



ELSEVIER

Biochimica et Biophysica Acta 1501 (2000) 99–115

BIOCHIMICA ET BIOPHYSICA ACTA

BBAwww.elsevier.com/locate/bba

Phosphatidylserine-dependent adhesion of T cells to endothelial cells

Jiamin Qu ^a, Jackie Adam ^b, David M. Bloxham ^c, K. Richard Bruckdorfer ^b,
Nigel G.A. Miller ^d, Nicola A. Parkinson ^{e,1}, Jack A. Lucy ^{a,*}

^a *Molecular Immunology Programme, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK*

^b *Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK*

^c *Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, UK*

^d *Technix, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK*

^e *Signalling Programme, The Babraham Institute, Babraham, Cambridge CB2 4AT, UK*

Received 25 November 1999; received in revised form 9 March 2000; accepted 22 March 2000

Abstract

Phosphatidylserine (PS) was exposed at the surface of human umbilical vein endothelial cells (HUVECs) and cultured cell lines by agonists that increase cytosolic Ca²⁺, and factors governing the adhesion of T cells to the treated cells were investigated. Thrombin, ionophore A23187 and the Ca²⁺-ATPase inhibitor 2,5-di-*tert*-butyl-1,4-benzohydroquinone each induced a PS-dependent adhesion of Jurkat T cells. A23187, which was the most effective agonist in releasing PS-bearing microvesicles, was the least effective in inducing the PS-dependent adhesion of Jurkat cells. Treatment of ECV304 and EA.hy926 cells with EGTA, followed by a return to normal medium, resulted in an influx of Ca²⁺ and an increase in adhering Jurkat cells. Oxidised low-density lipoprotein induced a procoagulant response in cultured ECV304 cells and increased the number of adhering Jurkat cells, but adhesion was not inhibited by pretreating ECV304 cells with annexin V. PS was not significantly exposed on untreated Jurkat cells, as determined by flow cytometry with annexin V-FITC. However, after adhesion to thrombin-treated ECV304 cells for 10 min followed by detachment in 1 mM EDTA, there was a marked exposure of PS on the Jurkat cells. Binding of annexin V-FITC to the detached cells was inhibited by pretreating them with unlabelled annexin V. Contact with thrombin-treated ECV304 cells thus induced the exposure of PS on Jurkat cells and, as Jurkat cells were unable to adhere to thrombin-treated ECV304 cells in the presence of EGTA, the adhesion of the two cell types may involve a Ca²⁺ bridge between PS on both cell surfaces. The number of T cells from normal, human peripheral blood that adhered to ECV304 cells was not increased by treating the latter with thrombin. However, findings made with several T cell lines were generally, but not completely, consistent with the possibility that adhesion to surface PS on endothelial cells may be a feature of T cells that express both CD4⁺ and CD8⁺ antigens. Possible implications for PS-dependent adhesion of T cells to endothelial cells in metastasis, and early in atherogenesis, are discussed. © 2000 Elsevier Science B.V. All rights reserved.

* Corresponding author. Present address: Department of Biochemistry and Molecular Biology, Royal Free and University College Medical School, Royal Free Campus, Rowland Hill Street, London NW3 2PF, UK. Fax: +44-1763-852284; E-mail: jacklucy@clara.net

¹ Present address: Department of Physiology, University College London, Rockefeller Building, University Street, London WC1E 6JJ, UK.

Keywords: Endothelial cell; T cell; Phosphatidylserine; Adhesion; Atherosclerosis

1. Introduction

An energy-dependent aminophospholipid translocase [1] is present in the plasma membranes of most cells, including endothelial cells [2], which transports the anionic phospholipid, phosphatidylserine (PS), from the outer to the inner leaflet of the phospholipid bilayer. Consequently, PS is normally found only in the inner leaflet of the plasma membrane [3–5] but, following the activation of scramblase activity by an increase in cytosolic Ca^{2+} , it rapidly appears on the cell surface [6–8] where it can mediate cell–cell interactions [9–11]. Since there is accumulating evidence for the importance of interactions between T cells and endothelial cells in the development and progression of atherosclerosis [12–14], we have characterised the adhesive response of Jurkat T cells to endothelial cell lines that have been treated with several agonists, including oxidised low-density lipoprotein (LDL), that increase cytosolic Ca^{2+} and expose PS at the cell surface. Experiments have also been undertaken on the mechanism of Jurkat cell adhesion to agonist-treated endothelial cells. Contact with thrombin-treated ECV304 cells was found to induce the exposure of PS on Jurkat cells and, since the adhesion of Jurkat cells was also Ca^{2+} -dependent, a Ca^{2+} bridge between PS on the surface of both cell types may be involved in their interaction. Other experiments with peripheral blood T cells and with a number of T cell lines provided some, but not conclusive, evidence for the possibility that adhesion to surface PS on endothelial cells may be a feature of double-positive $\text{CD4}^+/\text{CD8}^+$ T cells.

2. Materials and methods

2.1. Materials

Factor II (prothrombin), thrombin, factor Xa, gelatin, hematoxylin (Gill's No. 1), Histopaque 1077 and diaminobenzidine tetrahydrochloride (DAB) were from Sigma (Poole, Dorset, UK). Factor V was from Diagnostic Reagents Ltd. (Thame, Oxon, UK). Chromogenic substrate S-2238 (*H*-D-phenyl-

alanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride) was from Chromogenix AB (Mölnådal, Sweden). 5-Chloromethylfluorescein diacetate (CMFDA) and fluo-3-acetoxymethyl ester (fluo-3 AM) were from Molecular Probes Europe BV (Leiden, The Netherlands). Ionophore A23187 free acid, 2,5-di-*tert*-butyl-1,4-benzohydroquinone (BHQ), 4-bromo-A23187 and TNF- α were from Calbiochem–Novabiochem (Nottingham, UK). Recombinant annexin V, annexin V–FITC and propidium iodide (PI) were from Pharmingen (Cowley, Oxford, UK). Annexin V was purified to homogeneity ($M_r = 33\,000$) from chicken liver [15]: the protein (1100 nM) completely abolished the prothrombinase activity of sonicated vesicles of PS (0.25 μM) (not shown). Culture medium 199 with or without phenol red, RPMI 1640 medium, foetal bovine serum (FBS), penicillin, streptomycin and glutamine were from Sigma (Poole, Dorset, UK). Dulbecco's modified Eagle's medium (DMEM-HAT) containing 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine and 25 mM glucose was from Gibco BRL-Life Technologies (Paisley, UK). Lipid-free foetal calf serum was from Labtech International (Uckfield, Sussex, UK). Mouse anti-human monoclonal antibodies used to characterise HUVEC and ECV304 cells were as follows: anti-CD31, anti-CD62P (AK-4) and anti-CD106 (51-10C9) from Serotec (Kidlington, Oxford, UK), anti-CD54 (HA58) from Pharmingen (Cowley, Oxford, UK), MOPC-21 from Sigma (Poole, Dorset, UK); anti MHC-1 (W6/32) was a gift from Dr G.W. Butcher (Babraham Institute). Mouse anti-human monoclonal antibodies from Dako (Ely, Cambridge, UK) used to characterise purified human peripheral blood T lymphocytes and other T cell lines were as follows: anti-CD2 (MT910), anti-CD3 (UCHT1), anti-CD4 (MT310), anti-CD8 (DK25) and anti-CD45 (T29/33). Horseradish peroxidase-conjugated rat anti-mouse IgG1 (A85-1) and IgG2a (R19-15) monoclonal antibodies were from Serotec and Pharmingen. 'Red-Out', murine monoclonal antibody was from Robbins Scientific Corp. CA, USA. Diff-Quick was from Gamidor (Abingdon, Oxford, UK). DPX mounting medium was from BDH (Poole, Dorset, UK).

2.2. Endothelial cells and T cells

Human umbilical vein endothelial cells (HUVECs) were from TCS Biologicals (Buckingham, UK). Human umbilical cord ECV304 endothelial cells were a gift from the late Professor Irving B. Fritz. They were cultured (passage numbers 149–159) in medium 199 containing 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine at 37°C in 5% CO₂. Human EA.hy926 endothelial cells [16] were a gift from Dr C.-J.S. Edgell (University of North Carolina) and were cultured (passage numbers 232–240) in DMEM-HAT medium containing 10% (v/v) FBS, antibiotics and glutamine as above. 12×10^4 cells/ml (ECV304 and EA.hy926) were seeded into each well of a 24-well plate (2 cm²/well, Limbro plate) and cultured for 48 h prior to assay. Jurkat (E6.1) (human leukaemic T cell lymphoblast) and HPB-ALL (human acute lymphoblastic leukaemia T cell) cell lines were a gift from Dr D.R. Alexander (Babraham Institute). Other human T cell lines: D1.1 was from ATCC (American Type Culture Collection, Manassas, VA, USA), ACC282 (Jurkat sub-clone: human T cell leukaemia) was from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and MOLT-4 (acute T lymphoblastic leukaemia) was from ECACC (European Collection of Cell Culture, Salisbury, UK). T cell lines were cultured in RPMI 1640 medium containing 10% (v/v) FBS (5% FBS for HPB-ALL cells), antibiotics and glutamine as above.

