to deliver CDDP intraperitoneally (i.p.), resulting in improved survival compared with that achieved by CDDP delivered intravenously [7]. To keep the AUC of peritoneum/plasma high, the frequent or continuous intraperitoneal administration of CDDP was attempted, but this was very inconvenient for patients and caused catheter complications [14, 15]. Conversely, the intraperitoneal administration of CDDP in hypotonic solution resulted in a high AUC in plasma, a high intratumoral uptake, and prolonged survival in animal models of peritoneal metastasis; however, the AUC in the intraperitoneal fluid was low, and this strategy caused more renal toxicity [16, 17]. Although research is ongoing, the intraperitoneal administration of CDDP is still widely performed without any special artifice in drug delivery in clinical practice. Based on the theoretical rationale of intraperitoneal chemotherapy, further research is needed to find drug-delivery systems (DDS) that prolong the retention of drugs in the peritoneal cavity, that enhance the penetration of drugs into disseminated peritoneal nodules, and that prevent the adhesion of nodules in the peritoneal cavity [15, 18, 19].

Hydrogels are formed by cross-linking hydrophilic macromolecules. They swell with water absorption and shrink on degradation, and have been reported to be useful in prolonging drug retention in the peritoneal cavity and enhancing antitumor effects against peritoneal dissemination [20–22]. Stimuli-sensitive hydrogels, which are reversible polymer networks formed by physical interactions, exhibit a sol–gel phase transition in response to external stimuli such as temperature [21–24] or pH [25]. They have great potential in biomedical and pharmaceutical applications, especially in site-specific and controlled DDS [23, 26].

Hyaluronic acid (HA) is a well-known glycosaminoglycan that exists naturally in the human body and works as a lubricating agent in the peritoneal cavity [27, 28]. HA is widely and safely utilized as a biomaterial in the field of drug delivery [27, 29]. Reports have described in situ cross-linking hydrogels of HA, in which hydrogels were formed via the disulfide bond formation of thiolated HA (HA-SH) [30], ion-crosslinking [31], or imine formation [32, 33]. The HA hydrogel that we used in this study was described as a biocompatible material that prevents peritoneal adhesion after surgical processes [32, 33].

In this study, we encapsulated CDDP in HA polymers that were cross-linked in situ. Based on results from previous studies about antiadhesion materials [32, 33], we assumed the CDDP was gradually released from the HA hydrogel, and the HA gel was finally degraded by hyaluronidase (HAse) in the peritoneum, approximately 1 week after administration. Therefore, the sustained release of CDDP from the HA hydrogel could be a strategy to prolong the retention of this drug in the peritoneal cavity. The formation and swelling kinetics of hydrogels and the release kinetics of CDDP from hydrogels were studied in vitro. We studied the antitumor activity of intraperitoneal CDDP, delivered via in situ cross-linkable and HA-based hydrogels, to treat peritoneal dissemination of gastric cancer in vivo in a mouse model.

Materials and methods

Reagents and cell culture

HA (MW = 200 and 10 kDa) was kindly provided by Kikkoman Biochemifa (Tokyo, Japan). Adipic dihydrazide (ADH) was purchased from WAKO (Tokyo, Japan). 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide (EDC), sodium periodate, ethylene glycol, tert-butyl carbazate (t-BC), hyaluronidase, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxybenzotriazole (HOBt) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was purchased from WAKO (Tokyo, Japan).

The human gastric cancer line, MKN45P, which produces peritoneal dissemination, was established in our department [34]. It was cultured routinely in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (FBS) (Sigma-Aldrich) and 1 % antibiotics and antimyotics (Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5 % CO_2. Before reaching confluency, cells were subcultured by treatment with ethylenedinitrilo-tetraacetic acid (EDTA) and trypsin. CDDP was kindly provided by Nippon Kayaku Co., Ltd. (Tokyo, Japan).

Synthesis of the polymers and hydrogels

HA was modified to an aldehyde form (HA-CHO), as described previously [32, 35, 36]. Briefly, 1.5 g of HA (MW = 200) was dissolved in 150 ml water, then after 802 mg sodium periodate was added, it was stirred for 2 h. Finally, 200 μL of ethylene glycol was added to stop the reaction, and the mixture was dialyzed (MWCO 8000, Spectra/Por membrane) immediately against water. The purified product was freeze-dried and kept at 4 °C.

HA-adipic dihydrazide (HA-ADH) was synthesized as described previously [32, 35, 36]. Briefly, 0.5 g of HA (MW 10 kDa) was dissolved in water to make a 5 mg/ml solution, to which a 30-fold molar excess of ADH was added. The pH of the reaction mixture was adjusted to 6.8 with 0.1 M NaOH and 0.1 M HCl. We dissolved 0.78 g of EDC (5 mmol) and 0.77 g HOBt (5 mmol) in DMSO/H_2O (1:1 v/v, 5 ml each) and added this to the reaction mixture.

The pH of the solution was adjusted to 6.8 and maintained by adding 0.1 M HCl for at least 4 h. The reaction was allowed to proceed overnight. The pH was subsequently adjusted to 6.0 and exhaustively dialyzed (MWCO 8000, Spectra/Por membrane) against water. NaCl was then added to produce a 5 % (w/v) solution, and HA-ADH was precipitated in ethanol. The precipitate was re-dissolved in water and dialyzed again to remove the salt. The purified product was freeze-dried and kept at 4 °C.

Characterization of the polymers and hydrogels

We performed ^{1}H-NMR (Varian: Unity 300 spectrophotometer, Palo Alto, CA) spectroscopy of the 10 mg/ml solutions of HA-ADH and HA-CHO in D_2O. HA-CHO was analyzed after reacting with t-BC, as described previously [36]. The ^{1}H-NMR spectra of the polymers reacted with t-BC were measured in D_2O.

The gelation time was measured by the following previously described protocol [33]. First, 100 μl of aqueous 5, 10, 15, or 20 mg/ml HA-CHO solution that contained 0 or 0.02 mg/ml CDDP was added to 100 ml of the same concentration of aqueous HA-ADH solution, which contained the same concentration of CDDP as the HA-CHO. These solutions were then mixed with a magnetic stir bar on a petri dish at 300 rpm using a hot plate/stirrer (HS-300, As One, Tokyo, Japan). The gelation time, being the time required for the mixture to become a globule, was measured four times per sample.

Release kinetics of the CDDP and swelling kinetics of the hydrogels

HA-ADH and HA-CHO were dissolved in a CDDP solution of the same concentration (0, 0.02 mg/ml). The concentration of the polymers was set at 10 mg/ml. Each polymer solution was placed in separate sterile syringes, which were connected to a dual syringe applicator (Baxter, Chicago, IL, USA), and co-extruded into a silicon mold through a common needle. The liquid precursors started to gel instantly, and the gel was chilled at 4 °C for 1 h and then placed on a 35 mm dish. A total of 4 ml of either PBS or 10 U/ml HAse was added to each gel and incubated at 37 °C. At several time points from 0.5 to 100 h, the gel disks were weighed, the media were changed, and the concentrations of CDDP in the collected media were measured by atomic absorption spectrometry (AAS-Z2000, Hitachi, Tokyo, Japan), as described previously [37]. The furnace program was as follows: ramp from 25 to 80 °C, hold 2 s, ramp to 120 °C, hold 10 s, ramp to 1000 °C, hold 5 s, ramp to 2700 °C, hold 2 s, and cool to 25 °C over 20 s. The graphite partition tube was cleaned once every 40 samples by baking at 2800 °C for 7 s. Argon was used as the injection and carrier gas. We calculated the concentration of released CDDP at each time point and the % volume of the hydrogel, defined as the ratio of the volume of the hydrogel at each time point to the initial volume.

In vitro cell proliferation assay

To examine the cytotoxic effect on the in vitro growth of MKN45P, we used CDDP, HA-CHO, and HA-ADH for the cell proliferation assay. MKN45P (1×10^3 cells in 100 μl/well) was seeded into a 96-well microtiter flat-bottomed plate in DMEM containing 10 % FBS. Cells were cultured overnight at 37 °C in 5 % CO_2 to allow attachment. The medium was then aspirated and replaced with fresh medium containing 10 % FBS and varying concentrations (0, 0.01, 0.1, 1, 10 mg/ml) of each polymer. Each condition was run in three replicate wells. After incubation at 37 °C in 5 % CO_2 for 48 h, the number of living cells was counted using an MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) according to the manufacturer's protocols. The assay consisted of a tetrazolium compound (inner salt; 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS]) and an electron coupling reagent, phenazine methosulfate (PMS). MTS is converted by dehydrogenase enzymes into a formazan product, which is found in metabolically active cells and is soluble in tissue culture medium. Absorbance of the formazan at 490 nm was measured directly in a 96-well plate using a microtiter plate reader (ThermoFisher, Waltham, MA, USA), which is directly proportional to the number of living cells in the culture.

Animal experiments on the intraperitoneal administration of CDDP via HA hydrogels in a mouse model of peritoneal dissemination of gastric cancer

Six-week-old specific pathogen-free conditioned female BALB/c nude mice were purchased from Charles River Japan, Inc. (Yokohama, Japan) and fed in a temperature-controlled, light-cycled room. At 7 weeks of age, the mice were inoculated intraperitoneally with 1×10^6 MKN45P gastric cancer cells suspended in 1 ml of PBS. The mice were randomly divided into four groups of six: a PBS group, a Gel group, a PBS-CDDP group, and a Gel-CDDP group. On days 7, 14, and 21 after inoculation with MKN45P cells, the mice received an intraperitoneal injection of the respective drugs. The total amount of injected liquid was set at 1 ml to allow for better spreading of the drugs throughout the whole peritoneal cavity. The dose of CDDP was set at 1 mg/kg, and the polymer concentrations were set at 10 mg/ml, based on the previous studies [32, 33] and our preliminary experiment. In the Gel groups, CDDP was dissolved in 0.5 ml HA-ADH and 0.5 ml of HA-CHO, and the two polymers were placed in separate sterile syringes, which were connected to the dual

syringe applicator and injected into the peritoneal cavity through a common 23-gauge needle. On day 28 after inoculation with cancer cells, all mice were anaesthetized by an inhalation of diethyl ether, and a whole blood sample was collected by transthoracic cardiac puncture. The mice were then killed and a laparotomy was done to collect and weigh the peritoneal nodules. Serum was separated from the blood samples by centrifugation at 700*g* for 5 min at 4 °C. After separation, the levels of urea nitrogen (UN) were measured with a commercial kit, using the urease and indophenol method (Urea B test, Wako, Tokyo, Japan), and the level of creatinine was measured with a commercial kit, using the Jaffe method (LabAssay™ Creatinine, Wako, Tokyo, Japan) according to the manufacturer's protocols. All animal experiments were performed in accordance with the Guidelines of Animal Experiments of the University of Tokyo, and the protocols were approved by the animal care committee of the University of Tokyo.

