RESEARCH COMMUNICATION

Phosphatidylserine-mediated adhesion of T-cells to endothelial cells

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Phosphatidylserine was exposed on the surface of human umbilical endothelial cells (ECV304) a few minutes after adding thrombin *in vitro*, as monitored by prothrombinase assays with and without annexin V. Jurkat T cells adhered to the thrombin-

treated cells. The adhesion was inhibited by annexin V, indicating that it was mediated by exposed phosphatidylserine on the endothelial cells.

INTRODUCTION

Adhesive interactions arrest blood-borne leukocytes on the luminal surface of endothelial cells. Initially leukocytes roll on the endothelium. Subsequently, they may become attached and then extravasate. Selectins are the best-characterized mediators of the first step in the interactions of leukocytes with aortic endothelial cells [1–3]. However, other factors may contribute to the initial adhesion of T-cells to endothelial cells. An improved understanding of such interactions is important, since the attachment of T-cells to endothelial cells is central to lymphocyte targeting, the basis for immune surveillance, and is also an early manifestation of cellular dysfunction in atherosclerosis [4].

A role for phospholipids seems not to have been considered in this context, even though surface phosphatidylserine (PS) on apoptotic lymphocytes and aged erythrocytes mediates their interactions with macrophages [5,6], on tumour cells with monocytes [7], on apoptotic smooth-muscle cells with normal smooth muscle [8], and on sickled erythrocytes [9,10], oxidized erythrocytes [11] and symmetrical erythrocyte ghosts [12] with endothelial cells.

PS in the plasma membranes of most cells is present only in the inner monolayer [13,14], but previous studies have shown that procoagulant microvesicles, bearing exposed PS [15], are released from endothelial cells into the supernatant medium on stimulation by complement proteins C5b-9 [16] or thrombin [15]. With thrombin formed *in situ* during 15 min of incubation, exposed PS was observed only on the released vesicles and not on the treated cells; a similar finding was made with exogenous thrombin [15]. We have undertaken further experiments, without the intermittent, gentle whirling of the thrombin-treated cells used earlier [15], to determine whether PS is also exposed on the cell surface. It was found that PS is rapidly exposed on the surface of human umbilical endothelial cells after adding thrombin *in vitro*, and that Jurkat T-cells exhibit PS-mediated adhesion to the perturbed cells.

MATERIALS AND METHODS

Materials

Factor II (prothrombin), thrombin and factor Xa were from Sigma (Poole, Dorset, U.K.), and factor V was from Diagnostic

Reagents Ltd. (Thame, Oxon., U.K.). Chromogenic substrate S-2238 (*H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride) was from Chromogenix AB (Mölnådal, Sweden). 5,6-Carboxyfluorescein diacetate (CFDA) and 5-chloromethylfluorescein diacetate (CMFDA) were from Molecular Probes Europe BV (Leiden, The Netherlands). Human annexin V was from Alexis Corp. Ltd. (Bingham, Nottingham, U.K.). Annexin V was purified to homogeneity (M_r 33000) from chicken liver [17]; the protein (1100 nM) completely abolished the prothrombinase activity of sonicated vesicles of PS (0.25 μ M) (results not shown). *Ulex europaeus* agglutinin I was from Vector Laboratories Ltd. (Bretton, Peterborough, U.K.). Monoclonal antibodies OKT3 and OKT8 were from Ortho Diagnostic Systems (Amersham, Bucks., U.K.), and CD45.2 was a gift from Professor S. Meuer (German Cancer Research Centre, Heidelberg, Germany).

Cell cultures

Human umbilical-cord endothelial cells (ECV304) [18] (a gift from Professor I. B. Fritz, Babraham Institute, Babraham, U.K.) stained positively for *U. europaeus* agglutinin I, a marker for vascular endothelium in human tissues [19]. Cytoplasmic Weibel–Palade bodies, which were observed originally although their frequency was not reported [18], were present but were very infrequent. Human Jurkat T-cells (E6.1) were a gift from Dr. D. R. Alexander (Babraham Institute, Babraham, U.K.). They were characterized by the presence of surface markers CD3, CD8 and CD45 by flow cytometry, using OKT3, OKT8 and CD45.2 monoclonal antibodies respectively.

