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13. ABSTRACT ***(Maximum 200 Words)***

Our studies focused on (1) cloning and sequencing of wild-type endothelial DPP IV (wtDPP IV) and preparation of truncated DPP IV (tDPP IV); (2) identification of surface-associated fibronectin polymers (polyFN) as DPP IV ligand; (3) use of DPP IV$^{(-)}$ Fischer 344/CRJ rats as protein-knock-outs in metastasis; and (4) identification of the DPP IV/FN binding domains. A full-length clone of endothelial wtDPP IV was isolated from a rat lung cDNA library that was identical to hepatic DPP IV. Acid extraction of rat lung yielded a tDPP IV, which was an effective inhibitor of breast cancer cell adhesion to wtDPP IV and lung metastasis. The DPP IV ligand, polyFN, provides multiple binding sites for DPP IV, thereby allowing lung vascular arrest of cancer cells under hemodynamic conditions. Fischer 344/CRJ rats are unsuitable as DPP IV protein knock-out model, because lung endothelia leak expression of DPP IV and are able to support breast cancer cell arrest. The DPP IV binding domains of FN was localized to an N-terminal 30-kDa region and FN type III repeats 13 to 14. Three approximately equal size fragments of the extracellular domain of DPP IV were expressed as GST fusion proteins and are currently studied for FN binding.

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PROGRESS REPORT

THE ROLE OF DIPEPTIDYL PEPTIDASE IV IN LUNG METASTASIS OF BREAST CANCER CELLS

Introduction:

Our studies on "The Role of Dipeptidyl Peptidase IV in Lung Metastasis of Breast Cancer" have focused on the following aims: (1) the cloning and sequencing of wild-type endothelial DPP IV (wtDPP IV) from rat and the preparation of truncated DPP IV (tDPP IV); (2) the assembly of high-molecular-weight FN (HMW-FN) aggregates on breast cancer cell surfaces and the physical characteristics of these aggregates; (3) the evaluation of DPP $IV^{(-)}$ Fischer 344/CRJ for their suitability in breast cancer metastasis research; and (4) the identification of the DPP IV/FN binding domains.

Body:

Cloning and Sequencing of lung Endothelial DPP IV: Endothelial wtDPP IV has been identified as a 110-kDa transmembrane protein that shares its N-terminal sequence (30 amino acids) with that of the originally cloned hepatic DPP IV (Ref. 1). Using a cDNA library prepared from rat lungs and rat hepatic DPP IV cDNA as a probe, we have isolated, cloned, and sequenced the complete endothelial wtDPP IV cDNA. A full-length clone of 2.4 kb was obtained from the lung library and found to be identical to the published sequences for rat hepatic DPP IV (J02997, Hong and Doyle, PNAS 84:7962-7966,1987); J04591, Ogata et al., JBC 264:3596-3606,1989), except for a sequencing error at the C-terminus of J02997. This error occurred after the 765th codon TCC (Ser), where J02997 contains an extra C, resulting in a frame shift thereafter and extension of the C-terminus by additional 25 amino acids, instead of the TCC TTA CGC TAG (stop codon) (Ref. 2). Resequencing of the J02997 clone revealed that the published 25-amino acid extension does not exist. Differences in the amino sequences between endothelial wtDPP IV and the two hepatic DPP IV also exist at codons 38, 183, 332, 352, 392, 394, 562, 624, 767 [J022997: 183,392,562; J04591: 38,332,352,392,394,624] (Ref. 2). These differences are likely due to errors in the published sequences as we employed in our work high fidelity Pwo DNA polymerase and automated sequencing versus taq polymerase and manual sequencing in case of the published hepatic DPP IV cDNAs.

Isolation and Characterization of Truncated, Endothelial DPP IV (tDPP IV) and Functional Analyses: Extraction of rat lungs with acidified, deionized water at pH 4.0, followed by immunoprecipitation with anti-DPP IV mAb 6A3, resulted in the purification of a DPP IV species that had an electrophoretic mobility indistinguishable from that of wt DPP IV. However, N-terminal sequencing of this DPP IV species revealed that it was truncated, containing the complete extracellular domain of wtDPP IV, but lacking the amino acids MKTPWKVLL-GLLGVAALVTIITVPVVLLNK that together account for both the cytoplasmic and transmembrane domains of wtDPP IV (Ref. 2). The molecular mass of this truncated protein was estimated at 107 kDa. tDPP IV was readily soluble in detergent-free, physiological buffers and was used to advantage in anti-adhesion and anti-metastasis studies. When breast cancer cells were incubated with 100 µg/ml of tDPP IV, tDPP IV not only co-localized on the tumor cell surface with the FN aggregates, but was shown to be an effective, competitive inhibitor of breast cancer cell adhesion to wtDPP IV (100% inhibition) and lung metastasis (80% inhibition) (Ref. 2).

Characterization of Breast Cancer Cell Surface Associated Fibronectin as the DPP IV Ligand: The DPP IV ligand, identified as cell surface-associated FN, was further characterized by metabolic labeling of breast cancer cells and by incubating breast cancer cells in suspension culture with ^{125}I-plasma FN (pFN) [mimicking conditions of hematogenous dissemination] (Ref. 1). The data show that cellular fibronectin is assembled exclusively as a disulfide-bonded polymeric-FN (polyFN), while ^{125}I-pFN is first bound to the cancer cell surface in dimeric form, where it undergoes a conformational change from a V-shape to an extended linearized form. This immobilized, conformationally altered FN serves as a scaffold for rapid FN polymerization (self-association of FN molecules). These FN polymers are composed exclusively of FN and consist in part of disulfide-bonded and covalently bonded FN complexes. Functionally, these FN complexes provide multiple binding sites for endothelial DPP IV, thereby allowing lung vascular arrest of cancer cells under hemodynamic shear stresses.

Is the Fischer 344/CRJ Rat a "Protein-Knock-Out " Model for DPP IV-Mediated Lung Metastasis of Breast Cancer? The Fischer 344/CRJ rat harbors a G633R substitution in the DPP IV enzymatic domain (Tsuji et al., Biochemistry 31:11921-11927, 1992). This mutation leads to rapid degradation of the mutant protein in the endoplasmic reticulum. Thus, this rat may serve as a "DPP IV protein knock-out" model in our studies of DPP IV-mediated metastasis of breast cancer. A breeding colony of DPP IV $^{(-)}$ Fischer 344/CRJ was established in our laboratory and the rats extensively evaluated for DPP IV expression. In contrast to published data, Fischer 344/CRJ rats expressed DPP IV on lung capillary endothelia, albeit at greatly reduced levels when compared to normal Fischer 344 rats (Ref. 3). Mutant DPP IV protein was isolated from the lungs of 344/CRJ rats by immunoaffinity chromatography with anti-DPP IV mAb 6A3 and, as expected, found to lack the enzyme activity. However, the Fischer 344/CRJ-derived DPP IV maintained the FN-binding characteristics of wtDPP IV (Ref. 3), further supporting our previous finding that the enzyme substrate domain of DPP IV was not involved in the DPP IV/FN binding (Ref. 1). Expression of DPP IV in the lungs of 344/CRJ rats was sufficient to mediate lung metastasis by breast cancer cells, which was abolished when breast cancer cells were injected intravenously in the presence of soluble, truncated DPP IV (Ref. 3). Thus, Fischer 344/CRJ rats are not useful as a "DPP IV protein knock-out" model.

The DPP IV binding Domain of FN: Primary emphasis during the present funding period was on finding the DPP IV binding domain of fibronectin. Proteolytic FN-fragments and maltose binding protein (MBP)-FN fusion proteins were used in Far Western analyses and adhesion assays to home in on the binding domain. Positive binding reactions with DPP IV were obtained with the N-terminal 30-kDa (harboring the first FN heparin binding site; HepI) and the 70-kDa (harboring both the heparin and collagen binding sites) FN fragments, but only under reducing conditions, as well as the MBP-FN[III(8-15)] (FN type III repeats 8 to 15 harboring the second heparin binding site; Hep II). Further truncation of this fusion protein yielded a negative binding reaction for MBP-FN[III(8-11)] and a positive binding reaction for MBP-FN[III(12-15). MBP fusion proteins of the individual type III repeats 12, 13, 14, and 15 were all negative, presumably due to incorrect folding of the individual repeats. The shortest positive fusion protein was MBP-FN[III(13-14)]. Since FN[III(13-14)] harbors the second heparin binding and heparin effectively blocks DPP IV adhesion to this fragment, the Arg residues previously identified to be critical for heparin binding will be substituted with Ser, and the mutants tested for DPP IV binding.

The FN Binding Domain of DPP IV: Rat endothelial DPP IV is used in identifying its FN-binding domain. To that effect, three approximately equal size fragments of the extracellular domain of DPP IV have been expressed as GST fusion protein in bacteria and in HEK 293 cells. While bacteria were unable to synthesize a full-length fusion protein, expression of the fusion protein in HEK293 cells was satisfactory. Protein purification and binding to MTF7 rat breast cancer cells and/or fibronectin-coated dishes are currently in progress in static cell adhesion assays and by ELISA, respectively.

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1. Cheng HC, Abdel-Ghany M, Elble RC, Pauli BU. Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. J Biol Chem1998; 273(37): 24207-15.

2. Abdel-Ghany M, Cheng HC, Levine RA, Pauli BU. Truncated dipeptidyl peptidase IV is a potent anti-adhesion and anti-metastasis peptide for rat breast cancer cells. Invasion Metastasis 1998;18(1):35-43 .

3. Cheng HC, Abdel-Ghany M, Zhang SH, Pauli BU. Is the Fischer 344/CRJ rat a protein knock-out model for DPP IV-mediated lung metastasis of breast cancer? Clin. Exp. Metastasis (in press).

4. Cheng HC, Abdel-Ghany M, Levine RA, Pauli BU. Truncated dipeptidyl peptidase IV: a potent anti-adhesion and anti-metastasis peptide for rat breast cancer cells. Mol. Biol. Cell 9: 86a, 1998.

5. Cheng HC, Abdel-Ghany M, Elble RC, Pauli BU. Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. FASEB J. 12: A772, 1998.

APPENDED TO THE SUMMARY

Key research accomplishments:

- Cloning and sequencing of lung endothelial DPP IV
- Isolation and characterization of truncated, endothelial DPP IV and functional analyses
- Characterization of breast cancer cell surface associated FN as the DPP IV ligand
- The Fischer 344/CRJ rat is not a useful protein-knock-out model for DPP IV-mediated lung metastasis of breast cancer
- Identification of DPP IV binding domain of FN and FN binding domain of DPP IV

Reportable outcomes:

1. Cheng HC, Abdel-Ghany M, Elble RC, Pauli BU. Lung endothelial dipeptidyl peptidase IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin. J Biol Chem 1998; 273(37): 24207-15.

2. Abdel-Ghany M, Cheng HC, Levine RA, Pauli BU. Truncated dipeptidyl peptidase IV is a potent anti-adhesion and anti-metastasis peptide for rat breast cancer cells. Invasion Metastasis 1998;18(1):35-43 .

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Lung Endothelial Dipeptidyl Peptidase IV Promotes Adhesion and Metastasis of Rat Breast Cancer Cells via Tumor Cell Surface-associated Fibronectin*

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Endothelial cell adhesion molecules are partly responsible for the distinct organ distribution of cancer metastases. Dipeptidyl peptidase IV (DPP IV) expressed on rat lung capillary endothelia is shown here to be an adhesion receptor for rat breast cancer cells and to mediate lung colonization by these tumor cells. Fibronectin (FN) assembled on breast cancer cell surfaces into multiple, randomly dispersed globules from cellular and plasma FN is identified as the principal ligand for DPP IV. Ligand expression correlates quantitatively with the tumor cells' capabilities to bind to DPP IV and to metastasize to the lungs. DPP IV/FN-mediated adhesion and metastasis are blocked when tumor cells are incubated with soluble DPP IV prior to conducting adhesion and lung colony assays. Adhesion is also blocked by anti-DPP IV monoclonal antibody 6A3 and anti-FN antiserum. However, adhesion to immobilized FN is unaffected by soluble plasma FN and, thus, can happen during hematogenous spread of cancer cells at high plasma FN concentrations. The ability of many cancer cells to capture FN molecules on their surface and to augment such deposits by FN self-association during passage in the blood suggests that DPP IV/FN binding may be a relatively common mechanism for lung metastasis.

During the course of hematogenous metastasis, cancer cells escape from the primary tumor, enter the blood stream, arrest in the vasculature of a secondary organ, and extravasate to form new tumor colonies (reviewed in Ref. 1). The fate of tumor cells in the blood circulation has been traced by injecting labeled cells via intravenous and intracardiac routes. These studies conclude that cancer cells initially arrest in the microvasculature of the first organ they enter. Most tumor cells die in this location (2), and only a few succeed to form metastases or recirculate to colonize other organs in a tumor type-specific pattern (3). Clinical assessment supports these data indicating that some cancers favor select secondary locations for metastasis (4). For example, prostatic carcinomas and small cell carcinomas of the lungs preferentially colonize bones and the brain, respectively, while breast carcinomas most frequently metastasize to the lungs, but also to liver, bones, brain, and adrenals. There is mounting evidence that the initial selection of an organ for metastasis occurs at the time of attachment of blood-borne cancer cells to microvascular endothelia of that site. Vascular arrest appears to be mediated by "organotypic" molecules that are expressed on the endothelial cell surface of select vascular branches (*i.e.* postcapillary venules) (reviewed in Refs. 5–7). A specific example of such a molecule includes recent work in this laboratory detailing the isolation and characterization of the 90-kDa lung endothelial cell adhesion molecule-1 (Lu-ECAM-1)[1] (8–11). Lu-ECAM-1 selectively binds lung-metastatic melanoma cells, and its expression on endothelia of pulmonary venules correlates closely with the formation of melanoma metastases in these locations (11). Antiadhesive, anti-Lu-ECAM-1 monoclonal antibodies (mAbs) inhibit colonization of the lungs by lung-metastatic murine B16 melanoma cells but have no effect on lung colonization by other types of lung-metastatic cancer cells tested thus far (9).

In related work more recently, outside-out luminal membrane vesicles isolated from rat lung microvascular endothelia by *in situ* perfusion with a low strength paraformaldehyde solution were shown to bind in significantly larger numbers to lung-metastatic than to nonmetastatic rat breast carcinoma cells (12, 13). In contrast, vesicles prepared from the vasculature of a nonmetastasized organ showed no binding preference for either lung-metastatic or nonmetastatic mammary carcinoma cells. The mAb 6A3 generated against lung-derived endothelial cell membrane vesicles was shown to inhibit specific adhesion of lung endothelial vesicles to lung-metastatic breast cancer cells. The antibody identified a 110-kDa membrane glycoprotein of rat lung capillary endothelia, and N-terminal sequencing established identity with dipeptidyl peptidase IV (DPP IV; also known as CD26 or gp110) (13). Two basic properties of DPP IV may account for the putative ability to serve as an adhesion molecule for cancer cells. First, consistent with its enzymatic function (reviewed in Ref. 14), DPP IV may use its substrate binding domain to form transient, adhesive bonds with substrates associated with the tumor cell surface. Such binding might be mediated by x-proline dipeptide sequences (*e.g.* RP, KP, and GP) of the putative DPP IV substrate. However, there is no direct evidence that such a mechanism may lead to cancer cell binding, although this mode of action has

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[1] The abbreviations used are: Lu-ECAM-1, lung endothelial cell adhesion molecule-1; DPP IV, dipeptidyl peptidase IV; FN, fibronectin; pFN, plasma fibronectin; HEK293, human embryonal kidney cells; OG, octyl-β-glucoside; BME, β-mercaptoethanol; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; mAb, monoclonal antibody; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DOC, deoxycholate; FACS, fluorescence-activated cell sorting; HMW, high molecular weight.

been proposed for human immunodeficiency virus adhesion to and entry into T lymphocytes (15). Alternatively, the ability of DPP IV to bind to fibronectin (FN) via a domain distinct from its substrate recognition site (16) and the previously recognized association between cell surface expression of FN and lung metastasis of rhabdomyosarcoma cells (17) prompted us to investigate whether tumor cell surface-associated FN served as the ligand for DPP IV.

Here, we confirm that DPP IV is an endothelial cell adhesion molecule for rat breast cancer cells and mediates lung metastasis by these tumor cells. The DPP IV ligand is identified as tumor cell surface-associated FN, and concomitantly, a correlation between the level of FN expression and the tumor cells' ability to bind to DPP IV and metastasize to the lungs is established. DPP IV/FN-mediated adhesion and metastasis are blocked when tumor cells are incubated with soluble DPP IV prior to conducting adhesion and lung colony assays. Adhesion is also blocked by anti-DPP IV mAb 6A3 and by anti-FN antiserum but is unaffected by soluble plasma FN (pFN) and thus may readily happen during hematogenous spread of cancer cells *in vivo*.