Human peripheral T lymphocytes were prepared from freshly-drawn blood from healthy, adult donors by a method based on the protocol (Sigma) for Histopaque 1077. Briefly, platelets were removed by defibrination in roller tubes containing glass beads (2–3 mm diameter), 'Red-Out' murine monoclonal was used to remove erythrocytes, and polymorphonuclear leukocytes were removed by density gradient centrifugation with Histopaque 1077. Lymphocytes were finally resuspended in platelet-poor plasma (collected plasma that was centrifuged at 3400 rpm for 20 min) and used for adhesion assays after further washing within 1 h. The cells were roughly spherical with no evidence of shape changes, and they were 100% viable as judged by trypan blue exclusion. The purity of the cell preparations was determined with a Coulter

counter and by cytochemical staining with Diff-Quick, and the expression of cell surface markers was monitored by flow cytometry with monoclonal antibodies. The purest preparation contained 91.3% T lymphocytes, $0.7 \pm 0.2\%$ monocytes and $7.5 \pm 0.4\%$ neutrophils, with very low contamination by erythrocytes and platelets. 88% of the cells were positive for CD3, 47% for CD4 and 34% for CD8. Less than 1% of the cells were positive for CD14 (monocytes) and less than 4% for CD19 (B cells).

2.3. Surface markers on endothelial and T cells

HUVEC or ECV304 cells were passaged (passage 2–3 for HUVECs and passage 148–157 for ECV304) and grown to confluence on coverslips which had been coated overnight with 1% gelatin at room temperature. Confluent cell monolayers were stimulated with 30 nM thrombin for 10 min at 37°C, or 50–100 ng/ml of TNF-α for 4–6 or 16–18 h. After stimulation, the monolayers were washed in phosphate-buffered saline (PBS) and fixed in ice-cold 100% acetone for 3–5 min. Acetone was removed by washing with PBS, and the monolayers were incubated with mouse anti-human monoclonal antibodies (primary antibody) in PBS containing 1% human serum albumin (PBS-HSA) for 1 h at room temperature. After removal of unbound primary antibody by washing, monolayers were incubated with a second monoclonal antibody, horseradish peroxidase-conjugated rat anti-mouse IgG1 or IgG2a (1:100 dilution), in PBS-HSA for 1 h at room temperature. The monolayers were washed, and DAB was then added to develop the desired intensity of staining. The coverslips were counterstained with hematoxylin and mounted with DPX. For positive and negative controls, MHC-1 and MOPC-21 were used as primary antibodies under identical conditions to those described above.

Expression of cell surface markers on peripheral blood T lymphocytes and T cell lines was monitored by flow cytometry with fluorescent conjugated monoclonal antibodies. Prior to labelling, the cells were washed twice in cold PBS-HSA and labelled with appropriate FITC and RPE antibodies at room temperature in the dark for 15 min. Samples were then fixed with 1% formaldehyde and analysed using a standard Argon laser source on a Beckman Coulter

Epics Elite flow cytometer. Analysis regions were set using matched isotype controls.

2.4. Prothrombinase assay

The production of thrombin (prothrombinase activity) was used as an indirect assay for PS exposed on cell surfaces. Endothelial cells were treated with agonist for 10 min in medium 199 without phenol red containing 0.25% HSA (medium A) and the supernatant medium (first supernatant: S1) was removed. Clotting factors and prothrombin were added to it, and aliquots withdrawn over a period of 12 min to determine the prothrombinase activity of microvesicles released from the cells as described previously [17]. The final concentrations of reagents in the assay were 0.036 U/ml factor Xa, 1.09 nM factor Va, 0.36 μ M prothrombin, 1.8 mM Ca^{2+} and 150 μ M S-2238. Immediately after removing the first supernatant, the cells were washed and fresh medium A was added followed by clotting factors and prothrombin. Aliquots were withdrawn (second supernatant: S2) over 12 min to determine the thrombin generated as a measure of the prothrombinase activity of the treated cells and microvesicles additionally released from the cells.

Flow cytometry with FITC–annexin V was not used to determine the surface exposure of PS on endothelial cells because it requires the use of proteolytic enzymes or mechanical scraping to detach the cells from the culture dish. In varying degrees, these treatments might activate the cells (leading to surface exposure of PS), and/or damage the plasma membrane (making PS accessible in the inner monolayer).

2.5. Cytosolic Ca^{2+}

ECV304 and EA.hy926 cells were plated on 22 mm diameter coverslips for Ca^{2+} imaging in an inverted fluorescent Nikon Diaphot microscope. The cells were incubated with 5 μ M fluo-3 AM for 30 min at 37°C in 5% CO_2 in medium 199 without phenol red (medium B) supplemented with 10% heat-inactivated foetal calf serum. They were then gently washed three times in pre-warmed medium A to remove excess fluo-3. The coverslip was placed in a thermostatically controlled bath on the microscope

stage, and the cells were illuminated with a xenon UV lamp (excitation 490 nm). Emitted light at 520 nm was collected via an attached image intensified charge-coupled device camera using a MagiCal system and TARDIS software (Joyce-Loebel). Although great care was taken to avoid directly projecting media onto the cells, the loading procedure could lead to a rise in cytosolic Ca^{2+} resulting from mechanical shear stress on the cells whilst washing and manipulating the coverslip. This was particularly apparent in preliminary experiments with fura-2. After placing the cells on the microscope stage, they were therefore left for 10 min to allow cytosolic Ca^{2+} to return to basal levels after the loading procedure. For image acquisition, eight images were acquired that were averaged to produce a single image every 0.9 s. This procedure reduced background noise generated from stray light. Images were analysed as a self-ratio (F/F_0) (fluorescent intensity at time t /fluorescent intensity at zero time) to monitor changes in the concentration of cytosolic Ca^{2+} .

2.6. Adhesion of T cells

T cells and endothelial cells were cultured, the T cells labelled with CMFDA, and the adhesion of T cells to monolayers of endothelial cells was assessed qualitatively (by fluorescence microscopy) and quantitatively (by spectrofluorometry) as previously described [17]. After treatment with thrombin, or annexin V followed by thrombin, the endothelial cells were washed prior to the addition of Jurkat cells.

2.7. LDL and oxidised LDL (ox-LDL)

LDLs were prepared from healthy volunteers, and minimally oxidised or fully oxidised, as described previously [18]. LDL preparations were stored under nitrogen and used within 3–4 days of preparation. Control experiments with the final dialysate showed that diethylenetriaminepenta-acetic acid, used to protect LDL against oxidation during LDL preparation, did not induce a prothrombinase response in ECV304 cells.

2.8. Flow cytometry

HUVEC or ECV304 cells were grown to confluent

monolayers in 25 cm² flasks for 48 h, washed in pre-warmed medium B, and incubated with 30 nM thrombin in medium A for 10 min. Jurkat cells were washed in medium B to remove serum and suspended in medium A at a density of 4 × 10⁶ cells/ml. The endothelial cells were washed to remove thrombin; Jurkat cells were then added at 20 × 10⁶ per flask and incubated for 10 or 20 min at 37°C. Non-adherent Jurkat cells were removed in washes of RPMI 1640 culture medium containing 10% heat-inactivated foetal calf serum and 2 mM Ca²⁺ (medium C). Adherent Jurkat cells were detached by treatment with 5 ml of Hank's balanced salt solution containing 1 mM EDTA for a few seconds, and resuspended in medium C. Collected Jurkat cells were washed twice in cold PBS, and resuspended in 'binding buffer' (Pharmlingen) at a density of 1–5 × 10⁶ cells/ml. 5 µl of annexin V-FITC solution and 10 µl of PI (50 µg/ml) were added to 100 µl of cell suspension. The cells were gently mixed and incubated for 15 min at room temperature in the dark. Binding was terminated by adding 400 µl binding buffer, and the cells were then analysed immediately by flow cytometry

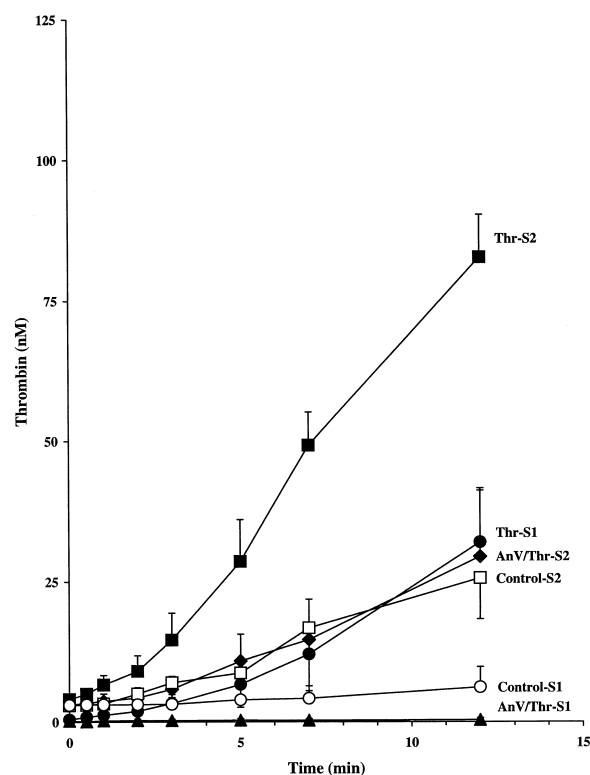
on a FACSCalibur (Becton Dickinson, San Jose, CA, USA). Each data file of 10 000 events was analysed with CELLQuest software. Annexin V-FITC and PI were detected through 530/30 and 585/42 band pass filters, respectively. For pretreatment with unlabelled annexin V, collected Jurkat cells were washed twice in cold PBS, resuspended in binding buffer at a density of 2–10 × 10⁶ cells/ml, and 50 µl of cell suspension was added to 50 µl of annexin V solution (1 mg/ml; recombinant or chicken annexin V) containing 2–4 mM Ca²⁺. After incubation for 15 min at room temperature, annexin V-FITC and PI were added as above and the incubation was continued for a further 15 min at room temperature in the dark prior to terminating the binding.

