Dipeptidyl Peptidase IV Overexpression Induces Up-Regulation of E-Cadherin and Tissue Inhibitors of Matrix Metalloproteinases, Resulting in Decreased Invasive Potential in Ovarian Carcinoma Cells

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ABSTRACT

Dipeptidyl peptidase IV (DPPIV/CD26) is a multifunctional cell surface aminopeptidase that is widely expressed in different cell types. Our previous study demonstrated a possible link between DPPIV expression and decreased i.p. dissemination and loss of invasive potential of ovarian carcinoma. In this report, we examined the mechanisms of the anti-invasive ability of DPPIV in greater detail. Expression of E-cadherin and β-catenin was positively correlated with DPPIV expression among five independent ovarian carcinoma cell lines. The introduction of DPPIV cDNA into an ovarian carcinoma cell line (SKOV3) with low DPPIV expression enhanced the expression of E-cadherin and β-catenin, with a cellular morphological change from a fibroblastic to motile phenotype to an epithelial phenotype. In addition, matrix metalloproteinase 2 and tissue inhibitors of metalloproteinases were up-regulated by DPPIV transfection. Furthermore, suppression of the phosphorylation levels of mitogen-activated protein kinase isoform, extracellular signal-regulated kinase, was observed in DPPIV-overexpressing cells. To our knowledge, this is the first evidence that increasing DPPIV expression may contribute to prolonged survival by up-regulation of E-cadherin and tissue inhibitors of matrix metalloproteinases.

INTRODUCTION

DPPIV3 is a 110-kDa glycoprotein expressed on various cell types, such as renal proximal tubules, intestinal epithelial cells, kidney, liver, placenta, and lung (1–3). DPPIV is also a cell surface aminopeptidase that was originally characterized as a T-cell differentiation antigen (CD26), and DPPIV cleaves NH2-terminal dipeptides from polypeptides with either l-proline or l-alanine in position 2 (4, 5). Therefore, DPPIV is capable of degrading various bioactive substances such as substance P and chorionic gonadotropin, tumor necrosis factor α, interleukin 2, and various chemokines including RANTES [regulated on activation, normal T-cell expressed and secreted (2, 6, 7)]. Recent reports have indicated that DPPIV has a variety of functions including regulation of inflammatory and immunological response, signal transduction, apoptosis, and interaction with molecules of the extracellular matrix besides its serine protease activity (8–10). Furthermore, a number of recent studies have provided evidence indicating that DPPIV may play a role in tumor progression such as cell adhesion, invasion, and cell cycle arrest (11–14). Previously, we demonstrated that DPPIV expression in ovarian carcinoma cell lines was negatively correlated with invasive potential. In addition, DPPIV overexpression in an ovarian carcinoma cell line induced a dramatic change in cellular morphology from a fibroblastic to an epithelioid pattern and a significant decrease in invasive potential in vitro and in vivo (15).

Caderhins are members of a large family of transmembrane glycoproteins that mediate calcium-dependent, homophilic, cell-cell adhesion (16). Cadherins also play a major role in various physiological processes, such as morphogenesis, signal transduction, and maintenance of cell polarity (17). E-cadherin has been thought to act as a tumor suppressor in the development of tumor invasiveness, and loss of E-cadherin expression is frequently found in various malignant tissues and correlates with invasiveness and metastatic potential (18, 19). Moreover, a recent report has provided evidence that restoration of a functional E-cadherin could reverse the invasive phenotype in E-cadherin-negative cell clones (20, 21). This study investigated whether DPPIV overexpression affected E-cadherin or catenin expression. Furthermore, we examined changes in MMP and TIMP expression in DPPIV-overexpressing cells because of the close association between E-cadherin expression and MMP activity or expression (20, 21).

MATERIALS AND METHODS

Cell Culture. Five human ovarian carcinoma cell lines (SKOV3, HRA, NOS2, NOS4, and TA0V) derived from serous cystadenocarcinoma were cultured and maintained as described previously (15). Five human ovarian carcinoma cell lines (SKOV3, HRA, NOS2, NOS4, and TA0V) were cultured and maintained as described previously (15). To generate stable cell lines expressing DPPIV, SKOV3 cells were transfected with eukaryotic expression vector pcDNA3.1(−) (Invitrogen, San Diego, CA; SKpCD26 cells) or pcDNA3.1(−) with DPPIV cDNA inserted (SKDPIV cells) as described previously (15). Briefly, transfections were carried out using LipofectAMINE (Life Technologies Inc., San Diego, CA) according to the manufacturer’s instructions, and stable transfectants were selected by growth in media supplemented with 400 μg/ml Genetin (Sigma, St. Louis, MO). Several hundred clones resistant to G418 were obtained. Polyclonal cells from these transfectants were used in the following experiments.

