Advances in Brief

Prolonged Survival and Decreased Invasive Activity Attributable to Dipeptidyl Peptidase IV Overexpression in Ovarian Carcinoma[1]

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Abstract

Dipeptidyl peptidase IV (DPPIV) is a multifunctional cell surface aminopeptidase with ubiquitous expression. Recent studies have suggested that DPPIV plays an important role in tumor progression in several human malignancies. In the present study, we investigated the correlation between DPPIV expression and progressive potential in ovarian carcinoma. We demonstrated that ovarian carcinoma cell lines with higher DPPIV expression were less invasive. Furthermore, DPPIV overexpression in SKOV3 cells, derived from serous cystadenocarcinoma, with little DPPIV expression induced a dramatic change in cellular morphology and a significant decrease in the abilities of both migration and invasion. In addition, we have also shown that nude mice inoculated with DPPIV-transfected SKOV3 cells showed significantly less peritoneal dissemination and longer survival time than those receiving the parental or vector-only transfected cells (mean survival time, 64.9 ± 4.7, 35.7 ± 2.8, and 36.6 ± 1.8 days, respectively). This evidence implies that DPPIV may functionally suppress peritoneal dissemination in ovarian carcinoma.

Introduction

Epithelial ovarian carcinoma is the leading cause of death from gynecological malignancy. Because ovarian carcinoma frequently remains clinically silent, the majority of patients with ovarian carcinoma have advanced intraperitoneal metastatic disease at diagnosis. Therefore, the 5-year survival rates for disseminated cases remain poor (1).

Peritoneal dissemination is the main metastatic process of ovarian carcinoma besides direct extension of the carcinoma into adjacent tissues and lymphatic dissemination. Peritoneal dissemination originates from carcinoma cells released in the ascites from the ovary. Once the carcinoma cells attach to mesothelial cells, these cells may invade into the mesothelial cell layer (2). Because extension to the peritoneum is found in >75% of all cases, it is necessary to understand the mechanisms of spread by exfoliative cells that disseminate and implant throughout the peritoneal cavity. However, the biology of this dissemination is still unknown.

DPPIV[3] is a cell surface aminopeptidase that was originally characterized as a T-cell differentiation antigen (CD26; Ref. 3) and has been reported to be present on epithelial cells of various tissues, including lung, liver, kidney, intestine, prostate, and placenta (4, 5). It has been reported that DPPIV has a variety of functions, not only serine protease activity, which liberates NH_2-terminal X-proline from peptides, but also various cellular processes such as regulation of immune response, signal transduction, and interaction with molecules of the extracellular matrix (6–9). A number of recent studies have provided evidence to indicate that DPPIV may play a role in tumor progression, such as cell adhesion and invasion (10, 11). Previous studies have also reported that DPPIV expression in melanoma cells has a suppressing effect on malignant phenotype (12) and, particularly, has anti-invasive function, which is related to neither protease activity locating at the extracellular domain nor the cytoplasmic domain possibly linked to signal transduction (13). However, there is no evidence to indicate the association between DPPIV expression in ovarian carcinoma and its progression. In the present study, we tried the DPPIV expression in various ovarian carcinoma cell lines and examined its effect on the progression of ovarian carcinoma *in vivo*. Our present data showed that DPPIV acts as a suppressor of ovarian carcinoma in peritoneal dissemination *in vitro* and *in vivo*.

Materials and Methods

Cell Culture. We used five human ovarian carcinoma cell lines (SKOV3, HRA, NOS2, NOS4, and TAOV) derived from serous cystadenocarcinoma. SKOV3 cells were generously donated by Memorial Sloan-Kettering Cancer Research Laboratory. HRA cells were kindly provided by Dr. Kikuchi (Defense Medical University, Tokorozawa City, Japan). NOS2, NOS4, and TAOV cells were established in our institute. These cell lines were maintained in RPMI 1640 supplemented with 10% FCS and penicillin-streptomycin. These cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Plasmid Construction and Transfection. Full-length cDNA for DPPIV was kindly provided by Dr. Ikehara (University of Fukuoka, Fukuoka City, Japan). The eukaryotic expression vector pcDNA 3.1(−) (Invitrogen) was used to drive the expression of inserted DPPIV cDNA. Transfections were carried out using Lipofectamine according to the manufacturer's instructions (Life Technologies, Inc., San Diego, CA). SKOV3 cells were transfected with pcDNA3.1(−) (SKpcDNA) or pcDNA3.1(−) with DPPIV cDNA inserted (SKDPIV). Stable transfectants were selected by growth in medium supplemented with 400 μg/ml of G418 (Sigma). Several hundred clones resistant to G418 were obtained. Because we hoped to eliminate any effects that could be attributed to clonal variation, polyclonal cells from these transfectants were used in the following experiments. The cells used in these experiments were within three passages after establishing the DPPIV-overexpressing cells.

