INTRODUCTION

The detection rate of early gastric cancer, and the overall survival rate of gastric cancer have been increasing because of improved diagnostic techniques and the promotion of routine mass screenings for gastric cancer. However, we still experience cases of far advanced gastric cancer with poor prognosis. Peritoneal dissemination is the most common form of recurrence in gastric cancer, and it is associated with a poor prognosis. Treatment of peritoneal dissemination is one of the most important challenges in the treatment of gastric cancer. However, no standard treatment for peritoneal dissemination has yet been proposed. Surgery alone or chemotherapy alone has no beneficial effect on survival. New anti-cancer drugs are being used for advanced and recurrent gastric cancer in many institutions [1]. A recent paper has suggested that neoadjuvant chemotherapy, such as preoperative intraperitoneal chemotherapy (IPC)
combined with systemic chemotherapy using S-1 and docetaxel may have a strong survival benefit for patients with scirrhus gastric cancer [2].

However, many questions remain unanswered, such as whether neoadjuvant chemotherapy is better than adjuvant chemotherapy, and whether IPC is better than systemic chemotherapy [2]. Moreover, there is as yet no effective treatment against peritoneal dissemination. The development of peritoneal metastasis is a multistep process, beginning with the detachment of cancer cells from the primary tumors, their attachment to peritoneal mesothelial cells, retraction of the mesothelial cells, and exposure of the basement membrane. After attachment to the basement membrane, the cancer cells degrade the extracellular matrix and proliferate [3]. Finally, the cancer cells induce angiogenesis and lymphangiogenesis. Many cytokines, adhesion factors, growth factors, matrix metalloproteinases (MMPs), and angiogenic factors play important roles in these steps. Briefly, cadherin and MMPs are related to the detachment of cancer cells from the gastric wall [4,5]. CD44 and integrin are important factors for the attachment of free cancer cells to the peritoneum, directly [6,7]. After attachment, migration factors such as c-met and autocrine motility factor (AMFR), and MMPs are needed for cancer cells to infiltrate the submesothelial matrix [5,8]. After infiltration, growth factors such as epidermal growth factor stimulate the growth of cancer cells, while simultaneously angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and IL-8 induce angiogenesis, and finally peritoneal dissemination is established [9-11]. Cyclooxygenase (COX) is a key enzyme in the conversion of arachidonic acid to prostaglandin, and COX-2 is significantly correlated with lymph node metastasis, stage, and prognosis in gastric carcinoma, suggesting that COX-2 might be correlated with invasion and metastasis of gastric carcinoma [12].

The second cascade of peritoneal metastasis is called trans-lymphatic metastasis. In this cascade, cancer cells migrate into the submesothelial lymphatic vessels through milky spots, stomata or initial lymphatics. There are four entrance gates of peritoneal free cancer cells into the submesothelial lymphatic vessels [13].

Recently, molecular targeting therapies combined with anti-cancer drugs are being utilized to treat various cancers and have shown a survival benefit for patients with cancer. The study of factors relevant to the development of peritoneal metastasis may have a significant impact on the treatment of gastric cancer. In the present study we compared cytokines, MMPs, angiogenic factors, invasion, adhesive ability, migration, and the expression of COX in the gastric cancer cell line MKN-45 and the high-potential peritoneal dissemination gastric cancer cell line MKN-45P which Miyagi et al. [14] established in our department.

MATERIALS AND METHODS

Cell lines

We used the high-potential peritoneal dissemination cell line MKN-45P, which was established from the human gastric cancer cell line MKN-45 (derived from a poorly differentiated adenocarcinoma in a 62-year-old woman; Health Science Research Resources Bank, Tokyo, Japan), in our institution as described previously [14]. Briefly, nude mice (BALB/c nu/nu) were subcutaneously inoculated with MKN-45 cells and the subcutaneous nodules were removed and injected into other mice intraperitoneally. The cancer cells from the peritoneal nodules were injected into the abdominal cavity of other mice. The process was continued through to a seventh generation. The resulting high potential peritoneal dissemination cell line was named MKN-45P. MKN-45 and MKN-45P cells were maintained in RPMI-1640 medium (Nihon Seiyaku Co., Komaki, Aichi, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Uxbridge, Middlesex, UK), 2mM-glutamine, and penicillin-streptomycin (50 IU/mL and 50 μg/mL, respectively) at 37.0°C in humidified air with 5% CO₂.

