Characterization of Adhesive Interactions between Human Endothelial Cells and Megakaryocytes

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Abstract

Cell–cell adhesion is essential for many immunological functions and is believed to be important in the regulation of hematopoiesis. Adhesive interactions between human endothelial cells and megakaryocytes were characterized in vitro using the CMK megakaryocytic cell line as well as marrow megakaryocytes. Although there was no adhesion between unactivated human umbilical vein endothelial cells (HUVEC) and megakaryocytes, treatment of HUVEC with inflammatory cytokines such as IL-1β, tumor necrosis factor alpha, INF-γ, or the phorbol ester phorbol myristate acetate (PMA) resulted in a time- and dose-dependent increase in adhesion. Stimulation of marrow megakaryocytes or CMK cells with the cytokines IL-1β, GM-CSF, IL-6, IL-3, or PMA augmented their adhesion to endothelium. Monoclonal antibodies against the LFA-1 subunit of the leukocyte adherence complex CD18 inhibited the binding of marrow megakaryocytes or CMK cells to HUVEC. Adhesion blocking experiments also demonstrated that the VLA-4/VCAM-1 pathway was important for megakaryocyte attachment to HUVEC. Adhesion promoted maturation of megakaryocytic cells as measured by increased expression of glycoproteins GpIb and GpIIb/IIIa and by increased DNA content. These observations suggest that alterations in megakaryocyte adhesion may occur during inflammatory conditions, mediated by certain cytokines, resulting in augmented megakaryocyte maturation. (*J. Clin. Invest.* 1993. 91:2378–2384.) Key words: integrins • cytokines • phorbol esters • megakaryocytopoiesis

Introduction

Adhesion molecules play a major part in cell–matrix and in cell–cell interactions (1). These adhesive interactions are important in the regulation of hematopoiesis and thymocyte maturation, the direction and the control of lymphocyte traffic and migration through tissues, and the development of immune and nonimmune inflammatory responses (2). Adhesion molecules belong to three different families: integrins (3), immunoglobulins (4), and selectins (5). Integrins are expressed by a large number of tissues whereas other adhesion molecule families are restricted to a smaller number of cell types. The leukocyte integrin family (3) is composed of $\alpha\beta$ heterodimeric membrane glycoproteins that share a common β subunit, designated CD18. The α subunits of each of the three members, lymphocyte function associated antigen-1 (LFA-1) (2), macrophage antigen-1 (Mac-1) and p150,95 are designated CD11a, b, and c respectively. These adhesion molecules play a critical part in the immune and inflammatory responses of leukocytes. Another integrin subfamily found on leukocytes is the very late after activation antigens (VLA) group (6). Members of this family function mainly as extracellular matrix adhesion receptors and are found both on hematopoietic and nonhematopoietic cells. A third integrin subfamily, the cytoadhesions, are receptors on platelets and endothelial cells, which bind extracellular matrix proteins (7, 8). The immunoglobulin superfamily of adhesion receptors, which include CD2 and ICAM-1, participate in T cell adhesive interactions and the antigen-specific receptors of T and B cells, CD4, CD8, and the MHC class I and II molecules (9). The third family of adhesion receptors is the selectins, characterized by a common lectin domain. Inducible endothelial leukocyte adhesion molecule-1 (ELAM-1) (10, 11) and granule membrane protein (GMP-140) (5, 12) are expressed on stimulated endothelial cells and activated platelets.

The integrin family consists of at least 17 distinct heterodimers formed by associations between 13 α subunits and 7 different β subunits, each with distinct ligand-binding properties (13). Among integrins, the VLA-5 complex is widely distributed and functions as a receptor for fibronectin (14, 15). In contrast, the VLA-4 complex is expressed at substantial levels on normal peripheral blood B and T- cells, thymocytes, monocytes, and some melanoma cells, as well as on marrow blast cells and erythroblasts (16, 17). Ligands for VLA-4 are vascular cell adhesion molecule-1 (VCAM-1), identified on the surface of activated endothelial cells (18–21) and CS-1, an alternately spliced domain within the Hep II region of fibronectin (22, 23).

The regulation of megakaryocyte proliferation and differentiation appears to be controlled by a number of soluble mediators as well as cell–cell interactions (24). The major stromal elements in the bone marrow microenvironment include cells of mesenchymal, endothelial, and macrophage origin. Systematic study of the effects of cytokines derived from each of these stromal elements, as well as adhesive interactions of these cell types with human megakaryocytes, may improve our understanding of regulation of megakaryocytopoiesis under physiological and disease conditions. In the bone marrow, megakaryocytes are located in the extravascular space applied to the abluminal surface of endothelium (25–27). In this location, megakaryocytes send cytoplasmic projections into the lumen, which could serve to anchor the cell to the endothelium (25, 26). The interaction of rat and guinea pig megakaryocytes with

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1. *Abbreviations used in this paper:* HUVEC, human umbilical vein endothelial cells; SCF-KL, stem cell factor/kit ligand.