MATERIALS AND METHODS

Rat Mammary Carcinoma Cells and Their Metastatic Potential—The rat breast carcinoma cell lines R3230AC-MET and R3230AC-LR were obtained from Dr. J. A. Kellen (Sunnybrook Medical Center, University of Toronto, Toronto, Canada) (18). The R3230AC-MET cell line was selected *in vivo* for high lung colonization. The R3230AC-LR cell line was concanavalin A- and wheat germ agglutinin-resistant and nonmetastatic. The lung-metastatic MTF7 clone of the rat mammary adenocarcinoma cell line 13762NF was received from Dr. D. R. Welch (Pennsylvania State College of Medicine, Hershey, PA) (19). RPC-2 cells were isolated from lung metastases of the *in vivo* transplantable Dunning R3327 rat carcinoma MatLyLu donated by Dr. J. T. Isaacs (Johns Hopkins Oncology Center, Baltimore, MD) (20). Detailed tissue typing of Dunning R3327 tumors suggest that these cancers are not, as originally thought, of prostatic origin but are likely derived from mammary epithelium (21). The metastatic potential of the four breast cancer cell lines was expressed as the median (range in parentheses) number of tumor colonies observed in the lungs 3 weeks after intravenous inoculation of 2×10^5 tumor cells into female Fischer 344 rats. R3230AC-MET produced 204 (176–237) lung colonies, R3320AC-LR 0 (0), MTF7 385 (312–397), and RPC-2 285 (78–327). Tumor cells were used for subsequent experiments within 10 passages following evaluation of their metastatic potential. They were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Inc.).

Transformed human primary embryonal kidney cells (HEK293) were used in DPP IV transfection experiments. HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS.

Antibodies—Anti-DPP IV mAb 6A3 was produced in Balb/c mice and purified from hybridoma supernatant by Protein G chromatography (RepliGen, Cambridge, MA) as described (13). mAb 6A3 (IgG_1) recognized rat DPP IV expressed by endothelia of lung capillaries, splenic sinusoidal venules, and renal vasa recta as well as by epithelia of bile canaliculi, kidney proximal tubuli, and small intestinal villi (13). Polyclonal anti-DPP IV antibodies CU31 were prepared in rabbits, using purified rat endothelial DPP IV. Control mAbs were directed against the endothelial cell adhesion molecule Lu-ECAM-1 (6D3; IgG_{2a}) (8, 11). Polyclonal antisera raised against rat pFN were obtained from Dr. J. L. Guan (22), and antisera against human pFN were from Life Technologies, Inc. A polyclonal, anti-peptide antiserum generated against human adenosine deaminase was a gift from Dr. J. W. Belmont (Institute for Molecular Genetics and Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX) (23).

Immunoaffinity Purification of Endothelial DPP IV—Thirty rat lungs, homogenized and washed in ice-cold 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, were extracted for 30 min at 4 °C with 150 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM benzamidine chloride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 0.27 trypsin inhibitory units/ml aprotinin, 30 μg/ml DNase, and 1% Nonidet P-40 (13). The lysates were first precleared on a 1-ml column containing nonimmune mouse IgG immobilized on Protein G-agarose (Life Technologies, Inc.), and the flow-through was directly applied onto a second 1-ml column of anti-DPP IV mAb 6A3 coupled to Protein G-agarose. Columns were washed and eluted as described in detail elsewhere (8, 10). The purity of the isolated DPP IV was monitored by SDS-PAGE (8% polyacrylamide) and visualized by silver or Coomassie Blue staining.

Truncated DPP IV missing the cytoplasmic and transmembrane domains was isolated from acid extracts of rat lungs as described by Yamaguchi *et al.* (24) and purified by immunoaffinity chromatography with anti-DPP IV mAb 6A3. This truncation did not affect the enzymatic and adhesion qualities of DPP IV (data not shown). Truncated DPP IV was preferred over detergent-extracted, full-length DPP IV in antimetastasis assays, since it was soluble in physiological, detergent-free buffers and did not cause any adverse reactions in injected animals.

Affinity Purification of the Metabolically Labeled DPP IV Ligand—Breast cancer cells in logarithmic growth phase were washed for 20 min at 37 °C in methionine-free RPMI 1640 medium and then metabolically labeled overnight at 37 °C with 0.4 mCi of [^{35}S]methionine in methionine-free RPMI 1640 medium containing 20 μM methionine and 10% dialyzed, FN-free FBS. To differentiate between labeled surface-associated and labeled cytoplasmic proteins, tumor cells were extracted in lysis buffer (30 min; 4 °C) either immediately or after treatment with α-chymotrypsin (10 μg/ml; 30 min; 37 °C) as suggested by Hynes (25). Extracts were cleared by centrifugation, and the DPP IV-tumor cell ligand was precipitated with DPP IV immobilized on Affi-Gel 10 beads (Bio-Rad Laboratories). DPP IV-ligand complexes were resolved by SDS-PAGE (5% polyacrylamide) under nonreducing and reducing (2% β-mercaptoethanol (BME)) conditions and visualized by autoradiography.

The FN nature of the DPP IV precipitate obtained from metabolically labeled MTF7 breast cancer cell extracts was further analyzed by autoradiography and Western blotting. In brief, proteins precipitated with DPP IV and separated under nonreducing conditions by SDS-PAGE (5% polyacrylamide) were cut from the gel and extracted in 50 mM ammonium carbonate, 0.1% SDS, and 1% BME overnight at 37 °C. Extracts were centrifuged, and the supernatant was supplemented with 100 μg/ml bovine serum albumin and incubated with a final concentration of 20% trichloroacetic acid for 10 h at 4 °C. The trichloroacetic acid precipitate was collected by centrifugation, washed with 100% cold ethanol, boiled in SDS sample buffer containing 1% BME (10 min), and then subjected again to SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes and probed with anti-FN antiserum (1:1000 in 5% skim milk) as described (10). Data from Western blots were contrasted with data from autoradiographs obtained from the same membranes or from gels prepared identically to those used for Western analysis.

Incorporation of ^{125}I-pFN into the Tumor Cell Glycocalyx—The ability of breast cancer cells to incorporate pFN into their surface coat was tested under conditions that mimicked hematogenous spread. In brief, tumor cells (5×10^6 cells) enzymatically released from their growth surface (0.25% trypsin in PBS; 5 min; 37 °C) were washed once in RPMI 1640 medium containing 10% FN-free FBS to stop the enzyme action and then incubated for various periods of time in rotating suspension cultures in RPMI 1640 medium supplemented with 10% FN-free FBS, 10 μg/ml unlabeled rat pFN (Life Technologies, Inc.), and 1 μg/ml ^{125}I-pFN labeled by the IODO-BEAD™ method as described by the manufacturer (Pierce). Cells were extracted for 30 min at 4 °C in either lysis buffer or 2% deoxycholate (DOC) in lysis buffer without Nonidet P-40 (26). Extracts were precipitated with immobilized DPP IV as described above, and the precipitates were subjected to SDS-PAGE (5% polyacrylamide) under both nonreducing and reducing conditions. The DOC-insoluble fraction was directly applied to SDS-PAGE. FN was visualized by autoradiography.

Plasmid Construction and Transfection—All transfection studies were performed with rat kidney DPP IV cDNA obtained from Dr. D. Doyle (State University of New York, Buffalo, NY) (27). The nucleotide sequence of kidney DPP IV cDNA was 100% identical to that of rat lung endothelial DPP IV cloned in our laboratory.[2] HEK293 cells were transiently transfected with DPP IV cDNA cloned into pRcCMV (Invitrogen, San Diego, CA), using Lipofectamine according to the manufacturer's instructions (Life Technologies). Control HEK293 cells were transfected with the pRcCMV vector alone.

Rosette Assay—A rosette assay was performed between DPP IV-transfected HEK293 cells and MTF7 breast cancer cells. MTF7 cells

[2] H.-C. Cheng, M. Abdel-Ghany, R. C. Elble, and B. U. Pauli, manuscript in preparation.

(1×10^4 cells/well) were seeded onto Lab-Tek® two-well chamber slides (Nunc Inc., Naperville, IL) and grown overnight at 37 °C in RPMI 1640 medium supplemented with 10% FN-free FBS. Cells were then labeled with the cytoplasmic dye Calcein-AM (Molecular Probes, Inc., Eugene, OR) as described by El-Sabban and Pauli (28). A 100-fold excess of either DPP IV- or mock-transfected HEK293 cells (1×10^6 cells/well) previously labeled with SNARF-1-AM (Molecular Probes) was seeded onto the adherent MTF7 breast cancer cells in serum-free medium. MTF7 and HEK293 cells were co-cultured under a gentle rocking motion for 30 min at 37 °C. Nonadherent HEK293 cells were removed by washing, and slides were examined under a fluorescent microscope (excitation, 490 nm; emission, 520–540 nm for Calcein and 550–650 nm for SNARF-1). Rosetting was indicated by the binding of six or more DPP IV-transfected HEK293 cells to MTF7 breast cancer cells. A total of 100 cells were counted in each of three experiments.

Flow Cytometry—Fluorescence-activated cell sorting (FACS) was performed to quantify FN expression on breast cancer cell surfaces. Tumor cells released from their growth surface and recovered as described above were suspended in 10% donkey serum in PBS for 15 min at 4 °C and then incubated with rabbit anti-rat pFN antiserum (diluted 1:100 in PBS) for 1 h at 4 °C. Cells were stained with fluorescein isothiocyanate-conjugated donkey anti-rabbit antiserum in PBS containing 10% donkey serum for 1 h at 4 °C and fixed in 2% paraformaldehyde in PBS. FACS analysis was performed on a Coulter Epics Profile (Coulter Electronics, Hialeah, FL). Nonspecific fluorescence was accounted for by incubating tumor cells with nonimmune serum instead of primary antibody.

A similar protocol was used to quantify the DPP IV expression on HEK293 cells transiently transfected with DPP IV cDNA, using anti-DPP IV mAb 6A3.

Enzyme-linked Immunosorbent Assay—Immulon® 4 Microtitration flat bottom plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with pFN (5 μg/ml in PBS) overnight at 4 °C. Immunopurified DPP IV (0, 0.1, 0.3, 0.5, 1.0, and 2.0 μg/ml) was added to the FN-coated wells in the presence or absence of 10 μg/ml pFN and incubated for 1 h at room temperature. Unbound DPP IV was removed by washing, and bound DPP IV was detected by enzyme-linked immunosorbent assay with anti-DPP IV antiserum CU31 (1:500 in PBS) (8).

Tumor Cell Adhesion Assay—Tumor cell adhesion assays were performed as described (9). The amount of DPP IV adsorbed per unit well surface area of Immulon® 4 Microtitration plates was determined from peptidase activity measurements relative to standard DPP IV enzyme activity curves (29). Assays were conducted in the presence or absence of the following components in PBS: (*a*) mAbs 6A3 and 6D3 (both tested at 50 μg/ml); (*b*) DPP IV substrate GPA and control peptide GGA (Sigma) (20 mM); (*c*) serine proteinase inhibitors 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Calbiochem) or phenylmethylsulfonyl fluoride (Sigma) (1–5 mM); (*d*) soluble DPP IV (wild-type DPP IV solubilized in 0.05% OG in PBS; truncated DPP IV in PBS alone) or glycophorin (50–1000 ng/ml); (*e*) soluble rat pFN (1–10 μg/ml); and (*f*) anti-FN antiserum (diluted 1:50 or 1:100). With the exception of soluble DPP IV and anti-FN antiserum, which were incubated with the tumor cells before addition to DPP IV-coated wells, these compounds were preincubated in DPP IV-coated wells for 1–2 h at room temperature and kept in the assay media throughout the tumor cell binding period unless indicated otherwise.

Lung Colony Assay—Breast cancer cells (2×10^5 cells/0.3 ml of PBS/rat) were inoculated via the lateral tail vein of 6-week-old, female Fischer 344 rats (Charles River Laboratories) to determine their metastatic potential. Rats were sacrificed 3 weeks after tumor cell injection, and lung colonies were counted using a dissecting microscope. Median and range of the number of lung colonies were determined for each cell line. Metastasis inhibition experiments were conducted only with MTF7 cells. For this purpose, MTF7 cells were incubated for 1 h at 37 °C in the presence or absence of purified, truncated DPP IV (80 μg/ml in PBS) prepared as described by Yamaguchi *et al.* (24) and then inoculated into rats as indicated above. Statistical comparisons between treatment groups were performed with Student's *t* test for unpaired data.

RESULTS

Lung Endothelial DPP IV Mediates Adhesion and Metastasis of Lung-metastatic Rat Breast Cancer Cells—Lung-metastatic rat mammary carcinoma cells (MTF7; R3230AC-MET; RPC-2) adhered to DPP IV isolated and purified from rat lungs (capillary endothelia) in a dose-dependent manner (Fig. 1). Adhesion of the three metastatic tumor cell lines plateaued at a coating concentration of 500 ng of DPP IV/50 μl of PBS/well (equal to 2.25 pmol of DPP IV bound per 1 mm^2 of well bottom surface), yielding adhesion values of 60–70% for MTF7 carcinoma cells and 40–50% each for R3230AC-MET and RPC-2 carcinoma cells. By comparison, adhesion of nonmetastatic R3230AC-LR tumor cells reached only 8–12% at the same DPP IV coating concentration. The specific adhesion of these cancer cells to DPP IV was inhibited in a statistically significant manner (approximately 95%) upon incubation of the DPP IV-coated wells with monospecific anti-DPP IV mAb 6A3 (50 μg/ml) (Fig. 2). Control mAbs of the same immunoglobulin class had negligible effects on specific tumor cell binding. Participation of the peptidase substrate domain in the DPP IV binding to breast cancer cells was ruled out when neither the peptide substrate GPA nor the serine proteinase inhibitor AEBSF had any inhibitory effect on the adhesion of lung-metastatic breast cancer cells to DPP IV-coated dishes (Fig. 2). The observed DPP IV binding characteristics were not the result of a possible coprecipitation of adenosine deaminase, since rat lung DPP IV preparations were free of detectable adenosine deaminase (30), as determined by both enzyme assay and Western blotting, and since purified, commercially supplied adenosine deaminase (Sigma) did not support adhesion to lung-metastatic breast cancer cells (data not shown).

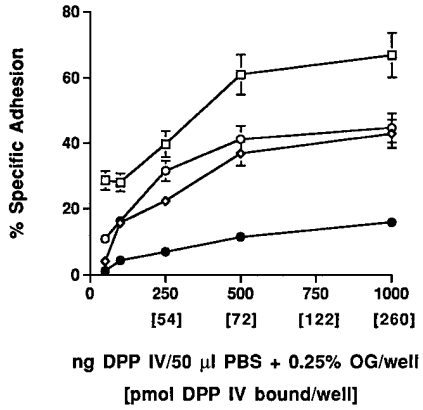

FIG. 1. **Lung-metastatic breast cancer cells adhere to DPP IV-coated wells in a dose-dependent manner.** Wells of Microtitration plates (Immulon® 4, Dynatech) were coated overnight at 4 °C with 50, 100, 250, 500, and 1000 ng of purified endothelial DPP IV, 50 μl of PBS, 0.25% OG/well, respectively. The pmol amounts of DPP IV adsorbed to the bottom surface of the wells were calculated as described under "Materials and Methods" and identified in *brackets* beneath the ng amounts of DPP IV contained in the coating solution. DPP IV-coated wells were seeded with 3×10^4 tumor cells (R3230AC-MET (○); R3230AC-LR (●); MTF7 (□); RPC-2 (◇)) per well, and an adhesion assay was performed as described in Ref. 9. Means ± S.D. are shown from six different experiments.

