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Extracellular Annexin II Is Associated With Divalent Cation-Dependent Tumor Cell–Endothelial Cell Adhesion of Metastatic RAW117 Large-Cell Lymphoma Cells

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Abstract Using fixed microvessel endothelial cell monolayers the molecules involved in the adhesion of liver-preferring murine RAW117 large cell lymphoma cells to murine liver-derived microvessel endothelial cells were identified by affinity isolation. Detergent lysates obtained from poorly (P) or highly (H10) liver-metastatic cells inhibited RAW117-H10 cell adhesion to hepatic sinusoidal endothelial (HSE) cell monolayers. Allowing detergent lysates of cell surface-labeled RAW117 cells to bind to fixed HSE cell monolayers and eluting the bound components indicated that several tumor cell surface molecules (~ 70, ~ 35, ~ 32, ~ 22, and ~ 14 kDa) might be involved in RAW117 cell–HSE cell adhesion. The ~ 35 kDa component was cation dependent in its binding to target HSE cells. Increasing detergent concentration had no effect on binding of the ~ 35 kDa component to HSE cell monolayers, whereas treatment with 0.5 M NaCl resulted in its selective elution from HSE cells. Incubation of the HSE cell monolayers with detergent lysates from cell surface-labeled RAW117-H10 cells resulted in selective depletion of the ~ 35 kDa component, suggesting that the binding is saturable. This divalent cation-dependent molecule is one of the major tumor cell surface components bound by several types of endothelial cells and murine hepatocytes, whereas there was poor binding of this component to unfixed or fixed human red blood cells. The purified, partially (~ 40%) sequenced molecule had amino acid sequence identity with murine but not bovine annexin II, indicating that it was not bound from the bovine serum used to grow RAW117 cells. Using antibodies specific for annexin II flow cytometery indicated equivalent amounts of annexin II are expressed on RAW117 cell surfaces in the absence or presence of excess EDTA, whereas annexin I was only found in low amounts on the surfaces of RAW117 cells. Annexin II antibodies inhibited by ~ 40–50% the adhesion of RAW117 tumor cells to live or fixed endothelial cells, and purified tumor cell surface fractions containing the ~ 35 kDa component partially inhibited (~ 35%) RAW117 cell–HSE cell adhesion. The data indicate that annexin II is expressed on the extracellular surface of RAW117 cells, and cell surface–annexin II mediates a portion of the Ca^{2+}-dependent RAW117 cell adhesion to liver microvessel endothelial cells.

Key words: calpactin, lipocortin, tumor metastasis, liver endothelium, tumor cell implantation, cell surface

Abbreviations used: BAE, bovine aortic endothelial cell; BSA, bovine serum albumin; DMEM, Dulbecco's Minimum Essential Medium; DME/F12, 1:1 (v/v) DMEM:F12 medium; DPBS, Dulbecco's phosphate buffered saline; FBS, fetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HSE, hepatic sinusoidal endothelial cell; LE, murine lung endothelial cell; MAb, monoclonal antibody; PBS, phosphate buffered saline; SDMEM, supplemented Dulbecco's Minimum Essential Medium; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis.

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The metastatic spread of tumors in the blood circulation to near and distant sites does not occur randomly. Certain tumors tend to metastasize to particular organ sites, and this appears to be due to differences in tumor cell and host organ molecular properties [Nicolson, 1988, 1989, 1991; Zetter, 1990]. Once in the blood, tumor cells can circulate to virtually every organ, yet metastases form only at certain sites [Nicolson, 1988]. Since the first barrier the circulating tumor cell encounters in homing to a specific organ site is the endothelial lining of the microvasculature, tumor cell–endothelial cell adhesion is the initial step in the process of nonrandom metastasis formation [Nicolson, 1982; Auerbach et al., 1987; Nicolson et al., 1989].

Multiple molecular interactions appear to be involved in tumor cell–endothelial cell adhesion [Nicolson, 1988, 1991; Behrens et al., 1992] as well as normal leukocyte–endothelial cell adhesion [Zimmerman et al., 1992; Vestweber, 1992]. Some of the tumor cell adhesion molecules function in the preferential adhesion of tumor cells to specific types of endothelial cells, whereas other molecules may be associated with the adhesion of tumor cells to various endothelial cells [Belloni et al., 1992]. Zhu et al. [1992] have identified a Ca^{2+}-dependent lung endothelial cell adhesion molecule that selectively promotes adhesion of lung-colonizing murine melanoma cells in a process that can be blocked by antibodies or soluble adhesion molecules. Other molecules strongly implicated in tumor cell–endothelial cell adhesion are selectins [Vestweber, 1992], CD-44 hyaluronate receptor [Behrens et al., 1992], and N-CAM [Zocchi et al., 1993]. Integrins are also involved in tumor cell–endothelial cell adhesion [Behrens et al., 1992]. We reported that RGD-containing peptide polymers inhibit the adhesion of RAW117 large-cell lymphoma cells to liver sinusoidal endothelial cells, suggesting that integrin-type molecules function in the adhesion of RAW117 cells to endothelial cells [Tressler et al., 1989].

We have identified nonintegrin tumor cell surface components that are associated with divalent cation-dependent adhesion of murine RAW117 large-cell lymphoma cells to murine microvascular endothelial cells [Tressler and Nicolson, 1992]. One of these components has been isolated, sequenced, and found to be an annexin, calcium-dependent, membrane- and cytoskeleton-binding proteins usually found inside cells [Burgoyne and Geisow, 1989] but also expressed on the surfaces of some normal [Campos-Gonzalez et al., 1990] and neoplastic cells [Yeatman et al., 1993]. Here we show that membrane-bound murine annexin is expressed at the surface of RAW117 large-cell lymphoma cells and can function as one of the types of molecules involved in tumor cell–endothelial cell adhesion.