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subendothelial extracellular matrix was associated with adhesion, platelet-like shape changes and thromboxane A_2 productions (28). However, characterization of these adhesion interactions in the marrow microenvironment is not well defined. To that end, we initially studied the interaction of human megakaryocytes with bone marrow fibroblasts and observed that a number of adhesion molecules on the surface of megakaryocytes may mediate interaction with bone marrow fibroblasts, particularly the c-kit protooncogene product on the megakaryocyte surface with the membrane-bound form of the stem cell factor/kit ligand (SCF/KL) expressed on the marrow fibroblast (29). We have now investigated the adhesive interactions between human megakaryocytes and endothelial cells. It appears that megakaryocyte–endothelial cell adhesive interactions differ in their nature compared with those with bone marrow stromal fibroblasts, suggesting that different stromal cell populations may have different roles in regulation of megakaryocytopoiesis.

Methods

Growth factors and reagents. Human SCF/KL was cloned and expressed in *Escherichia coli* and purified as previously described (30). Recombinant human SCF/KL, human IL-3, human GM-CSF, and human IL-6 were provided by Amgen Biologicals (Thousand Oaks, CA). Recombinant human IL-1β, INF-γ, and tumor necrosis factor-α (TNF-α) were provided by Dr. C. Dinarello (Tufts University, Boston, MA). These cytokines were determined to be free of endotoxin contamination. Plateau doses of each factor were determined from dose–response curves and used for culture at the following concentrations: 100 ng/ml SCF/KL; ng ml TNF-α 50; 1,000 ng/ml IFN-γ; 10 ng/ml IL-6; 10 ng/ml IL-1β; 10 ng ml IL-3; and 200 ng/ml GM-CSF. Cycloheximide and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO).

Megakaryocytic cells. Human bone marrow was obtained by aspiration from the iliac crest of normal donors who gave informed consent in a protocol approved by the New England Deaconess Hospital Institutional Review Board. The marrow was aspirated into preservative-free heparin (Sigma Chemical Co.) and separated by centrifugation through Ficoll-Hypaque (Pharmacia Inc., Piscataway, NJ) at 1,200 *g* at room temperature for 30 min. Human marrow megakaryocytes were isolated by a method using immunomagnetic beads using anti–human glycoprotein GpIIb/IIIa monoclonal antibody as described previously (31). All of the isolated cells were morphologically recognizable megakaryocytes. Growth factors were added to the cultures and were incubated for 16 h at 37°C in a 5% CO_2 humidified atmosphere.

The CMK cell line, provided by Dr. T. Sato, derived from a child with megakaryoblastic leukemia, has properties of cells of megakaryocytic lineage, including surface expression of glycoproteins Ib and IIb/IIIa, synthesis of platelet factor 4, platelet-derived growth factor, von Willebrand's factor, and becomes polyploid upon induction with phorbol esters (32, 33). No myeloid or lymphoid surface markers have been found on our cultured CMK cells. The CMK cell line was cultured in RPMI 1640 + 10% FCS. CMK-G is a subclone of CMK cells that expresses lower levels of glycoproteins GpIb and GpIIb/IIIa and was also cultured in RPMI 1640 + 10% FCS.

Viability and identification of megakaryocytes. Viability of separated cells was assessed by trypan blue exclusion (31). Megakaryocytes were identified by Wright's staining and flow cytometric expression of GpIIb/IIIa and GpIb (31). The maturation stages of the megakaryocytes were classified by cell size, nuclear morphology, and cytoplasmic staining (31).

Cell culture of human umbilical vein endothelial cells (HUVEC). HUVEC were obtained as primary cultures as previously described (34) (gift of M. Gimbrone, Jr., Brigham and Women's Hospital, Boston, MA). They were maintained at 37°C with 5% CO_2 in 80% nutrient medium 199 with 25 mM Hepes (M.A. Bioproducts, Walkersville, MD), 20% FCS (heated at 56°C for 30 min), and fresh 2 mM L-glutamine (Flow Laboratories, McLean, VA). The medium was supplemented with 100 μg/ml sodium heparin (porcine intestinal mucosa; Sigma Chemical Co.) and 50 μg/ml endothelial mitogen (Biomedical Technologies, Inc., Stoughton, MA). Tissue culture flasks were coated with 0.1% gelatin (Difco Laboratories, Inc., Detroit, MI) before passage of HUVEC. Second to fourth passage confluent cultures of HUVEC were used. HUVEC were allowed to grow to confluence on gelatinized 24-well culture plates ($\sim$ 250,000 HUVEC per well). Confluence was verified by phase-contrast microscopy before use in experiments.

HUVEC activation. The ability of various stimuli to induce adhesion of HUVEC to megakaryocytes was investigated by preincubating HUVEC with various activating stimuli at 37°C in a humidified atmosphere of 5% CO_2, 95% air for 24 h, unless otherwise indicated in the text. After this stimulation HUVEC were washed with warm (37°C) medium and 100 μl of cell suspension (5×10^4 CMK or isolated megakaryocytes) were added to each well and HUVEC and megakaryocytes were allowed to incubate for up to 3 h as indicated in the text. All experiments were performed in duplicate.

Flow cytometry. For flow cytometry, 5×10^5 cells were exposed to monoclonal antibodies (4°C, 30 min), washed three times, and followed by fluorescein-conjugated goat anti–mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) (1:50 dilution in HBSS with 0.1% BSA at 4°C for 20 min) and fixed in 1% paraformaldehyde in PBS.