3. Results

3.1. Exposure of PS on thrombin-treated HUVECs and PS-dependent adhesion of Jurkat cells

The development of a procoagulant response (prothrombinase activity), which provides an indirect as-

Fig. 1. Procoagulant response of HUVECs treated with thrombin. HUVECs were treated with 30 nM thrombin for 10 min at 37°C with or without 1100 nM purified chicken annexin V, and the supernatant medium (first supernatant: S1) was then removed. Clotting factors and prothrombin were added to it, and aliquots withdrawn over a period of 12 min to determine the thrombin produced by the prothrombinase activity of microvesicles that had been released from the cells. Immediately after removing the first supernatant, the cells were washed and fresh HSA medium was added followed by clotting factors and prothrombin. Aliquots were withdrawn (second supernatant: S2) over 12 min to determine the thrombin generated. This subsequent procoagulant response represented the prothrombinase activity of the treated cell monolayers and additionally released microvesicles. ○, control-S1 (first supernatant): untreated cells. ●, Thr-S1 (first supernatant): thrombin-treated cells. □, control-S2 (second supernatant): untreated cells. ■, Thr-S2 (second supernatant): thrombin-treated cells. ▲, AnV/Thr-S1 (first supernatant): annexin V-pretreated, thrombin-treated cells. ◆, AnV/Thr-S2 (second supernatant): annexin V-pretreated, thrombin-treated cells. The data points are mean values ± S.E.M. for three independent experiments, in each of which three replicate measurements were made of the thrombin generated at each time point. (Thr-S1 refers to thrombin generated additionally to the 30 nM thrombin already present in the first supernatant).



say for exposed PS, has previously been shown to be highly specific for PS [19,20]. HUVECs were treated with 30 nM thrombin for 10 min and the supernatant medium (first supernatant: S1) was removed to determine the prothrombinase activity (thrombin production) of microvesicles released from the cells. The cells were then washed, fresh medium was added followed by clotting factors and prothrombin, and aliquots were withdrawn (second supernatant: S2) to determine the prothrombinase activity of the treated cells and microvesicles additionally released from the cells.

The prothrombinase activity of microvesicles in the first supernatant from control cells that were incubated without thrombin was very low (Fig. 1: control-S1). The addition of clotting factors and prothrombin to monolayers of endothelial cells results in the formation of thrombin [21], and higher levels of thrombin were consequently observed in the second supernatant on subsequently incubating the control cells with clotting factors and prothrombin (Fig. 1: control-S2). Procoagulant microvesicles were released into the first supernatant from the thrombin-treated cells (Fig. 1: Thr-S1). The highest prothrombinase activity was observed with the second supernatants taken from thrombin-treated cells that were

subsequently incubated with clotting factors and prothrombin (Fig. 1: Thr-S2).

Annexin V binds with high specificity to PS via a Ca^{2+} -bridging mechanism, and this property has been employed in many studies as a probe for monitoring the surface exposure of PS. The addition of annexin V (1100 nM) 10 min before adding thrombin to HUVECs resulted in a lower prothrombinase activity of the first supernatant, confirming the presence of PS on the surface of the microvesicles (Fig. 1: AnV/Thr-S1). Since this procedure also decreased the procoagulant response when the thrombin-treated cells were incubated with clotting factors and prothrombin (Fig. 1: AnV/Thr-S2), surface PS must have been present on the surface of the cells where it bound annexin V. The bound annexin V then inhibited the formation of thrombin when the cells were incubated with clotting factors and prothrombin.

Jurkat T cells were labelled with the cytoplasmic fluorophore, 5-chloromethylfluorescein, and incubated at 37°C for 20 min with a monolayer of HUVECs that had previously been incubated with 30 nM thrombin for 10 min. Very few Jurkat cells adhered to untreated HUVECs (Fig. 2A) by comparison with the number that adhered to the thrombin-

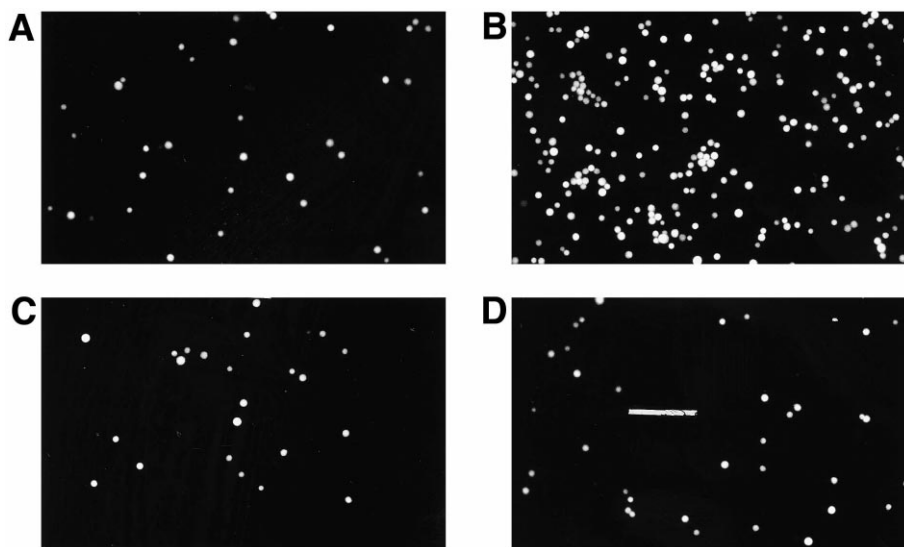


Fig. 2. Fluorescent micrographs of Jurkat cells, labelled with CMFDA, adhering to monolayers of HUVECs. The two cell types were treated as described in Section 2. (A) untreated HUVECs, (B) HUVECs treated with 30 nM thrombin, (C) HUVECs pre-incubated with purified chicken annexin V (1100 nM) in medium A for 10 min in 5% CO_2 before adding thrombin, (D) thrombin-treated HUVEC cells incubated with Jurkat cells in medium A containing 2.5 mM EGTA. (A–D) are at the same magnification: bar = 130 μm .

Table 1
Procoagulant response of endothelial cells treated with thrombin, ionophore A23187 and BHQ

Protocol	Cell line	Agonists (nM thrombin generated)		
		Thrombin	A23187	BHQ
First supernatant (S1) from treated cells	ECV304	2.1 ± 1.4 (n = 3)	20.6 ± 4.2 (n = 5)	0.3 ± 0.1 (n = 3)
	EA.hy926	4.1 ± 1.5 (n = 3)	15.0 ± 2.4 (n = 5)	0.2 ± 0.2 (n = 4)
Second supernatant (S2) from treated cells	ECV304	17.9 ± 2.0 (n = 3)	69.5 ± 7.6 (n = 5)	45.8 ± 10.3 (n = 3)
	EA.hy926	17.8 ± 7.0 (n = 3)	80.6 ± 7.1 (n = 5)	23.9 ± 12.3 (n = 4)

Cells were treated with 30 nM thrombin, 10 µg/ml A23187 or 50/100 µM BHQ (ECV304/EA.hy926 cells) for 10 min at 37°C to generate the first and second supernatants (S1 and S2) as described in Section 2. Mean values ± S.E.M. are given for the additional nM thrombin generated at the 7 min time points in prothrombinase assays for the treated cells, relative to the corresponding control values for the untreated cells, are from the number of independent experiments shown in each of which three replicate measurements were made.

treated endothelial cells (Fig. 2B). Jurkat cell adhesion to HUVECs that had been pre-incubated with 1100 nM chicken annexin V before the addition of thrombin was greatly decreased (Fig. 2C), showing that the observed adhesion was PS-dependent.

3.2. PS-dependent adhesion of Jurkat cells to agonist-treated endothelial cell lines

Cell lines, which behave more reproducibly than cells from umbilical veins, are widely used as models for endothelial cells. ECV304 and EA.hy926 human cell lines were therefore employed for quantitative experiments on the PS-dependent adhesion of Jurkat cells to thrombin-treated endothelial cells.

The ECV304 cells used were shown previously to stain positively for *Ulex europaeus* agglutinin I, a marker for vascular endothelium in human tissues, but they contained very few Weibel-Palade bodies [17]. In the present work, we compared the expression of four endothelial cell surface markers on

ECV304 cells with their expression on HUVECs. Untreated, thrombin-stimulated and TNF- α -stimulated ECV304 cells were all positive for the immunoglobulin superfamily adhesion molecules, ICAM-1 (CD54) and VCAM-1 (CD106), but these markers were strongly expressed on HUVECs only after stimulation with TNF- α . PECAM-1 (CD31) was not expressed on untreated, thrombin-stimulated or TNF- α -stimulated ECV304 cells, but was present on HUVECs that were stimulated with TNF- α , while P-selectin (CD62P) was expressed weakly on both cell types. It is thus apparent that ECV304 cells resemble but are not identical with HUVECs.

The procoagulant responses of ECV304 and EA.hy926 cells to thrombin, ionophore A23187 and BHQ are shown in Table 1. Each of these agonists increases cytosolic Ca²⁺. Thrombin, acting via Ins-(1,4,5)P₃ primarily mobilises Ca²⁺ from intracellular stores [22], A23187 primarily allows the entry of external Ca²⁺ [23], while BHQ inhibits the 97 kDa isoform of the Ca²⁺-ATPase of the endoplasmic retic-

Table 2
Effect of annexin V on the procoagulant response of endothelial cells treated with BHQ

Protocol	Thrombin generated (nM)	
	ECV304 cells	EA.hy926 cells
Second supernatant (S2): BHQ-treated cells	45.8 ± 10.3 (n = 3)	23.9 ± 12.3 (n = 4)
Second supernatant (S2): annexin V-pretreated, BHQ-treated cells	14.0 ± 7.0 (n = 3)	(n = 4) ^a

Endothelial cells were treated with 50/100 µM BHQ (ECV304/EA.hy926 cells), without or with 1100 nM purified chicken annexin V as described previously [17], to generate the second supernatants (S2) as described in Section 2. Mean values ± S.E.M. of the additional nM thrombin generated at the 7 min time points in the prothrombinase assays for the treated cells, relative to the corresponding control values for the untreated cells, are shown. The data are from three or more independent experiments, in each of which three replicate measurements were made.