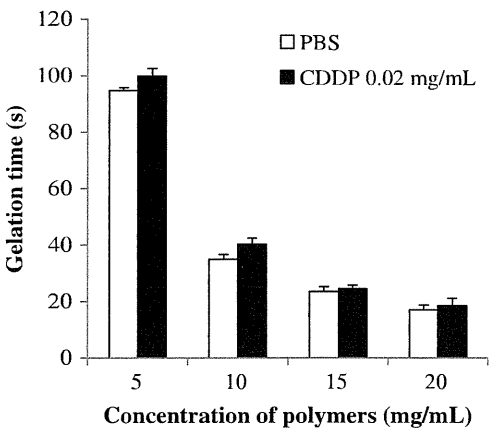

Fig. 1 Concentration of polymers and gelation time. Gelation time became longer as the concentration of polymers decreased, whereas the time did not vary much based on the concentration of CDDP

Statistical analysis

Results were examined statistically by an analysis of variance (ANOVA), followed by the Dunnett's method. Results are expressed as the mean ± SD. Throughout the experiments, differences with $p < 0.05$ were considered significant. All statistical analyses were performed using the JMP program, version 9.0 (SAS Institute, Cary, NC, USA).

Results

Synthesis and characterization of polymers and hydrogels

Synthesis of HA-ADH was confirmed by the methylene protons of the ADH (singlet peak at 1.62 ppm and the doublet peak at 2.25 and 2.38 ppm) [32, 35, 36]. The degree of modification, calculated from the ratio of the area of the peak for the *N*-acetyl-D-glucosamine residue of HA (singlet peak at 2.0 ppm) to that of the methylene protons of the adipic dihydrazide at 1.62 ppm, was found to be 23.7 %.

The gelation time is plotted in Fig. 1. As the concentration of the polymers increased, the gelation time decreased. The existence of CDDP did not affect the gelation time at any concentration of the polymers.

In vitro sustained release of CDDP in the hydrogel

Figure 2a shows the behavior of the sustained release of CDDP in vitro. CDDP was gradually released for more than 4 days, and HAse accelerated the degradation of the hydrogel.

Figure 2b shows the swelling kinetics of the hydrogels. The hydrogels swelled over 4 days without HAse, whereas they contracted with HAse.

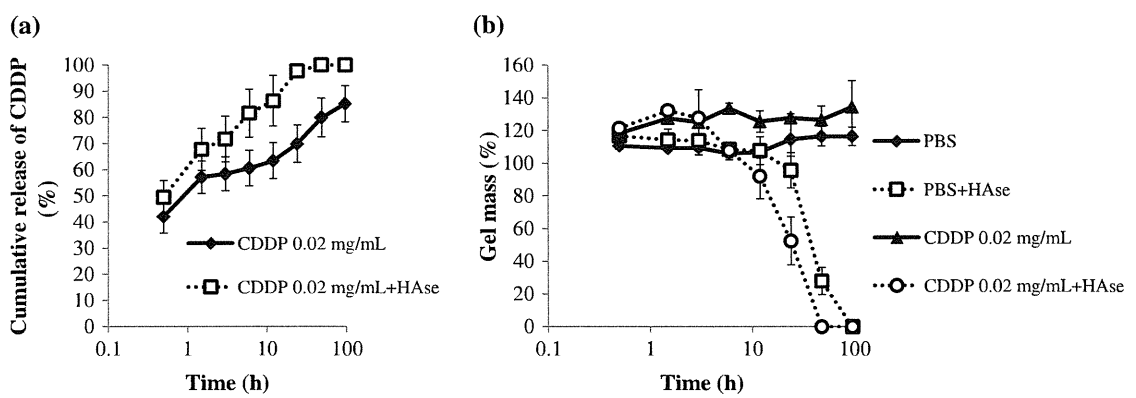

Fig. 2 Sustained release of CDDP from the hydrogel (**a**) and swelling kinetics of the hydrogel (**b**). CDDP was gradually released over more than 4 days, and HAse accelerated the degradation of the hydrogel (**a**). The hydrogels swelled over the 4 days without HAse, whereas they showed contraction with HAse (**b**)

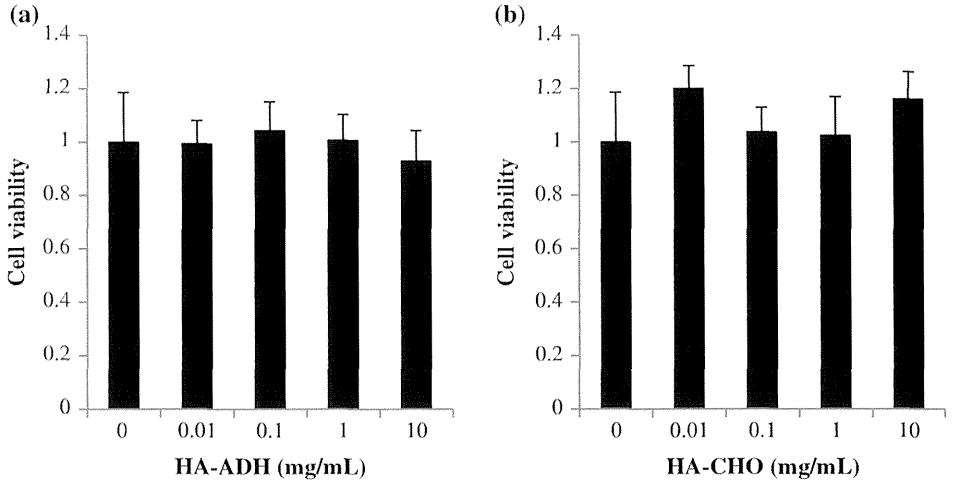

Fig. 3 In vitro cell proliferation assay. The polymers alone did not have cytotoxic activity (**a** HA-ADH, **b** HA-CHO)

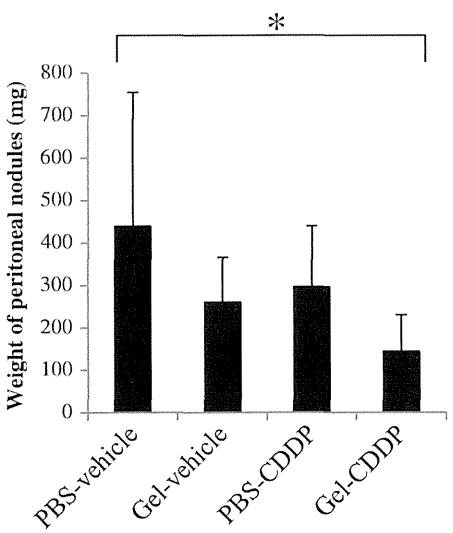

Fig. 4 Weight of the peritoneal nodules in the mouse model of gastric cancer. We observed significant reduction in the gel-CDDP group vs. the PBS-vehicle group, but no reduction in the PBS-CDDP group ($*p = 0.032$)

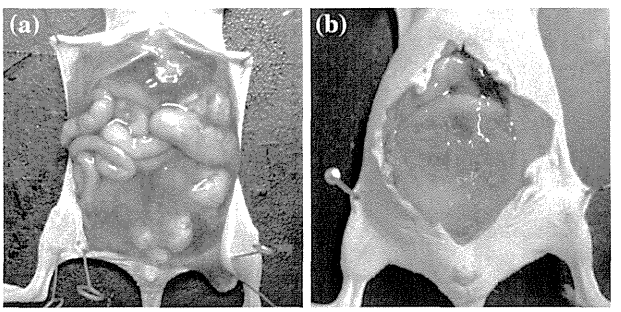

Fig. 5 In situ gelation and residual hydrogel in the peritoneal cavity. At dissection, very little residual hydrogel was observed in the peritoneal cavity when the concentration of HA polymers was set at 10 mg/ml (**a**), whereas massive residual hydrogel was observed when the concentration of HA polymers was set at 20 mg/ml (**b**) in a preliminary experiment

In vitro cell proliferation assay

The polymers alone did not show any cytotoxic activity at any concentration (Fig. 3a, b).

Enhanced antitumor effect of the intraperitoneal administration of CDDP via HA hydrogels in a mouse model of peritoneal dissemination of gastric cancer

By day 28 after the inoculation of MKN45P, two mice from the 1 mg/kg PBS-CDDP group had died, while all the mice in the other three groups were alive. In vivo gelation was successfully achieved. Figure 4 shows the weight of the peritoneal nodules. At the time of dissection, very little intraperitoneal 10 mg/ml gel remained (Fig. 5a).

Table 1 Renal function at the time of death (on day 28) in each group

	PBS-vehicle	Gel-vehicle	PBS-CDDP	Gel-CDDP
UN (mg/dL)	28.0 ± 3.9	36.3 ± 5.9	36.5 ± 2.0	38.6 ± 3.2
Cre (mg/dL)	0.34 ± 0.03	0.45 ± 0.04	0.44 ± 0.04	0.52 ± 0.03

Data are the mean ± SD. There were no significant differences between the treatment groups

UN urea nitrogen

Significant reduction in the weight of the peritoneal nodules was observed in the Gel-CDDP group vs. the PBS-vehicle group, whereas no significant reduction was detected in the PBS-CDDP group.

Table 1 shows the serum levels of UN and creatinine when the mice were killed. All values were within the normal limits.

