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Endothelial Cell Damage by Walker Carcinosarcoma Cells Is Dependent on Vitronectin Receptor-mediated Tumor Cell Adhesion

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The transport of cancer cells from blood vessels to extravascular tissue is a critical step in metastasis, where endothelial cells and the vascular basement membrane act as barriers to cell traffic. Because endothelial injury can facilitate the metastasis of intravascular cancer cells in vivo, ***the authors have studied*** in vitro ***the free-radical–mediated endothelial damage caused by the rat Walker 256 carcinosarcoma (W256) cell after stimulation with 10^{-6} mol/l (molar) phorbol ester. Here the authors have examined the hypothesis that W256 cell-mediated endothelial injury is dependent on adhesion between the effector and target cells. Attachment of phorbol 12-myristate, 13-acetate (PMA)-stimulated W256 cells to endothelial monolayers was increased 1.8 ± 0.1-fold and damage (^{3}H-2-deoxyglucose release from labeled endothelium) 1.4 ± 0.1-fold after 4-hour pretreatment of the endothelium with 10 ng/ml recombinant human interleukin-1α (rIL-1α). Under various assay conditions, the release of ^{3}H-2-deoxyglucose correlated directly with tumor cell adhesion ($r = 0.98$, $P < 0.005$). In the presence of a polyclonal anti-vitronectin receptor antiserum, adhesion of stimulated W256 cells to rIL-1α–treated monolayers was inhibited by 39% ± 2%, and ^{3}H-2-deoxyglucose release was inhibited by 53% ± 13%. Immunoblot analysis and immunofluorescence flow cytometry demonstrated that the endothelial cells but not the W256 cells expressed vitronectin receptor (VnR) on their cell surface. The surface expression of VnR by endothelial cells was increased 1.9 ± 0.1-fold after 4 hours' incubation with rIL-1α. The authors conclude that W256 cell-mediated endothelial damage is dependent on cell adhesion, which, in turn, is partly regulated by the expression of VnR on the endothelial cell surface. (Am J Pathol 1991, 138:1535–1543)***

The arrest of cancer cells in the capillary bed of target organs is an important step in the metastatic process.[1,2] Tumor cells first adhere to the endothelium, with contact between the underlying basement membrane and tumor cell occurring only 4 to 9 hours later.[3] Previous studies have shown that cytokine treatment of endothelial cell monolayers increases their adhesiveness for malignant melanomas and carcinoma cell lines.[4–8] These observations suggested that this increase in tumor cell attachment to endothelial cells might be mediated by the expression of an inducible adhesive moiety on the apical surface of the endothelium, as the responses were time dependent and required protein synthesis.[5,8] This laboratory has recently obtained evidence that the adhesive moiety is the vitronectin receptor.[9,10]

Studies *in vivo* have indicated that endothelial cell damage can promote the metastasis of circulating tumor cells.[11–13] In this context, we have observed that stimulated Walker 256 carcinosarcoma (W256) cells can damage endothelial cell monolayers by generating reactive oxygen species.[14] *In vitro*, this injury appeared to be dependent on cell contact, because the addition of cell-free supernatants from stimulated W256 cells did not cause damage (measured by ^{3}H-2-deoxyglucose release from labeled endothelium) and because pretreatment of the W256 cells with 1 μmol/l (micromolar) cytochalasin B inhibited W256-mediated isotope release, even while increasing tumor cell-generated chemiluminescence threefold. In this experiment, electron microscopy

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showed that fewer cytochalasin B-treated W256 cells were attached to the endothelial cell monolayer than were attached in untreated controls, suggesting that the adhesion of W256 cells was required to effect endothelial damage. Here we report that oxidative damage to the endothelium by W256 cells is promoted by vitronectin receptor-mediated tumor cell adhesion.

Materials and Methods

Materials

2-Deoxy-D-[1-^{3}H]glucose was obtained from Amersham Corporation, Oakville, Ontario, at a concentration of 1.0 mCi/ml (specific activity 17.0 Ci/mmole). ^{125}Iododeoxyuridine (2200 Ci/mmol) was obtained from duPont/New England Nuclear, Mississaga, Ontario. Two rabbit anti-human vitronectin receptor (VnR) polyclonal antisera were used. One of these was a gift from Dr. D. A. Cheresh (Scripps Clinic, La Jolla, CA) and has been characterized previously.[15] The other was purchased from Telios Biochemicals (San Diego, CA) and has been solid phase absorbed with normal human plasma proteins as well as with the fibronectin receptor. Rabbit anti-human fibronectin receptor (FnR) polyclonal antiserum also was obtained from Telios Biochemicals. Recombinant human interleukin-1α (rIL-1α) was a gift of Dr. P. Lomedico (Hoffman-La Roche, Nutley, NJ). All media were obtained from Gibco Laboratories (Burlington, Ontario). The phorbol ester, phorbol 12-myristate, 13-acetate (PMA), was dissolved in absolute ethanol to appropriate stock concentrations.

