The Protease Complex Consisting of Dipeptidyl Peptidase IV and Seprase Plays a Role in the Migration and Invasion of Human Endothelial Cells in Collagenous Matrices

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Abstract

Dipeptidyl peptidase IV (DPP4/CD26) and seprase/fibroblast activation protein α are homologous type II transmembrane, homodimeric glycoproteins that exhibit unique prolyl peptidase activities. Human DPP4 is ubiquitously expressed in epithelial and endothelial cells and serves multiple functions in cleaving the penultimate positioned prolyl bonds at the NH2 terminus of a variety of physiologically important peptides in the circulation. Recent studies showed a linkage between DPP4 and down-regulation of certain chemokines and mitogenic growth factors, and degradation of denatured collagens (gelatin), suggesting a role of DPP4 in the cell invasive phenotype. Here, we found the existence of a novel protease complex consisting of DPP4 and seprase in human endothelial cells that were activated to migrate and invade in the extracellular matrix in vitro. DPP4 and seprase were coexpressed with the three major protease systems (matrix metalloproteinase, plasminogen activator, and type II transmembrane serine protease) at the cell surface and organize as a complex in invadopodia-like protrusions. Both proteases were colocalized at the endothelial cells of capillaries, but not large blood vessels, in invasive breast ductal carcinoma in vivo. Importantly, monoclonal antibodies against the gelatin-binding domain of DPP4 blocked the local gelatin degradation by endothelial cells in the presence of the metallo- and serine protease systems that modified pericellular collagenous matrices and subsequent cell migration and invasion. Thus, we have identified a novel mechanism involving the DPP4 gelatin-binding domain of the DPP4-seprase complex that facilitates the local degradation of the extracellular matrix and the invasion of the endothelial cells into collagenous matrices. (Cancer Res 2006; 66(9): 4652-61)

Introduction

Proteolytic modification of the extracellular matrix is an absolute requirement for the migration and invasion of endothelial cells into the connective tissue during angiogenesis. A variety of angiogenesis models have addressed the role of different protease systems, components of which include matrix metalloproteinases (MMP-2, MMP-9, and MMP-14; refs. 1–3), plasminogen activator (PA)-plasmin (4, 5), and trypsinlike type II transmembrane serine proteases (6–8). Inhibition of the proteolytic activity in these systems, particularly in the context of angiogenesis, has become a key target in therapeutic strategies aimed at inhibiting tumor growth (9–12).

Recently, dipeptidyl peptidase IV (DPP4) and seprase, membrane-bound glycoproteins that cleave a conserved proline residue in prototypically resistant components (i.e., collagens), have been proposed to contribute to the invasive properties of different cell types within a microenvironment that exhibits elevated levels of MMP, PA-plasmin, and type II transmembrane serine proteases (13–15). Human DPP4 is ubiquitously expressed in epithelial and endothelial cells and has multiple functions in cleaving the penultimate positioned prolyl bonds at the NH2 terminus of physiologically important peptides in the circulation including chemokines and mitogenic growth factors (14, 15). In the endothelial cell models, neuropeptide Y was reliant on processing by DPP4 to become proangiogenic (16) and its product neuropeptide Y3-36 promoted the migratory activities of endothelial cells (17). On the other hand, DPP4 binds collagen (18, 19) and denatured collagen (gelatin; ref. 20). Our previous study showed that monoclonal antibody (mAb) inhibition of the binding of DPP4 to gelatin blocked subsequent migration of fibroblasts in collagenous matrices (20). In contrast to DPP4, seprase is undetectable in differentiated tissue cells (20–23). Seprase is transiently expressed in various cellular types and accumulated at invadopodial membranes where it is involved in gelatinolytic activity and cell invasion in collagenous matrices (24–29). However, the mechanism involving DPP4 and seprase in the cell invasion pathway of angiogenic endothelial cells remains to be elucidated.

To explore a potential function of DPP4 and seprase in the invasion of endothelial cells into the extracellular matrix, we used human endothelial cell culture models that include the promotion of migratory activities of endothelial cells on the two-dimensional substratum and the invasion of the cells into the three-dimensional collagenous matrices. Our goal is to define the role of DPP4 and seprase in the cell invasion pathway during the invasion of endothelial cells into collagenous matrices, an important process of angiogenesis. As αvβ3 integrin was shown to associate with invadopodial formation (30) and β3 integrins (31) and αvβ1 integrin (32) were adhesion molecules involved in angiogenesis, the role of DPP4 and seprase in the in vitro models was examined and compared with that of αvβ3 or αvβ1 integrins. We found that DPP4 and seprase were induced to form a protease complex at the invadopodia-like protrusions of endothelial cells involved in the invasion of collagenous matrices. mAbs against the gelatin-binding domain of DPP4 block matrix degradation, cell migration, and invasion in collagenous matrices. Because the DPP4-seprase complex is present at very low levels in differentiated endothelium...
and normal tissues but up-regulated in the invasive endothelial cells of human tumors, it makes an attractive therapeutic target for tumor angiogenesis.

