Human Scirrhouss Gastric Cancer: Chemotherapeutic Trials from Clinical and Basic Approaches

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Chapter I

Introduction

Gastric cancer has been declared as a Global Public Health Concern, with the World Health Organization (WHO) declaring that nearly 1 million new cases are diagnosed worldwide each year. Although the incidence is declining, gastric cancer is still globally the third-most common cause of cancer-related mortality (1). Two-thirds of gastric cancers occur in Eastern Asia, Eastern Europe, and South America, with a case fatality ratio of 78% versus 65% in the industrialized world (2). At diagnosis, virtually about half of gastric cancer patients present with an advanced stage of the disease, with a 5-year survival rate lower than 30% (3, 4). Although advanced gastric cancer is considered difficult to treat surgically, various novel anticancer drugs and several combination chemotherapy regimens have recently been shown to have significant anticancer effects, e.g., increased survival in patients with advanced disease.

Nevertheless, the prognosis of patients with scirrhous gastric cancer is still poor (5). Scirrhous gastric cancer is unique among gastric cancers: in this type, poorly differentiated carcinoma cells or signet-ring cells show diffuse infiltrative growth. Scirrhous gastric cancer is characterized by extensive areas of pronounced fibrous hyperplasia that results in significant narrowing of the gastric lumen. It is very difficult to identify this cancer in its early stage, and surgery in late stages does not increase survival (6). Owing to the low incidence of this cancer, only a few drug trials against it have been conducted to date (7). Further, the biological and drug-resistance mechanisms of scirrhous gastric cancer remain mostly unknown.

In this series of studies, I first retrospectively examined the outcome of patients who had been treated for Borrmann type 4 advanced gastric cancer at Kobe City General Center Hospital. Although Borrmann type 4 is often considered to be synonymous with scirrhous gastric cancer, and the two are currently considered to be nearly the same entity in most cases, there are,
however, a number of differences between them (8). The main characteristic of scirrhous gastric cancer is sclerosis or induration, whereas that of Borrmann type 4 advanced gastric cancer is a diffuse distribution. From a clinical perspective, it is helpful to consider Borrmann type 4 advanced gastric cancer as scirrhous gastric cancer in order to better understand its diagnosis and pathology. To briefly explain these similar cancer types, I retrospectively investigated patients with Borrmann type 4 advanced gastric cancer who had been pathologically diagnosed with scirrhous gastric cancer according to the endoscopic features described in Chapter II.

Second, drug resistance is a serious problem in chemotherapy and is one of the main causes of poor outcomes following cancer treatment, including that for scirrhous gastric cancer (9). Therefore, I examined the drug resistance of human scirrhous gastric cancer cell lines to various chemotherapeutic agents in vitro. The human scirrhous gastric cancer cell lines examined were established from pleural fluid or ascites fluid obtained from patients with scirrhous gastric cancer.

Third, as previously mentioned, the mechanism of action of chemotherapeutic agents and the responses of scirrhous gastric cancer to them are not well known. To develop new therapeutic approaches based on the characteristic biological features of scirrhous gastric cancer cells, I investigated the mechanisms underlying the cytotoxicity of various anticancer drugs against a scirrhous gastric cancer cell line, HSC-39, in vitro. Common features of scirrhous gastric cancer include invasive progression, remarkable fibrosis, and a high frequency of metastasis to the peritoneum or lymph nodes (10). HSC-39 is one of the human scirrhous gastric cancer cell lines that tend to metastasize to the peritoneum. Therefore, this cell line was chosen as a suitable one to examine the biological properties of drug resistance and metastasis. Moreover, I also examined the status of reactive oxygen species (ROS), which have been reported to be cytotoxic toward tumor cells, in HSC-39 cells. ROS, including non-radical hydrogen peroxide (H$_2$O$_2$), organic hydroperoxide (ROOH), and hypochlorous acid (HOCl) are generated from inflammatory immune cells such as activated
macrophages and neutrophils, which accumulate at sites of inflammation. ROS play important roles in the induction of apoptosis, not only in inflammatory cells but also in neighboring cells and tissues (11, 12). However, it is still largely unclear how anticancer drugs and ROS induce apoptosis of scirrhouss gastric cancer cells. Finally, I examined the biological effects of ROS on scirrhouss gastric cancer.

This thesis, in Chapters III-V, describes the drug resistance to and the biological effect of chemotherapeutic agents on human scirrhouss gastric cancer cell lines in vitro. The findings made in this study contribute to a better understanding of the mechanisms underlying the drug resistance of, and the effects of chemotherapeutic agents on, scirrhouss gastric cancer and should be valuable for the discovery and development of new treatments to improve the survival rate of patients with scirrhouss gastric cancer.
Chapter II.

The outcome of treatment for patients with Borrmann type 4 advanced gastric cancer

1. Introduction

Gastric cancer is the third most common cause of cancer-related death in the world (13). In 1926, Borrmann classified advanced gastric cancer into four gross types (14). Borrmann type 4 was characterized by less differentiation, usually presenting as poorly differentiated adenocarcinoma, and by invasion of the entire wall of the stomach. Borrmann type 4 advanced gastric cancer is defined as being a diffuse cancer with almost invisible ulcers and an ill-defined boundary. Despite recent advances in the treatment of gastric cancer, the prognosis of patients with Borrmann type 4 advanced gastric cancer remains extremely poor (15, 16); and the 5-year survival rate of patients with this advanced gastric cancer is significantly lower than that of those with other types of gastric cancer (17). This can be seen in a report by The Japan Gastric Cancer Association Registration Committee regarding the treatment results and causes of death in patients with primary gastric cancer who were treated in Japan. Of 13,002 patients with primary gastric cancer, the 5-year survival of patients with Borrmann type resected cases regardless of stage was as follows: type 0, \( n = 6869 \) (90.2%); type 1, \( n = 363 \) (65.5%); type 2, \( n = 1717 \) (60.4%); type 3, \( n = 2575 \) (46.0%); type 4, \( n = 923 \) (17.7%); and type 5, \( n = 339 \) (60.6%) (18,19). Survival rates at 5 years after curative gastrectomy have ranged from 11%–38.4% (20- 22). This has led to the conclusion that surgery is not a curative treatment for Borrmann type 4 advanced gastric cancer (23, 24). Indeed, no adequate therapeutic strategy for Borrmann type 4 advanced gastric cancer has yet been established.

In the 1990’s, S-1 (TS-1; Taiho Pharmaceutical, Tokyo, Japan), an oral derivative of 5-fluorouracil (5-FU), was developed for the treatment of gastric cancer (25, 26). In Japan, S-1
rapidly established itself as a standard treatment with a high response rate of 46% when used as a single agent. Phase III trials proved the non-inferiority of S-1 compared with infusional 5-FU in the advanced/metastatic setting (27). A phase III trial showed a better median survival time (MST) with the combination of S-1 plus cisplatin compared with S-1 monotherapy (28). In addition, the combination of irinotecan and S-1 achieved a longer median survival than S-1 monotherapy (29). In Japan, advanced gastric cancer is mainly treated with S-1 alone or S-1 combined with other drugs.

As treatment for gastric cancer advances, it is expected that treatment for Borrmann type 4 advanced gastric cancer will also improve. Owing to its low incidence, however, only a few trials for this condition have been conducted. Here, to assess survival in patients with Borrmann type 4 advanced gastric cancer, I retrospectively examined outcomes in these patients following chemotherapy.

2. Patients and Methods

2.1 Patient population and data collection

Data from patients diagnosed with Borrmann type 4 advanced gastric cancer as well as scirrhous gastric cancer at Kobe City General Center Hospital between July 2011 and June 2015 were retrospectively collected for this study. The inclusion criterion was histologically confirmed primary gastric cancer Borrmann type 4. The exclusion criteria were synchronous or metachronous cancers.

Clinical data were collected for each patient, including gender, age, and treatments. The 13th edition of the Japanese Classification of Gastric Carcinoma was used for data management (30). The date of last follow-up and mortality were collected for all patients.

This retrospective study was approved by the Institutional Review Board of Kobe City Medical Center General Hospital and was conducted in accordance with the ethical principles mandated in Japan’s Ethics Guidelines for Epidemiological Research.
2.2 Statistical considerations

Univariate and multivariate analyses were performed by using Cox’s proportional hazard model to identify risk factors affecting overall survival (OS). OS was defined as the period from the diagnosis of type 4 advanced gastric cancer to the date of death from any cause. Patients who remained alive at the time of the last record were censored at that time. Survival curves were generated by using the Kaplan-Meier method, and the difference was analyzed by the log-rank test. P values < 0.05 were considered statistically significant. Prognostic factors were evaluated by Cox’s proportional hazard model with a log-linear risk function. All statistical analysis was performed with Statistical Package for Social Science (SPSS) software (SPSS 23.0 Inc., Chicago IL, USA).

