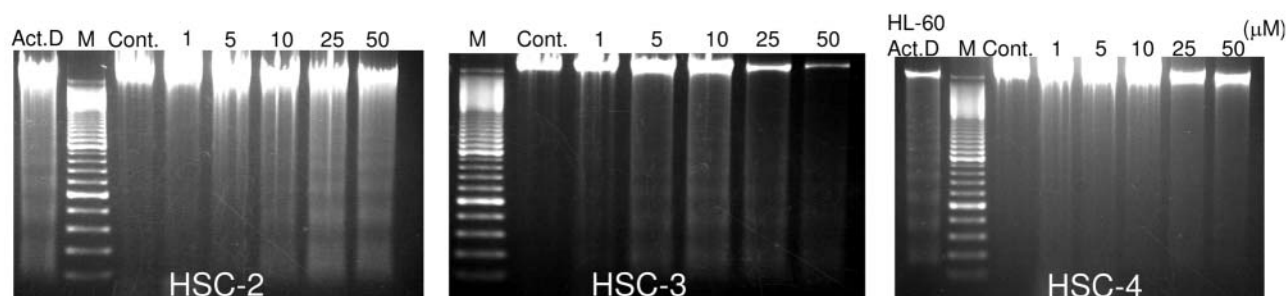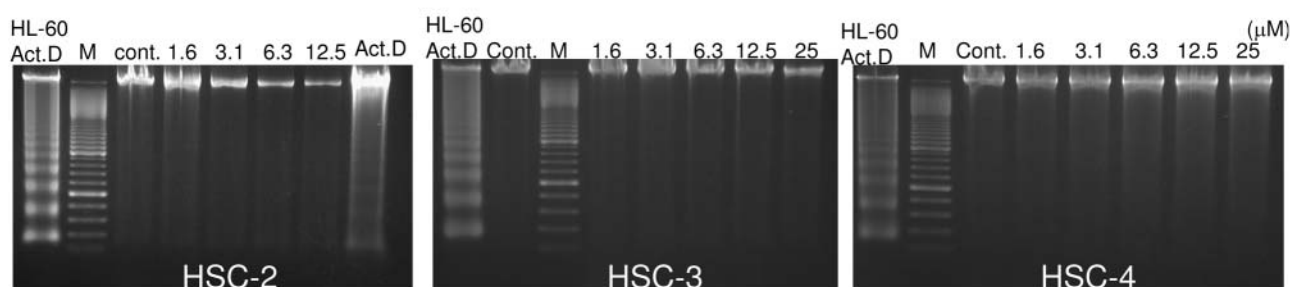

5-FU 48 hours

Figure 2. *Induction of DNA fragmentation by treatment with CDDP or 5-FU in HSC-2, HSC-3 and HSC-4 cells. Near confluent cells were incubated for 48 hours without (control) or with the indicated concentrations (μM) of CDDP or 5-FU and then assayed for DNA fragmentation. Act-D HL-60, DNA from apoptotic HL-60 cells induced by actinomycin D (1 μg/mL, 6 hours). Act-D, DNA from apoptotic HSC-2 cells induced by actinomycin-D (0.1 μg/mL, 24 hours).*

HSC-4 (1.4 to 1.8-fold). Treatment of HSC-4 cells with 5-FU (1, 10 μM) for 48 hours resulted in a 2.3- to 3.4-fold increase in the caspase-3 activity. 5-FU (1, 10 μM) activated caspase-3 to lesser extents in HSC-2 (1.5- to 2.9-fold) and HSC-3 cells (1.1- to 1.5-fold). Treatment of HSC-3 cells with CDDP (1, 5 μM) for 48 hours, activated caspase-8 (1.4- to 2.4-fold) and -9 (1.8- to 2.6-fold). 5-FU (1, 10 μM) did not clearly affect the caspase-8 or -9 activities in any cell line. But, 5-FU (10 μM) significantly activated the caspase-8 ($p < 0.05$) and -9 ($p < 0.01$) in HSC-2 cells, whereas 5-FU (both 1 and 10 μM) significantly ($p < 0.01$) activated caspase-8 in HSC-4 cells (Figure 3).

Time course of apoptosis induction. The time-course study showed that the exposure of HSC-2, HSC-3 and HSC-4 cells to CDDP for 12 hours and the subsequent 36 hours' incubation without CDDP induced comparable cytotoxic activity with that attained by continuous CDDP treatment for 48 hours (upper column in Figure 4). On the other hand, the continuous 48-hour exposure of these cells to 5-FU induced the highest cytotoxic activity, while the cytotoxic activity of 5-FU increased with increasing exposure time (lower panel in Figure 4).

