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Adhesion Molecules in Hematopoietic Cells

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ABSTRACT. **Interaction with stromal cells is known to be crucial for growth and differentiation of hematopoietic cells. To characterize adhesion molecules involved in this interaction, we examined adhesion of a panel of lymphoid, myeloid, and mast cell lines with stromal cells. We found that very late antigen-4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) were major adhesion molecules in lymphoid and myeloid cells, whereas myeloma cells adhered to stromal cells through hyaluronate. We investigated regulation of VLA-4 during differentiation of myeloid cells using a neutrophil precursor cell line, L-G3. Differentiation of neutrophils induced by granulocyte colony-stimulating factor was accompanied with down-regulation of VLA-4. Induced L-G3 cells adhered to stromal cells in proportion to the expression of VLA-4. Mast cells used two mechanisms to adhere to fibroblasts and stromal cells. They adhered to fibronectin through VLA-5 when stimulated with steel factor and also directly to membrane-anchored steel factor through c-*kit*.**

KEY WORDS. **Adhesion molecules — Very late antigen-4 — Vascular cell adhesion molecule-1 — Myeloid cells — Stromal cells**

INTRODUCTION

In hematopoiesis, stem cells reproduce themselves as well as give rise to cells of eight different types. During the course of differentiation, progeny of the pluripotent stem cells lose their multipotential capacity and differentiate into cells of more restricted potential, which eventually become committed to a single lineage [1]. This process is strictly regulated in the hematopoietic microenvironment. Recent progress in resolving this process by techniques of cell culture and molecular biology has led to the identification of a number of soluble factors, which act on multipotential precursors and restricted lineages of cells [2].

Another regulatory element of hematopoiesis is the stromal cell network. In the bone marrow, erythroblastic islands are often found where erythroid lineage cells are in close contact with macrophages or phagocytic reticulum cells [3]. Developing granulocyte lineage cells are associated with fibroblast-type, nonphagocytic reticulum cells [4].

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More understanding of bone marrow stromal cells has been achieved by the establishment of long-term bone marrow cultures, as described by Dexter and colleagues [5]. In this culture condition, multipotential stem cells colony forming unit-spleen (CFU-S), myeloid progenitors and differentiated cells are sustained by close physical association with a heterogeneous adherent stromal layer. Whitlock and Witte [6] developed a modified long-term bone marrow culture that supports B-lymphopoiesis in a stromal-dependent system. The stromal layers of both culture system are made up of fibroblasts, macrophages, endothelial cells, and so on. Cloned stromal cells have also been established, and most of them have been shown to support both myelopoiesis and lymphopoiesis [7, 8]. This simplification of the culture system made it possible to assess cytokine production from stromal cell clones and led to identification of important cytokines such as interleukin-7 (IL-7) [9] and stem cell factor (steel factor, mast cell growth factor, and *kit* ligand) [10–14]. Coculture of a multipotential stem cell clone or purified early lymphoid precursors and stromal cell clones has generated B cells, myeloid cells [15–17]. From these simplified culture systems, it was suggested that adhesive interactions of stromal cells and hematopoietic cells were crucial in establishing myelopoiesis and lymphopoiesis and that adhesion was required at an early stage of the differentiation.

To clarify the role of adhesion of hematopoietic cells to stromal cells, we analyzed adhesion of a panel of hematopoietic cells from different lineages to stromal cells, and examined what kind of adhesion molecules were involved and how they were regulated with hematopoietic growth factors.

MATERIALS AND METHODS

Cell Lines

The LyD9 hematopoietic progenitor cell line [18], the L-G3, L-GM3, and LS-1 myeloid progenitor cell lines [17], and the MC/9 mast cell line [19], were maintained in RPMI 1640 containing 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol, and 50 μg/ml gentamycin (complete medium) supplemented with 10% WEHI-3B-conditioned medium as a source of IL-3. Homogeneous cultured mast cells were established by maintaining nonadherent cells of bone marrow derived from BALB/c mice with the same medium as above and were used from 4 to 10 weeks after establishment. The 38 B9 pre-B-cell line [20] and the P3X63Ag myeloma cell line were cultured in RPMI 1640 containing 10% FCS and 50 μM β-mercaptoethanol (complete medium). The stromal cell lines, PA6 [21] and ST2 [22] were cultured in complete medium. NIH 3T3 cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% FCS.

Antibodies, Growth Factors, and Chemicals

The MFR-4A (2A9), MFR-4B (9C10), MFR-5 (5H10), MVCAM-A (429), and MVCAM-B (584) monoclonal antibodies (mAbs) were produced by immunizing Lewis rats (Charles River Laboratories, Wilmington, MA) with LyD9 for MFR-4A and 4B, with MC/9 for MFR-5, and with PA6 for MVCAM-A and B. Spleen cells were fused with the murine myeloma P3X63Ag8.653 and selected as described [23]. Detailed characterization of these mAbs will be published elsewhere. Anti-mouse VLA-4 mAb, R1-2 was described [24]. These mAbs were purified from culture supernatants by ammonium sulfate precipitation, followed by affinity chromatography on protein G-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ).

Ascites of ACK2, a mAb against murine c-*kit* [25, 26] was generously provided by Dr. Nishikawa (Kumamoto University, Kumamoto, Japan), murine steel factor (10^5 U/mg) was the kind gift of Dr. Gillis (Immunex Corp., Seattle). Purified murine IL-3, IL-4, and granulocyte colony-stimulating factor (G-CSF) were purchased from Genzyme, Boston, MA.

Rabbit anti-human fibronectin serum, human fibronectin, RGDS, and RGES peptides were purchased from Telios Pharmaceuticals, San Diego, CA. Mouse laminin and collagen type IV were purchased from Collaborative Research; genistein, phorbol 12-myristate 13-acetate (PMA) and cytochalasin B were from Calbiochem, San Diego, CA, and genestein and herbimycin A were purchased from Gibco BRL, Gaithersburg, MD.

Adhesion Assays

Adhesion assays with monolayers of stromal cells were performed in 96-well tissue culture plates (No. 25870, Corning, NY). Hematopoietic cells were labeled with 2′, 7′-*bis*-(2-carboxyethyl)-5 (and -6)carboxyfluorescein (BCECF; Molecular Probes, Inc., Eugene, OR) as described [27], and 5×10^4 cells in 100 μl were added to stromal layers prepared 1 day before the assay. After incubation at 37°C, 5% CO_2 for 30 minutes, culture medium in each well was aspirated with a needle connected to a suction pump, and warm culture medium was added and aspirated in the same way. This washing process was repeated four times before measurement of fluorescence with a Pandex reader. Binding was determined as a percentage of the input cells, as indicated by the ratio of the remaining fluorescent cells to the input level of fluorescent cells in wells that were not washed. Lipopolysaccharide (LPS) (011B4, Sigma, St. Louis, MO) was added at 2 μg/ml to stimulate stromal cells 16 hours before adhesion assays. In some experiments, the stromal monolayers were preincubated in Iscove's modified Eagle Medium containing hyaluronidase (200 U/ml, Boehringer Mannheim, Indianapolis, IN) at 37°C for 1 hour. The monolayers were washed three times before adhesion assays.

