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The Relative Roles of Vitronectin Receptor, E-selectin and $\alpha_4\beta_1$ in Cancer Cell Adhesion to Interleukin-1-treated Endothelial Cells

R.M. Lafrenie, S. Gallo, T.J. Podor, M.R. Buchanan and F.W. Orr

Adhesion of cancer cells to endothelium is thought to be a prerequisite to extravasation during the haematogenous phase of metastasis, and is enhanced after perturbation of the endothelium by interleukin-1 (IL-1). The inducible endothelial adhesion molecules, E-selectin, VCAM-1/$\alpha_4\beta_1$ and vitronectin receptor have been reported to mediate attachment of cancer cells to IL-1-treated endothelial cells. We have examined the relative contribution of these molecules by quantifying the adhesion of a panel of 22 human, ^{125}I-labelled cancer cells and the rat W256 tumour to untreated and IL-1-treated endothelial monolayers in the presence of relevant neutralising antibodies. Antibodies against E-selectin inhibited the adhesion of HL-60 leukaemia cells and two colon carcinomas. Anti-$\alpha_4\beta_1$ antibodies blocked adhesion of four melanomas, five sarcomas and one lung carcinoma. Anti-vitronectin receptor antibodies inhibited adhesion of 14 of the 22 human cell lines to IL-1-treated endothelial cells. Adhesion of seven cell lines was inhibited by more than a single antibody. In contrast, adhesion of one of the cancer cell lines was unaffected by any of the antibodies, suggesting involvement of other IL-1-inducible endothelial adhesion molecules. Moreover, none of the antibodies altered the attachment of cancer cells to unstimulated endothelial monolayers. We conclude that the mechanisms of cancer cell adhesion to the endothelium are influenced by endothelial activation and by the adhesive repertoire of the cancer cell.

Key words: endothelium, metastases
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INTRODUCTION

CANCER METASTASIS is a complex, multistep process involving properties of both the cancer cells and host tissues. Interactions between cancer cells and the microvasculature are particularly important since the bloodstream is a common pathway for the dissemination of cancer cells to distant sites [1]. Adhesion of intravascular cancer cells to the endothelium appears to precede their escape from the circulation [2]. Moreover, the endothelium has a regulatory role since, *in vivo*, experimental metastasis is enhanced by agents which perturb the endothelium, including the treatment of animals with purified cytokines prior to cancer cell injection [3–6]. *In vitro*, treatment of the endothelium with cytokines increases cancer cell adhesion, which has been correlated with increased expression or activation of surface adhesion molecules including E-selectin, VCAM-1 and vitronectin receptor [7–10]. E-selectin (ELAM-1) is not normally expressed by endothelial cells, but is induced by treatment of the endothelium, for 2–8 h, with interleukin-1 (IL-1)[3], tumour necrosis factor or bacterial endotoxin, when it can interact with carbohydrate ligands expressed on lymphocytes and some leukaemia and colon carcinoma cell lines, thereby mediating cell–cell adhesion [9,11]. Cytokine treatment for 4–48 h enhances VCAM-1 (INCAM-110) expression which can mediate adhesion of some lymphocytes, melanoma cells and sarcoma cell lines [7, 12–14]. VCAM-1-dependent adhesion is mediated through $\alpha_4\beta_1$ expressed on the lymphocyte or cancer cell, and melanoma cell adhesion is proportional to the amount of $\alpha_4\beta_1$ expressed by the cancer cell [12]. It has also been demonstrated that cells which express more $\alpha_4\beta_1$ are less invasive in matrigel [15], suggesting that the role adhesion molecules play during metastasis is complex, and involves more than cell–cell adhesion. Finally, IL-1 or the eicosanoid, 12(S) hydroxyeicosatetranenoic acid, can induce endothelial vitronectin receptor ($\alpha_v\beta_3$) expression which can mediate adhesion of human A549 lung carcinoma cells, W256 rat tumour cells and the murine B16a melanoma [10, 16, 17].

In this study, we examined the relative contribution of these three adhesion molecules to cancer cell/endothelial adhesion using a panel of diverse cancer cell lines, many of which have been derived from metastatic lesions and are commonly employed in studies of cancer cell biology. The results of these experiments confirm that cancer cell adhesion is influenced by endothelial activation, and demonstrate that endothelial molecules may act in synergy to effect adhesion, which is also dependent upon the adhesion molecule repertoire of the cancer cells. The results also suggest that other IL-1-inducible molecules, distinct from the ones studied, can be involved in adhesive interactions between cancer cells and the endothelium.

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MATERIALS AND METHODS

Materials

All cell culture media were obtained from Gibco (Burlington, Ontario, Canada) except for Ham's F-12 media which was obtained from BioFluids (Rockville, Maryland, U.S.A.). Pooled human sera (type AB) were supplied by the Canadian Red Cross (Hamilton, Ontario, Canada). The rabbit anti-human vitronectin receptor antiserum, anti-human fibronectin receptor antiserum and anti-α_4 (clone P4G9), anti-α_5 (clone P1D6) and anti-α_v (clone VNR147) monoclonal antibodies were obtained from Telios Biochemicals (San Diego, California, U.S.A). The anti-vitronectin receptor monoclonal antibodies, LM142 and LM609, were gifts from Dr D.A. Cheresh (Scripps Clinic, LaJolla, California, U.S.A). The anti-E-selectin monoclonal antibody (clone BBA2) was purchased from British Biotechnology (Oxford, U.K.). The anti-$\alpha_4\beta_1$ monoclonal antibody (clone HP2/1) was obtained from Bio/Can Scientific (Mississaga, Ontario, Canada). Normal rabbit serum and normal mouse ascites were obtained from Cedarlane Laboratories (Hornby, Ontario, Canada). Recombinant human interleukin-1α (IL-1) was a gift from Dr P. Lomedico (Hoffmann-LaRoche, Nutley, New Jersey, U.S.A.). [5-^{125}I]iododeoxyuridine (2200 Ci/mmol) was obtained from Dupont/New England Nuclear (Mississaga, Ontario, Canada). Translabel ([^{35}S]methionine and [^{35}S]cysteine, 1163 Ci/mmol) was obtained from Flow/ICN Biomedicals (Mississaga, Ontario, Canada). Goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate was purchased from Canadian Life Technologies (Burlington, Ontario, Canada). Goat anti-mouse IgG-Sepharose was obtained from Dimension Laboratories (Mississaga, Ontario, Canada).