^aProthrombinase activity not significantly different from corresponding control, untreated cells.

ulum and inhibits the re-uptake into internal stores of leaked Ca^{2+} [24]. As the treated cells very rarely became permeable to trypan blue, the procoagulant responses induced by the agonists were not the result of cell lysis. The high prothrombinase activities of the first supernatants (S1) from cells treated with A23187 showed that more PS-bearing microvesicles were immediately released into the medium by the ionophore than by the other two agonists. Very few procoagulant microvesicles were released into the first supernatants by BHQ. There was nevertheless a marked procoagulant response when clotting factors and prothrombin were added to BHQ-treated cells after removing the first supernatant (Table 1). This response (S2), which represents the prothrombinase activity of the treated cells themselves, and microvesicles additionally released from the cells, was inhibited by adding annexin V (1100 nM) to ECV304 cells 10 min before adding BHQ (Table 2). PS was therefore present on the surface of BHQ-treated cells where it bound the added annexin.

Jurkat cells adhered to ECV304 cells treated with thrombin, ionophore A23187 or BHQ. With all three agonists, the number of adhering Jurkat cells was decreased by pretreating the endothelial cells with 1100 nM annexin V prior to adding the agonist, showing that the adhesion was PS-dependent (Table 3). A23187 was the least effective of the three agonists in inducing Jurkat cell adhesion. This was presumably because a high proportion of the PS that was exposed on the surface of the cells treated with A23187 was immediately released from the cells into the medium in microvesicular form, as shown in Table 1. Comparable observations were made with EA.hy926 endothelial cells treated with thrombin, A23187 or BHQ although, in each case, the number of Jurkat cells adhering to the treated EA.hy926 cells

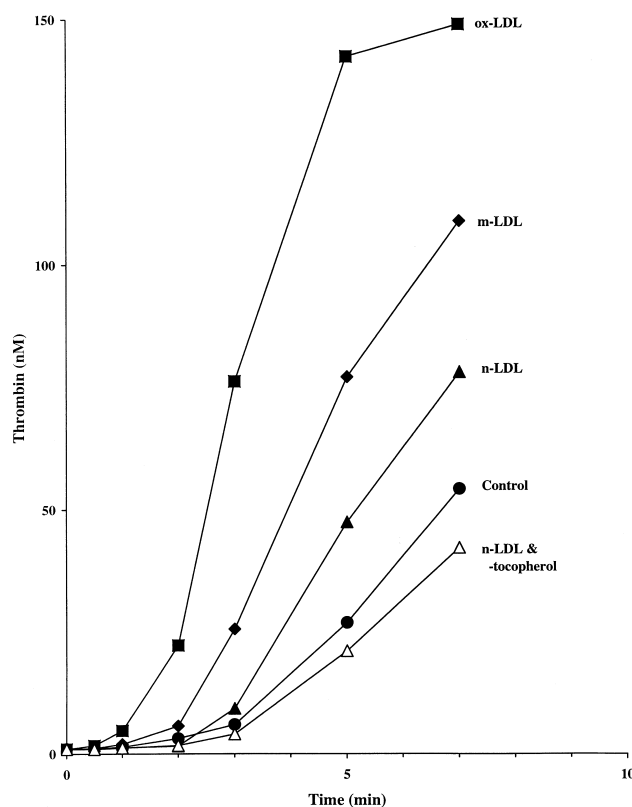


Fig. 3. Procoagulant responses of ECV304 cells treated with LDL. ECV304 cells were passaged and grown to confluence (24-well plate) for 46 h in medium 199 containing 10% regular, heated foetal calf serum as described in Section 2. The culture medium was removed, and the cells incubated at 37°C for 2 h in fresh medium 199 containing 10% heated, lipid-free foetal calf serum, and 50 $\mu\text{g}/\text{ml}$ of LDL (prepared as described in the text). The cell monolayers were washed prior to assays for prothrombinase activity. ●, no agonist (lipid-free foetal calf serum). ▲, n-LDL. △, n-LDL prepared from plasma containing 0.05 mg/ml of added α -tocopherol LDL. ◆, m-LDL. ■, ox-LDL. The data points are mean values of three replicate measurements.

Table 3

Adhesion of Jurkat T cells to ECV304 cells treated with thrombin, A23187 and BHQ

Protocol	Adherent Jurkat cells ($\times 10^{-3}$) (per well)		
	Thrombin 30 nM	A23187 10 $\mu\text{g}/\text{ml}$	BHQ 100 μM
No agonist	59 ± 7 ($n=7$)	38 ± 7 ($n=4$)	38 ± 7 ($n=4$)
Agonist treatment	228 ± 10 ($n=7$)	48 ± 20 ($n=3$)	163 ± 10 ($n=6$)
Annexin V pretreatment followed by agonist treatment	84 ± 10 ($n=7$)	18 ± 9 ($n=3$)	87 ± 20 ($n=6$)

ECV304 cells were treated with agonist for 10 min at 37°C with or without 1100 nM purified chicken annexin V. Jurkat cells were cultured, labelled with CMFDA and their adhesion to monolayers of ECV304 cells assessed spectrofluorometrically as previously described [17]. Mean values are shown \pm S.E.M. for n separate experiments (each of which was done in duplicate).

was approximately an order of magnitude less than to agonist-treated ECV304 cells. However, annexin V pretreatment was more effective with EA.hy926 cells than with ECV304 cells in inhibiting Jurkat cell adhesion.

3.3. Adhesion of Jurkat T cells to ECV304 cells treated with ox-LDL

Several publications, reviewed in [25], have indicated that ox-LDL may contribute to the rolling of leukocytes on the endothelium. Recently, treatment of HUVECs with ox-LDL, but not native LDL (n-LDL), for 5 min or 24 h was found to increase intracellular Ca^{2+} and affect cytoskeletal actin in HUVECs [26], while in platelets, ox-LDL (but not n-LDL) increases the level of intracellular calcium by directly inhibiting the plasma membrane Ca^{2+} -ATPase [27].

In view of these reports, we have investigated whether ox-LDL induces a procoagulant response in cultured endothelial cells, indicative of the surface exposure of PS, and whether this leads to a PS-dependent adhesion of Jurkat T cells. ECV304 cells were incubated for different lengths of time at 37°C

Table 4
Adhesion of Jurkat cells to ECV304 cells incubated with LDL

Protocol	Adherent Jurkat cells ($\times 10^{-3}$) (per well)
No agonist	2.7
n-LDL	2.9
n-LDL and annexin V	2.3
n-LDL plus α -tocopherol	2.5
n-LDL plus α -tocopherol and annexin V	2.7
m-LDL	3.8
m-LDL and annexin V	3.8
Ox-LDL	3.2
Ox-LDL and annexin V	3.4

ECV304 cells were passaged and grown to confluence (24-well plate) for 46 h in culture medium 199 containing 10% regular, heated foetal calf serum as described in Section 2. The culture medium was removed, and the cells incubated at 37°C for 2 h in fresh medium 199 containing 10% heated, lipid-free foetal calf serum, and 50 $\mu\text{g}/\text{ml}$ LDL (prepared as described in the text), with or without 1100 nM purified chicken annexin V. The cell monolayers were washed prior to assays for adhesion. Mean values are shown for eight determinations (four determinations in each of two experiments).

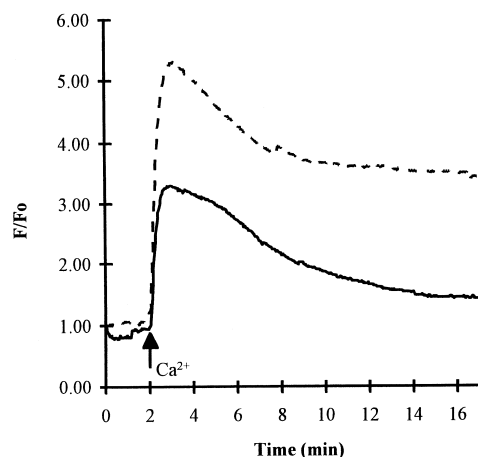


Fig. 4. Kinetics of changes in the fluorescence self-ratio F/F_0 for EGTA-pretreated cells. ECV304 cells were pre-incubated at 37°C for 10 min in medium A (1.8 mM Ca^{2+}) to which 5 mM EGTA had been added. 5 mM Ca^{2+} was then added, and the change in (F/F_0) for each of the cells assessed with time. Each trace is representative of the total population of 51 ECV304 cells (broken line) and 41 EA.hy926 cells (solid line) that were imaged as described in Section 2.

with 50 $\mu\text{g}/\text{ml}$ of LDL or modified LDL, with or without annexin V (1100 nM), in culture medium 199 containing 10% lipid-free foetal calf serum. After washing the cells, the prothrombinase activity of the second supernatant (S2) was determined. Treatment of ECV304 cells with extensively ox-LDL (50 $\mu\text{g}/\text{ml}$) for 10 min was without effect on the prothrombinase activity whereas, after 11 and 24 h of incubation, prothrombinase activity was induced equally by both n-LDL and ox-LDL. With 2 h of incubation, increasing procoagulant responses were observed with cells that were treated with n-LDL, mildly ox-LDL (m-LDL) and ox-LDL (Fig. 3). Similar data were obtained with 100 $\mu\text{g}/\text{ml}$ of LDL preparations (not shown). As might be anticipated, the addition of α -tocopherol (0.05 mg/ml) to plasma prior to isolation of the LDL decreased the ability of n-LDL to induce a procoagulant response (Fig. 3). None of the cells treated with preparations of LDL became permeable to trypan blue. Their procoagulant activity in the prothrombinase assay was therefore not the result of cell lysis.