Flow Cytometric Analysis. FACs analysis 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 analysis data were acquired on a FACS Calibur (Becton Dickinson, San Jose, CA), and analyzed using CELL Quest software (Becton Dickinson).

Immunohistochemical Staining. Immunohistochemical staining was performed using the avidin-biotin immunoperoxidase technique (Histofine SAB-PO kit; Nichirei, Tokyo, Japan) as described previously (22). As a first antibody for DPPIV staining, anti-DPP/CD26 monoclonal antibody (Ts-145) was used at a dilution of 1:100. This antibody was generously provided by Dr. Ryozu Ueda (Nagoya City University, Nagoya, Japan).

Immunofluorescence Staining. SKOV3, SKpCD26, and SKDPIV cells were grown in a chamber slide (Nalg Nunc International). They were fixed for 15 min with 4% paraformaldehyde and washed several times with PBS. Coverslips were incubated in blocking solution containing 2% BSA in PBS for 1 h and incubated with the appropriate primary antibodies for 1 h at room temperature. Mouse anti-E-cadherin and rabbit anti-β-catenin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used. After incubations with

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4 The abbreviations used are: DPPIV, dipeptidyl peptidase IV; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of MMP; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; MAPK, mitogen-activated protein kinase; FACS, fluorescence-activated cell-sorting; MT1-MMP, membrane type 1 MMP; EVT, extravillous trophoblast.

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appropriate secondary antibodies [E-cadherin, antimouse antibody (WAKO); β-catenin, antirabbit antibody (DAKO); H9252 - catenin, antirabbit antibody (DAKO)], fluorescence was visualized by epifluorescence microscopy (MRC1024; Bio-Rad).

Western Blot Analysis. Immunoblot analysis of E-cadherin, catenins, MMPs, and TIMPs was performed as described previously (23). Briefly, cells were grown to 70–80% confluence and treated with lysis buffer containing 1% Triton X-100 in PBS and protease inhibitor mixture tablets (Roche, Barcelona, Spain). Ten μg of total cell lysate were electrophoresed on a 10% (E-cadherin, catenins, MMPs) or 15% (TIMPs) SDS-polyacrylamide gel and transferred electrophoretically to Immobilon membranes (Millipore, Bedford, MA). After blocking in blocking solution (5% nonfat dry milk/0.1% Tween-20/PBS), the membranes were incubated overnight with a recommended dilution of primary antibodies. We used the following antibodies: anti-E-cadherin, anti-MMP-2, anti-α-catenin, anti-β-catenin, and anti-TIMP-2 (Santa Cruz Biotechnology); anti-MT1-MMP antibody (Chemicon); anti-TIMP-1 antibody (Oncogene Research Product); anti-ERK1/2 phosphospecific antibody (Biosource, Camarillo, CA); and anti-β-actin antibody (Sigma). The primary antibodies were washed in 0.05% Tween-20/PBS and then incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence reagent (Amersham Pharmacia Biotech) followed by exposure to X-ray film.

In Vivo Studies. Female nude mice (BALB/c) were provided at 6 weeks of age from Japan SLC (Nagoya, Japan). HRA, NOS2, NOS4, TAOV, SKOV3, SKpcDNA, and SKDPIV cells (1 × 10⁷ cells/0.5 ml media/mouse) were injected i.p. to examine their metastatic potential in vivo. Survival time was also examined among these seven groups.

Statistical Analysis. Spearman’s correlation test was used to compare the mean survival days among the seven groups of nude mice mentioned above and the mean fluorescence intensities of DPPIV among various ovarian carcinoma cell lines by FACS analysis. P < 0.05 was considered significant.