Adhesion assays with extracellular matrix proteins were performed in a 96-well polystyrene plate (Linbro-Titertek; Flow Laboratories, McLean, VA) coated with extracellular matrix proteins. Fibronectin, laminin, or collagen IV (100 μl of 100 μg/ml in RPMI 1640 supplemented with 10 mM HEPES, pH 7.4) was added to each well and incubated at 37°C for 2 hours or at 4°C overnight. Subsequent to experiments in Fig. 6 (later in this paper), fibronectin was used at 20 μg/ml. RPMI 1640 with 3% bovine serum albumin (BSA fraction V, Sigma Chemical Co., St. Louis, MO) was added for 1 hour at 37°C to block nonspecific binding sites. The wells were then washed four times with RPMI 1640 containing 10 mM HEPES (pH 7.4) and 0.03% BSA (the binding medium). MC/9 cells and cultured mast cells were labeled with BCECF, suspended in the binding medium, and transferred into coated wells (5×10^4 cells in 100 μl) with or without growth factors as indicated. The plates were incubated at 37°C for 30 minutes, and then unbound cells were washed away as described above.

When the effects of antibodies on adhesion were tested, labeled cells were pretreated with 100 μl of approximately diluted antibodies for 30 minutes at room temperature. For other inhibitors, BCECF-labeled mast cells were preincubated for 10 minutes with cytochalasin B, 1 hour with genestein, and 24 hours with herbimycin A. As a control for genistein, solvent (1% dimethylsulfoxide (DMSO)) was employed. Assays were performed in the presence of these inhibitors, except herbimycin A was washed away before the binding assay.

Purification and the binding assay for vascular cell adhesion molecule-1 (VCAM-1) was basically the same as described for intercellular adhesion molecule-1 (ICAM-1) [28] and will be published elsewhere. Briefly, VCAM-1 was purified from a lysate prepared from PA6 (5×10^9; lysis buffer contained 1% Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 tripsin inhibitor unit (TIU)/ml of aprotinin) with affinity chromatography using MVCAM-A mAb-sepharose. After washing the column with a 50-mM triethylamine (pH 10) solution containing 150 mM NaCl and 1% octylglucoside, VCAM-1 was eluted with 50-mM triethylamine solution (pH 12.5) containing 500 mM NaCl and 1% octylglucoside. Fractions containing VCAM-1 were stored at −100°C. Binding assays with purified VCAM-1 were performed as described for ICAM-1 [29].

Adhesion to Transfected COS Cells

COS cells were transfected 1 day after plating at 1.5×10^6 cells per 150-mm culture dish with murine full-length steel factor cDNA (KL-M1) in the transient expression vector pcDNA1 [30] or vector alone (mock) using diethylaminoethyl (DEAE)-dextran [31]. Twenty-four hours following transfection, COS cells were replated into six-well dishes (6×10^4 cells per well) and incubated for an additional 48 hours before use in adhesion assays. Mast cells (5×10^5 cells per well) were incubated in 2-ml complete medium with transfected COS cells at 37°C for 1.5 hours with occasional swirling. In some experiments, growth factors were added together with the mast cells. Unbound cells were washed away by aspirating and adding medium five times. Remaining cells were detached with trypsin/ethylenediamine tetraacetic acid (EDTA) and counted with a hemocytometer. ACK-2 ascites was used at 1/800 dilution. For determination of divalent cation dependency, transfected COS cells were fixed with 1% paraformaldehyde at 4°C for 1 hour. Without fixation, COS cells were dislodged from the dish in the absence of divalent cations. Fixed cells were washed five times with Hanks' balanced salts solution (HBSS) before use. Fixation prevented COS cells from detaching from the plate in the absence of divalent cations while keeping steel factor biologically intact. Mast cells were washed three times with HBSS and resuspended with HBSS containing 10% dialyzed FCS with 5 mM of EDTA, 1.5 mM of $MgCl_2$, 1.5 of mM $CaCl_2$ or both and subjected to adhesion assays.

Flow Cytomeric Analysis

Cells (5×10^5) were incubated with 50 μl of a saturating amount of antibody (1 μg for MFR-5, or 1:800 dilution for ACK2) at 4°C for 40 minutes and then washed twice with cold phosphate-buffered saline (PBS) containing 1% FCS. Cells were further incubated with 50 μl of a 1:40 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-rat IgG (Zymed Immunochemicals, San Francisco, CA) and washed under the same condition as above. Cells were analyzed using FACScan (Becton-Dickinson, Lincoln Park, NJ).

RESULTS

Adhesion of a Panel of Hematopoietic Cells and Stromal Cells

Two stromal cell lines previously shown to support hematopoiesis, PA6 and ST2 [21, 22], supported adhesion to the hematopoietic precursor cell line LyD9, B-cell lineage cell lines 38B9 and P3X63, granulocyte (LG3) and macrophage (LS-1 and LGM3) precursor cell lines, and a mast cell line (MC/9) (Fig. 1). Treatment with LPS augmented adhesion of ST2 to LyD9, 38B9,LS-1 and LG3 but did not affect adhesion of PA6. Very late antigen-4 (VLA-4) was involved in adhesion to cell lines examined except the P3X63 myeloma cell line and the MC/9 mast cell line, because the anti-VLA-4 mAb R1-2 reduced adhesion to PA6 by about 50% and to ST2 by about 80%.

To examine the involvement of proteoglycans in adhesion, stromal cells were treated with neuraminidase or hyaluronidase before the adhesion assay. Hyaluronidase treatment but not neuraminidase treatment inhibited adhesion of P3X63 cells to PA6 and ST2 (Fig. 2 and data not shown). Hyaluronidase treatment did not inhibit adhesion to LyD9 or MC/9 and augmented adhesion to LyD9 significantly. These results indicate that hyaluronic acid on stromal cells mediates adhesion to P3X63 myeloma cells. It has