Preparation of phorbol-12-myristate-13-acetate (PMA)–treated megakaryocytic cells. PMA (Sigma Chemical Co.) was dissolved in DMSO and stored at −80°C. Just before use, PMA was diluted in the RPMI culture medium. Cells were incubated with PMA at a concentration of 10 ng/ml in a 5% CO_2 humidified atmosphere as indicated.

Proliferation assays. Cell proliferation and viability were assessed by [^{3}H]thymidine incorporation and by trypan blue exclusion (0.4% trypan blue stain in 0.85% saline; Gibco Laboratories, Grand Island, NY). For [^{3}H]thymidine incorporation assays, cells were incubated at 37°C in a humidified atmosphere of 5.5% CO_2 for 48 h. Cells were pulsed with 0.5 μCi per well of [^{3}H]thymidine (DuPont–New England Nuclear, Boston, MA) and incubated for an additional 4 h. Samples were harvested onto glass fiber filters and counted by liquid-scintillation spectrometry.

Cell–cell adhesion assay. To measure cell–cell adhesion, 5×10^4 CMK cells or bone marrow megakaryocytes in 2 ml of DMEM 10% + FCS were added to HUVEC. The cells were allowed to settle for 3 h at 37°C. Nonadherent cells were then washed away in four changes of medium, centrifuged at 400 *g* for 10 min, and counted. The remaining adherent cells were collected by vigorously pipeting trypan blue. To confirm the adherence of megakaryocytes to HUVEC, in some experiments we used radiolabeling with ^{51}Cr. Primary megakaryocytes or CMK cells (10^6) cells were labeled with 100 μCi of $^{51}Cr\ Na_2{}^{51}CrO_4$ for 1 h at 37°C and then washed twice in PBS and resuspended at 10^6 cells/ml. ^{51}Cr-labeled cells were added to each well with HUVEC in a total of 100 μl and binding was allowed to occur for 3 h at 37°C. The nonadherent megakaryocytic cells were then removed by three washes with PBS, followed by lysis of bound cells with 0.1% NP-40. The radioactivity of each lysate was measured in a gamma counter, which was indicative of megakaryocyte cell adhesion.

For adhesion inhibition studies, we used the following monoclonal antibodies: TS1/22 (IgG_1) and TS1/18 (IgG_1), which recognize LFA-1α subunit and LFA-1β subunit, respectively (Dr. T. A. Springer, Center for Blood Research, Harvard Medical School, Boston, MA). Monoclonal antibodies to VCAM-1 (BBA-6) (IgG_1) were obtained from R&D Systems Inc. (Minneapolis, MN). Monoclonal antibodies for VLA-4 (CD49d) (IgG_1) were obtained from Immunotech International (Westbrook, ME). Antibody W6/32 (IgG2a, anti-HLA class I antigen), was obtained from American Type Culture Collection (Rockville, MD) and used as a control antibody; control IgG_1 ascites was also used. Monoclonal antibodies to human GpIb (IgG_1) and

GpIIb/IIIa (IgG_1) were obtained from Becton-Dickinson & Co. (Mountain View, CA). All monoclonal antibodies were used at a 1:100 dilution (final concentration of 10 μg/ml). To assay inhibition of adhesion, cells were treated (30 min, 37°C) with the relevant antibody, as indicated in the text. After 30 min, the megakaryocytic cells were washed 3× with PBS and then added to HUVEC monolayers for a 3-h incubation at 37°C. Nonadherent cells were removed and the remaining adherent cells were collected by vigorous pipeting and counted as described above.

Effect of temperature, cycloheximide, and actinomycin D on megakaryocyte adhesion to HUVEC. To examine the effect of temperature on CMK adhesion to HUVEC, HUVEC were first preincubated for 24 h at 37°C with either IL-1β (10 ng/ml), PMA (10 ng/ml), TNF-α (50 ng/ml), or IFN-γ (1,000 ng/ml). HUVEC were then washed with RPMI and CMK cells (5×10^4 cells) were then added to HUVEC for 3 h of adhesion at 4, 22, or 37°C. CMK adhesion was measured as indicated above.

To examine the effect of the protein synthesis inhibitor cycloheximide and the RNA synthesis inhibitor actinomycin D, HUVEC were pretreated for 30 min with cycloheximide (1 μg/ml) or actinomycin D (1 μg/ml) and then TNF-α (50 ng/ml) or PMA (10 ng/ml) was added for 24 h. HUVEC were then washed and CMK cells were added for 3 h. Adhesion of CMK cells was quantitated as described above.

Flow cytometric measurement of DNA content of primary megakaryocytes or CMK-G cells. CMK-G cells were plated in 24-well plates at 2×10^5/ml with 5% platelet-poor plasma for 5 d with or without IL-6 (10 ng/ml). In parallel, adhered CMK-G cells were cultured in 24-well plates containing unstimulated or TNF-α stimulated HUVEC at 2×10^5/ml, prelabeled with green fluorochrome, for 5 d as described (35). CMK-G cells were then collected and washed twice with HBSS, resuspended in nuclei isolation medium (NIM–0.2% BSA, 0.4% provided p40 and 10 mM Hepes pH 7.4 in HBSS) plus 54 Worthington units/ml RNase A at 2×10^6/ml, and an equal volume of nuclei isolation medium containing 25 μg/ml propidium iodide (Sigma Chemical Co.) was then added. Samples were kept in the dark at 4°C and analyzed the same day on a FACS® scan using Cell Fit software as previously described (32). Freshly prepared lymphocytes were used to mark the position of the 2N cells. The samples were analyzed for contamination with HUVEC cells by analyzing the CMK-G samples for green fluorochrome labeling.