Enzyme Activity Assay. DPPIV enzyme activity was measured spectrophotometrically using Gly-Pro-*p*-nitroanilide-tosylate (Peptide Institute, Inc.) as a DPPIV substrate. Whole-cell suspensions were prepared in test tubes and then washed with PBS. Thereafter, 5×10^5 cells were resuspended in 200 μl of PBS contained in each well of 96-well microtiter plates, and the substrate was added (final concentration, 0.24 mM Gly-Pro-*p*-nitroanilide-tosylate). DPPIV enzyme activity was measured at 405 nm by a microplate reader (Labsystems; Multiskan Bichromatic) after 60 min incubation at 37°C. We used both DFP (Wako, Japan) and DPA (Peptide Institute, Japan) as specific inhibitors of DPPIV enzyme activity.

Flow Cytometric Analysis. FACS was performed to quantify the expression levels of DPPIV on the cell surface of ovarian carcinoma cells. Then, the cells were incubated with phycoerythrin-conjugated monoclonal antibody specific for DPPIV (Pharmingen, San Diego, CA) for 30 min at 4°C and washed three times with PBS. FACS data were acquired on a FACS Calibur (Becton Dickinson, San Jose, CA) and analyzed using CELL Quest software (Becton Dickinson).

In Vitro **Cell Proliferation Assay.** SKOV3, SKpcDNA, and SKDPIV cells were plated in triplicate at a density of 1500 cells in a 100-μl volume in 96-well plates and cultured for 1–4 days. Cell viability was assayed using a modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay by CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Absorbance was measured at 490 nm by a microplate reader (Labsystems; Multiskan Bichromatic).

In Vitro **Invasion Assay.** Cell invasion was evaluated using 24-well Matrigel invasion chambers (Becton Dickinson Labware). Cells were suspended into the upper chamber at a final concentration of 75×10^4/ml in 200 μl of RPMI 1640 supplemented with 0.1% BSA. The lower chamber contained 700 μl of RPMI 1640 supplemented with 20 μg/ml of human fibronectin (Sigma) as a chemoattractant. After 16 h of incubation, the remaining tumor cells on the upper surface of the filters were removed by wiping with cotton swabs, and the invading cells on the lower surface were stained with May-Grünwald-Giemsa staining. The number of cells on the lower surface of the filters was counted under a microscope at $\times 20$, and we performed four individual experiments in the invasion assay in triplicate.

In Vitro **Migration Assay.** Cell migration was assayed in 24-well Transwell cell culture chambers (Costar). Cells were suspended into the upper chamber at a final concentration of 50×10^4/ml in 200 μl of RPMI 1640 supplemented with 0.1% BSA. The subsequent procedures were the same as those used for the invasion assay, except that the incubation time was 12 h.

Gelatin Zymography. The activities of matrix metalloproteases (MMPs) in the conditioned medium of SKOV3, SKpcDNA, and SKDPIV cells were assayed by zymography. These cells (1×10^5) were seeded into 24-well culture dishes and incubated in culture medium. After achieving confluency, cells were washed with serum-free medium and incubated for 24 h. After incubation, the conditioned medium was collected for zymography. Samples were electrophoresed on 10% SDS polyacrylamide gel containing 0.03% gelatin. After electrophoresis, the gel was washed with 2.5% Triton X-100 and incubated for 20 h at 37°C in reaction buffer [50 mM Tris-HCl, (pH 7.4), containing 10 mM CaCl$_2$]. The gel was then stained with a solution of 0.2% Coomassie Brilliant Blue in 50% methanol and 10% acetic acid for 1 h and washed with 20% methanol and 10% acetic acid. Gelatinase activities were detected as an unstained band.

In Vivo **Studies.** Female nude mice (BALB/c) at 6 weeks were provided from Chubu Kagaku (Nagoya, Japan). SKOV3, SKpcDNA, and SKDPIV cells (1×10^7 cells/0.5 ml of medium/mouse) were injected i.p. to examine their metastatic potential. Mice were sacrificed 30 days after carcinoma cell injection and evaluated for the formation of intraperitoneal dissemination. In addition, survival time was examined among these three groups.