Materials and Methods

Materials. Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (ATCC; Rockville, MD) and used within four passages. Endothelial cells were cultured in Medium 199 (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mmol/L t-glutamine, 1 unit/ml penicillin, 10 μg/ml streptomycin, 5 units/ml heparin, 200 μg/ml endothelial cell growth supplement (Collaborative Research, Inc., Bedford, MA), and 20% bovine serum (HyClone, Logan, UT). Rat mAbs E26, E19, and E3 are directed against human placental DPP4; rat mAbs D8 and D28 against human placental seprase (26, 33); rat mAb C27 against human melanoma β1 integrin; and rat mAb C37 against a 90-kDa cell-surface glycoprotein gp-90 (25). Common protease inhibitors, including EDTA (an inhibitor of metalloproteinases) and 4-(2-aminomethyl)benzenesulfonilfluoride (AEBSF; an inhibitor of serine proteases), were used to assist in identifying DPP4 and seprase in gelatin zymography and prolyl dipeptidase membrane overlay assay.

As an immunogen, the seprase-DPP4 complex was isolated from human placenta, and antibodies were produced as described (26, 33). mAbs E26, E19, and E3 belong to immunoglobulin G2a subclass and react with DPP4 but not with seprase. mAbs E26 and E19 block the binding of DPP4 and the DPP4-seprase complex to denatured type I collagen, gelatin degradation by the DPP4-seprase complex, and fibrillogenic invasion into the collagen gel, but mAb E3 does not (20). In addition, mAbs E19 and E26 are not directed against the catalytic domain of the enzyme and have no requirement for properly folded active site: E19 and E26 epitopes are not masked after cross-linking and after formalin fixation. Goat anti–urokinase-type plasminogen activator (uPA; C-20) antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-β1, and anti-β3 mAbs were from ATCC (clones L2.50 and AP-3, respectively).

Real-time reverse transcription-PCR analysis. Total RNA was purified from 1 × 10⁶ cells by RNasey Mini Kit (Qiagen, Valencia, CA). RNA (1.0 μg) was reverse transcribed by First Strand cDNA Synthesis Kit for reverse transcription-PCR (AMV, Roche, Mannheim, Germany) with random primers supplied by the kit. To measure mRNA levels, two primers each were designed for human seprase cDNA (5'-AGAAGAAAGACAGATGTG-3' and 5'-CACACCTTCTGCTTGGGAGGAT-3'), human DPP4 cDNA (5'-AGTACTACTGGCTGTTGGAGA-3' and 5'-CACACCTGAGGATCCTT-3'), and human MT1-MMP cDNA (5'-GAGAACGACCCGATTTGCTGC-3' and 5'-TGGTTGTATACAGGAACAGAGG-3'). To quantify β-actin mRNA, two primers were used (5'-AGATGACCAGCACTATGTTGCA-3' and 5'-GCACAGCTCTCCTTTAATGCTA-3') to use an endogeneous control with a PCR product of 300 bp. Real-time PCR reactions were conducted using the Quantitect SYBR Green PCR kit (Qiagen) according to the specifications of the manufacturer. Assays were carried out in the DNA Engine Option (MJ Research, Waltham, MA). The plasmids were used as template DNA at concentrations ranging from 1 ng to 10 fg to produce standard curves according to the recommended protocols of the manufacturer. Quantity calculation of the sample was achieved with Option Monitor software (ver. 2.02). Each sample was analyzed in five replicates per experiment. To correct the differences in both RNA quality and quantity between samples, data were normalized using the ratio of the target cDNA concentration to that of β-actin, a housekeeping gene.

Isolation of DPP4-seprase complex. HUVEC were cultured at low density to prevent stable formation of cell-cell contacts. To prepare cell lysates, each culture plate was washed thrice with PBS and extracted with 125 μl/cm² of a solution of PBS containing 1% Triton X-100. Extraction was done on a rotary shaker at 25 rpm (Bellco Orbital Shaker, Vineland, NJ) for 2 hours at 25°C. The cell layer and buffer were transferred to a 50-ml conical tube and incubated for 3 hours at 4°C with end-over-end agitation. The extract was clarified by centrifugation at 10,000 × g for 20 minutes at 4°C and the supernatant was used for immunoprecipitation reactions.

To prepare for immunofluorochromatography, purified rat mAbs (2.5 mg) were coupled to 1 ml CNBr-Sepharose 4 MB (Pharmacia Biotech, Inc., Piscataway, NJ). For each set of experiments, 0.25 ml of mAb beads was used to immunoprecipitate protein complexes from 25 ml of cell extract with end-over-end agitation for 12 hours at 4°C. After three washes in 25 ml of extraction buffer, the beads with coupled antibody-antigen complexes were resuspended in 0.25 ml of 0.1% glycine-HCl (pH 2.4) elution buffer and incubated for 5 minutes at 4°C. The sample was transferred to an Amicon filter insert (0.45 μm, 400 μl capacity) and centrifuged at 10,000 rpm in an Eppendorf microfuge for 10 minutes at 4°C. The filtrate was neutralized by addition of 2 mol/L Trizma base. To determine the subunit composition of isolated protein complexes, immunoprecipitates were analyzed by immunoblotting analysis using anti-seprase, anti-DPP4, and anti-α5, integrin mAbs as described (33).