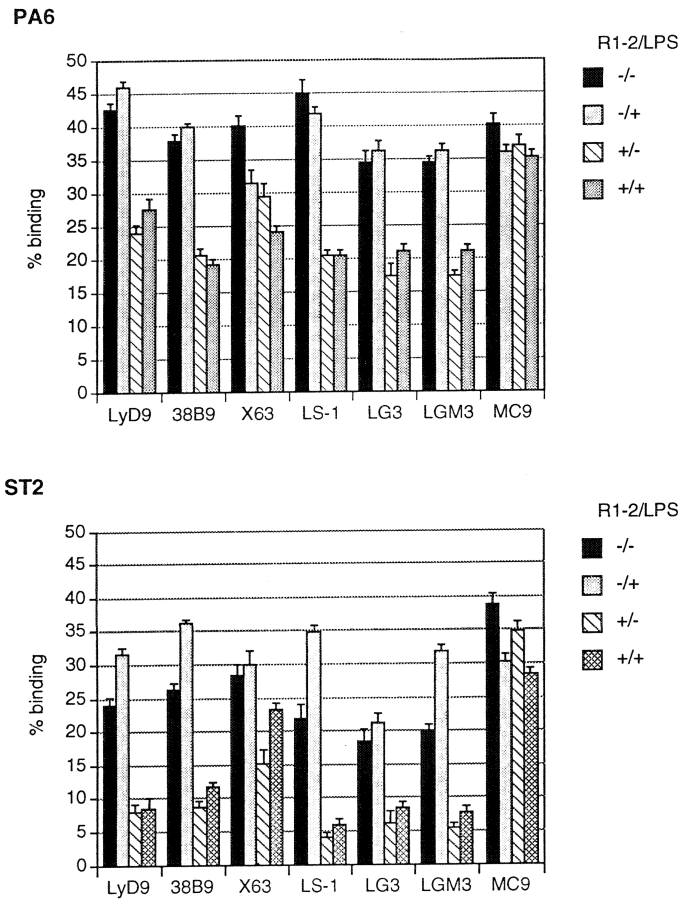

Fig. 1. Adhesion of a panel of hematopoietic cell lines and stromal cell lines. Adhesion assays were performed as described in Materials and Methods with unstimulated (−) or lipopolysaccharide (LPS)-stimulated (+) stromal cells in the absence (−) or presence (+) of R1-2 monoclonal antibody to very late antigen-4 (VLA-4) (20 μg/ml). The assays were performed in triplicate. Average bindings and SE are shown.

previously been shown that CD44 and hyaluronate are involved in the interaction of a B-cell hybridoma and a stromal clone [32].

Cell–Cell Adhesion Through VLA-4 and VCAM-1

Because a significant level of adhesion was observed in the presence of the anti-VLA-4 mAb R1-2, we made monoclonal antibodies that inhibited adhesion of LyD9 to PA6 to examine different components of adhesion. Rat hybridomas were produced by immunizing with LyD9 cells or PA6 cells and selected for the inability to block adhesion of LyD9 to PA6. Two mAb, MFR-4A (2A9) and MFR-4B (9C10), that recognize mouse

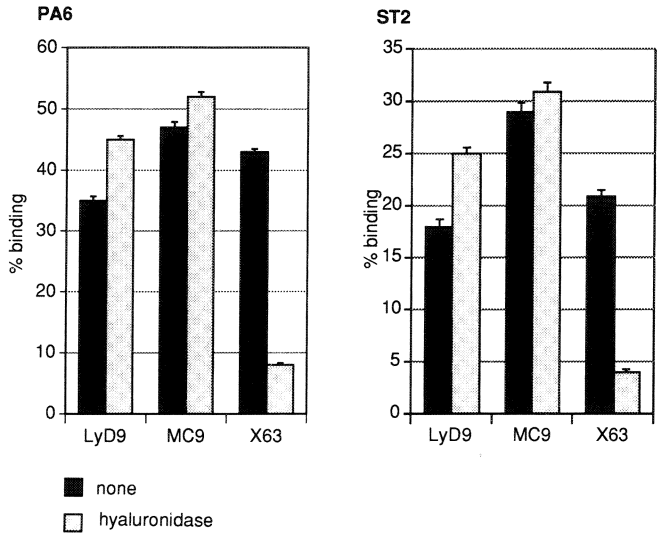

Fig. 2. The effect of the hyaluronidase treatment of stromal cells. Monolayers of stromal cells were treated with or without hyaluronidase before adhesion assays with LyD9, MC9, and P3X63Ag cells. The assays were performed in triplicate. Average bindings and SE are shown.

VLA-4 (personal communication, T. Kinashi and Y. St. Pierre) were characterized by the adhesion assay of LyD9 to PA6. MFR-4A almost completely abolished adhesion of LyD9 to PA6 (Fig. 3A). On the other hand, MFR-4B alone did not reduce adhesion significantly. In combination with R1-2, MFR-4B inhibited adhesion as much as MFR-4A. Two mAb, MVCAM-A and MVCAM-B, were made against PA6 cells. Both MVCAM-A and MVCAM-B recognized VCAM-1, a ligand for VLA-4 (personal communication, C.-H. Huang and T.A. Springer). Both partially inhibited adhesion of LyD9 to PA6 (Fig. 3), and combination of these did not further reduce adhesion (data not shown). Combination of MFR-4B and MVCAM-A or MVCAM-B was as effective as MFR-4A in blocking adhesion of LyD9 to PA6. These results suggest that R1-2 does not block VLA-4-dependent adhesion completely and that the majority of adhesion of LyD9 to PA6 is VLA-4-dependent. Because mAb against VCAM-1 only partially inhibited adhesion of LyD9, it remains unclear if VCAM-1 is the only ligand for VLA-4 in adhesion of LyD9 to PA6. The synergistic effect of R1-2 and MFR-4B could suggest that VLA-4 interacts with distinct ligands, that different epitopes of VCAM-1 contribute to VLA-4-dependent adhesion independently, or that neither mAb binds directly in the VCAM-1 binding site and that both are required to hinder adhesion to the binding site.

Adhesion to Purified VCAM-1

To examine the possibilities mentioned above, VCAM-1 was purified from PA6 with affinity chromatography on MVCAM-A mAb-sepharose. Purified VCAM-1 mediated

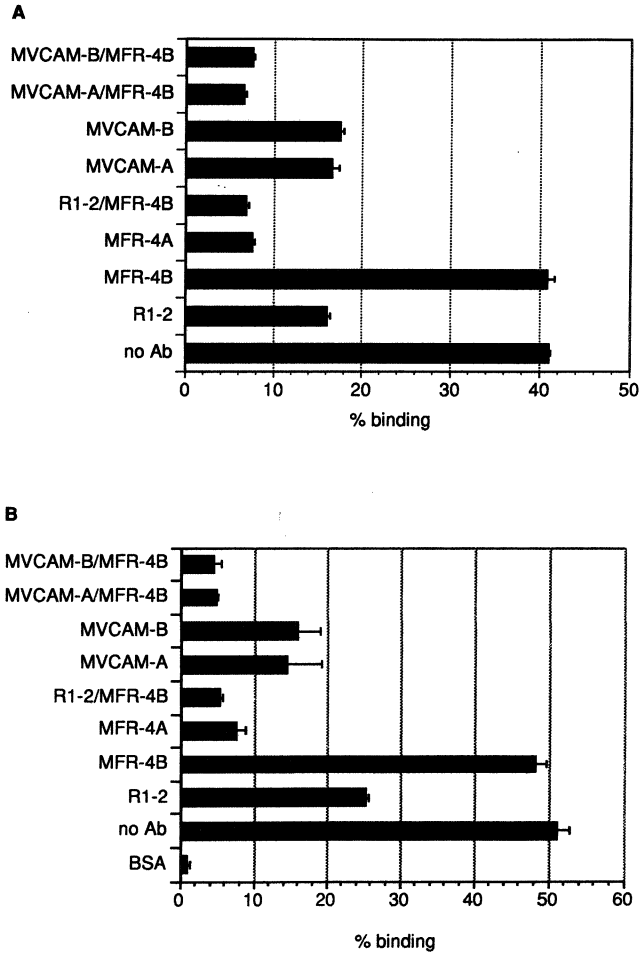

Fig. 3. The effect of monoclonal antibody (mAb) on adhesion of LyD9 cells to PA6 cells **(A)** or to purified vascular cell adhesion molecule 1 (VCAM-1) **(B).** LyD9 cells were preincubated with the indicated mAb at 20 μg/ml at room temperature for 30 minutes before transfer to the PA6 monolayers or VCAM-1-coated plates. Adhesion assays were performed as Fig. 1. (BSA, bovine serum albumin.)

