The vitronectin receptor ($\alpha_v\beta_3$) is implicated, in cooperation with P-selectin and platelet-activating factor, in the adhesion of monocytes to activated endothelial cells

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In this study we have investigated the presence on endothelial cells of potential glycoprotein receptors, other than P-selectin, which are involved in the adhesion of monocytes at the early stages of activation. We report that the majority of cells binding to thrombin-activated endothelial cells from a peripheral blood mononuclear cell (PBMC) preparation are monocytes. The adhesion of PBMC to thrombin-activated, but not resting, endothelial cells was inhibited (66%) by a monoclonal antibody (mAb) directed against $\alpha_v\beta_3$. Elutriated monocytes or a monocytic cell line (U937) were also inhibited by this antibody, its F(ab)'$_2$ fragments and three other anti-($\alpha_v\beta_3$) mAbs. $\alpha_v\beta_3$ isolated from endothelial-cell lysates significantly inhibited the adhesion of monocytes and U937 cells to endothelial cells. A peptide motif (RGDF) known to interact with $\alpha_v\beta_3$ inhibited U937 cell adhesion to activated endothelial cells by 53%. Finally, an anti-(P-selectin) mAb (LYP20) or a platelet-activating factor (PAF)-receptor antagonist (WEB 2086) inhibited monocyte adhesion to activated endothelial cells. This study shows for the first time that $\alpha_v\beta_3$ is implicated, in addition to P-selectin and PAF, in the adhesion of monocytes to activated endothelial cells.

INTRODUCTION

Leucocyte adhesion to the activated vascular endothelium cell surface is a critical event in the acute inflammatory response [1]. Such leucocyte–endothelial cell (EC) interactions can take place either within seconds or after a number of hours, depending on factors that induce inflammation and activation of vascular ECs [2]. Human umbilical vein ECs when treated with inflammatory agents, such as thrombin, histamine or phorbol esters, rapidly co-express P-selectin (GMP-140/PADGEM/CD62) [3] and platelet-activating factor (PAF) [4]. P-selectin is present in the boundary membrane of platelet α-granules and endothelial Weibel Palade bodies and is expressed on the surface of these cells within seconds of activation. This exocytosed glycoprotein, P-selectin, is thought to play a key role in targeting neutrophils or monocytes to sites of inflammation [4]. It has previously been reported, in a study similar to ours, that P-selectin and the CD-63-related antigen regulate the transiently increased neutrophil adhesion seen almost immediately after the endothelium has been exposed to thrombin, and also that PAF facilitates this reaction [5]. Moreover, PAF activates leucocytes by binding to a cell-surface receptor inducing up-regulation of the CD11a–CD18 and CD11b–CD18 integrin complexes on both monocytes and neutrophils [4]. CD11–CD18 [the main ligand for the cytokine-inducible EC receptor ICAM-1] is also reported to have at least one unidentified receptor on ECs. Activation of this integrin complex in turn allows it to adhere to this unidentified receptor [6,7]. Toothill et al. [5] have concluded that the vitronectin receptor may play a role in polymorphonuclear (PMN) cell adhesion to thrombin-activated endothelium.

In this study we have investigated the presence on ECs of potential glycoprotein receptors involved in the adherence of monocytes at early stages of the activation process. Results obtained in this work show, for the first time, that the EC vitronectin receptor ($\alpha_v\beta_3$) is implicated, in co-operation with P-selectin and PAF, in the adhesion of monocytes to thrombin-activated ECs.

MATERIALS AND METHODS

Materials

All chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise stated. Tissue-culture medium 199 and supplements [fetal-calf serum (FCS), horse serum, goat serum, penicillin/streptomycin, glutamine, sodium bicarbonate] were from Gibco Life Technologies Ltd. (Paisley, Scotland, U.K.). The PAF antagonist (WEB 2086) was a generous gift from Dr. Pistel and Dr. Duttmann (Boehringer, Ingelheim, Germany).

Peptides

The tetrapeptides Arg-Gly-Asp-Phe (RGDF), and a modified form Arg-Gly-Glu-Ser (RGES), were synthesized on an Applied Biosystem 431A peptide synthesizer (Paris, France) by Fmoc chemistry. These peptides were then purified by h.p.l.c. and their compositions were confirmed by amino acid analysis. Stock solutions were prepared in PBS (0.1 M, pH 7.2) and appropriate dilutions were made in RPMI medium (Flow Laboratories, Irvine, Scotland, U.K.) as described.