Measurement of cytokines in conditioned medium

For measurement of cytokines in conditioned medium, MKN-45 cells (1×10⁶ cells/10mL) or MKN-45P cells (1×10⁶ cells/10mL) were placed in 100 mm tissue culture dishes (IWAKI Co., Funabashi, Chiba, Japan) and cultured for 72 h in medium containing 10% FBS at 37.0°C in humidified air with 5% CO₂. The number of cells in each cell line was evaluated visually at 12, 24, 48, and at 72 h (values: mean of three fields). The supernatant was then collected, and the concentrations of IL-1β, IL-6, IL-8, IL-10, hepatocyte growth factor (HGF), transforming growth factor-β1 (TGF-β1), VEGF, MMP-2, MMP-9, and tissue inhibitor of metalloproteinases-1 (TIMP-1) proteins were measured using an enzyme-linked immunosorbent assay (ELISA) method (IL-1β, IL-8 and IL-10: Bio Source Europe S. A., Nivelles, Belgium; IL-6: Fujirebio Inc., Tokyo, Japan; HGF: Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan; TGF-β1 and VEGF: R & D Systems Inc., Minneapolis, MN, USA;
MMP-2, MMP-9 and TIMP-1: Daiichi Fine Chemical Co. Ltd., Takaoka, Toyama, Japan). Each cytokine was measured in 5 samples, and the means of these cytokines were compared between the MKN-45 cells and the MKN-45P cells.

**Invasion assay**

An invasion assay was performed in vitro to confirm interstitial invasion in the gastric cancer cell lines. Experiments were conducted using 24-well chemotaxicell chambers (Kurabo Industries Ltd., Osaka, Japan). Matrigel (BD Bioscience San Jose, CA, USA) was diluted in phosphate-buffered saline (PBS) to a concentration of 0.75 mg/mL, and 100 μL of this solution was placed into the chambers and air-dried overnight (Matrigel coating). Then, 200 μL of RPMI-1640 medium, containing 0.1% bovine serum albumin (BSA) was added to the Matrigel-coated chambers, and after allowing the film to swell (for 1 h), the MKN-45 or MKN-45P cell suspensions (2.5 × 10^4 cells) were placed in the chambers. Then, 700 μL of chemoattractant solution (adjusted to a fibronectin level of 10 μg/L with serum-free RPMI-1640) was added to the bottom chamber and the cells were cultured for 48 h in a 5% CO2 incubator. At 48 h, the cells on the upper surface of the filter were removed using a swab, and the cells that had infiltrated to the bottom surface of the filter were fixed in 70% ethanol for 30 min, subjected to Giemsa staining, and observed visually under a microscope (at 200× magnification). The number of invasive cells was the average of ten random views. The non-Matrigel-coated chambers were used as controls. Percent invasion was calculated as the mean of cells invading through the Matrigel insert membrane/mean of cells migrating through control insert membrane × 100.

**Adhesion assay**

Adhesion assay was performed to confirm cell-matrix adhesion in the gastric cancer cell lines. MKN-45 or MKN-45P cells (1 × 10^4 cells) were seeded on fibronectin, laminin or type IV collagen-coated 96-well plates, and were cultured for 6 h in a 5% CO2 incubator. At 6 h, after unattached cells were removed, Tetra Color ONE (Cell Proliferation Assay System) (Seikagaku Co., Tokyo, Japan) was added to each 96-well plate and the cells were incubated for 2 h at 37°C. Then the percent adhesion was calculated as absorbance at 450 nm using a 96-well microplate reader.

**Scratch wound assay**

Cells were grown to confluence in a 100 mm Tissue Culture Dish (Iwaki Co., Funabashi, Chiba, Japan). After 24 h, the monolayers were scratched using a 200 μL, sterile plastic pipette tip and washed twice with complete medium. The cells were allowed to migrate onto a plastic surface and photographed. Two random pictures under a microscope (at 4 × magnification) were taken for each wound immediately after the wound was inflicted to the cell monolayer and 12, 24 h.

**Western blotting**

Whole-cell lysates were prepared for Western blot analyses using a lysis buffer containing 0.75 mM Tris (pH7.5), 10% glycerol, and 2% SDS. After centrifugation the supernatant was collected as total proteins. Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay (Bio-Rad Laboratories, Richmond, VA, USA). Six μg of proteins were denatured in a SDS sample buffer [0.5 M Tris-HCl (pH6.8), 10% glycerol, 10% SDS, 10% mercaptoethanol, and 0.01% Bromophenol blue] at 100°C for 5 min. Samples were separated by a denaturing 10% SDS-polyacrilamide gel and transferred to polyvinylidene difluoride membranes (Immobilon, Bedford, MA, USA). Membranes were blocked with 1% skimmed milk in 0.5% Tween20 adding TBS (20 mM Tris-HCl (pH7.6), 137 mM NaCl) at 4°C overnight and incubated with primary polyclonal antibodies that recognize COX-1 (1 : 1,000, Santa Cruz, Inc. Santa Cruz, CA, USA), or COX-2 (1 : 1000, Santa Cruz). Secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Vector Laboratories, Inc. Burlingame, CA, USA) was used at 1 : 500 to detect primary antibodies, and enzymatic signals were visualized by enhanced chemiluminescence (ECL).

