

Regulation of Cell-Matrix Adhesion by Receptor Tyrosine Kinases

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Cell-cell and cell-matrix adhesive interactions mediated by integrins play crucial roles in leukocyte migration to inflamed tissues, and also in cell migration during embryogenesis. Much remains to be learned about the molecular mechanisms of regulation of adhesion mediated by integrins. Recently we found that steel factor and c-kit induce adhesion to fibronectin by VLA-5 in mast cells.¹ Activation of adhesiveness is transient, and occurs at concentrations of steel factor 100-fold lower than required for growth stimulation. This suggests that regulation of adhesion is an important biological function of steel factor and c-kit. Other receptor tyrosine kinases such as the PDGF receptor can substitute for c-kit. Signaling through receptor tyrosine kinases may offer a general mechanism for the regulation of integrin avidity.

KEY WORDS: Regulation Cell matrix-adhesion receptor

INTRODUCTION

Adhesion molecules are important for proper development and function of the organism,² particularly for regulation of migration and alteration in cell association. Although much progress has been made in identifying and structurally characterizing adhesion molecules, less is known about regulation of adhesion. T-cell receptor crosslinking stimulates T cells to adhere transiently to ICAM-1 through the integrin LFA-1. Transient adhesiveness of T cells through LFA-1 is not accompanied by any change in the density on the cell surface of LFA-1. Therefore the presence of adhesion molecules on a cell's surface is not sufficient for adhesion, but rather the affinity or some other qualitative change in LFA-1 that regulates adhesiveness is stimulated by T-cell receptor crosslinking. It is believed that cytoplasmic signals initiated by the T cell receptor crosslinking modulate the avidity of integrins (inside-out signaling). However, it is unclear which signaling pathways initiated by T-cell re-

ceptor are required to achieve avidity modulation of integrins.^{3–5}

Two strains of mutant mice drew attention to the hypothesis that tyrosine kinases might be involved in regulation of adhesion. Mutations at the *steel* (*Sl*) or *white spotting* (*W*) loci affect the development of several migratory cell lineages including primordial germ cells, neural crest-derived melanocytes, hematopoietic stem cells and mast cells.^{6,7} Mice bearing either mutation have similar phenotypes including sterility, severe anemia, white coat color and mast cell deficiency. The *W* locus encodes the proto-oncogene *c-kit*, which is a member of the transmembrane tyrosine kinase family that has homology to colony-stimulating factor 1 and platelet-derived growth factor receptors.^{8–10} The *Sl* locus encodes a ligand for c-kit^{11–13} which has been designated stem cell factor,^{13–15} mast cell growth factor^{11,16,17} and the kit ligand (KL);^{12,18} herein, we refer to the c-kit ligand as steel factor.¹⁹

The phenotypes of *Sl* and *W* mutation imply that steel factor and c-kit regulate either the development, the migration, or both development and migration of several migratory cell populations. Since cell migration is dependent on adhesive interactions with the extracellular matrix,²⁰ we have investigated how the steel factor/c-kit system reg-

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ulates cell-extracellular matrix adhesion using mast cells as a model system.¹

Adhesion of mast cells to extracellular matrix proteins. We tested the IL-3-dependent mast cell line MC/9 and bone-marrow derived mast cells for adhesion to extracellular matrix proteins. MC/9 cells adhered well to fibronectin and laminin, and to a lesser extent to vitronectin (Fig. 1A). Unstimulated bone-marrow derived mast cells did not bind significantly to any of the tested extracellular matrix proteins; however, steel factor but not IL-3 or IL-4 stimulated binding to fibronectin and less markedly to vitronectin (Fig. 1B). We confirmed that both IL-3 and IL-4 stimulated growth of our cultured mast cell cells (data not shown) as previously reported,²¹⁻²³ showing