Gelatin zymography and a prolyl dipeptidase substrate membrane overlay assay. The isolated proteins were examined for their peptidase and gelatinase activities using a prolyl dipeptidase substrate membrane overlay assay as described (26). Specifically, DPP4 activity was assayed using a substrate overlay membrane coupled with the fluorescent substrate Ala-Pro-4FC (Enzyme Systems Products, Dublin, CA) according to the instruction of the manufacturer. The membrane was moistened in 0.5 mol/L Tris-HCl (pH 7.8), placed against the gel, and incubated for 15 minutes at 37°C in a humidified chamber. The membrane was then removed from the gel and air-dried. The DPP4 activity of individual proteases was monitored by detecting AFC released from the substrate using a long-wavelength UV lamp. The protease specificity was determined by gelatin zymography with molecular mass markers used: thyroglobulin (66 kDa), apoferritin (443 kDa), myosin (220 kDa), phosphorylase b (97.4 kDa), and albumin (66 kD; Sigma, St. Louis, MO).

Microscopic colocalization of DPP4 and seprase. For direct immunofluorocolorization, purified rat mAb E19 against DPP4 was directly conjugated with FITC (FITC hydrochloride, 10% on Celite, Research Organics, Inc., Cleveland, OH) according to the instruction of the manufacturer. Anti-seprase mAb D28 was similarly conjugated with rhodamine (tetramethylrhodamine, 10% on Celite, Research Organics). Cells were cultured either on hydrated type I collagen films or in collagen gel, fixed, and immunolabeled in a single step using these directly conjugated mAbs as described (20). Cell samples were photographed using the Planap 25/12 objective on a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) under epifluorescence. Prepared cell samples were also observed under an Olympus Flview FX300 laser confocal microscopy (0.2-μm laser sections). Serial sections of paraffin-embedded tissue blocks, including both breast infiltrating ductal carcinomas and corresponding normal tissues from the same patients, were obtained from the Department of Pathology at Yamamashi Medical University, Japan. These tissues were fixed with 4% paraformaldehyde in PBS for 2 to 4 hours at 4°C, followed by paraffin embedding. Anti-seprase mAb D8 or D28 or anti-DPP4 mAb E19 or E26 or control antibody was added at a dilution of 1:10 to 1:25 of serum-free hybridoma supernatants to each section and incubated at 4°C overnight in a humidity chamber. Similarly, mouse mAbs against specific cell type markers, including CD31/platelet-endothelial cell adhesion molecule-1 endothelial cell marker (CD31; clone JC/70A, NeoMarkers, Fremont, CA) and cytokeratins 4, 5, 6, 8, 10, 13, and 18 (clone C-11, Sigma), were added to serial sections for marking specific cell types. Bound primary antibody was then detected by streptavidin-biotin-peroxidase technique (Dako, Carpenteria, CA) according to the instructions of the manufacturer using
diaminobenzidine (3,3′-diaminobenzidine tetrahydrochloride, Sigma) as a chromogen and counterstaining was done with hematoxylin.

**Assays for endothelial cell migration and gelatin degradation.** The methods for preparing fluorescently labeled type I collagen hydrated films or gel and for measuring gelatinase activity of migratory cells were previously described (20). Media containing control antibodies or inhibitory mAbs (300 μL/well) were added and their effects on cell migration in real time were observed using phase-contrast and fluorescence microscopy (Nikon Inverted Microscope). Cell migration and gelatin removal were quantified by measuring the areas of cell outgrowth and fluorescent gelatin removal by migratory cells using NIH Image 1.62b/fat analysis program.

**Matrix degradation assays using fluorescently labeled Matrigel and type I collagen gels.** The ability of endothelial cells to form networks and remodel Matrigel was accessed according to a previously described method with modification (20). Briefly, 50 μL/well of Matrigel (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA) or type I collagen were polymerized in a 96-well tissue culture plate (Nunc, Rochester, NY). Polymerized Matrigel and type I collagen gel were labeled with fluorescent dyes and evaluated by a previously described method (20). Less than 5% of peptides were released from FITC-Matrigel and TRITC-type I collagen independent of cells and were used as baseline values. In parallel, thymidine and leucine incorporations were used to determine the metabolic activities of cells under each culture condition, in which 150 μL/well of medium containing 2 μCi/mL [3H]thymidine or [3H]leucine were added into the culture and the cell layers were solubilized in scintillation fluid and counted in a scintillation counter (Beckman LS-7500).