Cell culture

Endothelial cells were obtained by collagenase treatment of human umbilical cord veins, plated on to T25 plastic culture flasks and grown in medium M199 supplemented with 20% pooled human serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 1% L-glutamine, 100 μg/ml pituitary-derived endothelial cell growth factor and 25 mM HEPES, pH 7.3 [10]. When confluent, the cells were harvested by trypsinisation, plated (1:3 dilution) on to 177 mm^2 fibronectin-coated plastic discs (Thermanox^{TM}) in 24-well Costar culture dishes and grown to confluent monolayers.

Cancer cell monolayers were cultured on 150-mm dishes in the recommended media containing 10% fetal calf serum, 1% L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (Table 1). HL-60 cells were grown in suspension cultures in RPMI 1640 supplemented with 10 mM HEPES, pH 7.3, 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin. W256 cells were maintained as an ascites tumour in adult, male Sprague–Dawley rats. The cancer cell ascites were harvested in Hanks' balanced saline solution (HBSS), pH 7.4, containing 5 U/ml heparin and erythrocytes were removed by three to four cycles of osmotic lysis as described [16].

Cancer cell [^{125}I]deoxyuridine labelling and adhesion assay

Cancer cell labelling and the cancer cell–endothelial cell adhesion assay were conducted as previously described [10, 16]. Subconfluent monolayer cell cultures were labelled for 24–48 h with 0.5 μCi/ml [^{125}I]deoxyuridine. HL-60 cells or W256 rat cancer cells grow as suspension cultures. For labelling, they were suspended at a concentration of 1×10^5 cells/ml and incubated with 0.5 μCi/ml [^{125}I]deoxyuridine in culture media for 24 h. Labelled cancer cell monolayers were harvested from the culture dish by incubation with 5 mM EGTA in HBSS, pH 7.4, and collected by scraping with a rubber policeman. The cells were washed with M199 and resuspended as single cells in M199 supplemented with 20% human serum at a concentration of 1×10^5 cells/ml.

Endothelial cell monolayers were incubated with 10 ng/ml IL-1 or suspending medium at 37°C for 4–6 h. The media were replaced with 0.5 ml of fresh supplemented culture medium and then 0.5 ml of the cancer cell suspension was added to each well. The cancer cells were incubated with the monolayer for 30 min at 37°C. Non-adherent cells were aspirated and the monolayers were washed three times with unsupplemented media. In some experiments, the antibodies were added to the medium and maintained for the duration of the assay. The effective concentrations of the various antibody solutions were determined by calculating the concentration required to give maximal inhibition of adhesion for at least one cancer cell line. Thus, the anti-E-selectin antibody was used at a concentration of 50 μg/ml and the anti-$\alpha_4\beta_1$ antibody and normal mouse ascites were added at a concentration of 5 μg/ml. The anti-vitronectin receptor antisera, anti-fibronectin receptor antisera and normal rabbit

Table 1. Cancer cell lines used in these experiments

Cell line	Source
Lung carcinomas	
A549 lung adenocarcinoma	J. Fogh*
L-9 lung carcinoma	W.A. Nelson-Rees†
Calu-1 lung carcinoma	J. Fogh
Calu-6 lung carcinoma	J. Fogh
Breast carcinomas	
MCF-7 breast carcinoma	R. Eisenberg‡
BT-20 breast carcinoma	ATCC§
Colon carcinomas	
HT-29 colon adenocarcinoma	ATCC
Colo-16 colon carcinoma	S.K. Liao‖
Oral carcinoma	
KB oral carcinoma	ATCC
Prostate carcinoma	
PC-3 prostate carcinoma	ATCC
Bladder carcinoma	
HT1376 bladder carcinoma	W.A. Nelson-Rees
Melanomas	
M6 malignant melanoma	S.K. Liao
M19 malignant melanoma	S.K. Liao
M22 malignant melanoma	S.K. Liao
M23 malignant melanoma	S.K. Liao
Glioblastoma	
LN18 glioblastoma	de Tribolet¶
LN140 glioblastoma	de Tribolet
Neuroblastoma	
SK-N-MC neuroblastoma	J. Fogh
SK-N-SH neuroblastoma	J. Fogh
Osteosarcoma	
SAOS-2 osteosarcoma	ATCC
U2OS osteosarcoma	ATCC
Leukaemia	
HL-60 leukaemia	F. Graham**
Other	
W256 rat cancer	F.W. Orr

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serum were added at a titre of 1:3000 [10]. The radioactivity associated with the monolayers was determined in a Packard gamma-counter.