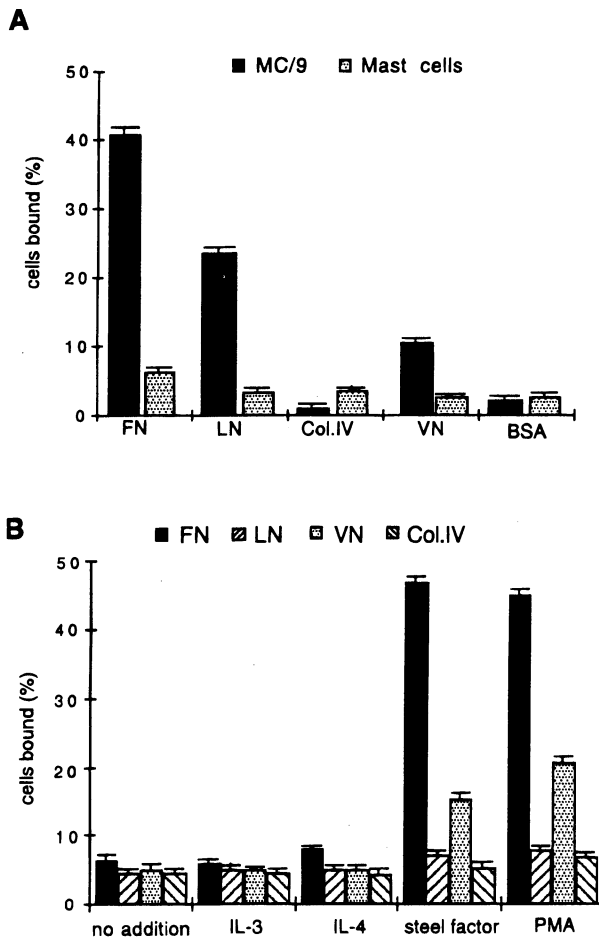


Figure 1 Adhesion of MC/9 and mast cells to extracellular matrix proteins. A. Cell binding was measured to fibronectin (FN), laminin (LN), collagen type IV (Col.IV), vitronectin (VN) and albumin (BSA) coated on polystyrene culture plates. B. Adhesion of mast cells stimulated with IL-3, IL-4, and steel factor (100 units/ml each) or PMA (10 ng/ml) to extracellular matrix proteins. The average and S.E. of triplicate determination are shown.

that their lack of effect on adhesion was not due to receptor modulation. Inability of IL-3 and IL-4 to stimulate adhesion suggests that signals for adhesion may be distinct from those for cell growth.

Consistent with this notion, stimulation of adhesiveness of cultured bone-marrow mast cells was exquisitely sensitive to steel factor. Half-maximal binding of mast cells to fibronectin was achieved with only 0.03 units/ml, whereas 10 units/ml was required to stimulate half maximal growth of mast cells (Fig. 2). Growth stimulating activity of steel factor for other hematopoietic cells is not very strong, and is best observed when combined with other cytokines.^{13,15} Adhesion-stimulating activity of steel factor may be a prime important activity *in vivo*.

Steel factor-stimulated adhesion to fibronectin is mediated through the murine homologue of VLA-5. A 120 kD fragment of fibronectin containing the classical RGD tripeptide cell attachment site²⁴ was able to substitute for native fibronectin in mediating steel factor-stimulated adhesion of mast cells (Fig. 3). MFR-5, a rat mAb that recognizes murine VLA-5, blocked adhesion to the 120 kD fragment and to intact fibronectin. The RGDS peptide but not the control RGEs peptide, completely inhibited the binding to the 120 kD fragment, indicating that the RGD cell attachment site is responsible for adhesion.

Transient adhesiveness to FN. 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 both steel factor and PMA was

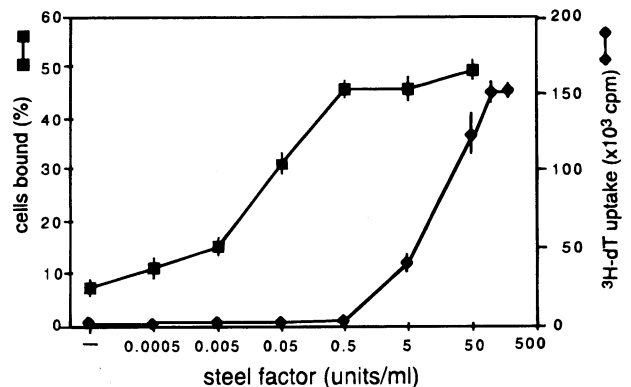


Figure 2 Comparison of adhesion-stimulating and growth-stimulating activities of steel factor. Stimulation of binding to fibronectin and ³H-thymidine uptake of mast cells was assayed in triplicate. Bars show the S.E.