Similarly, treatment of HSC-2 cells for 12 hours with CDDP (25 or 50 μM) and subsequent incubation for 36 hours without CDDP induced internucleosomal DNA fragmentation to a slightly lower extent than that attained by continuous 48-hour treatment with CDDP (Figure 5). Treatment of HSC-3 cells for 12 hours with CDDP (5 or 10 μM) and a subsequent 36-hour incubation without CDDP induced internucleosomal DNA fragmentation to an extent comparable with that attained by continuous 48-hour CDDP treatment (Figure 5).

Treatment of HSC-2 and HSC-4 cells for 12 hours with CDDP (5 or 10 μM) and a subsequent incubation for 36 hours without CDDP induced caspase-3 activation to the extent comparable with that attained by continuous 48-hour CDDP (5 or 10 μM) treatment (Figure 6). HSC-3 cells exhibit significantly greater activation after 48 hours of CDDP treatment.

Discussion

Many researchers have shown the apoptosis-inducing activity of CDDP and 5-FU in head and neck squamous cell carcinoma. CDDP and 5-FU have been reported to induce apoptosis *via* caspase activation. Caspase-9 has been
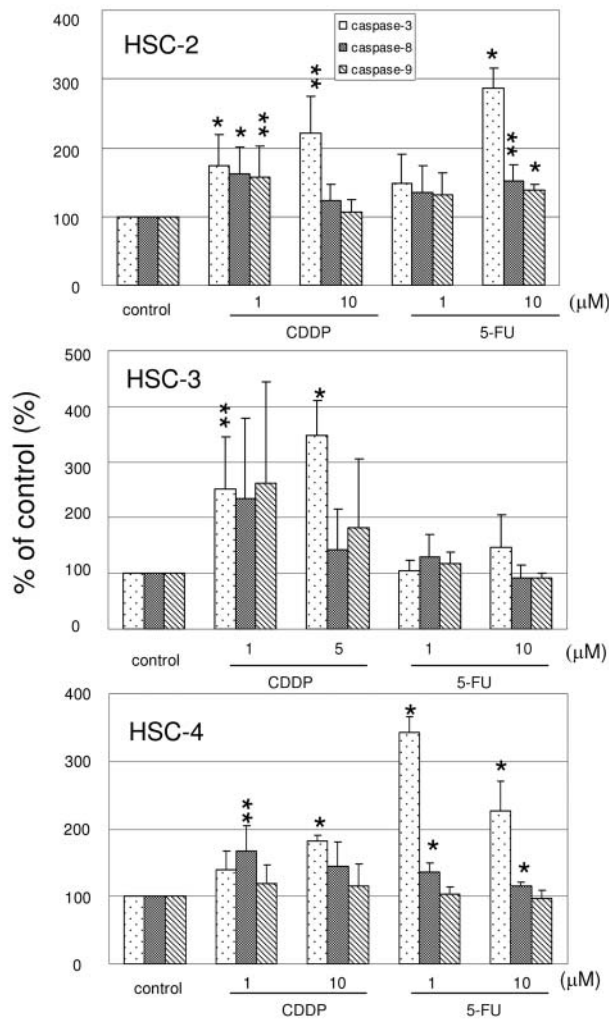
Figure 3. *Activation of caspase by CDDP or 5-FU in HSC-2, HSC-3 and HSC-4 cells. Near confluent cells were incubated for 48 hours without (control) or with the indicated concentrations of CDDP or 5-FU. Caspase activity was assayed and expressed as a % of that of the control. Each value represents the mean±S.D. from 3 independent experiments. Significance between CDDP or 5-FU and control: *p<0.01, **p<0.05.*

reported to be involved in the apoptosis induced by CDDP, in contrast to the involvement of caspase-1, -3 and -8 in 5-FU-induced apoptosis (5-7). On the other hand, CDDP and 5-FU have been reported to enhance the susceptibility of oral squamous cell carcinoma cell lines to the Fas-mediated apoptosis *via* the activation of caspase-3 and -8 (8). We established the optimum conditions for treatment with CDDP and 5-FU of oral squamous cell carcinoma cell lines. We found a relationship between the cytotoxic activity and caspase-3 activation in HSC-2, -3 and -4 cells induced by these agents for 48 hours (Figures 1 and 2). Because, treatment with CDDP for 48 hours induced the highest

cytotoxic activity and caspase-3 activation in HSC-3 cells, followed by HSC-2 and HSC-4 cells, whereas treatment with 5-FU for 48 hours induced the highest cytotoxic activity and caspase-3 activation in HSC-4 cells, followed by HSC-2 and HSC-3 cells. These data suggested that both CDDP and 5-FU induce apoptosis *via* caspase-3 activation.