Preincubation of lung-metastatic breast cancer cells with immunopurified, detergent-extracted DPP IV resulted in a dose-dependent reduction of the specific adhesion of the breast cancer cells to DPP IV-coated dishes (Fig. 3). For example, at a DPP IV concentration of 200 μg/ml in the adhesion assay medium, the specific adhesion of MTF7 breast cancer cells to DPP IV-coated dishes was inhibited by more than 80%. The control membrane protein glycophorin dissolved at the same concentrations in the same buffer as DPP IV had no effect on breast cancer cell binding to DPP IV. Identical adhesion inhibition data were obtained when acid-extracted, truncated DPP IV was used instead of detergent-extracted, full-length DPP IV (data not shown). In accordance with these data, MTF7 breast cancer cells incubated with truncated DPP IV (80 μg/ml in PBS; 1 h; 37 °C) prior to intravenous inoculation into Fischer 344 rats were greatly impeded in their ability to colonize the lungs (Table I). At an inoculation dose of 2×10^5 tumor cells/

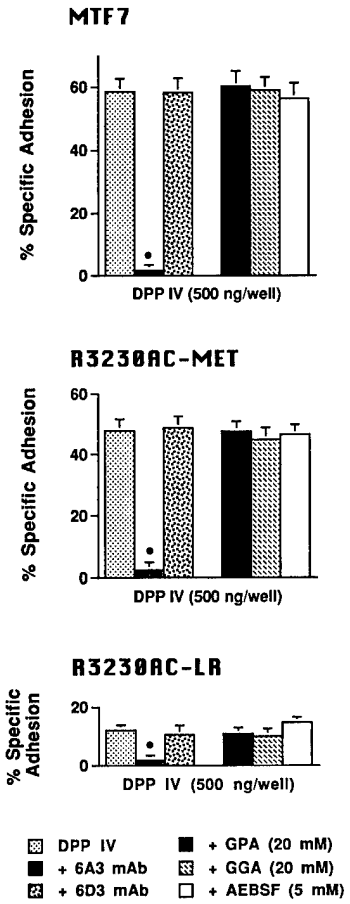

FIG. 2. **Adhesion of breast cancer cells to DPP IV is inhibited by anti-DPP IV mAb 6A3 but not by the DPP IV enzyme substrate GPA and the serine proteinase inhibitor AEBSF.** Microtitration wells were coated with 500 ng of DPP IV, 50 μl of PBS, 0.25% OG/well (overnight at 4 °C); incubated with 50 μg/ml mAb (6A3 and 6D3), 20 mM GPA or GGA, or 5 mM AEBSF in PBS (30 min at 37 °C); and then seeded with 3×10^4 tumor cells and incubated for 20 min at 37 °C. Antibodies, GPA, and GGA were kept in the assay medium throughout the adhesion assay, while AEBSF was removed before tumor cells were added to the wells. Control adhesion assays were performed in PBS alone. Coating and adhesion assay was conducted as described in Ref. 9. Means and S.D. are shown from three experiments. ●, $p < 0.01$.

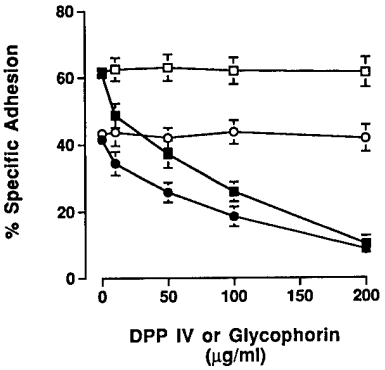

FIG. 3. **Soluble DPP IV competitively inhibits adhesion of lung-metastatic breast cancer cells (R3230AC-MET; MTF7) to DPP IV-coated dishes.** Microtitration wells coated as described in the legend to Fig. 2 were seeded with 3×10^4 tumor cells that had been preincubated for 30 min at 37 °C in various concentrations of purified DPP IV (0, 10, 50, 100, or 200 μg/ml in PBS containing 0.05% OG) and then washed three times with PBS. Control experiments were performed with tumor cells preincubated with glycophorin also dissolved in 0.05% OG in PBS. The adhesion assay was performed as described in Ref. 9. Means and S.D. values are shown from three experiments. Adhesion values between DPP IV- and glycophorin-treated cancer cells (10–200 μg/ml) were statistically significantly different ($p < 0.01$). ■, MTF7 and DPP IV; □, MTF7 and glycophorin; ●, R3230AC-MET and DPP IV; ○, R3230AC-MET and glycophorin.

TABLE I
Lung colonization by MTF7 breast cancer cells and its inhibition by DPP IV

Cell line	Rats with tumor	Lung colonies	
		Median	Range
MTF7	7/7	385[a]	312–397[a]
MTF7 + DPP IV[b]	7/7	79[c]	61–92[c]

[a] This number is low, since many colonies fused with each other, producing large tumor nodules.
[b] MTF7 cells are incubated with 80 μg/ml truncated DPP IV in PBS for 1 h at 37 °C and then washed with PBS and inoculated via the lateral vein of 6-week-old, female Fischer 344 rats.
[c] $p < 0.01$ (relative to MTF7).

rat, MTF7 cells incubated with buffer alone produced a median (range) of 385 (312–397) lung colonies, while DPP IV-decorated MTF7 cells generated only 79 (61–92) lung colonies, representing an 80% reduction in the number of lung metastases. DPP IV treatment of breast cancer cells had no effect on cell growth and viability. Similar metastasis inhibition studies were attempted with anti-DPP IV mAb 6A3. However, this antibody proved to be highly cytotoxic in Fischer 344 rats, causing severe pulmonary endothelial cell necrosis, edema, and hemorrhage, which often led to death of the rats within a few hours after intravenous inoculation of 100–500 μg of purified 6A3 antibody (24).

DPP IV-transfected HEK293 Cells Form Rosettes with Breast Cancer Cells—Adhesion of lung-metastatic MTF7 breast cancer cells to recombinant DPP IV was tested in a rosette assay, using MTF7 breast cancer cells and HEK293 cells transiently transfected with DPP IV cDNA (DPP IV-HEK293). MTF7 cells were allowed to spread for 12 h on a tissue culture plastic surface and labeled with the green fluorescent dye Calcein, then seeded with DPP IV- or mock-HEK293 cells tagged with the red fluorescent dye SNARF-1. DPP IV-HEK293 cells adhered in large numbers to MTF7 cells, forming multicellular aggregates (rosettes) of six or more cells around 32 ± 4% (S.D.) of the MTF7 cells. Most DPP IV-HEK293 cells adhered to the MTF7 cell body (Fig. 4*A*), but individual or rows of DPP IV-HEK293 cells were also bound along slender cytoplasmic processes of MTF7 cells. In contrast, mock-transfected HEK293 cells formed rosettes with only 2 ± 1% (S.D.) of the MTF7 breast cancer cells and, thus, were mostly removed from the dishes during the washing procedure (Fig. 4*B*). Adhesion between MTF7 and DPP IV-HEK293 cells correlated well with the amount of surface expression of recombinant DPP IV on HEK293 cells as assessed by FACS (Fig. 4*C*).

Tumor Cell Surface-associated FN Is Identified as the Ligand for DPP IV—MTF7 cancer cells, which produced the highest DPP IV adhesion and lung colonization values of the three lung-metastatic breast cancer cell lines, were used in the isolation and purification of the tumor cell ligand of endothelial DPP IV. Hence, extracts from metabolically labeled MTF7 cells, grown to approximately 70% confluence in RPMI 1640 medium containing 10% FN-free FBS, were precipitated with Affi-Gel 10-immobilized DPP IV. Upon SDS-PAGE, the DPP IV precipitate resolved as two high molecular weight (HMW) protein bands. The first and major band resided on top of the stacking gel and represented 95% of the DPP IV-precipitable radioactivity (Fig. 5*A*, *lane 1*, *arrowhead*). The second, minor protein band representing the remainder of the DPP IV-precipitable radioactivity was on top of the running gel (Fig. 5*A*, *lane 1*, *double arrow*). Both of these protein bands were reduced with BME to a single protein band of approximately 230 kDa

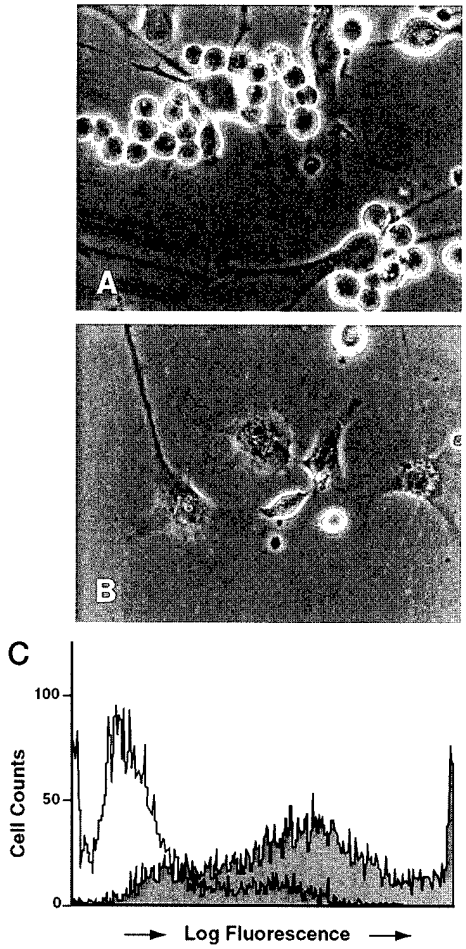

FIG. 4. **DPP IV-transfected HEK293 cells form rosettes with MTF7 breast cancer cells.** MTF7 cells were prepared for a rosette assay with DPP IV- or mock-transfected HEK293 cells as described under "Materials and Methods." *A*, DPP IV-transfected HEK293 cells form typical, multicellular rosettes of six or more cells around MTF7 breast cancer cells attached and spread on a plastic tissue culture surface. *B*, mock-transfected HEK293 cells are unable to adhere to MTF7 cells. Magnification is × 300. *C*, FACS analysis of mock- (*open area*) and DPP IV-transfected (*shaded area*) HEK293 cells.

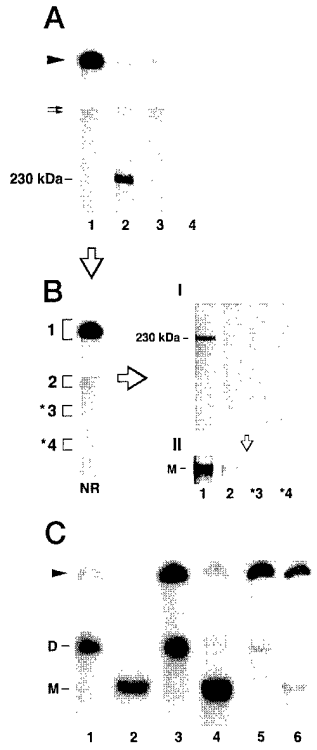

FIG. 5. **Breast cancer cell surface-associated FN is composed of intrinsic and extrinsic FN that is precipitable with DPP IV.** *A*, MTF7 cells were metabolically labeled with [^{35}S]methionine in RPMI 1640 medium in 10% FN-free FBS. Prior to extraction in lysis buffer, tumor cells were treated without (*lanes 1* and *2*) or with (*lanes 3* and *4*) 10 μg/ml α-chymotrypsin (30 min; 37 °C; cell viability >95%). Extracts were precipitated with Affi-Gel 10-immobilized DPP IV, and the precipitates were electrophoresed (5% polyacrylamide) under nonreducing (*lanes 1* and *3*) or reducing (*lanes 2* and *4*) conditions and visualized by autoradiography. *B*, immunoblots were prepared by cutting DPP IV-precipitated protein bands from the 5% polyacrylamide gel run under nonreducing conditions (*lane NR, bands 1* and *2*). Proteins were extracted from each of the two bands as described under "Materials and Methods," reelectrophoresed under reducing conditions (lane number corresponds to band number), and then subjected to autoradiography (*I*) and Western analysis with anti-FN antiserum (1:200 in PBS) (*II*). Both autoradiograph and Western blot reveal a single band in the FN monomer position. Although no signal was recorded in the nonreduced gel (*lane NR*), cuts were also made at the calculated dimeric (**3*) and monomeric (**4*) positions of FN, and the excised gel sections were processed for autoradiography and Western analysis in a manner identical to the HMW bands. *C*, a single-cell suspension of MTF7 cells was incubated for 4 h (*lanes 1–4*) or 12 h (*lanes 5* and *6*) at 37 °C in FN-rich medium (10 μg/ml pFN, 1 μg/ml ^{125}I-pFN, 10% FN-free FBS in RPMI 1640 medium) to allow incorporation of ^{125}I-pFN into the tumor cell surface coat and then extracted with 2% DOC (30 min; 4 °C) (*lanes 1–4*) or Nonidet P-40 lysis buffer (*lanes 5* and *6*). SDS-PAGE (5% polyacrylamide) was performed with the DOC-soluble fraction precipitated with immobilized DPP IV (*lanes 1* and *2*), the DOC-insoluble fraction (*lanes 3* and *4*), and the Nonidet P-40 extract precipitated with immobilized DPP IV (*lanes 5* and *6*). *Odd numbered lanes*, nonreducing; *even numbered lanes*, reducing; *M*, FN monomer; *D*, FN dimer; *closed arrowhead*, HMW FN on top of stacking gel; *, band positions calculated from standards.

that contained the sum of the radioactive counts present in the two HMW bands of the nonreducing gel (Fig. 5*A*, *lane 2*). The DPP IV-precipitated, labeled proteins were associated with the cell surface, since almost no DPP IV precipitate was obtained from tumor cells that had been treated with α-chymotrypsin prior to extraction (Fig. 5*A*, *lanes 3* and *4*). The exclusive composition of the DPP IV-precipitated HMW complexes by a 230-kDa protein was also demonstrated when DPP IV precipitates from surface-biotinylated MTF7 cell extracts were analyzed (data not shown).

To test whether the reported FN binding property of DPP IV was responsible for the precipitated protein, the HMW protein bands were cut from the nonreduced gels, extracted as described under "Materials and Methods," reelectrophoresed under reducing conditions, and analyzed by autoradiography and Western blotting with anti-FN antiserum. As expected, the two HMW protein bands depicted in Fig. 5*B* (*lane NR, bands 1* and *2*) resolved as single protein species of approximately 230 kDa by autoradiography (Fig. 5*B*, *I*, *lanes 1* and *2*). They were confirmed by Western analysis to be FN (Fig. 5*B*, *II*, *lanes 1* and *2*). The strongest signal in both the autoradiograph and the Western blot came from the band on top of the stacking gel (Fig. 5*B*, *NR, band 1*), reflecting the amount of radioactive counts extracted from this band. Although no proteins other than those in the HMW bands were resolved by the nonreduced gel (Fig. 5*B*, *lane NR*), gel sections corresponding to the calculated positions of dimeric and monomeric FN (Fig. 5*B*, *lane NR, *3* and **4*) were also excised and processed identically to the HMW materials. Except for an extremely faint FN monomer band in the Western blot prepared from the extracted gel section of the presumed dimeric FN position (Fig. 5*B*, *II*, *lane *3*), neither the autoradiograph nor the Western blot revealed any noticeable bands (Fig. 5*B*, *I* and *II*).

Conditions Mimicking Hematogenous Spread Promote pFN Incorporation into the Surface Coat of Metastatically Compe-

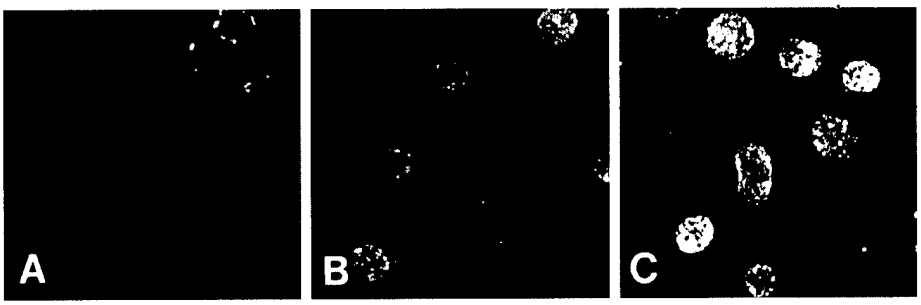

FIG. 6. **Immunocytochemistry with anti-FN antiserum reveals pFN incorporation into surface-associated FN globules of MTF7 cells in suspension culture.** *A*, cells freshly trypsinized and recovered in 10% FN-free FBS in medium (1 min); *B*, cells incubated for 4 h in medium plus 10% FN-free FBS; *C*, cells incubated for 4 h in medium, 10% FN-free FBS, 10 μg/ml pFN. Magnification is × 300.

tent Breast Cancer Cells and Enhance Adhesion to DPP IV—The possibility that blood-borne breast cancer cells use pFN to augment their FN surface coat and, by this action, increase their capability of adhering to DPP IV was tested under conditions that mimicked hematogenous dissemination of tumor cells. A single-cell suspension of MTF7 cells was prepared and incubated in pFN-rich medium (10 μg/ml pFN, 1 μg/ml ^{125}I-pFN, and 10% FN-free FBS in RPMI 1640) for a time period that was equivalent to the presumed time that cancer cells normally spend in circulation before entering and colonizing a secondary organ (up to 4 h). Over the 4-h incubation period in the FN-rich medium, MTF7 cells accumulated significant amounts of ^{125}I-pFN on their surfaces that could be harvested as DOC (2%)-soluble and DOC-insoluble fractions. The DPP IV-precipitate from the DOC-soluble fraction consisted primarily of dimeric FN and a small amount of HMW FN residing on top of the polyacrylamide stacking gel. Both of these FN forms reduced to monomeric ^{125}I-pFN in the presence of 2% BME (Fig. 5*C*, *lanes 1* and *2*). In contrast, the DOC-insoluble fraction consisted of prominent HMW (top of stacking gel) and dimeric ^{125}I-pFN bands. Although much of these materials were again reducible to monomeric FN, a significant portion of the HMW FN was resistant to reduction with 2% BME (Fig. 5*C*, *lanes 3* and *4*). With continued exposure of breast cancer cells to soluble pFN beyond the 4-h period, the HMW FN fraction precipitated with immobilized DPP IV from Nonidet P-40 extracts of MTF7 cells became more and more prominent, while the dimeric FN fraction decreased (Fig. 5*C*, *lanes 5* and *6*). This HMW FN fraction also became increasingly more resistant to reducing agents and was largely nonreducible after 12 h of incubation with ^{125}I-pFN (Fig. 5*C*, *lanes 5* and *6*). Identical results were obtained for the other lung-metastatic breast cancer cells used in this study (R3230AC-MET; RPC-2), while ^{125}I-pFN incorporation into the surface coat of nonmetastatic breast cancer cells (R3230AC-LR) was minimal (data not shown).