3. Results

3.1 Patient characteristics

Patient background at baseline is presented in Table 1. Forty-two cases were analyzed. The median age was 58 years (range, 32–81). Of the patients, 37 cases (88.0 %) had a baseline Eastern Cooperative Group performance status of 0–1. Thirteen cases (30.9 %) were locally advanced and 29 cases (69.0 %) were metastatic advanced. Fifteen cases (51.7 %) had peritoneal metastasis. As shown in Figure 1, among the 42 cases, 20 cases (52.3 %) were surgically resected. This included palliative resection in nine cases (one for bleeding from the primary lesion, five for stricture, and three for malnutrition) of metastatic advanced patients.
<table>
<thead>
<tr>
<th>Table 1. Patient characteristics ($n = 42$)</th>
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<td>Age, years</td>
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<td>Gender</td>
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<td>ECOG performance status</td>
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<td>Histological type</td>
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<td>HER 2 status</td>
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<td>Gastrectomy</td>
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<td>Degree of tumor extent</td>
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<td>Metastatic site</td>
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Figure 1. Diagram of the study

Borrmann type 4 advanced gastric cancer
42 cases

Locally advanced 13 cases

Metastatic advanced 29 cases

Not treated 2 cases
Resected 11 cases

Surgically resected 9 cases
No surgery 20 cases

Adjuvant/Recurrence
Recurrent

Chemotherapy 6 cases

Surgery only 3 cases
Chemotherapy 6 cases
Supportive care 8 cases
Chemotherapy 12 cases

Figure 2. Diagram of the chemotherapy treatment patients

Chemotherapy 24 cases

1st line chemotherapy 23 cases

Supportive care 13 cases

2nd line chemotherapy 10 cases

Supportive care 7 cases

3rd line Chemotherapy 3 cases

1 case transferred hospital

10 / 23 cases → 43%

3 / 10 cases → 33%
### 3.2 Patient outcome

Chemotherapy was planned for 24 patients. However, one patient was transferred to another hospital before chemotherapy was started (Figure 2), leaving chemotherapy as the first-line treatment in 23 patients. As shown in Figure 3, the first-line treatment regimen consisted of S-1 plus cisplatin (SP) in 16 patients (69.5%). Reasons for discontinuation of SP treatment in 10 patients were disease progression in nine (90.0%) and toxicity in one (10.0%). Other treatment regimens included capecitabine plus cisplatin (XP) in one patient (4.3%), S-1 plus oxaliplatin (SOX) in one (4.3%), S-1 monotherapy in two (8.6%), capecitabine monotherapy in one (4.3%), paclitaxel in one (4.3%), and docetaxel in one (4.3%). After discontinuation of first-line treatment in 12 patients due to disease progression and in one patient for toxicity, 13 patients (56.5%) received supportive care only and 10 patients (43.3%) received second-line chemotherapy. The 10 patients who received second-line treatment consisted of six patients who had received SP treatment (37.5% of SP treatment) and four who had received other treatments (57.1% of any other treatments). The second-line chemotherapy regimen consisted of paclitaxel in eight patients, irinotecan (CPT-11) in one, and S-1 monotherapy in one. After the discontinuation of second-line treatment due to disease progression, seven patients (70.0%) received supportive care only and three (30.0%) received third-line chemotherapy, two with docetaxel (66.6%) and one with S-1 plus paclitaxel (33.3%).

**Figure 3.** Chemotherapy treatment ratio

(A) 1st line chemotherapy \((n = 23)\)

- SP: 69.5%
- S-1: 8.6%
- Paclitaxel: 4.3%
- DOC: 4.3%
- XP: 4.3%

(B) 2nd line chemotherapy \((n = 10)\)

- Paclitaxel: 60.0%
- Trastuzumab + Paclitaxel: 10.0%
- S-1: 10.0%
- Irinotecan: 10.0%
- S-1 + Paclitaxel: 10.0%

SP: S-1 + cisplatin  XP: capecitabine + cisplatin  SOX: S-1 + oxaliplatin
Figure 4 shows the OS of locally advanced cases and metastatic advanced ones. The median OS of locally advanced cases was 29.6 months (95% confidence interval [CI]: 16.8–42.3 months), whereas that of the metastatic advanced ones was 11.5 months (95% CI: 2.1–20.8 months; p = 0.019). Among the locally advanced patients, one patient was stage I, two patients were stage II, and ten patients were stage III. Curative resection was performed in 11 of the locally advanced cases (84.6%). There was a statistically significant difference between the OS of locally advanced and metastatic advanced cases (p = 0.019).

In the study described in this chapter, the median OS for patients with peritoneal metastases was 8.9 months (95% CI: 0–21.9 months); whereas that for no peritoneal metastases was 11.5 months (95% CI: 0–29.0 months; p = 0.831; Figure 5), indicating that peritoneal metastases were unrelated to OS. The median OS for patients treated with first-line SP was 20.7 months (95% CI: 12.9–28.4 months); and that for those with any other type of chemotherapy was 19.3 months (95% CI: 5.1–33.4 months), p = 0.994 (Figure 6).

**Figure 4.** Overall survival of locally advanced cases and metastatic advanced cases (n = 42)
**Figure 5.** Overall survival of patients with and without peritoneal metastasis ($n = 29$)

**Figure 6.** Overall survival of patients with S-1 plus cisplatin treatment and patients on any other treatments ($n = 23$)
4. Discussion

In this study, Borrmann type 4 gastric cancer tended to affect younger patients, was particularly more prevalent in female patients (59.5%), and was mostly of the diffuse type (92.9%). Because the patients were mainly HER 2 status-negative (95.2%), they were not candidates for treatment with trastuzumab (31). In addition, at the time of diagnosis, most patients (69.0%) had unresectable disease, most with peritoneal dissemination (51.7%). These are the major reasons for the poorer prognosis of Borrmann type 4 advanced gastric cancer (32).

Compared with locally advanced cases, metastatic cases had a significantly lower median OS, i.e., 29.6 months vs. 11.5 months (p = 0.019), respectively. The OS benefit in locally advanced patients has been reported, but Borrmann type 4 advanced gastric cancer is seldom diagnosed at an early stage (stage IV vs. stage I–III was 69.0% vs. 30.9%).

Generally, a fluorinated pyrimidine plus platinum combination, S-1 plus cisplatin, was chosen as treatment for metastatic advanced cases. There were no significant differences in the median OS between patients treated with SP (20.7 months, 95% CI: 12.9–28.4 months) and those with any other type of chemotherapy (19.3 months, 95% CI: 5.1–33.4 months; p = 0.994). These results suggest that, compared with other treatments, SP does not have sufficient efficacy for the treatment of patients with Borrmann type 4 advanced gastric cancer.

Only 43% of the patients received second-line chemotherapy in this study (Figure 2). In contrast, 75% of patients in a recent Japanese phase III trial (SPIRITS; S-1 vs. S-1 plus cisplatin) for advanced gastric cancer received second-line treatment (28). It may be that patients with Borrmann type 4 advanced gastric cancer who progressed during first-line treatment were in poorer general condition, and so most of them might have missed the opportunity to receive the second-line chemotherapy.

Several limitations of this study require mention. The number of patients with scirrhous gastric cancer who had a relapse after surgery or who had metastasis at the time of initial diagnosis was too small to allow the comparison of OS with S-1 plus cisplatin treatment versus other treatments.
It is important for patients with Borrmann type 4 advanced gastric cancer to receive effective chemotherapy as first-line treatment to improve survival. Reasons for this include the unreliability of the administration and absorption of oral agents in these patients due to stricture, the high risk of complications, and the inability to receive the hydration that is required for renal protection from cisplatin. Oxaliplatin is a third-generation platinum compound that was developed to provide improved tolerability and administration over cisplatin. The non-inferiority of oxaliplatin-based regimens to cisplatin-based regimens was demonstrated in the Revised European-American Lymphoma (REAL)-2 study. This background therefore indicates the need for the development of novel chemotherapeutic regimens with non-oral agents, with no need for hydration, and with high feasibility for patients with this disease (33).

A previous clinical study demonstrated that gastric cancer patients with severe peritoneal metastases receiving 5-FU-based chemotherapy as first-line treatment had a median time to treatment failure (TTF) and OS of 1.9 and 4.6 months, respectively (34). 5-FU-based chemotherapy for gastric cancer patients with severe peritoneal metastases was feasible, but its efficacy was unsatisfactory. Recent phase I/II trials have demonstrated the non-inferiority of chemotherapy using oral fluoropyrimidines, such as capecitabine or S-1, compared to 5-FU-based chemotherapy (27, 34, 35). Oral fluoropyrimidine and cisplatin combination therapy is a standard regimen worldwide (15). Although 5-FU is one of the most commonly used drugs in patients with gastrointestinal cancers, systemic 5-FU chemotherapy has a limited response rate (36). A new treatment for Borrmann type 4 advanced gastric cancer, including 5-FU, is urgently required.

Given the above results, chemotherapy is expected to play an important role in the treatment of Borrmann type 4 advanced gastric cancer, because 69% of patients had metastasis at the time of initial diagnosis. SP treatment did not provide a significant improvement in OS compared with other treatments in this study, which result may have been due to the comparatively higher rate of metastatic cases and to the already poor prognosis at the time of diagnosis. Our results suggested that SP treatment might provide a beneficial effect on OS.
Further studies to reveal the mechanism of resistance of Borrmann type 4 advanced gastric cancer and to discover basic new strategies should improve OS and the efficacy of treatment for this condition.
Chapter III

Drug-resistance of human scirrhous gastric cancer cell lines to chemotherapeutic agents

1. Introduction

Although surgery currently represents the standard treatment for gastric cancer without distant metastasis, chemotherapy also appears to be a useful option in the treatment of advanced gastric cancer, as it gives survival benefits (37). Platinum-based chemotherapy containing cisplatin has shown promising results. Based on Japanese phase III trials for metastatic gastric cancer, S-1 plus cisplatin combination treatment has now been established as the standard first-line treatment throughout the world (28).

S-1 is an oral fluorouracil antitumor drug that combines three pharmacological agents: tagafur (FT), which is a prodrug of 5F-U; 5-chloro-2,4-dihydroxypyridine (CDHP), which inhibits dihydropyrimidine dehydrogenase (DPD) activity; and potassium oxonate, which reduces gastrointestinal toxicity (38). 5-Fluorouracil (5-FU) is a widely used anticancer agent and is currently considered to be the key drug in clinical chemotherapy for gastrointestinal cancers such as gastric cancer and colorectal cancer. DPD is associated with 5-FU catabolic action. This enzyme is present in large quantity in the human liver, and some reports suggest that 5-FU sensitivity is negatively correlated with DPD activity within tumor cells in many cancers (39, 40).

The anticancer activity of platinum arises from its ability to induce irreparable intra-strand DNA crosslinks/adducts, which lead to cell apoptosis (41) and oxidative and endoplasmic reticulum stress (42-44). On the other hand, various adaptive mechanisms work to induce platinum resistance in cancer, including reduced cellular uptake, increased DNA repair, tolerance (45), and inactivation by glutathione (46). In addition, some tumors acquire
resistance to chemotherapy, reducing its efficacy (47, 48).