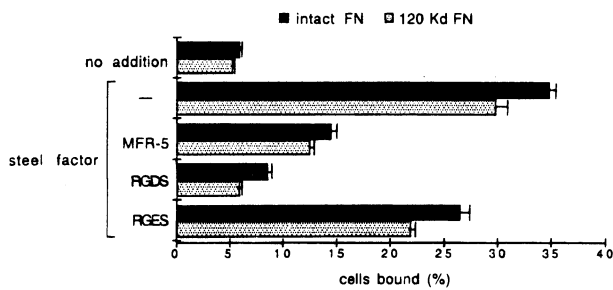


Figure 3 Adhesion to intact fibronectin or its 120 kD fragment was stimulated with steel factor in the presence of MFR-5, or 1.5 mM of RGDS or RGES peptide.

transient, and maximal adhesiveness was seen at about 10 minutes (Fig. 4), the earliest time point measurable after stimulation. Binding declined to background levels after 90 min. At 120 min., mast cells were re-incubated with either 100 units/ml steel factor or 10 ng/ml PMA; however, neither heterologous nor homologous 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 expression of VLA-5 on mast cells, as shown by staining with MFR-5 before and at several time points after stimulation with steel factor or PMA.¹ These findings suggest that the affinity or some other qualitative change in VLA-5 is stimulated by steel factor and PMA. This "inside-out" signaling is reminiscent of the increased adhesiveness in LFA-1 stimulated by T cell receptor crosslinking.²⁵ T cell receptor crosslinking was shown to result in transient adhesiveness of LFA-1, whereas adhesiveness stimulated by PMA was stable, showing both similarities and differences between the two systems.

W/W^v mast cells are defective in steel factor-stimulated adhesion but not in adhesion to steel factor. In order to investigate how *W* mutations affect adhesion, we characterized *W/W^v* mast cells. The *W* mutation results in a null phenotype, while the *W^v* mutation is a single amino acid substitution in the kinase domain of *c-kit* that is expressed comparably to the wild type,²⁶ (data not shown). Binding to FN of *W/W^v* mast cells stimulated with steel factor was dramatically reduced compared to wild type, whereas the responsiveness to PMA remained intact (Fig. 5A). This result shows that the tyrosine kinase activity of *c-kit* is crucial to induce adhesiveness to FN. The importance of the tyrosine kinase activity is also supported by experiments with the tyrosine-specific protein kinase inhibitors, genistein and herbimycin A, in which these inhibitors abolished the steel factor-induced FN binding whereas PMA-induced binding remained relatively intact (data not shown).

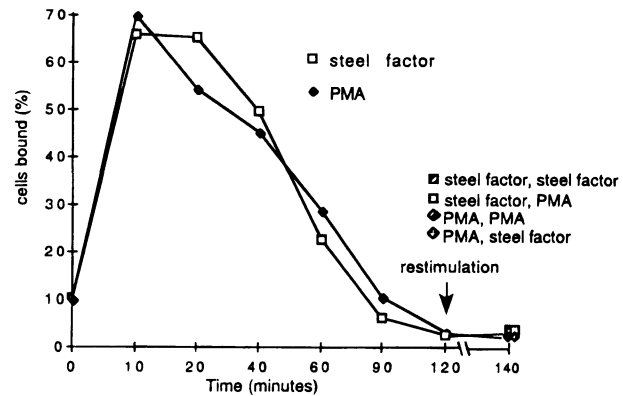


Figure 4 Kinetics of adhesion of mast cells stimulated with steel factor or PMA. Mast cells were stimulated with steel factor (10 units/ml) or PMA (10 ng/ml) in suspension for varying times before transfer into wells and centrifugation. The plate was then incubated for 6 minutes at 37°C before washing. Zero time indicates adhesion without stimulation. The time indicated includes all steps before washing. At 120 min., cells were restimulated with 100 units/ml steel factor or 10 ng/ml PMA in all four possible combinations as indicated in the figure. The average of triplicates is shown; the S.E. was less than 3%.

By contrast to adhesion stimulated by *c-kit*, direct adhesion through *c-kit* was unaffected by the *W^v* mutation. Mast cells from *+/+* and *W/W^v* mice adhered equally well to COS cells transfected with full length steel factor (Fig. 5B). This cellular adhesion was dependent on *c-kit*, because the blocking antibody to *c-kit*, ACK-2, abolished adhesion.