RESULTS

Correlation between DPPIV Expression and Survival Time in Vivo in Ovarian Carcinoma Cells. Fig. 1A shows the varying extent of DPPIV expression in ovarian carcinoma cell lines by FACS analysis. The mean fluorescence intensity of DPPIV in SKOV3, HRA, NOS2, TAOV, NOS4, SKpcDNA (mock transfected), and SKDPIV cells (DPPIV transfectant) was 12, 8, 651, 302, 421, 14, and 782, respectively. After inoculation, the mice were observed for survival. The mean survival of the inoculated mice was 35.7 ± 2.8 (SKOV3), 19 ± 1.5 (HRA), 64.3 ± 2.5 (NOS2), 62.6 ± 1.5 (TAOV), 60.7 ± 3.2 (NOS4), 36.6 ± 1.8 (SKpcDNA), and 64.9 ± 4.7 days. The various carcinoma cell lines with higher DPPIV expression described above showed significantly less metastatic potential in vivo (P < 0.05; r = 0.862).
fore, all mice developed peritonitis carcinomatosa and died. The mean survival of the inoculated mice was 35.7 ± 2.8 (SKOV3), 19 ± 1.5 (HRA), 64.3 ± 2.5 (NOS2), 62.6 ± 1.5 (TAOV), 60.7 ± 3.2 (NOS4), 36.6 ± 1.8 (SKpcDNA), and 64.9 ± 4.7 (SKDPIV) days. As shown in Fig. 1B, there is a positive correlation between mean fluorescence intensity and mean survival. We found that ovarian carcinoma cell lines with higher DPP IV expression have significantly less metastatic potential in vivo (P < 0.05; r = 0.862).

**Correlation between DPP IV Expression and E-Cadherin Expression.** We reported previously (15) that morphology in DPP IV-expressing cell lines tended to show an epithelioid pattern in contrast to that in non-DPP IV-expressing cell lines, which show a long spindle/bipolar pattern like fibroblasts. In the following study, we examined the levels of E-cadherin expression in ovarian carcinoma cell lines and also analyzed whether DPP IV expression was associated with E-cadherin expression. Fig. 2A shows immunoblot bands of E-cadherin expression in various ovarian carcinoma cell lines. In HRA and SKOV3 cells with limited DPP IV expression, E-cadherin expression was also slight. In contrast, E-cadherin was intensively expressed in NOS2, TAOV, and NOS4 cells that showed a high level of DPP IV expression. We next examined E-cadherin expression in DPP IV-overexpressing SKOV3 cells (SKDPIV). Although parental SKOV3 cells showed limited E-cadherin expression, E-cadherin expression was highly enhanced in SKDPIV cells (Fig. 2B). Furthermore, we found up-regulation of α- and β-catenin, which is usually sequestered in the E-cadherin adherens junction or in the tight-junction complex, by DPP IV transfection into SKOV3 cells (Fig. 2B). Fig. 2C shows the relative expression level of E-cadherin compared with that of SKOV3 cells by densitometric analysis. By DPP IV overexpression in SKOV3 cells, E-cadherin expression was enhanced about 8-fold. Interestingly, E-cadherin expression in these seven cell lines was significantly correlated with DPP IV expression, as shown by mean fluorescence intensity (P < 0.05; r = 0.945).

**Localization of E-Cadherin and β-Catenin in DPP IV-overexpressing SKOV3 Cells.** Fig. 3, A and B, shows the cellular morphology of parental SKOV3 cells and DPP IV-overexpressing SKDPIV cells, respectively. By DPP IV transfection, cellular morphology was significantly changed and became adherent, as shown in Fig. 3A and B. Furthermore, fluorescence signals specific to E-cadherin antibody were visualized as red in SKDPIV cells, and fluorescence signals specific to β-catenin as visualized green in SKDPIV cells. Enhancement of E-cadherin and β-catenin was also localized in cell-cell adherens junctions of SKDPIV cells together. E and F, immunohistological staining of DPP IV in surgically resected ovarian carcinoma tissues (serous cystadenocarcinoma).
chance between a fibroblastic, scattered pattern to an epithelioid, cobblestone pattern. In SKDPIV cells, DPPIV was localized in cell-cell adherens junctions (data not shown). Similarly, enhanced E-cadherin and β-catenin were also localized in cell-cell adherens junctions of SKDPIV cells (Fig. 3, C and D).