MATERIALS AND METHODS

Cell Culture

Poorly (RAW117-P) and highly (RAW117-H10) liver metastatic murine large cell lymphoma cell lines were grown as suspension cultures in plastic petri dishes (Falcon, Lincoln Park, PA) with SDMEM (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and 2.2 mM D-glucose without antibiotics [Brunson and Nicolson, 1978]. Cells were tested routinely for *Mycoplasma* by Hoescht staining and Gen Probe™ (Gen Probe, San Diego, CA) and were found to be negative. Primary cultures of bovine aorta (BAE), murine lung microvessel (LE), and murine hepatic sinusoidal endothelial (HSE) cells were isolated and characterized as described previously [Belloni et al., 1992]. Endothelial cells were cultured in 1:1 (v/v) DMEM/F12 (GIBCO) containing 2% horse serum (Hyclone), 5% FBS (Hazelton, Lenexa, KS), nonessential amino acids (GIBCO), 1 mM sodium pyruvate (GIBCO), 0.1 mM L-glutamine (GIBCO), and 0.1 mg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA) in gelatin-coated tissue culture dishes (Costar, Cambridge, MA) at 37°C in a 5% CO_2–95% air atmosphere.

Preparation of Radiolabeled Cell Extracts

Cell surface-specific iodination of proteins was performed with 1,3,4,5-tetrachloro-3-α-diphenylglycouril (3×10^7 cells/0.5 mCi/200 μg Iodogen; Pierce, Rockford, IL) according to Markwell and Fox [1978]. Cells were metabolically labeled with 10 μCi/ml [^{35}S]methionine (ICN, Irvine, CA; specific activity 400 Ci/mmol) for 5–8 h in methionine-free medium (GIBCO) plus 5% FBS (HyClone) at 37°C. The cells were then pelleted by centrifugation at 600*g*, washed 2–3 times in DPBS at room temperature, and solubilized for 15 min on ice in 2% CHAPS (Sigma, St. Louis, MO), 0.05 mM $CaCl_2$, 10 mM Tris, 10% sucrose, 5% glycerol, 1 mM $MgCl_2$, 1 μM PMSF (Sigma), 1 μM leupeptin (Sigma), at a concentration of 5×10^7 cells/ml. After solubilization, the detergent lysates were centrifuged at 11,000*g* for 5 min, and the supernatants were collected and used immediately or stored frozen at −80°C.

Gel Electrophoresis

Cellular extracts and endothelial cell–adherent tumor cell components were mixed with 0.5 vol of 6% SDS, 3% 2-mercaptoethanol, 1.5 mM EDTA, 30% glycerin, 187.5 mM Tris-phosphate buffer, pH 6.8 (3× sample buffer); the supernatants were collected after centrifugation and heated at 100°C for 10 min. SDS-PAGE was performed in 1 mm thick 5–15% linear gradient polyacrylamide gels as described previously [Tressler and Nicolson, 1992]. The gels were treated with EnHance (New England Nuclear, Boston, MA) and dried, and radioactive proteins

were identified by fluorography with Kodak (Rochester, NY) X-AR5 film.

Adhesion Assays

Cell adhesion assays were performed as described previously [Nicolson et al., 1989; Tressler and Nicolson, 1992]. RAW117 cells (2×10^6) in 30 ml of complete medium were labeled overnight with 0.5 μCi/ml [^{35}S]methionine (ICN), washed in serum-free medium three times, and added to the wells of confluent endothelial cells at a concentration of 2×10^5/well in HEPES-buffered DMEM plus 1% BSA (adhesion medium). In some experiments the endothelial cell monolayers were washed twice with DPBS and fixed prior to the adhesion assays with 0.0125% glutaraldehyde in DPBS for 45 min at room temperature, washed twice with DPBS, and blocked with 30 mM ethanolamine in DPBS for 1 h. The tumor cells were incubated on the endothelial monolayers at 37°C with or without shear and the number of bound cells was determined. Assays were performed in triplicate or quadruplicate, and standard error of the mean was calculated for each experiment. In some assays both Ca^{2+} and Mg^{2+} were depleted from the adhesion assays by washing the tumor cells three times with Ca^{2+}-Mg^{2+}-free PBS and resuspending the cells in Ca^{2+}-Mg^{2+}-free incubation medium [Tressler and Nicolson, 1992].

Inhibition of RAW117 cell adhesion to endothelial cell monolayers by tumor cell components was carried out as follows: CHAPS detergent lysates of RAW117 cells (100 μl) were incubated with fixed endothelial cell monolayers overnight at 4°C. The endothelial cell monolayers were then washed once with DPBS/0.05% Tween 20 and three times with DPBS alone, incubated 1 h at room temperature with 1 ml of adhesion medium, and used in adhesion assays with radiolabeled RAW117 cells. Control samples contained equimolar amounts of CHAPS solubilization buffer preincubated on the endothelial monolayers. Alternatively, divalent cation-reconstituted concentrates of endothelial cell–adherent tumor cell components isolated from 3 M KCl/EDTA-treated crude membrane preparations of RAW117-H10 cells were eluted from the fixed endothelial monolayers with Ca^{2+}-Mg^{2+}-free PBS plus 2 mM EDTA, reconstituted in DPBS with excess $CaCl_2$ and $MgCl_2$, incubated on fixed endothelial cell monolayers for 2 h at room temperature, and then used in the adhesion assays. Controls consisted of approximately equal total protein amounts of reconstituted endothelial cell–adherent RAW117 fractions depleted of the component of interest.

The effect of divalent cations on tumor cell–endothelial cell adhesion was assessed as follows: Radiolabeled RAW117 tumor cells were washed four times with Ca^{2+}-Mg^{2+}-PBS and added back into the adhesion assays in Ca^{2+}-Mg^{2+}-free adhesion medium. In some cases Ca^{2+} ($CaCl_2$) or Mg^{2+} ($MgCl_2$) was added back to the assay. The total cation concentration remained constant.

Purified MAb specific for annexins were obtained from Oncogene Science (Manhasset, NY). Anti-calpactin I (annexin II) was a gift of Dr. V. Roche (University of California, San Diego). Tumor cells (1×10^6) were preincubated for 1 h at 37°C with 100 μl of MAb in a total volume of 1 ml and then used in adhesion assays on fixed or viable endothelial cell monolayers.

