Identification of PECAM-1 in Solid Tumor Cells and Its Potential Involvement in Tumor Cell Adhesion to Endothelium*

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PECAM-1 (CD31/EndoCAM) is an adhesion molecule in the immunoglobulin supergene family that is expressed on endothelial cells, platelets, and some hematopoietic lineage cells. In this paper, using several polyclonal and monoclonal antibodies against PECAM-1, we identified PECAM-1 molecules on human, rat, and murine solid tumor cells. Immunocytochemical labeling and flow cytometric analysis using either polyclonal, monoclonal, or Fab portion of the antibodies against PECAM-1 detected a distinct distribution on tumor cell surface. Immunoblotting revealed proteins ranging from 120 to 130 kDa in tumor cells derived from different species. Immunoprecipitation and subcellular fractionation studies indicated that PECAM-1 is a constitutively expressed on the surface of human tumor cells (i.e. colon adenocarcinoma). The specificity of a major polyclonal anti-PECAM-1 used in the current study (i.e. SEW-3) was confirmed by the preabsorption studies. PECAM-1 molecule in tumor cells appears to bear terminal carbohydrate moieties (i.e. sialic acid residues) different from those on platelets, since neuraminidase treatment of tumor cells, unlike platelets, did not result in a mobility shift. Polymerase chain reaction (PCR) analysis of genomic DNA derived from tumor cell lines of different species revealed the presence of PECAM-1 gene in the genome. The mRNAs of PECAM-1 in tumor cells were detected by reverse transcription-PCR followed by Southern hybridization. Screening of more than 20 human, rat, and murine solid tumor cell lines indicated that PECAM-1 is widely expressed, although the level of expression varies considerably among different cell lines. The expression of PECAM-1 message in tumor cells was confirmed by Northern blotting. DNA sequencing of the PCR fragment revealed that human tumor cell PECAM-1 matches 100% to the human endothelial cell counterparts. Finally, it was demonstrated that tumor cell PECAM-1 is involved in mediating tumor cell adhesion to endothelium, as evidenced by the ability of anti-PECAM-1 antibodies to decrease the adhesion of unstimulated tumor cells to microvascular endothelial cells.

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** The abbreviations used are: Ig, immunoglobulin supergene family; mAb, monoclonal antibody; MEM, minimum essential medium; PAGE, polyacrylamide gel electrophoresis; pAb, polyclonal antibody; PECAM-1, platelet endothelial cell adhesion molecule-1; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); FBS, fetal bovine serum; kb, kilobase(s); BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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expression of PECAM-1 that is involved in tumor cell-tumor cell, as well as tumor cell-platelet-endothelial cell interactions. In 
model PECAM-1 is expressed in cultured human and rodent solid tumor cells. Furthermore, we will show that PECAM-1 functions in supporting nonstimulated tumor cell adhesion to vascular endothelium in vitro.

MATERIALS AND METHODS

Antibodies—Five antibodies against PECAM-1 were used in the present studies. Polyclonal anti-PECAM SEW-3 and SEW-16 (IgG) were generated in rabbit using affinity-purified human platelet PECAM-1 as the immunogen. SEW-3 was derived from the same batch of preparation as described previously (Albelda et al., 1991). SEW-16 was raised by once-weekly subdermal injections of 100-µg doses of immunoaffinity-purified PECAM-1 antigen (Newman et al., 1992). The Fab fragment of anti-PECAM-1 was derived from cleaving pAb SEW-3 with papain according to the product manual (Pierce Chemical Co.). Monoclonal anti-PECAM-1, mAb 1.3, (IgG1) was produced by immunizing mice with purified human platelet PECAM-1 protein (Albelda et al., 1991; Newman et al., 1992). Another mAb against PECAM-1, BBA-7, was purified by affinity chromatography on protein A-Sepharose using human umbilical vein endothelial cells as the immunogen and shown to be specific for PECAM-1 (R & D Systems, Minneapolis, MN).

Rabbit nonimmunized IgG or nonimmune rabbit serum (Cooper Biochemical, Malvern, PA) and mineral oil-elicited mouse ascites produced from MOPC tumor cell line (IgG1, κ chain, Sigma) were used as negative (antibody) controls in immunofluorescence studies, adhesion studies, and immunoblotting. Goat whole serum (Sigma) was used as negative (antibody) controls in immunofluorescence studies, and immunoblotting. Goat whole serum (Sigma) was used as negative (antibody) controls in immunofluorescence studies, adhesion studies, and immunoblotting. Goat whole serum (Sigma) was used as negative (antibody) controls in immunofluorescence studies, adhesion studies, and immunoblotting. Goat whole serum (Sigma) was used as negative (antibody) controls in immunofluorescence studies, adhesion studies, and immunoblotting. Goat whole serum (Sigma) was used as negative (antibody) controls in immunofluorescence studies, adhesion studies, and immunoblotting.

Cell Culture—Mouse microvascular endothelial cells, CD3, were isolated and characterized as described previously (Chopra et al., 1990). Large vessel endothelial cells, RAEC, were derived from rat (Sprague-Dawley) aortic rings (Diglio et al., 1989). These endothelial cells were routinely maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc.) and antibiotics (50 µg/ml gentamicin, 100 µg/ml penicillin G, and 2.5 µg/ml amphotericin B). Cells were cultured in a humified atmosphere with 5% CO₂ and the culture media were changed every 48 h. Endothelial cells were passaged with a mixture of EDTA (0.1%) and trypsin (0.05%). All cells used in this experiment were free of micoplasma infection.