In this study, I examined the drug resistance of scirrhouous gastric cancer cell lines to 5-FU and cisplatin *in vitro*.

### 2. Materials and methods

#### 2.1 Materials

5-FU was obtained from Kyowa Kirin (Tokyo, Japan); and cisplatin (CDDP), from Nichiiko (Toyama, Japan). WST-1 solution was purchased from Roche (Cell Proliferation Reagent WST-1).

#### 2.2 Cell culture

The human scirrhouous gastric cancer cell line used, HSC-39, had been established from the peritoneal ascites fluid of a 54-year-old male Japanese patient with scirrhous gastric cancer (49). The HSC-44PE cell line was established from the pleural fluid, obtained by thoracocentesis, of a 28-year-old female Japanese patient with scirrhous gastric carcinoma (Linitis plastica-type). The HSC-58 cell line was established from the ascites fluid, obtained by peritoneocentesis, of a 57-year-old male Japanese patient with scirrhous gastric carcinoma. The HSC-60 cell line was established from the ascites fluid, obtained by peritoneocentesis, of a 40-year-old male Japanese patient with scirrhous gastric carcinoma. Histopathologically, these primary tumors were all diagnosed as signet-ring cell carcinoma with a scirrhous stromal reaction (50, 51; Table 2). All of these cell lines were derived from patients who had not undergone chemotherapeutic treatments.

The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μg/ml streptomycin sulfate, and 50 U/ml penicillin G sodium (Nacalai Tesque, Kyoto). Media and sera were obtained from Gibco. The cells were seeded at low density in T-25 tissue culture flasks (Falcon) in standard medium containing 10% FCS and incubated at 37°C.
in 5% CO₂ - 95% humidity. They were then subcultured at a ratio of 1:10 every three days.

**Table 2. Biological characteristics of established gastric cancer cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Tumor source</th>
<th>Histological typing</th>
<th>Doubling time (hours)</th>
<th>Tumor markers</th>
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<td></td>
<td>Sex</td>
<td>Age</td>
<td>Original</td>
<td>Xenograft</td>
<td>CA19-9</td>
</tr>
<tr>
<td>HSC-39</td>
<td>Male</td>
<td>54</td>
<td>Pleural effusion</td>
<td>Sig (scirrhous)</td>
<td>28-30</td>
</tr>
<tr>
<td>(14)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-44PE</td>
<td>Female</td>
<td>28</td>
<td>Pleural effusion</td>
<td>Sig (scirrhous)</td>
<td>24</td>
</tr>
<tr>
<td>(15) (16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-58</td>
<td>Male</td>
<td>57</td>
<td>Ascitic tumor</td>
<td>Por2/ sig (scirrhous)</td>
<td>23</td>
</tr>
<tr>
<td>(15) (16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-60</td>
<td>Male</td>
<td>40</td>
<td>Ascitic tumor</td>
<td>Sig (scirrhous)</td>
<td>26</td>
</tr>
<tr>
<td>(15) (16)</td>
<td></td>
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**2.3 Morphologic observation**

Cells of the above cell lines were seeded into wells of a 96-well plate (Iwaki) at the concentration of 2×10³ cells/0.05 ml/well and incubated at 37°C for 1 hour. 5-FU or CDDP was added to the wells at final concentrations of 0, 0.1, 10 or 100 μM, and the cells were then incubated at 37°C for 72 hours. Thereafter, the cells were observed under a phase-contrast microscope (Diamat; Nikon, Tokyo, Japan); and photographs of random fields were taken.

**2.4 MTT assay for estimation of cell viability**

To evaluate cell viability by metabolic activity, the MTT assay was performed using a 96-well clustered plate (Iwaki), as described in Section 2.3. After incubation at 37°C for 72 hours, 100 μl of 10% WST-1 solution (Roche, Cell Proliferation Reagent WST-1) in the medium was added to each well, and the cells were incubated for a further 1 hour at 37°C. Finally, the absorbance at 450/630 nm was measured with a micro-ELISA reader (Multiscan...
FC; Thermo Scientific). Background activity, containing culture medium only without cells, was also measured in each plate for subtraction of background absorbance. Cell viability was estimated using the following formula:

\[
\% \text{ Viability} = \frac{(\text{Experimental activity}) - (\text{Background activity})}{(\text{Control activity without drug}) - (\text{Background activity})} \times 100
\]

For assessment of the cytotoxicity of 5-FU and CDDP toward scirrhous cancer cell lines, the 50% inhibitory concentration (IC\(_{50}\)) values and the 10% inhibitory concentration (IC\(_{10}\)) values were determined.

For determination of IC\(_{50}\) and IC\(_{10}\), different concentrations of the drug were plotted as log 10 of [concentrations of the drug] on the x-axis against % response from maximum on the y-axis. The curve falls from 100% (maximum response) to 0% (no response despite high concentration of drugs), enabling calculation of the concentration of a drug that produces a half-maximum effect.

\[
\text{IC}_{50} = 10^{\log_{10}(A/B) \times (50-C)/(D-C)+\log_{10}(B)}
\]

\[
\text{IC}_{10} = 10^{\log_{10}(A/B) \times (10-C)/(D-C)+\log_{10}(B)}
\]

A: Above concentration in the range of 50% or 10% inhibitory
B: Below concentration in the range of 50% or 10% inhibitory
C: Inhibitory ratio of B
D: Inhibitory ratio of A

2.5 Statistical Analysis

Results are shown as means for at least 2 experiments performed independently using separate cell preparations.
3. Results

3.1 Viability of scirrhous cancer cell lines treated with 5-FU or cisplatin

As shown in Figure 7, the four cell lines showed reduced cell viability with different profiles depending on the concentration of 5-FU (left graphs) and CDDP (right graphs) used. Among them, the HSC-39 cell line showed somewhat high sensitivity to 5-FU; whereas the HSC-58 and HSC-60 cell lines showed some viability even at 100 μM 5-FU. In response to CDDP, the HSC-39 cell line also showed high sensitivity, and the HSC-44PE cell line showed sensitivity similar to that of HSC-39. In contrast, the HSC-58 and HSC-60 cell lines were relatively resistant. None of the four cell lines survived at a CDDP concentration of 100 μM.

As shown in Table 3, the sensitivity of HSC-39 and HSC-44PE cells treated with 5-FU was reflected by $IC_{50} = 2.28 \mu M$ and $IC_{50} = 5.09 \mu M$, respectively. On the contrary, the $IC_{50}$ value of 5-FU for HSC-58 cells ($IC_{50} = 11.82 \mu M$) and HSC-60 cells ($IC_{50} = 11.12 \mu M$) were considerably higher, being approximately 2-5 times greater. It should be noted that $IC_{10}$, the value of inhibitory concentrations obtaining 10% of the control without drug, exceeded 100 μM for these cell lines, and it was more than seven times higher than that for the HSC-39 and HSC-44PE cell lines. These results suggest that the HSC-58 and HSC-60 cell lines would survive in the presence of 5-FU at 100 μM.

In contrast, the resistance of the HSC-39 and HSC-44PE cell lines to CDDP was $IC_{50} = 2.26 \mu M$ and $IC_{50} = 2.30 \mu M$, respectively. However, viability of both the HSC-58 ($IC_{50} = 12.21 \mu M$) and HSC-60 cells ($IC_{50} = 9.64 \mu M$) was 4-5 times greater than that of the HSC-39 and HSC-44PE cell lines. $IC_{10}$ values were 32.07 μM for HSC-58 cells and 66.51 μM for HSC-60 cells treated with CDDP.

Taken together, these findings show that the HSC-58 and HSC-60 cell lines were resistant to 5-FU and CDDP, the HSC-44PE cell line was moderately resistant, and the HSC-39 cell line is not resistant.
Table 3. Cytotoxicity of 5-FU or CDDP toward scirrhouos gastric cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>5-FU (μM)</th>
<th>CDDP (μM)</th>
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<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>IC&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
<tr>
<td>HSC-39</td>
<td>2.28</td>
<td>13.68</td>
</tr>
<tr>
<td>HSC-44PE</td>
<td>5.09</td>
<td>13.22</td>
</tr>
<tr>
<td>HSC-58</td>
<td>11.82</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>HSC-60</td>
<td>11.12</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>
**Figure 7. Viability of scirrhous cancer cell lines treated with 5-FU or CDDP**

Cell viability was examined by performing the MTT assay. For determination of cellular metabolic activity, the cells were treated with 5-FU or CDDP at various concentrations at 37°C for 72 hours, followed by the addition of 10% WST-1, as described in the text. Results are shown as metabolic activity relative to the control without chemotherapeutic agents and are given as the means of two independent experiments (n=2 for HSC-39 cells and HSC-58 cells; n=3 for HSC-44PE cells and HSC-60 cells).

**3.2 Morphological changes in scirrhous cancer cell lines treated with 5-FU or CDDP**

As HSC-58 cell lines showed high resistance to both 5-FU and CDDP (Figure 7, Table 3), the morphology of this cell line was observed at 72 hours (Figure 8). 5-FU reduced the number of intact cells (Figure 8b, c, d). However, some intact cells were present even after treatment with 5-FU at 100 μM (Figure 8d, arrow). There was a high association with the results of the MTT assay (Figure 7, left graphs), which showed that the HSC-58 cell line was highly resistant to 5-FU. In addition, HSC-58 cells were observed to remain intact at various concentrations of CDDP up to 10 μM (Figure 8, f and g). In contrast, the addition of 100 μM CDDP induced highly conspicuous changes in the cells (Figure 8h), such as agglutination.