Discussion

In this study, we performed in situ crosslinking of two HA polymers to achieve gelation. We showed in vitro that the speed of gelation and the period of sustained release of CDDP could be controlled by changing the concentration of the polymers. The in vivo release of CDDP from the HA hydrogel enhanced its antitumor effects against the peritoneal dissemination of gastric cancer.

To deliver drugs directly to disseminated peritoneal tumors, intraperitoneal administration has proven to be an effective regional therapy. Sustained exposure to drugs is believed to make therapy more effective by allowing deeper penetration of drugs into peritoneal nodules [15, 18]. There are three steps in our strategy. First, agents must be in liquid form to be infused intraperitoneally through a catheter; second, gelation must happen in situ; and third, the drugs must undergo sustained released from the gel as it degrades. We showed that two liquid HA precursor polymers can form a gel by mixing together, and that the gel was degraded by HAse, which is physiologically present in the peritoneal cavity.

We achieved in vivo gelation by administering two HA polymers via a dual syringe. The concentration of hydrogels was set at 10 mg/ml based on the previous studies [32, 33], and our preliminary experiment, which showed that the 20 mg/ml hydrogel remained in gel form in the peritoneum even 1 week after its administration; seemingly slow degradation (Fig. 5b). The intraperitoneal administration of CDDP with the hydrogel showed better antitumor effects than CDDP with PBS.

Bajaj et al. [38] used the same technique to achieve the sustained release of paclitaxel (PTX) from a hydrogel in a similar peritoneal tumor model of ovarian cancer. They mixed hydrogel and PTX micelles with Cremophor EL (named Taxol, 14 nm mean particle size) or a PTX derivative of microparticular size (named PTX-susp, 91 μm mean particle size). Prolonged retention of PTX in the peritoneal cavity was achieved only in the PTX-susp group when comparing groups treated with or without hydrogel. They stated that the size of the Taxol particles was too small for it to be sustained in hydrogel. Despite the prolonged retention, no enhanced antitumor effect was seen in the PTX-susp gel group, which they attributed to the fact that PTX might not be released from the hydrogel because of the large molecular size of the PTX-susp. In our present study, it is possible that part of the total CDDP was coordinated to the carbonyl groups of HA by a ligand exchange. Because of this coordination, part of the CDDP was not fully released from the hydrogels (Fig. 2). CDDP coordination might also have contributed to the prolonged retention and enhanced antitumor effects in vivo.

In our experiment, the HA hydrogel control showed unexpected antitumor effects, although they were not significant. Our results were consistent with those reported by Bajaj et al., who found a decreased number of peritoneal nodules of an ovarian cell line (SKOV3) in their gel-alone group compared with their PBS group, although again, not significant [38]. There is a speculation that HA hydrogels could inhibit cancer cell attachment to the peritoneum. HA is a ligand of CD44, known to be present in various cancer cells, including SKOV3 [39], MKN45 [40], and cancer stem cells, and to play various roles in molecular biology [41–43]. Therefore, the binding of HA to CD44 might be related to the antitumor activity of the hydrogel, but further studies are required to investigate this theory.

It is known that CDDP rapidly binds to albumin [44] and that the binding of CDDP to albumin may affect its antitumor effects; however, inside the gel network, the diffusivity of albumin is lower than that of CDDP. Therefore, based on the release kinetics of FITC-albumin from the same gel in a previous report [45], it should take 1 day for the albumin to penetrate the gel, whereas[45] in the present study, approximately 70 % of the CDDP was released in the first 24 h. We speculate that nearly all of the CDDP were released from the gel without binding to albumin.

One of the critical side effects of prolonged exposure to CDDP is renal toxicity [10]. In this study, the level of creatinine was slightly elevated in the hydrogel groups, but still within normal limits in all the groups. Sufficient and careful hydration may be important in the clinical use of hydrogels and CDDP.

The development of new formulations for intraperitoneal delivery of CDDP is desirable. The molecular interactions between HA and CDDP need to be investigated, to improve the release kinetics of CDDP from the HA hydrogels. CDDP is a metal complex; therefore, its conjugation by ligand exchange may be an effective approach to encapsulate it inside hydrogels. Additional effective strategies for the intraperitoneal delivery of CDDP, other than improving the controlled release of the drug by molecular interactions between HA and CDDP [46], could include achieving enhanced drug penetration of tumors utilizing nanotechnology [46, 47] and target-specific delivery through interactions between CD 44 and HA [43, 48, 49]. We plan to conduct further research into these areas, including modifications of this study.

In conclusion, we successfully achieved in situ gelation of biocompatible HA polymers and the sustained release of CDDP in the peritoneal cavity. The intraperitoneal administration of CDDP via the HA hydrogel showed enhanced antitumor activity against peritoneal dissemination in a mouse model, revealing a novel drug-delivery strategy for the treatment of peritoneal dissemination.

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Conflict of interest We declare no conflicts of interest.

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Original Article

Flow Cytometric Quantification of Intraperitoneal Free Tumor Cells in Patients with Peritoneal Metastasis

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Background: Peritoneal metastasis (PM) is the most life-threatening type of metastasis in abdominal malignancy. To improve the diagnostic accuracy of cytologic detection (CY) of free tumor cells (FTC) in the peritoneal cavity, we tried to quantify the FTC to leukocyte ratio using flow cytometry in patients with peritoneal metastasis.

Methods: Cells were recovered from ascites or peritoneal lavages from 106 patients who underwent abdominal surgery and additional 89 samples which were obtained from peritoneal catheter or access port in patients with PM (+) gastric cancer. The cells were immunostained with monoclonal antibodies to CD45 and to CD326 (EpCAM). Using flow cytometry, CD326 (+) and CD45 (+) cells were classified as either tumor cells (T) or leukocytes (L) and the T/L ratio (TLR) was calculated.

Results: In 106 samples obtained by laparotomy, Median (*M*) of the TLR of PM (+) patients was 1.39% (0–807.87%) which was significantly higher than PM (−) patients (*M*=0%, 0–2.14%, $P < 0.001$). In PM (+) patients, 86 CY (+) samples showed higher TLR than 61 CY (−) samples (*M*=2.81%, 0.02–1868.44% vs. *M*=0%, 0–3.45%, $p<0.0001$). In all of the 24 patients who were monitored for TLR before and after intraperitoneal (IP) chemotherapy, the TLR was reduced which was more dramatic than the results of the change in cytology.

Conclusions: TLR measured with FACS is an excellent reflection of the tumor spread in the peritoneal cavity and could be a reliable diagnostic biomarker to determine the severity of PM as well as effectiveness of IP chemotherapy.

Key words: peritoneal metastasis; intraperitoneal free tumor cell; peritoneal cytology; flowcytometry; CD326

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Peritoneal metastasis is one of the most frequent types of recurrent abdominal malignancy, especially as a result of gastric and ovarian cancer. It is most likely that peritoneal recurrence is caused by intraperitoneal free tumor cells (PFC), which have been exfoliated from the serosal surface of primary tumors (1,2). In fact, the detection of free tumor cells (FTC) by peritoneal cytology (CY) at the time of surgery has been reported to be one of the most reliable prognostic factors for peritoneal recurrence in gastric (3–6), colorectal (4,7), pancreatic (8), and gynecologic (9) malignancies. However, cytological diagnosis of peritoneal fluids is qualitative and

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largely dependent on institutions, as well as pathologists, which may result in confusion in the clinical evaluation of outcome of CY (+) patients (10,11).

More recently, molecular detection of tumor cell-specific mRNA, such as carcinoembryonic antigen (CEA) or cytokeratin (CK) 19 or 20 has been introduced, using real-time PCR, which improves sensitivity in detecting free intraperitoneal cancer cells (12-15). However, amplified mRNA can be derived from dead cells and CEA and CK can be expressed and released from hematopoietic cells in an inflammatory context (16), thus the clinical significance of false positive cases remains to be addressed. In this study, we report on our development of a new method to quantify the accurate volume of FTC in the abdominal cavity, using flow cytometry. We evaluate the clinical value of this method in patients with peritoneal metastasis.

MATERIALS AND METHODS

Patients

Ascites or peritoneal lavages were recovered from 106 patients who underwent abdominal surgery for gastric ($n = 90$) or colorectal cancer ($n = 16$) in the Department of Surgical Oncology between August 2008 and May 2013. All patients underwent open abdominal surgery and peritoneal washing was performed using 200 ml of normal saline. 100ml samples were obtained by lavage before operative manipulation. In cases of the presence of ascites, 20 ml of fluid from ascites was obtained soon after laparotomy. During the same period, 89 samples were obtained from intraperitoneal catheter or a subcutaneous intraperitoneal access port in patients who received intraperitoneal (IP) chemotherapy for peritoneal metastasis of gastric cancer. Pathologists evaluated peritoneal cytology for all samples. Informed consent was obtained in writing from all patients. This study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Tokyo.

MAbs

PE-conjugated mAb to CD326 (EpCAM) was purchased from Miltenyi Biotec (Auburn, CA). FITC-conjugated mAb to CD45, Fc-blocker and 7AA, as well as control mouse IgG1, were all purchase from Becton-Dickinson (San-Jose, CA).

Cell Processing and Flow Cytometry

After the centrifugation of ascites or peritoneal lavages at 1,500 rpm for 10min, the pellets were resuspended in PBS+0.02% EDTA and overlayed on Ficoll-Hypaque solution (Pharmacia Biotech, Piscataway, NJ). After centrifugation at 3,000 rpm for 10 min, the intermediate layer was taken and washed twice with PBS+0.02% EDTA. During the procedure, most of the cell clusters were dissociated to form single cell suspensions. The cells ($1x10^6$) were suspended in 100μl of PBS+0.02% EDTA, incubated with 10 μl of Fc-blocker for 20 min and then immunostained with FITC-conjugated CD45, PE-conjugated CD326 and 7AA for 30 min in 4°C as per the manufacturers' recommendation. After washing, 10^4 cells were acquired in the 7AA-negative area and analyzed for the expression of CD45 and CD326 with FACS-Caliber (Becton-Dickinson, San-Jose, CA). In some cases, the samples were observed with fluorescent microscopy Biozero (Keyence, Tokyo Japan).