**Results**

Enhanced expression of DPP4 and seprase in migratory endothelial cells. To determine if DPP4 and seprase contribute to matrix degradation and migration of endothelial cells on two-dimensional collagenous substratum, antibodies directed against DPP4 (E3, E19, and E26), seprase (D8, D28, and D43), and β1 integrin (C27) were used to examine the expression and localization of these transmembrane proteins in a HUVEC monolayer where all cells were in contact with each other and migration was minimal due to contact inhibition. In addition, distribution of proteins was examined in the endothelial cells that were induced to migrate by scratch wounding in the center of the monolayer (20). Figure 1A shows double-labeled immunofluorescence of migratory and quiescent endothelial cells in such an in vitro monolayer wounding that there is an increased expression of DPP4 and seprase (data not shown) in migratory endothelial cells at the wound edge (Fig. 1A, b and b′). In comparison, the common cell-surface adhesion receptor β1 integrins are highly expressed on the cell surface of the endothelial cells at the wound edge (Fig. 1Ac) and in the cell-to-cell junction of the monolayer (Fig. 1Ac′). The observation of relative distribution of DPP4 or seprase and β1 integrins in the endothelial cells at the wound edge and in the monolayer (Fig. 1A) corresponds with the inhibition data shown in Fig. 4 that DPP4 and seprase may act on the migratory process of endothelial cells whereas β1 integrins play a role in maintenance of the endothelial monolayer. Similar to β1 integrin, MT1-MMP was found evenly distributed on the cell surface of HUVEC at the wound edge and in stationary monolayers (data not shown).

HUVEC monolayers were also induced to migrate by passage to grow at low cell density on hydrated two-dimensional collagenous matrix (34, 35). The confluent endothelial cells were found to contain low levels of seprase and DPP4 proteins and their proteolytic activities were also low (Fig. 1B-D). However, growth of endothelial cells in sparse culture promotes cellular migratory activity on the two-dimensional collagenous substratum (data not shown); it also causes an increased expression of DPP4 and seprase proteins and their proteolytic activities (Fig. 1B-D). Using gelatin zymography (Fig. 1C) and prolyl dipeptidase substrate membrane overlay assay (Fig. 1D), proteolytic activities due to DPP4 and seprase were more evident in sparse (−) than confluent (+) HUVEC cultures. On gelatin zymography (Fig. 1C), DPP4 exhibits its diffuse proteolytic activity at 220 to 180 kDa and seprase shows its proteolytic activity at 170 to 160 kDa, which persist when gel was treated with a common metallo-protease inhibitor, EDTA (Fig. 1C, +EDTA) but disappear when gel was treated with a common serine protease inhibitor AEBSF (Fig. 1C, +25 μM AEBSF). On the prolyl dipeptidase substrate membrane overlay (Fig. 1D), DPP4 also exhibits its diffuse proteolytic activity at 220 to 180 kDa and seprase shows its proteolytic activity at 170- to 160-kDa.

In comparison, β1 integrin proteins (Fig. 1B) and EDTA-sensitive and AEBSF-resistant MMP (Fig. 1C) were similarly detectable in both confluent and sparse endothelial cultures. These results suggest that the migratory activity of the endothelial cells on the two-dimensional collagenous substratum is associated with an increased expression of DPP4 and seprase proteins and their proteolytic activities. Real-time PCR assays done on the endothelial cells show that there is a significant increase of DPP4 mRNA expression in sparse culture as compared with confluent culture (P = 0.0007), a moderate increase of MT1-MMP mRNA expression (P = 0.0043), and no change of seprase mRNA expression (P = 0.1041), suggesting that increased DPP4 and MT1-MMP mRNA expression occurred in migratory endothelial cells (Fig. 1E).

Association of the DPP4-seprase complex with invadopodia and lamellipodia of migratory endothelial cells. We found by coprecipitation, colocalization, and surface cross-linking experiments that like migratory fibroblasts (20), DPP4 and seprase in migratory HUVEC were present as a large complex. Aggregates of ~820-kDa mass in the nonionic detergent Triton X-100 (Fig. 2C) were shown as 170- to 220-kDa homodimers dissociated in the SDS ionic detergent, which were reactive on immunoblots to mAb D8 (against seprase) and mAb E19 (against DPP4; Fig. 2A). Immunoprecipitation of cell-surface proteins using mAb D28 (against seprase) and mAb E26 (against DPP4), followed by protein separation in SDS-PAGE and Western blotting, identified complexes of the 170-kDa seprase and the 220-kDa DPP4; however, a stable association between seprase or DPP4 and α5 or β3 integrins was not detected (Fig. 2A).

To examine the relationship of DPP4 and seprase to the invadopodia and lamellipodia of migratory endothelial cells on a two-dimensional collagenous substratum, double-label immunofluorescence experiments were done (Fig. 2B). We found that invadopodia and lamellipodia of endothelial cells migrating on the two-dimensional collagenous substratum were stained positively with FITC-mAb E19 against DPP4 (Fig. 2B, a and a′) and TRITC-mAb D28 against seprase (Fig. 2B, b and b′). The superimposed image also shows that seprase and DPP4 colocalize at the invadopodia and lamellipodia (Fig. 2B, c and c′). Furthermore, unlabeled mAbs D28 and E19 outcompeted the specific labeling of seprase and DPP4 (data not shown).