Animals and Cell Lines

Pathogen-free, adult (200 g) male Sprague-Dawley rats were maintained according to principles set out by McMaster University for the care and use of laboratory animals. The W256 cell line described previously[16] was maintained *in vivo* as an ascites tumor. For experiments, tumor ascites fluids were harvested in heparinized Hank's balanced salt solution (HBSS; 5 IU heparin/ml) and erythrocytes were removed by two to three cycles of osmotic lysis. Cell suspensions prepared by this method contained 98% ± 1% tumor cells identifiable on Wright-stained cytospin preparations with mean tumor cell viability of 95% after 6 hours as estimated by trypan blue exclusion and intact cell morphology.

Endothelial Cell Culture

Human umbilical cord endothelial cells were obtained by collagenase treatment and maintained in culture by a method modified from Jaffe et al.[17] Briefly, the cells were harvested by collagenase treatment and maintained in medium 199 plus 20% heat-inactivated human serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 100 μg/ml pituitary-derived endothelial growth factor. The cells were either passaged once into T25 flasks and then seeded onto fibronectin-coated plastic discs, contained in 24-well tissue culture dishes, or alternatively, seeded directly into the multiwell dishes after collagenase treatment. Cells were identified as being endothelial in origin by their cobblestone morphology as well as by their expression of von Willebrand factor. All cells used in experiments were at the first or second passage.

Adhesion Assay

W256 cells obtained from the ascites were cultured in BGJ medium (GIBCO, Burlington, Ontario, Canada) with 10% fetal calf serum and 1% antibiotics for 2 days before the experiment. The cells were radioactively labeled by the addition of 0.5 μCi/ml of ^{125}I-iododeoxyuridine to the culture medium on day 1. Endothelial cells were cultured to confluency on fibronectin-coated plastic discs, contained in 24-well tissue culture dishes.

Before the assay, some endothelial cell monolayers were pretreated for 4 hours with 10 ng/ml of rIl-1α at 37°C to induce expression of adhesion sites.[6,7] After rIL-1α stimulation, the medium was removed and 0.5 ml of ^{125}I-iododeoxyuridine–labeled W256 cells, suspended at a concentration of 5×10^6 cells/ml, was added. In some experiments the tumor cells were first stimulated by adding 1×10^{-6} mol/l (molar) PMA, washed twice, and then subsequently used in the assay. The labeled W256 cells were incubated for 1.5 hours at 37°C with the endothelial monolayers. Loosely adherent W256 cells then were removed from the endothelial cell monolayer by washing the plastic disc in three changes of unsupplemented M199. Each disc was then counted in a Packard gamma-counter (Hewlett Packard, Downers Grove, IL) and the number of adherent cells determined as described previously.[7,8] In some experiments antisera were added to the incubation medium before the assay.

Assays of Endothelial Damage

Confluent monolayers were labeled overnight by the addition of 1 μCi/well 2-deoxy-[1-^{3}H]-glucose-6-phosphate.[18] Eighteen to twenty-four hours later, the media were removed and the endothelial cell monolayers were washed four times with 1 ml of Roswell Park Memorial Institute (RPMI) medium 1640 (GIBCO, Burlington, Ontario, Canada) plus 0.5 mg/ml bovine serum albumin (BSA). One-millimolar buthionine sulfoximine was added

to the cells the night before the assay and then included at this same concentration in all washes and in the assay medium. The assay for endothelial cell damage was initiated by the addition of 5×10^{-6} W256 cells/ml, in a total volume of 0.5 ml RPMI plus 0.5 mg/ml BSA. In some experiments, the tumor cells were then stimulated by adding 1×10^{-6} mol/l PMA. After 90 minutes' incubation at 37°C in a humidified environment containing 5% CO_2, the medium was removed from each well. The endothelial cell monolayer was washed once with 0.5 ml RPMI medium plus 0.5 mg/ml BSA, and this was added to the medium. Any cells present in these supernatant fluids were removed by centrifugation for 3 minutes at 200*g*. The discs on which the endothelial monolayers had been grown then were removed from the culture dishes, and placed into 0.2 ml of Beckman tissue solubilizer. The radioactivity retained in the monolayer then was determined along with that in the medium using a Beckman LS 1801 scintillation counter. The release of the ^{3}H-2-deoxyglucose into the medium was calculated as a percentage of the total radioactivity in each well. In controls, considerable variation was found in the release of ^{3}H-2-deoxyglucose between different endothelial cell preparations. Therefore the percentage of specific release was determined by the formula:

$$(A - B)/B \times 100$$

where A equals the percentage release found under experimental conditions and B equals the percentage release found in controls, ie, medium without tumor cells. Percentage release was calculated as a mean of four to six replicates. Percentage specific release is given as a mean plus or minus standard error.[14]