adhesion to LyD9 as efficiently as the PA6 monolayer (Fig. 3B). The effects of inhibitory mAb on binding to purified VCAM-1 were similar to those to PA6 monolayers described above. MFR-4A abolished the binding of LyD9 to purified VCAM-1. R1-2 inhibited the binding partially. MFR-4B mAb alone did not reduce the binding significantly, but a combination of R1-2 and MFR-4B inhibited binding completely. MVCAM-A and MVCAM-B each partially blocked the binding of LyD9 to purified VCAM-1. The similarity to the results with binding to PA6 stromal cells does not provide support for the existence of VLA-4 ligands on PA6 other than VCAM-1.

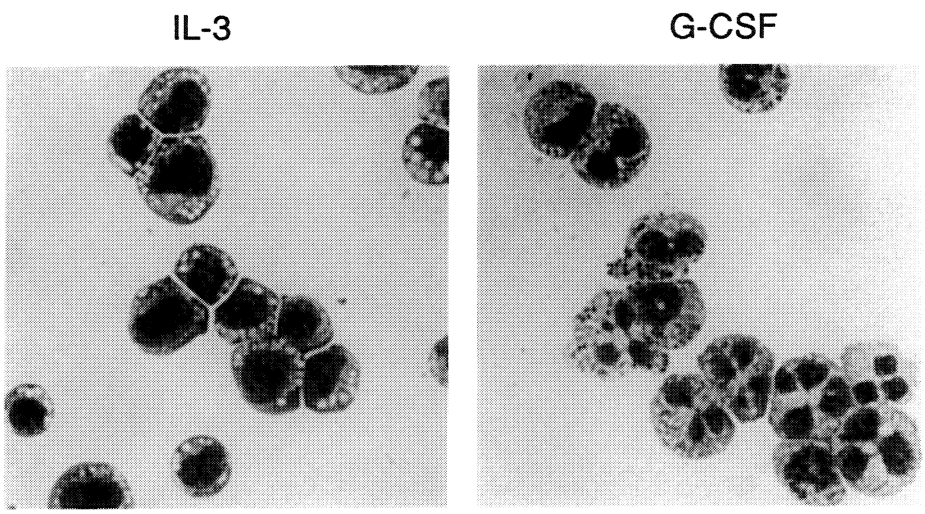

Fig. 4. Differentiation of L-G3 into neutrophils with granulocyte colony-stimulating factor (G-CSF). Immature L-G3 cells maintained with interleukin-3 (IL-3) (left) were induced to differentiate (right) by culturing them with G-CSF (20 U/ml) for 2 weeks.

Down-Regulation of VLA-4 During Differentiation of Neutrophils

Although association of hematopoietic progenitors with stromal cells is crucial for development of blood cells, de-adhesion of mature blood cells from stromal cells is also important to release mature blood cells into the blood stream. Because VLA-4 is a major adhesion molecule used by progenitor cells to adhere to stromal cells, we examined how the expression of VLA-4 is regulated during the differentiation of granulocytes. An IL-3-dependent cell line, L-G3 can be induced to differentiate into neutrophils with G-CSF [16] (Fig. 4). Immature L-G3 cells adhered to stromal cells through VLA-4 (Fig. 1). When induced with G-CSF, L-G3 completely down-regulated the expression of VLA-4, and partially up-regulated the expression of Mac-1 (Fig. 5A). Induced L-G3 adhered to stromal cells in proportion to their expression of VLA-4. L-G3 with a typical morphology of neutrophils after 14 days of culture with G-CSF did not adhere to stromal cells (Fig. 5B). This suggests that down-regulation of VLA-4 allows differentiated neutrophils to be dissociated from stromal cells and released into the blood stream.

*Steel Factor and c-*Kit *Regulate Cell–Matrix Adhesion of Mast Cells*

In contrast to the results seen with lymphoid and myeloid precursors, adhesion of the MC/9 mast cell line could not be ascribed to VLA4/VCAM-1 or hyaluronate. We therefore examined adhesion of MC/9 cells, and mast cells derived from bone marrow cells cultured with IL-3, to extracellular matrix. MC/9 adhered well to fibronectin and

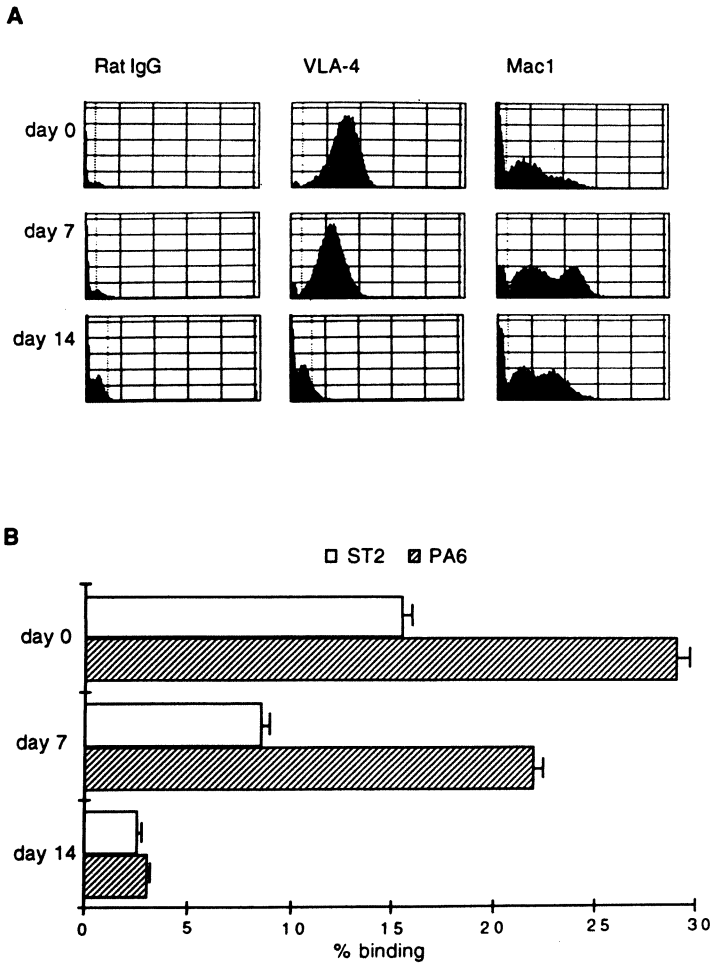

Fig. 5. A Down-regulation of very late antigen-4 (VLA-4) and upregulaton of Mac-1 in induced L-G3 cells. L-G3 cells cultured in interleukin-3 (IL-3) as control or with granulocyte colony-stimulating factor for 7 and 14 days were subjected to the flow cytometry (**A**) and adhesion assays with stromal cells (**B**). Cells were stained with rat IgG (control), R1-2 (VLA-4), or M1/70 (Mac-1) for the flow cytometry.

laminin (Fig. 6A). Unstimulated, bone marrow-derived mast cells did not bind significantly to any of the extracellular matrix proteins tested. When bone marrow-derived mast cells were stimulated with cytokines for 30 minutes, however, steel factor but not IL-3 or IL-4 stimulated binding to fibronectin (Fig. 6B). PMA also stimulated mast cells to bind to fibronectin. By contrast to mast cells cultured from bone marrow, the established mast cell line MC/9 showed only slightly augmented binding to fibronectin and laminin in the presence of steel factor (data not shown). Adhesiveness of MC/9 may be constitutively activated.

