

A Novel Experimental Mouse Model of Peritoneal Dissemination of Human Gastric Cancer Cells: Different Mechanisms in Peritoneal Dissemination and Hematogenous Metastasis

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We established a new cell line, AZ-P7a, with high peritoneal-metastatic potential in nude mice. AZ-P7a cells were derived from the human gastric carcinoma line AZ-521, which has low capacity for peritoneal dissemination. AZ-P7a cells developed peritoneal metastasis in 11/14 (78.6%) mice, whereas the parental AZ-521 cells developed metastasis in 2/6 (33.3%) mice. The metastatic foci in the peritoneum showed essentially the same histological appearance as those induced by parental cells. The tumorigenicity and the motile activity of AZ-P7a cells were stronger than those of the parental AZ-521 cells; in contrast, adhesion to the extracellular matrix and the production of vascular endothelial growth factor by AZ-P7a cells were decreased. In fluorescence-activated cell sorter (FACS) analysis, AZ-P7a cells expressed significantly greater levels of integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ and $\alpha v\beta 5$, as compared with AZ-521 cells. However, $\alpha 1$, $\alpha 4$, $\alpha v\beta 3$, hCD44H, hCD44v3, hCD44v6 and hCD44v10 were not expressed in either cell line. AZ-P7a cells developed no liver metastasis when administered by the intrasplenic injection method, though the highly liver metastatic cell line AZ-H5c showed the same rate of peritoneal dissemination as that exhibited by AZ-P7a cells after intraabdominal injection. These findings suggested that the mechanism of peritoneal dissemination differed from that of hematogenous metastasis. Moreover, the latter appears to be controlled by more complex mechanisms than the former. Thus, this cell line might be useful for investigating the mechanism of peritoneal dissemination of human gastric cancer.

Key words: Gastric carcinoma lines — Peritoneal dissemination model — Nude mice

Peritoneal dissemination is most frequently observed in human gastric cancer and is one of the most important causes of cancer deaths. Although some trials to prevent peritoneal dissemination of gastric cancer have been performed,¹⁾ including chemotherapy, hyperthermia and peritonectomy, no effective prolongation of survival has been attained in most patients.²⁾ The establishment of relevant animal models of metastasis is extremely important for the development of new therapeutic modalities for gastric cancer. To elucidate the molecular mechanisms of metastasis, several excellent hematogenous metastatic models have been established by intrasplenic injection^{3–5)} and the orthotopic implantation technique.^{6–8)} We also established and reported a highly liver metastatic cell line, AZ-H5c, derived from a human gastric cancer line, AZ-521.^{9,10)} However, only a few peritoneal dissemination models have been reported for human gastric cancer.¹¹⁾

In this study, we established a new cell line, designated as AZ-P7a, with a high potential of peritoneal dissemination in nude mice. Subsequently, we characterized the

tumorigenicity, the motility, the adhesive activity, the production of cytokines and the cell surface expression of adhesion molecules of this cell line, in comparison with those of the parental AZ-521 cell line, which has a low metastatic potential. Furthermore, to investigate the phenotypic difference of the cells in peritoneal dissemination and hematogenous metastasis, AZ-P7a cells were injected into the spleen and AZ-H5c cells were injected into the abdominal cavity of nude mice. A comparative analysis among the three cell lines, AZ-521, AZ-H5c and AZ-P7a, was also performed.

MATERIALS AND METHODS

Animals Athymic female BALB/c *nu/nu* mice, 6–7 weeks old and weighing 20–22 g, which originated from the Central Institute for Experimental Animals (Kawasaki), were purchased from CLEA Japan (Tokyo). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and provided with sterile food, water and cages.