The large heteromeric aggregates consisting of DPP4 and seprase were shown by serial procedures involving (i) cross-linking of cell-surface proteins with the irreversible cross-linker BS3, (ii) extraction of cellular proteins by nonionic detergents, and (iii) agarose-acrylamide electrophoresis, and then protein composition was analyzed by immunoblotting and the proteolytic activities by gelatin zymography or ala-pro-AFC (Fig. 2C). On the agarose-acrylamide gel, a large protein complex at ~820-kDa mass was identified to contain proteins recognized by both mAbs against...
seprase (D28) and DPP4 (E26). In three independent experiments involving the above agarose-acrylamide electrophoresis, a stable association of seprase and DPP4 was detected using mAbs against seprase and DPP4 but not those against uPA (Fig. 2C) and \( \alpha_\text{v}\beta_3 \) integrins (data not shown). Gelatin zymography of the surface cross-linked complex revealed gelatinase activity at \( \sim 820\)-kDa, a smear band ranging from 820 to 669 kDa, and homodimeric DPP4 and seprase at 220 to 160 kDa (see Fig. 2C, +EDTA, for better band resolution after inhibition of coisolated MMP). Similarly, prolyl dipeptidase substrate overlay assay shows that the 820-kDa protease complex and 220- to 160-kDa homodimeric DPP4 and seprase display the prolyl peptidase activity (Fig. 2C, Ala-pro-AFC).

Overall, gelatin zymography and the substrate overlay assay confirmed the above protein identification studies that in nonionic detergents a stable association between seprase and DPP4 homodimers occurred. As seprase contains a 97-kDa subunit and DPP4 a 110-kDa monomer, these data suggest that majority of DPP4 and seprase exist as DPP4-seprase complex at \( \sim 820\) kDa on the cell.

Role in migration of endothelial cells on the two-dimensional collagenous substratum.

Figure 1. Enhanced expression of DPP4 and seprase in migratory endothelial cells. A, intracellular DPP4 location of the endothelial cells in the monolayer (a'-d') compared with increased expression and localization of DPP4 to the cell surface of the endothelial cells migrating from the wound edge (a-d, large arrows). HUVEC monolayer was scratched by a pipette tip to create a wound area and cultured for three hours. Cells were fixed and stained with antibodies against DPP4 (E19) and \( \beta_1 \) integrin (C27). a, a', phase-contrast images. b, b', immunofluorescent red images for DPP4 localization that was labeled directly with TRITC-mAb E19 against DPP4. c, c', immunofluorescent green images for \( \beta_1 \) integrin localization in endothelial cells that were labeled directly with FITC-mAb C27 against \( \beta_1 \) integrin. d, d', superimposed image of b and c, as well as b' and c', showing that DPP4 colocalized with \( \beta_1 \) integrin in most cells of the wound edge (yellowish orange structures) but only a few spots of colocalization seen in the cells inside the monolayer (a', red and green structures). Bar, 50 \( \mu \)m. B, immunoblotting analysis of seprase and DPP4 expression in confluent (+) and sparse (-) HUVEC cultures. Protein expression was identified using anti-seprase (D28), anti-DPP4 (E26), anti-\( \beta_1 \) integrin (C27), and antibody IgG control. There is more seprase and DPP4 expressed in sparse (-) HUVEC cultures than confluent (+) cultures. However, there is little or no change in \( \beta_1 \) integrin expression detected in confluent (+) and sparse (-) conditions. IgG control was made with secondary horseradish peroxidase (HRP) antibody. Ib, immunoblotting. C, gelatin zymography showing that seprase activity is present in sparse (-) but not in confluent (+) HUVEC cultures. Seprase was shown by the 170-kDa lytic band in gelatin zymograms incubated in a buffer containing \( \text{Ca}^{2+} \) (+2 mM CaCl\(_2\)) or EDTA (+2 mM EDTA) but diminished in a buffer containing the serine protease inhibitor AEBSF (+25 \( \mu \)M AEBSF). The 170-kDa gelatinase (seprase) activity was elevated in sparse cultures whereas the 62-kDa MMP activity remained the same in confluent (+) and sparse (-) conditions. D, prolyl dipeptidase substrate membrane overlay assay showing prolyl dipeptidase activity in confluent (+) and sparse (-) HUVEC cultures. Cleavage of substrate ala-pro-AFC was seen due to dipeptidase activity at 220 to 180 kDa for DPP4 and that at 170 to 160 kDa for seprase derived from confluent (+) and sparse (-) HUVEC cultures. There is more cleavage of ala-pro-AFC in sparse (-) than confluent (+). E, quantification of seprase, DPP4, and MT1-MMP mRNA levels in confluent (6-cm) and sparse (15-cm) HUVEC cultures. Y-axis, ratio of seprase, DPP4, or MT1-MMP mRNAs in picograms and that of \( \beta\)-actin in nanograms. Columns, mean (n = 4); bars, SD.
seprase-DPP4 complex in cell migration and the localized degradation on the two-dimensional collagenous substratum, we developed a monolayer wound closure assay using HUVEC cultured on a two-dimensional hydrated type I collagen substratum (Fig. 3A). As described previously (20), a cell monolayer wound model was covered with a thin layer of fluorescent type I collagen gel for morphologic examination of cell migration and gelatin removal by cells (Fig. 3A). Cell migration and local collagen and/or gelatin (state of collagen is uncertain) removal by cells were measured by counting the area of cell migration or gelatin removal using image analysis in conjunction with phase-contrast and fluorescence microscopy. The fluorescent collagen gel, without cells and with the serine protease inhibitor AEBSF and the metalloproteinase inhibitor CT1847, released very little fluorescent peptides into the medium during the first 24 hours of incubation.