These biochemical data correlated well with the density and prominence of immunocytochemically detectable, cell surface-associated FN globules and the adhesion of MTF7 cells to DPP IV; *i.e.* immediately upon their removal from the growth surface by trypsin treatment and a brief wash in medium containing 10% FN-free FBS, MTF7 cells exhibited but a few, weakly stained FN globules on their surfaces and, accordingly, adhered poorly to DPP IV-coated surfaces (9.2 ± 2.2%) (Fig. 6*A*). MTF7 cells incubated in suspension for 4 h in medium supplemented with 10% FN-free FBS showed moderate expression of FN globules on their surfaces and adhered at intermediate levels to DPP IV-coated dishes (34.3 ± 3.1%) (Fig. 6*B*). In contrast, MTF7 cells incubated for the same period of time in pFN-rich medium exhibited prominent, surface-associated FN-globules by immunostaining and adhered in high numbers to DPP IV-coated dishes (63.7 ± 4.8%) (Fig. 6*C*). The amount of FN accumulated on MTF7 cells was quantified by FACS and compared with that on R3230AC-MET and R3230AC-LR. The lung-metastatic MTF7 and R3230AC-MET cancer cells both expressed significantly higher amounts of cell surface-associated FN than the nonmetastatic breast cancer cells R3230AC-LR (ratios of fluorescent intensities of MTF7: R3230AC-MET:R3230AC-LR = 6:5:1) (Fig. 7). Taken together, these findings imply that lung-metastatic breast cancer cells can accumulate significant amounts of pFN into their surface coat during hematogenous spread, thereby significantly increasing their ability to bind to endothelial DPP IV and their chance of lung vascular arrest.

The functional importance of the FN buildup on the tumor cell surface in the adhesion interaction with DPP IV was substantiated by a dose-dependent inhibition of adhesion of breast cancer cells to DPP IV with anti-FN antiserum (Fig. 8). At the highest anti-FN antiserum concentration (1:50) tested, the adhesion of lung-metastatic MTF7 and R3230AC-MET to DPP IV-coated dishes was blocked by more than 90%. This blocking of surface-associated FN was specific for the DPP IV/FN adhesion interaction and did not affect binding of MTF7 and R3230AC-MET cells to adhesion molecules other than DPP IV, *e.g.* the adhesion of these tumor cells to the endothelial cell adhesion molecule Lu-ECAM-1 (data not shown).

The DPP IV Binding Specificity for Immobilized FN Explains Tumor Cell Adhesion to DPP IV in the Presence of Excess Soluble pFN—If this newly discovered binding interaction between endothelial DPP IV and cell surface-associated FN is effective in causing vascular arrest of blood-borne breast cancer cells in the lungs, it must happen in an environment that is rich with soluble pFN (normal blood plasma pFN concentration: 300 μg/ml (31)). Hence, we tested the effect of increasing concentrations of soluble pFN on the adhesion of breast cancer cells to DPP IV in solid-state adhesion assays. None of the pFN test concentrations had any inhibitory effect on the tumor cell binding (Fig. 9). On the contrary, a slight increase in the tumor cell adhesion of both MTF7 and R3230AC-MET cancer cells to DPP IV was observed at higher pFN concentrations, suggesting ongoing incorporation of pFN molecules into the tumor cell surface-associated FN coat during the adhesion assay. These data reflect an inability of DPP IV to recognize and bind to "conformationally inadequate" pFN in solution. This select binding behavior of DPP IV was further exemplified when soluble pFN failed to inhibit the binding interaction between purified DPP IV and immobilized FN in an *in vitro* enzyme-linked immunosorbent assay (Fig. 9, *inset*).

DISCUSSION

Dipeptidyl peptidase IV (DPP IV; CD26) is a serine exopeptidase that was originally isolated and cloned from rat kidney (27) but is now recognized in a variety of tissues including capillary endothelia of the lungs (13, 32, 33). It is a transmembrane sialoglycoprotein that anchors to the plasma membrane by a hydrophobic domain near its N terminus such that the bulk of its molecular mass is exposed to the outside of the cell (27, 34). While this glycoprotein has been extensively investigated with respect to its enzyme and T-cell activation activities (reviewed in Ref. 14), little has been published on its adhesion

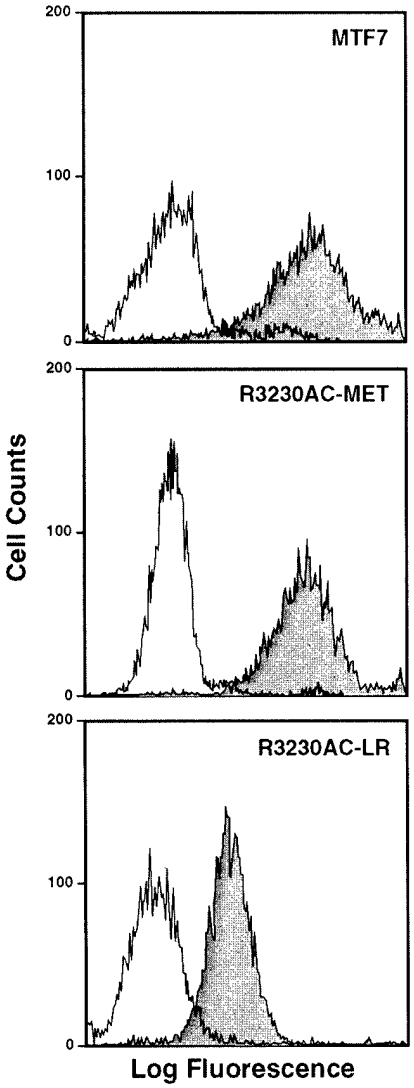

FIG. 7. **Quantification of cell surface-associated FN by FACS using anti-FN antiserum.** Lung-metastatic (MTF7; R3230AC-MET) and nonmetastatic (R3230AC-LR) breast cancer cells grown for 4 h in medium, 10% FN-free FBS, 10 μg/ml pFN were stained with anti-FN antiserum and processed for FACS analysis as described under "Materials and Methods." Histograms are from breast cancer cells stained with preimmune serum (*open area*) and anti-FN antibodies (*shaded area*), respectively. A representative experiment is shown (n = 2).

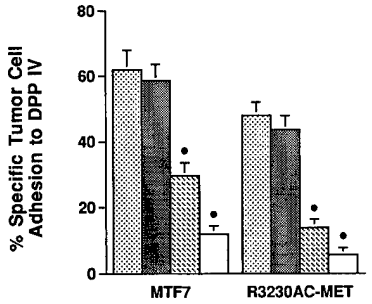

FIG. 8. **Anti-FN antibodies inhibit the binding of rat breast carcinoma cells to DPP IV.** A tumor cell adhesion assay was performed in DPP IV-coated plates (see Fig. 2) in the presence of anti-FN antiserum (1:50 and 1:100) or normal rabbit serum (1:50), as described under "Materials and Methods." Tumor cells used were MTF7 and R3230AC-MET. Means and S.D. were from three experiments. ●, $p <$ 0.01. *Dotted bar*, tumor cells alone; *shaded bar*, with nonimmune rabbit serum (1:50); *dashed bar*, with rabbit anti-FN antiserum (1:100); *open bar*, with rabbit anti-FN antiserum (1:50).

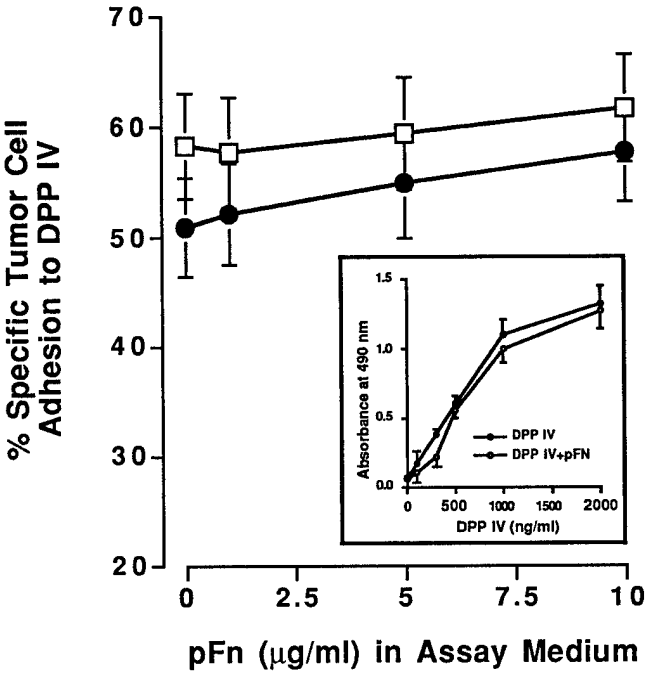

FIG. 9. **Soluble pFN does not inhibit breast cancer cell adhesion to DPP IV and is not recognized by DPP IV.** Lung-metastatic MTF7 (□) and R3230AC-MET (●) breast cancer cells were tested for their ability to bind to DPP IV-coated plates (see Fig. 2) in the presence of various concentrations of soluble rat pFN (0, 1, 5, and 10 μg/ml PBS). There was no inhibitory effect recorded. *Inset*, soluble pFN (10 μg/ml) fails to inhibit the *in vitro* binding interaction between purified endothelial DPP IV (0, 0.1, 0.3, 0.5, 1.0, and 2.0 μg/ml) and immobilized rat pFN (5 μg/ml). Shown is an enzyme-linked immunosorbent assay with rabbit anti-rat DPP IV antiserum CU31 (1:500).

properties although it is recognized as a collagen- and FN-binding protein (16, 35, 36). Here, we show that the FN binding property of DPP IV is responsible for the adhesion of lung-metastatic breast cancer cells, mediating lung vascular arrest and lung metastasis by these cancer cells. The binding interaction between tumor cell-surface associated FN and DPP IV occurs independently of the exopeptidase substrate domain of DPP IV but appears to be critically dependent upon the conformation of the FN substrate. Extensive DPP IV precipitation studies performed on extracts of breast cancer cells with metastatic and nonmetastatic phenotypes consistently show that the preferred FN form precipitated by immobilized DPP IV is cell surface-associated multimeric and dimeric FN. Evidence for this DPP IV/FN binding preference includes (*a*) the direct correlation between the number of lung-metastatic breast cancer cells that were able to bind to DPP IV-coated dishes and the number of tumor cells that expressed prominent FN globules on their surface; (*b*) the precipitation of various cell surface-associated FN forms from extracts of lung-metastatic breast cancer cells with immobilized DPP IV; (*c*) the direct interaction between soluble DPP IV and immobilized FN *in vitro*; (*d*) the formation of rosettes between lung-metastatic breast cancer cells expressing numerous FN globules on their surface and DPP IV-transfected HEK293 cells; (*e*) the specific inhibition of adhesion of lung-metastatic breast cancer cells to dishes coated with DPP IV by monospecific anti-DPP IV mAb 6A3, polyclonal anti-FN antisera, and immunopurified DPP IV; and (*f*) the inhibition of the DPP IV-mediated breast cancer cell adhesion and lung colonization after masking the DPP IV-binding sites on tumor cell surface-associated FN with soluble DPP IV.

The binding of DPP IV to immobilized FN and the inability of DPP IV to recognize soluble pFN implies that the DPP IV binding site is inaccessible in soluble pFN but becomes available when pFN binds to the cancer cell surface, uncoils, and

subsequently associates with other pFN molecules in forming linearized, supermolecular aggregates (31, 37, 38). Uncoiling and linearization of the FN structure and the associated gain in DPP IV binding avidity is reminiscent of the increasing binding avidities of collagen and heparin for their respective FN binding sites with progressive deletion of the FN peptide strand from the C to the N terminus (39) and, as reported more recently, of the binding of the III-1 FN peptide to the truncated (III-10A) but not the complete III-10 FN peptide (40). The process of uncoiling and linearization of pFN may occur under a variety of *in vivo* and *in vitro* conditions including the binding of pFN to cell surfaces (31, 41), gelatin-conjugated agarose beads (39), and even plastic surfaces (42)[3] and seems to proceed by the opening of the pFN arms from a V-shape to a more extended form that requires little energy and therefore can happen relatively easily (37, 43). In the absence of uncoiling, the FN V-shape may obscure the DPP IV-binding site, thereby making it impossible for DPP IV to interact with pFN in solution, even under the most sensitive assay conditions. For cancer metastasis, these binding properties of DPP IV imply that cancer cells expressing abundant uncoiled FN on their surface can dock to DPP IV-expressing lung endothelia although cancer cells are bathed during their hematogenous dissemination in high concentrations of blood pFN (300 μg/ml (31)).

The molecular basis of the initial immobilization of FN on the surface of breast cancer cells is poorly understood. It appears that rat breast cancer cells growing as a solid tumor mass in syngeneic animals support the synthesis of a modest FN-containing matrix.[4] As tumor cells escape the confinement of the primary mass, they enter neighboring blood vessels, thereby becoming blood-borne and traveling passively with the blood stream to other organ sites (1, 7). In the pFN-rich environment of the plasma, tumor cells decorated with a sparse coat of cellular FN use this FN scaffold to acquire pFN molecules for the buildup of a prominent FN coat that is visualized as multiple, densely distributed FN globules by immunocytochemistry. The presence of these randomly dispersed FN globules suggests that the initial FN binding to the cell surface occurs around focal adhesion points from which polymerization is then started by FN self-association (40, 44–47). Such adhesion points are proposed to be sites of integrin clusterings, most likely the classic FN receptor $\alpha5\beta1$ (38, 48, 49). Indeed, this receptor is strongly expressed by all lung-metastatic rat breast cancer cells tested[5] and could promote the initial immobilization of cellular FN molecules on the cancer cell surface. Alternatively, a cellular FN-$\alpha5\beta1$ complex could already form intracytoplasmically (*e.g.* in Golgi vesicles) that upon transport to and incorporation into the plasma membrane could serve as the necessary scaffold upon which self-assembly to supermolecular FN aggregates might occur.

The FN buildup on cancer cell surfaces is comparable with that reported first for normal fibroblasts incubated *in vitro* with ^{125}I-pFN (50); *i.e.* after a 4-h incubation period of lung-metastatic breast cancer cells with ^{125}I-pFN, the total cell surface-associated FN can be harvested as DOC-soluble and DOC-insoluble fractions. Although the 2% DOC extraction used in the present study did not allow a clear partition of the disulfide-bonded FN into the DOC-insoluble fraction as was achieved after extraction of ^{125}I-pFN-incubated fibroblasts with 1% DOC (50), the FN multimers observed on MTF7 breast cancer cells clearly increased with time of incubation with ^{125}I-pFN as reported for normal fibroblasts and hepatocytes (50, 51). However, the multimeric FN on breast cancer cells and normal hepatocytes, both grown in suspension, gradually converted to nonreducible, seemingly covalently bonded FN complexes (51) that were not observed on anchorage-dependent normal fibroblasts (50). Similar to hepatocytes, this conversion is perhaps mediated by a cancer cell-associated transglutaminase activity (51). The rapid accumulation of HMW FN on breast cancer cell surfaces, which plateaued after only 4 h of incubation with ^{125}I-pFN, might be essential for allowing blood-borne cancer cells to become arrested in the lung vasculature, since the large FN aggregates (globules) facilitate binding of multiple endothelial DPP IV molecules, thereby providing an adhesion strength between cancer cell and endothelial cell that can withstand the rigors of hemodynamic shear stresses.