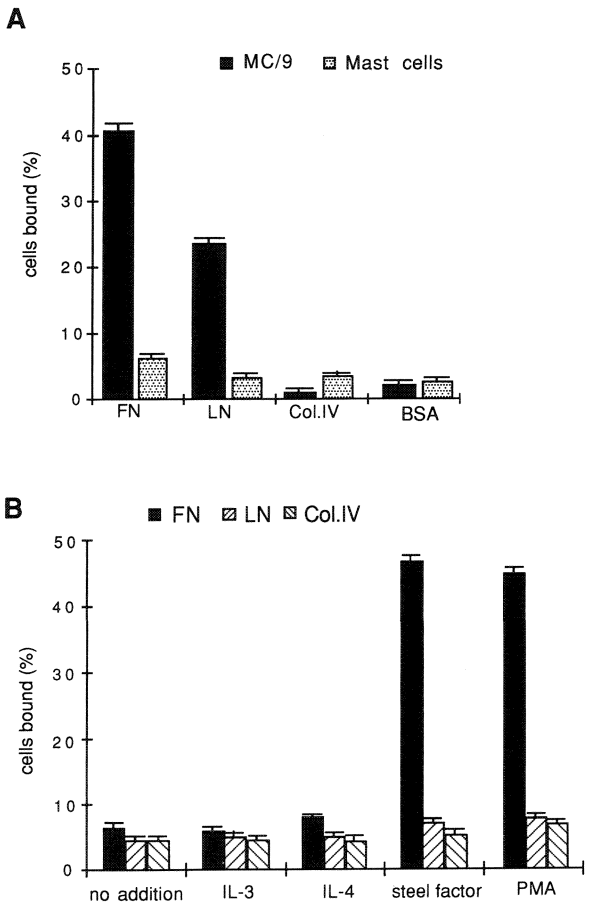

Fig. 6. Adhesion of MC/9 and mast cells to extracellular matrix proteins. **A** Cell binding was measured to fibronectin (FN), laminin (LN), collagen type IV (Col. IV), and albumin (BSA) coated on polystyrene culture plates and used for the binding assay. **B** Adhesion of mast cells stimulated with interleukin-3 (IL-3), IL-4, and steel factor (100 U/ml each) or phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) to extracellular matrix proteins.

Adhesion to Fibronectin Through the Murine Homologue of VLA-5 (α5β1)

To identify the receptor that mediates adhesion of mast cells to fibronectin, we prepared mAbs by immunizing rats with mast cell line MC/9. We identified one mAb, MFR-5, that reduced binding of steel factor-stimulated mast cells to fibronectin (Fig. 7A). MFR-5 recognize murine VLA-5 (manuscript in preparation). MFR-5 did not affect the binding of MC/9 to laminin (data not shown). Because MFR-5 did not inhibit mast cell binding as much as polyclonal anti-fibronectin serum (Fig. 7A), we tested the MFR-4A mAb to VLA-4. MFR-4A had no effect on adhesion of mast cells to fibronectin (Fig. 7A) and in combination with MFR-5 was not more inhibitory than MFR-5 alone (data not shown). A 120-kD fragment of fibronectin containing the classic RGD tripeptide cell attachment site substituted for native fibronectin (Fig. 7B). Steel factor stimulated mast cell binding to the 120-kD fragment, and MFR-5 blocked adhesion to the 12-kD fragment to a similar level as to native fibronectin. The RGDS peptide but

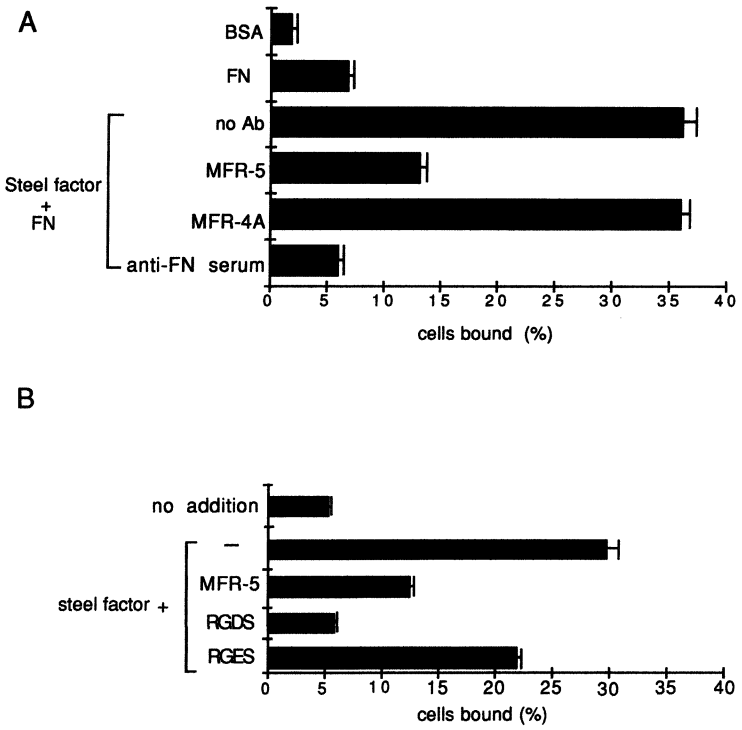

Fig. 7. A Inhibition of adhesion of mast cells to fibronectin with antibodies. Monoclonal antibodies MFR-5 (5H10), MFR-4A, or polyclonal anti-fibronectin antibody were used to inhibit adhesion of mast cells stimulated with steel factor (10 U/ml) to fibronectin. **B** Adhesion to the 120-kD fragment of fibronectin of mast cells stimulated with steel factor in the presence of MFR-5, or 1.5 mM of RGDS or RGES peptide.

not the RGES peptide almost completely inhibited the binding to the 120-kD fragment (Fig. 7B).