Cell-Affinity Binding and Amino Acid Sequencing

Confluent monolayers of BAE, HSE, LE, or murine hepatocytes grown in 12-well tissue culture dishes 2–3 days post confluency were incubated with DMEM/F12 plus 5% FBS, washed once with serum-free medium, and incubated for 3 h in serum-free medium (1 ml/well) at 37°C. The cells were fixed as described above. The endothelial cell monolayers were incubated with 1 ml of 1% BSA in DPBS for 1 h at room temperature, the medium was aspirated, 0.5 ml of DPBS, 0.05% Tween 20 was added to each well, and [^{35}S]methionine-labeled or ^{125}I-surface-labeled CHAPS detergent lysates of RAW117 cells (25 or 50 μl) were then added to each well. After incubation at room temperature or at 4°C for various times, the wells were washed four times with DPBS/Tween-20. The radiolabeled, adherent tumor cell components were eluted with 0.5% SDS and analyzed by SDS-PAGE. Alternatively, adherent tumor cell components were eluted by washing the cell monolayers with Ca^{2+}-Mg^{2+}-free PBS (with or without 2 mM EDTA) plus 0.05% Tween 20, or with DPBS plus 0.5 M NaCl. The eluted endothelial cell–adherent components were then analyzed by SDS-PAGE fluorography. In other experiments the EDTA eluates were reconstituted by adding back excess $CaCl_2$ and $MgCl_2$ in DPBS, and the 0.5 M NaCl eluates were desalted by gel filtration and multiple washes with DPBS over Amicon YM-10 filters. The reconstituted endothelial cell–adherent components were reincubated on

fixed endothelial cell monolayers. To deplete the endothelial cell–adherent components detergent lysates of RAW117 cells were incubated on fixed endothelial monolayers for various times at room temperature. The nonadherent tumor cell components were collected and reincubated on various fixed endothelial cell monolayers and eluted with 0.5% SDS, and the adherent components were analyzed by SDS-PAGE.

Crude RAW117-H10 membrane-KCl extracts were prepared by lysing the cells in 10 vol of ice-cold 0.5 M KCl, 20 mM [3-(N-morpholino)-propanesulfonic acid] (MOPS) (Calbiochem, La Jolla, CA), KOH, pH 7.2, containing 4 mM EDTA, 200 μM PMSF, and 40 μM leupeptin (KME) with ten strokes in a Dounce homogenizer. The suspension was centrifuged at 30,000*g* for 30 min at 4°C, and the pellet was resuspended in 10 vol of ice-cold KME with five strokes of a Dounce homogenizer and recentrifuged as before. The twice-extracted pellet was resuspended in 10 vol of ice-cold 10 mM CHAPS, in 100 mM NaCl, 10 mM MOPS, 1 mM PMSF, 40 μM leupeptin, pH 7.2, with ten strokes of a Dounce homogenizer, and the suspension was incubated at 0°C for 20 min before centrifuging as before. The supernatant (5 ml) of the CHAPS lysate was diluted to 170 ml with DPBS-Tween 20, and 5 ml of the diluted CHAPS lysate was added to each of 34 dishes (100 mm diameter) of fixed endothelial cell monolayers and incubated for 90 min at room temperature on a rotary shaker. The dishes were each washed twice with 5 ml of DPBS-Tween 20 and twice with 5 ml of DPBS before the dishes were incubated for 5 min with 4 ml of DPBS plus 0.5 M NaCl, 2 mM CHAPS, 200 μM PMSF, 40 μM leupeptin. The pooled eluates were concentrated by ultrafiltration (Centricon-30, Amicon) at 4°C and stored at −85°C. The concentrated eluate was diluted with 1 vol of reducing SDS-PAGE sample buffer and heated at 95°C for 10 min. Six equal aliquots were placed in the wells of a pre-electrophoresed 10% T, 2.5% C (piperazine diacrylamide, [PDA], BioRad) polyacrylamide gel that contained a buffer system that consisted of the [*bis*-(2-hydroxy-ethly)-imino-*tris*-(hydroxymethyl)-methane (Bis-Tris, ULTROL® grade, Calbiochem), HCl and 2-{[*tris*-(hydroxymethyl)-methyl]-amino}-ethanesulfonic acid (TES, ULTROL® grade, Calbiochem) [Moos et al., 1988]. The resolved proteins were electrophoretically transferred to a nitrocellulose sheet (0.1 μm pore size; Schleicher & Schuell, Keene, NH) in ice-cold transfer buffer containing 10 mM TES, 10 mM NaOH, 0.5 mM DTT, pH 8.0 (at 23°C) for 90 min at 250 mA constant current (10–15 V/cm). The blotted membrane was rinsed with H_2O, stained for 30–40 sec with 0.1% Ponceau S in 1% acetic acid, briefly destained with H_2O, and immediately photographed. Bands were carefully cut out with a clean scalpel and shipped moist in a cleaned, N_2-flushed, O-ring-sealed vial (1.5 ml cell-freezing vial, Sarsteadt) with dry ice to Dr. W.S. Lane, Harvard Microchemistry Facility, Boston, MA. Tryptic digestion, HPLC separation of peptides, and N-terminal sequencing of the separated peptides were performed by standard procedures.

Flow Cytometric Analysis

One million cells were suspended in 100 μl of medium in 1.5 ml microcentrifuge tubes and cooled to 4°C. To each tube, 100 μl of the appropriate antibody was added to yield a 200 μl suspension with a final antibody concentration of 2.5–5 μg/ml. Primary antibodies included biotinylated-murine MAb directed against p36/Calpactin I, p35/Calpactin II (Oncogene Science, Manhasset, NY) and annexins I, II, II (p11), IV, or VI (Zymed, So. San Francisco, CA). Cells were incubated with the primary antibody for 45 min at 4°C, washed twice in medium, and resuspended with either streptavidin-R-phycoerythrin (Molecular Probes, Eugene, OR) at a 1:100 final dilution or a secondary rabbit anti-mouse IgG (H+L)-F(ab')$_2$-phycoerythrin conjugate (Zymed) at a final dilution of 1:100 for 30 min at 4°C. Cells were then washed twice in medium and resuspended in 0.5 ml medium at 4°C prior to flow cytometric analysis with a Bectin-Dickinson model FACScan flow cytometer.