As described previously (20), inhibitors of metallo- and serine proteases inhibited the pericellular degradation of collagenous matrices by migratory fibroblasts; inhibition of gelatin degradation by mAbs E19 and E26 resulted from antibody effects on binding of the gelatin substrates to DPP4 in the DPP4-seprase complex. In a collagen gel, cells at the wound edge migrate into the gel and close the wound within 24 hours (Fig. 3A, a and b); however, these cells did not migrate in the presence of mAb E19 directed against the gelatin-binding domain of DPP4 (Fig. 3A, c and d). Importantly,
mAb C27 (against β1 integrins) is inhibitory to binding/attachment of cells to collagenous substrata; both mAb C27 and mAb E19 block the migration of endothelial cells during wound closure. In contrast, class-matched mAbs (IgG2a), including control IgG, mAb C37 (against a 90-kDa cell-surface glycoprotein gp-90), and anti-DPP4 E3 antibodies, do not block migration (Fig. 3B and C). There was an increase in inhibition with increasing amounts of mAb E19 and mAb C27 (Fig. 3B) and the antibody inhibitory effect

Figure 3. Role of the DPP4-seprase complex in the migration of endothelial cells on two-dimensional collagen substrata. A, morphology of HUVEC migration at time 0 (a and c) and 24 hours (b and d) after wounding of the monolayer. The wound was closed within 24 hours (b) and the cell migration could be blocked by mAb E19 against the gelatin-binding domain of DPP4 (d) B, dose-dependent inhibition of cell migration by mAb E19 (against DPP4; ○) and C27 (β1 integrin; △) but not by control mAb E3 against DPP4 (DPP4-; ▲) or C37 (against 90-kDa cell-surface glycoprotein gp-90; ●). Three experiments examining the monolayer wound closure model at 4 hours were carried out for each antibody. Cell migration was quantified by measuring the areas of cell advancement from the original wound edge. Points, mean; bars, SD. C, time course of antibody inhibition of cell migration. All antibodies, mAb E19 (against DPP4; ○), C27 (β1 integrin; △), E3 (DPP4-; ▲), C37 (glycoprotein gp-90; ●), or buffer alone (Control; ▯), were applied at 5 μg/mL. Experimental conditions were the same as in (B).
could be observed within 24 hours (Fig. 3C). Consistently, there was no significant alteration in [3H]thymidine uptake in cell cultures under parallel conditions (data not shown).

Role in cell migration and localized degradation in the three-dimensional collagenous matrices. The role of the DPP4-seprase complex in the matrix degradation and migration of endothelial cells in three-dimensional collagenous matrices was examined through the use of two collagen-based models, Matrigel (Fig. 4) and type I collagen gel (Fig. 5). Effects of mAbs E19 and E26 against the gelatin-binding domain of DPP4 and anti–β1 integrins and of protease inhibitors on matrix degradation and cell migration were accessed by adding agents into the models before or after initiation of cell migration.

When seeded as cell clusters in Matrigel, HUVEC migrated from cell clusters to form networks in 24 hours (Fig. 4A). Both mAbs E19 and E26 against the gelatin-binding domain of DPP4 blocked such cell migration and formation of cell networks (Fig. 4B). In the measurement of areas covered by the cell network, we were able to detect that anti-DPP4 mAb E19 or E26, anti–β1 integrin mAb C27, and MMP inhibitor CT1847 blocked migration and formation of the network by endothelial cells in Matrigel (P < 0.05; Fig. 4B). However, when added into preexisting cellular network, only anti–β1 integrin mAbs C27 and CT1847, but not the anti-DPP4 mAbs E19 and E26, perturbed the preexisting cellular network (Fig. 4C). None of the other mAbs to DPP4 and seprase affected formation of the cellular network in Matrigel. These data suggest that the DPP4-seprase complex is involved in sprouting of endothelial cells in Matrigel and the maintenance of spread morphology of endothelial cells, which requires continued action of MMP and β1 integrins.

