The Journal of Immunology

A Novel β_1 Integrin-Dependent Mechanism of Leukocyte Adherence to Apoptotic Cells

Barbara R. Schwartz, Aly Karsan, Thomas Bombeli and John M. Harlan

J Immunol 1999; 162:4842-4848; ;
http://www.jimmunol.org/content/162/8/4842

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Novel β_1 Integrin-Dependent Mechanism of Leukocyte Adherence to Apoptotic Cells[1]

Barbara R. Schwartz,[2]* Aly Karsan,[3]*† Thomas Bombeli,[4]* and John M. Harlan*†

Adherence of leukocytes to cells undergoing apoptosis has been reported to be dependent on a variety of recognition pathways. These include $\alpha_V\beta_3$ (CD51/CD61, vitronectin receptor), CD36 (thrombospondin receptor), macrophage class A scavenger receptor, phosphatidylserine translocated to the outer leaflet of apoptotic cell membranes, and CD14 (LPS-binding protein). We investigated the mechanism by which leukocytes adhere to apoptotic endothelial cells (EC). Peripheral blood mononuclear leukocytes and U937 monocytic cells adhered to human or bovine aortic EC induced to undergo apoptosis by withdrawal of growth factors, treatment with the promiscuous protein kinase inhibitor staurosporine, with the protein synthesis inhibitor and protein kinase activator anisomycin, or with the combination of cycloheximide and TNF-α. Expression of endothelial adherence molecules such as CD62E (E-selectin), CD54 (ICAM-1), and CD106 (VCAM-1) was not induced or increased by these treatments. A mAb to $\alpha_V\beta_3$, exogenous thrombospondin, or blockade of phosphatidylserine by annexin V did not inhibit leukocyte adherence. Further, leukocyte binding to apoptotic EC was completely blocked by treatment of leukocytes but not EC with mAb to β_1 integrin. These results define a novel pathway for the recognition of apoptotic cells. ***The Journal of Immunology*****, 1999, 162: 4842–4848.**

Programmed cell death, or apoptosis, is an active process involving specific cell membrane receptors and intracellular reactions that are now being elucidated (1, 2). Cells may undergo programmed cell death as a component of normal development, as in thymic involution (3), or in the progress of pathological conditions such as neoplasia (4) and vascular diseases, including atherosclerosis (5) and hypertension (6). However, the role of apoptosis in many pathological processes remains to be defined. Cells in tissue dying via apoptosis shrink without lysis and are rapidly engulfed by neighboring cells or by mononuclear phagocytes before cellular membranes break down. Apoptotic cells are thought not to release cellular contents and are therefore considered not to be proinflammatory (7). However, in contrast to cells in tissue, endothelial cells (EC)[5] undergoing apoptosis are directly exposed to plasma coagulation factors and circulating leukocytes. We propose that because of their unique location at the interface of blood and tissue, EC undergoing apoptosis are potentially proinflammatory and procoagulant. We have previously shown that HUVEC induced to undergo apoptosis by suspension or by treatment with staurosporine become procoagulant (8). In this study, we investigated whether apoptotic EC also promote leukocyte adherence.

Various models have been developed to study apoptosis in vitro, including deprivation of growth factors (9), ligation of TNF (10) and Fas (11) receptors, irradiation (12), and suspension of normally adherent cells (8, 13). Apoptosis can also be triggered by certain protein kinase inhibitors or activators. Staurosporine, a promiscuous inhibitor of protein kinases, has been used to induce apoptosis in a variety of cell lines (14–16), including EC (8). Anisomycin, an antibiotic that inhibits protein synthesis at translation and activates p38 and JNK kinases (17) and mitogen-activated protein kinase substrate protein kinase-2 (18) has been reported to induce apoptosis in HL-60 (19) and U937 cells (20). While TNF-α alone can induce apoptosis in some cell types when protein synthesis is prevented by inhibitors such as cycloheximide, TNF-α readily induces apoptosis in most cells including EC (21, 22). We induced apoptosis in EC including HUVEC, bovine aortic EC (BAEC), and human microvascular EC (HMEC), and in non-EC including Chinese hamster ovary (CHO) cells, human skin fibroblasts (HSF), and mouse embryo fibroblasts (MEF) by withdrawal of growth factors (23) and by treating cells with staurosporine (8), anisomycin, and the combination of cycloheximide and TNF-α (21). Adherent cells induced to undergo apoptosis became proadhesive for leukocytes.

Macrophage phagocytosis of apoptotic neutrophils (24, 25) and eosinophils (26) has been shown to involve macrophage $\alpha_V\beta_3$ integrin (CD51/CD61), extracellular thrombospondin-1, and CD36 (25). CD14, which binds bacterial LPS, has recently been shown to be involved in macrophage recognition and phagocytosis of apoptotic cells (27), although the ligand(s) being recognized remains unknown. The class A macrophage scavenger receptor has also been implicated in interactions between macrophages and apoptotic cells (28). Phosphatidylserine translocated to the external leaflet of the plasma membrane of apoptotic cells may also mediate phagocytosis by neighboring cells (29) and macrophages (30). In contrast to these situations, leukocytes bound to apoptotic EC, CHO, MEF, and HSF induced to undergo apoptosis by a variety of

Departments of *Medicine (Hematology) and †Pathology, University of Washington School of Medicine, Seattle, WA 98195

Received for publication August 3, 1998. Accepted for publication January 14, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by U.S. Public Health Service Grants No. HL18645 and HL03174. A.K. is the recipient of a Clinician-Scientist Award of the Medical Research Council of Canada.

[2] Address correspondence and reprint requests to Barbara R. Schwartz, Hematology Box 359756, Harborview Medical Center, 325 9th Avenue, Seattle, WA 98104. E-mail address: brs@u.washington.edu

[3] Current address: Department of Pathology and Laboratory of Medicine, University of British Columbia and St. Paul's Hospital, Vancouver, British Columbia, Canada.

[4] Current address: Department of Medicine, University Hospital of Zurich, Zurich, Switzerland.

[5] Abbreviations used in this paper: EC, endothelial cell(s); BAEC, bovine aortic endothelial cell(s); BD (*N*-tert-butoxy-carbonyl-asp-fluoromethylketone); CHO, Chinese hamster ovary cells; HMEC, human microvascular endothelial cells; HUVEC, human umbilical vein endothelial cell(s); MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ZIETD (*N*-carbobenzoxy-ile-glu-thr-asp-fluoromethylketone); ZVAD (*N*-carbobenzoxy-val-ala-asp-fluoromethylketone; HSF, human skin fibroblasts; MEF, mouse embryo fibroblasts.