RESULTS

The liver-metastatic RAW117 cells adhere at a significantly higher rate to liver sinusoidal endothelial (HSE) cell monolayers than to other organ-derived endothelial cell monolayers [Tressler et al., 1989]. All of the RAW117 cell lines were significantly more adherent to unfixed or fixed HSE and LE monolayers than to BAE monolayers [Tressler and Nicolson, 1992]. Although the rates of adhesion were lower with the fixed endothelial cell monolayers, the quantitative differences in tumor cell adhesion of the RAW117 cell sublines paralleled their degree of liver colonization [Tressler and Nicolson, 1992]. When Ca^{2+} and Mg^{2+} were depleted from the ad-

TABLE I. Effect of Ca^{2+} and Mg^{2+} on Adhesion of RAW117 Large-Cell Lymphoma Cells to Hepatic Sinusoidal or Lung Microvessel Endothelial Cells*

	Percent inhibition of tumor cell–endothelial cell adhesion		
Depleted cations	P[a]	H10[a]	L17[b]
None	0	0	0
Ca^{2+}	ND[c]	30	35
Mg^{2+}	ND	60	40
Ca^{2+} and Mg^{2+}	71	81	45

*Adhesion was measured at 120 min according to Materials and Methods.
[a]Adhesion of liver-metastasizing RAW117 cell lines was measured with HSE cells.
[b]Adhesion of lung-metastasizing RAW117-L17 cell line was measured with lung microvessel endothelial cells.
[c]ND, not determined.

hesion assays, RAW117 cell adhesion to endothelial cell monolayers was reduced by ~ 70–80%, respectively (Table I).

We previously found that detergent lysates of RAW117 cells could significantly inhibit the adhesion of RAW117 cells to fixed endothelial cell monolayers [Tressler and Nicolson, 1992]. Fixed HSE cell monolayers were incubated with ^{125}I-surface-labeled, CHAPS-solubilized RAW117-P or -H10 cell membrane components, the endothelial cell monolayers were rinsed with buffer and eluted with detergent, and the eluted components were analyzed by SDS-PAGE. Alternatively, the cell monolayers were eluted three times with Ca^{2+}-Mg^{2+}-free PBS or once with Ca^{2+}-Mg^{2+}-free PBS plus 2 mM EDTA prior to detergent elution, and the various eluted components were analyzed by SDS-PAGE. Divalent cation-dependent tumor cell components of ~ 32, ~ 35, and ~ 70 kDa bound to the HSE cell monolayers and were eluted in the Ca^{2+}-Mg^{2+}-free PBS containing EDTA (Fig. 1, cf. lanes 1,2 with lanes 3,4).

The eluted components in Ca^{2+}-Mg^{2+}-free buffer containing EDTA were reconstituted with excess $CaCl_2$ and $MgCl_2$ in DPBS, reincubated with HSE cell monolayers, and the endothelial cell–bound components were analyzed by SDS-PAGE. We found that the isolated ~ 32 kDa and ~ 35 kDa components rebound to the endothelial cells, whereas the ~ 70 kDa component did not (Fig. 2). The ~ 35 kDa molecule was selectively eluted along with a component of ~ 14 kDa by washing the endothelial monolayers with

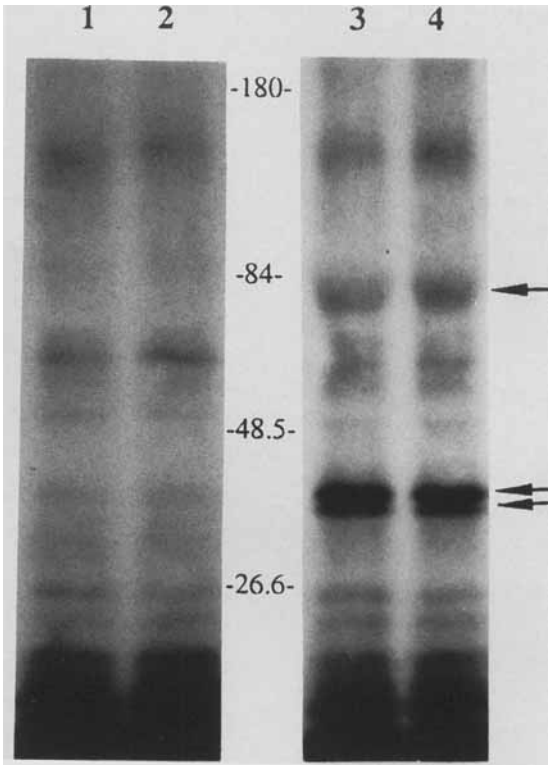

Fig. 1. Identification of RAW117-H10 cell surface-labeled components bound to HSE cell monolayers by SDS-PAGE. **Lanes 1, 2:** HSE-adherent components after elution with Ca^{2+}-Mg^{2+}-free EDTA/PBS solution. **Lanes 3, 4:** HSE adherent components after elution with Ca^{2+}-Mg^{2+}-containing PBS. Arrowheads indicate cation-dependent HSE-binding components. Molecular size of marker proteins indicated in kDa.

0.5 M NaCl in DPBS (Fig. 3). When the salt-eluted material was desalted, reconstituted in DPBS, and reincubated on fixed HSE cell monolayers, the ~ 35 kDa component was able to bind back to the endothelial cell monolayers, whereas the other low M_r proteins did not (Fig. 2).

The remaining endothelial cell–bound surface-labeled components after 0.5 M salt elution were then released from the endothelial cell monolayers with EDTA, reconstituted with Ca^{2+} and Mg^{2+}, and reincubated with HSE monolayers. The ~ 32 kDa component eluted from HSE cells bound back to the fixed endothelial cells (Fig. 2, lane 1). The ~ 35 kDa high-salt-sensitive molecule could also be eluted by EDTA, suggesting that the ~ 35 kDa and ~ 32 kDa molecules bind independently to endothelial cells and are not noncovalently associated in a heterodimeric adhesion complex.