Cell line and cell culture The AZ-521 human gastric carcinoma cells line was obtained from the Japanese Cancer

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Research Resources Bank (Tokyo). AZ-H5c is a highly liver metastatic cell line derived from AZ-521, and was previously reported.^{9,10} The cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma, St. Louis, MO), 2 ml L-glutamine, 50 units/ml penicillin and 50 mg/ml streptomycin (Gibco, Grand Island, NY) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The cells were passaged and expanded by trypsinization of cell monolayers followed by replating every 4–5 days. The culture medium was changed every 2–3 days. For mouse inoculation, the cells in log-phase growth were harvested by trypsinization, and a medium containing 10% FCS was added. The cells were washed three times with serum-free RPMI 1640, then resuspended in phosphate-buffered saline (PBS). They were kept at 37°C until inoculation into the mice.

Establishment of highly peritoneal-metastatic cell lines

The human gastric carcinoma cell line AZ-521 (1×10^7) was inoculated intraperitoneally into nude mice. After 3 weeks, the mice were killed and a few peritoneal-metastatic nodules were harvested aseptically. Single cell suspensions were made by mincing and trypsinization and were cultured *in vitro*. The cells in this culture were designated as AZ-P1a. The same procedure was repeated using AZ-1Pa cells, and the AZ-P7a cell line was established upon the seventh cycle of stepwise selection. Each resultant cell line at *in vitro* passages 3–7 was used for experiments.

Evaluation of the growth rate and metastatic potential of cell lines

To evaluate the *in vivo* tumorigenicity of the parental AZ-521 and metastatic AZ-7Pa cell lines, cultured cells (5×10^6 cells) were inoculated subcutaneously into three regions each of three nude mice. The mice were surveyed daily and the tumor was measured with calipers. Tumor volumes were estimated by using the following formula: $V = L \times W \times H / 2$ (V , volume; L , length; W , width; H , height). Next, using 12-well culture plates, 2×10^4 cells/well were seeded in standard culture medium supplemented with 10% FCS at 37°C to evaluate *in vitro* tumorigenicity. Every two days, from day 1 to day 7, cultured cells were detached by trypsinization and counted on a cell counter under a light microscope at a magnification of $\times 200$. To evaluate the peritoneal metastatic potential, cultured cells (1×10^7) from each cell line were inoculated into the peritoneal cavity of nude mice. Between 1 and 9 weeks after the inoculation, the mice were killed and examined macroscopically for the presence of peritoneal metastasis.

Motility assay A 24-well transwell cell culture chamber (Coster, Cambridge, MA) was used for the motility assay, as previously described.¹⁰ AZ-521 and AZ-P7a (2×10^4 cells/well) were suspended in serum-free RPMI 1640 medium and plated on the upper chamber. The lower chamber contained serum-free RPMI 1640 medium and

human cellular fibronectin (50 $\mu\text{g/ml}$). After incubation for 24 h at 37°C in a CO₂ incubator, the cells on the surface of the filter of the upper chamber were wiped off. Thereafter, cells were fixed with methanol and stained with Giemsa solution. Motile activity was quantified by counting cells that had migrated to the back surface of the filter. Five fields were counted in each filter under a light microscope at a magnification of $\times 125$. The experiment was performed in triplicate.

Adhesion assay To examine the effect of extracellular matrix (ECM) components and serum on cell proliferation, a 96-well Transwell cell culture chamber coated with fibronectin, type IV collagen and laminin (Becton Dickinson Labware, MA) was used for the adhesion assay, as previously described.³ AZ-521 and AZ-P7a cells (1×10^4 cells/well) were suspended in RPMI 1640 medium supplemented with 10% FCS and incubated for 1 h at 37°C. Unattached cells were removed gently by washing with PBS, and the number of attached cells was estimated using the MTT (3{4,5}-dimethylthiazol-2,5-diphenyl tetrazolium bromide; Sigma) assay, as previously described.^{12,13} The absorbance of each well was quantified with a Nova-Path microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. The experiment was performed in triplicate.