B16 amelanotic melanoma cell line (B16a), rat W256 carcinosarcoma (W256) cell line, and Lewis lung carcinoma (3LL) cell line were obtained from the Division of Cancer Treatment, National Institutes of Health (Frederick, MD) and adapted for cell culture as described previously (Chopra et al., 1988, 1990; Grossi et al., 1988; 1989; Tang et al., 1993b). B16a and 3LL cells were passaged with 2 ml EDTA in syringe (C57BL/6) male mice and cultured in either MEM (Life Technologies, Inc.) supplemented with 5% FBS (for B16a cells), or for Dulbecco's modified Eagle's medium supplemented with 10% FBS (for 3LL cells) and antibiotics (see above). 3LL cells were cultured in a humidified atmosphere with 5% CO₂. W256 cells were grown in MEM supplemented with 5% FBS and antibiotics and passaged with 2 ml EDTA. HEL (human erythroleukemia), clone A and DLD-1 (human colon carcinoma; Grossi et al., 1988; Tibbetts et al., 1977), M5751 (human cervical epidermoid carcinoma; metastasis to lymph node), TCCSUP (human primary bladder tumour, TCC-NU-LV, Nayak et al., 1977), ACHN (human renal carcinoma; originally derived from the malignant pleural effusion of a patient with widely metastatic renal adenocarcinoma), SK-HEP-1 (human liver carcinoma; Fogh et al., 1977), and SW900 (human lung squamous carcinoma; Fogh et al., 1977) cells were obtained from American Type Culture Collection. A series of human melanoma cell lines, WM35, WM15, WM164, WM226-4, WM793, WM983-A, and WM983-B were kindly provided by Dr. M. Herlyn (The Wistar Institute of Anatomy and Biology). These cell lines have not been extensively characterized in the literature. Human prostate adenocarcinoma Du145 (Stone et al., 1978) and PCC-1 (Brotzman et al., 1989), human head and neck squamous carcinoma (SSC-UM), human breast carcinoma (MCF-7; Soule et al., 1973), rat prostate adenocarcinoma (AT-3), and B16F10 and B16F10 murine melanoma cell lines were kindly provided by Drs. S. Wolman (Wayne State University), S. Fligiel (VA Medical Center, MI), A. Razz (Michigan Cancer Foundation), K. Pienta (Wayne State University), and J. L. Fidler (M. D. Anderson Hospital and Tumor Institute, TX), respectively. HEL, MCF-7, and AT-3 cells were cultured in RPMI medium plus 10% FBS. SW900 cells were grown in L-15 medium with 10% FBS and human melanoma cells of the WM series were cultured in MCDB/L-15 (4:1) supplemented with 2% FBS and 5 µg/ml of insulin. All of the remaining tumor cell lines were cultured in either MEM or Dulbecco's modified Eagle's medium containing 10% FBS and passed with 2 ml EDTA. A summary of the cell lines used in the present study is presented in Table I.

### Chemicals and Reagents—Protease inhibitors PMSF, leupeptin, antipain, aprotinin, chymostatin, and protein standard markers were obtained from Sigma. Immunoblotting detection kit (ECL system) was bought from Amersham Corp. Peroxidase-anti-peroxidase staining kit was purchased from Biogenex (San Ramon, CA). Protein kinase C activator TPA and eicosanoid 12(S)-HETE (i.e. 12(S)-hydroxyeicosatetraenoic acid) were purchased from Sigma and Cayman Chemical (Ann Arbor, MI), respectively. The RNA ladder (0.24-9.5 kb) and prestained protein standard SDS-7B (26.5–180 kDa) were obtained from Life Technologies, Inc. and Sigma, respectively.

#### Indirect Immunofluorescence—Cultured B16a, W256, 3LL, clone A, and B16F10 cells were dissociated from the tissue culture flasks with 2 ml EDTA and washed once with MEM and then fixed with 2% paraformaldehyde in PBS containing 1 mM CaCl₂, 1 mM MgCl₂, and 5% sucrose for 20 min at room temperature. CD3 endothelial cells were used as positive control cell line. Immunofluorescent labeling was performed essentially as described previously (Tang et al., 1992).

#### Table I Cell lines used in the present studies

| Species                  | Cell lines                  | Designation |
|--------------------------|-----------------------------|-------------|
| Mouse                    | Pulmonary endothelial cells | CD3         |
|                          | Melanoma                    | B16F10      |
|                          | Amelanotic melanoma         | B16a        |
|                          | Lewis lung carcinoma        | 3LL         |
| Rat                      | Aortic endothelial cells    | RAEC        |
|                          | Carcinosarcoma              | W256        |
|                          | Prostate adenocarcinoma     | AT-3        |
| Human                    | Erythroleukemia             | HEL         |
|                          | Colon carcinoma             | Clone A     |
|                          | Colon carcinoma             | DLD-1       |
|                          | Cervical carcinoma          | M5751       |
|                          | Bladder carcinoma           | TCC-SUP     |
|                          | Renal carcinoma             | ACHN        |
|                          | Liver carcinoma             | SK-HEP-1    |
|                          | Lung squamous carcinoma     | SW-900      |
|                          | Prostate adenocarcinoma     | PPC-1       |
|                          | Head/Neck squamous carcinoma| SSC-UM      |
|                          | Breast carcinoma            | MCF-7       |
|                          | Melanoma                    | WM series   |
1993a and 1993b). Briefly, for intracellular labeling, cells were permeabilized with HEPES-Tris buffer (20 mM HEPES, pH 7.6, 300 mM sucrose, 50 mM NaCl, 3 mM CaCl2, and 0.5% Triton X-100) for 3 min at room temperature. For surface labeling, cells were not permeabilized. All of the coverslips were incubated with 20% goat whole serum in 4% BSA-containing PBS for 20 min at 37°C to block nonspecific binding. Antibody reaction was performed by incubating coverslips with polyclonal (SEW-3; 30 μg/ml), monoclonal (mAb 1.3; 8 μg/ml), or Fab fragment (30 μg/ml) of anti-PECAM-1, or equivalent antibody controls for 60 min at 37°C, followed by washing (4 × PBS). Afterward, the cells were labeled with goat anti-rabbit or goat anti-mouse IgG-fluorescein isothiocyanate (FITC) or rhodamine (TRITC), and the primary antibodies used. Coverslips were mounted with glycerol and PBS (9:1) containing 0.1% N-propylgalact. Phase contrast and immunofluorescence pictures were taken with a Nikon Optiphot microscope. Transmission. light micrographs were taken with a Leitz Orthoplan microscope.