Abbreviations used: EC, endothelial cell; PAF, platelet-activating factor; vWF, von Willebrand factor; VCAM-1, vascular cell adhesion molecule; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; ECGF, endothelial cell growth factor; FCS, fetal-calf serum; FITC, fluorescein isothiocyanate; PMN, polymorphonuclear.

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Monoclonal antibodies (mAbs)

mAb LYP18 recognizes platelet $\alpha_{IIb}\beta_3$ and the $\alpha_v\beta_3$ complex expressed by ECs and melanoma cells [8]. Crossed-immunoelectrophoresis experiments indicate that LYP18 is directed against the heterodimer complex and not the isolated α or β subunits [9]. $F(ab)'_2$ fragments of LYP18 were prepared as described [10]. In contrast, LYP2 recognizes platelet $\alpha_{IIb}\beta_3$ complex but not $\alpha_v\beta_3$ [11]. Three other mAbs directed against $\alpha_v\beta_3$ were also used: 7E3 [12] (a generous gift from B. Coller, Stonybrook, NY, U.S.A.), and 13C2 and 23C6 [13] (generous gifts from M. Horton, London, U.K.). LYP20 is an anti-(P-selectin) mAb that is directed against a disulphide-dependent epitope and is known to inhibit platelet aggregation and platelet–monocyte interaction [14]. The antibodies directed against specific cell-surface antigens for B-lymphocytes (CD19:clone 4G7; CD20:clone L27), T-lymphocytes (CD2:clone S5.2; CD3:clone SK7) and monocytes (CD14:clone Mo/P9) were from Becton Dickonson (Mountain View, CA, U.S.A.). Antibody 60.3 is an IgG2a mAb, a generous gift from John Harlan (University of Washington), that precipitates all of the polypeptide chains of the CDw18 cell-surface glycoprotein complex [15]. All antibodies, with the exception of 13C2 and 23C6 which were present in a cell supernatant form, were used as purified immunoglobulins and diluted in RPMI medium as described.

Culture of human umbilical vein ECs

ECs were isolated and grown in culture as described by Jaffe et al. [16]. Briefly, cells were isolated from umbilical cords treated with collagenase type 2 (Worthington Chemical Company, Freehold, NJ, U.S.A.) and grown to confluence on fibronectin (Boehringer–Mannheim, Germany)-coated flasks in culture medium 199 at 37 °C in 5% CO_2. The culture medium was supplemented with 20% (v/v) human serum AB (CTS Beynost, France), 300 μg/ml endothelial cell growth factor (ECGF) (Boehringer–Mannheim), 5 mM $NaHCO_3$, 2 mM glutamine, 100 units penicillin/streptomycin and 1 mM sodium pyruvate. Cells were seeded either on to 24-well (Falcon Primarea, Meylan, France) or 96-well (Nunc Immunolon, Roskilde, Denmark) plates and left to grow to confluence (4×10^4 cells/96-well plate or 2×10^5 cells/24-well plate) before being used in e.l.i.s.a. or adhesion assays.

Isolation of peripheral blood mononuclear cells (PBMCs) or monocytes

Routine isolation of PBMCs was performed under sterile conditions using endotoxin-free Histopaque-1077 gradient medium in a gradient-density centrifugation technique [17]. Whole blood was drawn from donors in acid/citrate/dextrose and carefully layered on Histopaque-1077 medium. PBMCs present at the Histopaque interface, following centrifugation at 400 ***g*** for 30 min at 25 °C, were isolated and washed twice in 50 ml of PBS. Isolated PBMC pellets, gently resuspended in 1 ml of PBS, always showed more than 90% viability, normally more than 95%. Monocytes were isolated by elutrial centrifugation as previously described [18]. The preparation was > 90% pure, as determined by flow cytometry (FACScan; Becton Dickinson and Co., Mountain View, CA, U.S.A.) with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 antibody (LeuM3; Becton Dickinson and Co.) and by May–Grunwald–Giemsa staining. The monocyte population was > 90% $HLA\text{-}DR^+$ and < 1% $CD25^+$ and viability was more than 95%, as determined by Trypan Blue dye exclusion. U937 cells, a monocytic cell line expressing many of the monocytic-like characteristics exhibited by cells of histocytic origin [19] (European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts., U.K.), were cultured in RPMI tissue-culture medium (Flow Laboratories) supplemented with 10% FCS, 2 mM glutamine, 100 units penicillin/streptomycin, and 1 mM sodium pyruvate. Cells were suspended in RPMI/1% FCS for adhesion experiments. All cells were labelled by $Na^{51}CrO_4$ incorporation as described [20].