Statistical analysis. The results were expressed as the mean±SEM of data obtained from three or more experiments performed in duplicate. Statistical significance was determined using the Student's *t* test.

Results

Endothelial cell stimulation enhances adhesion for megakaryocytes. In the absence of activating stimuli, adhesion of primary marrow megakaryocyte or CMK cells to HUVEC was < 5% (Fig. 1). Treatment of HUVEC with IL-1β, TNF-α, IFN-γ, or PMA resulted in dose-dependent increases in adherence of megakaryocytic cells to HUVEC. Optimal binding of primary marrow megakaryocytes and CMK to HUVEC was induced, respectively, by 10 ng/ml IL-1β (35.1±9%; 39±7%), 1,000 ng/ml IFN-γ (37±10%; 42±8%), 50 ng/ml TNF-α (32±8%; 37±9%), and 10 ng/ml PMA (59±9%; 68±7%).

The adhesion kinetics of CMK megakaryocytic cells to HUVEC is shown in Fig. 2. Adhesion to HUVEC induced by the cytokines IL-1β, IFN-γ, or TNF-α was similar, being maximal by 6 h of incubation. Adhesion of megakaryocytic cells to PMA-treated HUVEC was more rapid, with a maximal increase at 60 min. Adhesion induced by all four stimuli gradually declined over 48 h.

The roles of protein and RNA synthesis in the adhesion process of HUVEC and megakaryocytes were evaluated. Experiments were performed in which HUVEC were treated for 30 min with the protein synthesis inhibitor cycloheximide (1 μg/ml) or the RNA synthesis inhibitor actinomycin D (1 μg/ml) and then incubated for 24 h without stimulus or after stimulation with TNF-α (50 ng/ml) or PMA (10 ng/ml). Neither cycloheximide nor actinomycin D affected the lack of adhesion of unstimulated HUVEC and CMK cells (6±3%). Cycloheximide modestly inhibited the level of adhesion induced by TNF-α (23±7% inhibition) but had almost no effect on PMA-induced adhesion (10±5% inhibition). Actinomycin D was more effective in blocking adhesion induced by TNF-α (52±10% inhibition) and by PMA (26±11% inhibition). Adhesion of CMK megakaryocytic cells to HUVEC was temperature dependent (37° > 22° > 4°C) (Fig. 3). Optimal adherence occurred at 37°C.

Stimulation of megakaryocytes enhances their adhesion to HUVEC. To demonstrate that various stimuli promote megakaryocytic cell adhesion to HUVEC, experiments were per-

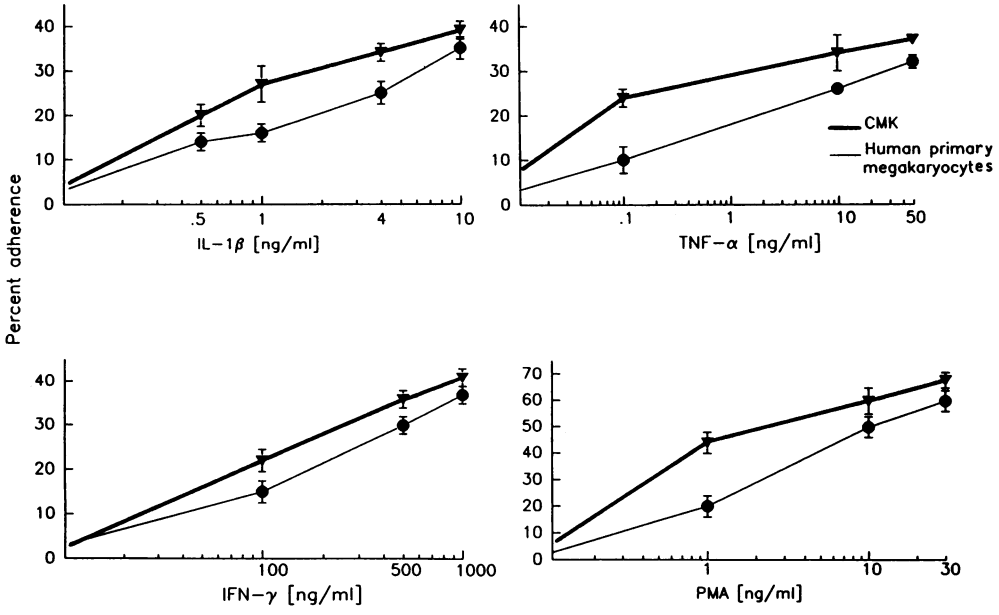

Figure 1. Adhesion of megakaryocytes to stimulated HUVEC. HUVEC were incubated with the indicated concentrations of IL-1β, TNF-α, IFN-γ or PMA for 24 h. Washed CMK cells or isolated marrow megakaryocytes were added and allowed to adhere to HUVEC for 3 h. Values represent the mean±SEM of duplicate determinations in three experiments.