Immunofluorescence Flow Cytometry

Nonadherent W256 cells were isolated by centrifugation, washed twice at 4°C in 1 × HBSS with 10 mmol/l HEPES buffer pH 7.3, 0.001% NaN_3, 2% BSA, and 2% goat serum (binding buffer). The cells then were resuspended in the latter to a cell concentration of 10^6 cells/ml. In some experiments, W256 cells were first stimulated with 10^{-6} mol/l PMA for 90 minutes before being isolated. Endothelial cell monolayers were washed twice at 4°C in 1 × Hanks' buffer with 0.1% ethylenediaminetetra-acetic acid, 0.001% NaN_3, and then incubated in the same for 15 minutes at 4°C. Detached cells then were washed twice at 4°C in l × HBSS with 2 mmol/l (millimolar) $CaCl_2$, 2 mmol/l $MnCl_2$, 0.001% NaN_3, and resuspended in binding buffer at a cell concentration of 1×10^6 cells/ml. In some experiments, endothelial cell monolayers were pretreated with 10 ng/ml rIL-1α for 4 hours before being isolated. Cell suspensions then were incubated with rabbit anti-human VnR antiserum at a titer of 1:200 as well as 5 μl of normal rabbit serum (1:200) at 4°C for 30 minutes with vigorous shaking. They were washed three times and resuspended in 1 ml of binding buffer at a concentration of 10^6 cells/ml. Fluorescein isothiocyanate–goat anti-rabbit gamma G immunoglobulin (IgG) then was added at a concentration of 0.5μg/ml. After a 30-minute incubation at 4°C, the cells were washed and resuspended in 1 ml of binding buffer. The samples were either analyzed immediately or fixed with 1% paraformaldehyde and analyzed within 4 days.[19]

Immunoblotting

Endothelial cells were detached from their culture dishes with 5 mmol/l ethylene glycol tetra-acetic acid in HBSS while Walker 256 cells were isolated by centrifugation. The cells were solubilized with 1% Nonidet P-40, 2 mmol/l phenylmethyl sulfonyl-flouride, and 0.1% soybean trypsin inhibitor in phosphate-buffered saline, pH 7.4. After centrifugation, the supernatants (100 μg of protein/lane) were electrophoresed on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose filters.[20,21] The filters then were incubated for 2 hours in 3% BSA, and 0.05% Tween-20 in TRIS-buffered saline pH 7.4 (TBST) and then incubated for another 2 hours with rabbit anti-human VnR antiserum (titer 1:2000) in 0.05% BSA and TBST. They then were washed with TBST and incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (titer 1:3000) and 0.5% BSA and TBST for 2 hours. Finally the filters were washed with TBST and reacted with 5 bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium chloride in 0.1 mol/l TRIS HCl, pH 9.5, to detect alkaline phosphatase activity.[22]

Antibody-binding Studies

Endothelial cell monolayers were grown to confluency in 96-well tissue culture plates. The monolayers then were incubated with 10 ng/ml rIL-1α or an equal volume of medium for the various periods indicated later. The monolayers then were washed twice with 100 μl of binding buffer at 4°C and incubated for 30 minutes, at 4°C, with 100 μl of binding buffer containing either rabbit anti-VnR polyclonal antiserum at a titer of 1:8000 or normal rabbit serum at an equivalent titre. After this incubation, the monolayers were washed twice with binding buffer and incubated another 30 minutes with ^{125}I-goat anti-rabbit $F(ab)_2$ fragments (1 mCi/ml). The monolayers then were washed three times with binding buffer, solubilized with 50 μL of 2% SDS in HBSS, and counted on a gamma-counter.

Results

Effect of PMA and rIL-1α on Tumor Cell Adhesion and ^{3}H-2-deoxyglucose Release

The adhesion of W256 cells to endothelial cell monolayers was dependent on tumor cell stimulation and on cytokine pretreatment of the endothelial cell monolayers (Table 1). The adhesion of unstimulated W256 cells was not significantly increased after pretreating the endothelium with 10 ng/ml of rIL-1α. When W256 cells were stimulated by the addition of 10^{-6} mol/l PMA, tumor cell adhesion to the unstimulated endothelial cell monolayers was increased from control values by 89% ± 35%, $P < 0.05$. Maximum values for adhesion were observed when PMA-stimulated W256 cells were incubated with endothelial monolayers that had been pretreated with rIL-1α; in this condition there was an increase in the adhesion of PMA-stimulated tumor cells by an additional 80% ± 13%, above that seen after stimulation of the cancer cell alone ($P < 0.005$). Scanning electron microscopy of adherent W256 cells demonstrated attachment of these cells to the endothelium. W256 cells were not seen to be adherent to the exposed matrix at areas of endothelial cell retraction (Figure 1).