Immunocytochemistry—Subconfluent endothelial cells and tumor cells were cultured for 18 h before being used for peroxidase-antiperoxidase staining. Cells were fixed and permeabilized as described for immunofluorescence. After washing, the coverslips were incubated with 3% H2O2 at room temperature for 5 min to eliminate endogenous peroxidase activity. Following primary antibody (SEW-3 or mAb 1.3) incubation, the coverslips were sequentially incubated with anti-rabbit or anti-rabbit IgG (corresponding to the primary antibodies used) and the peroxidase-anti-peroxidase complex for 60 min at 37°C each. The staining results were revealed by incubating cells with chromogen (ABC) for 15 min at 37°C.

Flow Cytofluorometry—Flow cytometric analysis was conducted as described previously (Tang et al., 1993a, 1993b). Briefly, suspended tumor cells (dissociated with 2 mM EDTA) were fixed with 2% paraformaldehyde (in PBS containing 0.1% BSA) for 20 min on ice. After washing, the cells were blocked with goat normal serum (in PBS containing 4% BSA) for 20 min on ice. Subsequently, without washing, cells were reacted with polyclonal anti-PECAM-1 (SEW-3) or nonimmune rabbit IgG (30 μg/ml), or mAb 1.3 or MOPC (10 μg/ml) for 45 min at room temperature, followed by washing (2 × PBS). Cells were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit or anti-mouse IgG (1:200) for 45 min at room temperature. Cell surface immunofluorescence was measured with Epics Profile II flow cytomter (Coulter Electronics, Hialeah, FL), operating at 200 milliwatts at 488 nm. The data analysis was performed as described previously (Grossi et al., 1988; Tang et al., 1993b).

Platelet Preparation—Human and mouse blood were collected with 3.8% sodium citrate and 4.5% dextrose in 0.9% physiological saline. Platelet-rich plasma was separated on 7.5% SDS-PAGE under reducing conditions. Gels were stained, dried, and exposed at −80°C using an intensifying screen.

Preadsorption Studies—To confirm the specificity of SEW-3, the major polyclonal antibody used throughout the current study, we preabsorbed this antiserum with PECAM-1 and then employed the preabsorbed antibody in the immunoblotting as well as immunostaining of tumor cells. L cells that do not express endogenous PECAM-1 were transfected with complete PECAM-1 cDNA sequence or the murine PECAM-1 cDNA (Albelda et al., 1991). One mg of the SEW-3 IgG was incubated with 5.6 × 106 of L cells transfected with either PECAM-1 or vector for 1 h at room temperature with mixing. The cells were then removed by centrifugation. This absorption step was repeated two more times. The final supernatant was filtered through a 0.2-μm filter and spun at 100,000 × g for 4 h at 4°C. The antibody was then tested by ELISA to determine the activity remaining using purified PECAM-1 plated in the microtiter wells. Dilutions of 1:10, 1:50, and 1:250 were used for the PECAM-1-absorbed antibody and the control-absorbed SEW-3 and normal rabbit IgG were used at 100, 50, and 25 μg/ml in the ELISA experiments. The absorbed and control-absorbed SEW-3 IgG were then used in immunofluorescent labeling and immunoblotting of tumor cells, as described above.

Tumor Cell Adhesion to Endothelium—In vitro cell adhesion assay was run to determine the potential functions of tumor cell PECAM-1 molecule. B16a or 3LL cells metabolically labeled with 0.1 μCi of 35S-methionine (37°C for 5 h in F-free MEM) were dissociated (with 2 mM EDTA) and washed twice with MEM. Then tumor cell adhesion to confluent CD3 cells in 24-well culture plates (Falcon) was performed according to the following three protocols: (a) tumor cells were first incubated with 40 μg/ml of polyclonal anti-PECAM-1 in MEM containing 4% BSA) for 30 min at 15°C and then added (100,000 cells/well) to CD3 monolayer; (b) CD3 monolayer was first treated with Ab (the same amount as in a) for 30 min at 15°C and then untreated tumor cells were added; and (c) tumor cells were suspended in the Ab solution and immediately added onto EC monolayer. Time course was run as described in c) for 10, 30, and 60 min following addition of tumor cells. These studies were performed using different concentrations of polyclonal anti-PECAM-1 (40, 20, and 10 μg/ml) or an equivalent amount of nonimmune rabbit whole serum. In some experiments, tumor cell adhesion to CD3 monolayers was performed by preincubating either tumor cells or endothelial cells with anti-PECAM-1 followed by washing to remove unbound antibodies. The adhesion was terminated by aspirating media and nondenatured adhered cells. The culture wells were rinsed with PBS (4 ×) and the contents harvested with a mixture of 0.1 N NaOH and 1% SDS. The number of adherent tumor cells was determined by counting the number of cells in three replicates. Triplicate experiments were performed, and the experiment was repeated three times with comparable results.
Polymerase Chain Reaction (PCR) Analysis of Genomic DNA and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Genomic DNA of cultured solid tumor cells was isolated using SDS/protease K method (Sambrook et al., 1987). PCR of genomic DNA was performed using 0.25 μg of DNA as the template in 50 μl of the following reaction mixture: 1× PCR buffer (Life Technologies, Inc.), 0.2 mM dNTPs, 20 units of RNase inhibitor (RNasin; Promega), 1 μM each primer (see primer B described below), and 200 units of M-MLV reverse transcriptase (Life Technologies, Inc.). RT-PCR was performed as described previously (Chen et al., 1992; Tang et al., 1993a and 1993b). RT-PCR was performed basically as described previously.