To measure the cell-associated matrix degradation in Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B), a microtiter plate format of the degradation assay was applied. HUVEC were seeded in low density (1.0 × 10^5 per well) in fluorescein-labeled Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B) in 96-well microtiter plates for 6 to 24 hours and matrix-degrading activities were measured by the release of fluorescent peptides from immobilized matrices using spectrofluorimetric analysis (Fig. 5A and B). These endothelial cells showed time-dependent degradation of Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B) from 6 to 24 hours in culture. The cell-associated degradation of Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B) was inhibited by AEBSF (20 μmol/L), CT1847 (50 nmol/L), and mAb E19 (against DPP4, 5 μg/mL), but not by the control mAb C37 (anti-gp-90, 5 μg/mL), within 24 hours of incubation. Cell metabolic activity remained unaltered in the presence of AEBSF, mAb E19, and control mAb C37 as indicated by [3H]leucine uptake by the endothelial cells in parallel experiments.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Role of the DPP4-seprase complex in the degradation of collagenous matrices by migratory endothelial cells in three-dimensional Matrigel and type I collagen gel. A, degradation of Matrigel by HUVEC cultured for 6 to 24 hours as described in Materials and Methods. Release of fluorescent peptides from Matrigel was inhibited by mAb E19 (against DPP4, 5 μg/mL), serine protease inhibitor AEBSF (20 μmol/L), and metalloproteinase inhibitor CT1847 (10 nmol/L) but not by control mAb E3 (anti-nonfunctional epitope of DPP4, 5 μg/mL) and control mAb C37 (against 90-kD cell-surface glycoprotein gp-90, 5 μg/mL). Columns, mean of three experiments for each antibody or inhibitor; bars, SD. B, histograms of degradation of fluorescent type I collagen gel by migratory endothelial cells. Gelatin degradation was measured by the release of fluorescent peptides from a TRITC-type I collagen gel by migratory endothelial cells. AEBSF (20 μmol/L), mAb E19 (against DPP4, 5 μg/mL), mAb C27 (against β1, 5 μg/mL), and CT1847 (MMP inhibitor, 10 nmol/L) inhibited the peptide release from type I collagen gel by migratory cells whereas control mAb E3 (anti-nonfunctional epitope of DPP4, 5 μg/mL) and control mAb C37 (anti-gp-90, 5 μg/mL) did not. Columns, mean of three experiments for each antibody or inhibitor; bars, SD. *P < 0.05, significant inhibition. C, colocalization of seprase and DPP4 in invadopodia-like protrusions of endothelial cells migrating in collagen gels as analyzed by confocal microscopy. Two boxed areas in each field are shown in higher magnification (3×). a, immunofluorescent image of seprase localization on invadopodia-like protrusions that were labeled with FITC-mAb E19 against DPP4. b, superimposed image of a and b, showing that seprase and DPP4 colocalized on invadopodia-like protrusions of endothelial cells invading collagen gels. Each selected plane was 0.2 μm in thickness. Bar, 10 μm.
Furthermore, immunofluorescent confocal microscopic analysis shows the colocalization of DPP4 and seprase in invadopodia-like membrane protrusions of endothelial cells invading surrounding type I collagen gel (Fig. 5A-c).

To test if the DPP4-seprase complex exists in active endothelial cells in vivo, the cellular distribution of DPP4 and seprase was examined in invasive breast ductal carcinoma. In serial sections from eight breast tumor specimens examined, there was no discernible antibody labeling for seprase and DPP4 in endothelial cells of large blood vessels within the tumor and adjacent normal tissue (Fig. 6). Interestingly, mAbs E19 and D8 labeled the DPP4-seprase complex present in the endothelial cells of capillary-like microvessels within the tumor (Fig. 6C and D, solid arrows) but the antibody labelings were not detectable in endothelial cells of large vessels (Fig. 6C and D, open arrows) or in adjacent normal skin from the same donor. These results strongly support the presence of the DPP4-seprase complex in the endothelial cells sprouting from blood vessels.

Discussion

In this report, we provide evidence of the physical and functional linkage of DPP4 and seprase at the invadopodia of endothelial cells migrating in collagenous matrix. Additionally, we elucidate the role of the noncatalytic domain of DPP4 in the protease complex in influencing extracellular matrix proteolysis and cellular migration on the two-dimensional substratum and invasion into the three-dimensional collagenous matrices. Moreover, we show a novel function of DPP4 gelatin-binding domain in bringing together gelatinous substrates with the DPP4-seprase complex to facilitate the pericellular proteolysis occurring during the invasion of endothelial cells into the extracellular matrices.

We also present new lines of evidence to show that the DPP4-seprase complex is operating at the cellular level. (i) We show by immunoprecipitation, colocalization, and cell-surface cross-linking experiments (Fig. 2) that mAbs E19 and E26 recognize the DPP4-seprase complex as a major form of DPP4 on surfaces of activated endothelial cells. (ii) We show by antibody inhibition experiments that mAbs E19 and E26 block the migration of activated endothelial cells on two-dimensional collagenous substratum (Fig. 3) and in three-dimensional Matrigel (Fig. 4). (iii) The cell-associated degradation of Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B) was inhibited by mAbs E19 and E26, but not by the control mAb C37 or mAb E3. (iv) Immunofluorescent confocal microscopic analysis shows the colocalization of DPP4 and seprase in invadopodia-like membrane protrusions of endothelial cells invading surrounding type I collagen gel (Fig. 5A-c).