Statistical Analysis. For data on cell proliferation, migration, and invasion, statistical comparisons among groups were performed with non-paired Student's *t* test. The Kaplan-Meier method was used to generate survival curves, and comparisons were performed by log-rank tests. $P < 0.05$ was considered significant.

Results

Correlation among DPPIV Expression, Invasive Potential, and Cellular Morphology. To evaluate the expression of DPPIV in various ovarian carcinoma cell lines, FACS analysis was performed. The mean fluorescence intensity for DPPIV in these cell lines was shown in Table 1. NOS2, NOS4, and TAOV cells were positive for DPPIV,

whereas SKOV3 and HRA cells were almost negative, which is consistent with data on enzyme activity analysis (data not shown). An invasion assay was performed in these cell lines to compare invasive potential and DPPIV expression. Table 1 also shows the correlation among mean fluorescence intensity of DPPIV in FACS, invasive potential, and cellular morphology in these ovarian carcinoma cell lines. The DPPIV expression in these cell lines was negatively correlated with invasive potential. Furthermore, the morphology in DP-PIV-expressing cell lines tended to show an epithelioid pattern in contrast to that in non-DPPIV-expressing cell lines, which show a long spindle/bipolar pattern-like fibroblasts.

Morphological Change after DPPIV Transfection. To investigate the effect of the DPPIV transfection in carcinoma cells, we overexpressed DPPIV into ovarian carcinoma SKOV3 cells (SKDPIV cells). Whereas both parental SKOV3 cells and vector-transfected SKpcDNA cells expressed little DPPIV on the cell surface, SKDPIV cells expressed a remarkably high level of DPPIV on FACS analysis (Fig. 1*A*). To confirm the enzyme activity of DPPIV protein expressed by the transfection, we also checked aminopeptidase activity. The DPPIV activity of SKDPIV cells was ~10 times higher than that of both parental SKOV3 cells and mock SKpcDNA cells in absorbance (Fig. 1*B*). The enzyme activity was almost completely inhibited by adding either 1 mM DFP or 100 μM DPA (data not shown).

Furthermore, a remarkable morphological change was observed in SKDPIV cells. Although both parental SKOV3 cells and SKpcDNA cells were a long-bipolar, spindle-shaped morphology-like fibroblasts with a scattered and unorganized growth pattern (Fig. 1, *C* and *D*), the shape of SKDPIV cells was round with a cobblestone-like appearance. In the case of SKDPIV cells, cell-cell adhesion seemed to be tighter than that in SKOV3 or SKpcDNA cells (Fig. 1*E*).

Functional Analysis of DPPIV *in Vitro*. We assessed the effect of DPPIV on cell proliferation, migration, and invasion. No significant differences among SKOV3, SKpcDNA, and SKDPIV were observed in the proliferation assay (Fig. 2*A*). As shown in Fig. 2*B*, the number of cells that had migrated on Transwell cell culture assay was significantly ($P < 0.0001$) reduced in SKDPIV cells compared with that in either parental SKOV3 or SKpcDNA cells. This reduction could not be restored by the addition of either 100 μM DPA or 1 mM DFP. As shown in Fig. 2*C*, the number of invading cells on Matrigel invasion assay was also reduced in SKDPIV cells compared with that in parental SKOV3 and mock SKpcDNA cells ($P < 0.0001$). This reduction also could not be restored by the addition of DPPIV-specific inhibitors. In gelatin zymography, pro-MMP-2 activity was remarkably reduced in SKDPIV cells, whereas SKpcDNA cells showed the same level of pro-MMP-2 activity as parental SKOV3 cells (Fig. 2*D*).

Effect of DPPIV on Peritoneal Dissemination *in Vivo*. We also investigated whether DPPIV suppresses the formation of peritoneal metastasis in ovarian carcinoma using nude mice. Peritonitis carcinomatosa was observed ~4 weeks after the inoculation of SKOV3 or SKpcDNA cells into mice. Fig. 3*A* shows the intraabdominal appearance of the mouse 30 days after inoculation of SKpcDNA cells. A number of disseminated tumors were observed throughout the whole peritoneal cavity, especially on the omentum, mesentery, and liver surface with a large amount of bloody ascites. A similar appearance was also observed in a mouse injected with parental SKOV3 cells (data not shown). In contrast, a mouse injected with SKDPIV cells macroscopically had no disseminated tumor with a small amount of ascites at the same time of autopsy (Fig. 3*B*).