RESULTS

Immunological Identification of PECAM-1 on Human, Rat, and Murine Solid Tumor Cells—Cultured tumor cells from different species were surface labeled with either pAb (Fig. 1), mAb (data not shown), or Fab fragment (Fig. 2) against PECAM-1. Immunodetection was conducted using immunofluorescence, peroxidase-anti-peroxidase staining, and flow cytometry. As shown in Fig. 1, tumor cells, like endothelial cells (Fig. 1a), expressed PECAM-1 molecules on their cell surface. The distribution pattern of the positive labels varied among different tumor cell lines. B16a (Fig. 1b) cells demonstrated a homogeneous surface labeling although heterogeneity existed among individual cells (i.e. some tumor cells expressed a much lower amount of PECAM-1 than others). B16F10 melanoma cells exhibited a similar staining pattern (Fig. 1c). In contrast, larger aggregates of positive label were detected on 3LL cells (Fig. 1c and h). On the other hand, clone A cells (human colon carcinoma) appeared to be enriched for PECAM-1 molecules at the cell periphery in subconfluent cultures (Fig. 1d) and cell borders in confluent cultures (data not shown). Peroxidase-anti-peroxidase staining revealed brownish granules on the cell surface of B16a (Fig. 1g) and also demonstrated a perinuclear region of 3LL cells (Fig. 1h). When cells were permeabilized with Triton-HEPES buffer, immunostaining with SEW-3 detected an intracellular pool of PECAM-1 molecules (data not shown). Staining with the Fab fragment of SEW-3 also detected cell surface labeling on B16a, 3LL, W256, clone A, and B16F10 cells (Fig. 2, a–e, respectively), excluding the possibility of nonspecific binding of intact antibody to Fc receptors. In addition, staining of 3LL cells with control antibodies, i.e. nonimmune rabbit IgG (Fig. 1, f and i, and Fig. 2f) or MOPC IgG1 (κ chain) monoclonal (data not shown), resulted in negligible staining. The specificity was confirmed by flow cytometric staining of clone A cells (mean fluorescence intensity = 61.1 ± 0.23 versus MOPC control 22.22 ± 0.2) and W256 cells (mean fluorescence intensity = 78.6 ± 0.34 versus MOPC control 18.7 ± 0.18) using mAb 1.3.

Biochemical Identification and Partial Characterization of PECAM-1 on Tumor Cells—Several PAbs and mAbs were used to detect PECAM-1 in solid tumor cell lines. PAb SEW-membrane 1 detected a 130-kDa protein, together with multiple lower bands, in human platelet lysates (Figs. 3, A and B). The lower bands represent either the degraded species or nonspecific staining since in other preparations of human platelets only the 130 kDa band protein was detected (e.g. see Figs. 3, C and Fig. 5D). When used in immunoblotting of solid tumor cells, SEW-3 detected a protein of approximately 125 kDa in HEL and W256 cells and a ~128-kDa protein in clone A (Fig. 3A) and DLD-1 (another human colon adenocarcinoma cell line)
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FIG. 1. Immunocytochemical identification of PECAM-1 molecules on solid tumor cells using immunofluorescence (a-f) and peroxidase-anti-peroxidase staining (g-i) with polyclonal anti-human platelet PECAM-1 (SEW-3). All micrographs represent cell surface labeling. a, CD3 (microvascular endothelial cells; used as control); b and g, B16a; c and h, 3LL; d, clone A; e, B16F10; f and i, 3LL cells stained with nonimmune rabbit IgG. a-g, ×660, h and i, 800.

FIG. 2. Identification of PECAM-1 on the surface of human (d, clone A), rat (c, W256), and murine (a, B16a; b, 3LL; and e, B16F10) tumor cells with the Fab antibody against human platelet PECAM-1 (SEW-3). Nonimmune rabbit IgG staining of 3LL cells was used as the control (f), ×600.

cells (data not shown). Rat aortic endothelial cells (RAEC) revealed a protein band of identical size to that of the W256 cells, i.e. 125 kDa (Fig. 3A). Rb IgG only stained some low molecular mass nonspecific protein bands (Fig. 3A). Two bands right below the 128 kDa band in clone A cells are probably the degradation products (see Fig. 3D and Fig. 5, C and D, for comparison). When used to stain murine cells, SEW-3 detected a 130-kDa protein in CD3 microvascular endothelial cells and 3LL tumor cells and a ~125-kDa protein in two melanoma cell lines, B16a and B16 F10 (Fig. 3B). 3LL cells also demonstrated two lower molecular mass bands which are probably the degradation products. Again, RbIgG only detected some low molecular mass nonspecific bands (data not shown). The reactivity of SEW-3 to PECAM-1 was, in a dose-dependent manner, blocked by preincubating this Ab with purified platelet membrane (Fig. 3D), thus providing indirect evidence for the specificity of SEW-3. Another pAb, SEW-16, also detected a ~128-kDa protein in clone A and DLD-1 cells (Fig 3E). Interestingly, this pAb does not recognize PECAM-1 in rodent tumor cells (data not shown). A mAb, BBA-7, detected similar protein bands in clone A and DLD-1 cells (Fig. 3F).

To unequivocally prove the specificity of SEW-3, we preabsorbed this polyclonal antibody with L cells transfected with PECAM-1 cDNA. As shown in Fig. 4a, SEW-3 IgG preabsorbed with PECAM-1-transfected L cells demonstrated little binding to immobilized PECAM-1 in ELISA experiments, in contrast to SEW-3 IgG incubated with control L cells (i.e. cells transfected with the vectors alone) or normal rabbit IgG. When used at a concentration of 20 μg/ml (1/50 dilution) in immunoblotting studies, control absorbed SEW-3 detected the expected PECAM-1 bands of 130 kDa (human platelet, Fig. 4b, lane 1), ~128 kDa (clone A and DLD-1 cells, Fig. 4b, lanes 2 and 3, respectively), and ~125 kDa (W256, Fig. 4, lane 4), along with some degradation and/or nonspecific bands. In contrast, PECAM-1-absorbed SEW-3 (Fig. 4b, lanes 1'-4') at the same concentration only apparently detected those low molecular mass species (suggesting that they are nonspecific products; also refer to Fig. 3A), while the detection of the expected PECAM-1 protein bands (i.e. ~125–130 kDa bands) was completely, or nearly completely blocked. Interestingly, those bands immediately below the ~125–130 kDa PECAM-1 bands were also undetectable using the PECAM-1-preabsorbed SEW-3 (Fig. 4b, lanes 1'-4'), suggesting that these bands represent the degradation products. Similar to the
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immunostaining results with native SEW-3 IgG (Fig. 1), control-absorbed SEW-3 antibody revealed prominent cell surface labeling on clone A (Fig. 4c), W256 (data not shown), and 3LL cells (Fig. 4d), while SEW-3 IgG preincubiated with L cells transfected with PECAM-1 cDNA completely lost its reactivity on tumor cell surface (e.g., Fig. 4e for clone A cells).