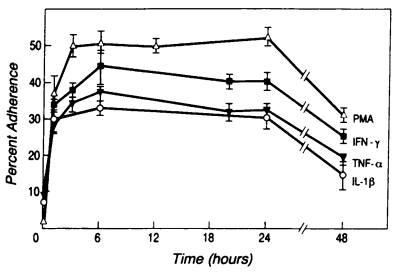

Figure 2. Kinetics of induction of adhesion of megakaryocytes to HUVEC. HUVEC were incubated with PMA (10 ng/ml), IFN-γ (1,000 ng/ml), TNF-α (50 ng/ml), or IL-1β (10 ng/ml) for the length of time indicated and then tested for adhesion of CMK megakaryocytic cells as in Fig. 1. Values represent the mean±SEM of duplicate determinations in three experiments.

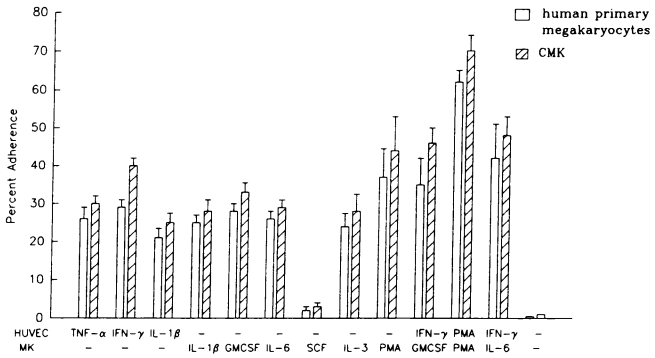

Figure 4. Megakaryocyte adherence to HUVEC after stimulation of both cell types. HUVEC were stimulated for 24 h. CMK or bone marrow megakaryocytes (*MK*) were stimulated for 16 h as indicated in the figure. Cells were exposed to TNF-α (50 ng/ml), IFN-γ (1,000 ng/ml), IL-1β (10 ng/ml), GM-CSF (200 ng/ml), IL-6 (10 ng/ml), SCF (100 ng/ml), IL-3 (10 ng/ml), or PMA (10 ng/ml), as indicated in the figure. Values represent the mean±SEM of duplicate determinations in three experiments.

formed with stimulated primary marrow megakaryocytes or CMK cells and HUVEC. Fig. 4 illustrates that although unstimulated megakaryocytes did not adhere to unstimulated HUVEC, stimulation of megakaryocytes with the cytokines IL-1β, GM-CSF, IL-6, IL-3, or PMA induced adhesion of megakaryocytes to unstimulated HUVEC with ≥ 20% adherence (Fig. 4). Activation of HUVEC with IFN-γ or PMA resulted in further enhancement of adhesion of cytokine-stimulated primary marrow megakaryocytes or CMK cells (Fig. 4).

FACS analysis of VLA-4 and LFA-1 expression on unstimulated CMK cells or CMK cells stimulated with IL-1β, GM-CSF, IL-6, IL-3, or PMA for 6 or 24 h revealed no changes in the level of expression of either adhesion molecule (with > 90% of cells expressing both LFA-1 and VLA-4). Endothelial cells can be induced to express ICAM-1 and VCAM-1, surface proteins that are involved in leukocyte adhesion (1). TNF-α or IL-1β treatment of HUVEC induced VCAM-1 and ICAM-1 expression (data not shown) as has been demonstrated previously (1, 36, 37).

We next investigated whether a subpopulation of megakaryocytic cells might be capable of adhesion to HUVEC on the basis of their degree of maturation. Thus, the stage of maturation of primary marrow megakaryocytes and CMK cells to HUVEC was compared with megakaryocytes that did not adhere. Expression of the surface molecules GpIb and GpIIb/IIIa and DNA content are markers of maturation, since expression and ploidy increase as immature megakaryocytes differentiate

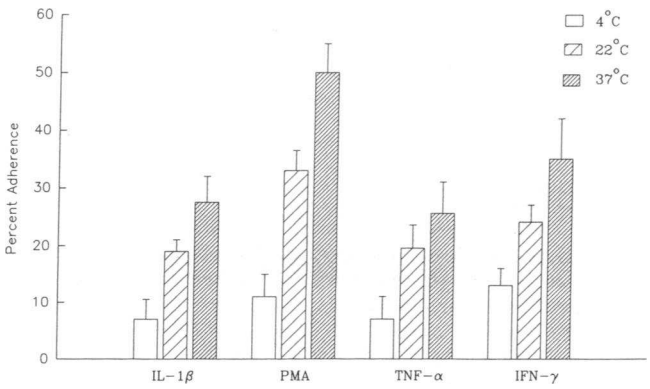

Figure 3. Temperature dependence of CMK adhesion to HUVEC. HUVEC were treated for 24 h as in Fig. 1 at 37°C. CMK cells cultured at 37°C were then added to HUVEC. The temperatures of the cultures were then altered to those indicated (4 or 22°C) or maintained at 37°C. Adhesion was measured 3 h later. Values represent the mean±SEM of duplicate determinations in three experiments.

and ultimately form platelets (24, 35). We did not observe different levels of expression of surface GpIb and GpIIb/IIIa by flow cytometric analysis of nonadherent megakaryocytes compared with the cells that adhered (data not shown). Ploidy analysis of nonadherent and adherent CMK megakaryocytic cells revealed no difference in distribution of DNA content (data not shown). These results indicate that adhesion of megakaryocytes to HUVEC was not dependent on the maturational stage of the megakaryocyte under the conditions of our in vitro cultures.