Statistics

In the comparison of the tumor cell/leukocyte ratio (TLR), *P*-value was calculated using Wilcoxon's nonparametric analysis with JUMP software.

RESULTS

Calculation of Tumor Cells/Leukocytes Ratio (TLR)

Figure 1 is representative of the FACS profiles of the cells recovered from ascites in a patient with peritoneal metastasis (Case 1, upper panel) and peritoneal lavage from a patient without peritoneal metastasis (Case 2, lower panel). Figures 1A-1E, 1K-1O show the results of staining with control mAbs, while Figures 1F-1J, 1P-1T show staining with FITC-conjugated anti-CD45mAb, PE-conjugated anti-CD326 mAb and 7AAD. First, 7AAD-positive areas were determined to be dead cells in Figures 1D, 1I, 1N, and 1S and excluded from the analysis. Then, in the 7AAD-negative region (R1), the FL-1 (FITC) and FL-2 (PE) intensities were plotted against SCC (Figs. 1B, 1G, 1L, and 1Q and Figs. 1C, 1H, 1M, and 1R). In Figures 1B and 1C and Figures 1L and 1M, negative areas were determined for CD45 and CD326, respectively. Usually, the threshold for fluorescein intensity increased as the SCC increased and the positive areas for CD45 (R2) and CD326 (R3) show the "sox like" shape. In Figures 1G and 1H, case 1, the number of CD45 (+) leukocytes (L) and CD326 (+) tumor cells (T) were calculated as the dot number located in the gated areas R1+R2 and R1+R3, respectively, and the tumor cell leukocyte ratio (TLR) was calculated as the relative frequency of FTC in the abdominal cavity. To make the calculation more accurate, the number of the cells located in the positive region in control IgG staining (Figs. 1B and 1C) was subtracted from those values to delete the cells with nonspecific binding. Thus, the TLR was calculated as the following formula.

$$\mathrm{TLR}\,(\%) = (\mathrm{PE-conjugated\ CD326(+)\ cells} - \mathrm{PE\text{-}conjugated\ mIgG\ (+)\ cells}) / (\mathrm{FITC-conjugated\ CD45\ (+)\ cells} - \mathrm{FITC-conjugated\ mIgG\ (+)\ cells}) \times 100$$

Then, the TLR in case 1 was calculated as (315-1)/(9157-18) x100 = 3.40 (%). Similarly, TLR in case 2 was calculated as (2-2)/(9348-71) x100 = 0(%), suggesting that no tumor cells were present in case 2.

FACS profiles of two additional cases with peritoneal metastasis (Cases 3 and 4) were expressed in Figure 2.

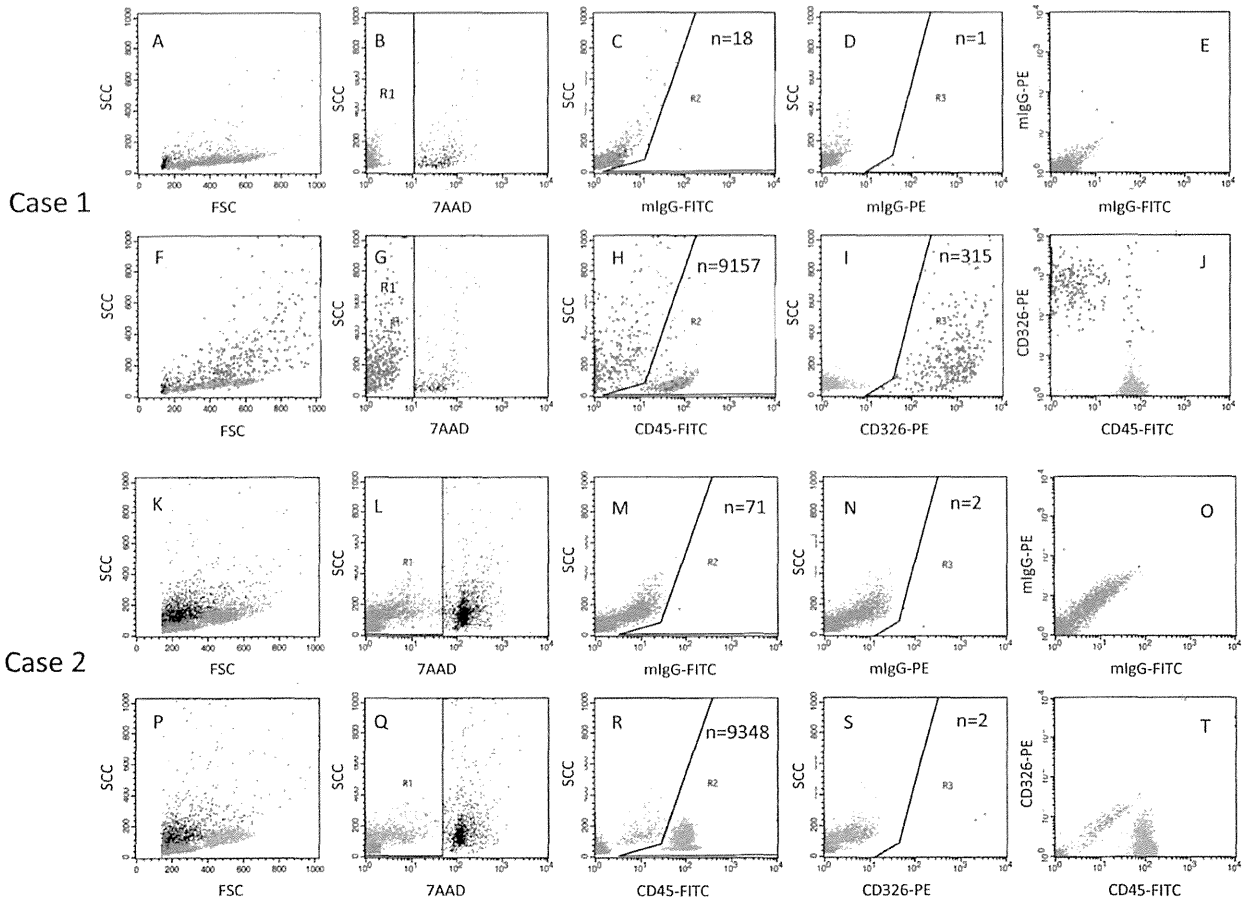

FIG. 1. FACS profiles of the patients with (Case 1, upper panels) or without (Case 2, lower panels) peritoneal metastasis. **A–E** and **K–O** show the staining with control mAbs, while **F–J** and **P–T** show the staining with FITC-conjugated anti-CD45 and PE-conjugated CD326. Figures A, F, K, and P show dot plots of FSC/SCC and E, J, O, and T show the FL1/FL2 profile. In figures B, G, L and Q of the FL3/SCC profile, 7AA negative cells were analyzed as living cells. Figures C, H, M and R show FL1/SCC and D, I, N and S show the FL2/SCC profile. CD45 and CD326-positive areas were defined as R2 and R3, respectively, and counted dot numbers were expressed. All the figures were expressed as multi-color plotting and green and red dots show CD45 (+) and CD326 (+) cells, respectively.

Although both cases were diagnosed as cytologically positive (CY+), TLR of case 3 was calculated as 1.84%, while that of case 4 was 190.15%. This indicates that the frequency of intraperitoneal tumor cells varied widely among patients with positive peritoneal cytology.

As shown in Figures 1F and 2D, CD326 (+) tumor cells (red spots) were generally distributed at relatively higher area in the FSC and SCC profile, as compared with CD45 (+) leukocytes (green spots). In addition, the expression of CD326 and CD45 were mutually exclusive in most cases (Figs. 1J, 2B, and 2E).

Observation with Fluorescent Microscopy

Figures 2C and 2F show the merged images of the same samples of Case 3 and 4, respectively, observed with immunofluoresence microscopy. These figures show that most of the cells in these samples consisted of single cells, which were stained with either FITC labeled anti-CD45 mAb or PE-labeled anti-CD326 mAb. The ratio of red tumor cells to green leukocytes was mostly consistent with TLR calculated with FACS analysis in both samples. The microscopic observation also indicates that tumor cells are relatively larger than leukocytes, which is consistent with the FSC/SCC profile of FACS analysis.

TLR of Peritoneal Fluid in Operative Patients

Figure 3A shows the TLR of the patients with or without peritoneal metastasis obtained at laparotomy. In general, samples derived from patients without peritoneal metastasis (PM-) contained few CD326-reactive cells and median (M) of TLR of the PM(-) 48 cases was 0% (0-2.14%). Indeed, the TLR of 30 of the 48 cases was 0% and less than 0.1% in the other 10. In contrast, the TLR of the samples recovered from the 58 patients with peritoneal metastasis (PM+) showed significantly higher