Human colon adenocarcinoma cells (i.e., clone A and DLD-1) were further studied by immunoprecipitation and subcellular fractionation (Fig. 5). It appears that PECAM-1 is constitutively expressed on the surface of clone A cells, since immunoprecipitation of radiiodinated clone A cells with both SEW-3 (Fig. 5A) and mAb 1.3 (Fig. 5B) resulted in the expected ~128 kDa protein band. Immunoblotting using membrane fractions readily detected PECAM-1 in both clone A and DLD-1 cells (Fig. 5C). In addition, it appears that the method of harvesting tumor cells (i.e., scraping versus EDTA dissociation) does not affect the detectability of PECAM-1 by SEW-3 (Fig. 5C). Treatment of clone A cells with TPA or 12(S)-HETE did not significantly alter the level of PECAM-1 associated with plasma membrane (Fig. 5D), although these two agents have been shown to increase the surface expression of integrin receptor αvβ3 (Tang et al., 1993a and 1993b).

PECAM-1 is a heavily glycosylated molecule where about 40% of the molecular mass is composed of carbohydrates. It is primarily N-glycosylated and has been demonstrated to possess terminal sialic acid residues (Newman et al., 1990). A preliminary characterization of tumor cell PECAM-1 molecules was performed using neuraminidase treatment. As presented in Fig. 6, 30-min treatment of human platelets with 1.0 unit/ml of neuraminidase resulted in a molecular mass reduction of approximately 5 kDa. In contrast, this decrease in the molecular weight, following neuraminidase treatment, was not observed with all of the tumor cell lines tested (i.e., B16a, W256, and 3LL; Fig. 6). This differential sensitivity of PECAM-1 to C. perfringens neuraminidase in platelets and tumor cells was observed following treatment of samples for up to 4 h (data not shown). Preliminary experiments with clone A cells revealed similar insensitivity of PECAM-1 to neuraminidase treatment (data not shown).

PCR of Genomic DNA, RT-PCR, Southern Hybridization, and Northern Blotting—PCR analysis of genomic DNA revealed the PECAM-1 genes in the genomes of several human, rat, and murine tumor cells (Fig. 7). The size (296 bp) of PECAM-1 fragments (which were confirmed by hybridization) in all cell lines examined was precisely the same as predicted from the size covered by the nested pair of primers which are based on the cDNA sequence, suggesting that this fragment represents a gene encoding segment (i.e., no intron is included in this fragment). All cell lines demonstrated the same size of PECAM-1 fragment (Fig. 7), suggesting the
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1.2
0.8
0.6
0.4
0.2
0.0
1/10
1/50
1/250
1/1250 concentration of IgG

Total SEW 3
SEW 3 absorbed with transfected L cells
SEW 3 absorbed with control L cells
Normal Rabbit IgG

100.00 20.00 4.00 0.80 optical density at 405 nm

FIG. 4. Preabsorption studies. a, ELISA using immobilized PECAM-1 purified from platelets to test the absorption of SEW-3 by L cells transfected with PECAM-1 cDNA sequence. b, Western blotting of human platelets (lanes 1 and 1'), clone A (lane 2 and 2'), DLD-1 (lanes 3 and 3'), and W256 cells (lane 4 and 4') using SEW-3 preincubated with either control L cells (lanes 1-4) or L cells transfected with PECAM-1 (lanes 1'-4'). The detected PECAM-1 proteins migrating at 130 kDa (for human platelet, lane 1), ~128 kDa (for clone A and DLD-1 cells, lanes 2 and 3), and ~125 kDa (for W256 cells, lane 4) were indicated by three arrowheads. c-e, immunofluorescent labeling of clone A (c and e) and 3LL cells (d) with SEW-3 preabsorbed with either control L cells (c and d) or L cells expressing transfected PECAM-1 (e). × 600.

genomic composition of PECAM-1 may be similar. PECAM-1 mRNA was examined by RT-PCR using PECAM-specific primers and DNA hybridization using PECAM-1 cDNA probes. The results of these experiments are depicted in Fig. 8. Interspecies and intraspecies differences in the amount, pattern, and sequence homology of PECAM-1 molecules were noted. For example, the PECAM-1 message of the predicted size (0.3 kb) was observed in two human tumor cell lines, i.e. HEL cells (Fig. 8, lane 3) and clone A cells (Fig. 8, lane 6). But HEL cells also expressed two larger forms of the message as confirmed by hybridization. On the other hand, murine endothelial cells (CD3; Fig. 8, lane 1), murine fibroblasts (Fig. 8, lane 2) and B16F10 melanoma cells (Fig. 8, lane 7) expressed abundant PECAM-1 message, while B16a cells (lane 4) and 3LL cells (lane 5) expressed lower PECAM-1 mRNA which was barely visible on RT-PCR by ethidium bromide staining but whose presence was confirmed by hybridization. This low amount may also result from the possibility that the PECAM-1 sequence of B16a and 3LL is divergent from both human and murine PECAM-1.

FIG. 5. A, immunoprecipitation of PECAM-1 from surface iodinated clone A cells using SEW-3. B, immunoprecipitation of PECAM-1 from surface iodinated clone A cells using mAb 1.3. HEL cells were used as the positive control cell line. The expected PECAM-1 band was indicated by arrows. C, cultured clone A or DLD-1 cells were either scraped using a rubber policeman or dissociated using 2 mM EDTA and then lysed in the hypotonic buffer for membrane preparation (see "Materials and Methods"). An equal amount of membrane protein was loaded onto 7.5% SDS-PAGE and the membrane was blotted using SEW-3. D, clone A cells were treated with either TPA or 12(S)-HETE (0.1 μM, 15 min), scraped off using a rubber policeman, and used for membrane preparation. Equal amounts of membrane proteins were separated on a 7.5% SDS-PAGE and the blotted membrane was stained using SEW-3. HP was included as a control.