When detergent lysates of ^{125}I-surface-labeled RAW117 cells were preincubated with various

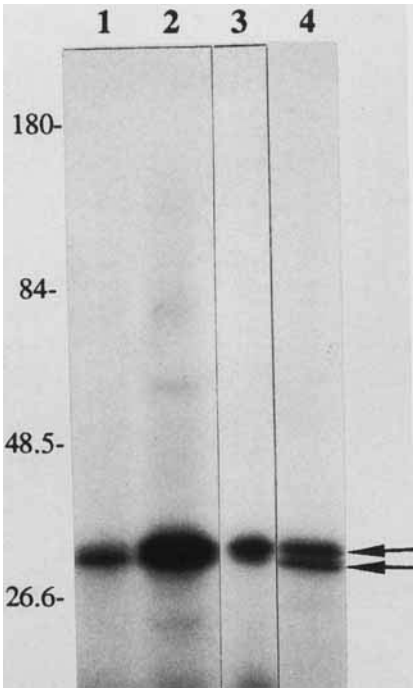

Fig. 2. Reincubation with HSE cell monolayers of RAW117-H10 cell surface-labeled components bound to HSE cell monolayers and then removed by various treatments. **Lane 1:** Removal of HSE-bound tumor cell components with 0.5 M NaCl followed by EDTA. After removal of HSE-bound tumor cell components from HSE cell monolayers with buffered EDTA, the solution was reconstituted with Ca^{2+}-Mg^{2+}-containing PBS and reincubated with HSE cell monolayers. After the incubation, the HSE cell monolayers were eluted with SDS sample buffer, and the solubilized components analyzed by SDS-PAGE. **Lane 2:** Removal of HSE-bound tumor cell components with EDTA. After removal of HSE-bound tumor cell components from HSE cell monolayers with buffered EDTA, the solubilized components were analyzed by SDS-PAGE. **Lane 3:** Removal of HSE-bound tumor cell components with 0.5 M NaCl. After removal of HSE-bound tumor cell components with 0.5 M NaCl, the solution was dialyzed to remove excess NaCl and reincubated with HSE cell monolayers. The HSE cell monolayers were then treated with SDS sample buffer to remove bound components. **Lane 4:** Same as lane 3, except the tumor cell components were eluted with EDTA, reconstituted with Ca^{2+}-Mg^{2+}-containing PBS, and reincubated on HSE cell monolayers, and the HSE-bound components were eluted with SDS sample buffer. Arrowheads indicate ~ 35 and ~ 32 kDa components. Molecular size of marker proteins indicated in kDa.

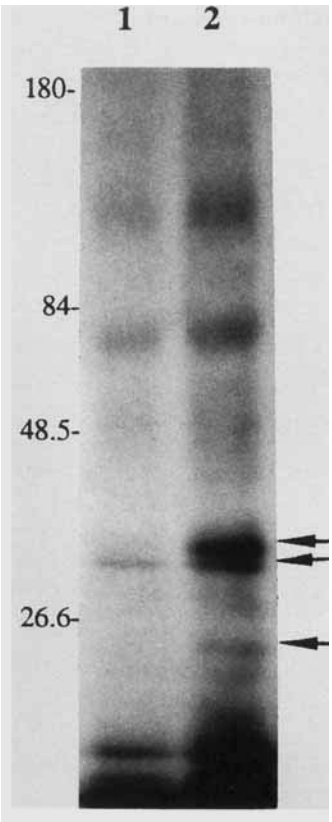

Fig. 3. Identification of HSE-adherent cell surface-labeled RAW117-H10 cell components after elution with PBS or PBS-buffered 0.5 M NaCl. **Lane 1:** HSE-bound components remaining after elution with 0.5 M NaCl. **Lane 2:** HSE-bound components remaining after elution with PBS. The HSE-adherent components were eluted with SDS sample buffer and analyzed by SDS-PAGE. Arrowheads indicate ~ 35 and ~ 14 kDa components. Molecular size of marker proteins indicated in kDa.

types of fixed endothelial monolayers for various times ranging from 30 min to overnight and then eluted from the enodthelial cells, SDS-PAGE analysis indicated that the ~ 35 kDa component was bound by several types (lung microvessel, brain microvessel, bovine aortic endothelial cells) of murine endothelial cells (data not shown). We found that the ~ 35 kDa component bound to murine hepatocytes but not to murine or human erythrocytes (data not shown). These data indicate that the ~ 35 kDa component is not strictly involved in organ-specific metastasis, but it contributes to endothelial cell adhesion. Preincubation of the RAW117 detergent cell lysates on BSA-coated petri dishes did not result in removal of the ~ 35 kDa molecule, indicating that nonspecific protein interactions did not result in removal of the ~ 35 kDa component.

When crude membrane fractions of RAW117-H10 cells were extracted with 3 M KCl and 8 mM EDTA, the ~ 35 kDa component remained with the membrane fraction, whereas the ~ 32 kDa molecule was released from the membranes. This suggested that the ~ 35 kDa but not the ~ 32 kDa molecule is an integral membrane component or its membrane association is divalent cation independent.

The ~ 35 kDa component was purified by cell affinity binding and EDTA elution and assessed for its ability to inhibit the adhesion of viable

Comparison of p35 Peptides with Human, Murine, and Bovine Annexin II

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murine-annexin II MSTVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAERDALNIETAIKTKGVDEVTIVNILTNRSNVQ
human-annexin II MSTVHEILCKLSLEGDHSTPPSAYGSVKAYTNFDAERDALNIETAIKTKGVDEVTIVNILTNRSNAQ
bovine-annexin II -STVHEILCKLSLEGDHSTPPSAYGSVKAYTNFDAERDALNIETAIKTKGVDEVTIVNILTNRSNEQ
p35 peptides                  LSLEGDHSTPPSAYGSVKPY                        KTKGVDEVTIVNILTNRSNV

murine-annexin II RQDIAFAYQRRTKKELPSALKSALSGHLETVILGLLKTPAQYDASELKASMKGLGTDEDSLIEIICSRTN
human-annexin II RQDIAFAYQRRTKKELASALKSALSGHLETVILGLLKTPAQYDASELKASMKGLGTDEDSLIEIICSRTN
bovine-annexin II RQDIAFAYQRRTKKELASALKSALSGHLETVILGLLKTPAQYDASELKASMKGLGTDEDSLIEIICSRTN
p35 peptides                                                TPAQYDASEL      GLGTDEDSLIEIICSR

murine-annexin II QELQEINRVYKEMYKTDLEKDIISDTSGDFRKLMVALAKGRRAEDGSVIDYELIDQDARELYDAGV
human-annexin II QELQEINRVYKEMYKTDLEKDIISDTSGDFRKLMVALAKGRRAEDGSVIDYELIDQDARDLYDAGV
bovine-annexin II QELQEINRVYKEMYKTDLEKDIVSDTSGDFRKLMVALAKGRRAEDGSVIDYELIDQDARDLYDAGV
p35 peptides                        TDLEKDIISDTSG      LMVALA