Megakaryocyte adhesion to HUVEC is LFA-1 and VLA-4 dependent. To address the question whether integrins expressed on megakaryocytes are involved in promotion of their attachment to HUVEC, we performed experiments in which megakaryocytic cells were treated with monoclonal antibodies directed against the common β subunit of the CD18 complex (TS1/22.1.1 13.3) and/or against the α subunit of LFA-1 (TS1/18.1, 2, 11.4). We initially determined that expression of LFA-1 and VLA-4 was abundant in CMK cells and human primary megakaryocytes with > 90% of cells expressing the surface molecules as detected by flow cytometry (Fig. 5). Antibodies to LFA-1 inhibited the adhesion of isolated bone marrow megakaryocytes (38±7%) or CMK cells (55±9%) to HUVEC. This indicated the involvement of the LFA-1 complex in adhesion of megakaryocytes and CMK to stimulated HUVEC. The adhesion of primary marrow megakaryocytes or CMK cells to HUVEC was also inhibited by anti-VLA-4 (43±10 and 74±8%, respectively). In addition, preincubation of HUVEC with VCAM-1 antibodies inhibited adhesion of HUVEC to primary marrow megakaryocytes and CMK cells by 28±7 and 56±10%, respectively (Fig. 6), indicating that VCAM-1 on HUVEC and VLA-4 on megakaryocytic cells play a role in the adhesion of megakaryocytes to HUVEC. Taken together, our results suggest that the β2 family of integrins (LFA-1), as well as the β1 family of integrins (VLA-4), play a role in the adhesion of marrow megakaryocytes and CMK cells to HUVEC.

Effect of adhesion on growth and differentiation of megakaryocytic cells. We next evaluated the proliferation and differentiation of megakaryocytes adhered to HUVEC. Marrow

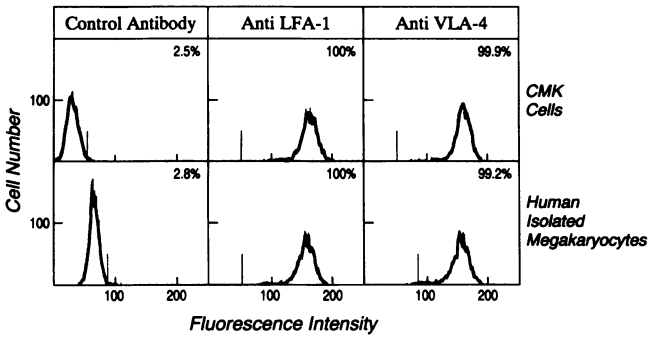

Figure 5. Immunofluorescence analysis using flow cytometry of CMK cells or isolated marrow megakaryocytes stained with specific antibodies for LFA-1 and VLA-4. CMK cells (10^5) or isolated bone marrow megakaryocytes (10^5) were incubated with purified control IgG ascites (1 μg/100 μl), control monoclonal antibodies (1 μg/100 μl), or monoclonal antibodies to LFA-1 (1 μg/100 μl) and VLA-4 (1 μg/100 μl). Cells were analyzed in each instance and fluorescence intensity was displayed in relative intensity on a logarithmic scale, as described in the Methods. Percentage of positive cells, calculated between channel numbers 50 and 200 are indicated. An unrelated FITC-labeled conjugate (swine anti-rabbit Ig) stained ~ 2% of each cell suspension (curves not shown).

megakaryocytes or CMK cells adhered to HUVEC did not proliferate under the various conditions of stimulation of HUVEC with TNF-α, IFN-γ, or PMA (data not shown).

CMK-G cells were found to have significantly increased expression of surface GpIb and GpIIb/IIIa after adhesion to HUVEC. The level of the increased expression of the adhered CMK-G was similar to CMK-G–stimulated cells with IL-6, a cytokine known to increase megakaryocyte differentiation (24, 32) (Table I). Furthermore, DNA analysis of adhered CMK-G cells revealed a significant increase in the percentage of polyploid cells. The distribution of DNA content of the unstimulated CMK-G is shown in Table II. Stimulation by IL-6 of CMK-G cells increased the percentage of polyploid cells (Table II). Unstimulated CMK-G after adhesion to TNF-α–stimulated HUVEC demonstrated a higher distribution of DNA content compared with nonadhered CMK-G cells treated with the cytokine IL-6 (Table II). Furthermore, IL-6–stimulated CMK-G cells adhered to TNF-α–stimulated HUVEC showed a further increase in the percentage of polyploid cells (Table II). These results indicate that increased maturation of megakaryocytes, as measured by expression of surface GpIb and GpIIb/IIIa and DNA content, may occur upon adhesion of cytokine-treated megakaryocytic cells with activated endothelial cells.