FIG. 6. Neuraminidase does not affect the mobility of murine and rat tumor cell PECAM-1 molecules. Nonidet P-40-extracted protein samples prepared from human platelets or from tumor cells were treated with 1.0 unit/ml neuraminidase derived from C. perfringens (marked by +) or with buffer alone (lanes marked by −) for 30 min on ice and then analyzed by immunoblotting using polyclonal anti-PECAM. Shown on the left are molecular mass in kDa. Note, the lower bands in 3LL lane are degradation products.
The expected 296-bp fragment of PECAM-1 was indicated by pattern. Detailed experimental protocol was given under "Materials and Methods." Lanes 1, clone A. Lanes 2, DLD-1. Lanes 3, PFC-1. Lanes 4, Du 145. Lanes 5, W256. Lanes 6, CD3. Lanes 7, B16a. Lanes 8, 3LL. Lanes 9, B16F1. Lanes 10, B16F10. Lanes 11, negative control. The expected 296-bp fragment of PECAM-1 was indicated by arrows. The molecular mass standards are EcoRV-restricted λ-phage fragments shown on the left in kb. Lane 1, CD3 endothelial cells; lane 2, 10T1/2 fibroblasts; lane 3, HEL; lane 4, B16a; lane 5, 3LL; lane 6, clone A; lane 7, B16F10, lane 8, RAEC endothelial cells; lane 9, W256; lane 10, AT 3.0; lane 11, negative control (i.e. RT-PCR without RNA). See "Materials and Methods" for description of cell lines.

human and other murine cell counterparts. Interestingly, B16a and 3LL also had an upper band that comigrated with one of the upper bands in HEL cells but it did not hybridize to PECAM-1 probes, suggesting that they might be amplified by-products. In contrast, all rat cell lines, including rat aortic endothelial cells (lane 8), rat carcinosarcoma cells (W256, lane 9), and AT 3.0 rat prostate carcinosarcoma cells (lane 10), expressed PECAM-1 mRNAs which were not amplified well by the human sequence-based PECAM-1 primers used in RT-PCR. However, the presence of the PECAM-1 message was corroborated by hybridization.

To further examine the expression of PECAM-1 in tumor cells, about 20 human tumor cell lines derived from different histological and pathological origins were screened for the expression of PECAM-1 message utilizing RT-PCR technique combined with Southern hybridization. The result of the screening (Fig. 9) indicated that all cell lines tested contained PECAM-1 message, although some tumor cell lines (e.g. MS751 human cervical carcinoma; lane 16) expressed little PECAM-1 mRNA. Again, heterogeneity was observed among these PECAM-1 messages, even among the tumor cells of the same histological and pathological source. A typical example was the WM series of melanoma cell lines (Fig. 9, lanes 1–7), some of which demonstrated an upper band. Surprisingly, none of these upper bands hybridized to the PECAM-1 cDNA probe. In contrast, the upper band in some other tumor cell lines (e.g. Du 145; lane 9) was positive by Southern blot.

The presence of PECAM-1 messages in tumor cells were further confirmed by Northern blotting analysis of human (HEL and clone A), rat (W256), and murine (3LL, B16F1, and B16F10) tumor cells (Fig. 10). The results revealed three bands, i.e. 3.7, 3.4, and 3.0 kb, for HEL cells, as reported by others (Zehnder et al., 1992). Hybridization revealed a single mRNA band of 3.3 kb (Fig. 10). The loading of samples was confirmed by rehybridization to β-actin cDNA probe.

Human Tumor Cell PECAM-1 Sequence Matches 100% to Human Endothelial Cell PECAM-1 Sequence—For final analysis, we obtained a partial cDNA sequence of PECAM-1 from a human tumor cell line, i.e. clone A colon carcinoma cells. Clone A cells were chosen because these cells are highly invasive, easily cultured, and express abundant amount of PECAM-1 protein (see Figs. 1–3). The DNA sequence ob-
Solid Tumor Cells Express Functional PECAM-1

PECAM-1 is widely expressed in human solid tumor cell lines. Representative solid tumor cell lines derived from different histological origins and pathological types were extracted for total RNA, which was used for RT-PCR. PCR-amplified fragments were then hybridized with PECAM-1 cDNA probe. Lanes 1–7, human melanoma cells (lane 1, WM35; lane 2, WM115; lane 3, WM164; lane 4, WM226-4; lane 5, WM793; lane 6, WM983-A, lane 7, WM983-B); lane 8, clone A (colon carcinoma); lane 9, Du145 (prostate carcinoma); lane 10, PPC-1 (prostate adenocarcinoma); lane 11, SSC-UM (head and neck squamous cell carcinoma); lane 12, ACHN (renal carcinoma); lane 13, SW900 (lung squamous carcinoma); lane 14, TCC-SUP (bladder carcinoma); lane 15, MCF-7 (breast carcinoma); lane 16, MS751 (cervical carcinoma); lane 17, SK-HEP-1 (hepatocellular carcinoma); lane 18, HEL; lane 19, Negative control. The molecular mass markers are the same as shown in Fig. 6. The expected PECAM-1 band is indicated by the arrow.

3.7 kb
3.4 kb
3.0 kb

β-Actin

4.1 kb

3.7 kb
3.4 kb
3.0 kb

Fig. 9. PECAM-1 is widely expressed in human solid tumor cell lines. Representative solid tumor cell lines derived from different histological origins and pathological types were extracted for total RNA, which was used for RT-PCR. PCR-amplified fragments were then hybridized with PECAM-1 cDNA probe. Lanes 1–7, human melanoma cells (lane 1, WM35; lane 2, WM115; lane 3, WM164; lane 4, WM226-4; lane 5, WM793; lane 6, WM983-A, lane 7, WM983-B); lane 8, clone A (colon carcinoma); lane 9, Du145 (prostate carcinoma); lane 10, PPC-1 (prostate adenocarcinoma); lane 11, SSC-UM (head and neck squamous cell carcinoma); lane 12, ACHN (renal carcinoma); lane 13, SW900 (lung squamous carcinoma); lane 14, TCC-SUP (bladder carcinoma); lane 15, MCF-7 (breast carcinoma); lane 16, MS751 (cervical carcinoma); lane 17, SK-HEP-1 (hepatocellular carcinoma); lane 18, HEL; lane 19, Negative control. The molecular mass markers are the same as shown in Fig. 6. The expected PECAM-1 band is indicated by the arrow.