murine-annexin II KRKGTDVPKWISIMTERSVCHLQKVFERYKSYSPYDMLESIKKEVKGDLENAFLNLVQCIQNKPLYFADR
human-annexin II KRKGTDVPKWISIMTERSVPHLQKVFDRYKSYSPYDMLESIRKEVKGDLENAFLNLVQCIQNKPLYFADR
bovine-annexin II KRKGTDVPKWISIMTERSVCHLQKVFERYKSYSPYDMLESIKKEVKGDLENAFLNLVQCIQNKPLYFADR
p35 peptides    KRKGTDVPKWISIMT                    SYSPYDMLESIK

murine-annexin II LYDSMKGKGTRDKVLIRIMVSRSEVDMLKIRSEFKRKYGKSLYYYIQQDTKGDYQKALLYLCGGDD
human-annexin II LYDSMKGKGTRDKVLIRIMVSRSEVDMLKIRSEFKRKYGKSLYYYIQQDTKGDYQKALLYLCGGDD
bovine-annexin II LYDSMKGKGTRDKVLIRIMVSRSEVDMLKIRSEFKKKYGKSLYYYIQQDTKGDYQKALLYLCGGDD
p35 peptides          KGKGTRDKVLIRIMV                              LYYYIQQDTKG
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Fig. 4. Amino acid sequence of murine (mu), human (hu), and bovine (bov) annexin II. The ~ 35 kDa cell surface-labeled RAW117 component isolated by adherence to HSE cell monolayers was digested with trypsin, separated by HPLC, and the selected peptides sequenced. The sequence data of peptides obtained from the [6did] f 35 kDa component are shown below the annexin sequences.

RAW117 cells to unfixed or fixed endothelial cell monolayers. The ~ 35 kDa component inhibited RAW117-H10 adhesion to HSE cells by ~ 34% at 45 min, reducing the percent cell adhesion of RAW117-H10 to HSE cell monolayers from 24.1 ± 1.0% to 16.0 ± 1.7%.

The endothelial cell adherent ~ 35 kDa component from RAW117 cells was purified by elution from the fixed HSE cell monolayers with NaCl, separation by SDS-PAGE, and transblotting onto nitrocellulose paper. The ~ 35 kDa band was removed from the blot and the amino acid sequence of several tryptic peptides determined. Sequence analysis of peptide fragments constituting ~ 40% of the protein indicated identity with murine but not bovine annexin II (Fig. 4). This indicated that the cell surface-associated annexin II was not derived from FBS in the medium.

When RAW117 cells were incubated with antibodies specific for annexin II prior to adhesion assays with fixed or viable endothelial cell monolayers, adhesion was inhibited by 40–50% (Fig. 5). Nonspecific antibodies had no effect on RAW117 cell adhesion to the endothelial cells. Fixation clearly reduces the ability of adhesion molecules to recruit receptors by laterial diffusion in the plane of the membrane and prevents distortion of cell surface structures—thus the lower rates of adhesion with the fixed cells (Fig. 5B). The anti-annexin II inhibited ahesion independent of whether the cells were viable or fixed.

Using MAb against annexin II cytofluorographic analysis of unfixed or fixed RAW117 cells indicated that H10 cells expressed slightly more cell surface annexin II than P cells (Fig. 6B), but they only expressed low amounts of cell surface annexin I (Fig. 6A). Although RAW117 cells express approximately equilivant total cellular amounts of annexin I and II, only the latter molecule is found in significant amounts on the cell surface of RAW117 cells, suggesting that the cell surface localization of annexin II is not due to a nonspecific mechanism. Moreover, excess EDTA did not remove annexin II molecules from the surfaces of RAW117 cells (Fig. 6C), suggesting that the cell surface-associated annexin II was not released from dead cells and bound nonspecifically to adjacent cells. Killing RAW117 cells by heat shock did not increase or decrease the amount of cell surface-associated annexin II (data not shown).

DISCUSSION

The attachmnent of circulating tumor cells to organ microvessel endothelial cells appears to involve specific tumor cell–endothelial cell interactions, and various adhesion molecules have

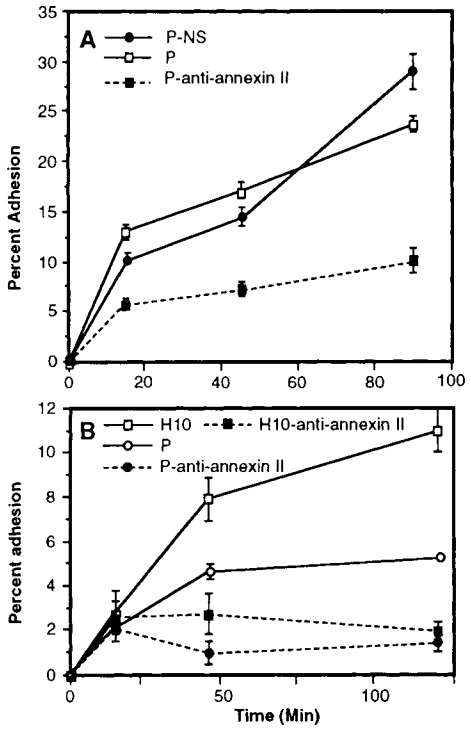

Fig. 5. Inhibition of RAW117 cell adhesion to unfixed or fixed HSE cell monolayers by anti-annexin II. **A:** Adhesion of RAW117-P (P) cells to unfixed HSE cell monolayers and its inhibition by anti-annexin II (P-anti-annexin II); NS, normal serum control. **B:** Adhesion of RAW117-P (P) or RAW117-H10 (H10) cells to fixed HSE cell monolayers and inhibition by anti-annexin II; NS, normal serum control. The data indicate quadruplicate samples ± S.D.

been implicated in this process [Nicolson, 1988, 1991; Tressler et al., 1989; Belloni and Tressler, 1990; McCarthy et al., 1991; Behrens et al., 1992; Zhu et al., 1992; Zocchi et al., 1993]. For example, the adhesion of murine RAW117 large-cell lymphoma cells to murine liver endothelial cells appears to involve several adhesion components. We reported that RGD-containing peptide polymers inhibited by ~ 40% the adhesion of RAW117 tumor cells to liver sinusoidal endothelial cells, suggesting that integrin-type molecules function as one of the important adhesion systems in RAW117 cell–endothelial cell attachment [Tressler et al., 1989]. Similar to our previous studies on the n-butanol extraction of adhesion components from RAW117 cells [Tressler and Nicolson, 1992] we have identified three divalent cation-dependent RAW117 cell surface components of ~ 70, ~ 35, and ~ 32 kDa that are also associated with endothelial cell adhesion. When one of these molecules was isolated and sequenced, it was identified as murine annexin II. Using specific antibodies against annexin II or addition of excess soluble annexin II, we were able to inhibit RAW117 cell adhesion to liver sinusoidal endothelial cells by 35–50%, suggesting that annexins may be only one of the classes of molecules involved in RAW117 cell–endothelial cell adhesion.