The three other cell lines showed a dose-dependent reduction in cell number as well as an increase in cell damage after treatment with 5-FU or CDDP (data not shown). These results suggest that these scirrhous gastric cancer cell lines showed morphological changes, including cell damage and aggregation, on treatment with 5-FU or CDDP, but that these changes varied in mode and extent.
Figure 8. Morphological changes in HSC-58 cells treated with 5-FU or CDDP

Morphology of HSC-58 cells was observed under a phase-contrast microscope after incubation with 5-FU (a-d) or CDDP (e-h) at 37°C for 72 h. Representative cells are shown in random fields of non-treated cells (a, e) and those treated with 1 μM (b, f), 10 μM (c, g) or 100 μM (d, h) 5-FU or CDDP. Original magnification ×200.
4. Discussion

In this study, human scirrhous gastric cancer cell lines were shown to have various degrees of resistance to 5-FU and CDDP, with HSC-58 and HSC-60 cell lines being the most resistant, followed by HSC-44PE cells with moderate resistance, and HSC-39 cells with no resistance to 5-FU or CDDP, as judged by the results of MTT assays. In particular, the HSC-58 and HSC-60 cell lines showed the growth of intact cells even after treatment with 5-FU at 100 μM. These results also suggest that some human scirrhous gastric cancer cell lines have natural resistance mechanisms.

Multidrug resistance (MDR) is a serious problem in chemotherapy, resulting in poor outcomes to cancer treatment (52). The MDR phenotype is often related to overexpression of drug-efflux pumps in cancer cells. P-glycoprotein (P-gp) encoded by the MDR1 gene is one of the best characterized drug-efflux pumps (53). Overexpression of P-gp on the surface of tumor cells allows the removal of cytotoxic drugs from the cells in an energy-dependent manner, thereby reducing drug accumulation and increasing multidrug resistance (54). However, there is evidence that P-gp-associated MDR cells develop other pathways in their instigation of chemotherapy resistance to P-gp-unrelated drugs such as 5-FU and CDDP (55). Drugs that have been shown to be not transportable by P-gp can induce minor improvements in P-gp overexpression. CDDP enters a cell and its chloride ligands are replaced by water, forming aquated that reacts with nucleophilic sites in cellular macromolecules. The presence of CDDP adducts in DNA is thought to trigger cell-cycle arrest and apoptosis. Knowledge of the mechanism of the action of CDDP has improved our understanding of drug resistance. Resistance to CDDP can arise as a result of decreased intracellular concentration due to decreased drug uptake, increased reflux or increased inactivation by sulfhydryl molecules such as glutathione (56).

Several mechanisms of resistance to 5-FU have been attributed to thymidylate synthase (TS; 57). Given that TS is the target of 5-FU and several folate-based TS inhibitors, TS expression is likely related to sensitivity to 5-FU (58) and the antifolates. My colleagues and I are currently
investigating the association of TS enzyme levels (TS activity, immunohistochemistry or mRNA), as well as those of DPD and other markers with drug resistance by using these scirrhous gastric cancer cell lines. Our hope is that these factors might be characterized as resistance markers in select patients and can therefore be used in personalized medicine.

In this chapter, I examined the resistance of human scirrhous gastric cancer cell lines to 5-FU and CDDP \textit{in vitro}. There were differences between the cell lines used, with two (HSC-58, HSC-60) showing high resistance to both 5-FU and CDDP. These results imply that the application of chemotherapy to individual scirrhous gastric cancer patients could be evaluated by personalized medicine via the analysis of genes of specimens from patients with drug-resistant marker molecules. These human scirrhous gastric cancer cell lines will provide useful information for the establishment of a panel of drug-resistance markers.

In the future, 5-FU and CDDP might be used simultaneously to evaluate drug resistance when used together. Furthermore, investigation is needed into the resistance of these cell lines not only to the combined use of 5-FU and CDDP but also to Paclitaxel, which is commonly used in second-line treatment.
Chapter IV.

Isolation and characterization of 5-FU and cisplatin-resistant mutants from a human scirrhous gastric cancer cell line, HSC-39

1. Introduction

Patients with gastric cancer usually have a good initial response to 5-FU-based or CDDP-based chemotherapy, but later they suffer a relapse due to the development of 5-FU and CDDP-resistance, either acquired or intrinsic, which reduces the clinical effectiveness of these drugs (59, 60). The phenomenon of acquired tumor resistance to chemotherapeutic agents has been recognized for decades (61). It is known that several cell membrane transporter proteins are responsible for this resistance. A number of cell lines have been isolated from human carcinomas with different levels of resistance to anticancer agents (62).

In this chapter, to develop new therapeutic approaches based on characteristic biologic features of cancer cells, I examined components of the mechanisms underlying the cytotoxicity of anticancer drugs by isolating 5-FU- and CDDP-resistant mutants from a human scirrhous cancer cell line.

Chapter III described how human scirrhous gastric cancer cell lines showed various degrees of resistance to 5-FU or CDDP. Because the HSC-39 cell line showed higher sensitivity than the other cell lines, it seemed rational to consider this cell line as a useful parental cell line for the generation of drug-resistant mutants among the four human scirrhous gastric cancer cell lines (HSC-39, HSC-44PE, HSC-58, and HSC-60 cells) used in my thesis. To reveal intrinsic resistance to 5-FU and CDDP and the development of high levels of resistance to these drugs, I established 5-FU- and CDDP-resistant mutant cell lines in vitro.

2. Materials and methods
2.1 Materials

5-FU was obtained from KYOWA KIRIN (Tokyo, Japan); and cisplatin (CDDP), from Nichiiko (Toyama, Japan). WST-1 solution was obtained from Roche (Cell Proliferation Reagent WST-1). Ethyl methanesulfonate (EMS) was obtained from Sigma. Methyl cellulose came from R&D Systems.

2.2 Cell culture

The HSC-39 cell line and the methods of cell culture were described in Chapter III. Before generating drug-resistant mutant cell lines, I established HSC-39 clone #3 (Cl#3), a subline of HSC-39 cells, which was selected as having stable growth and the highest drug-sensitivity among HSC-39 sublines, and used it as the parental cell line to generate drug-resistant mutants. Resulting clones were passaged routinely at the split ratio of 1:10. The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μg/ml streptomycin sulfate, and 50 U/ ml penicillin G sodium (Nacalai Tesque, Kyoto).

Cl#3 cells were seeded at 1×10^6 cells/ ml onto T-25 tissue culture flasks (Falcon #353108, Corning) in medium containing 10% FCS and incubated at 37°C in 5% CO2 - 95% humidified air for 1 hour.

This line was treated with ethyl methanesulfate (EMS) at a final concentration of 0.3 nM, and then the cells were incubated at 37°C for 2 days. After incubation, the cells were treated with 10 μM 5-FU or CDDP and then incubated at 37°C for 2 days. After incubation, the cells were washed with medium and incubated without 5-FU or CDDP at 37°C for 2 days. Then the resistant cells were applied to a methyl cellulose (R&D) assay for colony development. These colonies were then subjected to a second selection with CDDP or 5-FU, after which several clones were isolated and characterized by testing their drug-resistance.
2.3 Morphologic observation

Clonal sublines were seeded at $5 \times 10^4$ cells/ml onto a 96-well cluster plate (IWAKI #3860-096, Asahi Glass) and incubated at 37°C for 1 hour. The cells were then incubated with 5-FU or CDDP at a final concentration of 30 μM at 37°C for 5 days. Then, cells were observed under a phase-contrast microscope (Diamat; Nikon, Tokyo, Japan), and photographs of in random fields were taken.

2.4 MTT assay for estimation of cell viability

To evaluate cell viability by metabolic activity, I performed an MTT assay using 96-well plates as described in section 2.3. 5-FU or CDDP at final concentrations of 0-100 μM was added to the cells, which were then incubated at 37°C for 3 days. After incubation, 100 μL of 10% WST-1 solution (Roche, Cell Proliferation Reagent WST-1) in the medium was added to each well, and the cells were incubated for a further 1 hour at 37°C. Finally, the absorbance at 450/620 nm was measured with a micro-ELISA reader (Multiscan FC; Thermo Scientific).

Background activity, containing no cells but culture medium only, was also measured in each plate for the subtraction of background absorbance. Cell viability was estimated using the following formula:

\[
\text{% Viability} = \frac{\{(\text{Experimental activity}) - (\text{Background activity})\}}{(\text{Control activity without drug}) - (\text{Background activity})} \times 100
\]

For assessing the cytotoxicity toward cell lines, 50% inhibitory concentration ($IC_{50}$) values and 10% inhibitory concentration ($IC_{10}$) values were determined by the same method as that
described in Chapter III (2.4).

2.5 Flow cytometry

For cell-cycle arrest analysis, HSC-39 cells and drug-resistant mutants were seeded at $1 \times 10^6$ cells/mL/dish (Corning) and then treated with the final concentrations of either 30 μM 5-FU or 10 μM CDDP for 2 days at 37°C. The cells were thereafter collected into a 5-ml tube (FALCON) by pipetting with a micropipette, washed once with PBS (-), and then incubated with propidium iodide (PI; Sigma) at room temperature for 15 min in the dark. The cells were next mixed well with FACS Buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide). Finally, the cells were filtered through a 200-mesh nylon cloth and analyzed by using a cell sorter (FACSAria III; BD Biosciences, San José, CA, USA). Red-fluorescence emission from PI in the PerCP-Cy5.5 channel (BP695/40 filter) and data were processed with BD FACSDiva software (BD Biosciences).

2.6 Statistical Analysis

Results are shown as the means ± S.E.M. for 3 experiments performed independently by using separate cell preparations.

3. Results

3.1 Morphological changes in drug-resistant mutants treated with 5-FU or cisplatin

Morphology of HSC-39 cells and mutants was observed under a phase-contrast microscope after incubation of the cells with 30 μM 5-FU (A) or CDDP (B) at 37 °C for 5 days (Figure 10). Representative cells are shown in random fields of the cells (Wild type, WT; CDDP-R, CDDP-resistant mutant; and 5-FU-R, 5-FU-resistant mutant) left untreated or treated with 5-FU or CDDP. Original magnification ×200.
3.2 Drug-resistance of mutants treated with 5-FU or cisplatin

In the graphs and tables in Figure 11 are shown the drug resistance of wild-type and mutant cells based on IC50 and IC10 values. The IC50 values of the wild type (HSC-39 cells) and 5-FU-R cells were 0.23 μM and 0.75 μM, respectively (Figure 11A), the value for the 5-FU-R cells being 3.26-fold higher than that for the wild type. The IC50 values of the wild type and CDDP-R cells treated with CDDP were 3.41 μM and 14.8 μM, respectively (Figure 11B). The value for the CDDP-R was thus 4.34-fold higher than that for the wild type. Based on these IC50 and IC10 values, 5-FU- and CDDP-resistant mutant cell lines were isolated from human scirrhous gastric cancer cell line HSC-39.
Figure 11. Viability of drug-resistant mutants treated with 5-FU or CDDP

The cell viability was examined by performing the MTT assay. For determining cellular metabolic activity, the cells were treated with 5-FU or CDDP at various concentrations at 37°C for 3 days, followed by the addition of 10 % WST-1, as described in the text. The results are shown as the activities of WST-1 reduction to the control without drugs, and are the means of two independent experiments.