**DPPIV Expression in Ovarian Carcinoma Tissues.** We also confirmed DPPIV expression in surgically resected ovarian carcinoma tissues (serous cystadenocarcinoma). Although the intensity of immunohistochemical staining varied from tissue to tissue, DPPIV staining tended to be stronger in well-differentiated, low-grade ovarian carcinoma tissues (Fig. 3E) or noninfiltrating, opposing growing tissues than in poorly differentiated carcinoma (Fig. 3F). Furthermore, E-cadherin also showed positive staining in ovarian carcinoma tissues in accordance with the staining intensity of DPPIV (data not shown).

**DPPIV Overexpression Induces Down-Regulation of MMPs and Up-Regulation of TIMPs.** Metastatic potentials require proteolytic degradation of extracellular matrix, and imbalance between MMPs and TIMPs are thought to play an important role in tumor invasion and metastasis. Therefore, we investigated whether DPPIV expression also affects the MMPs and TIMPs. By immunoblotting, MMP-2 and MT1-MMP levels were found to be markedly lower for DPPIV-transfected cells (SKDPIV) than for parental or mock-transfected (SKpCDNA) cells. In contrast, up-regulations of TIMP-1 and TIMP-2 were observed in SKDPIV cells (Fig. 4).

**MAPK Cascade Activity of DPPIV-transfected Cells.** We next investigated whether the MAPK isoform, ERK, was involved in the action of DPPIV. Immunoblotting with the phosphospecific antibody showed that the phosphorylation levels of ERK were down-regulated by DPPIV overexpression in SKOV3 cells (Fig. 5).

**DISCUSSION**

We showed previously (15) that DPPIV overexpression induced a dramatic cellular morphological change and a significant decrease in invasive potential of SKOV3 cells with limited DPPIV expression both in vitro and in vivo. Extending our previous observations, the present data confirmed that the mean survival of nude mice inoculated with various ovarian carcinoma cell lines was positively correlated with the level of DPPIV expression (Fig. 1B). Moreover, we demonstrated for the first time that DPPIV expression was positively correlated with E-cadherin expression in ovarian carcinoma cell lines and that DPPIV overexpression in SKOV3 cells induced enhanced expression of E-cadherin and α- and β-catenin (Fig. 2, A and B). E-cadherin has been reported to play a central role in suppressing the invasive phenotype of a variety of carcinoma cells (19, 20). The present data suggest a possible link between decreased metastatic potentials attributable to DPPIV and those attributable to E-cadherin.

In the initial step of tumor metastasis, tumor cells must acquire motile properties and release proteolytic enzymes that can digest cell-cell contacts, allowing cells to detach from the primary lesions and invade local tissues (24). These processes require the reduction of E-cadherin expression to disrupt the adherens junction complex and the up-regulation of MMPs that proteolytically degrade the extracellular matrix complex. These two processes correlate closely with each other. Luo et al. (20) reported E-cadherin transfection in prostate carcinoma cells suppressed MMP-2 activity and subsequently led to reduced invasive potential in vitro. The present study shows that DPPIV overexpression in SKOV3 cells induced not only up-regulation of E-cadherin but also down-regulation of MMP-2 and MT1-MMP, which is known to activate pro-MMP-2. Furthermore, we also found that both TIMP-1 and TIMP-2 were suppressed by DPPIV overexpression (Fig. 4). TIMPs inhibit tumor invasion, angiogenesis, and metastasis, whereas MMPs promote these events. In the early steps of tumor invasion and metastasis, imbalance between MMPs and TIMP is now thought to promote the early events of tumor development (25, 26). Taken together, these findings show that DPPIV expression in SKOV3 cells normalizes aberrant regulation of E-cadherin and MMP-2 and TIMP expression, leading to the suppression of invasive potential. Although the precise mechanisms have not been clearly elucidated, the above-mentioned findings may partially explain the mechanisms of anti-invasive activity in DPPIV-overexpressing cells.