In conclusion, cell surface-associated FN is shown here to mediate lung vascular arrest by binding to endothelial DPP IV. The *in vivo* validity of this adhesion principle is underscored by a more than 80% competitive inhibition of lung metastasis when cancer cell surface-associated FN is masked by preincubation in a DPP IV solution. Given the ubiquity of cancer cell surface receptors (5, 7) that are able to bind FN on their surfaces, upon which FN self-association to HMW structures could occur during their dissemination in the blood, the DPP IV/FN binding mechanism may be a more frequent event in lung metastasis than currently realized (7). This notion is supported by a previously observed strong association between FN expression on the surface of rat rhabdomyosarcoma cell clones and the ability of these tumor cells to bind to lung endothelium and to colonize the lungs (17) and by a similar association between cell surface expression of globular FN complexes and DPP IV adhesion of Chinese hamster ovary cell variants.[3] Finally, the DPP IV/FN binding mechanism appears also to be relevant to lung vascular arrest of blood-borne human breast cancer cells, since various breast cancer cell lines currently investigated in our laboratory have been found to be decorated with FN and to adhere to endothelial DPP IV.

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[3] H.-C. Cheng and M. Abdel-Ghany, unpublished data.
[4] B. U. Pauli, unpublished data.
[5] B. U. Pauli, H.-C. Cheng, and M. Abdel-Ghany, unpublished data.

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Truncated Dipeptidyl Peptidase IV Is a Potent Anti-Adhesion and Anti-Metastasis Peptide for Rat Breast Cancer Cells

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Key Words
Endothelial DPP IV · DPP IV truncation · Breast cancer · Adhesion · Metastasis · Rat

Abstract
A novel adhesion receptor/ligand pair was shown recently to mediate lung vascular arrest and metastasis of rat breast cancer cells. The interacting adhesion molecules are endothelial dipeptidyl peptidase IV (DPP IV) and tumor cell surface-associated, polymeric fibronectin (FN). A truncated DPP IV (DPP IV$^{(31-767)}$: amino acids 31–767) in which the FN-binding site is preserved is shown here to mask the breast cancer cell surface-associated FN complexes, causing a dose-dependent inhibition of adhesion to endothelial DPP IV and impeding lung colony formation by approximately 80%. Since surface accumulation of FN is chiefly occurring during dissemination in the blood and since many cancer cell types have surface receptors by which they may initiate FN accumulation on their surfaces, the present anti-metastatic treatment modality may extend its efficacy farther than appreciated by this study.

Introduction

The colonization of remote, secondary organs by blood-borne cancer cells happens in a tumor type-specific, nonrandom fashion [1, 2]. Increasing evidence suggests that the initial organ selection for metastasis is realized by specific adhesion interactions between tumor cells and the endothelial lining of distinct vascular branches in that organ [3–6]. This interaction mediates vascular arrest of cancer cells under hemodynamic conditions [7] and appears to play a crucial role in initiating the signaling cascades that lead to extravasation of arrested cancer cells [8]. A tumor cell/endothelial cell adhesion receptor/ligand pair that

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mediates lung vascular arrest and promotes selective colonization of the lungs by rat breast cancer cells has recently been discovered and characterized by our laboratory [9–11]. This adhesion receptor/ligand pair consists of endothelial cell dipeptidyl peptidase IV (hereafter referred to as wild-type or wtDPP IV) and tumor cell-surface-associated fibronectin (FN) [10, 11]. DPP IV is abundantly, yet selectively expressed on the lumenal surface of endothelial cells that line lung respiratory capillaries [10, 12]. Its FN ligand consists of high-molecular-weight aggregates that are depicted immunocytochemically as multiple, randomly dispersed 'globules' and that are acquired by cancer cells during hematogenous dissemination from plasma FN (pFN) [11]. The number and prominence of these tumor cell surface-associated FN aggregates correlate well with the ability of rat breast cancer cells to bind to DPP IV and to metastasize to the lungs. This DPP IV binding interaction with cell-surface-associated FN is selective and is unaffected by high pFN concentrations [11].

Here we isolate by acid extraction from rat lungs a truncated form of wtDPP IV. N-terminal sequencing of the truncated DPP IV species and molecular cloning of wtDPP IV show that the truncated DPP IV lacks both the cytoplasmic and transmembrane domains, but contains the complete extracellular domain of wtDPP IV (amino acids 31–767 of wtDPP IV: DPP IV$^{(31-767)}$. When lung-metastatic MTF7 breast cancer cells are incubated with DPP IV$^{(31-767)}$, DPP IV$^{(31-767)}$ colocalizes with tumor cell-surface-associated FN globules, thereby acting as a competitive inhibitor of the MTF7/wtDPP IV adhesion and impeding lung colonization by these cancer cells.

Materials and Methods

Rat Breast Cancer Cells

The lung-metastatic MTF7 clone of the rat mammary adenocarcinoma cell line 13762NF was obtained from Dr. D.R. Welch (Pennsylvania State College of Medicine, Hershey, Pa., USA) [13]. Cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum (FBS).

Cloning and Sequencing of Rat wtDPP IV

A rat lung cDNA library was prepared in λZAP II (Stratagene) and screened with rat hepatocyte DPP IV cDNA (Dr. D. Doyle, SUNY Buffalo, Buffalo, N.Y., USA) [14] under high stringency conditions. Positive clones were converted to phagemids and 5′ ends of potentially full-length inserts (based on size) sequenced directly using the T3 primer site. Full-length endothelial cell wtDPP IV cDNA was sequenced using an automated system at the Biotechnology Resource Center of the Center for Advanced Technology of Cornell University. The BLAST program was used for comparison with published DPP IV (CD26) sequences [14, 15]. The Megalign of the DNAStar package (Lasergene) was employed for multiple sequence alignment.

Immunoaffinity Purification of Endothelial wtDPP IV

wtDPP IV was immunopurified with anti-DPP IV monoclonal antibody (mAb) 6A3 from lung extracts of 30 adult Fischer 344 rats as described [11]. The purified wtDPP IV fractions were collected in 100 m*M* Tris-HCl, pH 8.0, containing 150 NaCl, 100 m*M* glycine, and 0.5% octyl-β-glucoside (OG). Their purity was determined by SDS-PAGE, followed by silver staining and/or Western blotting [6, 11].

Immunoaffinity Purification of DPP IV$^{(31-767)}$

Purified DPP IV$^{(31-767)}$ was prepared from the lungs of 30 adult Fischer 344 rats by a modification of the method of Yamaguchi et al. [16]. In brief, lungs were washed in ice-cold, deionized water, then homogenized. The slurry was acidified with 1 *N* H_2SO_4 to pH 4.0 and incubated overnight at 37 °C. After neutralization and centrifugation, acid extracted DPP IV$^{(31-767)}$ was purified by immunoaffinity chromatography and its purity tested by SDS-PAGE and Western blot analysis as described for wtDPP IV. For N-terminal sequencing, the SDS-PAGE-separated DPP IV$^{(31-767)}$ was transferred to Immobilon-P membranes and Edman degradation performed at the Harvard University protein sequencing facilities.

Colocalization of DPP IV$^{(31-767)}$ and FN on MTF7 Breast Cancer Cell Surfaces

Subconfluent MTF7 breast cancer cells were removed from their growth surface by standard trypsin/EDTA treatment and recovered in suspension in DMEM containing 20% FBS for 1 h at 37°C. Cells were washed twice in PBS and incubated with 100 μg/ml biotinylated DPP IV$^{(31-767)}$ for 1 h at room temperature [11]. All subsequent incubations were carried out at room temperature. Washed MTF7 cells were incubated with anti-FN antiserum (1:100 in PBS) for 1 h, then washed again in PBS and incubated for 1 h with a mixture of FITC-conjugated donkey anti-rabbit antiserum (1:100 in PBS) and 4 μg/ml Texas Red-conjugated streptavidin (Jackson Laboratories) in PBS. After washing, tumor cells were fixed with 2% paraformaldehyde in PBS and immediately examined under a fluorescent microscope (absorption/emission: FITC, 490/520; Texas Red, 590/620).

Cell Adhesion and Adhesion Inhibition Assays

Adhesion assays were performed in 96-well microtitration plates (Immulon 4 flat bottom plate; Dynatech) essentially as described [5, 11]. Microtitration wells were coated overnight at 4°C with detergent-extracted wtDPP IV or acid-extracted DPP IV$^{(31-767)}$ (both at 50–1,000 ng/well: wtDPP IV in 50 μl PBS + 0.2% OG; DPP IV$^{(31-767)}$ in 50 μl PBS alone). Wells were blocked with 1% BSA in PBS for 2 h at 37°C, seeded with 3 × 10^4 MTF7 breast cancer cells in 100 μl DMEM in the presence or absence of 10% FBS to determine the effect of serum on tumor cell binding to wtDPP IV or DPP IV$^{(31-767)}$, and incubated for 30 min at 37°C. Unbound cells were removed by two cycles of flicking and washing in PBS. Adherent cells were stained with 0.5% crystal violet in 20% methanol, and the color read on a Microplate reader (Bio-Tek Instruments) at an adsorbance of 562 nm. The percent specific adhesion was determined as follows: 100 × [(adsorbance of cells bound to DPP IV or DPP IV$^{(31-767)}$) – (adsorbance of cells bound to 1% BSA)] / (adsorbance of cells bound to poly-*L*-lysine {100% binding}) – (adsorbance of cells bound to 1% BSA)] [5].

MTF7/wtDPP IV adhesion inhibition experiments were performed as follows: (a) in the presence of anti-DPP IV mAb 6A3 (0, 10, 50, 100 and 200 μg/ml in DMEM) or control anti-Lu-ECAM-1 mAb 6D3 (same immunoglobulin class as mAb 6A3; 200 μg/ml in DMEM) in the assay medium, and (b) with tumor cells preincubated for 1 h at 37°C with DPP IV$^{(31-767)}$ (0, 5, 20, 50 and 100 μg/ml in DMEM), or with DMEM containing 10% FBS.

Lung Colony Assays

MTF7 breast cancer cells (2 × 10^5 cells/0.3 ml DMEM/rat) previously incubated with DMEM for 1 h at 37°C were inoculated into 6-week-old, female Fischer 344 rats (Charles River Laboratories) via the lateral tail vein. Rats were sacrificed 3 weeks after tumor cell injection and weighed. Lungs were immediately removed and median and range of the numbers of lung colonies as well as the means of the lung weights and the tumor colony diameters (100–200 colonies measured) determined. Metastasis inhibition experiments were conducted with MTF7 cells preincubated for 1 h at 37°C with 100 μg/ml DPP IV$^{(31-767)}$ in DMEM. Anti-DPP IV mAb 6A3 (200–1,000 μg/ml in DMEM) coinjected with MTF7 tumor cells (2 × 10^5 cells/0.3 ml DMEM/rat) were also tested for metastasis inhibition. Data from animal groups were compared statistically by Student's t test for unpaired data ($p < 0.01$).

Results

Characterization of Rat Lung Endothelial wtDPP IV

Endothelial wtDPP IV was identified as a 110-kD transmembrane glycoprotein [10, 11] that shared its N-terminal sequence (30 amino acids) with that of the originally cloned hepatic DPP IV [14, 15]. Using a rat lung cDNA library and a rat hepatic DPP IV cDNA probe, a full-length wtDPP IV cDNA clone of 2.4 kb was isolated and found to be identical to the published sequences for rat hepatic DPP IV (J02997 [14]; J04591 [15]), except for a sequencing error at the C-terminus of J02997. This error occurred after the 765th codon TCC (Ser), where J02997 contained an extra C, resulting in a frame shift thereafter and an extension of the C-terminus by an additional 25 amino acids, instead of the TCC TTA CGC TAG (stop codon) (fig. 1A). Resequencing of the J02997 clone revealed that the published 25-amino acid extension did not exist. Differences in the amino acid sequences between endothelial wtDPP IV and the two hepatic DPP IV also

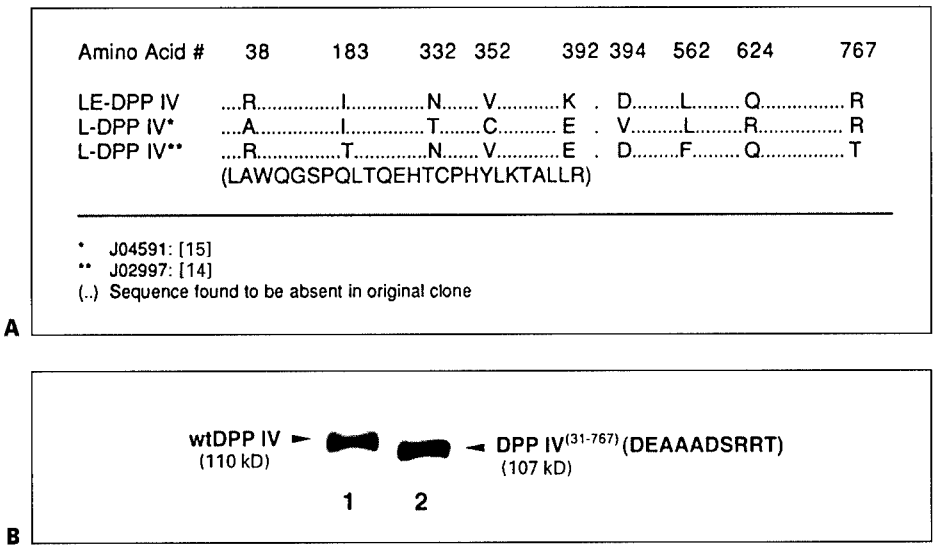

Fig. 1. Amino acid sequence analysis of wtDPP IV and DPP IV$^{(31-767)}$: **A** Amino acid sequence comparison between endothelial wtDPP IV (LE-DPP IV) and liver-derived DPP IV (L-DPP IV). **B** SDS-PAGE (5% polyacrylamide) of immunoaffinity-purified, Coomassie blue stained wtDPP IV (lane 1: MW, 110 kD) and DPP IV$^{(31-767)}$ (lane 2: MW, 107 kD, N-terminal sequence, DEAAADSRRT).

existed at codons 183, 392, and 562 of J02997 [14] and codons 38, 332, 352, 392, 394, and 624 of J04591 [15] (fig. 1A). These differences were likely due to errors in the published sequences as we employed in our work high-fidelity Pwo DNA polymerase and automated sequencing versus *Taq* polymerase and manual sequencing in case of the hepatic DPP IV cDNAs. These data are consistent with DPP IV being present as a single gene in the rat [14, 15].

Isolation and Characterization of the Truncated DPP IV Form (DPP IV$^{(31-767)}$)

Extraction of rat lungs with acidified, deionized water at pH 4.0, followed by immunoaffinity chromatography with anti-DPP IV mAb 6A3, resulted in the purification of a DPP IV species that had a slightly faster electrophoretic mobility than that of wtDPP IV [17, 18] (fig. 1B). N-terminal sequencing of this DPP IV species revealed that it was truncated, containing the complete extracellular domain of wtDPP IV (amino acids 31 to 767), but lacking the amino acids MKTPWKV-LL-GLLGVAALVTIITVPVVLLNK that together accounted for both the cytoplasmic and transmembrane domains of wtDPP IV (amino acids 1–30). The estimated molecular mass of the truncated protein, termed DPP IV$^{(31-767)}$, was 107 kD (fig. 1B).

Colocalization of DPP IV$^{(31-767)}$ and FN Complexes on Rat Breast Cancer Cells

The binding interaction between DPP IV$^{(31-767)}$ and MTF7 cell surface-associated FN was visualized by colocalization immunocytochemistry. Typically, the tumor cell surface-associated FN consisted of multiple, interconnected, brightly fluorescent 'globules'

Fig. 2. Colocalization of DPP IV$^{(31-767)}$ with cell surface-associated FN globules on an MTF7 breast cancer cell incubated with DPP IV$^{(31-767)}$ (100 μg/ml in PBS; 1 h at 37°C). **A** FN expression pattern. **B** DPP IV$^{(31-767)}$ colocalization pattern. ×900.

that were randomly distributed over the surface of MTF7 breast cancer cells and that corresponded biochemically to HMW aggregates of partly disulfide-bonded and partly covalently bonded FN molecules [11] (fig. 2A). This staining pattern was mirrored by that of MTF7-bound DPP IV$^{(31-767)}$ (fig. 2B). The strongest DPP IV$^{(31-767)}$ staining was observed in the center of the FN globules and was reminiscent of the adhesion of multiple DPP IV$^{(31-767)}$ molecules adhering to one FN complex.