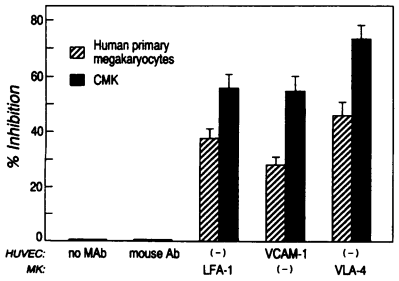

Figure 6. Effect of specific antibodies against LFA-1, VCAM-1, and VLA-4 on adhesion of megakaryocytes to HUVEC. HUVEC were stimulated with TNF-α for 24 h as in Fig. 1. HUVEC were incubated without antibodies or with monoclonal antibodies to VCAM-1 (1 μg/100 μl) or control antibody W6/32 (anti-HLA class I antigen) (1 μg/100 μl). CMK cells or isolated marrow megakaryocytes were incubated without antibodies, with monoclonal antibodies for LFA-1 (1 μg/100 μl), or with VLA-4 (1 μg/100 μl) for 30 min at 37°C. The cells were then washed and added to HUVEC for 3 h at 37°C. Values are mean±SEM of duplicate determinations in six experiments.

Table I. Immunofluorescence Analysis using Flow Cytometry of CMK-G Cells with Specific Antibodies for Glycoproteins GpIb and GpIIb/IIIa

Cell culture conditions	Fluorescence intensity of surface marker	
	GpIb	GpIIb/IIIa
CMK-G	13.1±0.4	75.1±1.1
CMK-G + HUVEC/TNF-α	17.1±0.1*	85.2±1.0*
CMK-G/IL-6	14.7±0.2	82.2±1.6
CMK-G/IL-6 + HUVEC/TNF-α	18.4±0.8‡	87.0±0.8‡

HUVEC were stimulated with TNF-α (50 ng/ml) for 24 h. CMK-G cells (10^5) unstimulated or IL-6 (10 ng/ml) stimulated for 16 h and some cultures were then incubated with HUVEC pretreated with TNF-α for 24 h. After adhesion to HUVEC, CMK-G cells were washed and incubated with control monoclonal antibodies or monoclonal antibodies for GpIb or GpIIb/IIIa for 30 min at 37°C. CMK-G cells were then washed and incubated for 30 min at 37°C with FITC-labeled conjugate (rabbit anti-mouse Ig). CMK-G cells were then washed, analyzed in each instance, and fluorescence intensity was displayed in relative intensity on a logarithmic scale. Percentage of positive cells, calculated between channel numbers 50 and 200, is indicated. CMK-G cells (10,000) were analyzed in each experiment. Results are given as means ± SEM of four independent experiments.
*Significantly elevated in the cultures of CMK-G + HUVEC/TNF-α compared with CMK-G alone ($P < 0.05$).
‡ Significantly elevated in the cultures of CMK-G/IL-6 + HUVEC/TNF-α. Compared with CMK-G/IL-6.

Table II. The Ploidy Distribution of Untreated Control CMK-G Cells, CMK-G Cells Treated with IL-6, and CMK-G Cells Adhered to TNF-α–Stimulated HUVEC

CMK-G cell culture conditions	Percent in each class		
	2N	4N	≥8N
CMK-G	54.2±0.8	13.0±1.3	1.3±0.3
CMK-G + HUVEC/TNF-α	54.4±0.6	31.7±2.3*	3.6±0.4*
CMK-G/IL-6	54.0±1.4	16.2±0.6	2.3±0.3
CMK-G/IL-6 + HUVEC/TNF-α	54.0±1.0	33.7±1.3‡	3.9±0.4‡

CMK-G cells (2×10^5/ml) were cultured for 5 d in RPMI + 5% platelet-poor plasma with or without IL-6 (10 ng/ml). CMK-G cells or IL-6–stimulated CMK-G cells were added to HUVEC pretreated for 24 h with TNF-α. After 5 d, CMK-G cells were stained with propidium iodide and analyzed for DNA content by fluorescence activated cell sorting scan using Cell Fit software, as described in Methods. The percentage of cells of 2N, 4N, and ≥8N does not add up to 100% due to the death of cells during the culture period. The results were obtained from analysis of 10,000 CMK-G cells. Results are given as mean ± SEM of four independent experiments.
* Significantly elevated in the cultures of CMK-G + HUVEC/TNF-α compared with CMK-G ($P < 0.05$).
‡ Significantly elevated in the cultures of CMK-G/IL-6 + HUVEC/TNF-α compared with CMK-G/IL-6 ($P < 0.05$).

We were technically unable to perform these maturation studies on primary marrow megakaryocytes for two reasons: isolation of marrow megakaryocytes uses immunomagnetic beads coated with antibody to GpIIb/IIIa, so all isolated cells express this surface structure; and ploidy analysis on marrow megakaryocytes is very difficult since the cells tend to spontaneously die after 4–5 d in vitro (the minimum time for DNA analysis). Techniques to isolate sufficient numbers of a purified population of less mature megakaryocytes (i.e., megakaryoblasts) are not yet established. Thus, cell lines of less maturity, such as CMK-G, provide a model for such maturational changes.