0022-1767/99/$02.00

different stimuli by a novel pathway of adhesion/recognition involving β_1 (CD29) integrin receptors on leukocytes and as yet unidentified ligand(s) on the apoptotic cells.

Materials and Methods

Cells

HUVEC were isolated and cultured as previously described (31) and were grown in RPMI 1640 with the addition of 2 mM glutamine, sodium pyruvate, nonessential amino acids, 10 mM HEPES, 100 U/ml penicillin, 100 U/ml streptomycin, 250 ng/ml fungizone (BioWhittaker, Walkersville, MD), 90 μg/ml heparin (Sigma, St. Louis, MO), bovine hypothalamic extract (gift of R. Ross, University of Washington, Seattle, WA), 10% bovine calf serum and 10% bovine calf serum supplemented with iron (HyClone, Logan, UT). HMEC-1 (32), the gift of E. Ades (Centers for Disease Control and Prevention, Atlanta, GA) and T. Lawley (Emory University, Atlanta, GA), were grown in EC basal medium (Clonetics Corporation, San Diego, CA) supplemented with 10 ng/ml epithelial growth factor (Life Technologies, Grand Island, NY), 1 μg/ml hydrocortisone (Sigma), and 10% FBS (HyClone). Human EC were cultured on surfaces coated with 2% gelatin (Sigma). BAEC (33) were isolated and grown as described. Additional BAEC were the gift of E. H. Sage (University of Washington). CHO cells were a gift of R. Lobb (Biogen, Cambridge, MA), and were grown in MEM-α containing deoxyribonucleosides (Life Technologies) with glutamine and 10% FBS. HSF were a gift of E. Raines (University of Washington), and MEF were obtained from the American Type Culture Collection (Manassas, VA) and were grown in DMEM (BioWhittaker) with glutamine and 10% FBS.

Leukocyte cell lines U937 (monocytic), K562 (multipotential hematopoietic), Ramos (Burkitt lymphoma; B cell), and THP-1 (monocytic leukemia) were obtained from American Type Culture Collection and were grown in Eagle's modified essential medium (BioWhittaker) supplemented with glutamine, pyruvate, nonessential amino acids, and 10% FBS.

Preparation of PBMC and neutrophils from peripheral blood

Peripheral blood was obtained from healthy donors with informed consent according to protocols approved by the Human Subjects Review Committee of the University of Washington. Blood was drawn into syringes containing heparin (10 U/ml). PBMC and neutrophils were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation. Red cells contaminating the neutrophil fraction were removed by lysis with cold red cell lysis buffer, 168 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3. Leukocytes were washed with PBS or HBSS (BioWhittaker). Mononuclear leukocytes were further separated into monocyte- and lymphocyte-enriched populations using Percoll (Pharmacia) gradient centrifugation. Additional monocytes prepared using OptiPrep (Life Technologies) were generously provided by W. Conrad Liles (University of Washington).

Abs

P4C10 (CD29, IgG1) and P1D6 (CD49e, IgG3) were purchased from Life Technologies. HP1/2 (CD49d, IgG1) was a gift of R. Lobb (Biogen). LM609 (CD51/CD61, IgG1) and P1E6 (CD49b, IgG1) were purchased from Chemicon (Temecula, CA), and G0H3 (CD49f, rat IgG2a) was obtained from Kamiya Biomedical Company (Seattle, WA). CY1787 (CD62E, IgG1) was a gift of L. Phillips and J. Paulson, Cytel Corporation (San Diego, CA). 4B9 (CD106, VCAM-1, IgG1) was prepared in our laboratory (34). 9.1G7 (CD54, IgG1) was also prepared in our laboratory. L230 (CD51, IgG1) was used as cell culture supernatant medium from hybridoma cells obtained from the American Type Culture Collection. FITC-labeled goat anti-mouse $F(ab')_2$ (Tago, Burlingame, CA) and peroxidase-labeled goat anti-mouse $F(ab')_2$ (Chemicon) were used as detecting Abs for flow cytometry and ELISA experiments, respectively.

Induction of apoptosis by chemical treatment

Cells were treated with staurosporine, anisomycin, or cycloheximide/TNF-α in defined medium consisting of RPMI 1640 (BioWhittaker) supplemented with glutamine, sodium pyruvate, nonessential amino acids, and HEPES, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite (Sigma), and 0.1% BSA (Sigma), with the addition of 4 ng/ml basic fibroblast growth factor-2 (R&D Systems, Minneapolis, MN) but without serum growth factors, or in one part defined medium and one part appropriate growth medium. For some experiments, EC were removed with versene (Life Technologies) or Triton X-100 (Sigma), and residual matrix washed with PBS/0.1% BSA (PBS/BSA) or defined medium before the adherence assay.

Adherence assays

Leukocytes were labeled with 2.5 μM calcein-AM (Molecular Probes, Eugene, OR) at room temperature for 20–40 min, washed, and resuspended in medium without phenol red. Preincubation of calcein-labeled leukocytes or treated or untreated EC with test reagents or Abs was conducted in the same medium. In some cases, unbound reagents were washed away from the cells before adherence. Leukocytes were allowed to adhere to control and treated EC or to EC matrix for 15–20 min at 37°C. Adherence was assessed in a Cytofluor Series 4000 fluorescence plate reader (PerSeptive Biosystems, Framingham, MA). Plates were scanned before and after washing for total and adherent cells, respectively, and calculations of percent adherence were performed using Excel (Microsoft, Redmond, WA). Statistical significance was determined using Student's *t* test.

Flow cytometry

For Ab binding, leukocyte cell lines were incubated with heat-inactivated adult bovine serum (HyClone) to control for nonspecific binding and then with primary Abs on ice. After washing, cells were incubated with FITC-labeled second Ab on ice. Cells were washed, fixed with 1% formaldehyde freshly diluted from 10% formaldehyde, methanol-free (Polysciences, Warrington, PA), and stored cold and dark until run on a Coulter EPICS XL FACS (Hialeah, FL). Analysis was performed with Reproman, a gift of D. Coder (University of Washington). Binding of annexin V and staining with propidium iodide in the presence of ribonuclease A for determination of DNA fragmentation were performed as previously described (8).