Prothrombinase assay

Confluent ECV304 cells (passage 140–160) in 24-well (2 cm²/well) plates, were washed at 37 °C in medium 199 without Phenol Red (medium 199) and incubated in 5% CO₂ for 10 min with 1.1 ml of this medium containing human serum albumin (HSA medium), with or without 30 nM thrombin. The effect of annexin V was investigated by pre-incubating cells for 10 min in 5% CO₂ with 1100 nM chicken annexin V in HSA medium before adding thrombin. A 1.0 ml aliquot of thrombin-containing or control medium (1st supernatants) was transferred to clean wells and

Abbreviations used: PS, phosphatidylserine; CFDA, 5,6-carboxyfluorescein diacetate; CMFDA, 5-chloromethylfluorescein diacetate.

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replaced by 1 ml of HSA medium after gently rinsing the monolayers twice in medium 199. A solution (50 μ l) containing factor Va (activated from factor V as described previously [20]) and factor Xa was added to the 1st supernatants and to the fresh media (2nd supernatants) above the cell monolayers, followed, after 2 min at 37 °C in a water bath, by an equal volume of prothrombin solution. The final concentrations of factors Va, Xa and prothrombin were 1.09 nM, 1.3 nM and 0.36 μ M respectively; the reaction mixture also contained 4 mM CaCl₂. At timed intervals, 20 μ l aliquots were withdrawn to plastic tubes containing 1 ml of Tris/EDTA buffer, and 180 μ l aliquots were then transferred in triplicate to 96-well plates. Chromogenic substrate S-2238 (20 μ l, 1.5 mM) was added, and the rate of increase in absorbance at 405 nm was measured. Thrombin concentrations were calculated from a standard curve obtained under identical conditions.

T-cell adhesion

Cultured Jurkat T-cells (E6.1) were washed in medium 199, resuspended in HSA medium at a cell density of 1×10^6 per ml, labelled for 20 min at 37 °C with 50 μ g/ml CFDA (from a 50 mg/ml solution in acetone) and washed by centrifugation. ECV304 cells were grown to confluence over 48 h on 22-mm-diam. glass coverslips, washed at 37 °C in medium 199 and incubated in 5% CO₂ for 10 min with 1 ml of HSA medium in a 33-mm-diam. dish, with or without 30 nM thrombin. As required, ECV304 cells were pre-incubated with chicken annexin V (1100 nM) in HSA medium for 10 min in 5% CO₂ before adding thrombin, or additionally incubated with EGTA (5 mM) for 5 min in 5% CO₂ before adding the annexin. ECV304 cells were gently rinsed with medium 199 after the thrombin treatment. Fluorophore-labelled T-cells were added to ECV304 cells at a density of 5×10^6 per cover slip in 1.5 ml of HSA medium, and the two cell types were incubated together for 20 min at 37 °C in 5% CO₂. The cells were shaken manually, with a reciprocating motion in successive washing aliquots (5 ml) of medium 199, until all unattached T-cells were removed, as monitored by phase-contrast microscopy. Adhering T-cells were revealed by inverted fluorescence microscopy.

For quantitative assays of cell adhesion, Jurkat cells were washed in medium 199, resuspended in HSA medium at a cell density of 2.5×10^6 per ml, labelled for 20 min at 37 °C with 2.3 μ g/ml CMFDA (from a 4.6 mg/ml solution in DMSO) and then washed by centrifugation. CMFDA leaked much more slowly from the labelled cells than did CFDA. The pelleted cells were resuspended in HSA medium at 37 °C for 30 min, washed three times and resuspended in HSA medium at a density of 3×10^6 cells/ml. ECV304 cells were grown to confluence for 48 h in 24-well plates, washed at 37 °C in medium 199 and incubated in duplicate in 5% CO₂ for up to 20 min with 1 ml of HSA medium, with or without 30 nM thrombin. Chicken annexin V was employed as described above. The cells were gently rinsed with medium 199 following thrombin treatment. After adding 1 ml of fluorophore-labelled T-cells per well, ECV304 and T-cells were incubated together for 20 min at 37 °C in 5% CO₂. Unattached T-cells were removed as above. Triton X-100 solution (10%; 1 ml) was then added to each well. After 30 min, the lysates were removed, the wells were washed with 0.5 ml of Triton solution, and the lysates and washings from duplicate wells were combined. The number of T-cells corresponding to the released fluorophore was determined from a standard curve obtained by lysis of fluorophore-labelled T-cells under similar conditions.

