RAPID COMMUNICATION

Integrins Involved in the Adhesion of Megakaryocytes to Fibronectin and Fibrinogen

By P.K. Schick, C.M. Wojenski, X. He, J. Walker, C. Marcinkiewicz, and S. Niewiarowski

We studied integrins involved in the adhesion of resting and activated megakaryocytes (MK) to fibronectin (FN) and fibrinogen (FGN). Guinea pig MK were isolated and in some experiments were activated by thrombin. MK adhering to FN or FGN coated on coverslips were quantitated by a computerized image analysis program. The binding of soluble human FN to MK was detected by Western blotting. Anti-integrin antibodies, disintegrins, and cyclic RGD peptides were used to identify integrins involved in the adhesion of MK to FN or FGN. Resting MK adhered to coverslips with immobilized FN. The adhesion of MK to FN was primarily inhibited by an anti-\(\alpha_5\) antibody and EMF-10, a disintegrin highly specific for \(\alpha_5\). However, the adhesion of MK to FN was not blocked by agents that inhibit \(\alpha_{IIb}\beta_3\), \(\alpha_5\beta_3\) or \(\alpha_4\beta_1\). A \(\beta_1\) activating antibody increased the number of MK bound to FN due to the activation of \(\alpha_5\). The binding of soluble FN was also primarily inhibited by agents that block \(\alpha_5\).

Resting MK did not adhere to FGN. However, MK activated by thrombin did adhere to FGN. This binding was mediated by \(\alpha_{IIb}\beta_3\), because binding was inhibited by bitistatin, a disintegrin, and a cyclic RGD peptide that are known to block this integrin. The binding of thrombin-activated MK to FN was mediated by both \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\) based on the additive effect of agents that inhibit these integrins. The study indicates that resting MK bind to FN but not to FGN and that \(\alpha_5\beta_1\) is the major integrin involved in the binding of MK to FN. Activated MK bind to FGN primarily by \(\alpha_{IIb}\beta_3\). However, the binding of activated MK to FN is due to both \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\). The demonstration that \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\) are involved in MK adhesion indicates that these integrins may have a role in MK maturation and platelet production.

\(\text{FIBRONECTIN (FN)}\) is a major component of bone marrow extracellular matrix and is considered to have an important role in hematopoiesis.\(^1\)\(^2\) Hematopoietic cells are found in areas of bone marrow rich with FN,\(^3\) and FN forms septa in bone marrow that may provide anchorage for migrating hematopoietic progenitor cells.\(^4\) The attachment of blood cell precursors to FN may modulate the migration and homing of cells to specific bone marrow regions that promote maturation of progenitors and the release of mature blood cells into the circulation.\(^5\)

A role for FN in megakaryocyte (MK) maturation and platelet production is suggested by the expression of FN in rat fetal liver MK before its expression in fetal hepatocytes.\(^6\) We have shown that guinea pig MK can synthesize FN,\(^7\) and a previous study has demonstrated FN mRNA in MK.\(^8\) The level of endogenous MK FN is 7.5-fold greater than FN in other bone marrow hematopoietic cells. In response to thrombin, FN is secreted and adheres to the surface of MK.\(^7\)

The purpose of the current study was to identify integrins involved in the binding of FN on the MK surface. \(\alpha_4\beta_1\) and \(\alpha_5\beta_1\) are the major integrins involved in the binding of FN in hematopoietic cells.\(^2\)\(^9\)\(^10\) \(\alpha_{IIb}\beta_3\) primarily reacts with fibrinogen (FGN) but can also bind FN.\(^11\) However, the activation state of these integrins, which determines their ability to bind ligands, can change during cell maturation. There is also evidence that the level of expression of \(\alpha_4\beta_1\) and \(\alpha_5\beta_1\) regulates the differentiation of erythroidic and myeloid precursors.\(^2\)\(^9\)\(^10\) Among the integrins known to bind FN, \(\alpha_5\), \(\alpha_{IIb}\beta_3\), \(\alpha_4\beta_1\), and \(\alpha_3\beta_3\) have been shown to be expressed and to be functional in MK.\(^12\)\(^13\)

We now report information about the binding of resting and activated MK to FN and FGN. We found that resting MK bind to FN but not to FGN and that \(\alpha_5\beta_1\) is the major integrin mediating the binding of MK to FN. In contrast, activated MK bind to FGN primarily through \(\alpha_{IIb}\beta_3\) to FN through both \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\). \(\alpha_3\beta_3\) and \(\alpha_4\beta_1\) do not seem to be involved in the binding of isolated recognizable MK to FN or FGN.