ELISA

Monolayers of HUVEC were incubated with primary Abs in RPMI/2% BCS for 45 min at 37°C. Following washing to remove unbound mAb, the cells were fixed with 0.05% glutaraldehyde (Eastman Kodak, Rochester, NY) freshly diluted in PBS. Ab binding was detected using peroxidase-labeled second Ab and quantitated using hydrogen peroxide and *O*-phenylenediamine (Sigma) in 0.1 M sodium citrate buffer at pH 4.5. Absorbance at 492 nm (reference, 650 nm) was determined on a Titertek Multiscan MCC/340 (Titertek, Huntsville, AL).

MTT assay

EC were treated as described. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution prepared in medium at fivefold concentration and filtered through a 0.45-μm filter was added to each well and the cells incubated at 37°C for 0.5–4 h. The medium was removed carefully, leaving behind MTT reaction product, which was then solubilized in DMSO. Absorbance at 570 nm (reference, 650 nm) was determined on a Titertek Multiscan MCC/340.

Reagents

Staurosporine, H7, KT5823, wortmannin, HA1004, cycloheximide, and anisomycin were obtained from Calbiochem (San Diego, CA). Staurosporine was also obtained from Kamiya, as were ZVAD (*N*-carbobenzoxy-val-ala-asp-fluoromethylketone), BD (*N*-tert-butoxy-carbonyl-asp-fluoromethylketone), and ZIETD (*N*-carbobenzoxy-ile-glu-thr-asp-fluoromethylketone). Genistein was obtained from Life Technologies. Annexin V and FITC-annexin V were the gifts of J. Tait (University of Washington). TNF-α, fibronectin, and laminin were obtained from R & D Systems. Cycloheximide, phospho-L-serine, phospho-D-serine, L-α-glycerophosphorylserine, propidium iodide, ribonuclease A, and MTT were obtained from Sigma. Thrombospondin was a gift of G. Liau (Genetic Therapy, Gaithersburg, MD). Reagents were prepared and stored according to suppliers' directions as stock solutions in water, alcohol, or DMSO and protected from light as necessary. Dilutions were prepared in medium daily.

Results

Leukocyte adherence to apoptotic cells

Staurosporine has been reported to induce apoptosis in a variety of cells, including EC (8). Staurosporine treatment induced dramatic morphological changes in monolayers including retracted cell bodies with cytoplasmic processes radiating from them, although staurosporine-treated HUVEC did not detach from the monolayer as did serum-deprived HUVEC. Oligonucleosome formation and hypodiploidy could be demonstrated in these cultures (Ref. 8 and data not shown).

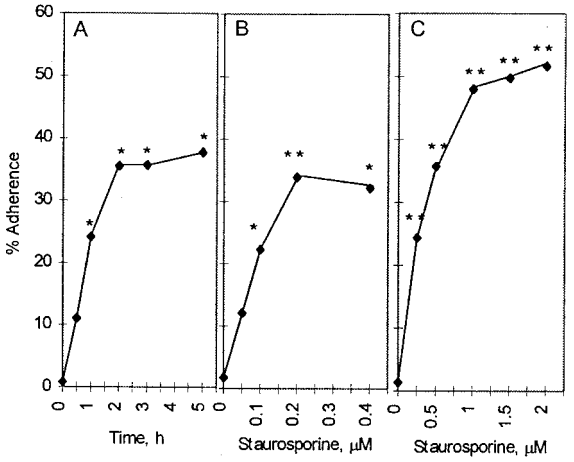

FIGURE 1. U937 adherence to staurosporine-treated HUVEC and BAEC is time- and dose-dependent. EC were treated with staurosporine in defined medium at 37°C at doses and times noted and washed twice before addition of calcein-labeled U937. *A*, HUVEC were treated with 200 nM staurosporine for various times. *B*, HUVEC were treated with various doses of staurosporine for 2 h. *C*, BAEC were treated with various doses of staurosporine for 2 h. Values represent means of four to nine experiments. *, $p < 0.05$; **, $p < 0.01$, compared with $t = 0$ or with no staurosporine.

EC treated with with staurosporine bound U937 in a time- and dose-dependent manner. Data for HUVEC and BAEC are presented in Fig. 1. Cycloheximide did not prevent staurosporine from inducing apoptosis or adhesiveness for U937 (data not shown). Staurosporine also induced adhesivity for U937 cells in the cell line HMEC-1. Adherence was 20 ± 11% to HMEC-1 treated with 200 nM staurosporine for 2–4 h vs 5 ± 2% to control HMEC-1 ($p < 0.05$, means ± SEM of six experiments). Staurosporine-treated CHO cells, MEF, and HSF also bound U937 cells (data not shown).

PBMC but not neutrophils increased adherence to staurosporine-treated HUVEC. Neutrophil adherence to control HUVEC was 1.8 ± 1.3% vs 1.2 ± 0.6% adherence to staurosporine-treated HUVEC ($p > 0.5$); PBMC adherence to control HUVEC was 2.9 ± 1.2% vs 9.4 ± 3.7% adherence to staurosporine-treated HUVEC ($p < 0.01$, means ± SEM, 4–11 experiments). Similarly, neutrophil adherence to control HUVEC was 1.1 ± 0.6% vs 1.7 ± 1.0% adherence to growth factor-deprived HUVEC ($p > 0.4$); PBMC adherence to control HUVEC was 5.0 ± 4.0% vs 13.2 ± 2.7% adherence to growth factor-deprived HUVEC ($p < 0.05$, means ± SEM, three to five experiments). All of the leukocytes adhered strongly to TNF-treated HUVEC (data not shown).

Treatment of EC with various reagents including genistein, herbimycin A, H7, wortmannin, KT5823, HA1004, and cycloheximide did not result in increased binding of leukocytes (data not shown). However, anisomycin, a protein synthesis inhibitor that also activates stress- and mitogen-activated kinases (19, 35) and induces apoptosis in other cells (19, 20) and in HUVEC (B.R.S. and J.M.H., unpublished observations), increased adhesiveness in HUVEC for U937 (Fig. 2) and PBMC but not for neutrophils (not shown). Anisomycin-treated BAEC also bound U937 (Fig. 2). Pretreatment of BAEC with 5 μg/ml cycloheximide for 30 min followed by addition of 50 ng/ml TNF-α and treatment for 17–19 h induced U937 to adhere. Adherence to cycloheximide/TNF-α-treated BAEC was 25 ± 10% vs 3.4 ± 2.7% to control BAEC ($p < 0.01$, means ± SEM for six experiments).