B-lymphocyte isolation

Tonsillar mononuclear cells were separated by a standard Ficoll/Hypaque gradient method and were next submitted to E rosetting with sheep erythrocytes. Non-rosetting cells were labelled with anti-(T cell) (CD2 and CD3) mAbs and anti-monocyte (CD14) mAb and subsequently incubated twice with magnetic beads coated with anti-(mouse IgG) antibodies (Dynal, Oslo, Norway). Residual non-B cells were removed by applying a magnetic field for 10 min. The purity of the B-cell populations obtained following this procedure was more than 95%, as estimated by FACScan immunofluorescence stainings performed with anti-CD2, -CD3, -CD14, -CD19 and -CD20 mAbs.

Immunostaining

ECs were grown to confluence on laminin-coated slides (Becton Dickinson), incubated with thrombin (2 units/ml) for 5–10 min, washed once with PBS and then incubated with either a PBMC preparation or purified B- or T-lymphocytes for a further 10 min. Slides were then washed three times to remove unbound cells and fixed for 5 min at 4 °C with methanol. Non-specific sites were blocked using blocking antibody provided by the manufacturer and mAbs (CD3, CD14 or CD19) that are specific for each of the aforementioned cell types (reviewed above) were added for a further period of 10 min and labelled using the universal LSAB kit (DAKO) according to the manufacturer's recommendations.

Flow cytometry

Flow cytometric analysis was carried out as described [21] to test for non-specific binding of mAbs to monocytes. Positive controls were also tested using human platelets as previously described [22].

Preparation of $\alpha_v\beta_3$

The cytoadhesin ($\alpha_v\beta_3$) was isolated on an LYP18/Protein A–Sepharose affinity column using EC lysates as described [10]. Bound material was removed by elution buffer over a stepwise pH regime, peaks detected at 280 nm and fractions analysed by gel electrophoresis and Western blot.

E.l.i.s.a. assays

Binding of mAbs to ECs, that were either resting or stimulated by an agonist, was performed according to the method of Pigott et al. [23]. Briefly, confluent ECs grown in 96-well microtitre plates were activated by thrombin for between 5 and 30 min. The 96-well plates were then gently emptied and washed three times with RPMI medium containing 2.5% (v/v) horse serum (RPMI/HS buffer). Sodium azide (0.1%) was included in this washing medium in order to kill the cells, thus maintaining P-selectin expression. ECs were then incubated with a mAb which was suspended in RPMI/1% FCS for 60 min at room temperature, washed, fixed for 5 min in phosphate-buffered saline (PBS) containing glutaraldehyde (0.025%) (Merck-Schuchardt, Munich, Germany), washed, and non-specific binding sites were

quenched by a 60 min incubation with PBS/HS. A goat anti-(mouse IgG)–horseradish peroxidase (Bio-Rad, Richmond, CA, U.S.A.), diluted to 1/3000 in PBS containing 10% (v/v) goat serum, was used to detect bound mAb.