To determine if damage to the endothelium correlated with tumor cell adhesion, W256 cells were incubated with ^{3}H-2-deoxyglucose–labeled human endothelial cell monolayers. The release of ^{3}H-2-deoxyglucose also was dependent on tumor cell stimulation and cytokine pretreatment of the endothelial monolayers (Table 1). When unstimulated W256 cells were added to the assay, pretreating the endothelial cell monolayers with rIL-1α had no significant effect on cell injury. The specific release of isotope was increased from 10% ± 3% to 20% ± 3% by stimulation of the tumor cells alone. Isotope release, however, was greatest when PMA-stimulated W256 cells were incubated with rIL-1α–pretreated monolayers (Table 1); in this case, stimulation of the endothelium increased the specific release of isotope from 20% ± 3% (stimulated W256 cells only) to 29% ± 2% ($P < 0.05$). In the absence of W256 cells, neither the addition of PMA nor the pretreatment of monolayers with rIL-1α induced a significant increase in the release of isotope (data not shown). The magnitude of ^{3}H-2-deoxyglucose release correlated directly with the magnitude of tumor cell adhesion ($r = 0.98$, $P < 0.005$).

Role of Vitronectin Receptor (VnR) in W256 Cell Adhesion to Endothelium

The adhesion of A549 human lung carcinoma cells to rIL-1α–pretreated monolayers had been found to be partially mediated by the expression of VnR.[9,10] Therefore we examined the effect of anti-human VnR antiserum on W256 cell adhesion. We observed that addition of anti-human VnR antiserum to the adhesion assay also inhibited W256 cell attachment to endothelial cell monolayers. Neither normal rabbit serum (NRS) nor anti-human FnR affected W256 cell adhesion to the endothelium (Table 2). The inhibitory effects of anti-human VnR antiserum on W256 cell adhesion to the endothelium were dose dependent (Table 3). Maximum inhibition was observed in the presence of anti-human VnR antiserum at a titer of 1:3000. At this concentration of antiserum, and after a 90-minute coincubation, the adhesion of unstimulated W256 cells to untreated endothelium was inhibited by 42% ± 14% and adhesion to rIL-1α–pretreated endothelium was inhibited by 47% ± 3%. The adhesion of stimulated W256 cells to untreated endothelium was inhibited by 27% ± 3%, whereas adhesion to rIL-1α–pretreated monolayers was inhibited by 39% ± 2% (Table 2).

Effect of Anti-human Vitronectin Receptor (VnR) Antisera on the Release of ^{3}H-2-deoxyglucose

Because W256 cell-dependent ^{3}H-2-deoxyglucose release had correlated directly with tumor cell adhesion, we examined the effect of anti-human VnR antiserum on the

Table 1. *The Effect of PMA and IL-1 on W256 Cell Adhesion and ^{3}H-2-Deoxyglucose Release*

Culture condition	Adherent W256 cells ($\times 10^4$ cells)*	Endothelial injury (% specific release)*
Medium only	—	0.0 ± 2.1
W256 cells only	1.48 ± 0.16	10.0 ± 2.7
IL-1–treated endothelium	1.76 ± 0.15	12.2 ± 3.3
PMA-treated W256 cells	2.79 ± 0.52†	20.1 ± 2.7
IL-1 + PMA treatment	5.03 ± 0.39‡	28.6 ± 2.4§

* Data are expressed as mean ± SEM of four independent experiments.
† $P < 0.05$ when compared with adhesion of unstimulated W256 cells to untreated monolayers.
‡ $P < 0.005$ when compared with adhesion of PMA-stimulated W256 cells to untreated monolayers.
§ $P < 0.05$ when compared with isotope release promoted by PMA-stimulated W256 cells from untreated monolayers.