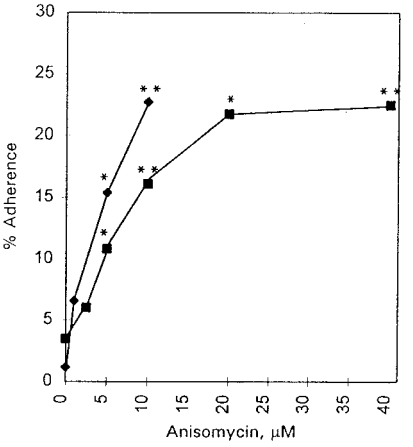

FIGURE 2. Anisomycin treatment of HUVEC and BAEC increases binding of U937. HUVEC (♦) were treated with anisomycin for 4–5 h, and BAEC (■) were treated for 18 h before washing and testing adherence of calcein-labeled U937. Values represent means of four to seven experiments. *, $p < 0.05$; **, $p < 0.01$, compared with no anisomycin.

Correlation of proadhesive changes with other markers of apoptosis

The induction of adhesiveness by staurosporine in HUVEC and in BAEC was an early event similar to increased annexin V binding, rather than a late event like DNA fragmentation (Ref. 8 and Fig. 3). Interestingly, incubation of EC before and during staurosporine treatment with IL-converting enzyme (caspase-1) inhibitor BD (100 and 200 μM), the caspase-8 inhibitor ZIETD (20 μM), or general caspase inhibitor ZVAD (20–200 μM) did not inhibit proadhesive changes (data not shown).

Phosphatidylserine, cytokine-inducible adherence molecules, and $\alpha_v\beta_3$ do not mediate leukocyte adherence to apoptotic HUVEC

Apoptotic cells translocate phosphatidylserine to the external leaflet of the plasma membrane, as demonstrated by increased binding of annexin V. Staurosporine treatment induced increased binding of annexin V by HUVEC (8) and BAEC (Fig. 3). Bennett et al. (29) reported significant inhibition of phagocytosis of apoptotic bodies by smooth muscle cells with 0.5 μM annexin V. However,

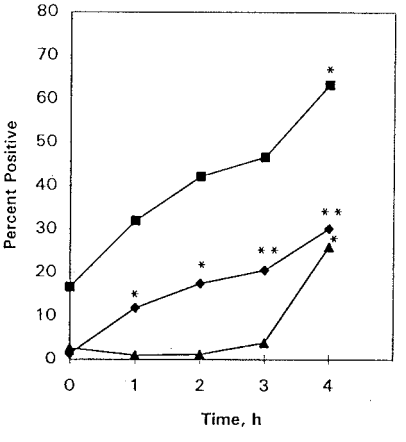

FIGURE 3. Proadhesive changes in BAEC follow a time-course similar to that of annexin V binding, while DNA fragmentation, scored as hypodiploidy by propidium iodide-staining, occurs later. ■, Percent of BAEC binding annexin V; ♦, percent U937 adherence; ▲, percent hypodiploid cells. Values represent means of two to four experiments. *, $p < 0.05$; **, $p < 0.01$.

Table I. *Inhibition of U937 adherence to staurosporine-treated EC by anti-integrin mAbs*

mAb(s)	Percent Specific Inhibition of Adherence[a]	
	HUVEC	BAEC
α_4	4 ± 13	29 ± 23*
α_5	7 ± 33	21 ± 28
α_6	0	14 ± 18
α_v	0	0
α_4, α_5	46 ± 18†	89 ± 10†
α_4, α_5, α_6	32 ± 20*	99 ± 4†
α_4, α_5, α_6, α_v	45 ± 22†	ND
$\alpha_v\beta_3$	0	ND
β_1	80 ± 16†	100 ± 6†

[a] U937 were incubated with the designated mAb(s) for 10 min at 37°C before assay of adherence to HUVEC treated with 200 nM staurosporine for 2 h or to BAEC treated with 2 μM staurosporine for 18 h. Percent specific inhibition was calculated as [(percent adherence with no mAB to treated EC − percent adherence with mAb(s) to treated EC)/(percent adherence with no mAb to treated EC − percent adherence with no mAb to control EC)] × 100%. Values represent means ± SEM of 3–11 experiments.

*, $p < 0.05$; †, $p < 0.01$, compared to adherence without mAb to staurosporine-treated EC.

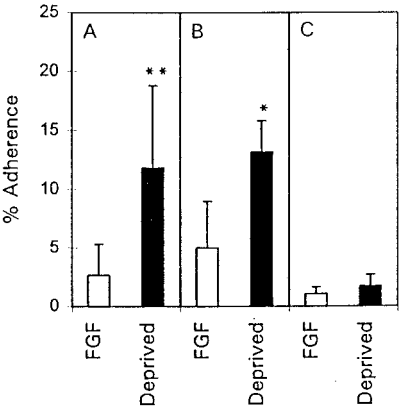

FIGURE 4. U937 binding to serum- and growth factor-deprived HUVEC is dependent on leukocyte but not on EC β_1 integrin. HUVEC were serum-deprived for 24 h and U937 adherence determined (*A*) in the absence or with either (*B*) HUVEC or (*C*) U937 pretreated with the anti-β_1 mAb P4C10. HUVEC or U937 were incubated with the anti-β_1 mAb for 10 min at 37°C, and unbound mAb was removed before the adherence assay was performed. Values represent means ± SEM for three experiments. Adherence to serum-deprived HUVEC (■) was different from adherence to fibroblast growth factor (FGF)-supplemented HUVEC (□) with no mAb pretreatment (*, $p < 0.05$) and with pretreatment of HUVEC (*, $p < 0.05$), but not with pretreatment of U937 ($p > 0.4$).