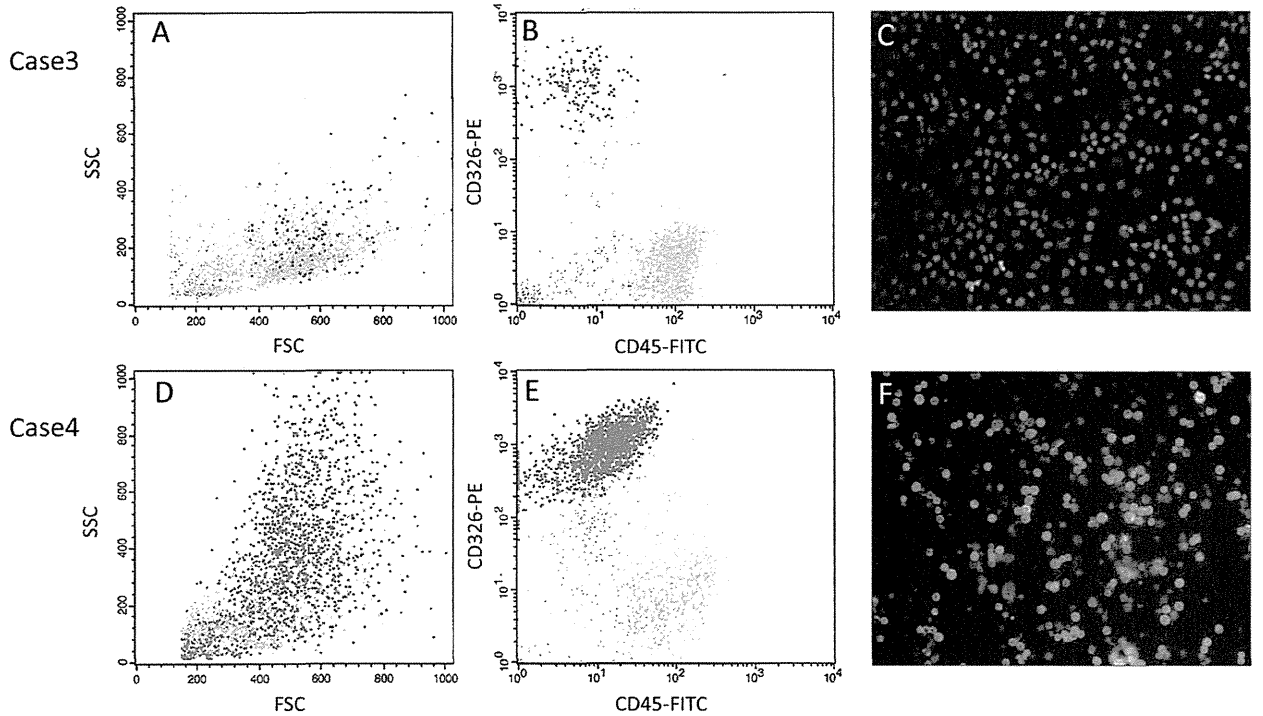

FIG. 2. FACS profiles of 2 additional cases (**A**, **B** and **D**, **E**) with peritoneal metastasis and positive cytology. Each figure was expressed in the same fashion as Figure 1. (**C**), (**F**): Samples of Case 3 (**C**) and Case 4 (**F**) were placed on a plastic plate and observed with Fluorescent microscopy. Green and red fluorescence were detected under the corresponding wavelengths and the two images were merged.

TLR (M= 1.39%, 0-807.87%) ($P < 0.001$). However, TLRs were highly variable among the samples from PM (+) patients. In the 58 patients, 7 cases contained extremely high levels of CD326 (+) cells with TLR over 100%, indicating that tumor cells are more predominant than leukocytes in the abdominal cavity. On the contrary, TLRs were less than 0.1% in 7 cases.

TLR of CY (+) and CY (−) Samples in Patients with Peritoneal Metastasis

Figure 3B shows the TLR of the samples derived from the patients with peritoneal metastasis, including 58 samples obtained at laparotomy and 89 samples obtained from a peritoneal catheter or access port. TLR and CY showed the good correlation (Table 1). All of the 86 CY (+) samples contained a significant number of CD326-reactive cells (M =2.81%, 0.02~1868.44%). In contrast, 61 CY (−) samples showed significantly lower TLR (M=0%, 0~3.45%, P <0.001). Their TLR was 0% in 36 cases and less than 0.1% in the remaining 6, which was mostly similar to TLR in samples obtained from the PM(-) patients at laparotomy.

TLR Before and After Intraperitoneal Chemotherapy

In 24 patients, the TLR was measured before and after IP chemotherapy. Cytology was initially positive in 21 and negative in 3 cases. As shown in Figure 4, TLR was reduced by IP chemotherapy in all cases including the 3 CY(-) cases. Among the 21 CY(+) cases, TLR was reduced to 0% in 10 cases after chemotherapy, which was accompanied with negative cytology after chemotherapy. In 11 other cases, however, TLR after IP chemotherapy was calculated to be between 0.016%~35.4% and 4 of the 11 samples were diagnosed as CY(+), even after chemotherapy. Even in those cases, however, we confirmed that the peritoneal lesions were partially reduced by laparoscopic findings, the change of TLR might be more sensitive than conventional cytology in terms of the response to chemotherapy.

DISCUSSION

Cytologic detection of FTC from a peritoneal lavage is now recognized as the most important determinant in the prediction of the development of peritoneal recurrence in patients with many types of abdominal malignancies (5,6) (3,4,7) (8) (9). However, conventional examination with Papanicoloaou staining is reported to lack the sensitivity, and thus immunostaining methods using specific mAbs to tumor cell-associated antigens have been used to increase sensitivity (17-19). In this study, we used the immunostaining method and tried to quantify the relative frequencies of FTC in the abdominal cavity using flow cytometry.

We used pan-leukocyte markers CD45 and CD326 (EpCAM), which are widely overexpressed in a variety

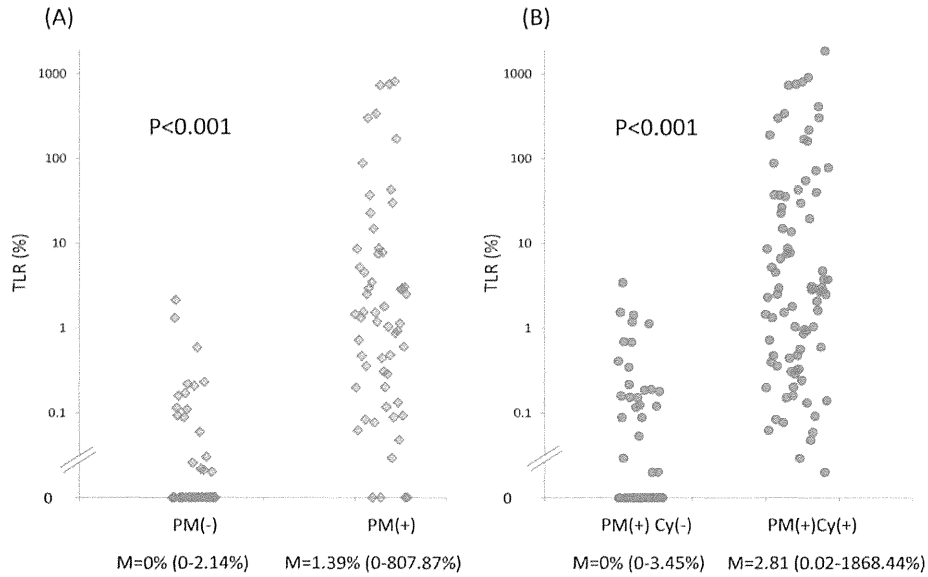

FIG. 3. **A:** TLR of the peritoneal fluids obtained from patients with ($n = 58$) or without ($n = 48$) peritoneal metastasis at laparotomy. **B:** TLR of peritoneal fluids with positive ($n = 86$) or negative ($n = 61$) cytology in patients with peritoneal metastasis. Out of 86 CY (+) samples, 49 were obtained at laparotomy and 37 from a peritoneal catheter or access port, while in 61 CY (−) samples, 9 were obtained intraoperatively and 52 from a port.

of human cancers (20–23) and are purportedly an ideal antigen for clinical application in diagnosis of circulating tumor cells. Since CD326 is not expressed in mesothelial cells (24), the numbers of CD326 (+) cells in peritoneal fluids are supposed to reflect the total number of tumor cells of epithelial origin in abdominal cavity. Since the cell density was highly variable in each sample recovered from peritoneal cavity, we needed to count other cell types as internal control. For this aim, we used pan-leukocyte marker CD45, since leukocytes were most predominant cell types in most cases and the number of the whole leukocytes was relatively stable, although changeable by peritoneal inflammation, than other cell types. Then, we counted the number of CD45 (+) leukocytes as well as CD326 (+) cells in total of 10^4 living cells with flow cytometry, and calculated the relative frequency of CD326 (+) tumor cells against CD45 (+) leukocytes.

Tumor cells are often detected as forming clusters in classical cytology examination. In fact, we found some CD326(+) clusters if we fixed the cell pellets just after centrifugation of peritoneal fluid. However, after repeated washing with EDTA-containing media and Ficoll treatment, few clusters were observed in all samples as shown in Figure 3. This suggests that most of the tumor cells in peritoneal fluids form clusters in Ca2+ dependent manner, which are easily dissociated to single cells during the staining procedure.

Another difficult problem in detecting the accurate number of tumor cells is defining the threshold for

Table 1
Correlation Between CY(+) and TLR(+)

	CY (−)	CY (+)
TLR (−)	34	0
TLR (+)	27	86

CY was determined by cytology.
TLR (−); TLR = 0 and TLR(+); TLR > 0.
$P < 0.001$ by chi-square test.

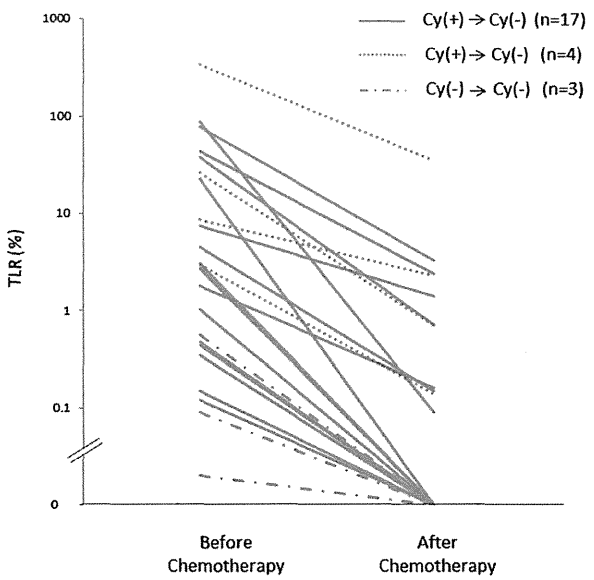

FIG. 4. The change of TLR before and after IP chemotherapy. In 24 cases, CY was initially positive in 21 and negative in 3 cases. In 17 of the 21 CY(+) cases (Blue continuous line), CY turned to be negative, while in 4 cases (Red dotted line) CY remained positive.

negative control for CD45 and CD326. This is especially important in quantitative analysis when the number of CD326 (+) cells is very low, compared with CD45 (+) cells. In our flow cytometric analysis, the borderline was usually defined as the value at which a positive result was detected in less than 0.3% of the total cell population for the negative control (mouse IgG). However, in our analysis of intraperitoneal cells, the threshold varies among the different cells. In fact, the level of binding of control murine IgG in tumor cells tended to be higher than that in leukocytes. In this study, therefore, we defined the threshold line in a FL1/SCC or FL2/SSC profile and the dot number was counted in regions that were positive for CD45 and CD326. By this method, cells were clearly divided into CD45 (+) leukocytes and CD326 (+) tumor cells, for most cases. In some cases, cells were located in CD45 (+) and CD326 (+) areas, although the number was very few. The double-positive cells may be so called "cancer-leukocyte fusion cells" (25), but further study is necessary to characterize the origin and pathological relevance of these cells.