Thus, steel factor binding to *c-kit* can both directly mediate adhesion, and stimulate integrin adhesiveness to fibronectin that is transient. Steel factor stimulation of integrin adhesiveness appears particularly relevant to the effect of the *W* and *Sl* mutations on migration of several cell lineages *in vivo*; adhesiveness is required for cell migration and this regulation of adhesiveness is thought to be closely coordinated with cell migration.^{2,27} The strong effect of *W* mutations that abolish tyrosine kinase activity on cell migration *in vivo* suggests that the stimulation of integrin adhesiveness, which requires this activity, rather than direct adhesion, which does not, is most relevant to the *in vivo* phenotype. *In situ* analysis of steel factor mRNA²⁸ shows that steel factor is expressed in tissues associated with the migratory pathways and homing sites of melanoblasts, germ cells and hematopoietic stem cells. Fibronectin is essential in migration of cell types including those derived from the neural crest;²⁰ however, fibronectin is much more widely distributed than steel factor. The localized distribution of steel factor may therefore be important in guidance of cells along the migratory pathway.

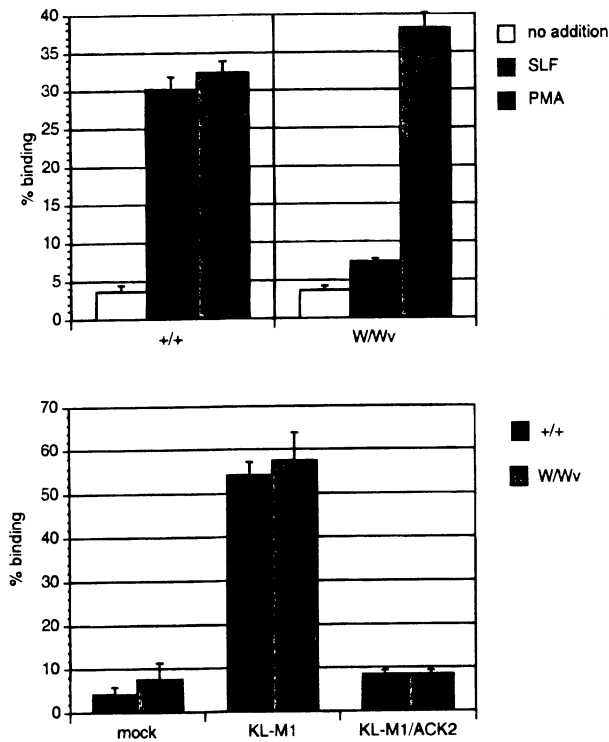


Figure 5 A. Effect of c-kit tyrosine kinase mutation on adhesion to fibronectin (A) or steel factor on the cell surface (B). A. Mast cells from +/+ and W/Wv mice were incubated with no addition (\blacktriangle), steel factor (\blacksquare), or PMA (\blacksquare), and assayed for adhesion to fibronectin. B. Adhesion of +/+ and W/Wv mast cells was assayed to COS transfected with a vector alone (mock), full-length steel factor cDNA (KL-M1), or full-length steel factor cDNA in the presence of anti c-kit antibody, ACK2 (KL-M1/ACK2).

Signals from c-kit for stimulation of adhesion to fibronectin.

The intermediates in the signal transduction pathway(s) for stimulation of integrin adhesiveness are poorly characterized. The fact that PMA mimics the effect of steel factor and is known to be a direct activator for protein kinase C (PKC) suggests that PKC is involved in stimulation of adhesion. To study how PKC contributes to stimulation of binding to FN, mast cells were pretreated with PMA to down-regulate PKC. This pretreatment down-regulates all PKC isotypes that are expressed in mast cells (data not shown). Mast cells pretreated with PMA failed to bind to FN in response to further PMA, but did respond to steel factor to a similar degree to untreated mast cells (Fig. 6a). This result suggests that activated c-kit stimulates through another pathway, which is resistant to the PMA treatment. Since phosphatidylinositol 3 kinase (PI3 kinase) is physically associated with activated c-kit, we examined the involvement of PI3 kinase by using wort-