preincubation of staurosporine-treated HUVEC or HMEC or of U937 with annexin V at concentrations up to 10 μM did not inhibit U937 adherence (data not shown). Similarly, preincubation of U937 with L-α-glycerophosphoryl serine or phospho-L-serine (or phospho-D-serine as a negative control) at concentrations of up to 1 mM did not inhibit U937 adherence to staurosporine- or anisomycin-treated HUVEC (data not shown), although L-α-glycerophosphoryl serine and phospho-L-serine at 100-fold lower concentrations have been reported to inhibit macrophage phagocytosis of apoptotic thymocytes (30). The question of whether leukocytes bound to apoptotic cells because of increased expression of known adhesion molecules was addressed. HUVEC deprived of growth factors or treated with staurosporine or anisomycin did not show increased expression of ICAM-1 (CD54) or induction of expression of E-selectin (CD62E) or VCAM-1 (CD106) (data not shown). $\alpha_V\beta_3$ integrin mediates interactions between mononuclear phagocytes and apoptotic neutrophils (24) or apoptotic eosinophils (26). However, the blocking anti-$\alpha_V\beta_3$ mAb LM609 did not inhibit U937 adherence to apoptotic EC (Table I). Similarly, inclusion of thrombospondin at concentrations to 40 μg/ml did not inhibit U937 adherence to apoptotic EC (data not shown).

Enhanced leukocyte adherence to apoptotic HUVEC involves leukocyte β_1 integrins

The blocking anti-β_1 integrin subunit mAb P4C10 markedly inhibited U937 adherence to serum-deprived (Fig. 4), staurosporine-treated (Table I), and anisomycin-treated (data not shown) HUVEC, and to staurosporine-, anisomycin-, and cycloheximide plus TNF-α-treated BAEC (Table I and data not shown). Initial experiments were conducted with mAb present during the entire assay. The observation that freshly prepared polymorphonuclear leukocytes, which do not express β_1 integrins, did not increase adherence to apoptotic HUVEC suggested that leukocyte rather than endothelial β_1 integrin(s) might be involved. This hypothesis was tested by preincubating leukocytes and EC separately with the anti-β_1 integrin subunit mAb and washing to remove unbound mAb before combining the cells for the adherence assay. U937 adherence to apoptotic HUVEC was inhibited when the leukocytes, but not when the EC, were preincubated with mAb (Fig. 4).

Freshly prepared PBMC adherence to staurosporine-treated BAEC was also β_1 integrin subunit-dependent. Monocyte-enriched populations increased adherence to staurosporine-treated BAEC by 41 ± 20%, and this increased adherence was 83 ± 19% inhibited by mAb to β_1 integrin subunit (mean ± SEM of seven experiments). Lymphocyte-enriched populations increased adherence to staurosporine-treated BAEC by 76 ± 56%; this increased adherence was 93 ± 28% inhibited by mAb to β_1 integrin subunit (mean ± SEM of five experiments).

Furthermore, in an attempt to obtain mAbs that inhibit leukocyte adherence to apoptotic EC, 720 wells were screened after the fusion of mouse myeloma cells with spleen cells from a mouse immunized with staurosporine-treated HUVEC and BAEC. One supernatant medium was identified that inhibited U937 cell adherence to staurosporine-treated BAEC. Further testing showed that this Ab did not bind to BAEC, but did inhibit adherence of U937 cells to staurosporine-treated EC after incubation with U937 and removal of unbound Ab. Because supernatant media had been decanted but not washed off the BAEC before addition of the U937 cells, a functional mAb to human β_1 integrin subunit was fortuitously obtained. This mAb was confirmed to be directed to human β_1 integrin subunit by its binding to CHO cells transfected with human β_1 integrin subunit (B. R. Schwartz, N. L. Kovach, and J. M. Harlan, unpublished observations).

Identification of α-subunits involved in adherence to apoptotic HUVEC

Attempts were made to define which α-subunit(s) were involved in the β_1-mediated adherence. In simple cases, where the cells expressed only one known integrin α-subunit, adherence could be ascribed to that subunit. For example, adherence to serum-deprived or staurosporine-treated HUVEC of K562 cells, which express only α_5 (CD49e), and of Ramos, which express only α_4 (CD49d), could be inhibited by the appropriate blocking anti-α-subunit mAb (Fig. 5). Because U937 cells express multiple α-subunits, mAbs to individual subunits and combinations of mAbs to α_4, α_5, α_6, and α_v were tested for effects on their adherence to apoptotic HUVEC and to apoptotic BAEC. With staurosporine-treated HUVEC, single mAbs did not inhibit adherence significantly, and even combinations of mAbs were not so effective as

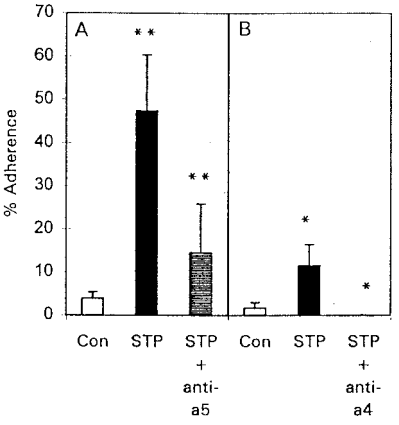

FIGURE 5. Blockade of α-subunits inhibits adherence to staurosporine-treated HUVEC. *A*, K562 were preincubated with the anti-α_5-subunit mAb P1D6; *B*, Ramos were preincubated with the anti-α_4-subunit mAb HP1/2, both before being tested for adherence to staurosporine (STP)-treated HUVEC. □, Control HUVEC (Con). ■, Staurosporine-treated HUVEC (STP). ▤, mAb-preincubated leukocytes to staurosporine-treated HUVEC. Values represent means ± SEM of three to five experiments. Staurosporine treatment of HUVEC induced adherence of K562 that was significant compared with adherence to untreated HUVEC (**, $p < 0.01$); this increased adherence was inhibited by pretreatment of K562 with anti-α_5 mAb (**, $p < 0.01$). Staurosporine-treated HUVEC bound more Ramos than untreated HUVEC (*, $p = 0.05$), and this adherence was inhibited by pretreatment of Ramos with anti-α_4 mAb (*, $p < 0.05$).

anti-β_1 mAb (Table I). However, the combination of anti-α subunit mAbs was nearly as effective as the anti-β_1 mAb in inhibiting U937 adherence to staurosporine-treated BAEC (Table I).