TLR calculated by this method was strongly correlated with the presence of peritoneal metastasis, especially according to cytology status. In the majority of the cases without peritoneal metastasis, TLR was calculated as 0% or less than 0.1%, except for one case with a TLR of 2.14%. However, this sample was obtained from a patient with advanced gastric cancer with serosal exposure and he developed peritoneal metastasis at 6 months after surgery, suggesting that the CY (-) diagnosed by pathology was a false negative.

In contrast, cases with peritoneal metastasis showed significantly higher TLR. Among them, TLR of CY (+) samples was significantly greater than that of CY (-) samples, which showed similar TLR as those obtained from the patients without peritoneal metastasis. However, even in CY (+) cases, TLR was largely different, ranging from less than 0.1% to more than 100%. This implies that the amount of FTC in the abdomen was highly variable among CY (+) cases with peritoneal metastasis and thus the quantification of FTC is clinically important. Follow-up of patient outcome may enable a new classification of disease severity in peritoneal metastasis by FTC. In addition, comparison of TLR in the same patients before and after IP chemotherapy showed significant reduction in all cases. It is notable that in 4 cases, cytology status remained CY (+) even after chemotherapy, despite the fact that the TLR had clearly decreased. This may be related the fact that diagnosis of cytology status after cytoreductive treatment is technically difficult and thus TLR should be more useful to monitor the effectiveness of chemotherapy.

Recent reports of tumor cells that lack the expression of CD326 have appeared, although the frequency generally seems to be rare (26-29). In our series, we stained some samples with anti-cytokeratin (CAM5.2), showing the presence of cytokeratin (+), CD45 (−), and CD326 (−) cells in peritoneal cells, especially in cases with high TLR (data not shown). These cells may be CD326 (-) tumor cells since they also lacked mesothelial antigens such as calretinin or HBME-1. Triple staining with CD326 and cytokeratin, as well as CD45, may provide more accurate information for FTC.

In summary, we have demonstrated a new method to quantify the relative frequency of FTC in PM(+) patients using flow cytometry. The TLR calculated in this method is highly reproducible and accurately reflects the volume of intraperitoneal FTC, and thus could be a useful biomarker to evaluate the effectiveness of IP chemotherapy, as well as to predict patient outcome. Recently, it has been reported that relative number of CD326 (+) tumor cells examined with a computerized image analysis system can be used for monitoring the efficacy of IP administration of catumaxomab for malignant ascites (30,31). Our method is based on the same concept. However, since immunostaining is performed in cell suspensions, the whole process is completed with about an hour. Moreover, analysis is possible without specialized equipment. Thus, it can be widely practiced in various institutes.

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ORIGINAL ARTICLE – GASTROINTESTINAL ONCOLOGY

Salvage Gastrectomy After Intravenous and Intraperitoneal Paclitaxel (PTX) Administration with Oral S-1 for Peritoneal Dissemination of Advanced Gastric Cancer with Malignant Ascites

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ABSTRACT

Background. Peritoneal metastasis of gastric cancer has extremely poor clinical outcomes. Recently, we developed a combination chemotherapy that used intraperitoneal (IP) paclitaxel (PTX) and produced excellent antitumor effects against peritoneal lesions. However, no information is available about the benefit of gastrectomy in cases with malignant ascites.

Methods. A total of 64 patients with severe peritoneal metastasis and ascites received IP PTX at 20 mg/m^2 via implanted subcutaneous peritoneal access ports as well as intravenous (IV) PTX at 50 mg/m^2 on days 1 and 8. S-1 was administered at 80 mg/m^2 day for 14 consecutive days, followed by 7 days of rest. In all patients, investigative laparoscopy was performed around the combination chemotherapy, and gastrectomy was performed on patients who showed apparent shrinkage of their peritoneal nodules as well as negative peritoneal cytology at the second laparoscopy.

Results. Gastrectomy was performed in 34 patients. The median course of chemotherapy before surgery was 5 courses (range 2–16). R0 operation was achieved in 22 patients (65 %), and grade 2 and 3 histological responses were obtained in 7 (21 %) and 1 (3 %) patient(s), respectively. The median survival time and 1-year overall survival of the gastrectomized patients were 26.4 months and 82 %, and those of the 30 patients who did not receive gastrectomy were 12.1 months and 26 %, respectively. Morbidity was minimal, and there was no mortality.

Conclusions. Salvage gastrectomy after chemotherapy of S-1 with IV and IP PTX is promising, even for patients with highly advanced gastric cancer and severe peritoneal metastasis and malignant ascites.

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Peritoneal metastasis is the most life-threatening type of metastasis and recurrence in patients with advanced gastric cancer. Despite recent advances in systemic chemotherapy, peritoneal dissemination due to gastric cancer still remains a dismal disease with an extremely short survival rate in patients. Moreover, peritoneal dissemination often causes ascites accumulation, intestinal obstruction, or hydronephrosis, all of which seriously impair patient quality of life (QOL).

Systemic chemotherapy for unresectable or recurrent gastric cancer has steadily progressed, and 5-Fu-based or cisplatin-based regimens are generally accepted as standard regimens worldwide.[1–4] Only a handful of phase II studies have examined the efficacy of these regimens for peritoneal metastasis, however. Additionally, no large-scale trials have been performed because of the difficulty in evaluating the responses of diffusely disseminated and unmeasurable peritoneal lesions. Thus far, the median survival time (MST) and 1-year survival of patients receiving systemic chemotherapy alone were 3.1–10.6 months and 16–40.7 %, respectively.[5–8]

In alternative treatment paradigms, cytoreductive surgery (CRS) combined with hyperthermic intraperitoneal chemotherapy (HIPEC) or early intraperitoneal (IP) chemotherapy have also been used by several institutes, mainly in Western countries. The MST and 1-year survival of these patients was reported to be 6.5–11 months and 29.4–49.1 %, respectively, however.[9–13] Moreover, the

high mortality and morbidity accompanied by these demanding procedures require strict patient selection.

IP administration of anticancer drugs is a reasonable method for treating peritoneal metastasis since it enables extremely high concentration of drugs to directly act upon and target cancer lesions in the peritoneal cavity. Paclitaxel (PTX) is known to inhibit cell division by accelerating the polymerization of microtubule proteins that results in excessive microtubule formation and stabilization. PTX has been shown to elicit antitumor activity on various cancers, including gastric carcinoma.[14] We recently developed a new treatment method for repeated IP PTX administration using an IP access port. In this regimen, we administered PTX from both intravenous (IV) and IP routes along with oral S-1 and determined that the optimum dose of IP PTX was 20 mg/m^2.[15] Consequently, we conducted a phase II study in gastric cancer patients with peritoneal metastasis and demonstrated excellent results with a 1-year OS rate of 78 % and a MST of 23.6 months.[16]

In those studies, we found that this regimen is feasible and fairly effective for patients with massive ascites.[17] In fact, some patients experienced clinical downstaging with complete disappearance of ascites after the initial course of combination chemotherapy and, consequently, underwent salvage gastrectomy. In this study, we summarized the profiles and outcomes of those patients who received the conversion gastrectomy after exhibiting a significant response against the malignant ascites.

PATIENTS AND METHODS

Patients

From October 2005 to June 2011, 64 patients with advanced gastric cancer were enrolled by the Department of Surgical Oncology, University of Tokyo. These patients were suspected to have peritoneal metastasis with malignant ascites by CT scan. In all cases, peritoneal metastasis was confirmed by staging laparoscopy under general anesthesia. Peritoneal lavages were also taken, and the peritoneal cytology was pathologically examined using Papanicolaou staining to determine peritoneal lavage (CY1). A peritoneal access port was then implanted in the subcutaneous space of the lower abdomen, and a catheter was placed in the pelvic cavity. For patients with massive ascites, a drainage catheter was inserted intraperitoneally, and PTX was administered. Staging laparoscopy and access-port implantation was then performed for these patients after the ascites shrank.

PTX was administered intravenously at a dose of 50 mg/m^2 and intraperitoneally at a dose of 20 mg/m^2 on days 1 and 8, according to the results of the phase I study.[15] PTX was diluted in 1 L of normal saline and administered through the implanted peritoneal access port over 1 h concurrent with an IV infusion after standard premedication. S-1 was administered twice daily orally at a dose of 80 mg/m^2 day for 14 consecutive days, followed by 7 days of rest. The treatment course was repeated every 3 weeks, until unacceptable toxicity, disease progression, or a response that enabled a macroscopically curative operation was observed. At that point, we performed a second laparoscopy and objectively evaluated any macroscopic appearance changes of the peritoneal metastases using video-recorded images. Gastrectomy was considered if the following criteria were reached: (1) no distant metastasis except in the peritoneal area, (2) negative peritoneal cytology, and (3) metastatic nodules in the peritoneal cavity were apparently reduced or under control. After the gastrectomy, we restarted and continued combination chemotherapy using the same regimen as long as it remained effective with tolerable toxicity.

Written informed consent was obtained from all patients. This study was carried out in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Tokyo.

Evaluation and Statistical Methods

Objective tumor responses were evaluated every 2 courses of treatment during the study and classified based on RECIST guidelines. To evaluate the antitumor effects of the treatment on peritoneal metastasis, the amount of malignant ascites and peritoneal cytology were also taken into account. In accordance with the Japanese Classification of Gastric Carcinoma, the amount of ascites was assessed by radiologists using CT.[18] Determining the cytology of the ascites and collecting the peritoneal lavage fluid occurred at the end of each treatment course. The peritoneal lavage fluid was collected through the peritoneal access port. Toxicity was monitored weekly and graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 3.0.