Antibodies and fluorescence-activated cell sorter (FACS) analysis

Expression of adhesion molecules on the surface of AZ-521 and AZ-P7a cells was analyzed by flow cytometry using various monoclonal antibodies (mAbs). Mouse anti- $\alpha 1$ chain (TS2/7), anti- $\alpha 2$ chain (IIE10), anti- $\alpha 3$ chain (IIF5), anti- $\alpha 4$ chain (B5G10), anti- $\alpha 5$ chain (A5-PUJ2) and anti- $\alpha 6$ chain (A6-ELE) mAbs were kindly provided by Dr. Martin E. Hemler (Dana-Farber Cancer Institute, Boston, MA). The mAbs against $\alpha v \beta 3$ and $\alpha v \beta 5$ were purchased from Chemicon (Temecula, CA), hCD44H, hCD44v3 and hCD44v5 were from R&D Systems (Abingdon, UK) and hCD44v6 and hCD44v10 were from Bender MedSystems (Vienna, Austria). The FACS analysis was carried out as previously reported.¹⁴ The experiment was performed in duplicate.

Production of cytokines AZ-521 and AZ-P7a cells were cultured in 96-well plates at a cell density of 1×10^4 cells/well in RPMI 1640 medium supplemented with 10% FCS for 24 h and the next day, culture media were changed to serum-free RPMI 1640. After 2 days, supernatants were collected and concentrations of vascular endothelial growth factor (VEGF), IL-6, IL-8 and IL-10 were measured using enzyme-linked immunosorbent assay kits (VEGF: IBL, Fujioka; IL-6 and IL-8: TFB, Tokyo; IL-10: Amersham, Buckinghamshire, England). The experiment was performed in duplicate.

Flow cytometric analysis of nuclear DNA content

Nuclear DNA content was assessed by flow cytometry. Approximately 2×10^4 cells stained with propidium iodide

were measured and histograms were generated for each cell line. Histograms with coefficients of variation (CV) of less than 5% were considered to be of good quality.

Intrasplenic injection of AZ-P7a and intraabdominal injection of AZ-H5c (cross injection) To elucidate the difference between hematogenous and peritoneal metastasis, AZ-P7a (5×10^6) was injected into the spleen and AZ-H5c (1×10^7) into the abdominal cavity of nude mice. The mice were killed either 3 or 5 weeks after the injection and the rate of metastasis of each cell line was evaluated.

Statistical analysis All data were analyzed using the unpaired *t* test and expressed as the mean \pm SE. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Macroscopic and histopathologic findings As shown in Fig. 1, the mice injected with AZ-P7a developed peritoneal metastases that appeared as numerous white nodules on the peritoneum and the abdominal organs; they were associated with bloody ascites. It was also observed that none of the cell lines induced liver or pulmonary metastasis within the experimental interval, in this study. The histopathological findings of these foci corresponded to poorly differentiated adenocarcinoma, with essentially the same appearance as the parental AZ-521 and AZ-P7a tumors (Fig. 2).

Tumorigenicity and peritoneal metastasis We first determined the *in vivo* tumorigenicity of the AZ-521 and AZ-P7a cell lines. AZ-521 and AZ-P7a cells were injected s.c into nude mice to determine their *in vivo* tumorigenicity. As shown in Fig. 3, AZ-P7a tumors grew faster than AZ-521 tumors from 5 days after inoculation. Conversely, the *in vitro* proliferation activity of AZ-P7a cells was lower than that of AZ-521 cells on day 7 of incubation

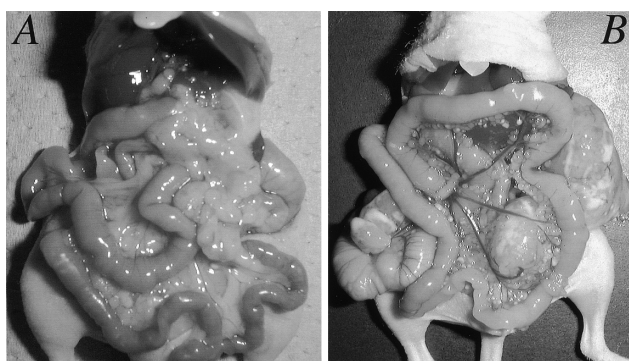


Fig. 1. Macroscopic views of peritoneal dissemination of cancer cells 3 weeks after intraabdominal injection. A, AZ-521; B, AZ-P7a. Extensive disseminated tumors and bloody ascites were observed with AZ-P7a, compared with AZ-521.