Indirect immunofluoresence flow cytometry

Untreated or IL-1-treated endothelial cell monolayers were harvested with 0.1% EDTA, 0.2 M urea and 0.02% NaN_3 in HBSS, pH 7.4. The cells were washed with 2 mM $CaCl_2$, $MgCl_2$ in HBSS, pH 7.4, and resuspended in binding buffer (HBSS containing 2% goat serum, 2% bovine serum albumin, 10 mM HEPES, pH 7.3, and 0.02% NaN_3) at a concentration of 10^6 cells/ml. The α_4, α_5, $\alpha_v\beta_3$ (LM609) or E-selectin antibody solutions were incubated with each cell suspension for 30 min at 4°C with constant mixing. The cells were then washed three times with binding buffer and resuspended in a volume of 1.0 ml. Twenty-five microlitres of goat anti-mouse IgG-FITC conjugate were added and then incubated for 30 min at 4°C. The cells were washed, resuspended in 1.0 ml binding buffer and fixed with 1% paraformaldehyde. The fluorescent profiles of the cell suspension were determined on an EPICS II profile analyser (Coulter Corporation) over a log scale of fluorescence intensity. The fluorochrome was excited with 15 mW of 488 nm light using an argon laser and light emission was detected on a photomultiplier tube using a bandpass filter (530/30).

[^{35}S]methionine labelling and immunoprecipitation

Metabolic labelling and immunoprecipitation analysis were carried out as described [10]. Endothelial cells were incubated with 100 μCi/ml [^{35}S]methionine in methionine-deficient MEM, containing 5% human serum, 100 μg/ml pituitary-derived endothelial cell growth hormone and 1% L-glutamine for 6 h in the presence or absence of 10 ng/ml IL-1. Cancer cells were labelled for 4 h with 100 μCi/ml [^{35}S]methionine in 10% dialysed fetal calf serum, 1% L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. The labelled cells were harvested by incubation in 5 mM EGTA in HBSS, pH 7.4, and then lysed in 200 mM octylglucoside, 0.1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 3 mM phenylmethylsulphonyl fluoride. An aliquot of the labelled cell suspension (normalised for cpm) was precleared by incubation with goat anti-mouse IgG-Sepharose and then co-incubated with the appropriate monoclonal antibody for 16 h at 4°C. Immune complexes were isolated by co-incubation with goat anti-mouse IgG-Sepharose for 2 h at 4°C. The beads were washed once with Tris-buffered saline, pH 7.4, containing 1 mg/ml bovine serum albumin and 0.1% Triton X-100, once with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml bovine serum albumin and 0.1% Triton X-100 and finally once with 150 mM NaCl, 25 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The washed beads were boiled in Laemmeli's sample buffer and the eluate was electrophoresed on 7.5% polyacrylamide gels containing sodium dodecyl sulphate. Finally, the gels were prepared for autoradiography and exposed to X-OMAT X-ray film. The intensity of the exposed bands was determined by densitometry.

Statistical analysis

All experiments were performed at least three times. Quantitative data in this paper represent the mean and standard error or mean and standard deviation, as indicated, of data from these experiments. Observations were analysed by Student's unpaired *t*-test. *P* values <0.05 were considered significant.

RESULTS

Effects of IL-1 on endothelial adhesion

The number of cells which attached to an untreated 1.77-cm^2 endothelial monolayer in a 30-min adhesion assay varied from approximately 850 to 2500 depending upon the cell line being examined. Treatment of the endothelial cell monolayers with 10 ng/ml IL-1 for 4–6 h before the adhesion assay caused a 1.2–4.8-fold increase in the subsequent attachment of 22/23 of the cancer cell lines (Table 2). Only adhesion of the PC-3

Table 2. The effects of antibodies on cancer cell adhesion to interleukin-1-treated endothelial cells

	Per cent inhibition of adhesion†				
	IL-1	Antibodies added			
Cancer cell	induction*	α-VnR	α-α_4	α-E-sel	α-FnR
Lung carcinomas					
A549	2.5 ± 0.2‡	40 ± 10§	4 ± 12	8 ± 12	2 ± 12
L-9	1.8 ± 0.1‡	18 ± 6§	3 ± 8	ND	−6 ± 16
CaLu-1	1.3 ± 0.1‡	5 ± 8	18 ± 8§	3 ± 8	ND
CaLu-6	1.6 ± 0.1‡	26 ± 8§	8 ± 10	ND	2 ± 10
Breast carcinomas					
MCF-7	1.9 ± 0.1‡	26 ± 7§	5 ± 12	21 ± 7§	5 ± 13
BT-20	1.8 ± 0.1‡	10 ± 12	6 ± 12	17 ± 12	10 ± 8
Colon carcinomas					
HT-29	4.8 ± 0.5‡	10 ± 13	ND	48 ± 10§	4 ± 13
Colo-16	3.7 ± 0.5‡	3 ± 14	ND	22 ± 14§	1 ± 19
Oral carcinoma					
KB	1.2 ± 0.1‡	11 ± 8§	−1 ± 7	2 ± 9	ND
Prostate carcinoma					
PC-3	1.1 ± 0.1	7 ± 19	11 ± 16	18 ± 21	11 ± 16
Bladder carcinoma					
HT1376	1.3 ± 0.1‡	20 ± 9§	ND	ND	ND
Melanomas				2 ± 9	
M6	4.8 ± 0.4‡	28 ± 8§	68 ± 8§	ND	−4 ± 9
M19	2.0 ± 0.1‡	12 ± 7§	27 ± 7§	ND	5 ± 7
M22	3.5 ± 0.2‡	9 ± 8	57 ± 13§	1 ± 12	3 ± 9
M23	3.0 ± 0.2‡	32 ± 10§	43 ± 7§		10 ± 12
Glioblastomas					
LN18	1.5 ± 0.2‡	14 ± 14	20 ± 15§	7 ± 14	ND
LN140	1.4 ± 0.1‡	29 ± 10§	13 ± 13	2 ± 16	−7 ± 16
Neuroblastomas					
SK-N-MC	1.5 ± 0.1‡	7 ± 9	34 ± 14§	ND	7 ± 9
SK-N-SH	2.4 ± 0.3‡	25 ± 10§	51 ± 18§	ND	6 ± 12
Osteosarcomas					
SAOS-2	1.7 ± 0.2‡	24 ± 16§	58 ± 16§	ND	6 ± 16
U2OS	1.3 ± 0.1‡	18 ± 9§	41 ± 17§	ND	−7 ± 15
Leukaemia					
HL-60	4.6 ± 0.4‡	2 ± 14	4 ± 12	57 ± 10§	ND
Other					
W256 rat¶					
−PMA	2.1 ± 0.3‡	38 ± 13§	ND	ND	13 ± 14
+PMA	4.3 ± 0.6‡	54 ± 16§	ND	ND	8 ± 12