In addition, the number of disseminated tumors at death in the group of mice injected with SKDPIV cells was obviously much less than the numbers in the other two groups. Only several disseminated tumors in this group of mice were observed, even 60 days after the inoculation of SKDPIV cells (Fig. 3*C*). Fig. 3*D* shows the survival

Table 1 *Correlation among DPPIV expression, invasive ability, and morphological characteristics in various ovarian cancer cell lines*

Cell line	DPPIV expression Mean fluorescence intensity[a]	Invaded cell counts[b]	Morphology
SKOV3	12	125 ± 23	Spindle/bipolar/scattered
HRA	8	152 ± 21	Spindle/bipolar/scattered
NOS2	684	11 ± 4	Epithelioid/cobblestone
NOS4	421	17 ± 6	Epithelioid/cobblestone
TAOV	293	22 ± 8	Epithelioid/cobblestone

[a] Mean fluorescence intensity by FACS analysis.
[b] By Matrigel invasion assay described in the text.

Fig. 1. The effect of DPPIV transfection on enzyme activities and cellular morphology. *A*, analysis of cell surface expression of DPPIV by FACS. *Right* and *left peaks* show SKOV3 and SKDPIV cells, respectively. *B*, increase of DPPIV enzyme activity in DPPIV-overexpressing ovarian carcinoma cells. Enzyme activity was determined by quantitating the enzymatic cleavage of the synthetic substrate Gly-Pro-*p*-nitoanilide. DPPIV activity of SKOV3, SKpcDNA, and SKDPIV cells is depicted as an increase in absorbance at 405 nm. *Bars*, SD. *C–E*, morphology of SKOV3, SKpcDNA, and SKDPIV cells, respectively. *Bar*, 15 μm. *SKpcDNA*, vector (pcDNA 3.1)-transfected SKOV3 cells; *SKDPIV*, DPPIV cDNA-transfected SKOV3 cells.
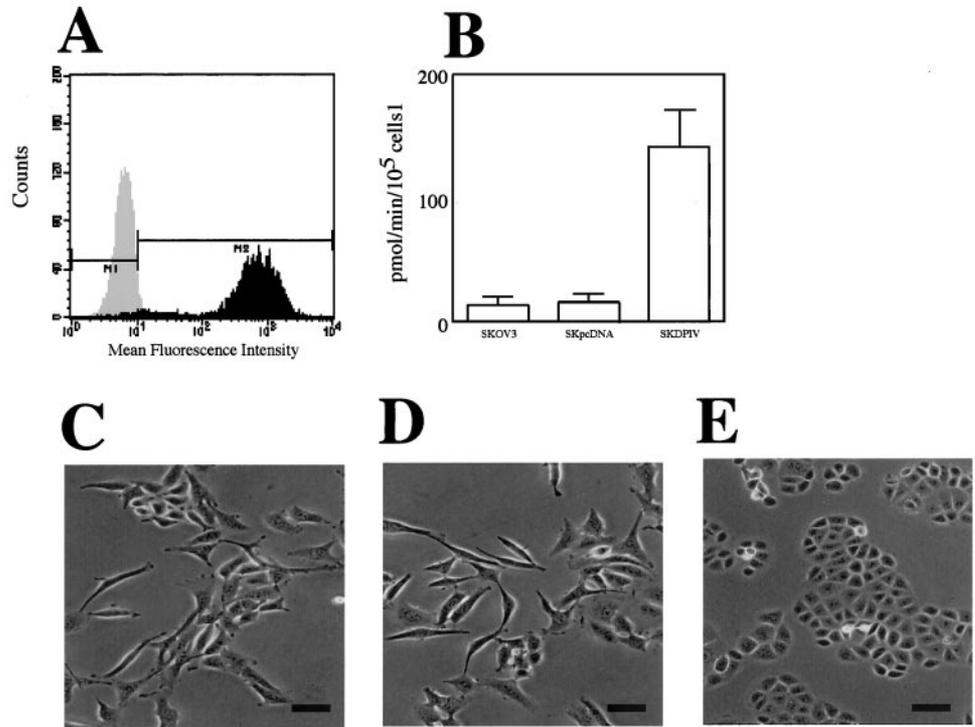
curves among these three groups. All mice finally died of peritonitis carcinomatosa. However, the mice injected with SKDPIV cells survived significantly longer than those injected with either SKOV3 cells or SKpcDNA cells [mean survival days: 64.9 ± 4.7 days (SKDPIV), 35.7 ± 2.8 days (SKOV3), and 36.6 ± 1.8 days (SKpcDNA), respectively; $P < 0.0001$].