3.3 Changes in the cell cycle of the drug-resistant mutants treated with 5-FU or CDDP

5-FU induced sub-G1/G1 cell-cycle arrest in the wild type (Figure 12 A). In contrast, the 5-FU-R mutant showed an interesting result in that the number for this sub-G1 population was reduced (Figure 12 B), probably through the escape from apoptosis caused by 5-FU. On the other hand, CDDP induced G2/M cell-cycle arrest in the wild-type cells (Figure 12C), but CDDP-R cells seemed to have escaped from this G2/M phase arrest (Figure 12D). These results obtained for 5-FU and CDDP treatment suggest that the cell cycle plays an important role in understanding the mechanism of drug-resistance.

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<th>Cell lines</th>
<th>5-FU (μM)</th>
<th>CDDP (μM)</th>
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<tr>
<td></td>
<td>IC(_{50})</td>
<td>IC(_{10})</td>
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<td>5-FU-R</td>
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Cell lines

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<td>IC(_{10})</td>
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<td>0.23</td>
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<tr>
<td>5-FU-R</td>
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### Changes in cell cycles of the mutants by CDDP or 5-FU

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<td>S</td>
<td>G2/M</td>
<td>Sub G1</td>
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<td>21.12</td>
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<tr>
<td>+ 5-FU</td>
<td>7.52</td>
<td>68.47</td>
<td>16.98</td>
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<td>Δ %</td>
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<td>+ 11.74</td>
<td>- 4.19</td>
<td>- 14.21</td>
<td>Δ %</td>
<td>+ 2.2</td>
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### Cell cycle (%)

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<th>G2/M</th>
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<th>S</th>
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<tr>
<td>Δ %</td>
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<td>- 38.28</td>
<td>- 0.52</td>
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<td>Δ %</td>
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### Cell cycle (%)

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<th>S</th>
<th>G2/M</th>
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<th>S</th>
<th>G2/M</th>
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<td>58.80</td>
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<td>19.28</td>
</tr>
<tr>
<td>Δ %</td>
<td>+ 2.33</td>
<td>- 20.18</td>
<td>+ 1.54</td>
<td>+ 16.11</td>
<td>Δ %</td>
<td>+ 2.33</td>
<td>- 20.18</td>
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</table>
Figure 12. Changes in the cell cycle of the wild type and drug-resistant mutant cells treated with 5-FU or CDDP

The wild type (HSC-39) and the drug-resistant mutants were incubated without or with 30 μM 5-FU (A and B) or with 10 μM CDDP (C and D) at 37°C for 2 days. The cells were then subjected to flow cytometric analysis with PI staining, as described in the text. The abscissa indicates the population of cells with PI, and the ordinate shows the intensity of the fluorescence of PI.

4. Discussion

In this chapter, I reported that 5-FU- and CDDP-resistant mutant cell lines were isolated from human scirrhous gastric cancer cell line HSC-39 and that they showed about three and four-fold higher resistance than did the wild type. The cell-cycle analysis of CDDP-R cells revealed that these mutants seemed to have escaped from G2/M arrest, whereas the analysis of 5-FU-R mutants revealed a cycle pattern similar to that of the wild type but with a reduced number of cells in the sub-G1 population, probably due to escape from apoptotic cell death caused by 5-FU. These results suggest that the combination treatment with the 5-FU analog with CDDP or other chemotherapeutic agents have possibilities to demonstrate retrospective study in Chapter II, because 5-FU and CDDP act at different phases of the cell cycle.

It was reported that CDDP exerts its cytotoxic effect through blockage of the cell cycle and is partly regulated by the p53 signaling pathway (63). The IC$_{50}$ value of the parental cell line, HSC-39 Cl #3, was 0.23 µM; and that of the original HSC-39 cells was 2.28 µM. Therefore, the IC$_{50}$ of this subline, HSC-39 Cl#3, was about one tenth of that of the original HSC-39, which reduced value might have been because the original HSC-39 cell line was a mixture of sublines of varied drug-resistances. Although the IC$_{50}$ values of these cell lines could not be compared directly, 5-FU-R mutant cells derived from HSC-39 Cl#3 acquired 3.26-fold higher resistance to 5-FU than the parental cell line.

It is known that several cell membrane transporter proteins are responsible for the resistance against many commonly used chemotherapeutic agents, and that they do this by affecting the
disposition of these drugs by the tumor cells. However, resistance to another group of chemotherapeutic agents, i.e. platinum-based anticancer agents, is caused by different mechanisms (61). Currently, my colleagues and I are investigating the expression of the genes responsible for the acquisition of drug resistance (data not shown), which should be closely linked to the actual mechanisms of drug resistance. To improve chemotherapy for scirrhous gastric cancer, studies on clinical samples as well as in vitro studies with suitable drug-resistant cell lines are necessary, as described in this chapter.
Chapter V.

**Effects of 5-fluorouracil, adriamycin, and irinotecan on HSC-39 cells**

1. **Introduction**

Scirrhous gastric cancer is unique and tends to spread over the peritoneum with rapid growth and early metastasis (5, 64-66). The prognosis of patients with scirrhous gastric carcinoma still remains very poor. The 5-year survival rate is low, although chemotherapy for other gastric cancers has been improved, with good results being obtained in Japan (18, 28, 67-69). In order to develop new therapeutic approaches based on the characteristic biological features of scirrhous cancer cells, we investigated the mechanisms underlying cytotoxicity of various anticancer drugs toward scirrhous cancer cell line HSC-39 *in vitro*.

HSC-39 cells already stated earlier are round, freely floating cells that tend to aggregate loosely in tissue culture medium; and they have characteristics similar to those of the original ascitic tumor cell phenotypes of a signet ring cell carcinoma (70). HSC-39 cells have a mutation in exon7 of their *p53* gene, which provides a possible selective advantage for tumor cell proliferation (71). Because it seems important to use gastric cancer cell lines with behavior similar to that of human scirrhous gastric carcinoma, including histologic characteristics and metastatic ability *in vitro*, I had chosen the HSC-39 cell line to examine its biological properties and also to develop new therapies for scirrhous cancer. Since these cells were derived from a scirrhous gastric carcinoma patient, they possess the appropriate phenotypes, including histological characteristics and metastatic ability *in vitro* on which to test new therapies for scirrhous gastric cancer.

The chemotherapeutic agents 5-fluorouracil (5-FU), adriamycin (ADR), and irinotecan (CPT-11), as well as reactive oxygen species (ROS), have all been reported to be cytotoxic
towards tumor cells. Among anticancer drugs for gastric cancer, S-1, a 5-FU analog, has recently become the standard first-line chemotherapeutic drug in Japan, whereas several other new drugs, including the topoisomerase I inhibitors CPT-11 and ADR, are less frequently used (72, 73). These drugs provide an improved prognosis for advanced gastric cancer (28, 73). ROS, including non-radical hydrogen peroxide (H$_2$O$_2$), organic hydroperoxide (ROOH), and hypochlorous acid (HClO), are generated from inflammatory immune cells such as activated macrophages and neutrophils, which accumulate at the sites of inflammation. ROS are important for the induction of apoptosis not only in inflammatory cells, but also in neighboring cells (11, 12). However, it is still largely unclear how anticancer drugs and ROS induce apoptosis in scirrhous gastric cancer cells. In the present study, I aimed to develop new therapeutic approaches based on the characteristic biological features of these cells. The effects of 5-fluorouracil, adriamycin, and irinotecan on HSC-39 cells were determined in order to investigate the mechanisms underlying the cytotoxicity of 5-FU, ADR, CPT-11 and ROS towards HSC-39 in vitro.

2. Materials and methods

2.1 Materials

5-FU, Adriamycin (ADR), and irinotecan (CPT-11) were obtained from Sigma; and cisplatin (CDDP) was purchased from Nichiiko (Toyama, Japan). Peroxynitrite and NOC18 came from DOJINDO (Kumamoto, Japan). H$_2$O$_2$ and HClO were purchased from Wako Pure Chemicals (Osaka, Japan). Primary antibodies, including rabbit antibodies against caspase-3, cleaved caspase-3, caspase-7, and cleaved caspase-7, and second antibody, anti-rabbit immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (HRP), were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.).

2.2 Cell culture

HSC-39 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM,
GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 μg/ml streptomycin sulfate, and 50 U/ml penicillin G sodium (Nacalai Tesque, Kyoto). Media and sera were obtained from GIBCO (Grand Island, NY). The cells were seeded at low density in 100-mm diameter dishes (IWAKI, Tokyo) or T-25 tissue culture flasks (Falcon) in standard medium containing 10 % FCS and incubated at 37°C in 5% CO₂ - 95 % humidified air.

2.3 Morphologic observation

Cells were observed under a phase-contrast microscope (Diamat; Nikon, Tokyo, Japan), and photographs of random fields were taken.