(Fig. 4). The characteristics of each of the stepwisely established cell lines are summarized in Table I. AZ-P7a cells disseminated within the peritoneal cavity in 11/14 (78.6%) mice, whereas parental AZ-521 developed in 2/6 (33.3%) mice 3 weeks after intraabdominal injection. The metastatic foci in the peritoneum showed essentially the same histological appearance as in the case of parental involvement. Consequently, AZ-P7a was the most highly peritoneal metastatic cell line.

Motile activity *in vitro* Since motile activity is reported to be an important factor in the process of tumor metastasis,¹⁵ we compared the motile activities of AZ-521 and AZ-P7a cells. The number of cells that migrated to the back surface of the filter was clearly higher in the case of AZ-P7a compared with AZ-521 cells in the presence of 50 μ g/ml human cellular fibronectin. The numbers of migrating AZ-521 and AZ-P7a cells were 0/field and 63.0 ± 35.2 /field, respectively ($*P < 0.005$) (Fig. 5).

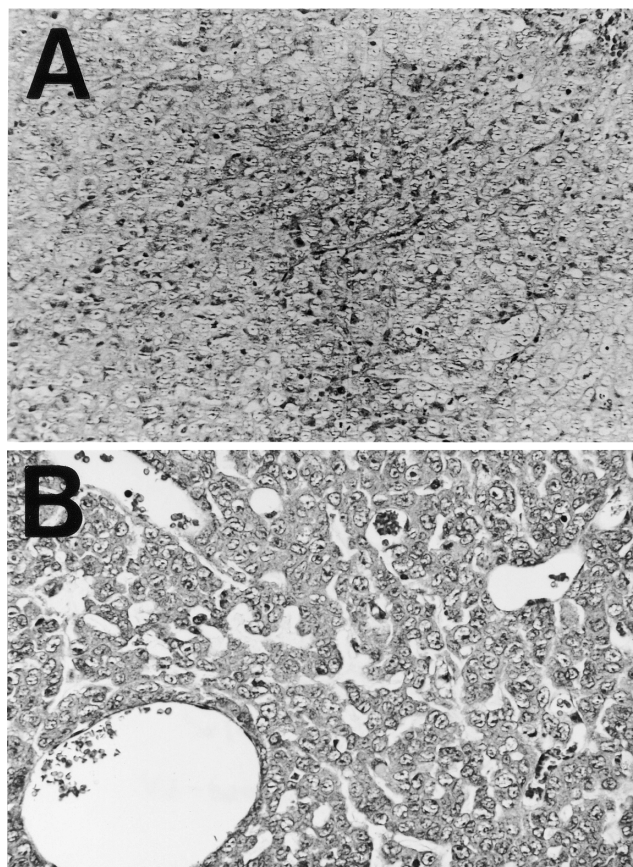


Fig. 2. Histopathological findings of disseminated tumors. A, AZ-521; B, AZ-P7a. Disseminated tumors showed findings compatible with those of poorly differentiated adenocarcinoma, and had essentially the same appearance in the case of both cell lines (A, $\times 100$; B, $\times 200$).

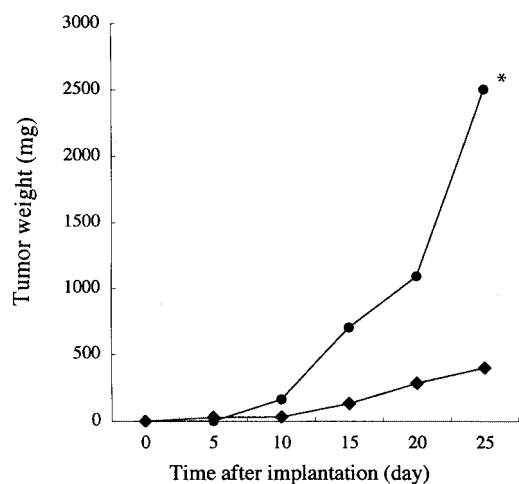


Fig. 3. *In vivo* growth analysis was performed by s.c. injection of cancer cell suspensions into nude mice. AZ-P7a cells (●) grew significantly faster than AZ-521 cells (◆) (**P*<0.005).