Compared with control ECV304 cells, there was an increase in the number of Jurkat cells adhering to ECV304 cells that had been incubated with ox-LDL, and a larger increase with m-LDL. However, neither of these increases in Jurkat cell adhesion re-

sulting from LDL treatment of the ECV304 cells was inhibited by pretreatment of the endothelial cells with annexin V (Table 4), indicating that it was not primarily mediated by PS.

3.4. Effects of EGTA and characterisation of Jurkat cell adhesion

By comparison with the response to thrombin alone, there was an increase in the number of adhering Jurkat cells when they were added (in the presence of Ca^{2+}) to ECV304 cells which had been treated simultaneously with 30 nM thrombin and 5 mM EGTA (Table 5). Monolayers of EA.hy926

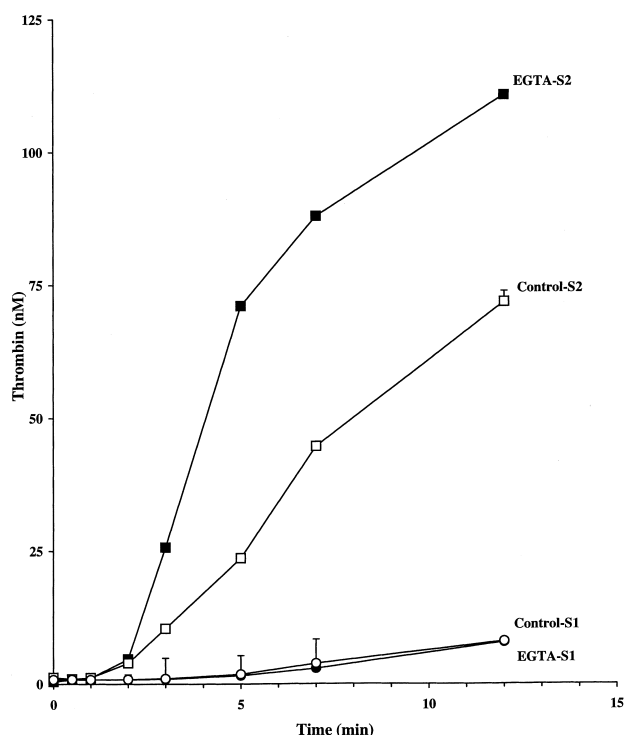


Fig. 5. Procoagulant response of ECV304 endothelial cells treated with EGTA. Cells were treated with 1 mM EGTA for 10 min at 37°C, and prothrombinase activities were determined as described in Section 2. ○, first supernatant: control cells. ●, first supernatant: 1 mM EGTA-treated cells. □, second supernatant: control cells. ◇, second supernatant: 1 mM EGTA-treated cells. The data points are mean values \pm S.E.M. for two/four independent experiments for the first and second supernatants, respectively, in each of which three replicate measurements were made of the thrombin generated at each time point. Standard errors which were smaller than the symbol points on the graph are not shown.

Table 5

Effects of EGTA on the adhesion of Jurkat cells to ECV304 cells

Protocols		Adherent Jurkat cells ($\times 10^{-3}$) (per well)
ECV304 cells	Jurkat cells	
No agonist	–	37 \pm 20
No agonist	EGTA	32 \pm 30
Thrombin	–	127 \pm 50
Thrombin and EGTA	–	234 \pm 20
Thrombin	EGTA	56 \pm 20
Thrombin and EGTA	EGTA	36 \pm 5

ECV304 cells were treated with 30 nM thrombin, with or without 5 mM EGTA, for 10 min at 37°C. Jurkat cells were cultured, labelled with CMFDA, and their adhesion in the presence or absence of 5 mM EGTA to washed monolayers of ECV304 cells was assessed spectrofluorometrically as previously described [17]. Mean values are shown \pm S.E.M. for three separate experiments (each of which was done in duplicate).

cells responded similarly. Experiments were undertaken to determine whether EGTA increased cytosolic Ca^{2+} and rendered the endothelial cells procoagulant, thereby resulting in Jurkat cell adhesion. Cultures of ECV304 cells were incubated with 5 mM EGTA. When 5 mM Ca^{2+} was subsequently added, an immediate increase in cytosolic Ca^{2+} was observed with both cell lines (Fig. 4). EA.hy926 cells responded similarly. An increased procoagulant response (supernatant S2) relative to control ECV304 cells was also observed following incubation of the cells for 10 min in medium A (1.8 mM Ca^{2+}) containing added 1 mM EGTA. This presumably resulted from an influx of Ca^{2+} during the prothrombinase assay (Fig. 5). (The relatively high prothrombinase activity of the second supernatant (S2) from the control cells was due to the known formation of thrombin [21] when clotting factors and prothrombin are added to monolayers of endothelial cells). Very few procoagulant vesicles (supernatant S1, assayed in the presence of 3 mM Ca^{2+}) were released from the EGTA-treated cells (Fig. 5), and control experiments showed that the low procoagulant activity of supernatant S1 was not due to inhibition of the prothrombinase assay.

By contrast to the above observations, the presence of 5 mM EGTA markedly inhibited cell–cell

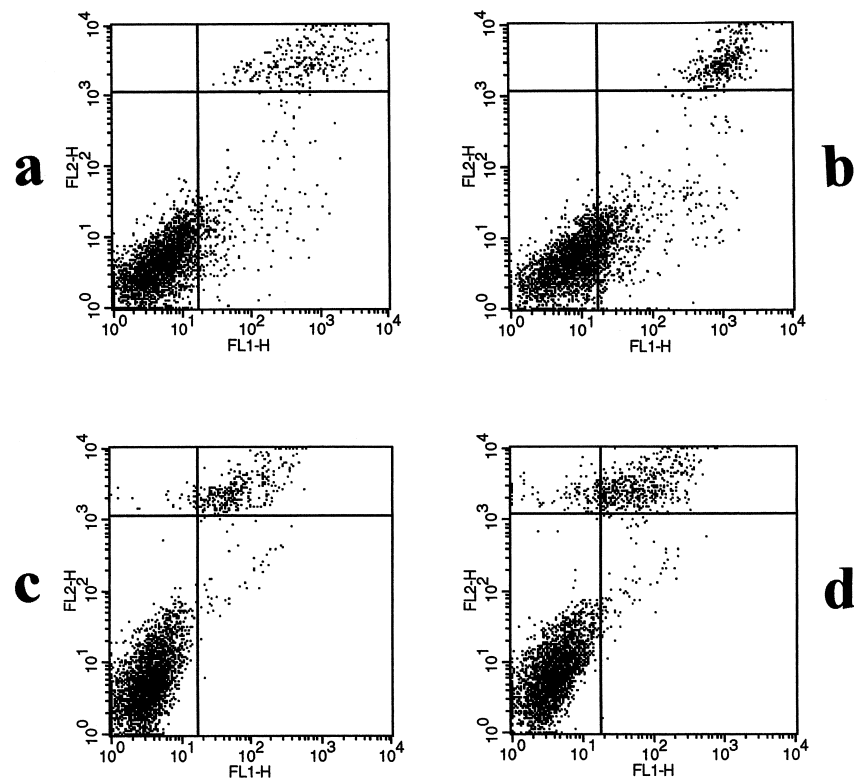


Fig. 6. Exposure of surface PS on Jurkat cells after adhesion to ECV304 cells. Jurkat cells were prepared for flow cytometry and stained with annexin V–FITC and PI as described in Section 2. (a) Control Jurkat cells (treated with 1 mM EDTA), (b) Jurkat cells that had adhered to thrombin-treated (30 nM) ECV304 cells for 10 min at 37°C, and were then detached in 1 mM EDTA, (c) and (d) Jurkat cells as in (b) but which were pretreated with (unlabelled) recombinant annexin V, or unlabelled purified, chicken annexin V, respectively. (a) and (b) are representative of 10 separate experiments, while (c) and (d) are representative of two and four experiments, respectively.

adhesion when Jurkat cells were added to monolayers of thrombin-treated ECV304 cells (Table 5). The adhesion of Jurkat cells to thrombin-treated HUVECs was similarly inhibited by the presence of 2.5 mM (compare Fig. 2D with Fig. 2B) and 5 mM EGTA. The adhesion of Jurkat cells to thrombin-treated endothelial cells is therefore Ca^{2+} -dependent.

The ability of Jurkat cells labelled with CMFDA to adhere to PS-coated glass coverslips, compared with uncoated coverslips, was investigated by fluorescence microscopy. No increased adhesion of the cells to PS-coated glass was observed, and it appears that the PS-dependent adhesion to thrombin-treated endothelial cells may involve an essential, ancillary factor(s) on the endothelial cell surface, or that there are topological or conformational differences between PS deposited on glass compared with PS that is exposed on the surface of endothelial cells.

3.5. Surface exposure of PS following cell–cell contact

A phospholipid scramblase has been reported to be constitutively high in Jurkat cells [28]. In our hands, however, the prothrombinase activity of untreated Jurkat cells at a density of 3×10^5 cells/ml was negligible, although there was some activity with higher cell densities (6×10^5 and 12×10^5 cells/ml). We therefore employed flow cytometry with annexin V–FITC and PI to investigate the effects of contact with endothelial cells on the surface exposure of PS on Jurkat cells. In the absence of previous contact with ECV304 cells, the majority of the Jurkat cells were not stained by annexin V–FITC and were impermeable to PI, and they thus appeared in the lower left quadrant of Fig. 6a. Surface PS was present only on a few PI-impermeable cells (positive

staining with FITC; lower right quadrant of Fig. 6a). When Jurkat cells were allowed to adhere to thrombin-treated ECV304 cells for 10 min and then detached by treatment with 1 mM EDTA for a few seconds (which did not release any ECV304 cells from the endothelial monolayer), there was an increased number of cells with PS on the surface which still excluded PI (lower right quadrant of Fig. 6b). Binding of annexin V–FITC to this population of cells was prevented by pretreating the detached Jurkat cells with (unlabelled) recombinant (Fig. 6c) or with purified chicken annexin V (Fig. 6d) prior to flow cytometry. Hence it is apparent that close apposition of the Jurkat cells and ECV304 cells resulted in surface exposure of PS on the T cells. Such an exposure of PS on Jurkat cells may actually be required for adhesion. Thus, whereas the adherent Jurkat cells exhibited an increased exposure of PS on the surface, flow cytometry in 10 separate experiments showed that the level of surface PS on the population of PI-impermeable Jurkat cells that failed to adhere to the thrombin-treated ECV304 cells in 10 min was no greater than on Jurkat cells that had not been in contact with the endothelial cells.