CDDP induced DNA fragmentation only in HSC-2 and HSC-3 cells, whereas 5-FU did not induce the DNA fragmentation in all cells (Figure 2). These data demonstrate that three oral squamous cell carcinoma (HSC-2, HSC-3, HSC-4) cell lines showed different sensitivity to CDDP and 5-FU and that these agents failed to induce internucleosomal DNA fragmentation in HSC-4 cells.

Whether these agents induce non-apoptotic cell death such as autophagy in HSC-4 remains to be investigated. The sensitivity to mitomycin C, bleomycin or peplomycin were in the following order (from sensitive to resistant): HSC-2 > HSC-3 > HSC-4 (9). It has been reported that *p53* (tumor suppressor gene) mutation is involved in the sensitivity of cancer cells to anticancer agents (10, 11). Hepatoma cells expressing wild-type *p53* were found to be sensitive to bleomycin, CDDP and methotrexate, to accumulate the *p53*, to express Fas, and were committed to apoptosis, whereas hepatoma cells containing mutated *p53* were resistant (10). HSC-4 cells, but not HSC-2 and HSC-3 cells have been reported to express *p53* (12), whereas HSC-3 and HSC-4 cells have been reported to express mutated *p53* (11). Thus, we compared that the sensitivity of HepG2 cells (with wild-type *p53*) and HSC-2, HSC-3 and HSC-4 cells to CDDP and 5-FU (Figure 1). The sensitivity to CDDP was in the following order (from sensitive to resistant): HSC-3 > HepG2 > HSC-2 > HSC-4. The sensitivity to 5-FU was in the following order (from sensitive to resistant): HSC-4 > HSC-2 > HSC-3 > HepG2. These results suggested that the sensitivity of these cell lines was not strictly determined by *p53* mutation, but rather affected by the type of anticancer agents.