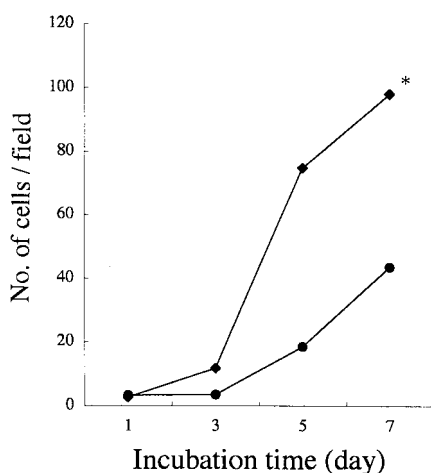


Fig. 4. *In vitro* growth analysis of AZ-P7a cells (●) and AZ-521 cells (◆). The growth of AZ-P7a cells was less than that of the parental AZ-521 cells (**P*=0.0005).

Adhesion activity *in vitro* Among the sequential steps of tumor metastasis, the adhesion of tumor cells to ECM is known to be critical.^{2,16)} Therefore, we determined whether AZ-521 and AZ-P7a could adhere to such ECM components as fibronectin, type IV collagen and laminin. As shown in Fig. 6, the *in vitro* adhesive activity of AZ-P7a cells to ECM was significantly lower than that of AZ-521 cells (**P*<0.0001).

Expression of cell adhesion molecules as assessed by FACS analysis To clarify the involvement of cell surface antigens in the process of metastasis, the expression of several cell adhesion molecules on the cell surface was

Table I. Metastasis after the Intraabdominal Injection of Gastric Cancer Lines

Gastric carcinoma lines	Cell dose	Autopsy (w)	No. of mice with peritoneal metastasis/total no. of mice (%)
AZ-521	5×10 ⁶	7	2/14 (14.3)
AZ-521	1×10 ⁷	3	2/6 (33.3)
AZ-P1a	1×10 ⁷	4.5	1/6 (16.7)
AZ-P2a	1×10 ⁷	4.5	3/4 (75)
AZ-P3a	1×10 ⁷	9	1/2 (50)
AZ-P4a	1×10 ⁷	3	3/4 (75)
AZ-P5a	1×10 ⁷	4.5	3/5 (60)
AZ-P6a	1×10 ⁷	3.5	4/5 (80)
AZ-P7a	1×10 ⁷	1	3/4 (75)
AZ-P7a	1×10 ⁷	2	3/4 (75)
AZ-P7a	1×10 ⁷	3	11/14 (78.6)

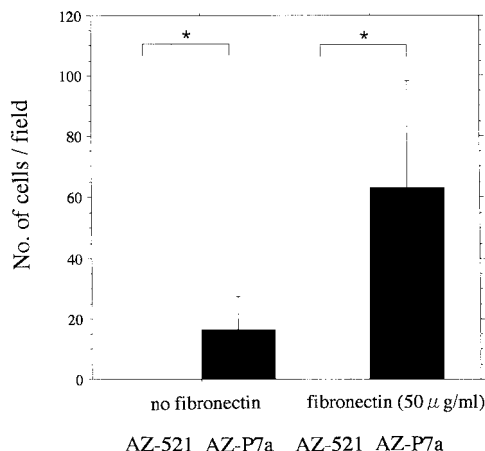


Fig. 5. *In vitro* motile activity of AZ-521 cells and AZ-P7a cells (black columns). The number of migrating AZ-P7a cells was significantly larger than that of AZ-521 cells with or without a chemoattractant, fibronectin (**P*<0.005). Bars represent mean±standard error.

compared in AZ-521 and AZ-P7a cells by indirect immunofluorescence flow cytometry. Fig. 7 shows the percentages of cells expressing each cell adhesion molecule. As compared with AZ-521 cells, AZ-P7a cells showed up-regulation of integrins α2, α3, α5, α6 and αvβ5. However, α1, αvβ3, hCD44H, hCD44v3, hCD44v6 and hCD44v10 were not expressed in any of the cell lines.