U937 adhere to apoptotic EC and not to exposed EC matrix

Because β_1 integrins recognize multiple matrix components, it was important to ascertain that adherence to the treated EC themselves was being measured and not adherence to the matrix exposed by retracting EC. The U937 cell line was selected for these experiments because unless they are activated by phorbol ester these cells do not exhibit significant adherence to matrix or to components of matrix. U937 were tested for adherence to matrix prepared from staurosporine- or anisomycin-treated HUVEC or BAEC by removal of cells using EDTA chelation (followed by washing in the presence of divalent cations) or Triton-X-100, as well as to purified fibronectin or laminin bound to plastic. Adherence of U937 to EC matrix or the matrix components was low and, furthermore, was not increased by staurosporine or anisomycin treatment. Table II shows data for U937 adherence to BAEC vs matrix of staurosporine-treated BAEC.

Table II. *U937 adhere to staurosporine- or anisomycin-treated BAEC but not to matrix of treated BAEC*

	U937 Adherence[a]	
	Expt. 1	Expt. 2
Untreated BAEC	2.5 ± 0.8	3.5 ± 0.5
Staurosporine-treated BAEC	39 ± 7†	36 ± 1†
Untreated matrix	1.0 ± 0.3	0.6 ± 0.1
Staurosporine-treated matrix	0.8 ± 0.1*	0.6 ± 0.1
Untreated BAEC	2.5 ± 0.6	2.4 ± 0.9
Anisomycin-treated BAEC	12 ± 1†	37 ± 5†
Untreated matrix	0.8 ± 0.2	0.6 ± 0.1
Anisomycin-treated matrix	0.7 ± 0.2	0.5 ± 0.1†

[a] U937 were tested for adherence to BAEC that had been treated for 17 h with 2 μM staurosporine or 20 μM anisomycin or to matrix from which treated BAEC had been removed with EDTA followed by washing with divalent cations. Values represent mean percent adherence ± SD of four to six wells for BAEC and 12 wells for matrix in each experiment.

*, $p < 0.05$; †, $p < 0.01$, compared to basal.

Discussion

Under conditions of inflammation or infection in vivo, nearby EC are stimulated by chemicals released by injured cells (e.g., IL-1) or infecting organisms (e.g., LPS) to become proadhesive and procoagulant. The specificity of leukocyte emigration is conferred by the particular molecules that the EC present to the circulating leukocytes. Apoptosis has been distinguished from other forms of cell death by the maintenance of cellular membrane integrity (7). Pieces of cytoplasm of apoptotic cells still bounded by cell membrane, known as apoptotic bodies, have been observed, rather than release of cellular contents. Cells that die in tissue via apoptosis are engulfed by neighboring cells or by resident phagocytes and do not induce significant inflammatory infiltrates. Apoptotic cells in tissue apparently transmit signals different from those of injured cells, and apparently do not activate EC to recruit inflammatory cells to the region. However, when EC themselves undergo apoptosis, their neighbors are other EC and the leukocytes in the passing blood. We have shown that serum-deprivation, or treatment with staurosporine or anisomycin, induces apoptosis and can cause the EC to become procoagulant (8) and proadhesive (this report). In this report, serum-deprivation and unrelated chemical reagents, staurosporine, a promiscuous protein kinase inhibitor, anisomycin, a protein synthesis inhibitor and protein kinase activator, and the combination of cycloheximide and TNF-α were used to induce apoptosis in EC and other adherent cells in culture. Characteristics of apoptotic cell death were confirmed by several criteria including oligonucleosome formation (DNA "ladders") and hypodiploidy and translocation of phosphatidylserine to the outer leaflet of cell membranes detected by increased binding of annexin V (8). Induction of apoptosis in vitro by these treatments caused EC to bind mononuclear leukocytes, either freshly prepared from peripheral blood or as cell lines, but not to bind neutrophils.

Leukocyte adherence to serum-deprived HUVEC was low, which may reflect in part the entry into a sequence of events leading to apoptosis of only part of the population at any given time. By the time adhesivness for leukocytes was tested, after an overnight incubation in serum- and growth factor-free medium, many of the apoptotic EC had already detached from the substratum and were not included in the adherence assay, and some of the EC had not yet entered the apoptotic program. We propose that the remaining adherent cells undergoing early stages of apoptosis were recognized by the leukocytes, which adhered to them. The use of chemical reagents to induce apoptosis allowed development of more uniform populations of treated EC and other cultured cells, independent of serum- or growth factor-dependent variables. Induction of apoptosis in HUVEC occurred more rapidly and uniformly with staurosporine and anisomycin treatments, with assessment of U937 adherence usually occurring within 2–4 h.

Staurosporine induction of proadhesive changes was an early event in apoptosis. Adherence of U937 to BAEC was observed to parallel increased binding of annexin V, which occurred hours before DNA fragmentation (Fig. 3). This is consistent with the appearance of proadhesive changes in serum-deprived HUVEC before detachment from the monolayer.

Clearance of apoptotic neutrophils and eosinophils has been demonstrated to be mediated in part by macrophage (24) and fibroblast (36) vitronectin receptor ($\alpha_v\beta_3$, CD51/CD61) and CD36 (25, 26). Macrophage recognition of apoptotic neutrophils by $\alpha_v\beta_3$/CD36 involves thrombospondin (25, 37), which may act as a

bridge between the two cell types. Phosphatidylserine on the surface of apoptotic leukocytes, demonstrated by increased binding of annexin V, appears to be another signal recognized by macrophages (30) and neighboring cells (29). The phosphatidylserine receptor/ligand is unknown. Fibroblast phagocytosis of apoptotic neutrophils is also inhibited by monosaccharides, suggesting a lectin-carbohydrate ligand interaction (36). Engulfment of murine thymocytes by macrophages was inhibited by a mAb that recognizes macrophage class A scavenger receptors and by scavenger receptor ligands, suggesting a role for the class A scavenger receptor (28). CD14 on macrophages can also participate in recognition and phagocytosis of apoptotic cells (27), with the ligand on the apoptotic cells still unknown.