2.4 LDH assay for estimation of cellular cytotoxicity

The amount of lactate dehydrogenase (LDH) released into the culture medium from the injured cells was measured for estimation of cell damage. Briefly, HSC-39 cells were seeded at 4×10⁵ cells/ml into wells of a 48-well multiplate (Coaster) and treated with 5-FU, ADR or CPT-11 for 48 hours or incubated with peroxynitrite, NOC 18, H₂O₂ or HOCl for 18 hours. The resultant supernatants were collected and assayed for LDH by using an LDH-Cytotoxic Test Wako (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer’s protocol. Results were expressed as the percentage release, according to the formula below, where the total activity was obtained by treatment of the same number of the cells with 0.1 % Triton X-100 (SIGMA). The culture supernatant was collected at time 0 of incubation to determine the background release of LDH.

\[
\text{\% Release} = \frac{(\text{Experimental release}) - (0 \text{ time release})}{(\text{Total release}) - (0 \text{ time release})} \times 100
\]

2.5 MTT assay for estimation of cell viability

For evaluation of viable cell number and cellular metabolic activity, the MTT assay was performed by using a Cell Titer 96 kit (Promega). Briefly, 2×10³ cells/ well were seeded onto
wells of 96-well cluster plates (0.1 ml/well) and incubated at 37°C for 72 hours in the presence or absence of various concentrations of anticancer drugs. Then, 100 μl of WST-1 solution (Roche, Cell Proliferation Reagent WST-1) was added to each well, and the cells were incubated further at 37°C for 1 hour. The absorbance at 450/630 nm was measured with a micro-ELISA reader (Bio-Rad, Hercules, CA). Background activity, containing no cells but culture medium only, was also measured in each well for subtraction of the background. Cell viability was estimated by the following formula:

\[
\% \text{ Viability} = \frac{\text{(Experimental activity)} - \text{(Background activity)}}{\text{(Control activity without drug)} - \text{(Background activity)}} \times 100
\]

2.6 Flow cytometry

For flow cytometric analysis, HSC-39 cells were seeded at 1×10^6 cells/ml/dish (Corning Inc., Corning, NY, USA), and treated with 5-FU, ADR or CPT-11 for 20-24 hours, or with peroxynititne, NOC-18, H_2O_2 or HClO for 18 hours at 37°C. The cells were collected into a 5-ml tube (Corning Falcon) and washed once with PBS (-) before the reagents of an apoptosis kit, Annexin V-FITC kit (MEBCYTO®; MBL, Nagoya, Japan), were added to detect early-stage apoptotic cells. Cells were suspended in Annexin V-FITC and propidium iodide (PI), and incubated at room temperature for 15 min in the dark. The cells were then mixed well with fluorescence-activated cell sorting (FACS) buffer (PBS containing 0.1% bovine serum albumin and 0.1% sodium azide), filtered through a 200-mesh nylon cloth, and analyzed by a cell sorter (FACSAria III; BD Biosciences, San Jose, CA, USA). Signals from Annexin V-FITC were detected by the FITC channel (BP530/30 filter); and those from PI, by the PerCP-Cy5.5 channel (BP695/40 filter). Data were processed with BD FACSDiva software (BD Biosciences).

2.7 SDS-PAGE and Western blot analysis

HSC-39 cells were seeded at 1 × 10^6 cells/60-mm/dish (Iwaki #3000-035) and treated either with CPT-11 and incubated for 4 hours or with 5-FU and ADR for 24 hours at 37°C. The cells
were then chilled on ice and washed twice with PBS by centrifugation at 4°C and at a speed of 200 × g for 10 min. The final cell pellets were suspended in 100 μl of lysis buffer comprising 10 mM EDTA (pH 8.0), 0.5% Triton X-100, and 10 mM Tris-HCl buffer (pH 7.4). After having been stood on ice for 10 min, the cells were centrifuged at 11,000 × g for 5 min at 4°C, and the resultant supernatants were used as cell extracts.

SDS-PAGE/Western blotting was performed as described previously (74). In brief, 15-μg aliquots of the cell extracts were electrophoresed through a 5–20% gradient polyacrylamide gel (ATTO, Tokyo, Japan), and the proteins were subsequently transferred to Immobilon polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, U.S.A.) for Western blotting. The membranes were blocked with 30 mg/mL milk casein (Megmilk Snow Brand, Tokyo, Japan) in a rinse buffer comprising 0.1% Triton X-100, 0.1 mM EDTA, and 0.8% NaCl in 10 mM Tris–HCl buffer, pH7.4. After blockage, the blots were first reacted at 4 °C overnight with a primary antibody (Cell Signaling Technology, Danvers, MA, USA), anti-caspase-3 (1: 1000), anti-cleaved caspase-3 (1: 1000), anti-caspase-7 (1: 1000) or anti-cleaved caspase-7 (1: 1000), followed by reaction with a secondary antibody, anti-rabbit IgG antibody conjugated with HRP (1: 1000). Chemiluminescence was generated by using Pierce Western blotting Substrate (Thermo Fisher Scientific) and detected by using an LAS 3000 Mini Image Analyzer (FUJIFILM, Tokyo, Japan). The results were analyzed by using Image J software (Ver. 1.48V). Quantitative data were obtained by using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.8 Statistical Analysis

Results are shown as means for at least two experiments performed independently using separate cell preparations. Statistical analysis and estimation of the significance of differences between groups with comparable variance were performed by one-way or two-way ANOVA analysis and the Tukey-Kramer’s post hoc test. Statistical analysis was made by using EZR software.

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3. Results

3.1 Morphological changes in HSC-39 cells treated with chemotherapeutic reagents

The morphology of the HSC-39 cells was observed under a phase-contrast microscope after treatment of them with anticancer reagents at 37°C for 48 hours. The addition of 100 μM 5-FU (Figure 13 C) induced the most marked changes in the cells, such as agglutination and cell-membrane rupture; whereas treatment with a much lower dose, 10 μM 5-FU (Figure 13 B), resulted in no remarkable changes. When the cells were treated with 1 μM ADR (Figure 13 F) or 10 μM CPT-11 (Figure 13 H), the number of both intact and apoptotic cells were decreased. Additionally, the cells treated with 1 μM ADR (Figure 13 F) or 10 μM CPT-11 (Figure 13 H) were round, floating freely, and tended to be larger than the non-treated cells. Fragmentation of the cells was frequently observed after treatment with 100 μM CPT-11 (data not shown). These results suggest that treatment of HSC-39 cells with these anticancer reagents led to a decrease in the number of living cells with a concomitant increase in the number of either apoptotic cells with 5-FU treatment or aponecrotic cells when the cells were treated with ADR or CPT-11.
Figure 13. Morphological changes in HSC-39 cells effected by 5-FU, ADR or CPT-11

Cell damages were observed in HSC-39 cells under the phase-contrast microscope after incubation with 5-FU, ADR or CPT-11 at 37°C for 48 hours. (A) None, (B) 10 μM 5-FU, (C) 100 μM 5-FU, (D) 1000 μM 5-FU, (E) 0.1 μM ADR, (F) 1 μM ADR, (G) 1 μM CPT-11, and (H) 10 μM CPT-11. Original magnification, ×400.
3.2 Cytotoxic effects of chemotherapeutic agents on HSC-39 cells as revealed by changes in LDH release

Apoptotic stimuli induce necrosis of cells through depletion of cellular ATP. The type of cell death varies depending on the mechanisms and processes that lead to these responses in cells and tissues (75). LDH release from cells principally results from rupture of the cell membrane. LDH was significantly released from the cells after treatment with 5-FU at 10 μM or higher in a dose-dependent manner (Figure 14 A). In contrast, significant release of LDH was predominantly observed only after treatment with a high concentration of ADR (10 μM; Figure 14 B) or CPT-11 (100 μM; Figure 14 C).

Similarly, viability, as estimated by the MTT assay using WST-1 as a substrate, differed between the treatment groups. 5-FU at 1 μM attenuated WST-1 reduction, which was more pronounced and significant at higher doses (Figure 15 A). In contrast, ADR and CPT-11, which exerted similar effects on cell viability, only significantly attenuated WST-1 reduction at 10 μM and 100 μM, respectively (Figure 15 B and C). These results show that the effect of 5-FU treatment, i.e., cell damage and decreased cell viability, as measured by LDH release and MTT assay was different compared with that of ADR or CPT-11. Such data suggest that 5-FU may induce cell damage via a different mechanism than cell-membrane disruption for LDH release, indicating different functions in apoptotic cell death.
**Figure 14. LDH release from HSC-39 cells treated with various chemotherapeutic agents**

LDH release was examined as a marker of cell damage of HSC-39 cells after treatment with various concentrations of 5-FU (A), ADR (B) or CPT-11 (C). LDH release was quantitated as described in the text, and the results are the means ±S.E.M. for three independent experiments. (*P<0.05, **P<0.01, one-way ANOVA and the Tukey-Kramer’s post hoc test.)
Cell viability was examined by performing the MTT assay. For determination of cellular metabolic activity, the cells were treated with 5-FU (A), ADR (B) or CPT-11 (C) at various doses at 37°C for 72 hours, after which WST-1 substrate was added, as described in the text. The results are shown as the activities of WST-1 reduction relative to the control without any chemotherapeutic reagents.