Experiments were performed in triplicate and each value is the mean of at least two independent experiments. * Magnitude of increase in the number of cancer cells which adhered to interleukin-1-treated endothelial monolayers compared to untreated endothelial monolayers. † Per cent inhibition ±S.D. was calculated by determining the number of cells which adhered to an interleukin-1-treated monolayer in the absence and presence of the indicated antibody solution. See Materials and Methods for experimental details. ‡ Enhancement in response to interleukin-1 treatment was significant, $P < 0.05$. § Inhibition by the antibody was significant, $P < 0.05$. ND, not determined. ¶ The adhesion of W256 cells to endothelial monolayers was measured in the absence or presence of 10^{-6} M phorbol ester. See [16] for details.

prostatic cancer cell line was not enhanced by prior IL-1 treatment of the endothelium.

Vitronectin receptor-dependent adhesion

The adhesion of 15/23 cancer cell lines to IL-1-treated endothelial cells was inhibited by 11–54% in the presence of an anti-vitronectin receptor polyclonal antiserum (Table 2). Adhesion of A549 adenocarcinoma cells, M6 melanoma cells and M19 melanoma cells was also inhibited by the anti-vitronectin receptor monoclonal antibody, LM609, but not by antibody LM142 (Figure 1). LM609 is specific for the RGD-dependent binding site of $\alpha_v\beta_3$ (and does not recognise $\alpha_{11b}\beta_3$, $\alpha_v\beta_1$ or $\alpha_v\beta_5$) while the LM142 monoclonal antibody binds to the α_v subunit and does not block RGD-dependent adhesion via $\alpha_v\beta_3$ [18]. Vitronectin receptor-dependent adhesion of cancer cells to IL-1-treated endothelial cells did not correlate with their expression of vitronectin receptor. For example, the adhesion of CaLu-1, BT-20, SK-N-MC cells, which expressed vitronectin receptor (Table 3) was not inhibited by anti-vitronectin receptor antibodies. Alternatively, the adhesion of A549 cells and W256 rat cancer cells, which do not express vitronectin receptor, was vitronectin receptor-dependent. This observation suggested that vitronectin receptor-dependent adhesion is mediated via vitronectin receptor expressed on the endothelial cells. Consistent with this possibility, treatment of the endothelial cells with 10 ng/ml IL-1 for 6 h increased the amount of vitronectin receptor on the cell surface by 1.6-fold as measured by immunofluorescence flow cytometry (Figure 2) and by 3.5 ± 0.3 fold when measured by immunoprecipitation analysis (Figure 3).

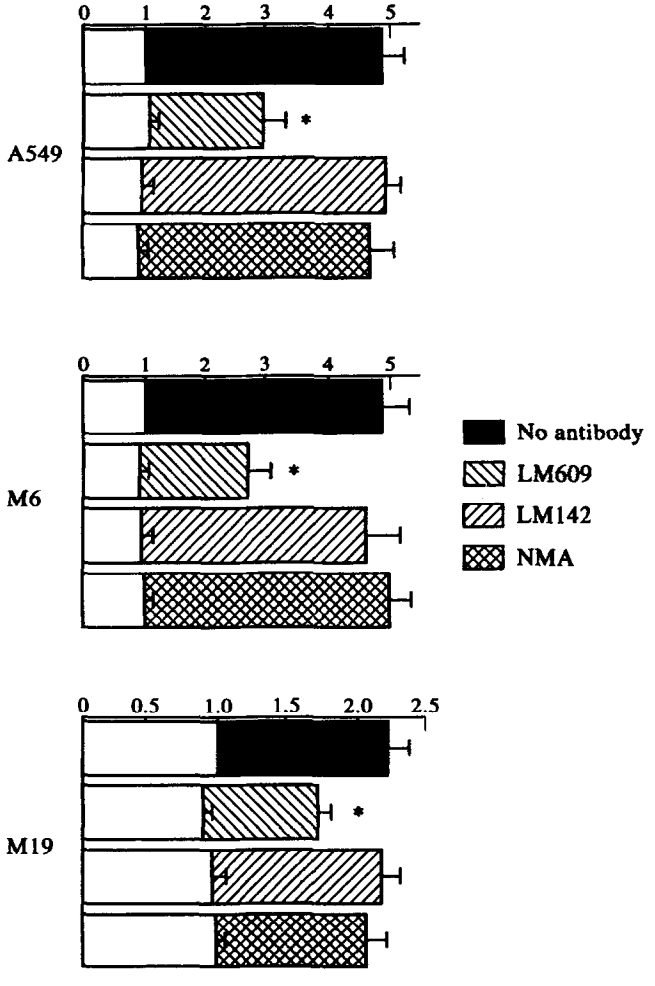

Figure 1. The effect of vitronectin receptor monoclonal antibodies on cancer cell adhesion to untreated and IL-1-treated endothelial cells. An [^{125}I]deoxyuridine-labelled cancer cell suspension (0.5 ml; 10^5 cells/ml) was added to an IL-1-treated endothelial cell monolayer in the presence of LM142 and LM609 anti-vitronectin receptor monoclonal antibodies or normal mouse ascites (NMA) solutions and incubated at 37°C. Each bar represents the relative increase in adhesion of cancer cells in response to IL-1 treatment of the endothelial cells ± standard error where 1 represents the number of cells adhered to an untreated monolayer. Statistical analysis was performed using Student's *t*-test and *P*-values < 0.05 are marked by an *.