Adhesion assays

^{51}Cr-labelled PBMC, monocytes, or U937 cells were assayed for their adhesion to unstimulated or stimulated ECs following the method, with minor modifications, previously described [4]. Briefly, fully confluent EC were stimulated with thrombin (1–2 units/ml) for a period of 5–10 min and the reaction terminated by plate inversion and washing, or by the addition of a thrombin antagonist, D-phenylalanyl-L-prolyl-L-argininechloromethane (PPACK: Calbiochem, La Jolla, CA, U.S.A.) (10^{-6} M final concentration). ECs were then immediately incubated with RPMI/1% FCS in the presence or absence of mAbs or their F(ab)'$_2$ fragments (LYP18, LYP20, 13C2, 23C6, and 7E3) for 5–10 min at room temperature followed by the addition of PBMC, elutriated monocytes or U937 cells [(4–6) × 10^5/well] for 10 min. For the isolated $\alpha_v\beta_3$, the PAF antagonist (WEB 2086) or the RGDF and RGES peptides there was no incubation period with ECs before addition of monocytes. Most experiments were carried out at room temperature (preliminary experiments at 37 °C yielded similar results) under static or rotating (64 rev./min) conditions and inhibitors were left in wells throughout monocyte incubation. All wells were checked microscopically during the experiments to ensure that the integrity of the EC monolayers was not disrupted by added inhibitors. Following the incubation period, wells were then emptied by inversion and non-adherent or loosely adherent cells were removed by washing two or three times with RPMI/1% FCS. Well contents were solubilized with 50 mM Tris/0.1% SDS, pH 8.4, and radioactivity in cell lysates measured. For all experiments, the binding of PBMC, elutriated monocytes or U937 cells to activated control in the absence of antibody or inhibitor was normalized to 100% and the reduction in binding caused by the inhibitor was calculated as a percentage of this.

Analysis of data

Each experiment was carried out in replicate as described and the mean calculated. S.D. values were calculated from the mean of each experiment. The significance of the difference between mean values was determined using Student's t test.

RESULTS

mAbs LYP20 and LYP18 bind to ECs

Studies were initially performed to examine the conditions under which the anti-(P-selectin) and anti-($\alpha_v\beta_3$) mAbs, the two principal mAbs used in this study, bound to ECs. Parameters giving the best P-selectin expression by activated ECs were then subsequently used in adhesion assays. ECs were exposed to thrombin because of their ability to induce rapid P-selectin expression [3] and increase leucocyte adherence [5], for periods of time varying between 5 and 30 min. Optimal fixation of LYP20 occurred after exposure to thrombin at a concentration of 2 units/ml for 5–10 min (Table 1), which is in accordance with what has been previously reported [4,24]. These treated ECs were observed by light microscopy to change from a longitudinal flat structure to a more plump and cuboidal shape, consistent with what has been described previously [25], in addition to the appearance of microstructures on their surface (results not shown). LYP18 was observed to bind avidly to resting ECs, as cultured ECs expose $\alpha_v\beta_3$ on their luminal surface [26], and this binding was increased following thrombin activation (Table 1). Such observations may be a result of the EC undergoing shape change following stimulation, allowing LYP18 to bind to the basal side of the cell, as previously reported [27].

Table 1 Binding of monoclonal antibodies LYP20 or LYP18 to resting or activated endothelial cells

Human umbilical vein endothelial cells were seeded on 96-well e.l.i.s.a. plates, grown to confluence and treated with thrombin (2 units/ml for 5–10 min). Anti-(P-selectin) mAb (LYP20) was then added (5 μg/ml) and binding measured as described in the Materials and methods section. Binding was significantly increased ($P < 0.001$) on these thrombin-treated wells compared with non-treated wells. The high S.D. values are due to variations in basal levels of P-selectin. An anti-$\alpha_v\beta_3$ mAb (LYP18, 2 μg/ml) bound avidly to resting cells. Binding of LYP18 was significantly increased ($P < 0.001$) on thrombin-activated cells. Data represent mean ± S.D. of triplicate determinations from four independent experiments.

Antibody	Resting cells (A_{490})	Activated cells (A_{490})
LYP20	0.14 ± 0.15	0.20 ± 0.21
LYP18	0.77 ± 0.09	0.87 ± 0.06

Table 2 Effect of monoclonal antibodies LYP20 or LYP18 on mononuclear cell adhesion to endothelial cells

Human umbilical vein endothelial cells were stimulated with thrombin (2 units for 5–10 min) and adhesion assays carried out as described in the Materials and methods section. In these experiments 9 ± 2% of added monocytes bound to activated control, normalized to 100%, and inhibitory effects of antibodies were calculated as percentages of this. Also shown is the relative percentage adhesion of PBMC to resting endothelial cells. Data represent the mean ± S.D. of triplicate determinations from three independent experiments. *$P < 0.001$ as determined by Student's t test.