3.3 Induction of apoptosis and/ or necrosis in HSC-39 cells

To examine apoptotic cell death, I examined, by flow cytometry, the cell-surface binding of Annexin V, a phosphatidylinerine (PS)-binding protein, and staining of the cells with PI. Translocation of PS to the external cell surface is not unique to apoptosis, as it also occurs
during cell necrosis. The difference between apoptosis and necrosis is in the integrity of the cell membrane. That is, during the initial stages of apoptosis the cell membrane remains intact; whereas for necrosis, the cell membrane becomes leaky, allowing access of PI to the nucleus, in which the nucleic acids there are stained. The Annexin V assay allows for the detection of the early phase of apoptosis, before the loss of cell-membrane integrity, and permits assessment of apoptotic death. As shown in Figure 16 A, the control cells no fluorescein fluorescence. In contrast, cells treated with 10 µM 5-FU for 20-24 hours displayed significant binding of Annexin V-fluorescein to the membrane surface (Figure 16 B), indicating apoptosis of the cells. To distinguish between apoptotic and potentially necrotic or lysed cells that may also have exposed their PS due to loss of membrane integrity, I concomitantly monitored the Annexin V (+) and PI (+) cells, which indicate aponecrosis of HSC-39 cells (Figure 16 B). Annexin V (+) and PI (+) cells were also observed after treatment with 1 µM ADR or 10 µM CPT-11 (Figure 17), suggesting that these drugs induced both apoptosis and aponecrosis.
Figure 16. **Induction of apoptosis and/or necrosis in HSC-39 cells treated with 5-FU**

HSC-39 cells were incubated without (A) or with 10 μM 5-FU (B) at 37°C for 48 hours. Then the cells were subjected to flow cytometric analysis with annexin V/PI, as described in the text. The abscissa indicates the staining intensity of the cells positive for annexin V; and the ordinate, that for PI. As shown below the control graph, area “c,” both negative, corresponds to intact cells; “d,” to apoptosis; “a,” to necrosis; and “b,” to apo-necrosis.
Figure 17. Induction of apoptosis and/or necrosis in HSC-39 cells treated with 5-FU, ADR or CPT-11

The cells were treated with various concentrations of 5-FU, ADR or CPT-11 at 37°C for 20-24 hours. Then they were examined after staining with Annexin V/PI, as described in the legend to Figure 16. On the ordinate, the population (%) of the cells are shown as Intact (Annexin V (-)/PI (-)), Annexin V (+)/PI (-), Annexin V (-)/PI (+), and Annexin V (+)/PI (+). The results are the means ±S.E.M. for three independent experiments. For comparison of more than two groups with comparable variances, one-way ANOVA and the Tukey-Kramer’s post hoc test were carried out. Statistical analysis was performed by using EZR software.

3.4 Caspase-3- and caspase-7-mediated apoptosis in HSC-39 cells

Caspase family members play important roles in the progression of apoptosis in various cells. Among them, caspases-3 and -7 are the effector caspases activated by apical caspase and cleavage of cellular death substrates. Therefore, I examined which caspase was involved in the induction of cytotoxicity. The level of cleaved caspase-3, its activated form, was much higher than that of cleaved caspase-7 after treatment with 10-100 μM 5-FU for 48 hours.
(Figure 18) as compared with their untreated control. Similar results were obtained after treatment with 1-10 µM ADR or CPT-11. These results suggest that both caspase-3 and caspase-7 were involved in the progression of chemotherapeutic drug-induced apoptosis in HSC-39 cells.

**Figure 18. Activation of caspase-3 and caspase-7 in HSC-39 cells treated with 5-FU, ADR or CPT-11**

The cells were treated with various concentrations of 5-FU, ADR or CPT-11 at 37°C for 48 hours. Cell extracts were prepared and then subjected to SDS-PAGE/Western blotting. The activation of caspases -3 and -7 was judged by their cleavage, as described in the text. The relative ratio was calculated from the densities. Quantitative data were obtained by using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

3.5 Cytotoxic effects and induction of apoptosis of HSC-39 cells by ROS

ROS have been shown to induce apoptosis in many different cell systems (76). In the present study, I examined the cytotoxic effects of ROS on HSC-39 cells. These cells released a significant amount of LDH upon treatment with 0.5 mM H₂O₂, or higher, with > 60 %
release significantly occurring at 1 mM and greater (Figure 19 A). In contrast, HSC-39 cells showed less sensitivity to HClO (Figure 19 B) or NOC 18 (Figure 19 D). Little to no LDH release was induced by peroxynitrate (Figure 19 C).

Next I examined the induction of apoptosis and/or necrosis of HSC-39 cells by performing Annexin V and PI staining and flow cytometric analysis. Treatment with 0.1 mM H₂O₂ induced Anexin V (+) and PI (-) cells, indicating early-stage apoptosis, whereas treatment with 0.2-0.5 mM H₂O₂ or 2-5 mM NOC18 dose dependently induced Annexin V (+) and PI (+) cells, indicating necrosis or apo-necrosis. HSC-39 cells showed high sensitivity to H₂O₂ and NOC18, significantly different from the controls. At the low doses, HClO had no effect on the cell membrane; but at high doses (5 mM) it induced Annexin V (+) and PI (-) cells and Annexin V (+) and PI (+) cells, indicating the induction of apoptosis and apo-necrosis, respectively (Figure 19 A and B). Similarly, the cells were also induced after treatment with peroxynitrate (Figure 19 C). In addition, the pattern of cytotoxicity appeared to change over time in all treatment groups, suggesting that induction of apoptosis occurred first, followed by necrosis. However, treatment with H₂O₂ or HClO did not result in typical apoptosis (data not shown).

These results suggest that HSC-39 cells showed high dose-dependent sensitivity to H₂O₂ and NOC18, but low sensitivity to HClO and peroxynitrate, suggesting that the cell damage may be linked to the degree of membrane permeability induced by the various ROS.
Figure 19. Cytotoxic effects of ROS on HSC-39 cells monitored by LDH release

The cells were incubated at 37°C for 18 hours with various concentrations of ROS, including \( \text{H}_2\text{O}_2 \) (A), HClO (B), peroxynitrite (C), and NOC18 (D). The cell death was quantitated by performing the LDH release assay described in the text. The results are the means ± S.E.M. for three independent experiments. (*P<0.05, **P<0.01, one-way ANOVA and the Tukey-Kramer’s post hoc test.)
a: vs. 0 - 0.2 mM H$_2$O$_2$ (p < 0.01), b: vs. 0 - 2.0 mM HClO (p < 0.01)
c: vs. 0 - 2.0 mM Peroxynitrite (p < 0.01),
d: vs. 0 - 0.2 mM NOC18 (p < 0.01), vs. 0.5 mM NOC18 (p < 0.05)
e: vs. 0 - 2.0 mM NOC18 (p < 0.01), f: vs. 0.2 mM HClO (p < 0.01)
g: vs. 0.2 mM Peroxynitrite (p < 0.01), h: vs. 0.2 mM NOC18 (p < 0.01)
i: vs. 0.5 mM HClO (p < 0.01), j: vs. 0.5 mM Peroxynitrite (p < 0.01)
k: vs. 0.5 mM NOC18 (p < 0.01), l: vs. 2 mM HClO (p < 0.01)
m: vs. 2 mM Peroxynitrite (p < 0.05), n: vs. 5 mM HClO (p < 0.01)
o: vs. 5 mM Peroxynitrite (p < 0.01)
**Figure 20. Induction of apoptosis and/or necrosis of HSC-39 cells by ROS**

The cells were incubated with various concentrations of \( \text{H}_2\text{O}_2 \), HClO, peroxynitrite or NOC18 at 37 °C for 18 hours. Then the cells were harvested, washed, and stained for flow cytometric analysis with annexin V/PI. The results are shown as in Figure 17, with the population (%) of the cells as Intact (Annexin V (-)/PI (-)), Annexin V (+)/PI (-), Annexin V (-)/PI (+) or Annexin V (+)/PI (+). The results are the means ±S.E.M. for three independent experiments. (two-way ANOVA and the Tukey-Kramer’s *post hoc* test.)

**4. Discussion**

In this chapter, I examined the mechanisms underlying the cytotoxicity of anticancer drugs and reactive oxygen species (ROS) toward a human scirrhous cancer cell line, HSC-39, in vitro. I demonstrated that 5-FU induced apoptosis and ADR and CPT-11 induced necrosis and/or apo-necrosis of the HSC-39 cells. 5-FU reduced cell viability and caused little LDH release at low doses, whereas ADR and CPT-11 lowered cell viability and induced LDH release at high doses. In phase II trials, the 5-FU analog S-1 showed a 33% response rate against scirrhous gastric cancer. Due to the reported promising effects of S-1 for neoadjuvant chemotherapy against scirrhous gastric cancer in a pilot study, a new phase II trial was planned to determine the survival benefit of S-1 treatment.

Apoptosis is a crucial mechanism for many different circumstances. The apoptotic process is characterized by distinct morphological features. In HSC-39 cells, typical structural changes and related alterations in cell functions of the apoptotic pathway were observed, including cell rupture, translocation of PS to the outer layer of the plasma membrane, and altered mitochondrial metabolic activity.

Several chemotherapeutic drugs have been evaluated for their antitumor effect. Compared with the best supportive care, the survival benefit of 5-FU has been reported based on
chemotherapy for metastatic gastric cancer (77). In the present study, 5-FU, ADR, and CPT-11 dose dependently induced apoptosis and/or apo-necrosis in HSC-39 cells, as assessed by flow cytometric analysis (Figure 16 and 17). 5-FU effectively reduced cell viability even at low doses, although little LDH release was observed; whereas ADR and CPT-11 inhibited WST-1 reduction only at high doses, where LDH release occurred (Figure 14 A and Figure 15 A). A previous study indicated that the administration of 5-FU results in an increase in the S-phase fraction in human gastric carcinoma, which is coincident with the appearance of apoptosis-positive cells (78). Moreover, TS activity is immediately and markedly suppressed. These findings suggest that induction of apoptosis and inhibition of DNA synthesis, both induced by 5-FU, may be closely associated with its antitumor effect. At a low dose, 5-FU inhibits energy metabolism, resulting in decreased ATP levels to impair membrane barrier function, rather than inducing direct damage to cells (79). In contrast, ADR induces an increase in c-jun and ATF3 mRNA levels in the mitogen-activated protein kinase (MAPK) pathway, followed by apoptosis (80). This finding may explain why ADR predominantly induced apoptosis and LDH release in HSC-39 cells at the high dose. The mechanism underlying the similar actions of CPT-11 remains unclear.

We also demonstrated that the progression of apoptosis upon treatment with 5-FU, ADR or CPT-11 was accompanied by cleavage of not only caspase-3 but also caspase-7 (Figure 18). Caspase-3 normally exists in the cytosol fraction as an inactive precursor that becomes activated through cleavage in apoptotic cells (81). Caspase-7, but not caspase-3, undergoes proteolytic activation during lovastatin-induced apoptosis, an effect prevented by mevalonate, and was identified as a possible mediator of lovastatin-induced apoptosis (82). The activation of both caspase-3 and caspase-7 during the apoptosis of HSC-39 cells (Figure 18), as demonstrated in the present study, may indicate a new pathway in the apoptotic cascade in scirrhus gastric cancer cells.