$\alpha_4\beta_1$-dependent adhesion

Adhesion of all four melanoma cell lines, the U2OS, SA0S-2, LN18, SK-N-SH and SK-N-MC sarcoma cell lines and the CaLu-1 lung carcinoma cell line to IL-1-treated endothelial cells was partially inhibited by the anti-$\alpha_4\beta_1$ antibody, clone HP2/1 (Table 2). This monoclonal antibody blocks epitopes on $\alpha_4\beta_1$ which are required for binding to VCAM-1. Alternatively, the anti-$\alpha_4\beta_1$ antibody, clone P4G9, directed against the RGD-independent fibronectin-binding domain did not block adhesion of M6 or M22 melanoma cells to IL-1-treated endothelial cells (data not shown). These data suggest that the anti-$\alpha_4\beta_1$ antibody blocks VCAM-1-dependent adhesion. Neither of these anti-$\alpha_4\beta_1$ antibodies interacted with untreated or IL-1-treated endothelial cells in fluorescence cytometry (Figure 2) or immunoprecipitation experiments (Figure 3). However, these anti-$\alpha_4\beta_1$ antibodies did recognise material on the melanoma cells, U2OS, SA0S-2, LN18, SK-N-SH and SK-N-MC sarcoma cells and CaLu-1 carcinoma cells (Table 3).

Table 3. The expression of vitronectin receptor, fibronectin receptor and $\alpha_4\beta_1$ by cancer cells employed in this study

Cell line	Fibronectin receptor	Vitronectin receptor	$\alpha_4\beta_1$
Carcinomas			
A549 lung carcinoma	+	–	–
L-9 lung carcinoma	+	+	–
CaLu-1 lung carcinoma	+	+	+
MCF-7 breast carcinoma	+	+	–
BT-20 breast carcinoma	+	+	–
KB bladder carcinoma	+	+	–
PC-3 prostate carcinoma	+	+	–
Melanomas			
M6 malignant melanoma	+	+	+++
M19 malignant melanoma	+	–	++
M22 malignant melanoma	+	+	++
M23 malignant melanoma	+	+	++
Sarcomas			
SAOS-2 osteosarcoma	+	+	+
U20S osteosarcoma	+	+	+
SK-N-MC neuroblastoma	+	+	+
SK-N-SH neuroblastoma	+	+	+
LN18 glioblastoma	+	+	+
Leukaemia			
HL-60 leukaemia	–	–	–
Other			
W256 rat carcinosarcoma	–	–	ND

The presence of visually detectable immunoreactive material for fibronectin receptor at 150 and 120 kDa, for vitronectin receptor at 160 and 90 kDa and for $\alpha_4\beta_1$ at 150 and 120 kDa is indicated by +. The absence of reaction is indicated by –.