It has been reported that when the treatment time with CDDP was prolonged from 1 to 8 hours, the cytotoxic activity of CDDP became detectable at a concentration of 5 μg/ml, similar to that observed after 1-hour treatment with CDDP (50 μg/ml) against asynchronous human lymphoma cells (13). Therefore, we performed a time-course study to establish the optimum treatment conditions for CDDP and 5-FU. We found that the treatment of HSC-2, HSC-3 and HSC-4 cells with CDDP for 12 hours (and subsequent 36-hour incubation without CDDP) produced the comparable magnitude of cytotoxicity with that attained by continuous 48-hour CDDP treatment, and was caspase activated. HSC-2 and -3 cells displayed internuclesomal DNA fragmentation under both conditions. These results indicate that the optimal treatment with CDDP for any of HSC-2, HSC-3 or
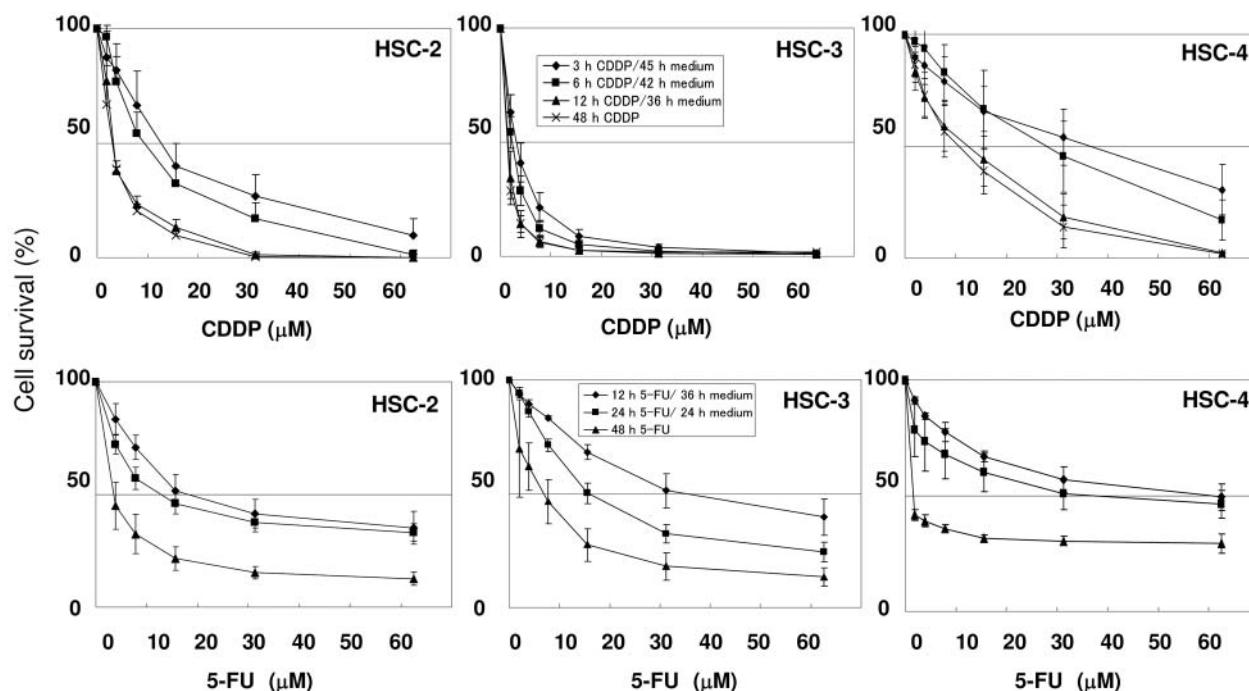
Figure 4. *Time course of cytotoxic activity induction after treatment with CDDP or 5-FU in HSC-2, HSC-3 and HSC-4 cells. Near confluent cells were treated for 3, 6, 12 or 48 hours without (control) or with the indicated concentrations of CDDP, washed once, and then incubated further for 0, 36, 42 or 45 hours in fresh drug-free medium (total incubation time =48 hours). The cells were also treated for 12, 24, 48 hours without (control) or with the indicated concentrations of 5-FU, washed once, then incubated further for 0, 24 and 36 hours in fresh drug-free medium. The relative viable cell number (% of control) was determined by MTT assay.*

HSC-4 cells was 12 hours. On the other hand, the cytotoxic activity of 5-FU depended on the dose and exposure times. Our findings are in agreement with previous findings that administration of a lower dose of 5-FU for longer time-periods is more effective in producing direct cytotoxic activity againt human tumor cells (colon adenocarcinoma, cervix and ovary carcinoma cell lines) than the treatment with higher doses for shorter times (14).

The different effect of CDDP and 5-FU may be due to their different sites of action. CDDP is known to intercalate into DNA at any phase of the cell cycle (13, 15, 16), causing the cytotoxic effect and reducing the cellular viability almost to base line (Figure 1). On the other hand, 5-FU is known to inhibit the function of RNA or DNA synthesis depending on the concentration (17, 18) and cause the cytostatic effect accompanied by the growth arrest at the G1/S boundary of the cell cycle (19), reducing the viability to 25% of control after 48 hours (Figure 1). There are very few studies which determine the optimum treatment time for CDDP. The present study demonstrates that the minimum exposure time to CDDP required for the induction of the plateau level of cytotoxicity is 12 hours in all three human oral squamous cell lines. The shorter the treatment time, the fewer side-effects of CDDP to be expected. This information may serve as a

basis of establishing more efficient treatment conditions, in combination with other chemotherapeutic agents, for the oral squamous cell carcinoma.