**Statistical analysis**

Statistical tests were performed by Student’s t-test and χ² test. P values <0.05 were considered significant.

## RESULTS

**Measurement of cytokines in condition medium**

The number of MKN-45 or MKN-45P cells was counted at 24, 48 and 72 h. There was no difference in the number of cancer cells between the two cell lines. The concentration of cytokines in conditioned media from MKN-45 or MKN-45P were shown in Table 1. The concentrations of IL-6, IL-8, VEGF and MMP-2 protein in the culture supernatants of MKN-45P were significantly higher than those of MKN-45 (M*= 0.045, P= 0.011, P= 0.013, and P= 0.021, respectively) (Table 1).
Invasion assay

The mean number of invasive MKN-45 and MKN-45P cells was counted by invasion assay. There was no significant difference in percent invasion between the MKN-45 cells and the MKN-45P cells ($P = 0.7579$).

Adhesion assay

Adhesion assay was performed to compare the adhesive ability of MKN-45 cells and MKN-45P cells to matrix. In the type IV collagen or laminin-coated groups there was no significant difference in percent adhesion between the MKN-45 cells and the MKN-45P cells ($P = 0.2562$ and $P = 0.6236$). However, in the fibronectin-coated group, percent adhesion of MKN-45P cells was significantly higher than that of MKN-45 cells ($P = 0.0009$) (Fig. 1).

Scratch wound assay

Using the scrape wound-healing migration assay to compare migration and motility, there were no differences in migration between the MKN-45 cells and the MKN-45P cells at 12 h and 24 h.

Western blotting

Western blotting was performed to compare the

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**TABLE 1.**

Comparison of cytokines between MKN-45 and MKN-45P

<table>
<thead>
<tr>
<th></th>
<th>IL-1β (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-8 (pg/mL)</th>
<th>VEGF (pg/mL)</th>
<th>MMP-2 (ng/mL)</th>
<th>TIMP-1 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKN-45</td>
<td>0.9±0.7</td>
<td>1.2±0.7</td>
<td>381.9±147.1</td>
<td>1335.0±624.3</td>
<td>0.3±0.1</td>
<td>2.7±1.8</td>
</tr>
<tr>
<td>MKN-45P</td>
<td>0.4±0.2</td>
<td>2.9±0.6</td>
<td>891.4±210.2</td>
<td>3806.0±229.8</td>
<td>0.7±0.5</td>
<td>6.0±4.0</td>
</tr>
<tr>
<td>P value</td>
<td>0.109</td>
<td>0.045</td>
<td>0.011</td>
<td>0.013</td>
<td>0.021</td>
<td>0.126</td>
</tr>
</tbody>
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**Fig. 1.** The result of adhesion assay. In the type IV collagen or laminin coating group, there was no significant difference in the %adhesion between the MKN-45 cells and the MKN-45P cells ($P = 0.0616$ and $P = 0.6236$). However, in the fibronectin group, the %adhesion in the MKN-45P cells was significantly higher than that in the MKN-45 cells ($P = 0.0239$).

**Fig. 2.** COX-1 and COX-2 protein expression on Western blotting. The expressions of COX-1 and COX-2 were detected in both cell lines.

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**Fig. 2.** COX-1 and COX-2 protein expression on Western blotting. The expressions of COX-1 and COX-2 were detected in both cell lines.
expressions of COX-1 and COX-2 in MKN-45 cells and MKN-45P cells. We observed the expression of COX-1 and COX-2 in both cell lines (Fig. 2).

DISCUSSION

We compared the concentration of cytokines in culture supernatant of the gastric cancer cell line MKN-45 with that in the high-potential peritoneal dissemination gastric cancer cell line MKN-45P which had been established from MKN-45. The concentrations of IL-6, IL-8, VEGF and MMP-2 protein were significantly higher in the supernatant of culture medium of MKN-45P than in MKN-45.

IL-6 is a 26 kDa molecular weight protein that is produced by monocytes, macrophages, T-lymphocytes, and endothelial cells. It is related to hemopoiesis, immunity, and inflammation, and is a multifunctional regulator in the nervous and endocrine systems [15]. Moreover, IL-6 has been reported as a prognostic factor in gastric carcinoma, and was significantly correlated with the incidence of lymph node metastasis and of liver metastasis [15]. IL-8 is an 8kDa molecular weight polypeptide and is one of the α-chemokines which belong to the C-X-C family, which are produced mainly by monocytes, macrophages, and endothelial cells, and is related to the migration of white corpuscles and inflammation [16]. Moreover, IL-8 has been reported as a prognostic factor in gastric carcinoma, and was significantly correlated with the depth of invasion and vessel infiltration [16]. IL-6 and IL-8 are related to the accomplishment of peritoneal dissemination by inducement of angiogenesis [10,11].

