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) (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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† 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)benzene-sulfonyl fluoride; mAb, monoclonal antibody; PBS, 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 antisera 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 (Sunbury 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 non-metastatic. 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 transformed 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 105 tumor cells into female Fischer 344 rats. R3230AC-MET produced 204 (176–237) lung colonies, R3230AC-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.).

**Transfected HEK293 Cells**—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 re-prepared over a detergent-extracted, full-length DPP IV in antimitastasis 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 [35S]methionine in methionine-free RPMI 1640 medium containing 20 μg/ml 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 a-mercaptoethanol (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 protein G-agarose (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 anti-DPP IV mAb 6A3 and separated under nonreducing conditions by SDS-PAGE and separated under nonreducing conditions by SDS-PAGE were cut from the gel and extracted in 50 μl 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 antisera (1:1000 in 5% skim milk) as described (10). Data from Western blots were contrasted with data from autoradiographs obtained from the same tissue extracts or from gels prepared identically to those used for Western analysis.

**Incorporation of 35S-labeled pFN into the Tumor Cell Glycolcay—**The ability of breast cancer cells to incorporate pFN in their surface coat was tested under conditions that mimicked hematogenous spread. In brief, tumor cells (5 × 106 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 35S-labeled 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 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 pCR3 (Invitrogen, San Diego, CA), using Lipofectamine according to the manufacturer’s instructions (Life Technologies). Control HEK293 cells were transfected with the pCRCMV vector alone.

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

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with the cytoplasmic dye Calcein-AM (Molecular Probes, Inc., Eugene, OR) and grown overnight at 37 °C in RPMI 1640 medium supplemented with 10% FBS. Cells were then labeled with the cytoplastic dye Calcein-AM (Molecular Probes, Inc., Eugene, OR) and grown overnight at 37 °C. A 100-fold excess of either DPP IV- or mock-transfected HEK293 cells (∼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 540 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). Non-specific 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—Immunol® 4 Microtitration flat bottom plates (DynePhate Laboratories Inc., Chantilly, VA) were coated with pFN (50 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 biotinylated anti-DPP IV mAb 6A3.

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 Immunol® 4 Microtitration plates was determined from peptide activity measurements relative to standard DPP IV enzyme activity curves (9). 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 μM); (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 glyorphin (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 the lungs were removed. 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 mm² 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 peptide 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).

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 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 glyrophin 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/
of the MTF7 cells. Most DPP IV-HEK293 cells adhered to the MTF7 cell body (Fig. 4A), 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. 4B). 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. 4C).

**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 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 running gel (Fig. 5A, lane 1, arrowhead), and represented radioactivity (Fig. 5A, lane 1, arrowhead). The second, minor protein band represented the remainder of the DPP IV-precipitate radioactivity (Fig. 5A, lane 1, double arrow). Both of these protein bands were reduced with BME to a single protein band of approximately 230 kDa.
that contained the sum of the radioactive counts present in the two HMW bands of the nonreducing gel (Fig. 5, lane 1). 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. 5A, 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. 5A, lanes 1 and 2) resolved as single protein species of approximately 230 kDa by autoradiography (Fig. 5B, I, lanes 1 and 2). They were confirmed by Western analysis to be FN (Fig. 5B, 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. 5B, 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 nonreducing gel (Fig. 5B, lane NR), gel sections corresponding to 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 p FN, 1 μg/ml 125I-pFN, 10% FN-free FBS in RPMI 1640 medium) to allow incorporation of 125I-pFN into the tumor cell surface coat and then extracted with 2% DOC (30 min; 4 °C) (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.

**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 [35S]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 conditions (lane 1 and 3) or reducing (lanes 2 and 4) 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 autoradiographs 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 125I-pFN, 10% FN-free FBS in RPMI 1640 medium) to allow incorporation of 125I-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.

**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.

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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 125I-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 125I-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 125I-pFN in the presence of 2% BME (Fig. 5C, lanes 1 and 2). In contrast, the DOC-insoluble fraction consisted of prominent HMW (top of stacking gel) and dimeric 125I-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. 5C, 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. 5C, 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 125I-pFN (Fig. 5C, 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 125I-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, weekly stained FN globules on their surfaces and, accordingly, adhered poorly to DPP IV-coated surfaces (9.2 ± 2.2%) (Fig. 6A). MTF7 cells incubated in suspension for 4 h in medium supplemented with 10% FN-free FBS showed moderate expression of FN globules on their surface and adhered at intermediate levels to DPP IV-coated dishes (34.3 ± 3.1%) (Fig. 6B). 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. 6C). 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...
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 monoclonal anti-DPP IV mAb 6A3, polyclonal anti-FN antiserum, 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

![Image 1](http://www.jbc.org/)

**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).

![Image 2](http://www.jbc.org/)

**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).

![Image 3](http://www.jbc.org/)

**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).
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 α5β1 (38, 48, 49). Indeed, this receptor is strongly expressed by all lung-metastatic rat breast cancer cells tested5 and could promote the initial immobilization of cellular FN molecules on the cancer cell surface. Alternatively, a cellular FN-α5β1 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 125I-pFN (50); i.e. after a 4-h incubation period of lung-metastatic breast cancer cells with 125I-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 125I-pFN-incubated fibroblasts with 1% DOC (50), the FN multimers observed on MTF7 breast cancer cells clearly increased with time of incubation with 125I-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 125I-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 vitro 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.6 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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DPP IV/FN Adhesion in Breast Cancer Metastasis

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