3.6. Peripheral blood T cells and T cell lines

In three separate experiments, it was found that, unlike Jurkat cells, the number of peripheral blood T cells that adhered to ECV304 endothelial cells was

not increased by the exposure of PS on the endothelial cell surface induced by thrombin.

In view of this, we have investigated a number of T cell lines for comparison with Jurkat cells. As with the Jurkat cells, treatment of ECV304 cells with thrombin increased the number of adhering HPB-ALL and MOLT-4 T cells. Both of these cell lines were double-positive for CD4 and CD8, while Jurkat E6.1 cells were strongly positive for CD8 and weakly positive for CD4 (Table 6). Annexin V inhibited the increase by varying extents: 90% with Jurkat E6.1 cells, 67% with HPB-ALL cells and 22% with MOLT-4 cells (Table 6). Although the number of adhering single-positive ACC282 cells (CD4⁺/CD8⁻) was markedly increased by the thrombin treatment, the increase was minimally inhibited (14%) by pretreatment of ECV304 cells with annexin V (1100 nM, 10 min). Adhesion of double-negative (CD4⁻/CD8⁻) D1.1 cells to ECV304 endothelial cells was not enhanced by treating the endothelial cells with thrombin (30 nM, 10 min). These findings are generally, but not completely, consistent with the possibility that adhesion to PS on the surface of thrombin-treated endothelial cells may be a feature of T cells that express both CD4 and CD8.

4. Discussion

PS that is exposed on the cell surface is known to

Table 6
Adhesion of T cell lines to ECV304 cells treated with thrombin

Protocol	Cell line				
	Jurkat E6.1	HPB-ALL	MOLT-4	ACC282	D1.1
	Adherent cells ($\times 10^{-3}$) (per well)				
No agonist	68 \pm 6	140 \pm 4	60 \pm 5	79 \pm 20	244 \pm 10
Thrombin	228 \pm 10	263 \pm 10	218 \pm 18	197 \pm 15	255 \pm 20
Annexin V followed by thrombin	84 \pm 10	180 \pm 8	184 \pm 10	180 \pm 15	238 \pm 19
CD marker	Cells bearing marker (%)				
CD3	80	87	89	72	86
CD4	7.9	88	40	44	0.6
CD8	98	88	69	0.7	0.1

ECV304 cells were treated with 30 nM thrombin, without or with 1100 nM purified chicken annexin V as described previously [17]. Cultured T cells were labelled with CMFDA, and their adhesion to monolayers of ECV304 cells was assessed spectrofluorometrically as previously described [17]. Mean values are shown \pm S.E.M. for three separate experiments for all data except for Jurkat E6.1 cells where the mean values \pm S.E.M. are for seven separate experiments (each experiment was done in duplicate). CD markers were assessed by flow cytometry with monoclonal antibodies.

mediate various cell–cell interactions. For example, surface PS on apoptotic lymphocytes and aged erythrocytes mediates their interactions with macrophages [9,10], and on tumour cells with monocytes [11]. However, although much information has been obtained on the protein-dependent interactions of leukocytes with endothelial cells, little consideration has been given to the possibility that phospholipids might also be involved. In initial work, we showed that human ECV304 cells exhibit a procoagulant response on treatment with thrombin, and that Jurkat T cells adhere to the thrombin-treated cells in a PS-dependent manner [17]. These experiments raised the possibility that trauma to endothelial cells which occurs *in vivo* to expose PS might contribute to the adhesion of T cells to endothelial cells, since perturbed endothelial cells express tissue factor and factor XII activator on the cell surface which can initiate the formation of thrombin at the site of perturbation [29]. This thrombin could induce a local procoagulant response that might be relevant, for example, to the formation of arterial microthrombi on arterial endothelial cells [30]. Local procoagulant responses in endothelial cells may also occur as a result of the continued, marked generation of thrombin that lasts for at least 2 weeks following balloon angioplasty of coronary arteries [31]. We have now further investigated the exposure of PS at cell surfaces, and the PS-dependent adhesion of T cells to endothelial cells, in experiments with HUVECs, two endothelial cell lines and peripheral blood lymphocytes.

The human ECV304 cell line, which we used as one of the culture models for human endothelial cells, was initially reported to be a line of spontaneously transformed HUVECs [32]. Very recently, during the course of our work, the DSMZ (German Culture Collection) reported that this cell line has the same DNA fingerprint as the T-24 cell line (human bladder carcinoma). DNA fingerprinting and multiplex PCR analysis by ECACC (European Collection of Cell Cultures) has further shown that ECV304 and T24/83 cells are derived from the same source and are fundamentally the same cell line. It is thought that ECV304 is the false line because T-24 was established in 1970 compared to 1984 for ECV304. However, ECV304 cells have been used by many investigators as a model for human endo-

thelial cells in a very large number of studies from which it is clear that the cells do express endothelial properties. Following the recent karyotyping analysis of ECV304 cells, the properties of this cell line have been reviewed in the context of work on HUVECs and other primary culture endothelial cells (W. Joan Abbot: manuscript in preparation). It has been concluded that ECV304 cells express a remarkably ‘endothelial’ phenotype, and that the cells have played and will continue to play an important role as a useful working endothelial model.

The surface markers found on ECV304 cells in the present investigation confirm the endothelial nature of the cells used in our laboratory for the present work. The other cell line which we have employed, EA.hy926, is a hybrid derived from HUVECs and a cell line (A549) from a human lung carcinoma [16]. In our experiments, ECV304 and EA.hy926 cells showed similar changes in cytosolic Ca^{2+} and procoagulant behaviour in response to thrombin, A23187, BHQ and EGTA. Furthermore, HUVEC and ECV304 cells exhibited similar procoagulant responses to thrombin. Jurkat T cells also adhered in a PS-dependent manner to agonist-treated HUVEC, ECV304 and EA.hy926 cells. These several findings indicate that the behaviour of ECV304 cells reported here is typical of endothelial cells.

Treatment of ECV304 and EA.hy926 cells with 5 mM EGTA, followed by a return to normal medium, resulted in an influx of Ca^{2+} and an increase in adhering Jurkat cells. This entry of external Ca^{2+} is consistent with the sustained Ca^{2+} inward current found when the Ca^{2+} pools of mast cells were depleted by treating the cells with EGTA [33], and with a considerable increase in cytosolic Ca^{2+} seen in Jurkat cells when extracellular Ca^{2+} was complexed with excess EGTA, followed by a subsequent re-addition of Ca^{2+} [34].

Ox-LDL is present in extracts of human atherosclerotic lesions [35], and it has been shown to increase cytoplasmic Ca^{2+} in endothelial cells [26]. We therefore investigated the potential ability of ox-LDL to induce the surface exposure of PS on endothelial cells and the PS-dependent adhesion of lymphocytes. Ox-LDL is cytotoxic to a number of cell types, including endothelial cells, and it has been suggested that lesion prone areas may be more sensitive to the toxicity of ox-LDL than quiescent cells [36]. Never-

theless, the exposure of PS on the surface of ECV304 cells treated with oxidised preparations of LDL for 2 h in our experiments (Table 4) was not accompanied by permeability to trypan blue. We were encouraged to investigate the possibility that ox-LDL might induce a PS-dependent adhesion to T cells by the observation that treatment of endothelial cells with ox-LDL increased the adhesion of U937 monocyte-like cells, without involving P-selectin or E-selectin [37]. An enzymatically degraded but non-oxidised form of LDL (E-LDL) has also been shown to promote the adhesion of T lymphocytes to monolayers of endothelial cells [38]. However, although increasing pro-coagulant responses were observed with ECV304 cells on treatment with n-LDL, m-LDL and ox-LDL, and there was an increase in the number of Jurkat cells adhering to the m-LDL- and ox-LDL-treated cells, the increase in adhesion was not primarily PS-dependent.

The interaction of exposed PS on one cell with a receptor for PS on a different cell mediates the recognition of apoptotic lymphocytes by macrophages [39] and of neoplastic cells by monocytes [11]. Relatively non-specific, homotypic adhesion, involving exposed PS on two apposed surfaces, can also facilitate adhesion in the presence of Ca^{2+} . Although hydrated bilayers of phospholipid normally repel each other strongly as a consequence of hydration repulsion arising from solvation of the polar head groups, Ca^{2+} ions interact so strongly with PS on adjacent bilayers that they displace surface water and enable the bilayers to achieve direct molecular contact via PS- Ca^{2+} -PS bridges [40]. In our experiments, Jurkat cells were unable to adhere to thrombin-treated HUVEC and ECV304 cells in the presence of EGTA. Taken together with the observation that the adhesion of Jurkat T cells to thrombin-treated ECV304 cells resulted in the exposure of PS on the Jurkat cells, this indicates that adhesion of the two cell types may involve a Ca^{2+} bridge between PS on the surface of both cells, rather than being receptor-mediated. Whether sufficient PS is exposed on thrombin-treated cells for such a mechanism is not clear however since, unlike experiments with erythrocytes, a comparison of the prothrombinase activities of thrombin-treated and lysed endothelial cells cannot be used to estimate the fraction of exposed PS on the treated cells because PS is present on

membrane-bound organelles within lysed endothelial cells. PS may, in fact, mediate the observed cell-cell interactions in combination with other factors. This would be consistent with Jurkat cells being unable to adhere to PS-coated glass. On the other hand, there may be topological or conformational differences between PS deposited on glass compared with PS exposed on endothelial cells.