Control or antibody	PBMC adhesion (%)
Activated control	100 ± 22
LYP20 (30 μg/ml)	45.4 ± 10.6*
LYP18 (20 μg/ml)	33.3 ± 4.6*
Resting	19.3 ± 12.0*

LYP20 and LYP18 inhibit PBMC adhesion to activated but not resting ECs

PBMC bound to resting ECs but there was a significant increase in binding when layered on thrombin-activated cells. This adhesion to activated ECs was reduced by the anti-(P-selectin) mAb LYP20 and the anti-$\alpha_v\beta_3$ mAb LYP18 (Table 2). LYP18 has been previously shown to inhibit platelet aggregation, platelet–melanoma interaction and melanoma tumour growth in nude mice [9]. Preliminary experiments at different mAb concentrations demonstrated consistent and maximum inhibition of PBMC adhesion in the presence of 30 μg/ml of LYP20 and 20 μg/ml of LYP18, and these concentrations were used in all assays. LYP18 and LYP20, when added together in the assays, appeared to have no additive or synergistic effect, the percentage inhibition being the same as that for LYP18 alone. Moreover, neither antibody inhibited the adhesion of monocytes to resting cells (results not shown).

Table 3 Effect of anti-$\alpha_v\beta_3$ monoclonal antibody LYP18 on monocyte adhesion to endothelial cells

Human umbilical vein endothelial cells were stimulated with thrombin and adhesion assays were carried out as described in the Materials and methods section. In these experiments 20.13±2.36% of added monocytes bound to activated control compared with 14.6±1.6% on resting cells. Data represent the mean±S.D. of triplicate determinations from three independent experiments. *$P < 0.001$ as determined by Student's t test.

Control or antibody	Monocyte adhesion (%)
Activated control	100
LYP18 (20 μg/ml)	65±6.5*
F(ab)′$_2$ (20 μg/ml)	70±1.7*

Table 4 Effect of (i) anti-$\alpha_v\beta_3$ or anti-(P-selectin) monoclonal antibodies, (ii) purified $\alpha_v\beta_3$, or (iii) RGDF peptide on the adhesion of U937 cells to thrombin-activated endothelial cells

Human umbilical vein endothelial cells were stimulated with thrombin and adhesion assays were carried out as described in the Materials and methods section. In these experiments 12.9±6.5% of U937 cells bound to activated endothelial control cells compared with 8.1±5.2% on resting cells. (i) Adhesion of U937 cells to ECs in the presence of anti-$\alpha_v\beta_3$ mAbs and anti-(P-selectin) mAb LYP20. (ii) Purified $\alpha_v\beta_3$ was isolated from ECs as described in the Materials and methods section and added to assays at a concentration of 20 μg/ml. Also shown is the effect of the dialysed Lubrol control. The difference between the two, $\alpha_v\beta_3$ + Lubrol versus Lubrol alone, was statistically significant ($P < 0.001$). (iii) U937 cells were layered on thrombin-activated ECs in the presence or absence of RGDF or RGES peptide (2 mM). Each value represents the mean±S.D. of duplicate determinations from three independent experiments. Abbreviation: NS, not significant.

Control or inhibitor	U937 cell adhesion (%)	Significance
Activated control	100	
(i)		
LYP18 (20 μg/ml)	47.5±10.6	$P < 0.020$
LYP18 F(ab)′$_2$ (20 μg/ml)	60.0±7.7	$P < 0.019$
7E3 (20 μg/ml)	45.5±8.6	$P < 0.010$
13C2 (20 μg/ml)	49.0±7.7	$P < 0.009$
23C6 (20 μg/ml)	63.5±9.2	$P < 0.030$
LYP20 (30 μg/ml)	39.0±7.1	$P < 0.007$
(ii)		
$\alpha_v\beta_3$	22.0±7.0	$P < 0.001$
Lubrol control	75.0±22	NS
(iii)		
RGDF	47.0±7.1	$P < 0.009$
RGES	89.0±4.0	NS

Majority of cells adhering to ECs from a PBMC preparation are monocytes

As the ratio of lymphocytes to monocytes in the PBMC preparation is about 5:1 we investigated which cell type bound to the thrombin-activated EC. By immunostaining techniques using antibodies directed against antigens unique to each cell type we observed that the majority of cells from these PBMC preparations that bound to activated ECs were monocytes. An mAb marker (CD14:clone Mo/P9) specific to monocytes was observed to intensely label cells bound to activated ECs. In contrast, when an anti-(B lymphocyte) marker (CD20:clone L27) or an anti-(T lymphocyte) marker (CD3:clone SK7) was used on the same PBMC population, incubated simultaneously and for the same time period, no staining was observed. Pure B-lymphocyte and T-lymphocyte populations which were used as controls and added to activated ECs showed no binding.