Transient Activation of VLA-5

To investigate the kinetics of adhesiveness, mast cells were stimulated for varying time periods in suspension, centrifuged onto the fibronectin substrate for 1 minute, and incubated on fibronectin for 6 minutes at 37°C before washing. The adhesiveness stimulated by steel factor was transient, and maximal adhesiveness was seen at about 10 minutes, the earliest time point measurable after stimulation (Fig. 8). Binding declined to background levels after 90 minutes. At 120 minutes, mast cells were restimulated with either 100 U/ml steel factor or 10 ng/l PMA; however, neither restimulation had any further effect on adhesiveness. Cell viability after restimulation was more than 95% as judged by trypan blue exclusion. Transient adhesiveness was unrelated to the level of

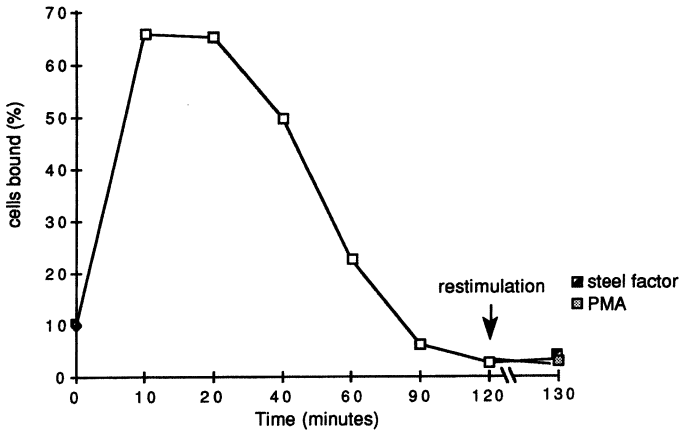

Fig. 8. Kinetics of adhesion of mast cells stimulated with steel factor. Mast cells were stimulated with steel factor (10 U/ml) in suspension for the time indicated. Zero time means adhesion without stimulation. The experiments were performed in triplicate. The average binding is shown. SEs were less than 3%.

expression of the fibronectin receptor on mast cells, as shown by staining with MFR-5 before and at several time points after stimulation with steel factor or PMA (data not shown).

*Tyrosine Kinase Activity of c-*Kit *is Required for Activation of VLA-5*

To explore the mechanisms linking steel factor/c-*kit* with VLA-5, the adhesion assay was performed in the presence of inhibitors of various protein kinases. Genistein, a tyrosine-specific protein kinase inhibitor [33], inhibited steel factor-stimulated binding of mast cells to fibronectin in a dose-dependent manner but had much less effect on PMA-stimulated binding (Fig. 9A). Herbimycin A, another specific inhibitor of tyrosine kinases [34], also inhibited adhesion of mast cells stimulated with steel factor to fibronectin completely, whereas it reduced the PMA-stimulated binding to half at most (Fig. 9B).

We studied the expression of the fibronectin receptor and the expression and tyrosine kinase activity of c-*kit* on mast cells treated with these drugs. Genistein did not affect the cell surface expression of the fibronectin receptor identified with the mAb MFR-5 or c-*kit* (Fig. 10). Pretreatment with herbimycin A reduced c-*kit* expression 10-fold, whereas it had no effect on the expression of the fibronectin receptor. Herbimycin A, thus, may exert its inhibitory effect by down-regulating c-*kit* expression, while genestein suppresses the tyrosine kinase activity without affecting the expression of c-*kit* and VLA-5.

*Steel Factor and c-*Kit *as Adhesion Molecules*

Mast cells specifically bind to COS cells transfected with the membrane form but not the soluble form of steel factor [30] and to fibroblasts from wild-type but not Sl^d mice

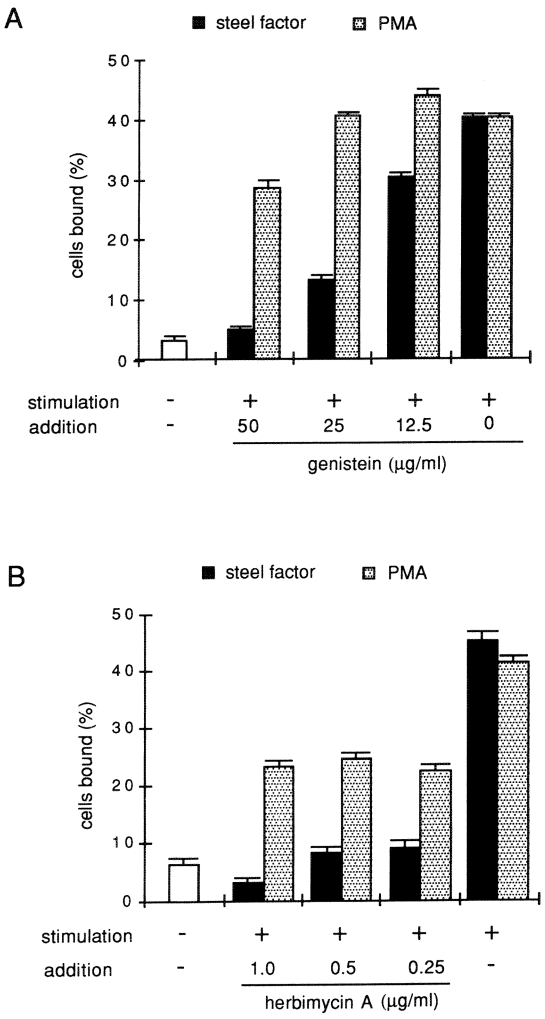

Fig. 9. The inhibitory effects of tyrosine kinase inhibitors on adhesion of mast cells stimulated with steel factor (solid bar) or phorbol 12-myristate 13-acetate (PMA) (dotted bar) to fibronectin. The experiments were performed in triplicate, and average bindings were shown ± SE.

[35]. To determine whether the membrane form of steel factor acts as an adhesion molecule recognized by c-*kit* or induces or activates a distinct adhesion molecule, we characterized adhesion of mast cells to COS cells transfected with the cDNA of steel factor.

We confirmed that mast cells bound to COS cells transfected with the membrane form of steel factor but not mock-transfected cells (Fig. 11A and C). The anti-fibronectin receptor antibody, MFR-5, and a polyclonal anti-human fibronectin antibody failed to inhibit the binding of mast cells to COS cells transfected with steel factor, whereas the mAb to c-*kit* inhibited adhesion completely (Fig. 12A). We further examined the effect of adding soluble steel factor or IL-3 (100 U/ml) in the adhesion assay (Fig. 11B). Interleukin-3 did not affect adhesion of mast cells to mock or steel-transfected COS cells. By contrast, soluble steel factor reduced adhesion to COS cells transfected with