A Pearson's Chi square test was used for statistical analyses of the differences between groups. P values <0.05 were considered statistically significant. The 1-year survival rate was estimated using the Kaplan–Meier method. All statistical analyses were performed using the JMP program, version 8.0 (SAS Institute, Cary, NC).

RESULTS

Responses to the Combination Chemotherapy

At the initial laparoscopy, peritoneal metastases (P1) were identified as the presence of small white nodules on the peritoneal surface, especially at the subdiaphragm,

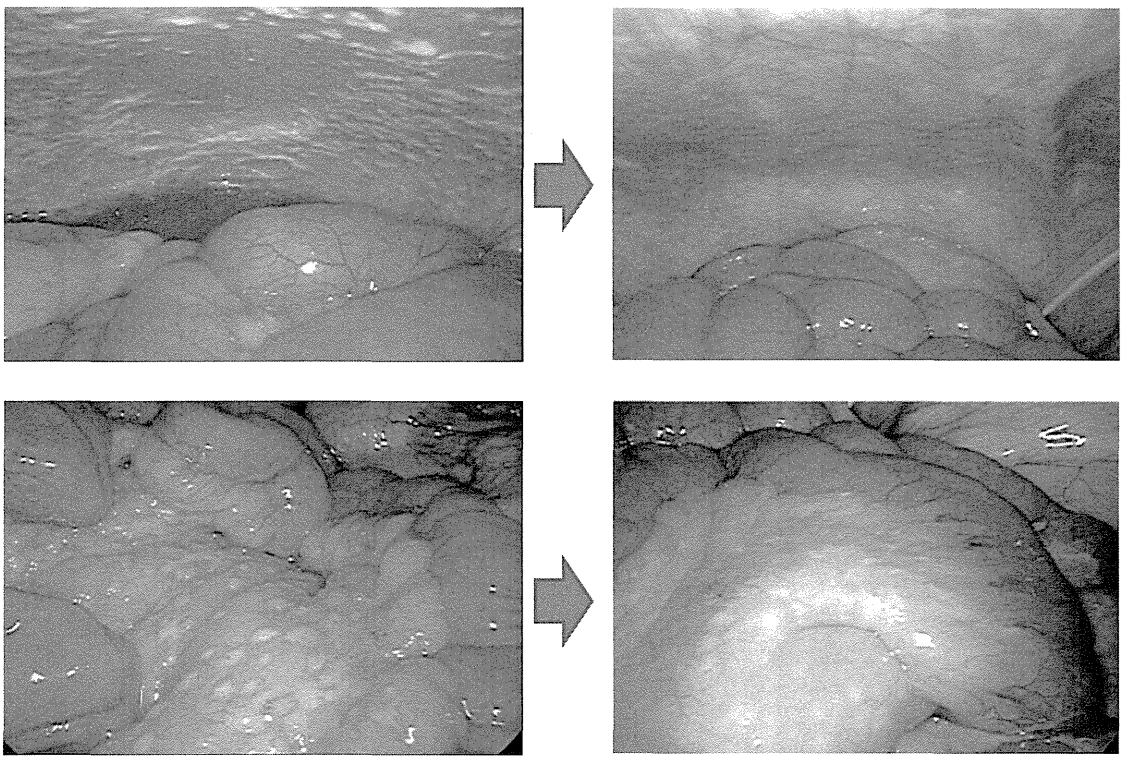

FIG. 1 Laparoscopic appearance of metastatic nodules in the Douglas Pouch (*upper*) and mesenteric surface (*lower*) at the initial laparoscopy before chemotherapy (*left*) and at the second laparoscopy performed after 9 cycles of IV and IP PTX and S-1 administration (*right*)

Douglas pouch, mesentery, and omentum. Significant amounts of malignant ascites were detected in all cases. Carcinoma cells were detected in the ascites or CY1 in 58 cases. After several courses of the combination chemotherapy, however, the second laparoscopy revealed that the number and size of peritoneal metastases were significantly reduced with a scarlike appearance in 42 patients (66 %). Laparoscopic findings in representative cases are shown in Fig. 1. Peritoneal cytology turned negative in 45 of 58 CY positive cases (78 %). In 21 cases that showed measurable lesions by RECIST, partial response (PR) was detected in 9 cases, and the response rate was 43 %. When examined by CT, ascites completely disappeared in 8 cases (Fig. 2) and were significantly reduced in an additional 20 cases.

Patient Characteristics

We performed salvage gastrectomy in 34 patients who showed significant responses in both peritoneal nodules and cytology. Time to surgery from the initiation of chemotherapy depended on the response in each case. The median course of chemotherapy before surgery was 5 courses (range, 2–16 courses), and 10 patients received chemotherapy for more than 10 courses. The characteristics of the patients who did and did not undergo gastrectomy are summarized in Table 1. The majority of the patients with peritoneal involvement were type 4 with undifferentiated histology. No significant differences were observed for age, gender, performance status, macroscopic and histologic types, depth of tumor invasion, and nodal status between the 2 groups. Although the majority of the patients had a high peritoneal cancer index (PCI) score for both groups, patients who received gastrectomy tended to have relatively limited peritoneal metastasis without positive cytology. Notably, all 6 CY negative patients at initial examination underwent gastrectomy.

Operative Results

For 34 patients, total and distal gastrectomies were performed in 30 and 4 cases, respectively, with D1 or D2 nodal dissection in 21 and 13 cases, respectively (Table 2). Combined resection of the spleen, pancreas, colon, small intestine, and adnexa was performed in selected cases, and R0 operation was achieved in 22 cases (65 %). We did not perform peritonectomy or HIPEC in order to avoid peritoneal adhesion that may seriously impair the effectiveness of the continued IP chemotherapy. Pathological examination of the resected stomach and lymph nodes revealed that grade 2 and grade 3 histological responses were obtained in 7 (21 %) and 1 (3 %) case(s), respectively. The postoperative complications anastomotic leakage and pancreatic

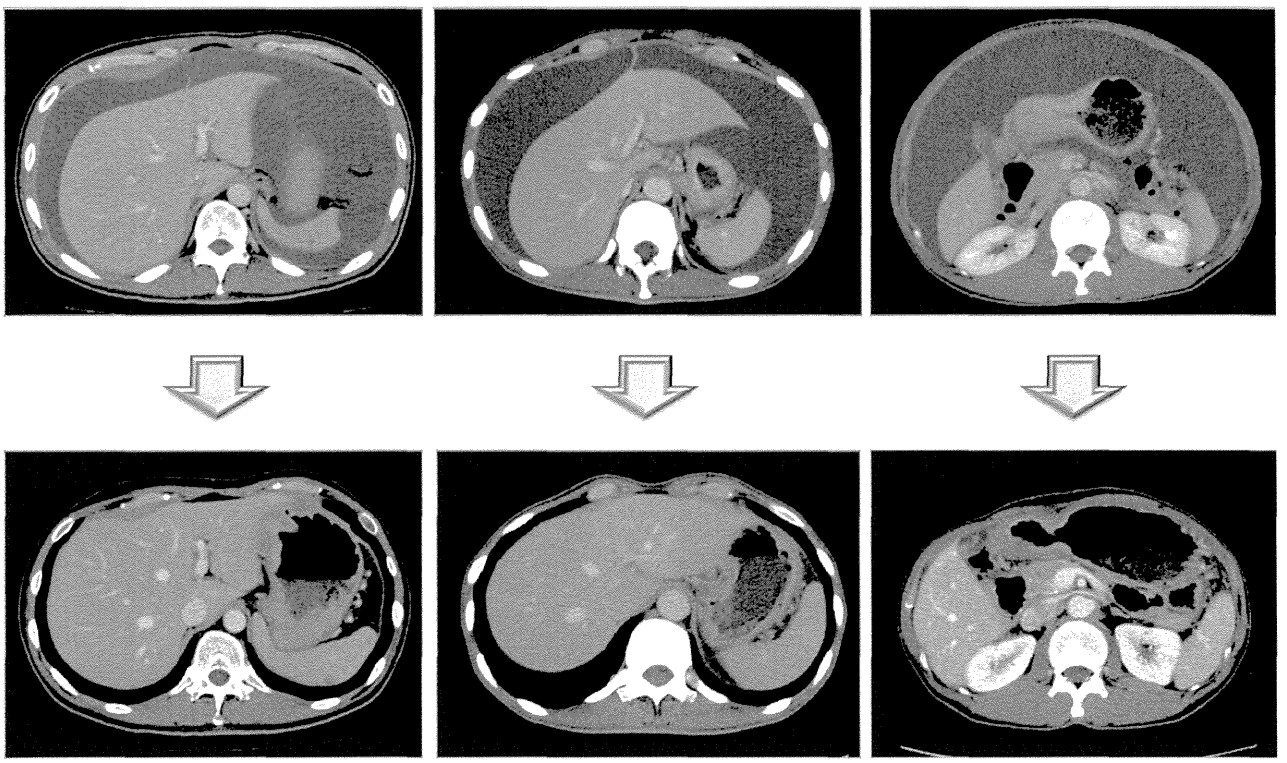

FIG. 2 CT images of ascites before and after induction chemotherapy. Ascites completely disappeared after 3, 5, and 9 cycles of combination chemotherapy in those 3 different cases

fistula developed in 1 case each and were completely addressed by conservative treatment. No mortality was observed.

Overall Survival

Overall survival curves of all the patients and patients with or without surgery are shown in Fig. 3. The MST for all patients was 16.6 months at the median follow-up period of 36 months (range, 2–202 months). In particular, the MST and 1-year survival of 34 patients who received gastrectomy reached 26.4 months and 82 % at the median follow-up period of 36 months (range, 6–202 months). On the other hand, the other 30 patients who did not receive gastrectomy because of poor responses had a MST of 12.1 months and a 1-year survival of 26 %.