Comparison of Breast Cancer Cell Adhesion to wt DPP IV and DPP IV$^{(31-767)}$

The adhesion qualities of DPP IV$^{(31-767)}$ were tested in a standard solid-state adhesion assay, and the data compared with those obtained for wtDPP IV. MTF7 breast cancer cells adhered in slightly higher numbers to DPP IV$^{(31-767)}$ than wtDPP IV, yielding specific adhesion values of 91.5 ± 6.3 for DPP IV$^{(31-767)}$ and 76.5 ± 5.4% for wtDPP IV at a coating concentration of 1 μg/well of wtDPP IV and DPP IV$^{(31-767)}$, respectively (fig. 3A). The MTF7/wtDPP IV adhesion was unaffected when adhesion assays were conducted in the presence of 10% FBS in the assay medium, clearly supporting our previously published observation that pFN was unable to interfere with adhesion of breast cancer cells to wtDPP IV in vivo [11]. Identical adhesion characteristics were recorded for other lung-metastatic rat breast cancer cells (R3230AC-MET; RPC-2), while the wtDPP IV-adhesion values for nonmetastatic breast cancer cells (R3230AC-LR) were unremarkable (data not shown).

The adhesion interaction between MTF7 and wtDPP IV was blocked in a dose-dependent manner by anti-DPP IV mAb 6A3, but was unaffected by the control mAb 6D3 (fig. 3B). At an anti-DPP IV mAb 6A3 concentration >100 μg/ml, MTF7 adhesion to wtDPP IV was totally blocked. MTF7/wtDPP IV adhesion was also blocked when cancer cells were incubated with soluble DPP IV$^{(31-767)}$ prior to conducting an adhesion assay. Blocking again occurred in a dose-dependent manner, causing an approximately 90% adhesion inhibition when MTF7 cells were preincubated with 100 μg/ml DPP IV$^{(31-767)}$ for 1 h at 37°C (fig. 3B). Again, the presence or absence of 10% FBS in the assay medium had no effect on this competitive blocking of the MTF7/wtDPP IV adhesion (data not shown).

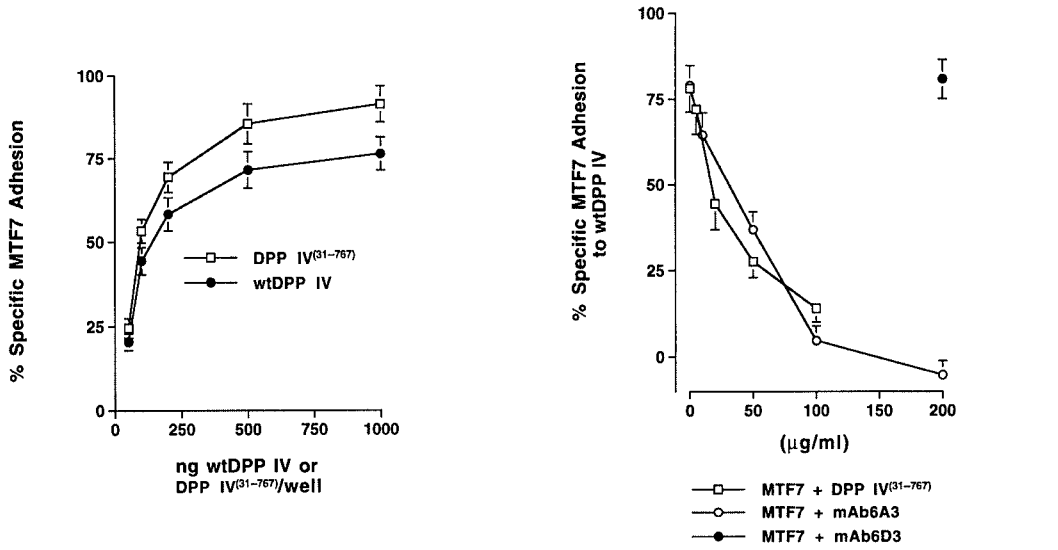

Fig. 3. MTF7/DPP IV adhesion and adhesion inhibition with DPP IV$^{(31-767)}$. **A** Dose-dependent adhesion of MTF7 cells to wtDPPIV (●) and DPP IV$^{(31-767)}$ (□). **B** Dose-dependent inhibition of MTF7 adhesion to wtDPP IV with DPP IV$^{(31-767)}$ (□) and anti-wtDPP IV mAb 6A3 (○); ● = adhesion conducted with MTF7 cells in the presence of control mAb 6D3 (tested only at 200 μg/ml). Data represent means from 3 separate experiments. Standard deviations are < 10%.

Table 1. Inhibition of lung colonization by MTF7 breast cancer cell treatment with DPP IV$^{(31-767)}$

MTF7 treatment[a]	Rats with tumors	Rat weight, g[b]	Lung weight, g[b]	Lung colony diameter, mm[b]	Lung colonies[c]
Experiment 1					
None	6/6	318.2 ± 5.4	5.00 ± 0.34	2.00 ± 0.10	304 (224–434)
DPP IV$^{(31-767)}$	6/6	350.0 ± 3.3*	1.44 ± 0.13*	1.29 ± 0.07*	71 (49–93)*
Experiment 2					
None	7/7	317.3 ± 10.9	5.31 ± 0.93	ND	315 (257–353)
DPP IV$^{(31-767)}$	7/7	341.2 ± 12.1*	1.40 ± 0.32*	ND	56 (23–94)*

* $p < 0.01$ (Student's t test). ND = Not determined.

[a] MTF7 breast cancer cells were incubated with 100 μg/ml DPP IV$^{(31-767)}$ in DMEM for 1 h at 37°C, then washed with DMEM and immediately injected into the lateral tail vein of 6-week-old female Fischer 344 rats at 2×10^5 cells/0.3 ml DMEM/rat. Control rats were inoculated with MTF7 cells incubated for the same period of time in DMEM alone (MTF7 treatment: none) before intravenous injection.

[b] Mean ± SE.

[c] Median (range).

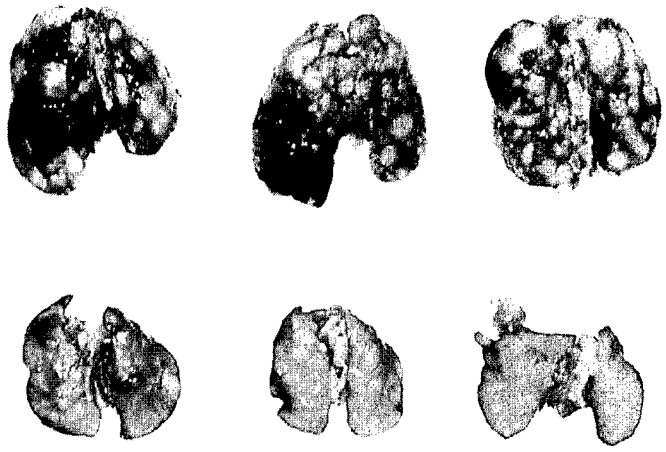

Fig. 4. Inhibition of MTF7 lung colonization by DPP IV$^{(31-767)}$. Top row: rat lungs colonized by MTF7 cells (2×10^5 cells/rat) incubated with DMEM + 100 µg/ml BSA for 1 h at 37 °C prior to tail vein inoculation of Fischer 344 rats (note: multiple, coalescent tumor nodules occupy the entire rat lung). Bottom row: rat lungs colonized by MTF7 breast cancer cells incubated with DMEM + 100 µg/ml DPP IV$^{(31-767)}$ for 1 h at 37 °C prior to tail vein inoculation of Fischer 344 rats.

DPP IV$^{(31-767)}$ is an Anti-Metastatic Compound for Rat Breast Cancer Cells

MTF7 breast cancer cells injected intravenously at a dose of 2×10^5 tumor cells/0.3 ml DMEM/rat generated a median number of 304 and 315 lung colonies per rat in two independent experiments (table 1). Tumor colonies were randomly distributed throughout the lung tissue and measured 2.00 ± 0.10 (SEM) mm in diameter. Colonies often coalesced with each other forming large multinodular aggregates (fig. 4, top row). The weight of the tumor-bearing lungs was 5.00 ± 0.34 and 5.31 ± 0.93 g, respectively, compared to 1.28 ± 0.11 g for lungs of normal, age-matched control rats. By comparison, MTF7 breast cancer cells incubated with 100 µg/ml DPP IV$^{(31-767)}$ prior to intravenous inoculation into Fischer 344 rats produced only a median number of 71 and 56 lung colonies per rat, respectively (table 1). Typically, the tumor colonies were significantly smaller, displaying an average colony diameter of 1.29 ± 0.07 mm. There were few, if any, colony aggregates (fig. 4, bottom row). The average lung weight was 1.44 ± 0.13 and 1.40 ± 0.32 g for the two experiments. These data represent a reduction in lung colonization by approximately 80% from untreated MTF7 cells to MTF7 cells treated with DPP IV$^{(31-767)}$. Treatment of cancer cells with DPP IV$^{(31-767)}$ had no effect on tumor cell viability and growth. Moreover, the enzymatic activity of DPP IV$^{(31-767)}$ was not affecting lung colonization as similar data were obtained with boiled DPP IV$^{(31-767)}$ that expressed no enzyme activity (data not shown).

Discussion

A novel adhesion principle that mediates vascular arrest of blood-borne rat breast cancer cells in rat lungs has recently been discovered by our laboratory [10, 11]. The interacting adhesion molecules are lung endothelial cell DPP IV (wtDPP IV) and breast cancer cell surface-associated, polymeric FN. Endothelial wtDPP IV is expressed at high levels on the lumenal surface of lung capillary endothelia and is identical to the DPP IV isolated and cloned from rat liver (bile canaliculi) [10].

It is a sialoglycoprotein that anchors to the plasma membrane by a hydrophobic domain near its N-terminus such that the bulk of the molecular mass is exposed to the outside of the cell and that harbors distinct collagen and FN binding sites [14, 17, 18]. The wtDPP IV ligand is assembled on tumor cell surfaces by FN self-association, predominantly from plasma FN acquired during the journey of rat breast cancer cells in the blood circulation [11]. Biochemical analyses indicate that the tumor cell surface-associated FN is nonreducible by standard reducing agents such as β-mercaptoethanol and, thus, is considered to consist of largely covalently bonded FN [11]. These aggregates impress immunohistochemically as FN globules that are randomly distributed over the cancer cell surface. Their large size allows multiple binding interactions with endothelial wtDPP IV, providing a sufficiently strong cell-to-cell adhesion that can withstand the physiologic, hemodynamic shear stresses to which cancer cells are subjected in the lung vasculature [4].

The involvement of tumor cell surface-associated FN aggregates in lung metastasis of rat cancer cells seems to be widespread. In the rat breast cancer cell model, the amount of surface expression of FN is correlated quantitatively with the adhesion of breast cancer cells to immobilized wtDPP IV and the propensity with which the tumor cells colonized the lungs; i.e., highly lung-metastatic breast cancer cells such as MTF7, R3230AC-MET, and RPC2 cells express numerous, prominent FN globules on their surface and consequently adhere in large numbers to immobilized wtDPP IV, while nonmetastatic breast cancer cells (e.g., R3230AC-LR) express little FN on their surface and adhere poorly to wtDPP IV [9–11]. A similar correlation between cell surface expression of FN and lung metastasis was established for various cell clones derived from a rat rhabdomyosarcoma cell line [19]. In this model, clones with high surface expression of FN were highly metastatic, while those with low surface expression of FN were poorly metastatic. Given the ubiquity of cancer cell surface receptors that are able to bind FN to their surfaces, upon which FN self-association to high-molecular-weight structures can occur during dissemination of tumor cells in the blood [3, 4], the wtDPP IV/FN binding mechanism may be a more frequent principle in lung metastasis than currently realized.

Disruption of the wtDPP IV/FN binding interaction by a truncated DPP IV variant (DPP $IV^{(31-767)}$) whose FN binding domain has been preserved, is an effective method in controlling tumor cell colonization of the lungs in an experimental animal model. The DPP $IV^{(31-767)}$ used in the present study is readily isolated by acid extraction from rat lungs and consists of the complete extracellular domain of endothelial wtDPP IV [16]. The truncated molecule is soluble in detergent-free, physiological buffers and, thus, is superior to detergent-soluble wtDPP IV for use in both in vitro adhesion and in vivo metastasis inhibition experiments as it is superior in the coating of polystyrene dishes used in adhesion assays and as it generates no adverse host reactions under the conditions applied here. In contrast, an anti-metastatic therapy based on anti-DPP IV mAb 6A3 failed due to the severe cytotoxicity exerted by the antibodies, causing massive edema and hemorrhage of the lungs and death of rats within minutes to several hours following antibody injection. This problem could not be overcome by adapting rats to increasing concentration of anti-DPP IV mAb prior to the intravenous challenge with lung-metastatic breast cancer cells [16].

Masking of a tumor cell surface-associated adhesion molecule with a peptide derived from the extracellular domain of the interacting endothelial cell adhesion receptor is a nov-

el concept in anti-metastasis treatment. Such therapy effectively blocks arrest of cancer cells in the vasculature of the target organ for metastasis, causing rapid elimination of cancer cells from the blood circulation [20]. Moreover, it prevents cancer cells from initiating signaling cascades, readily triggered in conjunction with adhesion to endothelium, that promote extravasation and establishment of micrometastases in the subendothelial tissue [8].

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Is the Fischer 344/CRJ rat a protein-knock-out model for dipeptidyl peptidase IV-mediated lung metastasis of breast cancer?

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Abstract

Fischer 344/CRJ rats harbor a G633R substitution in dipeptidyl peptidase IV (DPP IV) that leads to retention and degradation of the mutant protein in the endoplasmic reticulum (Tsuji et al. [1]). However, when these rats were used as a 'protein knock-out' model in further evaluating the previously established role of DPP IV in metastasis, lung colonization of the highly metastatic MTF7 rat breast cancer cell line was reduced by only 33% relative to normal Fischer 344 rats. To examine whether lung endothelia leak expression of mutant DPP IV and whether mutant DPP IV exhibits the same adhesion qualities as wild type DPP IV, detailed immunohistochemical, biochemical, transfection, and FACS analyses were performed to assess the surface expression of mutant DPP IV on lung endothelia and transfected HEK293 cells and adhesion assay to compare the adhesion qualities of wild-type and mutant DPP IV. Both endothelial and transfected HEK293 cells expressed mutant, enzymatically inactive DPP IV on their surfaces, albeit at greatly reduced levels when compared to expression of wild type DPP IV. Purified mutant DPP IV had identical adhesion qualities for lung-metastatic MTF7 cells as wild type DPP IV, and competitive inhibition of MTF7 lung colonization by truncated DPP IV confirmed involvement of mutant DPP IV in lung metastasis of Fischer 344/CRJ rats. Although metastasis appears to be mediated by several, often parallel mechanisms involving multiple tumor and host factors, these data indicate that altered expression of a single component can drastically change the outcome of metastatic disease.

Abbreviations: DPP IV, dipeptidyl peptidase IV; FN^{SAP}, cell surface-associated, polymeric fibronectin; DPP $IV^{(31-767)}$, soluble polypeptide encompassing amino acids 31 to 767 of wild type DPP IV; CRJ, Charles River Japan;HEK293, human embryonal kidney cells; OG, octyl-β-glucoside; FACS, fluorescent activated cell sorting; ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator.

Introduction

Dipeptidyl peptidase IV (DPP IV) has recently been reported to play an important role in the colonization of the lungs by rat and, possibly, human breast cancer cells [2, 3]. Its prominent expression on endothelia of lung respiratory capillaries accounts for arrest of blood-borne breast cancer cells in the lung vasculature by way of a high affinity binding interaction with cancer cell surface-associated, polymeric fibronectin (FN^{SAP}) [3]. The affinity of this binding interaction correlates quantitatively with the surface accumulation of FN^{SAP} on breast cancer cells and parallels the propensity with which these cancer cells metastasize to the lungs. Accordingly, DPP IV/FN^{SAP}-mediated adhesion and metastasis are blocked when tumor cells are incubated with soluble, truncated DPP IV (DPP $IV^{(31-767)}$) prior to conducting adhesion and lung colony assays [3, 4]). This adhesion is also blocked by anti-DPP IV monoclonal antibody 6A3 and anti-FN antiserum, but is unaffected by peptide substrates (e.g., Gly-Pro-Ala) of the DPP IV exopeptidase activity and by soluble plasma FN (pFN). These adhesion inhibition data imply that the DPP IV/FN^{SAP} binding interaction occurs independent of the exopeptidase substrate domain of DPP IV, but is critically dependent upon the conformation of FN [3]. The select adhesion of DPP IV to FN^{SAP}, present as globular supermolecular FN aggregates on breast cancer cell surfaces, and the inability to engage in binding interactions with pFN, allow the DPP IV/FN^{SAP} binding interaction to occur *in vivo* in the presence of high blood plasma concentrations of pFN. The ability of many cancer cells to capture pFN molecules on their surfaces and to augment their FN^{SAP} deposits by FN-self-association during passage in the blood suggests that the DPP IV/FN^{SAP} binding may be a relatively common mechanism for lung metastasis [3, 5].