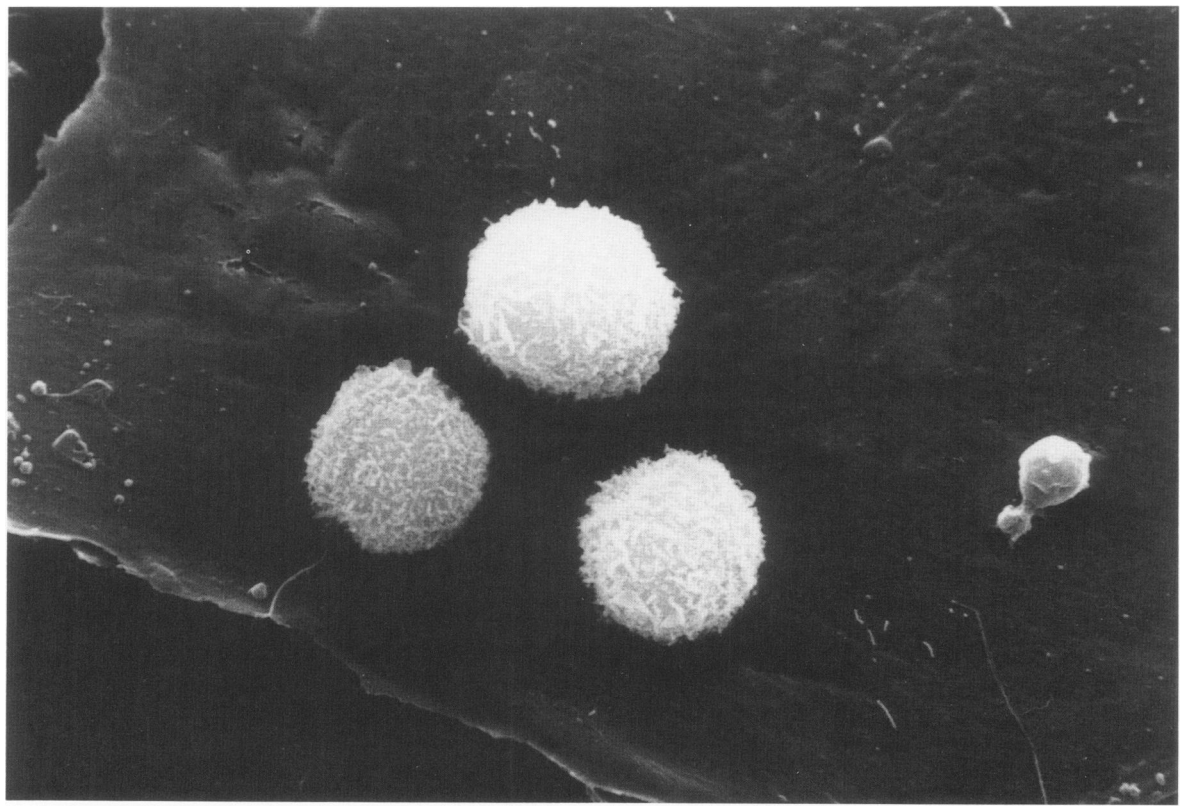

Figure 1. *Scanning electron micrograph of PMA-stimulated W256 cell adherent to rIL-1α—pretreated endothelium. W256 cells were pretreated with 10^{-6} mol/l PMA, washed, and incubated with endothelial cell monolayers for 90 minutes. The discs on which the endothelial cell monolayers were grown were then removed, washed to remove nonadherent W256 cells, and fixed in 2% glutaraldehyde and 0.1 mol/l cacodylate buffer in preparation for electron microscopy (×2500).*

release of isotope from prelabeled monolayers. In these experiments, a 1:3000 dilution of antiserum completely inhibited the isotope release caused by unstimulated W256 cells, whether or not the endothelium had been preincubated with rIL-1α. The release of isotope from unstimulated endothelium, caused by PMA-treated W256 cells, was inhibited by 66% ± 22% and the (maximal) damage, seen when PMA-stimulated W256 cells were incubated with rIL-1α—pretreated endothelial cells, was inhibited by 53% ± 13%. W256 tumor cell–promoted damage was not affected by the addition of either NRS or anti-human FnR antiserum at equivalent titers (Table 4).

Modulation of Endothelial Vitronectin Receptor Expression by rIL-1α

Immunofluorescence flow cytometry demonstrated that only the endothelial cells and not the W256 cells expressed detectable VnR on their cell surface (Figure 2).

Table 2. *The Effect of Anti-vitronectin Receptor Antiserum on W256 Tumor Cell Adhesion*

	Adherent W256 Carcinosarcoma Cells (×10^4)			
	W256 cells only	rIL-1–α–treated endothelium	PMA-treated W256 cells	rIL-1α + PMA treatment
Experiment 1				
Control	1.72 ± 0.12	1.96 ± 0.15	2.15 ± 0.28	5.12 ± 0.29
Anti-VnR (1:3000)	0.99 ± 0.24*	1.03 ± 0.07*	1.57 ± 0.17*	3.14 ± 0.11*
Experiment 2				
Control	0.67 ± 0.11	1.53 ± 0.34	4.22 ± 0.36	5.77 ± 1.28
NRS (1:3000)	0.98 ± 0.20	1.44 ± 0.32	4.23 ± 0.78	6.08 ± 1.45
Experiment 3				
Control	1.10 ± 0.15	1.23 ± 0.22	3.15 ± 0.59	3.87 ± 0.66
Anti-FnR (1:3000)	0.84 ± 0.03	1.08 ± 0.09	3.04 ± 0.47	3.98 ± 0.29

* $P < 0.05$ when compared with tumor cell adhesion from corresponding positive controls.