References

1   Rosenberg B, Van Camp L and Krigas T: Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature *205*: 698-699, 1965.
2   Heidelberger C, Chaudhuri NK, Danneberg P, Mooren D and Griesbach L: Fluorinated pyrimidines, a new class of tumor-inhibitory compounds. Nature *179*: 663-666, 1957.
3   Okamura M, Hashimoto K, Shimada J and Sakagami H: Apoptosis-inducing activity of cisplatin (CDDP) against human hepatoma and oral squamous cell carcinoma cell lines. Anticancer Res *24*: 655-662, 2004.
4   Okamura M, Kobayashi M, Suzuki F, Shimada J and Sakagami H: Induction of cell death by combination treatment with cisplatin and 5-fluorouracil in a human oral squamous cell carcinoma cell line. Anticancer Res *27*: 3331-3338, 2007.
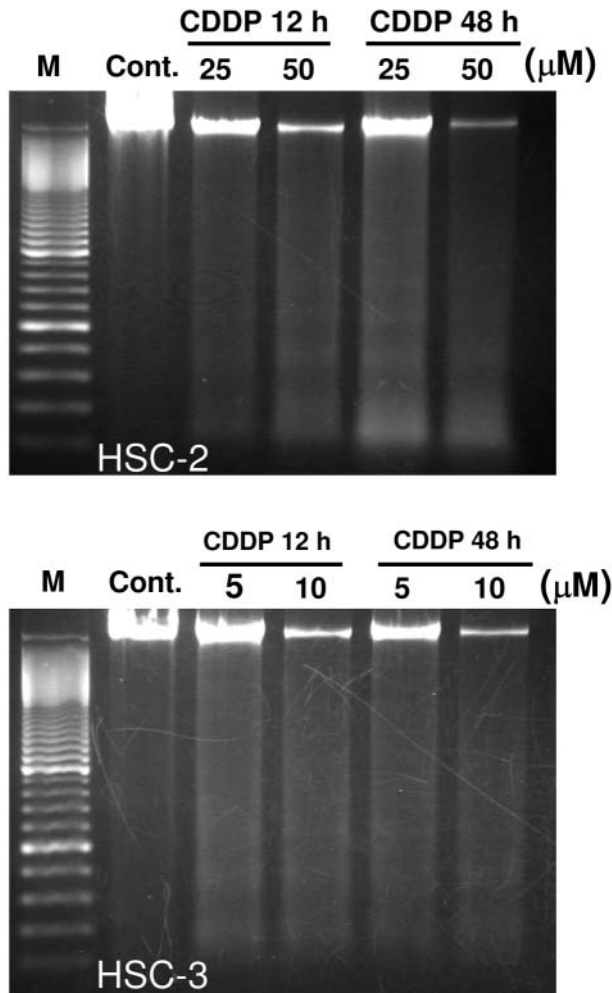
Figure 5. *Time course of induction of DNA fragmentation after treatment with CDDP or 5-FU in HSC-2 and HSC-3 cells. Near confluent cells were treated for 12 or 48 hours with the indicated concentrations of CDDP (μM), washed once, then incubated further for 0 or 36 hours in fresh drug-free medium and then assayed for DNA fragmentation.*
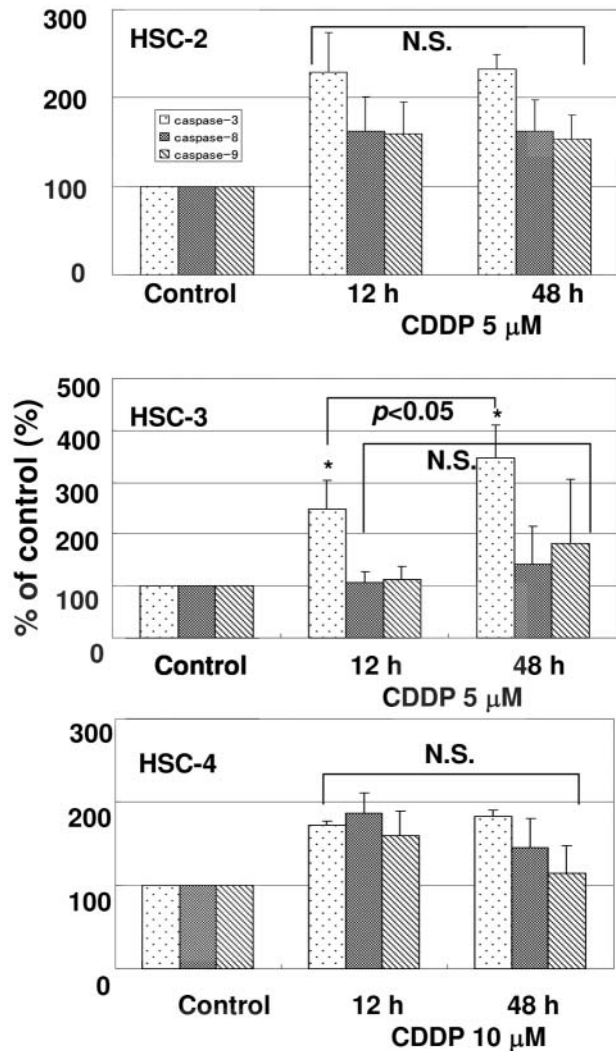