HSC-39 cells also showed high sensitivity to H2O2 (Figure 19 A), as indicated by LDH
release and also by Annexin V and PI staining. $H_2O_2$ has strong intracellular cytotoxic effects due to its high membrane permeability. Furthermore, $H_2O_2$ can induce apoptosis in neutrophils; and this can be prevented by catalase, an enzyme that also prevents spontaneous neutrophil apoptosis. This finding suggests that $H_2O_2$ may be an important triggering mechanism responsible for the short life-span of mature neutrophils. Caspase-3, but not other caspases, is required for commitment to ROS-induced apoptosis (83).

The susceptibility of HSC-39 cells to 5-FU and $H_2O_2$ suggests that there may be common mechanisms underlying the cytotoxicity of these reagents. Manganese superoxide dismutase (Mn-SOD) negatively regulates 5-FU-mediated apoptosis induction in squamous carcinoma cells (84), and 5-FU increases cellular accumulation of $H_2O_2$ in CT26 colon cancer cells (85). These studies suggest that 5-FU induces an increase in cellular $H_2O_2$ levels, which action may lead to decreased metabolic activity, as was indicated by the WST-1 assay (Figure 15A), and to increased apoptotic and subsequent necrotic changes in (Figure 17), as well as increased LDH release by (Figure 14 A) HSC-39 cells, as observed in the present study.

HSC-39 cells undergo apoptosis when treated with TGF-β under serum-free culture conditions, which process is mediated by activation an apoptosis signal transduction pathway (86, 87). Therefore, it may be of interest to examine the interaction between HSC-39 cells and TGF-β-producing cells such as activated fibroblasts or macrophages with respect to peritoneal metastasis. Furthermore, it may be beneficial to examine whether various cytokines act as transcriptional regulators in TGF-β-mediated apoptosis. My colleagues and I are now the process of investigating this point.

Cisplatin ($cis$-diamminedichloroplatinum) was developed by Rosenberg (88) in the 1960’s and was initially used in the treatment of head and neck, uterine, and bladder cancers (89). Cisplatin is one of the most important drugs for the treatment of gastric cancer, for it has demonstrated a high positive-response rate. Although combination treatment with 5-FU and cisplatin has demonstrated a significantly increased cancer-free survival compared with treatment with 5-FU alone, no significant differences have been observed in overall survival
between the two treatments (90). This lack of survival difference, however, may have resulted from chance in conducting the subgroup analysis and may have had limited influence on the interpretation of the primary conclusion of that study.

In conclusion, I demonstrated that 5-FU induced apoptosis of HSC-39 cells, reduced cell viability, and triggered little LDH release at low doses. In contrast, ADR and CPT-11 induced necrosis and/or aponecrosis of HSC-39 cells, inhibited cell viability, and induced LDH release at high doses. The present study provides important insights into the underlying mechanisms of apoptosis aside from the cytotoxicity toward scirrhous gastric cancer. Furthermore, consistent with the widening acceptance of combination chemotherapies for use in clinical practice, such as agents, cisplatin, irinotecan, and the taxanes, my findings suggest that 5-FU has potential efficacy and that use of 5-FU in combination with other chemotherapeutic agents that attack the membrane barrier may be a successful chemotherapy regimen for scirrhous gastric cancer.
Chapter VI.

Conclusions

In this thesis, I first described the results of a retrospective study of the outcome of treatment for patients with Borrmann type 4 advanced gastric cancer in Chapter II. The data revealed that the median overall survival (OS) of patients with locally advanced cancer was longer than that for those with metastatic advanced cancer (29.6 and 11.5 months, respectively). The presence or absence of peritoneal metastases did not affect survival (8.9 and 11.5 months). In the 23 patients who received chemotherapy, S-1 plus cisplatin was prescribed as first-line treatment in 16 cases (69.5%), but this treatment did not significantly improve OS compared with other chemotherapeutic regimens in these patients with Borrmann type 4 advanced gastric cancer. The results of this retrospective study indicate the need for new approaches, including combination chemotherapy, to improve the efficacy of treatment and OS in these patients.

Second, I found it necessary to study the susceptibility and resistance of scirrhous cancer to various chemotherapeutics. I therefore examined the resistance of human scirrhous gastric cancer cell lines to 5-FU and CDDP in vitro, based on the results of clinical treatments in which the most commonly used drugs were S-1 plus cisplatin. There were differences in the degree of drug resistance among the cell lines used, some of which (HSC-58, HSC-60) showed high resistance to both 5-FU and CDDP. These results imply that the application of chemotherapy to individual patients with scirrhous gastric cancer could be evaluated by personalized medicine using genetic analysis of specimens from these patients with regard to marker molecules of natural drug resistance.

Third, I isolated 5-FU- and CDDP-resistant mutants from the human scirrhous gastric cancer cell line HSC-39, which was shown to have higher sensitivity to these drugs than other scirrhous gastric cell lines (91). Somatic cell mutagenesis was used to isolate and establish drug-resistant mutant cell lines, and 5-FU-resistant mutant (5-FU-R) and CDDP-
resistant mutant (CDDP-R) cells were obtained (92). Cell-cycle analysis of CDDP-R cells revealed that this cell line appeared to have escaped from G2/M arrest, whereas that of 5-FU-R ones revealed a similar pattern of cell cycle but with a much smaller sub-G1 population, suggesting escape of the 5-FU-R cells from apoptotic cell death. These results suggest that the combination of a 5-FU analog with CDDP or other chemotherapeutic agents for the treatment of gastric scirrhou sheep cancer is a reasonable one.

Fourth, in Chapter V, I examined the mechanisms underlying the cytotoxicity of anticancer drugs and reactive oxygen species (ROS) toward HSC-39 cells in vitro in order to develop new therapeutic approaches based on the characteristic biologic features of these cancer cells. Anticancer drugs such as 5-FU, adriamycin (ADR), and CPT-11, as well as ROS, were shown to have important cytotoxic effects on these tumor cells. 5-FU effectively and dose-dependently induced apoptosis and necrosis in HSC-39 cells, whereas adriamycin and CPT-11 induced apoptosis, necrosis, and/or apo-necrosis. 5-FU effectively inhibited WST-1 reduction in the MTT viability assay, even at the low doses in which LDH release was not observed. In contrast, ADR and CPT-11 inhibited WST-1 reduction only at the high doses at which LDH release was induced. Further, HSC-39 cells showed high sensitivity to H2O2 and NOC18 but less sensitivity to other ROS, suggesting a link between cell damage and membrane permeability changes induced by H2O2 or NOC18, or by related oxygen radical species such as OH• or •O2. Given the role of ROS in the regulation of apoptosis induced by exposure to 5-FU, this mechanism might be useful for reducing the cancer cell number. These results again suggest that combination treatment with a fluoropyrimidine such as 5-FU along with other chemotherapeutics is a reasonable chemotherapy for scirrhou sheep gastric cancer.

Some randomized trials have failed to demonstrate the superiority of 5-FU-based combination treatment as compared with 5-FU monotherapy (33, 93, 94). One randomized controlled trial suggested that three commonly used combination regimens, 5-FU/adriamycin/MTX (FAMTX), 5-FU/cisplatin (FP), and etoposide/leucovorin/5-FU (ELF), showed only modest activity, with no significant differences in overall survival among these
regimens (95). Further, infusional 5-FU, in combination with cisplatin and epirubicin (ECF), showed significant superiority over FAMTX in terms of response rate, quality of life, and survival, indicating that ECF might represent a new standard treatment. With regard to median survival time in these trials, however, there was little substantial difference among the various regimens used. In addition, the SPIRITS trial showed that median survival was significantly longer for patients who had received a S-1 plus cisplatin combination than for those who had received S-1 alone (28). Therefore, in general, S-1 plus cisplatin as a standard treatment for advanced gastric cancer is reasonable.

However, such regimens can be tolerated only by those patients with adequate organ function. This limitation renders many patients with scirrhouous gastric cancer unsuitable, because of their poor performance status at initial diagnosis. Therefore, further investigation with effective but less toxic regimens are warranted in patients with scirrhouous gastric cancer. A combination of leucovorin-modulated weekly bolus 5-FU and weekly paclitaxel (FLTAX) was reported, along with its toxicity profile (96). Due to the efficacy of this regimen and tolerance to its toxicity, FLTAX holds promise for the treatment of scirrhouous gastric cancer. A multicenter phase II/III study of the FLTAX regimen is now underway in patients with diffuse peritoneal spread of gastric cancer. I think it is important to assess the roles of drugs from the viewpoint of how we can maximize the potential value of each drug or combination regimen in disease-specific clinical situations.

For patients with scirrhouous gastric cancer, it is difficult to diagnosis and to evaluate the efficacy of chemotherapy against peritoneal dissemination in clinical trials as well as in clinical practice, because disseminated tumor cells do not form a measurable mass but rather constitute diffuse lesions. Clinicians must diagnose and assess the efficacy of each treatment and the disease status of individual patients based on the integration of clinical information, such as radiology imaging, tumor markers, and clinical symptoms. For these reasons, a surrogate marker would be useful and is needed. My colleagues and I are now investigating an antigen specific to scirrhouous gastric cancer.
Personalized medicine has recently become a focus of interest, particularly with regard to several aspects of medical treatment (97, 98). Many basic and clinical studies have been conducted to realize the idea of treating patients with the most effective chemotherapeutic agents, including the prediction of responses through *in vitro* and *in vivo* assays. However, these approaches have not been sufficient to satisfactorily cure scirrhou s gastric cancer, and new methods and trials are thus required to intensify the efficacy of such prediction. It is my hope that new strategies for scirrhous gastric cancer, together with those described in this thesis, will be established in the near future.
Chapter VII

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Chapter VIII.

List of publications

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The Outcome of Treatment for Patients with Borrmann Type 4 Advanced Gastric Cancer. *Journal of Cancer Therapy*, 7, 953-962 (2016)

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Chapter IX.

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