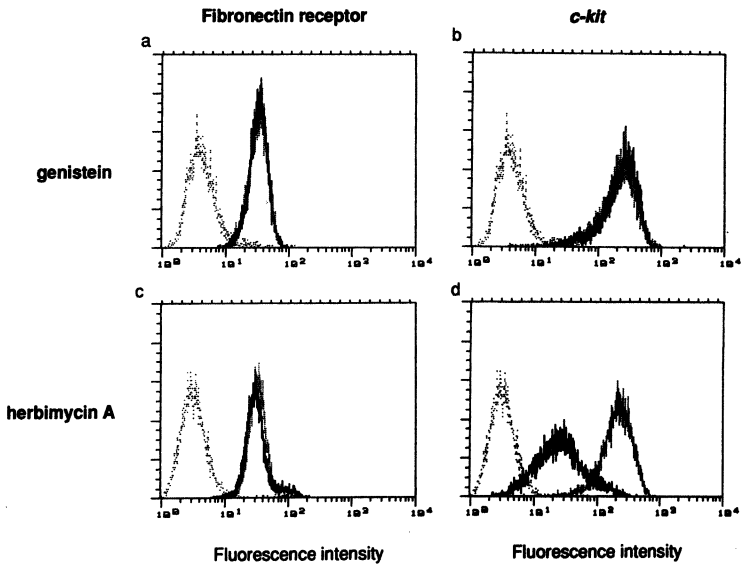

Fig. 10. The expression of the fibronectin receptor and c-*kit* of mast cells treated with genistein or herbimycin A. Mast cells treated with genistein (50 μg/ml) or herbimycin A (1.0 μg/ml) were stained with MFR-5 or ACK2 and fluorescein isothiocyanate (FITC)-labeled anti-rat IgG as the second antibody. Second antibody only (dotted line), without inhibitors (grey line), with inhibitors (black line). The treatments with inhibitors were performed as described in experimental procedures.

transmembrane steel factor to about 50% of those without steel factor and augmented adhesion to mock-transfected COS cell cultures (Fig. 12B). Microscopic inspection showed that in the presence of exogenous steel factor, mast cells did not adhere to either mock or steel-transfected COS cells, but instead adhered to the substrate (Fig. 11B and D). These results indicate that exogenous soluble steel factor competed with cell-surface steel factor and abolished adhesion of mast cells to COS cells transfected with steel factor cDNA. Adhesion to regions between COS cells probably reflects the binding of mast cells stimulated with steel factor to extracellular matrix components deposited by COS cells. These results ruled out the possiblity that cell–cell interactions between mast cells and COS cells expressing steel factor were mediated by fibronectin and the fibronectin receptor and suggest that cell-surface steel factor and c-*kit* are directly involved in adhesion.

Adhesion of mast cells to COS cells expressing steel factor was characterized for dependence on temperature, a functional cytoskeleton, energy, tyrosine kinase activity, and divalent cations (Fig. 12C). Mast cells adhered poorly to COS cells expressing steel factor at 4°C. Cytochalasin B reduced the binding to 5% of the specific binding. When the assay was performed with 2-deoxy D-glucose and sodium azide to deplete the amount of adenosine triphosphate (ATP), specific binding was reduced to 17% of the control. Genistein was used to study the requirement for the tyrosine kinase activity of

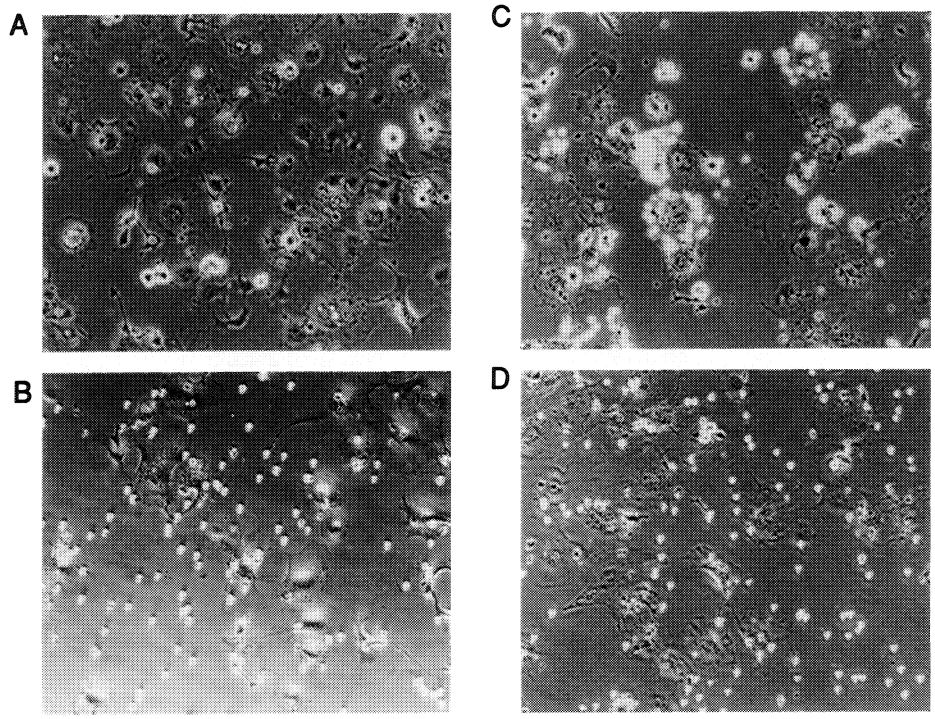

Fig. 11. Exogenous steel factor abolishes mast cell adhesion to COS cells transfected with cDNA encoding the full-length steel factor (KL-M1) and activates binding to the substrate. Mast cells were allowed to adhere to COS cells transfected with vector alone in the absence **(A)** or presence **(B)** of soluble steel factor (100 U/ml), or to COS cells transfected with KL-M1 in the absence **(C)** or presence **(D)** of soluble steel factor. Cultures were washed five times to remove nonadherent cells and were photographed with phase microscopy. Note that in the presence of steel factor mast cells adhered not to COS cells but to the substrate. Scale bar is 100 μm.

c-*kit*, because it inhibited the c-*kit* kinase activity without affecting c-*kit* expression. Genistein did not inhibit mast cells from binding to COS cells transfected with steel factor. This finding suggests that the tyrosine kinase activity of c-*kit* was not necessary for adhesion of COS cells transfected with steel factor. Finally, adhesion between mast cells and fixed COS cells expressing steel factor was not dependent on divalent cations, because no significant inhibition of adhesion was observed in the presence of EDTA (Fig. 12D). Because fixation of COS cells still allowed adhesion to mast cells, it is unlikely that membrane-bound steel factor on COS cells modulated other adhesion molecules to bind to mast cells. These results indicate that membrane-bound steel and c-*kit* act as adhesion molecules without requiring divalent cations and c-*kit* kinase activity. Adhesion between them, however, is temperature- and energy-dependent and requires a functional cytoskeleton.