Figure 6. *Time course of activation of caspase-3, caspase-8 and caspase-9 after treatment with CDDP or 5-FU in HSC-2, HSC-3 and HSC-4 cells. Near confluent cells were treated for 0 (control), 12 or 48 hours with the indicated concentrations of CDDP (μM), washed once, then incubated further for 0, 36 or 48 hours in fresh medium, respectively. Caspase activity was then assayed and expressed as a % that of the control. Each value represents the mean±S.D. of three independent experiments. Significance between 12- and 48-hour treatment with CDDP, *p<0.05.*

5   Kuwahara D, Tsutsumi K, Kobayashi T, Hasunuma T and Nishioka K: Caspase-9 regulates cisplatin-induced apoptosis in human head and neck squamous cell carcinoma cells. Cancer Letters *148*: 65-71, 2000.

6   Kuwahara D, Tsutsumi K, Oyake D, Ohta T, Nishikawa H and Koizuka I: Inhibition of caspase-9 activity and Apaf-1 expression in cisplatin-resistant head and neck squamous cell carcinoma cells. Auris Nasus Larynx *30*: 585-588, 2003.

7   Ohtani T, Hatori M, Ito H, Takizawa K, Kamijo R and Nagumo M: Involvement of caspases in 5-FU induced apoptosis in an oral cancer cell line. Anticancer Res *20*: 3117-3122, 2000.

8   Iwase M, Watanabe H, Kondo G, Ohashi M and Nagumo M: Enhanced susceptibility of oral squamous cell carcinoma cell lines to Fas-mediated apoptosis by cisplatin and 5-fluorouracil. Int J Cancer *106*: 619-625, 2003.

9   Sasaki M, Okamura M, Ideo A, Shimada J, Suzuki F, Ishihara M, Kikuchi H, Kanda Y, Kunii S and Sakagami H: Re-evaluation of tumor-specific cytotoxicity of mitomycin C, bleomycin and peplomycin. Anticancer Res *26*: 3373-3380, 2006.

10 Muller M, Strand S, Hug H, Heinemann E-M, Walczak H, Hofmann WJ, Stremmel W, Krammer PH and Galle PR: Drug-induced apoptosis in hepatoma cells is mediated by the CD95(APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. J Clin Invest *99*: 403-413, 1997.

11 Kamiya Y and Ohshima T: The individual cell properties of oral squamous cell carcinoma and *p53* tumor suppressor gene mutation. Oral Science Int *2*: 104-117, 2005.

12 Kebusa Y: Relationship between p53 and expression of Fas and Fas ligand proteins in squamous cell carcinoma of the oral cavity. Meikai Univ Dent J *32*: 205-216, 2003 (in Japanese).

13 Drewinko B, Brown BW and Gottlieb JA: The effect of *cis*-diamminedichloro platinum(II) on cultured human lymphoma cells and its therapeutic implications. Cancer Res *33*: 3091-3095, 1973.

14 Paula M, John E, John F and Thomas R: Time-dose relationships for 5-fluorouracil cytotoxicity against human epithelial cancer cells *in vitro*. Cancer Res *42*: 4413-4420, 1982.

15 Zwelling LA and Kohn KW: Mechanism of action of *cis*-dichlorodiammineplatinum (II). Cancer Treat Rep *63*: 1439-1444, 1979.

16 Takahashi K, Ebihara K, Honda Y, Nishikawa K, Kita M, Oomura M and Shibasaki C: Antitumor activity of cis-dichlorodiammineplatinum(II) and its effect on cell cycle progression. Gan To Kagaku Ryoho *9*: 624-631, 1982 (in Japanese).

17 Spiegelman S, Sawter R, Nayak R, Ritzi E, Stolfi R and Martin D: Improving the anti-tumor activity of 5-fluorouracil by increasing its incorporation into RNA *via* metabolic modulation. Proc Natl Acad Sci USA *77*: 4966-4970, 1980.

18 Parker WB and Cheng YC: Metabolism and Mechanism of action of 5-fluorouracil. Pharmac Ther *48*: 381-395, 1990.

19 Li MH, Ito D, Sanada M, Odani T, Hatori M, Iwase M and Nagumo M: Effect of 5-fluorouracil on G1 phase cell cycle regulation in oral cancer cell lines. Oral Oncol *40*: 63-70, 2004.