Published human endothelial cell PECAM-1 sequence (Albelda et al., 1991), the tumor cell sequence was found to be identical (Fig. 11), therefore providing the conclusive evidence that solid tumor cells express PECAM-1.

PECAM-1 Is Involved in Tumor Cell Adhesion to Vascular Endothelium—Homologous in vitro cell adhesion assay was performed to examine the function of tumor cell PECAM-1 molecules. Radiolabeled tumor cells (i.e. B16a and 3LL) were coincubated with confluent murine microvascular endothelial cells (CD3) in the presence of anti-PECAM (SEW-3, mAb 1.3, or Fab Ab) or non-immune rabbit IgG or MOPC ascites (as the Ab control). Fig. 12a demonstrated that all three Abs could inhibit B16a cell adhesion to endothelium, although in general the pAb SEW-3 demonstrated the strongest inhibitory effect. An inhibition of approximately 40% was obtained with all of the antibodies. The adhesion-blocking effect was observed 10 min following addition of the antibody and persisted throughout the experimental period (up to 60 min). The inhibition by the pAb of 3LL cell adhesion to CD3 appeared to be greater than the inhibition with B16a (Fig. 12b). Dose studies indicated that SEW-3 exhibited a dose-dependent inhibition of B16a adhesion to endothelium (Fig. 12c). Incubation with antibody for a shorter time period (i.e. 10 min) appeared to give rise to a greater inhibition than observed with a longer time period (i.e. 20 min; Fig. 12c). When either tumor cells or endothelial cells were individually treated with antibodies (after which Abs were washed off) and then used in the adhesion assay (see "Materials and Methods" for details), inhibition of adhesion was also observed (data not shown). Collectively, these data suggest that tumor cells express functional surface PECAM-1 molecules which are involved in tumor cell adhesion to endothelium.
DISCUSSION

PECAM-1 is a member in the Ig family of adhesion molecules. A large array of Ig family adhesion molecules have been implicated in tumorigenesis and cancer metastasis. For example, both N-CAM and Ng-CAM have been detected in neuroblastoma and phaeochromocytoma and found to be related to tumor cell invasion (Brackenbury, 1985). Vascular cell adhesion molecule-1 expressed on cytokine-activated endothelial cells have been demonstrated to mediate tumor cell adhesion to the vascular monolayer via binding to VLA-4 (Martin-Padura et al., 1991; Taichman et al., 1991). Carci-noembryonic antigen gene family are expressed on solid tumor cell lines such as colon carcinoma and breast cancers and mediate either Ca2+-dependent (Turbide et al., 1991) or Ca2+-independent homotypic tumor cell aggregation or heterotypic cell-cell adhesion. Another Ig family member, ICAM-1 (intercellular cell adhesion molecule-1), which is normally expressed on activated endothelium, has been observed to be expressed on solid tumor cells and its expression is correlated with metastatic potential (Johnson et al., 1989). Based on the above observations, we hypothesized that some solid tumor cells may express PECAM-1 and that PECAM-1 may be involved in tumor cell-endothelial cell interactions. Hence we undertook the studies presented in this paper.

Tumor cell PECAM-1 molecules were shown to be expressed on the cell surface, as indicated by immunocytochemical surface labeling, flow cytometry, immunoprecipitation of surface iodinated cells, as well as by subcellular fractionation studies using diverse antibodies. Tumor cells from different histological origins appear to exhibit different topological distribution patterns of PECAM-1 on the surface, since some tumor cells demonstrate homogeneous labeling (e.g. B16a melanoma), while others (e.g. 3LL lung carcinoma) demonstrate larger surface “granules” (or aggregates), and still others (e.g. clone A colon carcinoma) appear to be enriched for PECAM-1 molecules at the cell periphery (i.e. cell-cell contact zones; see Fig. 1). The heterogeneity in PECAM-1 expression is also observed within a specific tumor cell type, i.e. the level of expression is not homogeneous among all cells in a population. Labeling of permeabilized tumor cells also reveals an intracellular pool.

Western blotting using two pAbs and a mAb (Fig. 4) demonstrated that tumor cell PECAM-1 migrates in the range of 120–130 kDa, a molecular mass similar to PECAM-1 expressed in platelets and endothelial cells (Muller et al., 1989; Albelda et al., 1990; Newman et al., 1990; Albelda et al., 1991; this study). The specificity of a major polyclonal antibody used in the current study, i.e. SEW-3, was confirmed indirectly by preabsorbing this antibody with purified platelet membrane as well as directly by preincubating the antibody with PECAM-1-transfected cells. The molecular weight of tumor cell (i.e. clone A) PECAM-1 is not affected by reduction (data not shown), suggesting that this protein, like endothelial cell PECAM-1, is made up of a single polypeptide. The Western blotting results were confirmed by immunoprecipitation and subcellular fraction studies. PECAM-1 appears to be constitutively expressed on the surface of some tumor cells, e.g. clone A cells. Several lines of experimental data support this conclusion. First, immunofluorescence, peroxidase-anti-peroxidase staining, and flow cytometric analysis all detected the surface labeling. Second, immunoprecipitation with surface-radiolabeled cells resulted in the protein band. Third, immunoblotting using membrane fraction revealed the PECAM-1 band. Finally, non-treated clone A cells and clone A cells treated with TPA or 12(S)-HETE do not demonstrate a difference in terms of the amount of membrane-associated PECAM-1. TPA and 12(S)-HETE, by activating protein kinase C, have previously been shown to increase the surface expression of integrin αvβ3 in endothelial cells (Tang et al., 1993a, 1993b). Therefore, the observations in the present