In the present study, we make use of a rat substrain, Fischer 344/CRJ (Charles River Japan) [6–8, 11], that har-

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bors a mutant DPP IV to further explore the role of DPP IV/FN^{SAP} in metastasis. The Fischer 344/CRJ rat substrain contains a G to A transition at nucleotide 1897 of the DPP IV cDNA sequence [9] which leads to a G633R substitution in the DPP IV enzymatic domain G^{629}-W-S-Y-G^{633} [1]. This mutation has been shown to cause rapid degradation of the initially synthesized 103-kDa protein product that is rich in mannose-type oligosaccharide sidechains, thereby preventing processing to the mature 109-kDa DPP IV [1]. Thus, the Fischer 344/CRJ rat can be conceived as a 'DPP IV knock-out' (at the protein level only) model that should not allow metastasis by a DPP IV mechanism. However, our initial lung colony assay involving the highly lung-metastatic breast cancer cell line MTF7 revealed only a 33% reduction in the number of metastases from those observed in normal Fischer 344 rats. Detailed immunohistochemical and biochemical analyses, supplemented by DPP IV-transfection experiments as well as adhesion and lung colony assays, showed that Fischer 344/CRJ rats supported breast cancer metastasis by a DPP IV/FN^{SAP} mechanism. Although the amount of mature, mutant DPP IV expressed by lung capillary endothelia was greatly diminished in Fischer 344/CRJ rats, it was sufficient to cause arrest of a respectable number of breast cancer cells in the lung vasculature and to promote the generation of lung colonies.

Materials and methods

Animals and cell culture

Normal female Fischer 344 rats, 6 weeks old, were purchased from Charles River Laboratories (Wilmington, MA) and Fischer 344/CRJ of equal sex and age from Charles River Japan (Atsugi, Japan). The lung-metastatic MTF7 clone of the rat mammary adenocarcinoma cell line 13762NF was obtained from Dr D.R. Welch (Pennsylvania State College of Medicine, Hershey, PA) [10] and grown in DMEM containing 10% heat-inactivated fetal bovine serum (FBS). Transformed human embryonal kidney (HEK293) cells were obtained from the American Type Culture Collection (Rockville, MD) and grown according to the supplier's instruction.

Immunohistochemistry of rat lung

Lungs from Fischer 344 and 344/CRJ rats were perfused via the right heart ventricle with 4% paraformaldehyde in PBS, pH 7.4, and embedded in paraffin. Deparaffinized lung sections (3-μm thick) were treated with 0.3% H_2O_2 in methanol (15 min; 4 °C) and blocked with 10% normal goat serum (30 min; room temperature). DPP IV was visualized by a standard immunoperoxidase technique using mouse anti-rat DPP IV mAb 6A3 (10 μg/ml), horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:500), and HRP substrate 3,3″-diaminobenzidine tetrachloride. Control sections were stained similarly but mouse non-immune IgG1 (immunoglobulin isotype of mAb 6A3) substituted for mAb 6A3.

Surface-biotinylation by in situ *vascular perfusion*

Fischer 344 and 344/CRJ rats were given i.p. injections of 0.5 ml of 20% sodium citrate, sacrificed with an overdose of barbiturate, and prepared for immediate vascular perfusion as described [11, 12]. Perfusates entered through the pulmonary artery and exited from the left heart atrium. Lung vascular beds were first flushed extensively with PBS, pH 7.4, containing 1 mM $CaCl_2$ and 0.5 mM $mgCl_2$ at a flow rate of 5 ml/min for 10 min at 37 °C. Flushing was followed by perfusion of the vasculature with 100 μg/ml biotin (Biotin NHS, Vector Laboratories, Burlingame, CA) in PBS at a flow rate of 0.25 ml/min for 30 min at 37 °C. Lungs were then flushed again with PBS (10 min; 37 °C), removed from the cadavers, and immediately processed for immunoprecipitation with anti-DPP IV mAb 6A3 as described in detail [3]. Immunoprecipitates were resolved by SDS-PAGE, blotted to nitrocellulose, and probed with horseradish peroxidase-conjugated streptavidin and ECL and by Western with mouse anti-rat DPP IV mAb 6A3 and rabbit anti-rat DPP IV polyclonal antibody CU31.

Immunoaffinity purification, immunoprecipitation, and western blotting of endothelial DPP IV

Wild-type and mutant endothelial DPP IV were immunopurified with anti-DPP IV mAb 6A3 from lung extracts of 15 adult Fischer 344 rats and 15 Fischer 344/CRJ, respectively, as described [3]. The purified DPP IV fractions were collected in 100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 100 mM glycine, and 0.5% octyl-_-glucoside (OG). Their purity was determined by SDS-PAGE, followed by silver staining and/or Western blotting with rabbit anti-rat DPP IV antiserum CU31 [3]. DPP IV-immunoprecipitation and Western analyses were as described by Cheng et al. [3]. Truncated DPP IV (DPP IV$^{(31-767)}$) was prepared from 15 lungs of Fischer 344 rats as described by Abdel-Ghany et al. [4].

Plasmid construction and transfection

All transfection studies were performed with rat kidney DPP IV cDNA obtained from Dr D. Doyle, State University of New York at Buffalo [13]. The nucleotide sequence of kidney DPP IV cDNA was 100% identical to that of rat lung endothelial DPP IV cloned in our laboratory [4]. HEK293 cells were transiently transfected with DPP IV cDNA cloned into pcDNA3.1 (Invitrogen, San Diego, CA), using LipofectAMINE according to the manufacturer's instructions (Life Technologies). Control HEK293 cells were transfected with the pcDNA3.1 vector alone. A DPP IV cDNA construct that accounted for a G^{633} (GGA) to R (AGA) substitution was generated by overlap extension PCR as described [14] and cloned into the *Not*I site of the pcDNA3.1 vector using *Not*I site-incorporated PCR primers. Correct sequence was verified by sequencing. Transfection into HEK293 cells was as described above.

Flow cytometry

Fluorescent activated cell sorting (FACS) was performed to quantify the expression of wild-type and mutant DPP IV on HEK293 cell surfaces, 48-h after initiation of transfection with the respective DPP IV cDNA. Transfected HEK293 cells were released from their growth surface with 0.05% trypsin/0.02% EDTA, recovered in DMEM containing 10% FBS (30 min; 37 °C), and incubated in 10% goat serum in PBS for 15 min at 4 °C. Cells were then treated with mouse anti-rat DPP IV mAb 6A3 (diluted 1:100 in PBS) for 1 hr at 4 °C, followed by FITC-conjugated goat anti-mouse antiserum in PBS containing 10% goat serum for 1 hr at 4°C. After cell fixation in 2% paraformaldehyde in PBS, FACS analysis was performed on a FACS Calibur System (Becton Dickinson, San Jose, CA). Non-specific fluorescence was accounted for by incubating cells with non-immune serum instead of primary antibody.

Enzyme assay

The DPP IV enzyme activity was assayed by measuring the cleavage of Gly-Pro nitroanilide (Sigma, St. Louis, MO) as described by Nagatsu et al. [15]. Various concentrations of immunopurified wild-type or mutant DPP IV were incubated in 250 μl 75 mM glycine-NaOH buffer, pH 9.0, containing 1 mM Gly-Pro nitroanilide at 37 °C for 30 min and the reaction stopped with 750 μl of 1 M acetate buffer (pH 2.8). The activity was determined by measuring the absorption at 385 nm.

Cell adhesion and adhesion inhibition assays

Adhesion assays were performed in 96-well microtitration plates (Immulon 4 Flat Bottom Plate; Dynatech) essentially as described [3, 16]. Microtitration wells were coated overnight at 4 °C with detergent-extracted wild-type or mutant DPP IV (both at 500 ng/well in 50 μl PBS+0.2% OG). Wells were blocked with 1% BSA in PBS for 2 h at 37 °C, seeded with 3×10^4 MTF7 breast cancer cells in 100 μl serum-free DMEM, and incubated for 30 min at 37 °C. Unbound cells were removed by two cycles of flicking and washing with PBS. Adherent cells were stained with 0.5% crystal violet in 20% methanol, and the color read on a Microplate reader (Bio-Tek Instruments) at an absorbance of 562 nm. The percent specific adhesion was determined as follows: 100×[(Absorbance of cells bound to wild-type or mutant DPP IV) − (Absorbance of cells bound to 1% BSA)] / [(Absorbance of cells bound to poly-L-lysine {100% binding}) − (Absorbance of cells bound to 1% BSA)] [16]. MTF7/DPP IV adhesion inhibition experiments were performed in the presence of anti-DPP IV mAb 6A3. Controls were run in the presence of non-immune mouse IgG1 (immunoglobulin isotype of mAb 6A3). Both mAb 6A3 and mouse IgG1 were used at a concentration of 100 μg/ml in DMEM assay medium.

Table 1. Colonization of the lungs of Fischer 344 and 344/CRJ rats by MTF7 breast cancer cells.

Rat strain	Rats with tumors	Lung weight (g)[a]	Number of lung colonies[b]
Fischer 344	13/13	5.17 ± 0.66	309 (224–434)
Fischer 344/CRJ	9/9	4.41 ± 0.57	206 (130–226)*

[a]Mean ± SEM.
[b]Median (range).
*$P < 0.01$ (Student's *t*-test).

Lung colony assays

MTF7 breast cancer cells (2×10^5 cells/0.3 ml DMEM/rat) previously incubated with DMEM for 1 h at 37 °C were inoculated into 6 weeks old, female Fischer 344 or 344/CRJ rats via the lateral tail vein. Rats were sacrificed 3 weeks after tumor cell injection. Lungs were immediately removed and median and range of the lung colonies as well as the means of the lung weights and the tumor colony diameters (100 to 200 colonies measured) determined. Metastasis inhibition experiments were conducted with MTF7 cells preincubated for 1 h at 37 °C with 100 μg/ml DPP IV$^{(31-767)}$ in DMEM [4]. Data from animal groups were compared statistically by Student's *t*-test for unpaired data ($P<0.01$).

Results

Lung colonization of Fischer 344/CRJ rats with MTF7 breast cancer cells

Lung-metastatic rat breast cancer cells (MTF7; R3230AC-MET; RPC-2) injected intravenously at a dose of 2×10^5 cells/0.3 ml DMEM/rat have been reported to colonize the lungs of normal Fischer 344 rats in large numbers [3]. Typically, MTF7 breast cancer cells produce a median and range of 309 (224–434) lung colonies [4]. Tumor colonies are distributed randomly throughout the lungs and often coalesced with each other to form large multinodular tumor aggregates, causing death within 4 weeks following tumor cell injection. When normal Fischer 344 rats are substituted in the above lung colony assay with Fischer 344/CRJ rats, which harbor a G633R substitution in DPP IV and lack expression of the mature membrane protein [1], only a moderate reduction in the number of MTF7 lung colonies is recorded. Median and range are 206 (130–226) lung colonies (Table 1). These data are unexpected and may have two possible explanations. First, lung metastasis in Fischer 344/CRJ rats is mediated by an adhesion mechanism other than DPP IV/FN^{SAP}; and second, lung endothelia differ from other cell types in their processing of mutant DPP IV 'leaking' expression of mature protein on their lumenal surfaces that is sufficient to cause vascular arrest of blood-borne breast cancer cells and to promote lung metastasis. To explore these possibilities, systematic analyses of the surface expression of DPP IV in lung endothelia and of the mechanism of lung metastasis in female Fischer 344/CRJ rats have been initiated here.

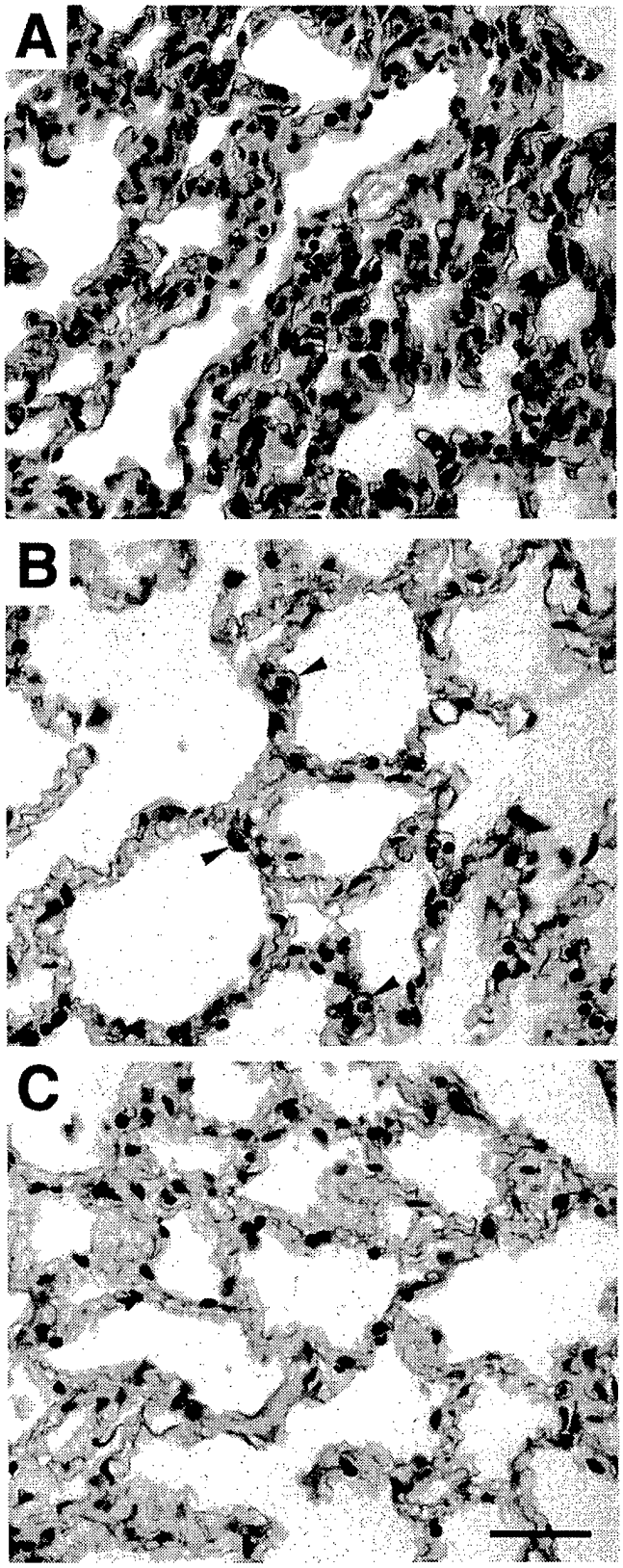

Figure 1. Lung sections from Fischer 344 (A) and 344/CRJ (B and C) rats stained with mouse anti-rat DPP IV mAb 6A3 and HRP-conjugated goat anti-mouse secondary antibody. (A) Prominent staining of endothelia of respiratory capillaries in normal Fischer 344 rat; (B) Weak staining reaction along the endothelial lining of respiratory capillaries and cytoplasmic staining of endothelia (arrow head) in Fischer 344/CRJ rat; (C) Control staining with mouse IgG1. Bar: 100 μm.

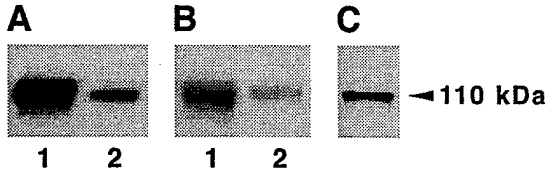

Figure 2. Immunoprecipitated wild-type and mutant lung endothelial DPP IV: Immunoprecipitates from lung extracts of Fischer 344 and 344/CRJ rats (A and B) and Fischer 344/CRJ rats, whose vascular lining was surface-surface biotinylated by *in situ* vascular perfusion (C), were prepared using anti-DPP IV mAb 6A3. SDS-PAGE-resolved immunoprecipitates were probed by Western blotting using anti-DPP IV mAb 6A3 (A) and anti-DPP IV polyclonal antibody CU31 (B) and by streptavidin-HRP (C). Lane 1: Fischer 344 rats; and lane 2: Fischer 344/CRJ.