This report and previous publications implicate the involvement of the DPP4-seprase complex in tumor angiogenesis. Active endothelial cells from various tissues express various cell-surface proteases, including seprase and DPP4, during vascular morphogenesis and angiogenesis (7). This article shows that the DPP4-seprase complex is involved in the invasion of HUVEC into collagenous gels. Human dermal microvascular endothelial cells can also produce the DPP4-seprase complex functioning in a manner similar to HUVEC (4). Importantly, immunohistochemical studies of human invasive ductal breast carcinoma specimens have resulted in the demonstration that DPP4 and seprase are colocalized on endothelial cells of capillary-like microvessels but not large vessels within the tumor (Fig. 6). Attempts to examine the role of these serine proteases in animal tumor angiogenesis models are hampered by the fact that mouse endothelial cells are not recognized by mAbs specific for human DPP4 and seprase.

Additionally, we identified the involvement of DPP4 and seprase in the proteolytic assays on gels and in cell models through uses of broad-spectrum MMP and serine protease inhibitors (CT1847, EDTA, and AEBSF) in combination with mAbs specific for DPP4 and seprase. We found that the large heteromeric aggregates were composed of DPP4 and seprase (Fig. 2C). On the agarose-acrylamide gel, a large protein complex

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4. G. Ghersi, unpublished data.
at ~ 820-kDa mass was identified to contain proteins recognized by both mAbs against seprase (D28) and DPP4 (E26). Such a heteromeric complex was also shown by the proteolytic activities of the cross-linked protease complex (Fig. 2C, Zy, Ala-pro-ACF). In addition, the cell-associated degradation of Matrigel (Fig. 5A) and type I collagen gel (Fig. 5B) was inhibited by AEBSF (20 µmol/L), CT1847 (50 nmol/L), and mAb E19 (against DPP4; 5 µg/mL), but not by the control mAb C37 (anti-gp-90; 5 µg/mL). Together, our data support the notion that the DPP4-seprase complex is operating at the cellular level to promote the migration and invasion of endothelial cells.

The ability of DPP4 to bind to multiple molecules including seprase and gelatin substrates allows not only activation of both DPP4 and seprase but also their cooperative degradation of the extracellular matrix at the cellular invasion front. These localized proteolytic events could explain the dramatic effect of mAbs E19 and E26 on the degradation of gelatinous matrices by migratory endothelial cells comparable to the effect exhibited by inhibitors of PA-plasmin, type II transmembrane serine protease, and MMP systems (Fig. 5A and B). This is consistent with previous findings, which suggest that the DPP4-seprase complex must interact with major protease systems in the regulation of biological processes, such as tumor angiogenesis (1–5). It is possible that MMP and serine proteases are required for the proteolytic modification of the extracellular matrix that defines the overall growth and migration of tissue cells. The modified matrices may then become susceptible to degradation by the DPP4-seprase complex on the invadopodia of endothelial cells, thereby facilitating angiogenesis. In this study, we provide two lines of evidence to support this hypothesis: (i) inhibition of the gelatin-binding domain of DPP4 shows that the DPP4-seprase complex has an essential role in the gelatin degradation and invasion of endothelial cells into the extracellular matrix, and (ii) the cross-linking experiments show that the 820-kDa DPP4-seprase complex does not associate stably with PA or MMP systems (Fig. 2C).

Considering that the DPP4-seprase complex is absent in normal differentiate endothelia, blood and stroma (20–23), our data suggest that the DPP4-seprase complex generated in migratory endothelial cells could be a positive regulator for the localization of extracellular matrix proteolysis occurring at the invasion front of angiogenic microvessels. DPP4 has consistently been shown to exhibit a unique activity in that it is able to bind to collagen (18–20) and gelatin (20); formation of the DPP4-seprase complex occurs in fibroblasts during the early stage of wound closure and is known to promote localized pericellular proteolysis and fibroblastic migration in collagenous matrices (20). In addition, DPP4 itself may also possess a seprase-like gelatinolytic activity (36). Taken together, DPP4 may play an important role in the pericellular matrix degradation that occurs in invasive tumors (37–40).

Of note is the speculation surrounding DPP4 and its role as a tumor suppressor. In experimental melanoma progression models, a decline in DPP4 expression correlated with transformed phenotypes, including an increase in tumorigenicity, anchorage-independent growth, and cell survival independent of exposure to exogenous growth factors (41). Recently, a study by Wesley et al. (42) further showed a correlation between DPP4 expression and suppression of basic fibroblast growth factor production and phenotypic changes of human prostate tumor cell lines. In contrast, some malignant tumors, including prostate cancer, were shown to express high levels of DPP4 (37–40). Based on these contradictory reports, the role of DPP4 in tumor suppression warrants further study using alternative functional approaches.

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