**Fig. 11.** Partial cDNA sequence of human colon carcinoma cell (clone A) PECAM-1. A segment encompassing a region of the extracellular domain and part of the transmembrane domain of PECAM-1 was PCR-amplified in the first round by primers A and B. The amplified PCR fragment (solid line) was purified and sequenced using dideoxynucleotide termination method. The nucleotide sequence and derived amino acid sequence of part of the PCR products were further amplified in the second round by primers C and D. The amplified PCR fragment (solid line) was purified and sequenced using dideoxynucleotide termination method. The nucleotide sequence and derived amino acid sequence of part of this fragment are shown below. This nucleotide sequence matches 100% to the corresponding region (nucleotide 1660–1895) of the published human endothelial cell sequence (Newman et al., 1990; Albelda et al., 1991).
Solid Tumor Cells Express Functional PECAM-1

The presence of PECAM-1 on solid tumor cells is confirmed by detection of PECAM-1 gene in tumor cell genomes using the PCR technique. The presence of PECAM-1 message in tumor cells was investigated by RT-PCR followed by hybridization of PCR-amplified fragments. Both PCR analysis of genomic DNA and RT-PCR of cellular RNA revealed a PCR fragment of the same size which was confirmed to be PECAM-1 fragment by subsequent hybridization. These observations suggest that this segment of PECAM-1 molecule as defined by the nested pair of primers does not encompass an intron. Several lines of experimental data exclude the possibility that the PECAM-1 fragment detected by RT-PCR is due to contaminated genomic DNA. First, the RT reaction buffer was treated by DNase prior to the initiation of RT reaction (see “Materials and Methods”). Second, in another set of experiments, the RT buffer was pretreated with RNase prior to RT and the subsequent PCR reaction did not reveal any product. Third, the PCR and hybridization patterns of genomic PCR and RT-PCR are significantly different. For instance, in RT-PCR B16a and 3LL cells and all rat cell lines do not demonstrate a well-defined PECAM-1 band (see Fig. 8). However, these cell lines demonstrate a strong PECAM-1 band in genomic PCR (Fig. 7). Another example is that hybridization of some RT-PCR-derived fragments gives two or more bands (e.g. in Du 145 cell line, see Fig. 9) while hybridization of genomic PCR-derived fragments all result in a single predicted band (Fig. 7).

Previously, Simmons et al. (1990) detected PECAM-1 transcripts in a metastatic colon carcinoma and the authors concluded that the transcripts might come from tumor-infiltrated macrophages. These authors did not detect PECAM-1 mRNA on other solid tumor cells. From our own experiments, we suspect that the negative results obtained by these authors are due to insensitivity of Northern blotting using total cellular RNA. Using purified mRNA for Northern blotting, we detected the PECAM-1 message in solid tumor cells. This provides substantive corroboration for the RT-PCR data. Three messages are detected in HEL cells. This observation is consistent with our RT-PCR data revealing the presence of multiple PECAM-1 mRNAs and with the Northern blotting results of others (Zehnder et al., 1992). In clone A, W266, 3LL, and B16F10 cells, only a single species of message is observed. The size of PECAM-1 mRNA in 3LL cells appears to be smaller than that in other cells and identical to the second species of mRNA in HEL cells (i.e. 3.3 kb). This difference in the size of PECAM-1 message in different tumor cell lines may represent cell type-specific alternative splicing. The molecular biology experiments we performed allow us to conclude: (a) PECAM-1 appears to be expressed on many human solid tumor cells, although the level of expression varies greatly among different cell lines; (b) human tumor cell PECAM-1 sequence is identical or highly homologous to that of endothelial cell PECAM-1; and (c) rodent solid tumor cells also express PECAM-1.

The significance of the detection of PECAM-1 on solid tumor cells is reinforced by the fact that it is widely expressed and involved in mediating tumor cell adhesion to vascular endothelium, one of the most important steps leading to organ preference of metastasis (Pauli et al., 1990; Honn and Tang, 1992). Adhesion assays either in the presence or absence (but with pretreatment of tumor cells with anti-PECAM-1) of Abs has consistently demonstrated that PECAM-1 is functional in mediating tumor cell adhesion to unstimulated endothelium.

considering that tumor cells are reported to possess aberrant glycosylation of surface glycoproteins.

The form of PECAM-1 present on some tumor cells may have different biochemical properties than its platelet counterpart. Neuraminidase treatment does not result in any alterations in the molecular mass of tumor cell PECAM-1, although an approximate 5 kDa decrease is observed with the platelet PECAM-1 following neuraminidase treatment. Two possibilities arise from this observation. Tumor cell PECAM-1 molecules may not be significantly sialylated in the termini of their carbohydrate chains. Alternatively, tumor cells may possess aberrant or abnormal terminal sialyl residues which are not cleaved by neuraminidase from C. perfringens. This possibility is especially tempting and more plausible when considering that tumor cells are reported to possess aberrant glycosylation of surface glycoproteins.

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\[^2\] D. G. Tang and K. V. Honn, unpublished observations.
ECAM-1 has been shown to be enriched at the cell-cell borders of confluent endothelial cells in culture and of the vessel wall in situ ( Muller et al., 1989; Albeida et al., 1990; Newman et al., 1990). Of interest, morphological studies on tumor cell-endothelial cell adhesion frequently indicate that adhesion preferentially occurs at the apoposis zone between neighboring endothelial cells (Pauli and Lee, 1988). Thus, it is tempting to speculate that PECAM-1, among other adhesion molecules, may mediate early phase (i.e., "locking"; see Honn and Tang, 1992) tumor cell adhesion to unstimulated endothelium, when activation-dependent adhesion molecules such as ICAM-1, E-selectin, P-selectin, and vascular cell adhesion molecule 1 are not available. Interestingly, Lee et al. (1992) recently reported that adhesion of melanoma cells to cultured human microvascular endothelial cells is independent of vascular cell adhesion molecule 1, E-selectin, and ICAM-1. It was hypothesized that some novel microvessel-related adhesion proteins are involved. Our results suggest that PECAM-1 may be one of these "novel" adhesion molecules.

It is worthwhile to point out that we did not examine whether all available tumor cell lines express PECAM-1 protein although RT-PCR results indicated that most of these cells express readily detectable message. In situ hybridization experiments are underway to determine the expression of PECAM-1 mRNA in tumor cells in vivo.

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