It should be added that, since 2 mM EGTA for 15 min reverses the exposure of PS on DO11.10 T lymphocyte hybridoma cells 6 h after the induction of apoptosis by anti-CD3 antibody [41], the inability of Jurkat cells to adhere to thrombin-treated HUVEC and ECV304 cells in the presence of EGTA might possibly have resulted from PS returning to the inner leaflet of the plasma membrane of the treated endothelial cells.

The T cells that adhered to thrombin-treated endothelial cells in the work reported here are all tumour cells. This raises the possibility that, in addition to the factors already known to be involved in metastasis [42–45], surface PS may contribute to adhesive interactions between endothelial cells and tumour cells prior to the extravasation of malignant cells. It is relevant that contact between tumour cells and endothelial cells results in a rise of intracellular Ca^{2+} in the endothelial cells which enhances cell-cell adhesion [46,47] and mediates the transendothelial migration of tumour cells [47]. Electron microscopy has shown that vesicles are formed on, and shed from, endothelial cell ‘sprouts’ under the influence of cells from a mammary tumour, and that these vesicles have procoagulant activity [48]. Contact with tumour cells may thus lead to the exposure of surface PS on endothelial cells, via an increase in their cytoplasmic Ca^{2+} , which could facilitate adhesive interactions with tumour cells. PS- Ca^{2+} -PS bridges may be involved in such interactions since PS has also been observed on the surface of tumour cells [11,49]. As our observations indicate that adhesion to PS on the surface of thrombin-treated endothelial cells may be a feature of T cells that express both CD4 and CD8 antigens, it is also interesting that pleural dissemination of an invasive thymoma, accompanied by a marked increase in the number of CD4⁺/CD8⁺ T cells in peripheral blood, has also been reported [50].

Our finding that Jurkat T cells, but not peripheral

blood T cells, adhere in a PS-dependent manner to thrombin-treated endothelial cells is reminiscent of work reported by Bernard et al. [51]. They found that anti-E2 (CD99) monoclonal antibodies induced the homotypic aggregation of Jurkat cells and CD4⁺/CD8⁺ human thymocytes. Normal peripheral T cells did not aggregate. The antibodies in question were reported previously to cause the exposure of PS on the surface of Jurkat cells and human thymocytes [52] but, surprisingly, neither exogenous PS nor antibodies directed against PS inhibited the homotypic aggregation. Our experiments have yielded rather similar results since they were generally, but not completely, consistent with adhesion to PS on the surface of thrombin-treated endothelial cells being a feature of double-positive (CD4⁺/CD8⁺) T cells. Normally, only 2–3% of circulating human T cells express both CD4 and CD8 antigens. However, there is evidence for increased numbers of double-positive CD4⁺/CD8⁺ T cells in the peripheral blood of patients with infectious diseases. CD4⁺(dim)/CD8⁺(bright) T cells (cf. Jurkat E6.1 cells, Table 6) have been identified cytofluorometrically in peripheral blood from patients with EBV-infectious mononucleosis [53], and from a renal transplant recipient with a cytomegalovirus infection [54]. 10–12% of CD4⁺/CD8⁺ T cells have been observed in the peripheral blood of a patient with pulmonary tuberculosis [55], and a very high level of double-positive CD4⁺/CD8⁺ peripheral T cells (>40% of all the lymphoid cells) in a HIV patient has been briefly reported [56]. Our observations, albeit made *in vitro*, indicate that double-positive CD4⁺/CD8⁺ T cells in individuals bearing infections may adhere to damaged endothelial cells in a PS-dependent manner. This may contribute to atherosclerosis since there is growing evidence that infection is a risk factor [57–59], and many workers regard atherosclerosis as an inflammatory, immunological disease [60,61].

Acknowledgements

We thank the British Heart Foundation and the Central Research Fund, University of London, for awards that supported this work. We are grateful to Dr G.W. Butcher and Dr G.V.W. Stevenson for their helpful comments on the manuscript.

References

- [1] C. Diaz, A.J. Schroit, Role of translocases in the generation of phosphatidylserine asymmetry, *J. Membr. Biol.* 151 (1996) 1–9.
- [2] M. Julien, J.-F. Tournier, J.-F. Tocanne, Basic fibroblast growth factor modulates the aminophospholipid translocase activity present in the plasma membrane of bovine aortic endothelial cells, *Eur. J. Biochem.* 230 (1995) 287–297.
- [3] P.F. Devaux, A. Zachowski, Maintenance and consequences of membrane phospholipid asymmetry, *Chem. Phys. Lipids* 73 (1994) 107–120.
- [4] P. Williamson, R.A. Schlegel, Back and forth: the regulation and function of transbilayer phospholipid movement in eukaryotic cells, *Mol. Membr. Biol.* 11 (1994) 199–216.
- [5] R.F.A. Zwaal, A.J. Schroit, Pathophysiologic implications of membrane phospholipid asymmetry in blood cells, *Blood* 89 (1997) 1121–1132.
- [6] J. Dachary-Prigent, J.-M. Pasquet, J.-M. Freyssinet, A.T. Nurden, Calcium involvement in aminophospholipid exposure and microparticle formation during platelet activation: a study using Ca²⁺-ATPase inhibitors, *Biochemistry* 34 (1995) 11625–11634.
- [7] P. Comfurius, P. Williamson, E.F. Smeets, R.A. Schlegel, E.M. Bevers, R.F.A. Zwaal, Reconstitution of phospholipid scramblase activity from human blood platelets, *Biochemistry* 35 (1996) 7631–7634.
- [8] Q.S. Zhou, J. Zhao, J.G. Stout, R.A. Luhm, T. Wiedmer, P.J. Sims, Molecular cloning of human plasma membrane phospholipid scramblase - A protein mediating transbilayer movement of plasma membrane phospholipids, *J. Biol. Chem.* 272 (1997) 18240–18244.
- [9] V.A. Fadok, D.R. Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, P.M. Henson, Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages, *J. Immunol.* 148 (1992) 2207–2216.
- [10] J. Connor, C.C. Pak, A.J. Schroit, Exposure of phosphatidylserine in the outer leaflet of human red blood cells. Relationship to cell density, cell age, and clearance by mononuclear cells, *J. Biol. Chem.* 269 (1994) 2399–2404.
- [11] T. Utsugi, A.J. Schroit, J. Connor, C.D. Bucana, I.J. Fidler, Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumour cells and recognition by activated human blood monocytes, *Cancer Res.* 51 (1991) 3062–3066.
- [12] S. Haraoka, T. Shimokama, T. Watanabe, Participation of T lymphocytes in atherogenesis - sequential and quantitative observation of aortic lesions with diet induced hypercholesterolemia using en face double immunostaining, *Virchows Arch. Int. J. Pathol.* 426 (1995) 307–315.
- [13] D.B. Cines, E.S. Pollak, C.A. Buck, J. Loscalzo, G.A. Zimmerman, R.P. McEver, J.S. Pober, T.M. Wick, B.A. Konkle, B.S. Schwartz, E.S. Barnathan, K.R. McCrae, B.A. Hug, A.M. Schmidt, D.M. Stern, Endothelial cells in physiology