Discussion

Our present study showed the relationship among DPPIV expression, invasiveness, and morphological changes in various ovarian carcinoma cell lines (Table 1). Invasiveness was negatively correlated to the DPPIV expression levels, suggesting that DPPIV should be

Fig. 2. The effect of DPPIV transfection on cellular functions. *A*, effect of DPPIV transfection on cell proliferation in a 4-day modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Analysis of the proliferation rate was performed as described in "Materials and Methods." Data are expressed as the means of three independent experiments; *bars*, SD. *B*, effect of DPPIV transfection on cell migration. Migration of parental SKOV3, SKpcDNA, and SKDPIV cells was examined as described in "Materials and Methods." In addition, whether the migration rate was affected in the absence or presence of two kinds of specific inhibitor for DPPIV activity was also examined in this experiment. *DPA*, 100 μM DPA; *DFP*, 1 mM DFP. ∗, $P < 0.001$ *versus* SKOV3 cells and *versus* SKpcDNA cells. *C*, effect of DPPIV transfection on Matrigel invasion in the absence or presence of above two kinds of specific inhibitor for DPPIV activity. †, $P < 0.0001$ *versus* SKOV3 cells and *versus* SKpcDNA cells. *D*, enzymatic activity of pro-MMP-2 (72-kDa) and pro-MMP-9 (92-kDa) was studied in conditioned medium from SKOV3 (*Lane 1*), SKpcDNA (*Lane 2*), and SKDPIV (*Lane 3*) cells by gelatin zymography.
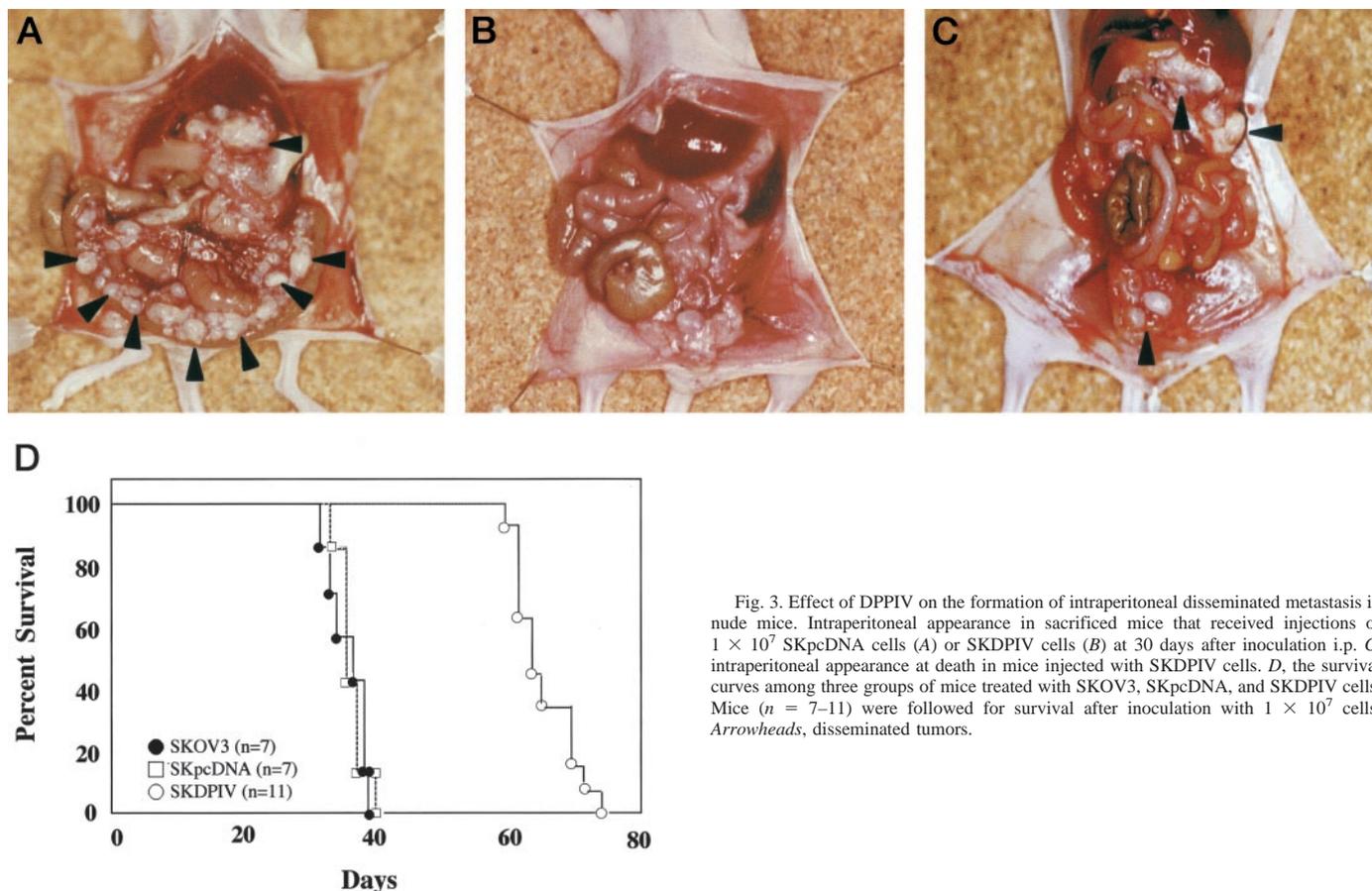
2755
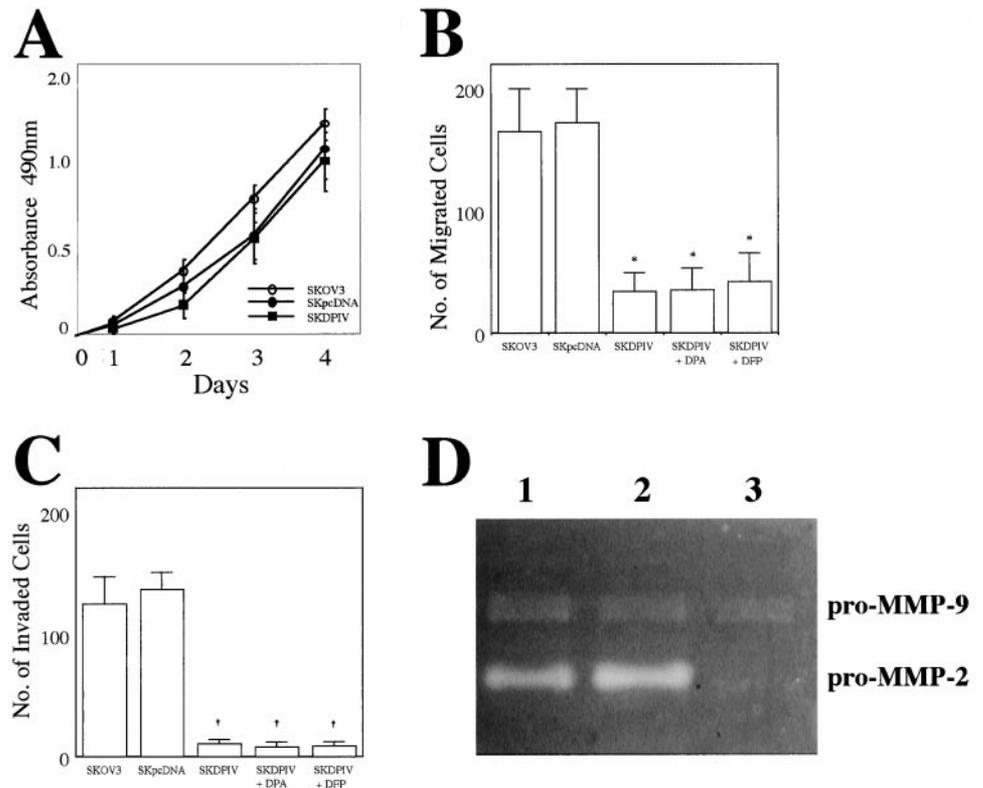
Fig. 3. Effect of DPPIV on the formation of intraperitoneal disseminated metastasis in nude mice. Intraperitoneal appearance in sacrificed mice that received injections of 1×10^7 SKpcDNA cells (*A*) or SKDPIV cells (*B*) at 30 days after inoculation i.p. *C*, intraperitoneal appearance at death in mice injected with SKDPIV cells. *D*, the survival curves among three groups of mice treated with SKOV3, SKpcDNA, and SKDPIV cells. Mice (*n* = 7–11) were followed for survival after inoculation with 1×10^7 cells. *Arrowheads*, disseminated tumors.