Expression of mutant DPP IV by lung endothelia

Expression of mutant DPP IV in lung endothelia has first been examined immunohistochemically. Lung tissue sections are prepared from Fischer 344 and 344/CRJ rats and stained with mAb 6A3 generated against immunopurified rat endothelial DPP IV. As reported previously [2], staining of normal Fischer 344 rat lungs generates a strong staining reaction with the inner surface (endothelial lining) of respiratory capillaries (Figure 1A). This staining reaction is selective and is absent in other vascular channels or, for that matter, any other lung tissue components. In contrast, the lungs of Fischer 344/CRJ rats generate a much weaker staining reaction with anti-DPP IV mAb 6A3, but respiratory capillary endothelia consistently are DPP IV-positive. Sparse staining is noticed not only along the inner surface of respiratory capillaries but also in the cytoplasms of some capillary endothelial cells (Figure 1B, arrowhead). By contrast, sections stained with non-immune mouse IgG1 (IgG isotype of mAb 6A3) are non-reactive (Figure 1C). The foregoing immunohistochemical data are confirmed biochemically by isolating full-length, endothelial cell DPP IV from the lungs of Fischer 344/CRJ rats. Lungs from both rat substrains contain a full-length DPP IV of the expected molecular mass of 110 kDa (Figures 2A and B). However, the amount of protein harvested from Fischer 344/CRJ rats is significantly lower than that harvested from normal Fischer 344 rats. To prove that a portion of the 110-kDa DPP IV protein isolated from 344/CRJ rats is surface-expressed by lung endothelia, we have surface-biotinylated the lumenal membranes of lung endothelia by *in situ* vascular perfusion. Mutant DPP IV was immunoprecipitated, resolved by SDS-PAGE, and blotted immunoprecipitates probed with streptavidin-HRP. A single distinct band is observed at the 110-kDa position (Figure 2C).

Surface-expression of Mutant DPP IV in transfected HEK293 cells

To obtain a quantitative impression of the surface expression of mutant DPP IV, we have transfected HEK293 cells with wild-type and mutant (G^{1897} substituted with A) DPP IV cDNA and have subjected transfected cells 48 h later to biochemical and FACS analyses. The biochemical data show surface expression of a biotinylated protein product of 110-

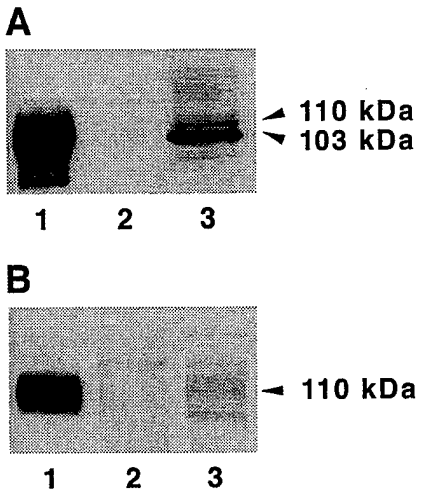

Figure 3. Immunoprecipitated DPP IV from transfected HEK293 cells: Monoclonal antibody 6A3-immunoprecipitates prepared from extracts of untreated (A) or surface-biotinylated (B) HEK293 cells transfected with wild-type DPP IV (lane 1), vector alone (lane 2), or mutant DPP IV (lane 3) were resolved by SDS-PAGE, blotted onto nitrocellulose, and probed with mAb 6A3 (A) or streptavidin-HRP (B).

kDa molecular mass that by Western blotting is confirmed as DPP IV in both HEK293 cells transfected with wild-type and mutant DPP IV (Figure 3). In contrast to rat lung, where the principal protein products of both wild-type and mutant DPP IV have molecular masses of 110 kDa (Figure 2), transfected HEK293 cells, in which DPP IV is placed under the control of a strong viral promoter (CMV), express both the precursor (103-kDa) and mature (110-kDa) DPP IV protein products. In HEK293 cells transfected with wild-type DPP IV, these two protein products are present in large amounts and are poorly resolved, while in HEK293 cells transfected with mutant DPP IV the bulk of the DPP IV protein is present in the 103-kDa form with only a minor band at the 110-kDa position (Figure 3A, lanes 1 and 3). Quantification of the surface-expressed, mutant DPP IV is accomplished by FACS, using cells stained successively with mAb 6A3 and FITC-conjugated secondary antibody. Histograms prepared from mock-, mutant DPP IV-, and wild-type DPP IV-transfected HEK293 cells reveal the following. Transfection with wild-type DPP IV generates HEK293 cells with a broad spectrum of DPP IV expression, ranging from equally large populations of weakly, moderately and intensely stained cells (Figure 4). In contrast, transfection with mutant DPP IV generates mostly weak DPP IV expressers (approximately 40% of the total cell population) in addition to a few moderate expressers (approximately 5% of the total cell population). Mock-transfection produces mostly non-expressers.

Functional analyses of mutant DPP IV

All *in vitro* functional analyses are conducted with DPP IV immunopurified from Fischer 344 and 344/CRJ rats. The first assay measures the enzymatic activity of the two DPP IV preparations. As expected, a dose-dependent increase is registered for wild-type DPP IV, while mutant DPP IV exhibits no detectable enzyme activity at any of the concentra-

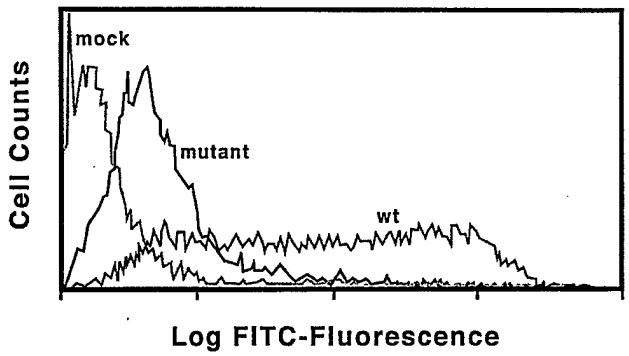

Figure 4. FACS quantification of surface-expressed DPP IV: HEK293 cells transfected with vector alone (mock), mutant DPP IV (mutant), or wild-type DPP IV (wt) were stained subsequently with anti-DPP IV mAb 6A3 and FITC-conjugated secondary, then washed, fixed, and subjected to FACS analysis.

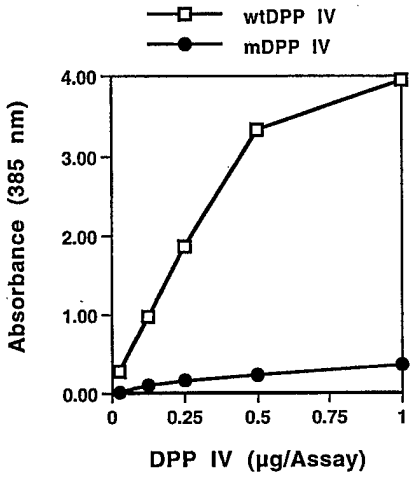

Figure 5. DPP IV enzyme activity: The enzyme activities of DPP IV immunopurified from lung extracts of Fischer 344 and 344 CRJ rats were measured as described in 'Materials and methods'.

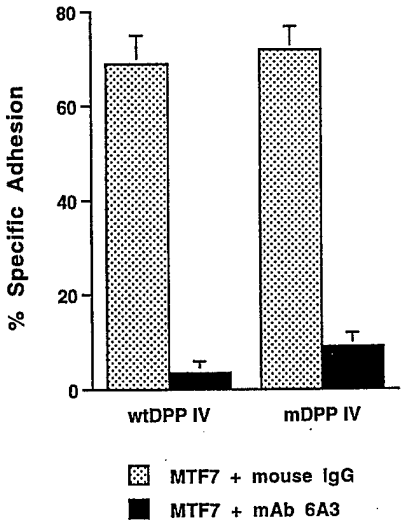

Figure 6. Adhesion of MTF7 breast cancer cells to wild-type and mutant DPP IV. Adhesion assays were conducted in microtitration plates in the presence of mAb 6A3 or non-immune mouse IgG1 as described by Cheng et al. (1998). The specific adhesion was calculated as described in 'Materials and methods'. The data represent averages of 3 separate experiments.

Table 2. Inhibition of lung colonization of Fischer 344/CRJ rats by MTF7 breast cancer cells treated with DPP IV^{31-767}

MTF7 treatment[a]	Rats with tumors	Lung weight (g)[b]	Lung colony diameter (mm)[b]	Number of lung colonies [c]
None	8/8	5.12 ± 0.61	2.30 ± 0.58	205 (120–224)
DPP $IV^{301-767}$	6/6	2.34 ± 0.55*	1.87 ± 0.49	39 (15–73

*P < 0.01 (Student's *t*-test).
[a]MTF7 breast cancer cells were incubated with 100 μg/ml DPP IV^{31-767} for 1 h at 37 °C, then washed with DMEM and immediately injected into the lateral tail vein of 6-week-old female Fischer 344/CRJ rats at 2×10^5 cell/0.3 ml DMEM/rat. Control trats were incubated for the same time period of time in DMEM alone (MTF7 treatment: none) before intravenous injection.
[b]Mean ± SEM.
[c]Median (range).

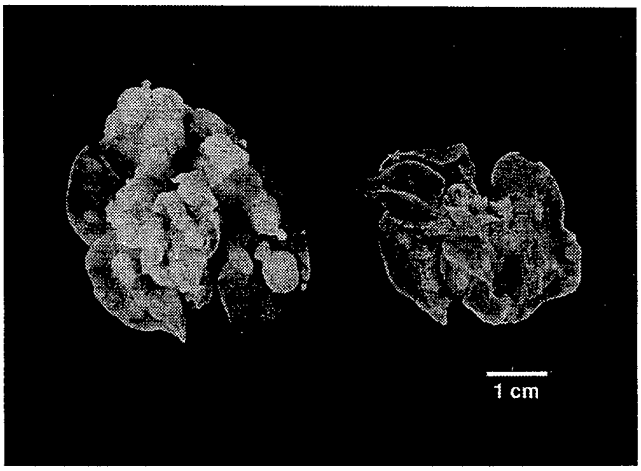

Figure 7. Inhibition of MTF7 lung metastasis in Fischer 344/CRJ rats by truncated DPP IV (DPP $IV^{(31-767)}$): Rats were injected via the lateral tail vein with 2×10^5 MTF7 cells incubated for 1 h in DMEM alone or in DMEM containing 100 μg/ml truncated DPP $IV^{(31-767)}$. Rats were sacrificed 3 weeks after tumor cell injection and lungs removed. Left lung (ventral view): Representative rat injected with MTF7 cells treated with DMEM alone; Right lung: Representative rat injected with MTF7 cell treated with DPP $IV^{(31-767)}$ (100 μg/ml in DMEM; 1 h; 37 °C).

tions tested (Figure 5). Static adhesion assays are conducted next in microtitration plates coated with the same protein concentrations of either wild-type or mutant DPP IV. The adhesion values for MTF7 breast cancer cells are 69±6% and 72±5%, respectively (Figure 6). Adhesion is blocked almost totally in the presence of 100 μg/ml anti-DPP IV mAb 6A3, yielding adhesion values of 4±3 and 9±3%, respectively. The ultimate test for a DPP IV/FN^{SAP} involvement in lung metastasis of MTF7 breast cancer cells in Fischer 344/CRJ rats is brought about by a lung colony assay, in which the DPP IV/FN^{SAP} binding interaction is competitively blocked by truncated DPP IV (DPP $IV^{(31-767)}$) (Table 2). These data show a reduction of metastasis by more than 80%, yielding a median and range of 205 (130±224) lung colonies for untreated MTF7 cells and 39 (15–73) for MTF7 cells treated with 100 μg/ml DPP $IV^{(31-767)}$. The drastic reduction in metastatic disease is also exemplified by a reduction in the average lung weight from 5.12±0.61 (SEM) gm in rats injected with untreated MTF7 cells versus 2.34±0.55 g in rats injected with MTF7 cells treated with 100 μg/ml DPP $IV^{(31-767)}$ (Figure 7).

Discussion

The Fischer 344/CRJ rat carries a missense point mutation in its DPP IV gene. This mutation has been localized to nucleotide 1897 of the cloned DPP IV cDNA and identified as a G to A transition [1]. It is responsible for a G633R substitution within the protein consensus sequence G^{629}-W-S-Y-G^{633}proposed as the active-site sequence of the DPP IV serine exopeptidase [17]. This substitution results in a complete loss of the DPP IV enzyme activity and leads to rapid degradation of the primary translation product of DPP IV in the endoplasmic reticulum (ER) [1]. Our intention in this study has been to use the Fischer 344/CRJ rat as a 'DPP IV protein knock-out' model in further analyzing the DPP IV/FN^{SAP}-mediated colonization of the lungs by lung-metastatic rat breast cancer cells [3]. However, the MTF7 breast cancer cell line, which expresses the highest lung-metastatic potential of our collection of rat breast cancer cell lines, colonizes the lungs of Fischer 344/CRJ rats with an efficiency that is only 33% lower than that in normal Fischer 344 rats. Detailed immunohistochemical, biochemical, and molecular studies reveal that lung endothelia continue to express the mature DPP IV protein product on their lumenal surface, albeit at greatly reduced levels relative to normal Fischer 344 rats. Seemingly, the amount of DPP IV expressed by lung capillary endothelia is sufficient to cause vascular arrest and colonization of the lungs by a respectable number of intravenously injected MTF7 breast cancer cells. Proof that metastasis of MTF7 breast cancer cells to the lungs of Fischer 344/CRJ rats is mediated by DPP IV/FN^{SAP} and not by some other unknown mechanism is obtained by an effective inhibition of metastasis with truncated DPP IV (DPP $IV^{(31-767)}$) previously shown to inhibit lung metastasis of the same cancer cells in normal Fischer 344 rats [4].

Expression of low levels of endothelial cell surface-associated mutant DPP IV in Fischer 344/CRJ lungs is documented here by biotinylation of the lumenal membranes of lung endothelia by *in situ* vascular perfusion and is corroborate by biochemical and FACS analyses of HEK293 cells transfected with mutant DPP IV. These data are at variance with those of Tsuji et al. [1], who reported retention in the ER and total failure of surface incorporation of mutant DPP IV protein in Fischer 344/CRJ hepatocytes and in COS-7 cells transfected with mutant DPP IV cDNA. Although there is ample documentation that the endoplasmic reticulum (ER) contains a series of chaperons and folding factors that safeguard against migration of malfolded, faulty proteins further along the secretory pathway [18, 19, 20]), this 'biosynthetic gate' is by no means impervious as has been demonstrated for the ?F508 mutation of the cystic fibrosis transmembrane conductance regulator CFTR [21]. Leakage seems to depend upon a dynamic equilibrium between intermediate and mature folded states, as proposed for ΔF508 CFTR [22] and H^+-ATPase [23], with the more severe mutations causing kinetic traps that favor non-productive off-pathway folding

intermediates. According to this model, the population of mutant protein that attains normal conformation can exit the ER, while the malfolded forms are retained [23]. At the moment, it is unclear whether the DPP IV mutants capable of reaching the plasma membrane are malfolded forms that escape the ER quality control 'gate', or whether some proportion of the mutant proteins eventually manage to fold correctly [23, 24]. Studies by Gossrau et al. [7] seem to indicate that mutant, enzymatically inactive DPP IV is expressed at different levels in different tissues. For example, while most known DPP IV-expressing tissues have been judged negative for mutant DPP IV expression in Fischer 344/CRJ rats, hepatocytes as well as acinar or capillary endothelial cells have been immunoreactive with anti-DPP IV antibodies, indicating that mutant DPP IV is expressed by select cell types but at lower concentrations. In this context, the thought that endothelial cells either may exert a less stringent quality control over which mutant proteins are allowed to progress further along the secretory pathway and which ones are retained and degraded in the ER or may be capable of correcting malfolding of mutant proteins, is intriguing and may warrant further investigation.

Although several, often parallel mechanisms involving multiple tumor and host factors mediate metastatic spread of cancer cells [25, 26], our data show that reduced expression of DPP IV in lung capillary endothelia of Fischer 344/CRJ rats significantly affects lung colonization by MTF7 breast cancer cells. The importance of DPP IV in lung metastasis is underscored by an almost complete inhibition of MTF7 lung colonization in both normal Fischer 344 rats [4] and Fischer 344/CRJ rats by a soluble DPP IV polypeptide (DPP $IV^{(31-767)}$). The Fischer 344/CRJ rat model also provides compelling evidence that the enzymatic activity of DPP IV is irrelevant for proper adhesion and lung-colonization functions.

Acknowledgements

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TI Truncated dipeptidyl peptidase IV: A potent anti-adhesion and anti-metastasis peptide for rat breast cancer cells.
AU Cheng-H-C. Abdel-Ghany-M. Levine-R-A. Pauli-B-U.
JT Molecular Biology of the Cell.
SO Molecular Biology of the Cell. 9 (SUPPL.). Nov., 1998. 86A.
IS ISSN 1059-1524.
LG English (EN).
AB No Abstract available.
MJ Cell Biology; Enzymology (Biochemistry and Molecular Biophysics); Tumor Biology.
AN PREV199900016985.

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TI Lung endothelial DPP IV promotes adhesion and metastasis of rat breast cancer cells via tumor cell surface-associated fibronectin.
AU Cheng-H-C. Abdel-Ghany-M. Elble-R-C. Pauli-B-U.
JT FASEB Journal.
SO FASEB Journal. 12 (5). March 20, 1998. A772.
IS ISSN 0892-6638.
LG English (EN).
AB No Abstract available.
MJ Cell Biology; Tumor Biology.
AN PREV199800202865.

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