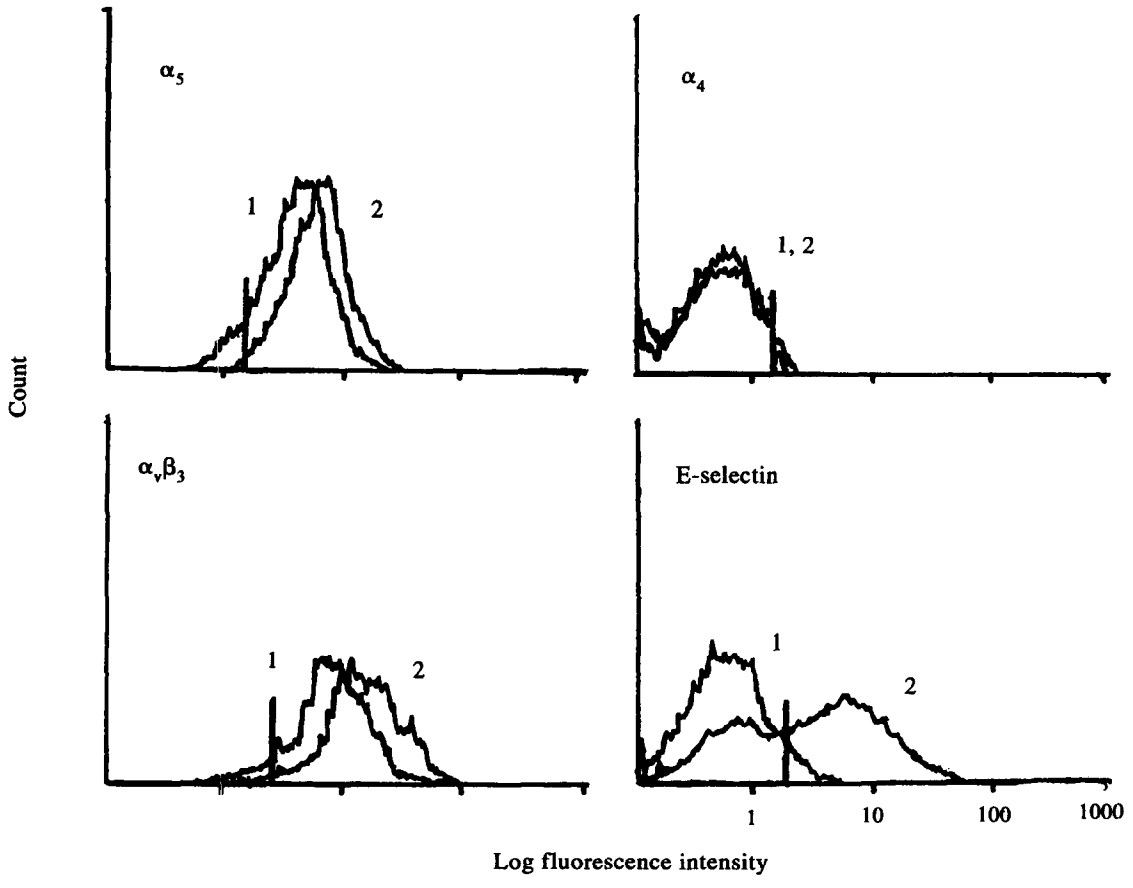

Figure 2. Indirect immunofluorescence flow cytometry of untreated or interleukin-1-pretreated endothelial cells. The cells were incubated with anti-fibronectin receptor monoclonal antibody (α_5), anti-vitronectin receptor monoclonal antibody LM609 ($\alpha_v\beta_3$), anti-E-selectin monoclonal antibody (E-selectin) or anti-α_4 monoclonal antibody (α_4) solutions for 30 min, at 4°C. The bar in each panel marks the position which includes 95% of cells incubated with normal mouse ascites as the primary antibody. 1, No IL-1; 2, IL-1 treated.

Effects of anti-E-selectin

An anti-E-selectin monoclonal antibody partially inhibited adhesion of HL-60 leukaemia cells, MCF-7 breast carcinoma cells and two colon carcinoma cell lines to IL-1-treated endothelial cell monolayers (Table 2). E-selectin-dependent adhesion was observed only after IL-1 treatment of the endothelial cells. Similarly, by flow cytometry and immunoprecipitation, E-selectin was not detectable on untreated endothelial cells but this was dramatically increased by IL-1 treatment (Figures 2 and 3).

Anti-fibronectin receptor antibodies

Anti-fibronectin receptor antibodies (α_5) did not block the adhesion of any cancer cells to either untreated or IL-1-treated endothelial cells (Table 2), although treatment of the endothelial cells with IL-1 caused them to express more fibronectin receptor than untreated cells (Figures 2 and 3).

Effects of multiple antibodies

Adhesion of MCF-7 cells was partially inhibited by both anti-E-selectin and anti-vitronectin receptor antibodies. Both anti-vitronectin receptor and anti-$\alpha_4\beta_1$ antibodies partially inhibited adhesion of M6, M19 and M23 melanoma cells to IL-1-treated endothelial cells. Addition of both inhibitory antibodies blocked cell adhesion to a greater extent than did addition of one antibody alone (Figure 4).

Adhesion not dependent on antibodies

None of the monoclonal or polyclonal antibodies had an effect on the attachment of the cancer cell lines to untreated endothelium, suggesting this adhesion is mediated by molecules distinct from those induced by IL-1 (data not shown). Further, even though the adhesion of BT-20 breast carcinoma cells was enhanced by 1.8-fold by IL-1 pretreatment of the endothelium, none of the antibodies were able to significantly inhibit this enhanced adhesion in four independent experiments.

DISCUSSION

While there is controversy concerning the role of endothelial adhesion molecules in the arrest of circulating intravascular cancer cells [1], studies *in vivo* suggest that attachment of metastatic cells to the endothelium precedes endothelial cell retraction, adhesion to the basement membrane and entry into extravascular tissue to form metastases [2]. Constitutively expressed endothelial adhesion molecules may partially contribute to target organ preferences for metastasis *in vivo* (reviewed in [19]). Endothelial cells are also susceptible to perturbation by stimulants such as IL-1, histamine, tumour necrosis factor, transforming growth factor-β and thrombin. Responses to these cytokines include synthesis and/or upregulation of a complex repertoire of surface adhesion molecules including the integrins and members of the selectin family. These molecules are a major focus of current research activity because of their ability to regulate the adhesion of inflammatory cells facilitating leucocyte polarisation and migration [20]. Similarly, the inducible endothelial adhesion molecules, vitronectin receptor, VCAM-1 (INCAM-110) and E-selectin, have been shown to mediate the adhesion of cancer cells to cytokine-treated endothelial cells *in*