The interaction between mononuclear leukocytes and apoptotic EC appears to involve a different mechanism(s) from those already described. Adherence of U937 to HUVEC rendered apoptotic by serum-deprivation or by treatment with staurosporine or anisomycin was inhibited by anti-β_1 mAbs but not by mAb to $\alpha_v\beta_3$. Anti-β_1 mAbs also inhibited U937 adherence to staurosporine-, anisomycin-, and cycloheximide/TNF-α-treated BAEC. Freshly prepared mononuclear leukocytes, both monocytes and lymphocytes, also increased adherence to staurosporine-treated BAEC in a β_1 integrin subunit-dependent manner. In addition, an attempt to obtain mAbs that inhibit leukocyte adherence to apoptotic EC by immunizing with treated EC yielded an anti-human- β_1 integrin subunit mAb. Of interest, in separate studies, we found that unactivated platelets also bind to apoptotic HUVEC by a β_1-dependent mechanism (38), whereas activated platelets bind to normal HUVEC via a bridging mechanism involving platelet GPIIbIIIa (CD41a/CD61), different adhesive proteins, and three different EC counter-receptors (39).

The identity of the α-subunit(s) involved was not clear. In cases such as K562 and Ramos, where only one α-subunit known to mediate cell-cell interactions is expressed, mAb specific for that subunit did inhibit. In U937, several α-subunits are expressed, and the mixture of anti-α_4 plus anti-α_5 mAbs inhibited adherence to a greater extent than either mAb alone. However, even combinations of mAbs were not so effective in inhibiting adherence to apoptotic HUVEC as anti-β_1 mAb alone. This suggests the presence of other, as yet unidentified, α-subunit(s) capable of mediating leukocyte adherence to apoptotic HUVEC, or more likely, that several $\alpha\beta_1$ heterodimers will recognize the apoptotic ligand(s). The differences in efficacy of combinations of α-subunit mAbs in inhibiting U937 adherence to apoptotic BAEC compared with apoptotic HUVEC may also indicate differences in EC ligand(s) expressed by the apoptotic BAEC and HUVEC that are recognized by the various integrins on human leukocytes. Further elucidation of the latter question awaits the identification of the ligand(s) induced by these treatments.

Cells expose subendothelial matrix as they retract, as in the case of staurosporine treatment, or retract and then detach, as in the case of serum-deprivation. Because the exposure of matrix can be dependent on dose of reagent and time of exposure, it was important to distinguish leukocyte adherence to affected cells from adherence to the exposed matrix. For example, neutrophil adherence to EC cultures exposed to photodynamic treatment, which was dose- and time-dependent, was found to be due to adherence to the exposed subendothelial matrix (40). Similarly, we have observed human erythroleukemia (HEL) cells to adhere to matrix avidly, so that some reagents that induced HUVEC to retract appeared to induce HEL adherence to the treated cells, but actually induced HEL adherence to the exposed matrix. U937 cells, on the other hand, did not adhere to matrix unless they were activated by phorbol esters. However, they did adhere to EC induced to express adhesion molecules by treatment with cytokines or by the induction of apoptosis.

The apoptotic EC ligand(s) for leukocytes is not one of the known inducible EC adhesion molecules—E-selectin, ICAM-1, or VCAM-1. Serum-deprivation, staurosporine, and anisomycin all failed to induce or increase expression of these adhesion molecules. As noted in other systems (41), induction of apoptosis caused translocation of phosphatidylserine to the outer leaflet of plasma membrane, detected by increased binding of annexin V (Ref. 8 and Fig. 4). Others have found that the presentation of phosphatidylserine by cells undergoing apoptosis signaled other cells to bind and to phagocytose them (29, 30). However, phosphatidylserine exposure was not a signal for mononuclear leukocytes to bind to apoptotic EC, as demonstrated by the failure of annexin V (29), L-α-glycerophosphorylserine, and phospho-L-serine (30) to inhibit adherence of U937 to staurosporine- or anisomycin-treated EC.

Because anisomycin, a protein synthesis inhibitor, induced adhesion, the apoptotic ligand is not the result of de novo protein synthesis. Cycloheximide did not prevent staurosporine from inducing an adhesive phenotype, indicating de novo protein synthesis was not required. On the other hand, the presence of cycloheximide assured that treatment with TNF-α would result in apoptosis rather than induction of known adherence molecules.

We conclude that EC induced to undergo an apoptotic death present a neo-ligand(s) for leukocyte β_1 integrins as the result of mobilization of preexisting molecules to the cell surface or of conformational changes of molecules already present on the cell surface. Induction of a proadhesive phenotype was not inhibited by incubation of EC before and during staurosporine-treatment with caspase inhibitors, perhaps indicating that this early apoptotic change is upstream of caspase activation. Finally, the identification of the apoptotic ligand(s) on EC is under investigation.

Acknowledgments

We thank Thomas Eunson for skillful technical assistance and Jia-Ling Teo for HUVEC cell culture.

References

1. Yang, E., and S. J. Korsmeyer. 1996. Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood 88:386.*
2. White, E. 1996. Life, death, and the pursuit of apoptosis. *Genes Dev. 10:1.*
3. Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkinson, and J. J. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature 337:181.*
4. Strattin, P., J. E. Damber, L. Karlberg, H. Nordgren, and A. Bergh. 1996. Bcl-2 immunoreactivity in prostate tumorigenesis in relation to prostatic intraepithelial neoplasia, grade, hormonal status, metastatic growth and survival. *Urol. Res. 24:257.*
5. Geng, Y.-J., and P. Libby. 1995. Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1B-converting enzyme. *Am. J. Pathol. 147:251.*
6. Hamet, P., D. deBlois, T. V. Dam, L. Richard, E. Teiger, B. S. Tea, S. N. Orlov, and J. Tremblay. 1996. Apoptosis and vascular wall remodeling in hypertension. *Can. J. Physiol. Pharmacol. 74:850.*
7. Wyllie, A. H., J. F. R. Kerr, and A. R. Currie. 1981. Cell death: the significance of apoptosis. *Int. Rev. Cytol. 68:251.*
8. Bombeli, T., A. Karsan, J. F. Tait, and J. M. Harlan 1997. Apoptotic vascular endothelial cells become procoagulant. *Blood 89:2429.*
9. Araki, S., Y. Shimada, K. Kaji, and H. Hayashi. 1990. Apoptosis of vascular endothelial cells by fibroblast growth factor deprivation. *Biochem. Biophys. Res. Commun. 168:1194.*
10. Liu, Z.-G., H. Hsu, D. V. Goeddel, and M. Karin. 1996. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. *Cell 87:565.*
11. Castro, J. E., J. A. Listman, B. A. Jacobson, Y. Wang, P. A. Lopez, S. Ju, P. W. Finn, and D. L. Perkins. 1996. Fas modulation of apoptosis during negative selection of thymocytes. *Immunity 5:617.*
12. Langley, R. E., E. A. Bump, S. G. Quartuccio, D. Medeiros, and S. J. Braunhut. 1997. Radiation-induced apoptosis in microvascular endothelial cells. *Br. J. Cancer 75:666.*