Various calcium-dependent cell adhesion molecules, such as the P-, E-, and N-cadherins, have been shown to be important in embryogensis [Aebersold et al., 1987], neural development [Cheney and Willard, 1989], and the segregation of organ tissues [Erikson and Erikson, 1980]. Cadherins have been found to decrease in their cell surface expression with progression to the metastatic phenotype in prostate [Bussemakers et al., 1992] and head and neck carcinomas [Schipper et al., 1992].

In addition to cadherins, the integrin superfamily of adhesion molecules represents a broad class of adhesion components that are divalent cation dependent, and such molecules play an important role in cell-cell and cell-matrix adhesion [Erikson et al., 1984; Hynes, 1992]. Although the adhesion properties of integrins are divalent cation dependent, integrins are composed of subunits of much higher M_r than the cell surface components found here associated with endothelial cell adhesion. Because they are complex integral cell surface molecules requiring subunit interactions, it is unlikely that integrins would be isolated by the procedures used here to isolate endothelial cell adhesion molecules from tumor cells.

Using adhesion of solubilized tumor cell surface molecules to endothelial cell monolayers, we isolated and sequenced a molecule subsequently identified as an annexin. The annexins are known for the most part to be expressed intracellularly, not extracellularly, although there are reports that annexins may be expressed extracellularly [Forbush, 1982; Campos-Gonzalez et al., 1990; Yeatman et al., 1993]. We have used specific antibodies to annexins I–VI to examine the cell surface expression of annexins on various rodent and human tumor cell lines and have found that various types of adenocarcinoma cells but not melanoma cells express cell surface annexins [Yeatman et al., 1993]. In addition, using intact cells and fluorescent-labeled anti-annexins we found puntate ring fluorescense at the cell surface, indicating that annexins are expressed on the surfaces of tumor cells [Yeatman et al., 1993].

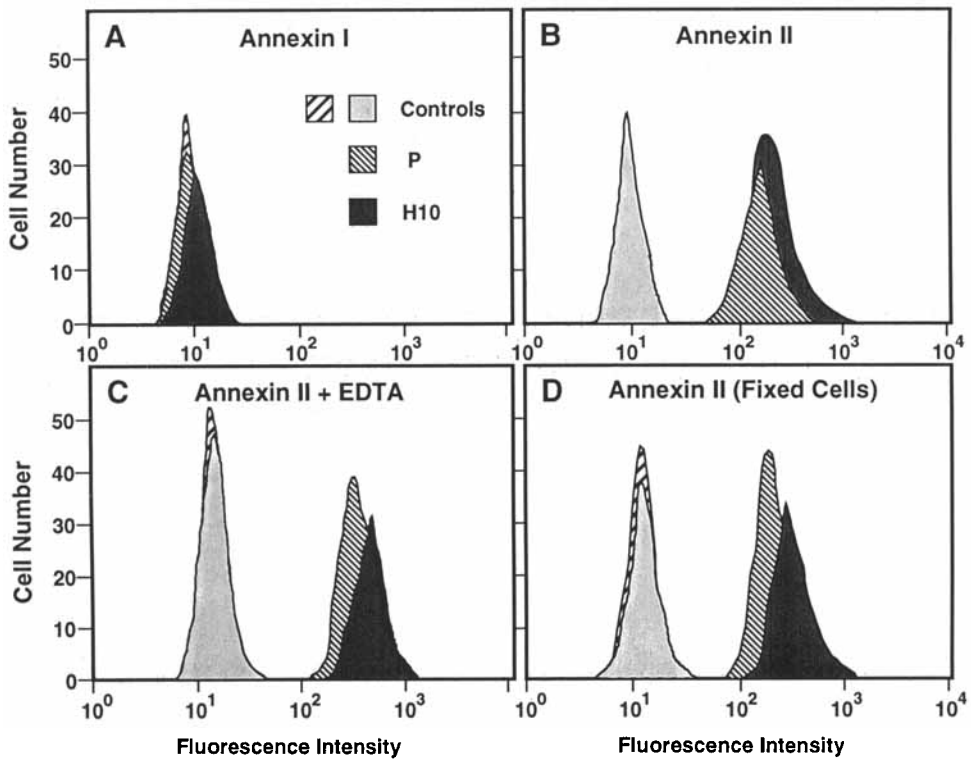

Fig. 6. Cytofluorometric analysis of RAW117-P and -H10 cell surface expression of annexins I and II. See Methods for procedures. **A:** Anti-annexin I. **B:** Anti-annexin II. **C:** Anti-annexin II plus EDTA. **D:** Anti-annexin II, fixed RAW117 cells. The peaks to the left represent the control fluorescence with second antibody only.

Although the annexins do not contain the usual signal sequence for secretion, certain cells secrete specific annexins. For example, the human prostate gland selectively secretes high concentrations of annexin I but not annexin IV [Christmas et al., 1991]. Alternatively, there exists a possibility that the labeling procedures used here did not exclusively label cell surface components. That intracellular cell surface-associated annexins, such as lipocortin I, calpactins, or calpains, were labeled by our procedures was investigated by use of an alternative procedure utilizing a modification of the water-soluble Bolton-Hunter reagent [Glenney, 1986]. When iodination with Bolton-Hunter reagent was performed, the ~ 35 kDa component was prominently labeled and retained its endothelial cell–binding properties. The ~ 32 kDa was also labeled, but less intensely, by this procedure. Similar results were obtained by cell surface labeling with a biotinylation reagent. Thus it is likely that the ~ 35 kDa component is expressed on the cell surface and plays a role in RAW117 cell–endothelial cell adhesion.