Table 3. *The Effect of Anti-vitronectin Receptor Antiserum Titre on W256 Tumor Cell Adhesion*

Condition	Titre of anti-VnR	Adherent W256 Cells ($\times 10^4$) Duration of Adhesion 45 minutes	90 minutes
W256 cells only	Control	0.09 ± 0.01	1.72 ± 0.12
	1:5000	0.08 ± 0.01	1.43 ± 0.07
	1:4000	0.10 ± 0.01	—
	1:3000	0.08 ± 0.01	0.99 ± 0.24*
IL-1–treated endothelium	Control	0.18 ± 0.02	1.96 ± 0.15
	1:5000	0.16 ± 0.04	1.58 ± 0.15
	1:4000	0.17 ± 0.02	—
	1:3000	0.14 ± 0.02	1.03 ± 0.07*
PMA-treated W256 cells	Control	0.54 ± 0.06	2.15 ± 0.28
	1:5000	0.48 ± 0.05	2.06 ± 0.07
	1:4000	0.38 ± 0.04*	—
	1:3000	0.26 ± 0.04*	1.57 ± 0.17*
IL-1 + PMA treatment	Control	2.04 ± 0.10	5.12 ± 0.29
	1:5000	1.70 ± 0.19	4.49 ± 0.69
	1:4000	1.45 ± 0.04*	—
	1:3000	1.20 ± 0.23*	3.14 ± 0.11*

* $P < 0.05$ when compared with tumor cell adhesion from corresponding positive controls.

W256 cells still failed to express VnR on their cell surface after a 90-minute incubation with 10^{-6} mol/l PMA (data not shown). However rIL-1α pretreatment of the endothelial cells for 4 hours was found to increase VnR expression 1.30 ± 0.03-fold (mean of three experiments).

Immunoblot analysis confirmed that only the endothelial cells and not the W256 cells contained immunoreactive material to VnR antiserum (molecular weight 160, 95 kd; Figure 3). Neither unstimulated (lane 3) nor PMA-stimulated W256 cells (lane 4) expressed immunoreactive material. Densitometric analysis of the immunoblots indicated that rIL-1α pretreatment of the endothelium had increased VnR expression by 1.8 ± 0.2-fold (lane 1 *versus* 2).

Antibody-binding studies showed that the expression of VnR increased in a time-dependent manner after incubation of the endothelium with 10 ng/ml rIL-1α (Figure 4). At 4 hours, the expression of VnR had increased 1.9 ± 0.1-fold above control values. Maximum expression of VnR was observed after an 8-hour incubation of the endothelial cell monolayers with 10 ng/ml rIL-1α, at which time VnR expression had increased 2.6 ± 0.2-fold.

Discussion

There is growing evidence that adhesive interactions between the endothelium and cancer cells can regulate the metastatic process. In addition to those adhesion molecules that are constitutive in expression,[25] other molecules appear to be variably expressed, depending on the endothelial activation state. Examples of these include ELAM-1 and InCAM-110.[5] Recent evidence has suggested that VnR, an Arg-Gly-Asp-dependent adhesion receptor,[23] also may mediate the attachment of some tumor cells to stimulated endothelial cells. Specifically, work in this laboratory indicates that the adhesion of A549 human lung carcinoma cells and M6 human melanoma cells to endothelium is enhanced after 4 hours' incubation of the endothelium with rIL-1α. This adhesion

Table 4. *The Effect of Anti-vitronectin Receptor Antiserum on W256 Cell-promoted ^{3}H-2-Deoxyglucose Release*

Culture condition	% Specific Release No antiserum	Normal rabbit serum	Fibronectin receptor antiserum	Vitronectin receptor antiserum
Medium only	0.0 ± 5.3	0.0 ± 2.1	0.0 ± 2.5	0.0 ± 1.9
W256 cells only	9.4 ± 2.6	11.2 ± 5.3*	10.8 ± 6.6*	0.0 ± 1.8†
IL-1–treated endothelium	7.9 ± 3.5	2.6 ± 4.0*	11.3 ± 5.3*	0.0 ± 2.7†
PMA-treated W256 cells	19.6 ± 3.5	17.2 ± 3.8*	18.9 ± 5.4*	6.7 ± 4.4†
IL-1 + PMA treatment	27.3 ± 3.2	20.9 ± 5.1*	22.3 ± 5.3*	12.9 ± 3.5†

* $P > 0.1$ when compared with release of isotope from corresponding positive controls.
† $P < 0.05$ when compared with release of isotope from corresponding positive controls.