LYP18 and LYP20 inhibit adhesion of elutriated monocytes and U937 cells to thrombin-activated ECs

The adhesion of elutriated monocytes to thrombin-activated ECs was inhibited by LYP18 and its F(ab)′$_2$ fragments (Table 3). Flow cytometric analysis of elutriated monocytes showed that LYP18 does not bind to monocytes. Adhesion assays using U937 cells were subsequently carried out to determine whether anti-$\alpha_v\beta_3$ or anti-(P-selectin) mAbs would have any effect. mAbs LYP18, LYP20 and the three other anti-$\alpha_v\beta_3$ antibodies (7E3, 13C2 and 23C6) inhibited adhesion of U937 cells to activated ECs (Table 4). F(ab)′$_2$ fragments of LYP18 also inhibited this adhesion. Flow cytometric analysis of both resting and phorbol ester-activated U937 cells showed no binding of LYP18 (results not shown). Adhesion assays carried out under rotating conditions (64 rev./min) showed quantitatively similar adhesion to that found under static conditions on thrombin-activated ECs ($P < 0.985$).

Purified $\alpha_v\beta_3$ inhibits adhesion of U937 cells to ECs

We used the purified $\alpha_v\beta_3$ sample, in RPMI/1% FCS at a Lubrol concentration of 0.025%, to test whether $\alpha_v\beta_3$ could inhibit adhesion of U937 cells to ECs. The $\alpha_v\beta_3$ sample and the control, containing only the detergent in RPMI/1% FCS, were extensively dialysed (18 h) to reduce the detergent level. Table 4 shows that $\alpha_v\beta_3$ dramatically inhibited U937-cell adhesion to ECs in contrast to the control.

RGDF peptide inhibits U937-cell adhesion to ECs

$\alpha_v\beta_3$ on activated ECs may either directly interact with receptors present on adhering monocytes or via ligands such as von Willebrand factor (vWF), vitronectin, fibrinogen, fibronectin or thrombospondin. To determine whether these ligands were involved in the interaction we used a peptide motif (RGDF) which is expressed by the above-mentioned adhesive ligands in the adhesion assays. A modified form of this peptide (RGES) was also used as control. Table 4 shows results of the peptide assays at a fixed concentration of 2 mM, showing that RGDF significantly inhibits U937-cell adhesion in contrast with RGES.

PAF antagonist inhibits monocyte adhesion to thrombin-activated ECs

One of the most interesting findings in this study was the fact that the anti-$\alpha_v\beta_3$ antibodies only inhibited monocyte adhesion to activated, but not to resting, ECs. We suspected that EC activation might result in the expression of receptors which could in some way induce activation of complexes on the monocyte surface, as previously reported [4], allowing them to interact with $\alpha_v\beta_3$. PAF, which is known to be rapidly expressed with P-selectin on the surface of activated ECs, is also known to activate the CD11–CD18 complex [9]. By blocking the activation of monocyte cell-surface complexes we predicted that we could affect the adhesion of monocytes via this pathway. Using a PAF-receptor antagonist (WEB 2086) inhibited monocyte adherence to thrombin-activated ECs in a concentration-dependent manner

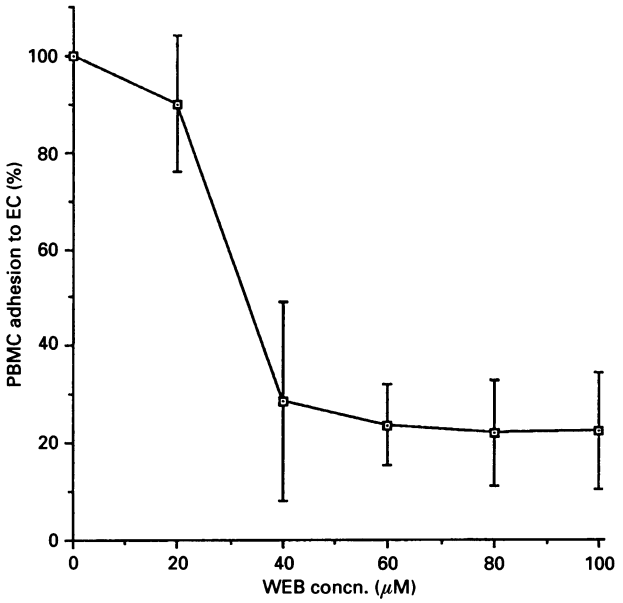

Figure 1 Effect of PAF receptor antagonist (WEB 2086) on adhesion of monocytes to endothelial cells