involved in carcinoma invasion. However, to our knowledge, there have been no reports about DPPIV in ovarian carcinoma, although DPPIV is a multifunctional cell surface aminopeptidase that is expressed on various types of carcinoma (10, 14, 15). We confirmed DPPIV expression in various ovarian carcinoma tissues by immunohistochemical staining using DPPIV-specific monoclonal antibody provided by Dr. Ueda (Nagoya City University, Nagoya City, Japan), and the intensity of immunohistochemical staining varied from tissue to tissue (data not shown). Thus, it is beneficial to make a DPPIV overexpression subline to clarify a role of DPPIV *in vitro* and *in vivo*.

In this study, we showed that the overexpression of DPPIV in SKOV3 cells results in remarkable morphological change from spindle-like appearance to cobblestone in aggregation with the increase of enzyme activity (Fig. 1). Furthermore, not only migration but also invasion of SKOV3 cells was markedly reduced by the transfection of DPPIV. However, there was no difference in cell proliferation between SKOV3 and SKDPIV cells (Fig. 2). Because these reductions were not restored by the addition of DPPIV inhibitors such as DFP and DPA, the enzyme activity of DPPIV might not be involved in migration or invasion of SKDPIV cells. Pethiyagoda *et al.* (13) also showed that DPPIV had an anti-invasive activity in melanoma cells, and this effect was not restored by the deletion mutant of the active site of enzyme activity of DPPIV.

Recent studies on cadherins have reported observations similar to our findings. The overexpression of cadherins in carcinoma cells resulted in the adherence of cells to each other and formation of compact aggregation of cells in epithelial-like cell clusters. In addition, the cadherin-overexpressing cells showed reduction of both invasive and migratory potential and therefore reduced tumorigenicity, although the mechanisms differed among reports (16–18). It is

reasonable to speculate that the tight adhesion between carcinoma cells is contradictory to the invasiveness of carcinoma cells. The mechanisms of low migration potential and morphological change in DPPIV-overexpressing cells are not clear; however, tight cell-cell adhesion caused by DPPIV may inhibit cell motility and invasiveness.

Our data showed that DPPIV overexpression in ovarian carcinoma cells results in the reduction of both intraperitoneal dissemination of carcinoma cells and prolongation of survival time *in vivo* (Fig. 3). Although the loss of DPPIV expression was related to the progression of melanoma, the transfection of DPPIV into melanoma cells resulted in a loss of tumorigenicity and therefore a marked decrease in the tumor size of s.c. injected melanoma in nude mice (12).

The mechanisms of tumor metastasis consist of various steps such as adhesion, migration, and invasion of carcinoma cells in metastasized organs. DPPIV in lung endothelial cells also acts as an adhesion molecule for the metastasis of breast carcinoma cell (11). Indeed, we found that the adhesion of SKOV3 cells to mesothelial monolayer cells is slightly increased by the transfection of DPPIV into SKOV3 cells (data not shown). However, the results that much fewer disseminated tumors were observed 60 days after inoculation with DPPIV-overexpressing cells than those of parental and vector-transfected cells suggested that most adherent cells could not grow or were eliminated because of loss of migration and invasive potential by DPPIV overexpression.

There have been many reports that various bioactive substances in malignant ascites such as interleukin 1β, tumor necrosis factor, and various chemokines, all of which might be the candidates for substrate of DPPIV, were associated with both progression and survival of carcinoma (12, 19, 20). Therefore, this enzyme may have an effect on the progression of carcinoma *in vivo* in relation to these bioactive

substances. In addition, pro-MMP-2 activity was remarkably reduced in DPPIV-transfected cells in gelatin zymography. Because MMPs are involved in carcinoma invasion, low invasive potential in DPPIV-transfected cells might be partially attributable to reduced MMP-2 activity.

In conclusion, the present *in vitro* and *in vivo* data provide a possible link between DPPIV and decreased intraperitoneal dissemination of ovarian carcinoma attributable to both invasion and migration of ovarian carcinoma cells. The potential of DPPIV for treating intraperitoneal metastatic carcinoma including gene therapy seems promising, although extensive work is required before this enzyme can become available for clinical use.

Acknowledgments

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