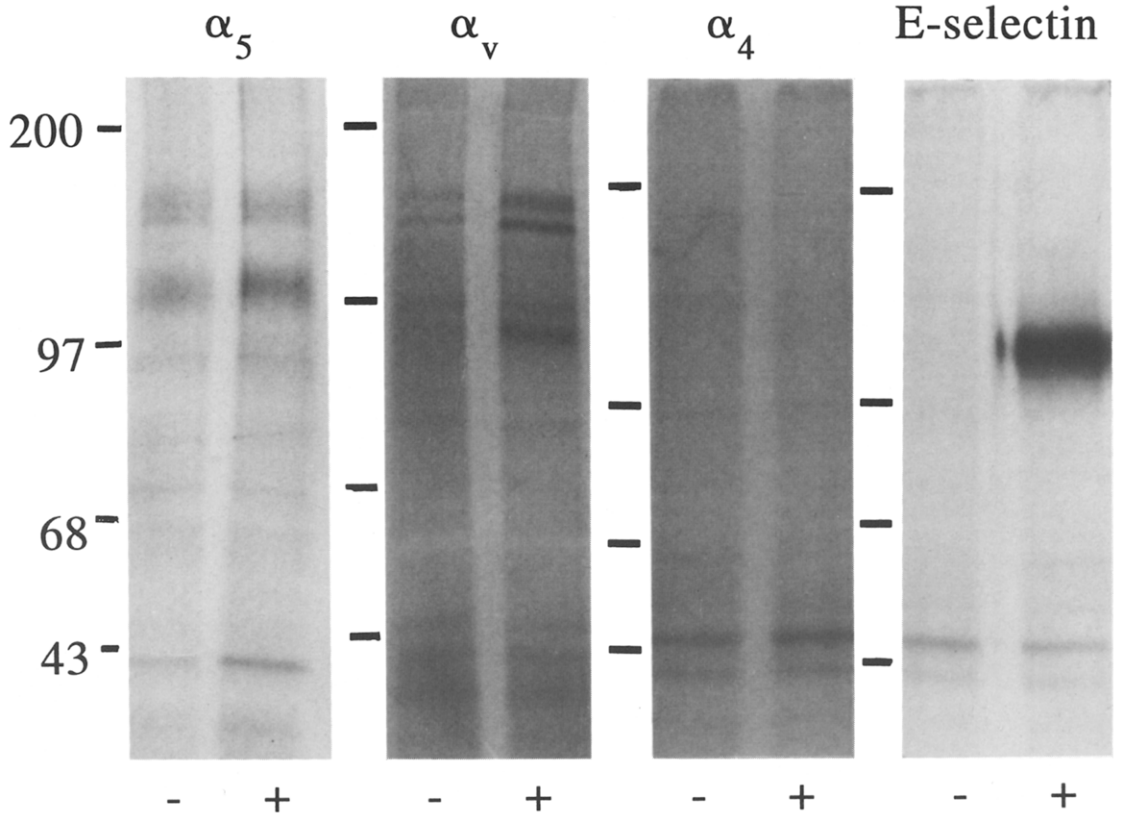

Figure 3. Effect of IL-1 treatment on expression of vitronectin receptor, fibronectin receptor, E-selectin and $\alpha_4\beta_1$ by endothelial cells. Endothelial cells were labelled with [^{35}S]methionine in the absence (−) or presence of IL-1 (+) and then immunoprecipitated with monoclonal antibody against fibronectin receptor (α_5), vitronectin receptor (α_v), $\alpha_4\beta_1$ (α_4) or E-selectin, as indicated. The α_5 antibody immunoprecipitated material at 150 kDa (α_5) and 120 kDa (β_1); the α_v antibody immunoprecipitated material at 160, 150 kDa (the mature and precursor forms of α_v) and 90 and 100 kDa (the mature and precursor forms of β_3); and the E-selectin antibody immunoprecipitated a 90-kDa band.

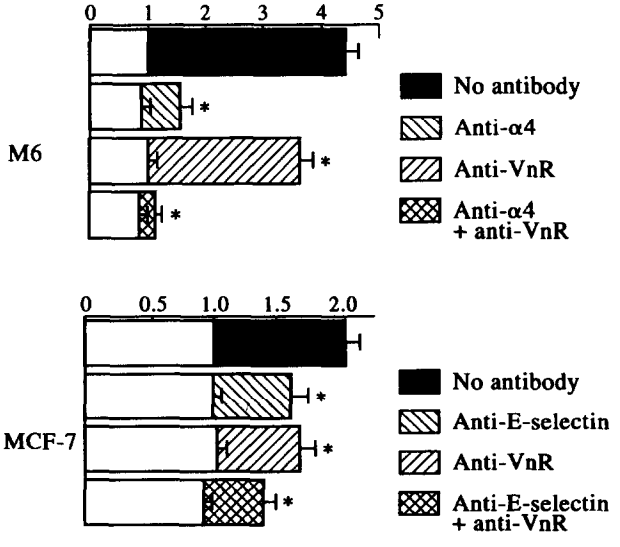

Figure 4. The effect of combinations of monoclonal antibodies on cancer cell adhesion to untreated and IL-1-treated endothelial cells. Each bar represents the relative increase in adhesion of cancer cells in response to IL-1 treatment of the endothelial cells ± standard error, where 1 represents the number of cells adhered to an untreated monolayer. Statistical analysis was performed using a Student's *t*-test. Combination of antibodies inhibited adhesion to a greater extent ($P < 0.01$) than did a single antibody ($P < 0.05$).

vitro [7, 8, 10, 12, 21]. There is evidence that such molecules might influence metastasis *in vivo* [3–6].

Since the adhesive interactions between leucocytes and endothelium can involve more than one of the inducible adhesion molecules, we postulated that the inducible adhesive interactions between cancer cells and the endothelium might also involve more than one of the known receptor–ligand interactions. The availability of a large panel of human tumour cell lines enabled us to confirm that the adhesiveness of the endothelium for cancer cells is dependent upon its activation status. By using a panel of antibodies related to the reported inducible endothelial adhesion molecules, we were able to demonstrate that attachment of cancer cells to activated endothelial cells can involve more than one adhesion molecule, since adhesion of seven of the 22 cell lines examined was inhibited by more than one of the antibodies employed in the study. Moreover, since inducible adhesion of one of the cell lines was not inhibited by the antibodies used in the study, it seems possible that other molecules than the ones examined here may be involved in some interactions.

Our data suggest a promiscuous role for vitronectin receptor since anti-vitronectin receptor antibodies inhibited attachment of 15 of the 23 cell lines to the activated endothelium. Untreated endothelial cells express vitronectin receptor on the abluminal surface, where it is involved in endothelial cell adhesion to the subendothelial matrix [22] as well as in an 'inactive' form on the apical surface [23]. However, adhesion of cancer cells to the apical surface of unstimulated endothelial cells does not appear to be influenced by this receptor since anti-vitronectin receptor

antibodies do not alter their binding. These data support previous observations that IL-1 and eicosanoids induce the activity, synthesis or relocalisation of endothelial vitronectin receptor to the cell surface where it can mediate adhesion of cancer cells [10, 17].