Production of cytokines To investigate the role of the metastasis-related cytokines such as VEGF and IL-8, in peritoneal dissemination, we determined the concentrations of VEGF, IL-6, IL-8 and IL-10 produced by AZ-521 and AZ-P7a cells. As shown in Fig. 8, VEGF production

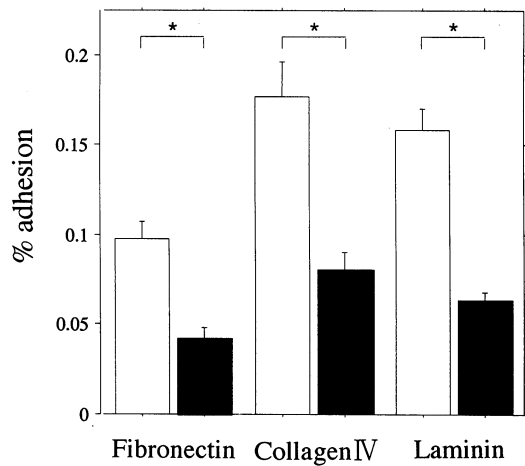


Fig. 6. *In vitro* adhesive activities of AZ-521 (open columns) and AZ-P7a (black columns) cell lines. The adhesive ability of AZ-P7a cells to ECM was significantly reduced compared with that of AZ-521 cells (* $P < 0.0001$). Bars represent mean \pm standard error.

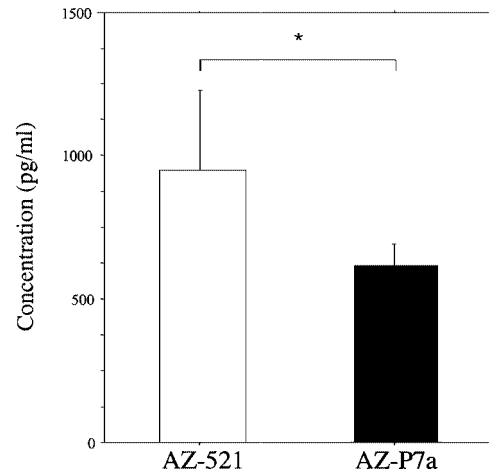


Fig. 8. VEGF production by AZ-521 (open columns) and AZ-P7a (black columns) cells. VEGF production by AZ-P7a cells was significantly lower than that by AZ-521 cells (* $P < 0.01$). Bars represent mean \pm standard error.

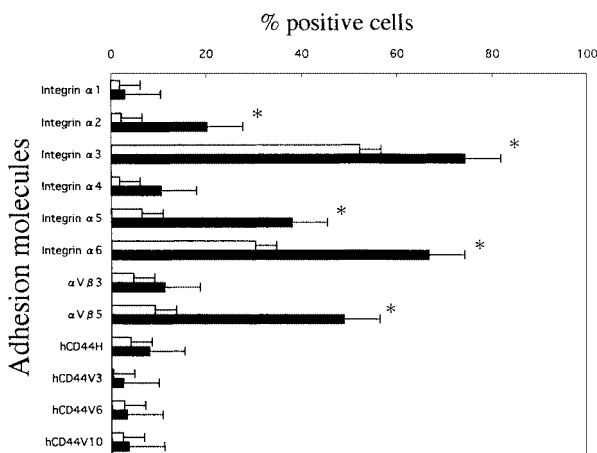


Fig. 7. Fluorescence-activated cell sorter (FACS) analysis of the cell surface expression of adhesion molecules on AZ-521 (open columns) and AZ-P7a (black columns) cells. The cell samples were stained with monoclonal antibodies as described in "Materials and Methods." Significant increases of $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ and $\alpha v \beta 5$ integrins in AZ-P7a cells were observed (* $P < 0.001$). Bars represent mean \pm standard error.