RESULTS AND DISCUSSION

Prothrombinase activity

Exposed PS provides a procoagulant surface that facilitates the conversion of prothrombin to thrombin. The production of thrombin (prothrombinase activity) can thus be used as an indirect assay for exposed PS [21].

Following a 10 min incubation at 37 °C of ECV304 cells with medium 199, the supernatant medium was removed. This supernatant generated a very low level of thrombin during a 12 min incubation with factors II (prothrombin), Va, and Xa (Figure 1, Control-S1). When the cells were subsequently incubated for 12 min with the three clotting factors, they released more procoagulant activity into the medium (Figure 1, Control-S2), confirming a previous report [15]. By comparison, the supernatant from cells incubated for 10 min with thrombin generated a relatively high level of thrombin when added to the clotting factors (Figure 1, Thr-S1). The magnitude of the procoagulant activity released into the medium when the thrombin-treated cells were subsequently incubated with clotting factors (Figure 1, Thr-S2) is consistent with the proposal [15] that membranous vesicles are released from unstimulated cells incubated with

Figure 1 Prothrombinase activity of vesicles released from endothelial cells

ECV304 cells were treated with 30 nM thrombin for 10 min, and the prothrombinase activity of vesicles released into the supernatant was determined as described in the Materials and methods section. ○, Control-S1 (1st supernatant): control cells. ●, Control-S2 (2nd supernatant): control cells. □, Thr-S1 (1st supernatant): thrombin-treated cells. ■, Thr-S2 (2nd supernatant): thrombin-treated cells. △, AnV/Thr-S1 (1st supernatant): annexin V-pretreated, thrombin-treated cells. ▲, AnV/Thr-S2 (2nd supernatant): annexin V-pretreated, thrombin-treated cells. The data have been normalized, relative to curve Thr-S2, for three independent experiments, means $\pm$ S.E.M., in which 62, 111 and 135 nM thrombin was generated in 12 min in the 2nd supernatant from thrombin-treated cells. (Curves Thr-S1 and AnV/Thr-S1 refer to thrombin generated additionally to the 30 nM thrombin already present in these supernatants.)

Figure 2 Adhesion of Jurkat T-cells to thrombin-treated endothelial cells

Fluorescent micrographs of T-cells attached to ECV304 cells. The two cell types were treated as described in the Materials and methods section. (a) ECV304 cells treated with 30 nM thrombin; (b) untreated ECV304 cells; (c) ECV304 cells pre-incubated with chicken annexin V (1100 nM) in HSA medium for 10 min in 5% CO₂ before adding thrombin; (d) ECV304 cells additionally pretreated with EGTA before adding the annexin, after which thrombin was added. (a)–(d) are at the same magnification: the bar represents 110 μ m.

clotting factors (cf. Control-S2) as a result of thrombin production *in situ*.

The high affinity of annexin V for PS in the presence of physiological concentrations of Ca²⁺ has been used in several recent studies as an alternative probe for monitoring the surface exposure of PS [8,22,23]. Figure 1, curve AnV/Thr-S1 (compared with Thr-S1), shows that adding annexin V (1100 nM) 10 min before adding thrombin to ECV304 cells resulted in a lower procoagulant activity of the supernatant medium. In addition, Figure 1, curve AnV/Thr-S2 (compared with Thr-S2), shows that this procedure decreased the procoagulant activity released into the medium when the cells were subsequently incubated with clotting factors. The latter observation indicates that PS was present on the surface of the thrombin-treated cells. Binding of annexin V to the exposed PS thus inhibited subsequent vesicle release (as in experiments on platelets [24]) and/or the procoagulant activity of the released vesicles. Only very occasional cells that had been incubated for 10 min at 37 °C with 30 nM thrombin were permeable to Trypan Blue, indicating that the observed procoagulant activity was not the result of cell lysis. Consequently, treatment of ECV304 endothelial cells with thrombin appears to result in translocation of PS to the cell surface, where it may cause the plasma membrane to vesiculate. This is consistent with the recently reported presence of aminophospholipids on the surface of activated platelets [24,25].