- and in the pathophysiology of vascular disorders, *Blood* 91 (1998) 3527–3561.
- [14] O.J. DeBoer, A.C. VanderWal, C.E. Verhagen, A.E. Becker, Cytokine secretion profiles of cloned T cells from human aortic atherosclerotic plaques, *J. Pathol.* 188 (1999) 174–179.
- [15] C.M. Boustead, R. Brown, J.H. Walker, Isolation, characterization and localization of annexin V from chicken liver, *Biochem. J.* 291 (1993) 601–608.
- [16] C.J.S. Edgell, C.C. McDonald, J.B. Graham, Permanent cell line expressing factor VIII related antigen established by hybridization, *Proc. Natl. Acad. Sci. USA* 80 (1983) 3734–3737.
- [17] J.M. Qu, L.A. Conroy, J.H. Walker, F.B.P. Wooding, J.A. Lucy, Phosphatidylserine-mediated adhesion of T-cells to endothelial cells, *Biochem. J.* 317 (1996) 343–346.
- [18] K.M. Naseem, A. Goodall, K.R. Bruckdorfer, Differential effects of native and oxidatively modified low-density lipoproteins on platelet function, *Platelets* 8 (1997) 163–173.
- [19] P.F.J. Verhallen, E.M. Bevers, P. Comfurius, R.F.A. Zwaal, Fluoride-dependent calcium induced platelet procoagulant activity shows that calpain is involved in increased phospholipid transbilayer movement, *Biochim. Biophys. Acta* 942 (1988) 150–158.
- [20] L.Y. Song, Q.F. Ahkong, J.M. Baldwin, R. O'Reilly, J.A. Lucy, Divalent cations, phospholipid asymmetry, and osmotic swelling in electrically-induced lysis, cell fusion, and giant cell formation with human erythrocytes, *Biochim. Biophys. Acta* 1148 (1993) 30–38.
- [21] P. Schoen, C. Reutelingsperger, T. Lindhout, Activation of prothrombin in the presence of human umbilical-vein endothelial cells, *Biochem. J.* 281 (1992) 661–664.
- [22] M. Si-Tahar, P. Renesto, H. Falet, F. Rendu, M. Chignard, The phospholipase C protein kinase C pathway is involved in cathepsin G-induced human platelet activation: Comparison with thrombin, *Biochem. J.* 313 (1996) 401–408.
- [23] W. Seiss, Molecular mechanisms of platelet activation, *Physiol. Rev.* 69 (1989) 58–178.
- [24] J.-M. Pasquet, J. Dachary-Prigent, A.T. Nurden, Calcium influx is a determining factor of calpain activation and microparticle formation in platelets, *Eur. J. Biochem.* 239 (1996) 647–654.
- [25] J.A. Berliner, J.W. Heinecke, The role of oxidized lipoproteins in atherogenesis, *Free Radic. Biol. Med.* 20 (1996) 707–727.
- [26] B. Zhao, W.D. Ehringer, R. Dierichs, F.N. Miller, Oxidized low-density lipoprotein increases endothelial intracellular calcium and alters cytoskeletal f-actin distribution, *Eur. J. Clin. Invest.* 27 (1997) 48–54.
- [27] B. Zhao, R. Dierichs, F.N. Miller, W.L. Dean, Oxidized low density lipoprotein inhibits platelet plasma membrane Ca^{2+} -ATPase, *Cell Calcium* 19 (1996) 453–458.
- [28] J. Zhao, Q. Zhou, T. Wiedmer, P.J. Sims, Level of expression of phospholipid scramblase regulates induced movement of phosphatidylserine to the cell surface, *J. Biol. Chem.* 273 (1998) 6603–6606.
- [29] G.M. Rodgers, Hemostatic properties of normal and perturbed vascular cells, *FASEB J.* 2 (1988) 116–123.
- [30] J.B. Duguid, *The Dynamics of Atherosclerosis*, Aberdeen University Press, Aberdeen, 1976, pp. 1–78.
- [31] R. Gallo, A. Padurean, V. Toschi, J. Bichler, J.T. Fallon, J.H. Chesebro, V. Fuster, J.J. Badimon, Prolonged thrombin inhibition reduces restenosis after balloon angioplasty in porcine coronary arteries, *Circulation* 97 (1998) 581–588.
- [32] K. Takahashi, Y. Sawasaki, J.-I. Hata, K. Mukai, T. Goto, Spontaneous transformation and immortalization of human endothelial cells, *In Vitro Cell. Dev. Biol.* 25 (1990) 265–274.
- [33] M. Hoth, R. Penner, Depletion of intracellular calcium stores activates a calcium current in mast cells, *Nature* 355 (1992) 353–356.
- [34] A.H. Guse, C. DeWit, T. Klokow, K. Schweitzer, G.W. Mayr, Unique properties of the capacitative Ca^{2+} entry antagonist LU52396: its inhibitory activity depends on the activation state of the cells, *Cell Calcium* 22 (1997) 91–97.
- [35] D. Steinberg, A critical look at the evidence for the oxidation of LDL in atherogenesis, *Atherosclerosis* 131 (1997) S5–S7.
- [36] W.M. Zhu, P. Roma, A. Pirillo, F. Pellegatta, A.L. Catapano, Human endothelial cells exposed to oxidized LDL express hsp70 only when proliferating, *Arterioscler. Thromb. Vasc. Biol.* 16 (1996) 1104–1111.
- [37] J.A.M. Maier, L. Barenghi, F. Pagani, S. Bradamante, P. Comi, G. Ragnotti, The protective role of high-density lipoprotein on oxidized- low-density-lipoprotein-induced U937/ endothelial cell interactions, *Eur. J. Biochem.* 221 (1994) 35–41.
- [38] M. Klouche, A.E. May, M. Hemmes, M. Messner, S.M. Kanse, K.T. Preissner, S. Bhakdi, Enzymatically modified, nonoxidized LDL induces selective adhesion and transmigration of monocytes and T-lymphocytes through human endothelial cell monolayers, *Arterioscler. Thromb. Vasc. Biol.* 19 (1999) 784–793.
- [39] R.A. Schlegel, M. Callahan, S. Krahling, D. Pradhan, P. Williamson, Mechanisms for recognition and phagocytosis of apoptotic lymphocytes by macrophages, *Adv. Exp. Med. Biol.* 406 (1996) 21–28.
- [40] R.P. Rand, V.A. Parsegian, Hydration forces between phospholipid bilayers, *Biochim. Biophys. Acta* 988 (1989) 351–376.
- [41] B. Verhoven, S. Krahling, R.A. Schlegel, P. Williamson, Regulation of phosphatidylserine exposure and phagocytosis of apoptotic T lymphocytes, *Cell Death Differ.* 6 (1999) 262–270.
- [42] L. Mendoza, E. Olaso, M.J. Anasagasti, A.M. Fuentes, F. Vidal-Vanaclocha, Mannose receptor-mediated endothelial cell activation contributes to B16 melanoma cell adhesion and metastasis in liver, *J. Cell. Physiol.* 174 (1998) 322–330.
- [43] N. Jahroudi, J.S. Greenberger, The role of endothelial-cells in tumor invasion and metastasis, *J. Neuro-Oncol.* 23 (1995) 99–108.
- [44] R.M. Lafrenie, S. Gallo, T.J. Podor, M.R. Buchanan, F.W. Orr, The relative roles of vitronectin receptor, E-selectin and

- $\alpha(4)\beta(1)$ in cancer cell-adhesion to interleukin-1-treated endothelial-cells, *Eur. J. Cancer* 30A (1994) 2151–2158.
- [45] T. Krause, G.A. Turner, Are selectins involved in metastasis?, *Clin. Exp. Metastasis* 17 (1999) 183–192.
- [46] R. Pili, S. Corda, A. Passaniti, R.C. Ziegelstein, A.W. Heldman, M.C. Capogrossi, Endothelial cell Ca^{2+} increases upon tumor cell contact and modulates cell–cell adhesion, *J. Clin. Invest.* 92 (1993) 3017–3022.
- [47] J.-M. Lewalle, D. Cataldo, K. Bajou, C.A. Lambert, J.M. Foidart, Endothelial cell intracellular Ca^{2+} concentration is increased upon breast tumor cell contact and mediates tumor cell transendothelial migration, *Clin. Exp. Metastasis* 16 (1998) 21–29.
- [48] H.H.C. Lichtenbeld, A.D. Muller, M.C.E. van Dam-Mieras, G.H. Blijham, Tumor spheroid-induced vesicle formation on endothelial cells is associated with procoagulant properties, *J. Cell Sci.* 106 (1993) 657–662.
- [49] M. Sugimura, R. Donato, V.V. Kakkar, M.F. Scully, Annexin V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces, *Blood Coagul. Fibrinolysis* 5 (1995) 365–373.
- [50] J. Tamaoki, A. Chiyotani, A. Nagai, K. Konno, Invasive thymoma with $\text{CD4}^+\text{CD8}^+$ double-positive cell, *Respiration* 64 (1997) 176–178.
- [51] G. Bernard, D. Zoccola, M. Deckert, J.-P. Breittmayer, C. Aussel, A. Bernard, The E2 molecule (CD99) specifically triggers homotypic aggregation of $\text{CD4}^+\text{CD8}^+$ thymocytes, *J. Immunol.* 154 (1995) 26–32.
- [52] C. Aussel, G. Bernard, J.-P. Breittmayer, C. Pelassy, D. Zoccola, A. Bernard, Monoclonal antibodies directed against the E2 protein (MIC2 gene product) induce exposure of phosphatidylserine at the thymocyte cell surface, *Biochemistry* 32 (1993) 10096–10101.
- [53] C. Ortolani, E. Forti, E. Radin, R. Cibin, A. Cossarizza, Cytofluorimetric identification of two populations of double positive (CD4^+ , CD8^+) T lymphocytes in human peripheral blood, *Biochem. Biophys. Res. Commun.* 191 (1993) 601–609.
- [54] R.J. Rentenaar, P.C. Wever, F.N.J. vanDiepen, P.T.A. Schellekens, P.M.E. Wertheim, I.J.M. tenBerge, $\text{CD4}(\text{dull})\text{CD8}(\text{bright})$ double-positive T-lymphocytes have phenotype of granzyme B(pos) $\text{CD8}(\text{pos})$ memory T-lymphocytes, *Nephrol. Dial. Transplant.* 14 (1999) 1430–1434.
- [55] K. Ohnishi, Expansion of $\text{CD4}^+\text{CD8}^+$ T lymphocytes in the peripheral blood of a patient with pulmonary tuberculosis, *Rinsho Byori* 40 (1992) 959–964.
- [56] V. Ribrag, D. Salmon, F. Picard, M. Guesnu, D. Sicard, F. Dreyfus, Increase in double-positive $\text{CD4}^+\text{CD8}^+$ peripheral T-cell subsets in an HIV-infected patient, *AIDS* 7 (1993) 1530.
- [57] G. Noll, Pathogenesis of atherosclerosis a possible relation to infection, *Atherosclerosis* 140 (1998) S3–S9.
- [58] M. Seigneur, J. Constans, A. Blann, M. Renard, J.L. Pellegrin, J. Amiral, M. Boisseau, C. Conri, Soluble adhesion molecules and endothelial cell damage in HIV infected patients, *Thromb. Haemost.* 77 (1999) 646–649.
- [59] J.L. Mehta, T.G.P. Saldeen, K. Rand, Interactive role of infection, inflammation and traditional risk factors in atherosclerosis and coronary artery disease, *J. Am. Coll. Cardiol.* 31 (1998) 1217–1225.
- [60] T. Watanabe, S. Haraoka, T. Shimokama, Inflammatory and immunological nature of atherosclerosis, *Int. J. Cardiol.* 54 (1996) S25–S34.
- [61] R. Ross, Mechanisms of disease - Atherosclerosis - An inflammatory disease, *N. Engl. J. Med.* 340 (1999) 115–126.