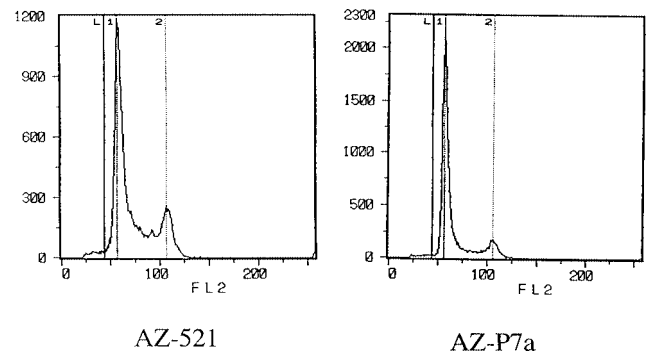


Fig. 9. There was no virtual difference in DNA ploidy pattern.

by AZ-P7a was significantly lower than that by AZ-521; however, IL-6, IL-8 and IL-10 were produced by neither of the two cell lines.

Flow cytometric analysis of DNA ploidy pattern Since a recent study revealed that the nuclear DNA content in human neoplasms was correlated with their malignant

potential and prognosis,¹⁷⁾ we examined the DNA content of this cell line. There were no clear differences in DNA histograms between AZ-521 and AZ-P7a cells, with both lines showing a diploid pattern (Fig. 9).

Cross injection We investigated the phenotypic differences between peritoneal dissemination and hematogenous metastasis. As shown in Table II, at 3 weeks after intrasplenic injection of AZ-H5c cells, eleven out of fourteen mice (78.6%) showed multiple liver metastatic foci. In the case of AZ-P7a cells, even at 5 weeks after intrasplenic injection, only 33.3% of the mice showed solitary liver metastatic foci. Six of eight mice (75%) developed peritoneal metastasis after intraabdominal injection of AZ-H5c cells, and a similar rate was seen with AZ-P7a cells (Table III). Although AZ-P7a cells did not metastasize to the

Table II. Metastasis after Intrasplenic Injection

	Cell dose	Liver metastasis		Total (%)
		3 w (%)	5 w (%)	
AZ-521	5×10 ⁶	0/6 (0)	2/14 (14.3)	2/20 (10.0)
AZ-P7a	5×10 ⁶	0/4 (0)	1/3 (33.3)	1/7 (14.3)
AZ-H5c	5×10 ⁶	11/14 (78.6)	8/11 (72.7)	19/25 (76.0)

Table III. Metastasis after Intraabdominal Injection

	Cell dose	Peritoneal dissemination		Total (%)
		3 w (%)	5 w (%)	
AZ-521	1×10 ⁷	2/6 (33.3)	ND	2/6 (33.3)
AZ-P7a	1×10 ⁷	8/10 (80.0)	ND	8/10 (80.0)
AZ-H5c	1×10 ⁷	3/4 (75.0)	3/4 (75.0)	6/8 (75.0)

ND, not determined.

liver after intrasplenic injection, numerous metastases were observed in the peritoneum after intraabdominal injection of AZ-H5c cells.

DISCUSSION

We established a new cell line, AZ-P7a, of human gastric carcinoma with high peritoneal-metastatic potential, by repeated intraabdominal injection starting with the parental AZ-521 cells. To investigate the mechanism of peritoneal metastasis, several animal models have been established and characterized.^{17, 18)} However, the mechanism has not been extensively studied as yet, in comparison with that of hematogenous metastasis.^{19, 20)} At present, peritoneal dissemination is thought to involve several sequential steps²⁰⁾; namely, exfoliation, adhesion of free cancer cells to the peritoneum, invasion, proliferation around the vessels and angiogenesis. At each step, the tumor cells interact with a variety of MMPs (matrix metalloproteinases), adhesion molecules and cytokines.^{21–26)} Since our model starts with intraabdominal injection, it may reflect the sequential steps after the free cancer cell step, in the abdominal cavity. However, patients with free cancer cells in the abdominal cavity do not always develop peritoneal metastasis clinically, so it is important to investigate the behavior of free cancer cells in the abdominal cavity.