During the follow-up period, recurrence was observed in 22 of the 34 patients with gastrectomy. Recurrent sites are peritoneum in 14: lymph nodes in 7, ovary in 2, meninges in 2 patients, and bone, pleura, and adrenal gland are involved in 1 patient.

Next, we examined outcome of the patients who received gastrectomy after 1–5, 6–10, and >10 courses of the combination chemotherapy. However, we found no survival differences among the 3 groups (Fig. 4). Similarly, no difference was observed in overall survival between R0 and R1/2 patients (Fig. 5). These data were not enough for selection of candidate for salvage gastrectomy, but rather suggest that the timing and curability of gastrectomy are not critical for the outcome of the patients with salvage gastrectomy.

DISCUSSION

Peritoneal metastasis is a frequent pattern of metastasis and recurrence for advanced gastric cancer, especially with serosal exposure and undifferentiated histology.[19–21] Various methods have been used to treat peritoneal dissemination, including systemic chemotherapy, IP chemotherapy, hyperthermia, immune therapy, and aggressive surgery. None of these treatments have provided satisfactory clinical outcomes, however. Recently, we found that repeated administration of PTX from both IV and IP routes combined with oral S-1 elicited remarkable antitumor effects for peritoneal metastasis of gastric cancer. Additionally, this treatment resulted in excellent survival with an MST of around 2 years.[15,16]

PTX is absorbed slowly through the lymphatic system after IP administration because of its large molecular weight and fat solubility. Moreover, because of its strong antiproliferative activity, IP PTX infusions rarely cause peritoneal adhesion that allows for repeated IP

TABLE 1 Clinical features

	Surgery (+) ($n = 34$)	Surgery (−) ($n = 30$)	P value
Gender			0.48
Male	14	15	
Female	20	25	
Age	55 (29–72)	57 (39–76)	0.88
PS			0.47
0	24	17	
1	9	11	
2	1	2	
Macroscopic type			0.42
3	11	7	
4	23	23	
Histologic type			0.67
Differentiated	2	3	
Undifferentiated	29	23	
Mixed	3	4	
Depth of tumor			0.85
T3	30	26	
T4	4	4	
Nodal metastasis			0.17
N0	13	15	
N1	13	8	
N2	5	1	
N3	3	6	
Peritoneal cytology			0.0043
CY0	6	0	
CY1	28	30	
Degree of peritoneal metastasis			0.11
Low (P1)	3	0	
Median (P2)	5	3	
High (P3)	26	27	
Ascites			
+	26	16	
++	4	3	
+++	4	11	
Ovarian metastasis			0.34
−	25	25	
+	9	5	
Hydronephrosis			0.38
−	29	23	
+	5	7	
Intestinal stricture			0.15
−	27	19	
+	7	11	

The amount of ascites (+, ++, +++) and hydronephrosis were defined on CT scan images by radiologists. Intestinal stricture was defined by enema study

TABLE 2 Operative procedures and results

Procedure	No. cases
Chemotherapy course before surgery	
2–5	13
6–9	10
>10	11
Type of gastrectomy	
Total gastrectomy	30
Distal gastrectomy	4
Lymph node dissections	
D1+a	21
D2	13
Combined resections	
Spleen	9
Distant pancreas	3
Small intestine	1
Colon	10
Adnexa	7
Resections	
R0	22 (65 %)
R1	6 (18 %)
R2	6 (18 %)
Histological effects	
Grade 1a	16 (47 %)
Grade 1b	10 (29 %)
Grade 2	7 (21 %)
Grade 3	1 (3 %)
Postoperative complications	
Anastomotic leakage	1
Pancreatic fistula	1

administration. Therefore, PTX is an ideal drug for IP chemotherapy. However, when administrated into abdomen only once, the depth of drug infiltration from the peritoneal surface was limited, thereby restricting the clinical efficacy of IP chemotherapy.[22–25] Therefore, we expected repeated IP administration to improve the antitumor effects of PTX against peritoneal metastasis.

Additionally, we administrated PTX at the same time via intravenous infusion. We predicted that the administration of PTX from 2 different routes may significantly improve the efficacy of PTX delivery to peritoneal lesions. In murine experiments, we confirmed that IV PTX was distributed around the tumor vessels in peritoneal nodules, while IP PTX directly infiltrated into the central area of peritoneal nodules from the tumor surface, particularly in hypovascularized areas.[16,26] The unique distribution of PTX in metastatic nodules may be critical for the marked shrinkage of peritoneal metastases that is observed. In fact, it has been recently reported that the combination of IP and

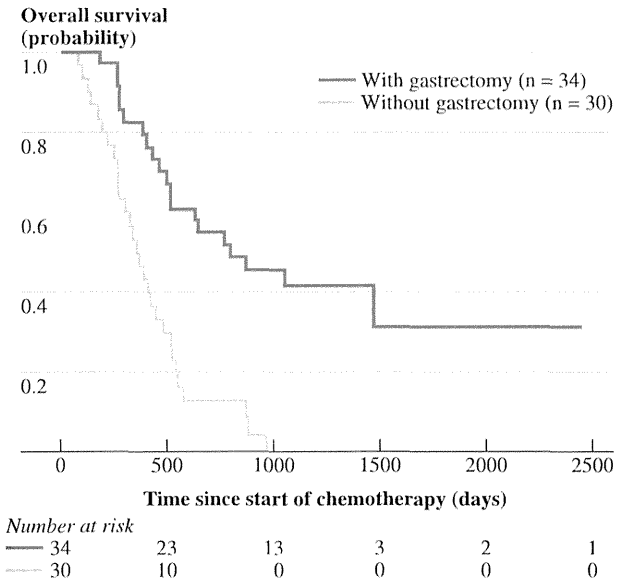

FIG. 3 Kaplan–Meier plot for overall survival in 34 patients who received conversion gastrectomy and 30 patients who did not

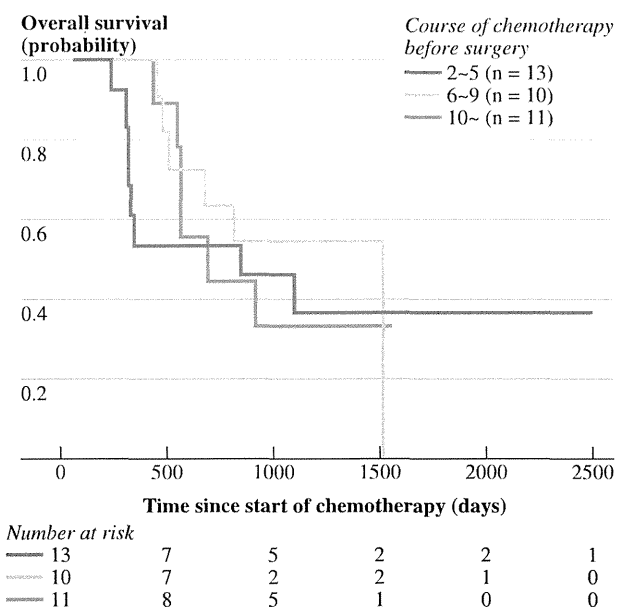

FIG. 4 Kaplan–Meier plot for overall survival of the patients who received gastrectomy after 2–5, 6–9, and ≥10 courses of combination chemotherapy

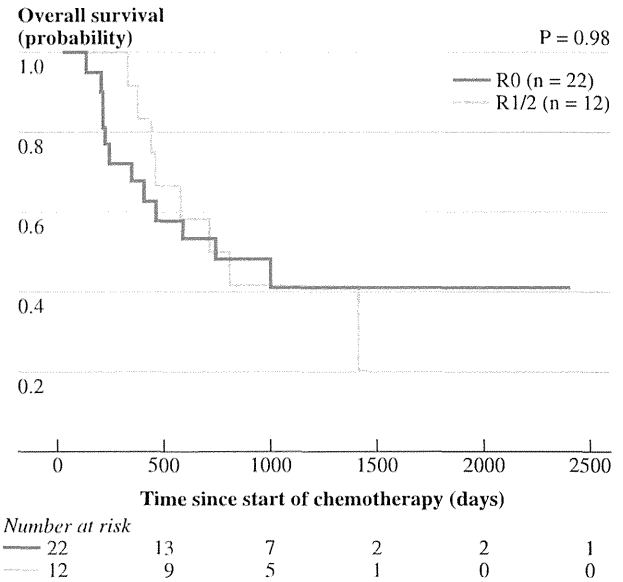

FIG. 5 Kaplan–Meier plot for overall survival of the patients who received R0 or R1/2 gastrectomy

IV chemotherapy, termed "bidirectional chemotherapy," showed excellent clinical effects for peritoneal malignancy.[27,28]

In this study, we demonstrated that our treatment could control peritoneal lesions even in highly advanced gastric cancer cases with malignant ascites. Laparoscopy revealed that peritoneal metastases were dramatically reduced, and peritoneal cytology turned negative in many cases with high PCI. Additionally, the MST of all the patients was remarkably better than those observed in previous reports.[6–8,10,11,13] Furthermore, we found apparent down-staging of peritoneal lesions in more than half the cases, and we successfully performed gastrectomy on those patients. The resulting MST for those patients was more than 2 years. In contrast, the MST of patients who did not undergo gastrectomy was around 1 year. Since the gastrectomy was performed on only good responders, comparing only MST is not adequate, and a comparative study will be necessary to determine the real efficacy of salvage gastrectomy. However, pathological examination revealed that most of the resected primary gastric tumors were categorized in histological grade 1, although the peritoneal lesions were completely replaced by fibrosis with no remaining cancer cells, suggesting that primary tumors are most resistant for this chemotherapy. In our experience, primary gastric tumors tend to have resistance and often show regrowth during the course of combination chemotherapy. This remains true even in cases where other metastases, including peritoneal legions, completely regress. Therefore, we suppose that gastrectomy may somehow contribute to the excellent survival in those patients.

The most important issue was determining reasonable criteria for performing gastrectomy in these patients. Thus far, we consider 3 clinical factors for gastrectomy: peritoneal cytology, macroscopic appearance of peritoneal metastasis at the second laparoscopy, and distant metastasis except in the peritoneal area. If cytology of the peritoneal