MATERIALS AND METHODS

Antibodies, cyclic peptides, and disintegrins. SAM-1, an \(\alpha_5\) blocking antibody (Immunotech, Westbrook, ME); HP2/1, an \(\alpha_4\) blocking antibody (Immunotech); LM609, an \(\alpha_3\) blocking antibody (Chemicon International, Temecula, CA); monoclonal antibody (MoAb) IST-4 (Sigma, St Louis, MO), which detects human FN; and anti-\(\alpha\)-actinin antisera (Sigma) and anti-FN EIIIB antibody prepared by Dr Vickie Bennet,\(^7\) which cross-reacts with guinea pig FN, were used. MoAb 8A2 was kindly donated by Dr Nicholas Kovach.\(^15\) Whole mouse IgG and isotype-specific IgG were used as controls for the blocking and enhancing antibodies and did not have any effect. All antibodies that were used were anti-human. MK0852, a cyclic RGD peptide, and bitistatin, a disintegrin, were used to demonstrate the activity of \(\alpha_{IIb}\beta_3\) and to FN through both \(\alpha_5\beta_1\) and \(\alpha_{IIb}\beta_3\). \(\alpha_3\beta_3\) and \(\alpha_4\beta_1\) do not seem to be involved in the binding of isolated recognizable MK to FN or FGN.

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novel disintegrin isolated from *Eristochophis macmahoni* venom. It is a heterodimer composed of two subunits A and B that are structurally almost identical to eristocophin I and II, the sequence of which has been previously reported by Siddiq et al.²⁰ EMF10 was isolated by reverse-phase high-performance liquid chromatography (HPLC) using a Vydac C18 column and eluted at 41% acetonitrile gradient. EMF10 has been characterized and the molecular mass of this protein, determined by mass spectrometry, was 14,974 Daltons (Marcinkiewicz et al, manuscript in preparation). EMF10 can inhibit the activity of α5β1 and αIIbβ3, but α5β1 activity is considerably more sensitive to this agent than is αIIbβ3, because inhibition of platelet aggregation required 1,600 nmol/L EMF10. The inhibitory effects of EMF10 on cell adhesion to various ligands were determined in solid phase assays, as shown in Table 1.

Disintegrins and cyclic peptides can be used to identify integrins involved in binding of FN and other ligands, but higher concentrations of these agents can inhibit several integrins. Antibodies are more specific for integrin subunits, but some anti-integrin antibodies do not cross-react with guinea pig tissues. However, disintegrins and cyclic peptides can be used to inhibit and study integrins in guinea pig MK.

**Source of MK.** Guinea pig MK were isolated to approximately 90% purity by cell size and greater than 98% purity by protein content, because MK are considerably larger than other bone marrow cells, as previously described.²¹ Approximately 1 × 10⁶ cells are isolated by this procedure and their viability is approximately 89%.

**Adhesion assays.** In preparation for the adhesion assays, FN- and FGN-coated glass coverslips were prepared by coating the coverslips with a 1% solution of gelatin (Bio-Rad, Melville, NY) for 2 hours at 37°C and then with 50 µg/mL bovine plasma FN (Sigma) or 250 µg/mL purified human plasma FGN (kindly provided by Dr Jose Martinez) overnight at 4°C.

MK were pretreated with varying amounts of antibodies, disintegrins, or peptides in Dulbecco’s phosphate-buffered saline (DPBS) containing Ca²⁺ and Mg²⁺ (GIBCO BRL, Grand Island, NY) for 10 minutes at room temperature (RT) before aliquots were placed on the coverslips. Control samples were pretreated with whole IgG or type-specific IgG (5 to 20 µg/mL). In some experiments after this 10 minutes of incubation, MK were activated with thrombin (0.5 U/mL) for 3 minutes at RT, followed by the addition of hirudin (0.5 U/mL; Sigma) to stop thrombin activity.

Aliquots (100 µL) containing 1 to 2 × 10⁴ MK were overlaid in triplicate onto FN- or FGN-coated coverslips and incubated at 37°C for 30 minutes. After incubation, the coverslips were rinsed twice with DPBS, followed by fixation with 3.7% formaldehyde in DPBS for 20 minutes at room temperature. After washing, the cells on the coverslips were stained with Leukostat Solution (Fisher, Pittsburgh, PA), and cell adhesion was quantitated using computerized image analysis (NIH Image version 1.60 software; National Institutes of Health, Bethesda, MD).