WEB 2086 was added to endothelial cells immediately before incubation with mononuclear cells as described in the Materials and methods section. Inhibition of adhesion occurred in a concentration-dependent manner. Adhesion of mononuclear cells to resting endothelial cells was between 25 and 30% relative to control. Each point represents the mean ± S.D. of duplicate determinations from three independent experiments.

(Figure 1). Moreover, the addition of an anti-CD18 mAb (60.3) at a concentration of 30 μg/ml inhibited U937 cell adhesion by 22.4% ($P < 0.001$) only on thrombin-activated ECs and not on resting ECs (results not shown).

DISCUSSION

In this study we show for the first time that $\alpha_v\beta_3$ expressed by ECs is involved, in co-operation with P-selectin and PAF, in the adhesion of monocytes to thrombin-activated ECs. Several lines of evidence implicate $\alpha_v\beta_3$ in the adherence of monocytes: (1) an anti-$\alpha_v\beta_3$ mAb (LYP18) and its F(ab)′$_2$ fragments inhibits the adhesion of monocytes and the monocytic cell line (U937) to activated but not resting EC. Three other mAbs also directed against $\alpha_v\beta_3$ show the same inhibitory effect. Absence of binding of LYP18 or LYP20 to monocytes or U937 cells indicate an absence of $\alpha_v\beta_3$ expression by these cells and no platelet contaminants. (2) A peptide motif (RGDF), present on adhesive ligands (vWF, vitronectin, fibrinogen, fibronectin or thrombospondin) [28] and known to interact with $\alpha_v\beta_3$, significantly inhibits monocyte adhesion to activated ECs. (3) $\alpha_v\beta_3$ isolated from EC lysates and added to the adhesion assays dramatically inhibits monocyte adhesion to ECs.

The effect of mAbs LYP18 and LYP20 was first observed using a PBMC preparation on activated ECs. Since the proportion of lymphocytes to monocytes is much higher (approx. 5:1) in PBMC preparations we investigated whether or not B or T lymphocytes, in addition to monocytes, were bound to thrombin-activated ECs. Our immunostaining studies demonstrate the adhesion of monocytes but not B- or T-lymphocytes. Our findings are in accordance with those observed by a number of groups [3,29–31] who reported that leucocytes, U937 and HL60 but not Jurkatt (human T-cell line), Daudi (human B-cell line) or lymphocytes bind to P-selectin. In contrast, Moore and Thompson [32] specifically implicated memory cells (CD45RO$^+$), in the CD4$^+$, CD8$^+$ subpopulation in binding to isolated P-selectin. In addition, it has been reported that T lymphocytes (natural killer cells and an undefined subpopulation of T lymphocytes present in the CD4$^+$, CD8$^+$ subclass) bind to activated platelets [33]. Discrepancy between these observations may well be due to the fact, as shown by Damle et al. [34], that CD4$^+$ T lymphocytes have to be antigen-primed and chronically stimulated to bind to P-selectin. In the present study binding of T lymphocytes to ECs was not observed since T cells were not activated. Alternatively, T-lymphocyte binding may have remained undetected due to a specific subclass, representing a small percentage of the total T-lymphocyte population, adhering to ECs and not being identified by the markers used. Finally, monocytes may compete off lymphocytes for P-selectin sites on ECs as they appear to do for P-selectin sites on activated platelets [35].