The annexin II found at the surfaces of RAW117 cells could have been released from dying cells and bound nonspecifically to the cell surfaces of RAW117 cells. This is considered unlikely because 1) annexin II but not appreciable amounts of annexin I was found on RAW117 cells, although both annexins are present inside these cells at approximately similar concentrations, 2) EDTA did not remove cell surface-bound annexin II, suggesting that its attachment is not mediated by divalent cations, 3) our RAW117 cell preparations did not contain significant numbers of dead cells, and 4) we could not detect the presence of released, soluble annexins in our RAW117 cell suspensions.

We have identified the ~ 35 kDa molecule expressed on the RAW117 cell surface as annexin II. Binding of the ~ 35 kDa molecule to endothelial cells was rapid, specific, and saturable, and a cell-affinity isolated fraction containing the ~ 35 kDa component significantly inhibited the adhesion of RAW117-H10 cells to HSE cell monolayers. Thus this molecule may function in the divalent cation-dependent adhesion

of RAW117 cells to microvessel endothelial cells, suggesting that it is probably one of the common RAW117–endothelial cell adhesion molecules. Lim et al. [1990] reported that a high M_r (~ 67,000) annexin-related molecule was associated with cell-CAM105 at the cell surface of rat hepatocytes, and this molecule may function in cell adhesive interactions. Another annexin-related molecule, anchorin II, is present on the surface of chondrocytes and has been shown to bind to collagen [Pfäffle et al., 1988]. We identified an annexin associated with the adhesion of RAW17 tumor cells to endothelial cells, and this and related molecules may play a role in the cell-cell adhesion of various cell types.

Annexins have been localized to the intracellular cell membrane surface in a variety of cell types [Courtneidge et al., 1983; Nigg et al., 1983; Greenberg and Edelman, 1983]. Using normal and RSV-transformed NRK cells, Courtneidge et al. [1983] demonstrated that portions of annexin II behave as peripheral or integral membrane proteins; however, they were unable to distinguish between vesicular entrapment of annexin II or an integral membrane association of annexin II. The mechanism of outer plasma membrane association of annexin II is unknown. Annexin II does not contain a hydrophobic sequence that could serve as a transmembrane domain, and it does not have an N-terminal hydrophobic sequence for secretion or endoplasmic reticulum translocation. Recently Christmas et al. [1991] reported that annexin I was secreted by human prostate gland epithelial cells using a novel pathway that did not involve targeting to the endoplasmic reticulum. Other metastatic systems have been found to express annexins. Kumble et al. [1992] noted that the proliferating regions of primary tumors and especially their metastases expressed high amounts of annexin II.

The mechanism of cell surface-annexin attachment to RAW117 cell membranes is not known. A calcium-independent, membrane-bound form of annexin I that behaves as an integral protein was described by Sheets et al. [1987], who found that sonication of high-salt- and EDTA-treated membranes did not release annexin in a soluble form. They concluded that annexin I must behave as an integral membrane component. There is a cysteine residue in the carboxy terminus of murine annexin II that could serve as a site for myristoylation. Wice and Gordon [1992] have described a myristoylated annexin of ~ 35 kDa that is expressed in a membrane-associated form in the gut epithelium.

We found that RAW117 cells express several cation-dependent endothelial cell–binding components. It is likely that some of these are also annexins, such as the ~ 32 kDa and ~ 70 kDa components. We have preliminary data suggesting that the ~ 70 kDa component is an annexin (VI), and we have been able to partially inhibit RAW117 cell–endothelial cell adhesion with antibodies against annexin VI (R. Tressler and G.L. Nicolson, unpublished data). There are at least two other cell surface molecules of ~ 70 kDa on RAW117 cells. One is the RNA tumor virus envelope glycoprotein gp70 [Reading et al., 1980], and the other is an antiproliferative molecule that inhibits the mitogen-induced proliferation of normal spleen cells [Joshi et al., 1991]. Previously we found that gp70 was expressed in much lower amounts on the highly metastatic H10 subline [Reading et al., 1980]. Since the ~ 70 kDa component that is adherent to microvessel endothelial cells appears to be expressed on both poorly and highly metastatic RAW117 cells, and the highly metastatic H10 subline adheres at higher rates to HSE cells [Nicolson et al., 1989; Tressler and Nicolson, 1992; Tressler et al., 1989], the ~ 70 kDa component is probably annexin VI. Cell surface-bound annexin II and annexin VI appear to be acting as tumor cell–endothelial adhesion molecules, and they may also be involved in hepatocyte adhesion. Öbrink and collaborators have described a cation-dependent ~ 70 kDa molecule that is involved in rat hepatocyte cell-cell adhesion [Öbrink et al., 1976; Ocklund et al., 1984]. This component could also be similar to the ~ 70 kDa cell surface-bound annexin VI on RAW117 cells. Therefore, it is likely that the major cation-dependent RAW117 components involved in HSE and other adhesion events are cell surface-bound annexins.

Although it is an important event, the adhesion of RAW117 cells to hepatic microvessel endothelial cells is not the only property important in RAW117 metastasis to liver. Highly metastatic RAW117 cells invade target organ (liver) tissues at higher rates than do poorly metastatic RAW117 cells [Nicolson et al., 1989], suggesting that the highly metastatic cells express higher levels of degradative enzymes, and RAW117 cells responded better to target-organ-derived motility factors [Hamada et al., 1992]. The highly metastatic RAW117 cells were also stimulated to grow at higher rates in the presence of target-

organ-conditioned medium [Nicolson, 1987]. Finally, RAW117 cells of high metastatic potential are less susceptible to host-mediated effector systems, such as activated macrophages [Miner and Nicolson, 1983; Reading et al., 1983]. Collectively, these all of these properties are probably important in determining whether tumor cells metastasize successfully to specific sites.

NOTE ADDED IN PROOF

Cytofluorographic evidence indicates that RAW117 cells express annexin VI. Antibodies against annexin VI also inhibit RAW117 cell adhesion to HSE cells, suggesting that both annexins II and VI serve as adhesion components for microvessel endothelial cells when expressed at the cell surface of RAW117 cells.

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