In initial studies, we determined the *in vivo* and *in vitro* growth activity of the two cell lines. AZ-P7a cells demonstrated higher growth capability than the parental AZ-521 cells *in vivo*. In contrast, the *in vitro* proliferation activity of AZ-521 cells was higher than that of AZ-P7a cells. Thus, it appears that the host organ microenvironment can profoundly influence the growth of metastatic tumor cells.

Increased motility and adhesion to ECM are important factors in the cancer metastatic process.^{2, 15, 27)} We found that the motile activity of AZ-P7a cells *in vitro* was significantly stronger than that of AZ-521 cells and even than that of AZ-H5c cells (data not shown). A recent study revealed that lymphoid tissue (so-called milky spots) was found on the peritoneum and free cancer cells in the peritoneal cavity could infiltrate the peritoneum through this tissue in the early stages of peritoneal dissemination.²⁸⁾ Furthermore, the distribution of milky spots was not homogeneous; that is, they were numerous in the omentum, whereas only a few were found in other tissues.²⁹⁾ Thus, free cancer cells in the peritoneal cavity might have increased motility to explore the environment for a suitable site for adhesion and/or invasion.

It is well known that various cytokines are produced by tumor cells and they mediate the interaction between host cells and tumor cells in the microenvironment. Some of them promote fibrosis and angiogenesis, while others inhibit tumor growth.²²⁾ VEGF is a cytokine essential for neovascularization in tumor proliferation and is associated with hematogenous metastasis.³⁰⁾ In our study, the production of VEGF by AZ-P7a cells was significantly lower than that by AZ-521 and AZ-H5c cells (data not shown). These findings suggested that in the case of peritoneal dissemination, increased neovascularization might be less important than it is in hematogenous metastasis. Enhanced motile activity might play an important role in the dissemination of tumor cells in the abdominal cavity.

Although very little is yet known about the implication of integrins in peritoneal dissemination in gastric cancer, some authors have reported that increased expression of integrins $\alpha 2$ and $\alpha 3$ and CD44H is closely associated with peritoneal dissemination, while expression of $\alpha 5$ integrin and tumorigenicity are inversely related.^{20, 24)} The expression of integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ and $\alpha v\beta 5$ was significantly increased in AZ-P7a cells as compared with AZ-521 cells, while integrins $\alpha 2$, $\alpha 3$ and $\alpha 5$ were expressed highly in AZ-H5c cells.⁹⁾ Thus, integrin $\alpha 6$ and/or $\alpha v\beta 5$, in addition to integrins $\alpha 2$, $\alpha 3$ and $\alpha 5$, might play important roles in peritoneal dissemination of human gastric cancer cells. The hCD44 series was not expressed in either of the two cell lines.

The flow cytometric analysis of DNA content of several human cancers is known to correlate with malignant potential and is thus of prognostic value.³¹⁾ DNA aneuploidy was found in many cancer patients with high malignancy. However, in our study, no clear differences were seen in DNA histograms between AZ-521 and AZ-P7a cells, with both lines showing a diploid pattern.

The results of cross injection were interesting. Hematogenous metastasis was not observed after intrasplenic injection of the highly peritoneal-disseminating cell line AZ-P7a, while marked peritoneal dissemination occurred

after intraabdominal injection of the highly hematogenous metastatic cell line AZ-H5c. These findings suggest that the mechanism of peritoneal dissemination differs from that of hematogenous metastasis and might be subject to tighter control.

In conclusion, we have established a new cell line of human gastric carcinoma with high potential for peritoneal dissemination after intraabdominal injection. Increased motility and enhanced expression of integrins $\alpha 2$, $\alpha 3$, $\alpha 5$, $\alpha 6$ and $\alpha v\beta 5$ were observed in the peritoneal dissemination model. Our model could be useful for investigating

the molecular mechanism of peritoneal dissemination and the biological differences from hematogenous metastasis in human gastric cancer.

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