Discussion

Very little information has been obtained on cell–cell interaction between megakaryocytes and endothelial elements. Cultured HUVEC as well as cultured bovine endothelial cells produce an extensive underlying extracellular matrix that closely resembles the vascular subendothelial basal lamina in its origins and chemical composition (25). Levine et al. (28) previously reported the use of cultured bovine endothelial cells as a model to study the interaction of megakaryocytes with the subendothelium. Bovine vascular endothelial cells were found to induce human platelet adhesion, aggregation, thromboxane A_2 formation, and the release reaction in a manner similar to that observed with human platelets flowing over subendothelium from human arteries or veins (28). HUVEC may also provide a model of bone marrow microvascular endothelial cells. Lichtman et al. (26) observed that an intimate relationship exists between the megakaryocyte and the abluminal surface of the endothelial lining cell. At the time of platelet release, the megakaryocyte cytoplasm invaginates and penetrates the endothelial lining cell; the penetrating cytoplasm is then detached and enters the marrow circulation as a packet of platelets that undergoes further fragmentation in the circulation. In addition, electron microscopic studies (25, 27) indicate that megakaryocytes in the extravascular compartments are preferentially located in the subendothelial region. In this location, they can send numerous organelle-free projections, which serve to anchor the cell to the endothelium, into the lumen. These observations indicate that megakaryocyte adhesion to endothelial cells in vivo is an important physiological process for platelet formation (25). Therefore, characterization of adhesive interactions between megakaryocytes and endothelium should provide insights into thrombopoiesis under normal and disease states. Our studies suggest that inflammatory cytokines such as IL-1β, TNF-α, or IFN-γ, which are known to activate endothelium, can promote such adhesion. These cytokines that induce adhesion of HUVEC for megakaryocytes also induce adhesion of HUVEC for basophils (36) and for other granulocyte subpopulations, including neutrophils and eosinophils (37–39). The kinetics of megakaryocyte adhesion and the induction of adhesion in HUVEC for megakaryocytes resemble that for granulocytes, and appears to require both RNA and protein synthesis, suggesting that megakaryocytes may be binding to the same inducible surface structures previously described for neutrophil, eosinophils, and basophil adherence (36–39). Furthermore, cytokines known to promote megakaryocyte growth and/or differentiation such as IL-3, GM-CSF, and IL-6 appear to augment the attachment of the megakaryocyte to endothelium.

We have demonstrated that incubation of megakaryocytes with antibodies directed against the common β subunit or against the α subunit of the CD18 complex lead to marked inhibition of adhesion of megakaryocytes to HUVEC. The finding that blockade of the β and α subunits with specific antibodies does not completely inhibit megakaryocyte adherence to stimulated HUVEC indicates that additional cell-surface structures are important for endothelial cell–megakaryocytic interactions. In this regard, we observed that megakaryocytes expressed VLA-4, which interacted with VCAM-1 expressed on the surface of HUVEC. This is in addition to the β2 integrins that interact with ICAM-1 expressed on activated HUVEC.

The VLA-4/VCAM-1 adhesion pathway has been implicated in important physiological processes involving migration and localization of immune cells (18, 19, 40, 41) and some tumor cells (17). In the present study, we observed that this pathway can also play a role in mediating adhesion of megakaryocytes to endothelial cells. Our studies also identified the specific β2 integrins, the LFA-1 complex, as involved in megakaryocyte adhesion. Although participation of VLA-4 and β2 integrins accounted for most of the adhesion, other adhesive molecules may also contribute to this process. Further experiments using multiple antibody combinations may help resolve this question.

Our characterization of megakaryocyte adhesion to two major marrow stromal elements, fibroblasts and endothelial cells, suggests that there may be alternative pathways of cytoadhesion involved in megakaryocytopoiesis. In a previous study, we observed that megakaryocytes adhered to marrow stromal fibroblasts constitutively, using in part the membrane form of SCF/KL on the fibroblast and its cognate c-kit receptor on the megakaryocyte. Increased megakaryocyte replication occurred with such adhesion to mesenchymal cells (29). Maturational, rather than proliferative changes were observed upon megakaryocyte adhesion to endothelial cells. There may be a branch point in the pathway of megakaryocyte replication versus maturation that depends on interaction with stromal fibroblasts versus endothelium.

During inflammatory conditions, thrombocytosis due to increased megakaryocytopoiesis is often observed (24). Activation of endothelial cells by inflammatory cytokines such as IL-1β, TNF-α, or IFN-γ could potentiate megakaryocyte adhesion to endothelium. IL-6, also a cytokine released during inflammation, resulted in increased differentiation of HUVEC-adhered megakaryocytic cells as measured by DNA content compared with nonadherent IL-6–treated cells. Thus, certain members of the family of inflammatory cytokines may modulate megakaryocyte maturation via enhancing adhesive interactions with endothelial cells. This model of enhanced interaction between endothelium and megakaryocytes during inflammation merits further studies, potentially in recently established small animal models of human hematopoiesis that are amenable to cytokine treatment and challenge with inflammatory stimuli (42).

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