13. Meredith, J. E., Jr, B. Fazeli, and M. A. Schwartz. 1993. The extracellular matrix as a cell survival factor. *Mol. Biol. Cell 4:953.*
14. Bertrand, R., E. Solary, P. O'Connor, K. W. Kohn, and Y. Pommier 1994. Induction of a common pathway of apoptosis by staurosporine. *Exp. Cell Res. 211:314.*
15. Jarvis, W. D., A. J. Turner, L. F. Povirk, R. S. Traylor, and S. Grant. 1994. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Res. 54:1707.*
16. Weil, M., M. D. Jacobson, H. S. R. Coles, T. J. Davies, R. L. Gardner, K. D. Raff, and M. C. Raff. 1996. Constitutive expression of the machinery for programmed cell death. *J. Cell Biol. 133:1053.*
17. Stein, B., H. Brady, M. X. Yang, D. B. Young, and M. S. Barbosa. 1996. Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade. *J. Biol. Chem. 271:11427.*
18. Cano, E., Y. N. Doza, R. Ben-Levy, P. Cohen, and L. C. Mahadevan 1996. Identification of anisomycin-activated kinases p45 and p55 in murine cells as MAPKAP kinase-2. *Oncogene 12:805.*
19. Polverino, A. J., and S. D. Patterson. 1997. Selective activation of caspases during apoptotic induction in HL-60 cells: effects of a tetrapeptide inhibitor. *J. Biol. Chem. 272:7013.*
20. Kochi, S. K., and R. J. Collier. 1993. DNA fragmentation and cytolysis in U937 cells treated with diphtheria toxin or other inhibitors of protein synthesis. *Exp. Cell Res. 208:296.*
21. Pohlman, T. H., and J. M. Harlan. 1989. Human endothelial cell response to lipopolysaccharide, interleukin-1, and tumor necrosis factor is regulated by protein synthesis. *Cell Immunol. 119:41.*
22. Polunovsky, V. A., C. H. Wendt, D. H. Ingbar, M. S. Peterson, and P. B. Bitterman. 1994. Induction of endothelial cell apoptosis by TNFα: modulation by inhibitors of protein synthesis. *Exp. Cell. Res. 214:584.*
23. Karsan, A., E. Yee, G. G. Poirier, P. Zhou, R. Craig, and J. M. Harlan. 1997. Fibroblast growth factor-2 inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms. *Am. J. Pathol. 151:1775.*
24. Savill, J., I. Dransfield, N. Hogg, and C. Haslett. 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature 343:170.*
25. Savill, J., N. Hogg, Y. Ren, and C. Haslett. 1992. Thrombospondin cooperates with CD36 and the vitronectin receptor in macrophage recognition of neutrophils undergoing apoptosis. *J. Clin. Invest. 90:1513.*
26. Stern, M., J. Savill, and C. Haslett. 1996. Human monocyte-derived macrophage phagocytosis of senescent eosinophils undergoing apoptosis. Mediation by $\alpha_v\beta_3$/CD36/thrombospondin recognition mechanism and lack of phlogistic response. *Am. J. Pathol. 149:911.*
27. Devitt, A., O. D. Moffatt, C. Raykundalia, J. D. Capra, D. L. Simmons, and C. D. Gregory. 1998. Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature 392: 505.*
28. Platt, N., H. Suzuki, Y. Kurihara, T. Kodama, and S. Gordon. 1996. Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. USA 93:12456.*
29. Bennett, M. R., D. F. Gibson, S. M. Schwartz, and J. F. Tait 1995. Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ. Res. 77:1136.*
30. Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol. 148:2207.*
31. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest. 52:2745.*
32. Ades, E. W., F. J. Candal, R. A. Swerlick, V. G. George, S. Summers, D. C. Bosse, and T. J. Lawley. 1992. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J. Invest. Dermatol. 99:603.*
33. Schwartz, S. M. 1978. Selection and characterization of bovine aortic endothelial cells. *In Vitro 14:966.*
34. Carlos, T. M., B. R. Schwartz, N. L. Kovach, E. Yee, M. Rosso, L. Osborn, G. Chi-Rosso, B. Newman, R. Lobb, and J. M. Harlan. 1990. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. *Blood 76:965.*
35. Zinck, R., M. A. Cahill,. M. Kracht, C. Lachsenmaier, R. A. Hipskind, and A. Nordheim. 1995. Protein synthesis inhibitors reveal differential regulation of mitogen-activated protein kinase and stress-activated protein kinase pathways that converge on Elk-1. *Mol. Cell. Biol. 15:4930.*
36. Hall, S. E., J. S. Savill, P. M. Henson, and C. Haslett. 1994. Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin. *J. Immunol. 153: 3218.*
37. Navazo, M. D., L. Daviet, J. Savill, Y. Ren, L. L. Leung, J. L. McGregor. 1996. Identification of a domain (155–183) on CD36 implicated in the phagocytosis of apoptotic neutrophils. *J. Biol. Chem. 271:15381.*
38. Bombeli, T., B. R. Schwartz, and J. M. Harlan. 1999. Endothelial cells undergoing apoptosis become proadhesive for non-activated platelets. *Blood. In press.*
39. Bombeli, T., B. R. Schwartz, and J. M. Harlan. 1998. Adhesion of activated platelets to endothelial cells: evidence for a GPIIbIIIa-dependent bridging mechanism and novel roles for endothelial intercellular adhesion molecule 1 (ICAM-1), $\alpha_v\beta_3$ integrin, and GPIbα. *J. Exp. Med. 187:329.*
40. de Vree, W. J., A. N. Fontijne-Dorsman, J. F. Koster, and W. Sluiter. 1996. Photodynamic treatment of human endothelial cells promotes the adherence of neutrophils in vitro. *Br. J. Can. 73:1335.*
41. Martin, S. J., C. P. M. Reutelinsperger, A. J. McGahon, J. A. Rader, R. C. A. A. van Schie, D. M. LaFace, and D. R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med. 182:1545.*