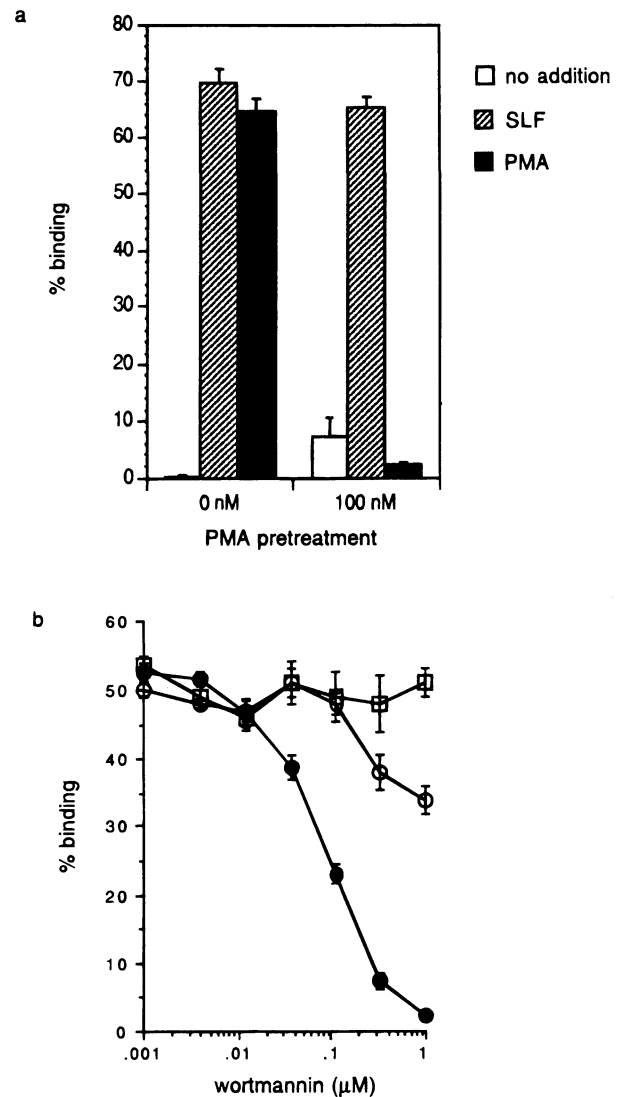


Figure 6 Adhesion to fibronectin of mast cells treated with PMA and wortmannin. a. Effect of the PMA pretreatment on adhesion. Mast cells were pretreated with or without PMA (100 nM) for 2 days before the adhesion assay. Mast cells were stimulated for adhesion with steel factor (hatched bar), PMA (closed bar), or were unstimulated (open bar) prior to the adhesion assay. b. Effect of wortmannin on adhesion. Mast cells that were untreated or pretreated with PMA (100 nM) for 2 days were incubated with various amounts of wortmannin as indicated for 10 min. at room temperature, transferred into fibronectin-coated plates together with wortmannin, stimulated, and assayed for binding to fibronectin. Mast cells were stimulated with PMA (open square), steel factor (open circle), or cells pretreated for 2 days with PMA were stimulated with PMA (closed circle).

mannin, a specific PI3 kinase inhibitor.²⁹ Wortmannin had no effect on steel factor or PMA-induced adhesion to fibronectin. However, when mast cells were pretreated with PMA, wortmannin inhibited adhesion to fibronectin in a dose dependent manner (Fig. 6b).

Our results suggest that there are two independent pathways leading from c-kit to integrin avidity regulation. One pathway is sensitive to the PMA pretreatment and probably mediated by PKC, and the other is PMA resistant, but sensitive to wortmannin, indicating that PI3 kinase is likely involved in this pathway. Thus far each pathway appears to stimulate similar efficiency of adhesion and cell spreading on fibronectin, although there might be unidentified differences in effects on each pathway. Phospholipase C γ -1 (PLC γ) and PI3 kinase are known to physically associate with the activated PDGF receptor as well as c-kit.³⁰ As expected, the PDGF receptor can substitute for c-kit (submitted for publication). Studies using mutant PDGF receptors confirmed that there are two pathways in which PLC γ -PKC and PI3 kinase are involved, respectively. It should be noted that a mutant PDGF receptor with no binding sites for PLC γ and PI3 kinase loses adhesion-stimulating activity but still retains the ability to stimulate cell growth. Signals for adhesion are thus dissociated from those for cell growth in this case. PLC γ and PI3 kinase are activated when the T-cell receptor and B-cell receptor are cross-linked.³¹ Tyrosine kinases are thus likely to trigger these cascades. The signaling pathways for adhesion described above may thus be common among different families of receptors.

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