Thrombin activation of the EC monolayer resulted in a 4-fold increase of adhering PBMC, similar to what has been previously reported by Toothill et al. [5] who used PMN instead of monocytes on ECs pretreated with thrombin in a similar manner. This adhesion was blocked by an anti-(P-selectin) antibody (LYP20) and also by an anti-$\alpha_v\beta_3$ antibody (LYP18). Neither antibody inhibited PBMC adhesion on resting ECs, indicating that P-selectin and PAF expression were essential for this $\alpha_v\beta_3$-mediated adhesion. The adhesion of elutriated monocytes to activated ECs was similarly inhibited by LYP18 and its F(ab)′$_2$ fragments. Flow cytometric analysis showed the absence of an LYP18 determinant on elutriated monocytes and no adhering platelets. The use of EDTA during the elutriation step eliminated platelet contamination. The main difference between elutriated monocytes and those present in PBMC preparations was the increased binding of elutriated monocytes to resting ECs, most probably due to monocyte activation during the extended manipulations, as previously reported [36]. Our results with U937 cells, using LYP18 and three other mAbs also directed against $\alpha_v\beta_3$, show similar inhibition of adhesion to activated ECs, thus supporting our findings with monocytes. The role of $\alpha_v\beta_3$ as one of the receptors involved in mediating monocyte adhesion to activated ECs is further confirmed by the inhibitory effect of RGDF peptide and purified $\alpha_v\beta_3$. Previous studies have shown that certain tumour cell lines adhere to activated ECs via $\alpha_v\beta_3$ [37,38].

Adhesion of leucocytes to activated ECs, prior to extravasation and homing to a site of inflammation, is now known to be mediated by a number of adhesion receptors that are expressed or up-regulated by activated ECs (see review) [39]. In this study we concentrated specifically on the very early stages of activation and adhesion and the receptors involved in these interactions. It has been well documented that following stimulation by fast-acting activators such as thrombin or histamine there occurs a rapid degranulation of the Weibel Palade bodies resulting in the transient co-expression of P-selectin and PAF. Recent studies have shown that P-selectin tethers leucocytes, thus allowing PAF to induce their activation, which in turn results in strengthened adherence [4,40]. As PAF is known to activate the CD11–CD18 complex we predicted that by blocking the PAF receptor, expressed by activated ECs, the monocyte CD11–CD18 complex would not be activated and a reduced number of adhering monocytes would be observed. Our results in the presence of PAF-receptor antagonist (WEB 2086), using exactly the same EC activation protocol described [4,40], show that the number of monocytes adhering to activated ECs is greatly reduced and is below that observed for resting cells. WEB 2086 has been shown

to significantly inhibit PMN adhesion to thrombin-activated ECs [5]. The authors concluded that whereas PAF production by ECs may facilitate the enhanced adhesion induced by thrombin, it is not sufficient to explain the reaction.

Because LYP18 and the anti-CD18 mAb only inhibited adhesion on thrombin-activated but not resting EC, we speculate that this activation is essential for $\alpha_v\beta_3$ involvement and that P-selectin, PAF and $\alpha_v\beta_3$ somehow act in concert. At least one unidentified counter-receptor for the PAF-activated monocyte CD11–CD18 heterodimers complex is thought to be present on ECs [6,7]. It is conceivable that this counter-receptor on the activated EC surface may be the $\alpha_v\beta_3$ complex acting via adhesive ligands (fibrinogen, fibronectin, vWF, thrombospondin) bearing an RGD peptide motif. The potential role of $\alpha_v\beta_3$ has previously been suggested by Toothill et al. [5]. When using an mAb (C17) directed against the β-chain on platelets and ECs they found that it dose-dependently inhibited PMN adhesion to thrombin-activated ECs. Moreover, they also found that a peptide containing an RGD sequence also inhibited this adhesion whereas the RGES inactive peptide had no effect. Fibrinogen has been shown to bind to leucocyte CD11b–CD18 [41] and a recent study reported that fibrinogen mediates leucocyte adhesion to ECs through an intracellular adhesion molecule 1 (ICAM-1)-dependent pathway [42]. This adhesion was up-regulated following EC stimulation. Moreover, mAbs to CD11–CD18 have been shown to inhibit monocyte adherence to ECs [43]. The inhibition we observed with an anti-CD18 mAb (60.3) could possibly have been increased had we used greater concentrations of mAb or used it together with an anti-CD11 antibody.

In conclusion, we show in this report that the vitronectin receptor ($\alpha_v\beta_3$) is involved in the adhesion of monocytes to ECs following thrombin activation of ECs. This adhesion works in concert with P-selectin and PAF which are rapidly expressed on the EC surface following stimulation. Work is currently underway to determine the counter-receptor on the monocyte surface responsible for this interaction.

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