Our results support the evidence that $\alpha_4\beta_1$-dependent adhesion to IL-1-treated endothelial cells depends on α_4 expression by the neoplastic cell and that $\alpha_4\beta_1$ is expressed by non-epithelial neoplasms (melanoma and some sarcoma cells) and few other cancer cell types [7, 13, 14]. Martin-Padura and associates have shown that adhesion of melanoma cells to the cytokine-activated endothelium is proportional to the amount of $\alpha_4\beta_1$ expressed on the cell surface [12].

E-selectin expression appears to be exclusive to vascular endothelial cells; cancer cells do not express E-selectin [24, 25]. Anti-E-selectin monoclonal antibodies have been reported to inhibit adhesion of leucocytes and neutrophils to IL-1- and tumour necrosis factor-treated endothelial cells [24]. Data from our study indicate that E-selectin-dependent adhesion to activated endothelial cells may not be limited to leucocytic cells since anti-E-selectin antibodies blocked attachment of breast and colon cancer cell lines as well as that of HL-60 leukaemic cells.

The fibronectin receptor is important for the adhesion of cells to subendothelial matrices [22], and is thought to be important in cancer cell adhesion to denuded blood vessels. However, anti-fibronectin receptor antibodies did not inhibit adhesion of any of the cancer cells to either untreated or IL-1-treated endothelial cells. Thus, although treatment of the endothelial cells with IL-1 caused them to express more fibronectin receptor than untreated cells, our data suggest that endothelial fibronectin receptor did not mediate cancer cell adhesion.

None of the antibodies tested significantly affected the adhesion of BT-20 breast carcinoma cells, although their adhesion of the endothelium was increased by IL-1 pretreatment of the endothelium. (Unlike MCF-7 breast cancer cells, BT-20 cell adhesion was not significantly inhibited by anti-E-selectin or anti-vitronectin receptor antibodies.) This suggests the existence of the additional cytokine-inducible endothelial adhesion molecules which are able to mediate adhesion of cancer cells.

These experiments demonstrate that cancer cells can use a variety of molecules to adhere to activated endothelial cells. For example, melanoma cells and some sarcoma cells express $\alpha_4\beta_1$ and adhere via the VCAM-1 adhesion molecule, while many cancer cells do not. The organ distribution of constituitive and inducible adhesion molecule expression might explain why cancer cells adhere at different sites within the vasculature and therefore, might explain their consequent sites of metastasis *in vivo*. Treatment of animals with IL-1 enhances experimental metastasis in different experimental systems and, in some cases, this enhancement appears to have an organ-specific component [4]. For example, treatment of animals with IL-1 increases the number of lung tumours formed by cancer cells which normally colonise the lungs, but does not induce tumour formation by cancer cells which normally do not form lung tumours [3]. Further, Arguello and colleagues [5] have shown that IL-1 treatment of mice increases B16 metastasis to bone/bone marrow, but not to other organs. This suggests either enhancement of adhesion molecules by cytokine treatment is organ-specific or that other factors, not altered by cytokine treatment, are required for organ-specificity of metastasis, for example, adhesion molecules, specific growth factors or receptive tissue environments.

These studies have used a static adhesion assay. This assay may not represent an accurate model of metastasis, since *in vivo* adhesion would take place in the presence of a shear force. Finally, it should be noted that umbilical vein endothelial cells, representing large vessel endothelium, were used. These endothelial cells may not be optimal for studies on metastasis which is likely to occur in the microvasculature. There are a number of significant antigenic and metabolic differences between large vessel and microvessel endothelial cells [26, 27]. The reproducible localisation of metastatic cancer cells to specific organ microvasculatures is well known, suggesting the existence of organ-specific (microvasculature specific) adhesion molecules. It is not known if the inducible receptors examined here might be regulated differently between different microvasculatures, although VCAM-1 and E-selectin have been identified histologically in various inflammed tissues [28], and a recent report by Tang and Honn documents induction of vitronectin receptor ($\alpha_v\beta_3$) on lung microvascular cells [19].

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Expression of π-Glutathione S-transferase Gene (GSTP1) in Gastric Cancer: Lack of Correlation With Resistance Against Cis-diamminedichloroplatinum (II)

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Class π-glutathione S-transferase (GSTP-1) is one of several factors proposed to affect drug sensitivity to cis-diamminedichloroplatinum (II) (CDDP). It has also been investigated as a potential marker for the serodiagnosis of various types of cancers. In this study, attempts were made to quantify mRNA levels of the enzyme in healthy and cancerous gastric mucosa specimens, and to evaluate their significance in inherent drug resistance to CDDP. Thirty gastric cancer specimens were analysed by northern blotting with radiolabelled GSTP1 cDNA. Of these, the chemosensitivities of 22 specimens were evaluated by the succinic dehydrogenase inhibition (SDI) test. GSTP-1 mRNA was detected in all the specimens, with slightly increased, but non-significant expression in the neoplasms. Comparison of these drug sensitivities with results of northern blotting analysis showed no inverse correlation, as was expected from the widely investigated role of the enzyme in drug resistance.

Key words: glutathione S-transferase, drug resistance, gastric cancer, cis-diamminedichloroplatinum (II)
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INTRODUCTION

THE EFFECTIVENESS of several clinically useful anticancer drugs can be severely limited by drug resistance, which appears to be intrinsic to some tumours. According to studies carried out using cultured tumour cell models and other systems, a variety of mechanisms can contribute to drug resistance [1–3]. The present study deals with one such mechanism—glutathione S-transferase (GST) which is a group of isozymes that is known to play an important role in the resistance of cells to anticancer agents, including alkylating agents [4, 5] and cis-diamminedich-