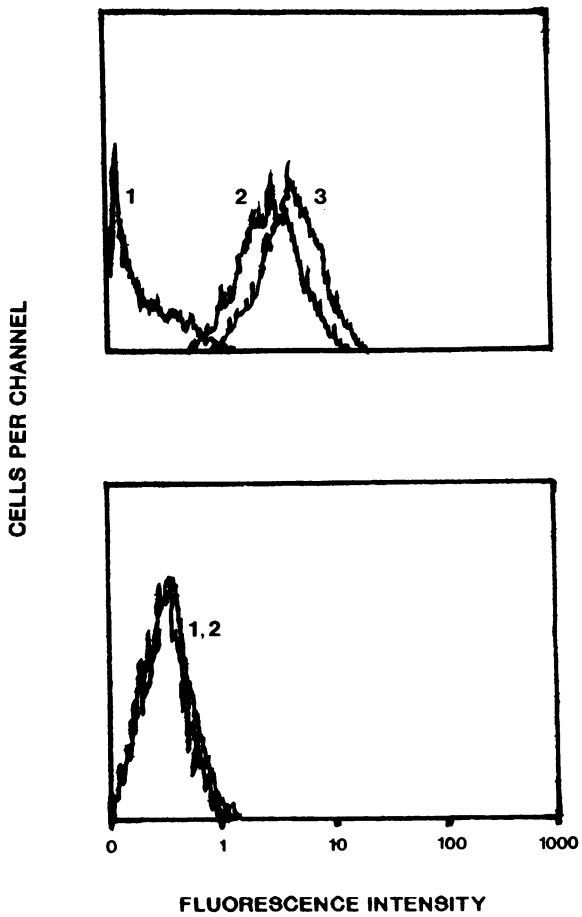

Figure 2. *Increased vitronectin receptor expression on rIL-1α–pretreated endothelial cells as measured by immunofluorescence flow cytometry. In the upper panel, endothelial cells were grown to confluency in 75-cm² flasks. Some monolayers were pretreated with 10 ng/ml rIL-1α for 4 hours before harvesting and processing the cells for immunofluorescence flow cytometry. In the lower panel, Walker 256 cells were maintained in BGJ medium with 10% fetal calf serum and 1% antibiotics for 2 or 3 days before the experiment. Some W256 cells were stimulated with 1 × 10⁻⁶ mol/l PMA for 90 minutes before harvesting and processing the cells for immunofluorescence flow cytometry.* **Upper panel:** *peak 1, untreated endothelial cells with normal rabbit serum; peak 2, untreated cells with anti-VnR antiserum; peak 3, rIL-1α–pretreated cells with anti-VnR antiserum.* **Lower panel:** *peak 1, untreated W256 cells with normal rabbit serum; peak 2, untreated cells with anti-VnR antiserum.*

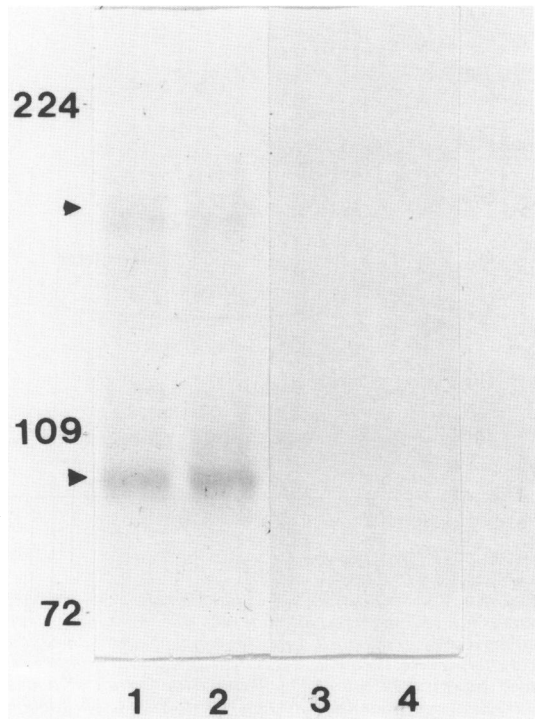

Figure 3. *Immunoblot of VnR extracted from human endothelial cells and W256 cells. VnR was extracted from* **(A)** *human endothelial cells and* **(B)** *Walker 256 carcinosarcoma cells by the addition of 1% Nonidet P-40. The solubilized extracts were electrophoresed (100 μg/lane) on 7.5% SDS-PAGE and transfered to nitrocellulose. The blots were blocked by incubation with 3% BSA in TBST and then incubated with rabbit anti-VnR polyclonal antiserum in TBST containing 0.5% BSA. The blots then were incubated with goat anti-rabbit IgG alkaline phosphatase conjugate and reacted with BCIP/NBT in 0.1 mol/l TRIS-HCl, pH 9.5. Lane 1, endothelial cells; lane 2, rIL-1–pretreated endothelial cells; lane 3, W256 cells; lane 4, PMA-stimulated W256 cells.*