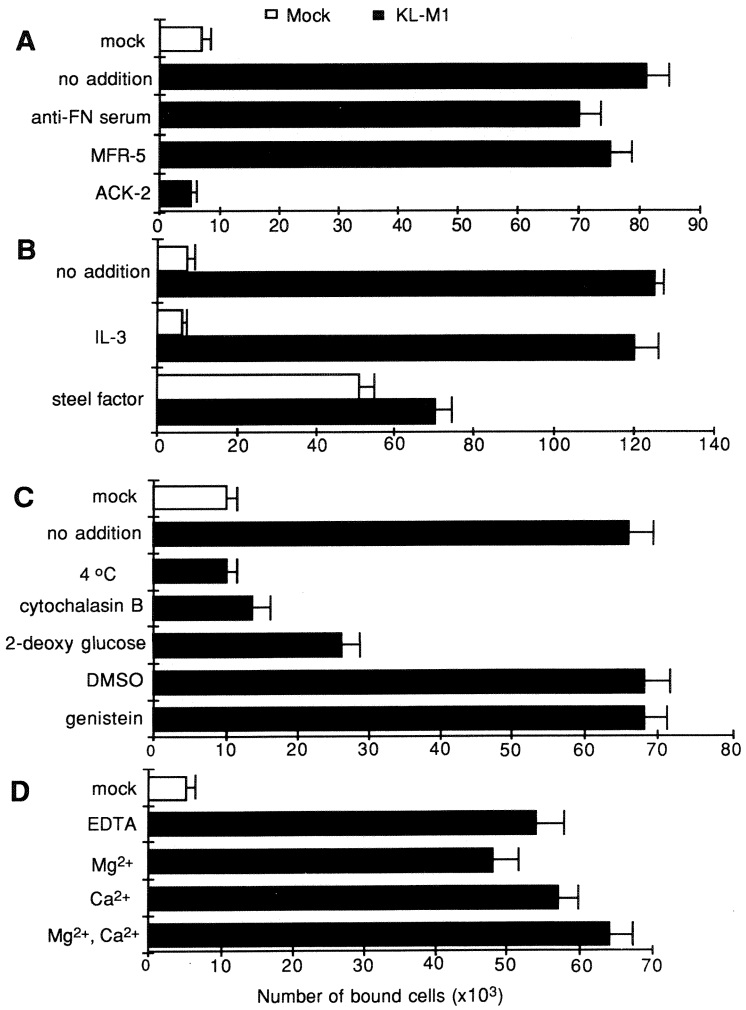

Fig. 12. Characterization of adhesion of mast cells to COS cells transfected with full-length cDNA encoding steel factor (KL-M1) and to the intervening substrate. **A** The effects of MFR-5, ACK-2, or anti-fibronectin antibody on adhesion. **B** Adhesion of mast cells stimulated with interleukin-3 (IL-3) (100 U/ml) or steel factor (100 U/ml). **C** Adhesion of mast cells treated with cytochalasin B (20 μM), 2-deoxy D-glucose (50 mM) + sodium azide (10 mM), DMSO (1%), or genistein (50 μg/ml). **D** Divalent cation-independent adhesion of mast cells to transfected COS cells. Adhesion assays were performed with fixed COS cells in the presence of 5 mM ethylenediamine tetraacetic acid (EDTA), 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, or 1.5 mM $MgCl_2$ + $CaCl_2$. All experiments were performed in triplicate. Average number of bound cells is shown with SE. The number of input cells was 5×10^5 cells.

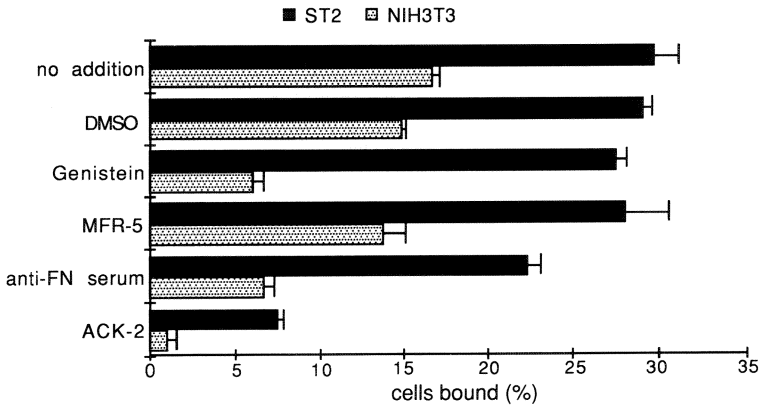

Fig. 13. Adhesion of mast cells to fibroblast cells or stromal cells. Adhesion assays with monolayer of NIH3T3 or ST2 were performed with DMSO (1%), genistein (50 μg/ml), MFR-5, anti-fibronectin antibody, or ACK-2.

Adhesion of Mast Cells to Fibroblast and Stromal Cells

The NIH3T3 fibroblast cell line and the ST2 stromal cell line can support mast cell growth in the co-culture system [36]. Adhesion of mast cells to monolayers of these cell lines was inhibited by mAb to c-*kit*, suggesting direct adhesion mediated by cell–surface steel factor and c-*kit* or adhesion mediated by other molecules such as fibronectin and fibronectin receptor activated by steel factor (Fig. 13). Adhesion to ST2 was not affected by genistein and mAb to the fibronectin receptor, although antibody to fibronectin slightly reduced adhesion. Adhesion to NIH3T3 cells was reduced by half in the presence of genistein or antibody to fibronectin, indicating that adhesion of mast cells stimulated with steel factor to fibronectin was involved. However, mAb to the fibronectin receptor was ineffective, suggesting the involvement of other adhesion molecules that recognize fibronectin. These results suggest that steel factor binding to c-*kit* is responsible for the major component of adhesion between mast cells and ST2 and that adhesion of mast cells to NIH3T3 resulted in c-*kit* kinase-stimulated binding to fibronectin. The different mechanisms of adhesion in ST2 and NIH3T3 could be due to the differing expression of the membrane form of steel factor or cell-associated fibronectin.

SUMMARY

We show that hematopoietic cells use different adhesion molecules to interact with stromal cells and extracellular matrix components. Our studies show that multiple adhesion molecules are expressed on stromal cells and hematopoietic cells, and we demonstrate that preferential use of specific adhesion pathways can be regulated by the expression level of adhesion molecules or kinase-dependent stimulation of adhesion with growth factors. It remains to be seen how adhesion regulated by the above

mechanisms influences growth and differentiation of hematopoietic stem cells. We have preliminary results that suggest that adhesion molecules directly deliver signals to progenitor cells and modulate their response to hematopoietic growth factors. "Cross talk" between adhesion molecules and growth factor receptors could play a pivotal role in hematopoiesis.

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Discussion

NIENHUIS — You've shown the interaction of SCF and its ability to induce adhesive molecules in mast cells. I wonder if there are any data that you or others have on the effect of the cytokine to stimulate adhesion in primitive progenitor populations?

KINASHI — To my knowledge, little is known about progenitor cells with regard to regulation of adhesion molecules, but there are a lot of examples in leukocytes, like T cells and neutrophils, and in these cells adhesion is dependent on T-cell receptor cross-linking or chemoattractant binding. To my knowledge, how adhesion molecules are regulated in the progenitor cells is totally unclear.

QUESENBERRY — Is there any evidence that if you take cell lines and synchronize them that the binding is dependent on the phase of cell cycle?

KINASHI — No, I haven't done that kind of experiment.

YODER — Have you isolated the 46-kD VCAM molecule and examined how it works in combination with IL-3 or SCF to support cell growth?

KINASHI — I tried very hard, but I haven't succeeded in isolating P46 in purified form. It's contaminated by a lot of the junk proteins, so I didn't do any experiments with the small molecular weight form of VCAM.