VEGF is a 34-45 kDa molecular weight typical angiogenic factor which is secreted mainly from monocytes, macrophages, pituitary cells, cancer cells, and smooth muscle cells, and is significantly related to angiogenesis in gastric cancer [17]. VEGF, as well as functioning as a growth factor, is able to function as a vascular permeability factor. Increased permeability of blood vessels facilitates the extravasation of proteins and the formation of ascites [18]. In previous reports, the expression level of VEGF has been found to be directly associated with the production of ascites and carcinomatosis [18,19]. Aoyagi et al. [20] reported that immunohistochemical expression of VEGF in gastric cancer tissue was correlated with peritoneal metastasis from gastric cancer, and that the expression of VEGF in cancer cells was a useful indicator of peritoneal recurrence. Moreover, Imaizumi et al. [21] reported that bevacizumab, which is a humanized monoclonal antibody against VEGF, suppressed peritoneal dissemination from gastric cancer in a peritoneal metastasis model. These studies provide clear evidence that VEGF is an essential element in the development of peritoneal metastasis and support the result of our in vitro study.

Degradation of the extracellular matrix is considered to be a prerequisite for peritoneal metastasis, and MMPs are thought to play an important role in this process [22,23]. There are many reports that highly invasive cancer cells with a high potential for metastasis stimulate the production of MMPs [22], and MMP-2 is significantly correlated with depth of invasion, lymph node metastasis, and distant metastasis of gastric cancer [24].

A loss in balance or a disequilibrium between MMPs and TIMPs, which inhibit the activity of MMPs, is thought to be a major factor leading to invasion and metastasis [25]. Some studies have reported that increased TIMP-1 or administration of TIMP-1 inhibited peritoneal invasion [25,26]. Miyagi et al. [14] reported that the TIMP-1 gene was transferred to MKN-45P by an adenoviral vector, and that peritoneal dissemination was significantly inhibited in a TIMP-1 transfected group compared with a non-virus group and a Lac-Z transfected group, using a peritoneal metastasis model.

The development of peritoneal metastasis is a multistep process, beginning with the detachment of cancer cells from the primary tumor, attachment to peritoneal mesothelial cells, retraction of the mesothelial cells and exposure of the basement membrane, degradation of the extracellular matrix, proliferation of the cancer cells, and angiogenesis, and it is clear that many agents are involved at the various stages of this process [3]. Therefore, the invasion, adhesion, and migration abilities of cancer cells are very important to the accomplishment of peritoneal dissemination. The present study found no significant difference in invasion or migration between MKN-45 and MKN-45P cells. However, the adhesion of MKN-45P to fibronectin was significantly higher than that of MKN-45. Integrins play an important role in the attachment of gastric cancer cells to the submesothelial basement membrane of the peritoneum. Over-expression of integrin-α2β1 and α3β1 are recognized in MKN-45P and OCUM-2MD3, which are high frequency peritoneal metastasis gastric cancer cell lines, and anti-α2β1- and α3β1-integrin antibody significantly reduced the number of cancer cells on the peritoneum in nude mice [7,27]. Moreover, α3β1-integrin mediates cell-binding to fibronectin, which is a component of basement membrane, and these are thought to be important for attachment of free gastric cancer cells to
the peritoneum [7].

COX is a key enzyme in the conversion of arachidonic acid to prostaglandin, and two isoforms of COX, namely COX-1 and COX-2, have been identified [28]. COX-1 is constitutively expressed in many tissues and is considered to be involved in various physiological functions, whereas COX-2 is induced by pathological stimuli, such as inflammation, various growth factors and cytokines produced by tumor cells [28]. Increased COX-2 expression has been reported in colorectal, pancreatic, hepatocellular and other cancers [29]. Li et al. [29] reported that COX-2 expression in gastric adenocarcinoma was higher than that in the paracancerous tissues, and was related to lymph node metastasis and the depth of invasion, suggesting COX-2 might be correlated to the occurrence and advancement of gastric carcinoma. Leung et al. [30] reported that overexpression of COX-2 in gastric cancer is associated with up-regulation in VEGF and angiogenesis. However, in this study, we observed the expression of COX-1 and COX-2 in both cell lines on Western blotting.

These results suggested that the secretion of IL-6, IL-8, VEGF and MMP-2 from cancer cells was related to the establishment of peritoneal dissemination by promoting angiogenesis and degradation of the extracellular matrix, and by increasing the ability of cancer cells to adhere to the peritoneum.

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REFERENCES