is inhibited by the addition of GRGDS peptide,[8] anti-human VnR polyclonal antiserum, and by monoclonal antibody (MAb) LM609.[9,10] The latter MAb specifically recognizes the 135/115-kd receptor ($\alpha_v\beta_3$) on the surface of human umbilical vein endothelial cells, which mediates endothelial cell attachment to vWF, fibrinogen, and vitronectin.[23]

In the present study, rIL-1α pretreatment of endothelial cell monolayers was shown to increase the adhesion of PMA-stimulated rat W256 cells and to increase the expression of VnR on the endothelial cell surface. This increase in tumor cell adhesion was shown to be at least partially dependent on increased VnR expression, as W256 cell adhesion could be blocked by anti-human VnR polyclonal antiserum. Evidence that the tumor cells were adherent to the endothelium and not to underlying extracellular matrix included 1) scanning electron micrographs demonstrating attachment of the W256 cells to the endothelium but not to the basement membrane (Figure 1); 2) analysis of VnR expression by flow cytometry and immunoblotting demonstrated VnR on the endothelial cells only (and not the W256 cells). Anti-VnR antiserum blocked W256 cell adhesion in the endothelial cultures. Because VnR is an integral membrane protein and not a basement membrane constituent,[24] we concluded that tumor cell attachment was to the endothelium and not to the underlying basement membrane.

We have not determined why rIL-1α pretreatment of the endothelium increased the adhesion of PMA-stimulated W256 cells without increasing the adhesion of unstimulated W256 cells. In addition to inducing oxygen radical release,[14,16] PMA stimulation of W256 cells has been shown to increase W256 cell adhesion to noncellular foreign surfaces, such as plastic culture dishes and nylon fibers, as well as endothelial cell monolayers.[26] This

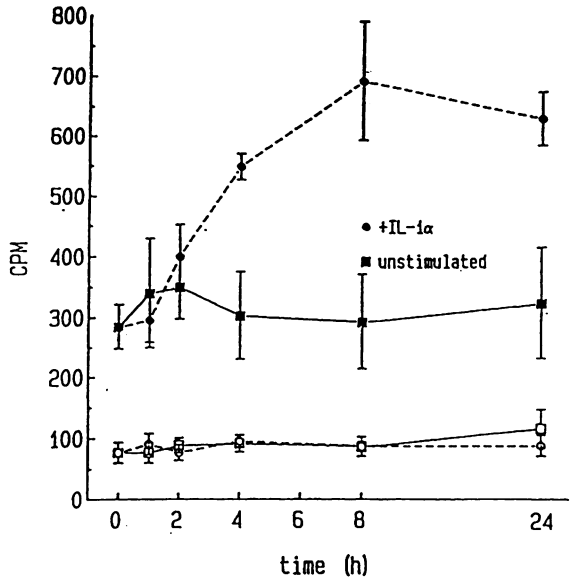

Figure 4. *Time course for rIL-1α induction of vitronectin receptor expression on human endothelial cells. The monolayers were incubated with 10 ng/ml rIL-1α or an equal volume of medium for various periods. Then they were incubated for 30 minutes at 4°C with either rabbit anti-VnR polyclonal antiserum at a titer of 1:8000 (closed symbols) or normal rabbit serum (open symbols) at an equivalent titer. After this incubation, the monolayers were incubated a further 30 minutes with ^{125}I-goat anti-rabbit F(ab)$_2$ fragments (1 mCi/ml) as described in Materials and Methods.*

response has been shown to be an energy-dependent process involving cytoskeletal rearrangements, but is independent of protein synthesis. It would be attractive to speculate that W256 cells express the ligand for vitronectin receptor binding on PMA stimulation.

In vivo, drug-, neutrophil-, or x-ray–induced endothelial injury are known to promote tumor cell metastasis.[11,12,27] We have previously demonstrated that such damage can be caused by the release of oxygen radicals from stimulated W256 cells.[14] These oxygen metabolites, although highly reactive, are very short lived and susceptible to the action of free radical scavengers. In the present study we have demonstrated that cancer cell-mediated endothelial damage is promoted by adhesive interactions between the two cell types. We suggest that the expression of adhesive moieties on the cell surface of the endothelium may promote the metastasis of circulating cancer cells by facilitating cancer cell-mediated endothelial damage, possibly by creating a decreased diffusion distance between the W256 cell and the endothelial cell, preventing the action of free radical scavengers.

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