Adhesion of Jurkat T-cells

Jurkat T-cells were labelled with a cytoplasmic fluorophore, and incubated at 37 °C for 20 min with a monolayer of ECV304 cells that had been incubated with 30 nM thrombin for 10 min. Large numbers of T-cells adhered to thrombin-treated ECV304 cells (Figure 2a), while very few cells (Figure 2b) adhered to untreated ECV304 cells. Adhesion to ECV304 cells that had been pre-incubated with 1100 nM chicken annexin V (Figure 2c), or with 1100 nM human annexin V (results not shown), before the addition of thrombin was greatly decreased. The inhibitory effect

Table 1 Adhesion of Jurkat T-cells to thrombin-treated endothelial cells

ECV304 cells were treated with 30 nM thrombin for 5–20 min, and the adhesion of Jurkat T-cells to thrombin-treated ECV304 cells was measured as described in the Materials and methods section. Mean values are shown $\pm$ S.E.M. for three independent experiments (each of which was done in duplicate).

Length of thrombin treatment (min)	$10^{-3} \times$ cells per well
0	12.0 ± 2.0
5	87.5 ± 14.0
10	163 ± 23.5
15	132 ± 18.0
20	97.5 ± 12.0

Table 2 Effect of annexin V on adhesion of Jurkat T-cells to ECV304 cells

The adhesion of Jurkat T-cells to ECV304 cells, which had been pre-incubated with different concentrations of annexin V for 10 min before adding 30 nM thrombin and incubating for a further 10 min, was measured as described in the Materials and methods section. The value for 'untreated cells' is the number of Jurkat cells that adhered to untreated ECV304 cells. Mean values are shown $\pm$ S.E.M. for three independent experiments (each of which was done in duplicate).

Concentration of annexin V (nM)	$10^{-3} \times$ cells per well
0	163 ± 23.5
138	50.0 ± 16.0
275	42.5 ± 12.5
550	25.0 ± 7.0
1100	12.5 ± 3.5
Untreated cells	12.0 ± 2.0

of chicken annexin V was overcome by the presence of 5 mM EGTA (Figure 2d).

In quantitative experiments, Triton X-100 was added to solubilize T-cells adhering to ECV304 cells, and the fluorophore thus released was assayed as a measure of adhering T-cells. A 5 min treatment of ECV304 cells with 30 nM thrombin resulted in an increase in the number of T-cells adhering to the endothelial cells. The number of adhering cells reached a maximum with a 10 min thrombin treatment (Table 1). The apparent decrease in T-cell adhesion to ECV304 cells that had been treated with thrombin for longer times reflected detachment of the endothelial cells from the substrate. When ECV304 cells were pre-incubated with 138 nM chicken annexin V before the addition of thrombin, the number of adhering T-cells was decreased markedly, and increasing concentrations of annexin V further decreased the number of adhering T-cells (Table 2). The inhibitory effects of pre-incubating ECV304 cells with 1100 nM concentrations of human annexin V (results not shown) and chicken annexin V, before the addition of thrombin, were similar. These several observations indicate that Jurkat T-cells adhere to thrombin-treated ECV304 cells via PS on the surface of the endothelial cells.

Although P-selectin is mobilized from Weibel–Palade bodies to the surface of endothelial cells within minutes of activation by thrombogenic and inflammatory mediators, including thrombin [26], it seems unlikely to be involved in the adhesion of Jurkat cells reported here because, although certain T-cell subsets bind to P-selectin [27,28], Jurkat T-cells do not [29]. Furthermore, very few Weibel–Palade bodies were present in the ECV304 cells. However, the mechanism by which PS mediates the adhesion of Jurkat T-cells to ECV304 cells has yet to be elucidated.

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 [30]. This thrombin is considered to contribute to the progression of arteriosclerotic lesions via multiple effects [31]. Our finding that T-cells exhibit PS-mediated adhesion to endothelial cells within a few minutes of treating the latter with thrombin, albeit with cell lines, raises the possibility that exposure of PS on the surface of thrombin-perturbed endothelial cells might facilitate the initial adhesion of T lymphocytes in atherogenesis. It remains to be seen if PS-mediated interactions apply beyond the system studied here, and whether they are modulated by factors involved in the aetiology of atherosclerosis. Should this be the case, the inhibition of such interactions might have therapeutic potential in the longer term.

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