To our knowledge, there are no reports suggesting a positive correlation between DPPIV and E-cadherin expression in the field of cancer research. However, possible linkage between their expressions seems to be present in human placentation. Cytotrophoblast is the stem cell located in the villous lining that differentiates villous trophoblast or EVT in early pregnancy (27). Although villous trophoblast differentiates directly into syncytiotrophoblast, EVTs acquire an invasive phenotype and infiltrate into decidual tissue and maternal arteries in the first trimester of pregnancy. Therefore, EVTs located in the proximal part of the cell column and deep portion of maternal tissues have a noninvasive phenotype; in contrast, EVTs migrating in the decidual tissue have an invasive phenotype. Previous reports demonstrated that DPPIV expression was well detected in the proximal region of the cell column and disappeared on cells migrating into decidual tissues by immunohistochemical staining. Thus, the EVTs that stained positively for DPPIV belong to the noninvasive phenotype. On the other hand, the EVTs of the invasive phenotype are negative for DPPIV staining (28). The E-cadherin expression pattern in EVTs during early gestation was similar to that of DPPIV (28, 29). This evidence suggests that a change in the localization pattern of DPPIV and E-cadherin in EVTs was coincident according to phenotypic changes.

Whether DPPIV protease activity is necessary in this relationship remains an important issue. In DPPIV-overexpressing SKOV3 cells or NOS2 cells that natively express a considerable amount of DPPIV, neither additional morphological change nor alteration of E-cadherin expression was demonstrated in the presence of DPPIV-specific inhibitors such as diprotin A or diisopropyl fluorophosphate in culture media (data not shown). Our previous report also showed that the lower invasive potential in DPPIV-overexpressing cells could not be...
restored by the addition of either diprotin A or diisopropyl fluorophosphate (15). Although the hypothesis that DPPIV activity is involved in enhancing the effects of E-cadherin appears true, we could not obtain any evidence to support that theory in this study. DPPIV has a variety of substrates such as chemokine and bioactive peptides (30). However, there is no report indicating the presence of previously identified substrates of DPPIV that intensively promote cellular invasive potential and reduce E-cadherin expression. Therefore, we speculate that the E-cadherin-promoting effect of DPPIV is due, at least in part, to another mechanism independent of its enzyme activity.

In the present study, we confirmed DPPIV expression in ovarian carcinoma tissues and found that DPPIV tended to be expressed in well-differentiated tissues that were rich in glandular structures (Fig. 3E). Moreover, the DPPIV-expressing carcinoma tissues tended to show apressive growth pattern toward surrounding stromal tissues (Fig. 3F). The immunohistochemical findings seem to be consistent with the less invasive potential in ovarian carcinoma cells expressing DPPIV.

ERK, a downstream factor in the MAPK cascade, is phosphorylated and activated by MAPK kinase. The ERK pathway has been found to play a critical role in the control of cell survival, proliferation, and differentiation when activated by cellular stimulation via peptide growth factor receptors or adhesion molecules (31). Previous reports have suggested that the ERK cascade was correlated with the formation of cellular morphology via adherens junction molecules such as E-cadherin and β-catenin (31, 32). Ara et al. (31) demonstrated that MT1-MMP expression and pERK declined in E-cadherin-transfected squamous carcinoma cells and that inhibition of E-cadherin by neutralization antibody decreased pERK expression. Lu et al. (32) reported that suppression of the basal ERK activity by transfection of an ERK-inhibitory mutant in PC12 cells resulted in the formation of calcium-dependent aggregates where adherens junction protein such as E-cadherin and β-catenin was up-regulated. Thus, E-cadherin and ERK cascade may be closely linked to each other for epithelial-mesenchymal transition. Wilding et al. (34) reported that E-cadherin overexpression down-regulated the epidermal growth factor receptor and induced a less invasive phenotype of human papilloma virus-transfected keratinocytes. The evidence suggested that many of the alterations that characterize the cellular phenotype including invasiveness or altered E-cadherin/catenin expression are potentially attributable to a range of growth factor receptor signals, such as epidermal growth factor receptor, which could be upstream signals of the ERK cascade. In the current examinations, we also demonstrated that pERK was decreased in DPPIV-overexpressing SKOV3 cells (Fig. 5). Although the detailed mechanism is not clear at present, DPPIV may also be involved in these linkages through other unknown adaptor molecules or signal pathway in DPPIV-overexpressing SKOV3 cells.

In conclusion, we hypothesize that disruption of the coordinated regulation of DPPIV and E-cadherin promotes the progression of some carcinoma cells. Reintroduction of this coordination may facilitate control of invasion and metastasis in ovarian carcinoma. Further work is required to elucidate the molecular mechanisms of DPPIV in tumor cell biology; however, the present study indicates that DPPIV may become an important prognosis marker or a target for therapeutic intervention in ovarian carcinoma.

REFERENCES


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