**Results**

Unstimulated guinea pig MK adhered to FN coated over gelatin onto glass coverslips. The adherence was maximal in the presence of both calcium (1.0 mmol/L) and magnesium (0.5 mmol/L) and was blocked by EDTA (10 mmol/L). MK did not adhere to gelatin-coated coverslips.

The adhesion of MK to FN-coated coverslips resulted in changes in morphology most likely representing cytoskeletal reorganization. Figure 1 shows that unstimulated MK adhering to FN put out blebs or pseudopods along their entire circumference in contact with the FN demonstrated by the immunostaining of α-actinin and visualization by confocal microscopy. These changes were not seen with the adhesion of MK to polylysine, because MK retained their round shape under these conditions. Thus, these changes were specific to MK adhering to FN.

An α5-blocking antibody, SAM-1, inhibited MK adherence to FN in a dose-dependent manner, with an IC50 of 0.6 µg/mL (Fig 2), whereas an α4-blocking antibody, HP2/1, and an αvβ3 blocking antibody, LM609, did not inhibit adhesion at 20 µg/mL (data not shown). Both HP2/1 and LM609 have previously been shown to cross-react with integrins in guinea pig tissues.²²,²³ We found by flow cytometry that 85% of guinea pig MK stained positively with each of these antibodies (data not shown). This information is similar to the finding that greater than 90% of isolated human MK express α4β1.¹³ α4β1 was also found to be expressed in human MK grown in culture, but there was less α4β1 in mature than in immature MK.¹² Thus, α4β1, α5β1, and αvβ3 are expressed in mature guinea pig MK. Also, HP2/1 (5 µg/mL) caused 83% ± 9% inhibition of the binding of guinea pig MK to vascular cell adhesion molecule (VCAM), and LM609 (5 µg/mL) caused 61% ± 1% inhibition of binding to vitronectin (mean ± SD; n = 3), indicating that these antibodies are functional against guinea pig integrins.

To confirm that α5β1 mediated the adherence of MK to

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<th>Integrin</th>
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<td>αIIbβ3</td>
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<td>αvβ3</td>
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<td>α4β1</td>
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Cultured cells labeled with CFMDSA were incubated with ligands immobilized on microplates with various concentrations of EMF10. Cells attached to the plate were lysed by detergent and fluorescence intensity was measured using a microtiter plate reader as previously described.³⁴ CH0 cells transfected with αIIbβ3 and with αvβ3 were kindly provided by Dr Mark Ginsberg (Scripps Research Institute, La Jolla, CA). Jurkat cells expressing predominantly αvβ1 and K562 cells expressing predominantly α5β1 were purchased from ATCC (Rockville, MD). Recombinant VCAM-1 was a generous gift from Dr Mark Renz (Genentech, San Francisco, CA).
immobilized FN, we tested a newly characterized disintegrin, EMF10. As shown in Fig 3, EMF10 inhibited MK adherence to FN in a dose-dependent manner, with an IC50 of 4.5 nmol/L, which was very similar to its ability to inhibit α5β3-mediated binding of K562 cells to FN, as shown in Table 1. Inhibition of adhesion at this concentration of EMF10 is not likely due to an effect on αIIbb3, because much higher concentrations are needed to inhibit αIIbb3 mediated binding to FGN, as shown in Table 1.

MoAb 8A2, which activates integrins containing the β1 subunit, enhanced the binding of MK to FN, and the binding was concentration dependent, as shown in Fig 4.

SAM-1 (5 µg/mL) inhibited the MoAb 8A2-enhanced binding of MK to FN by 62% ± 6% (mean ± SD), whereas HP2/1 (20 µg/mL) alone or in combination with SAM-1 had no effect (n = 3).

HP2/1 (5 µg/mL) did block the MoAb 8A2-enhanced binding of MK to V-CAM by 80% (data not shown). This information indicates that α5β1 was the primary integrin involved in the MoAb 8A2-augmented binding of MK to FN.

To obtain specific information about the role of αIIbb3, we used bitistatin, a disintegrin, and MK0852, a cyclic RGD peptide. The IC50 for inhibition of MK adherence to FN was greater than 1 µmol/L for bitistatin and greater than 5 µmol/L for MK0852 (data not shown). Thus, αIIbb3 did not appear to mediate the adhesion of resting MK to FN. These agents can inhibit the activity of guinea pig αIIbb3, because we found that they inhibit ADP- and collagen-induced aggregation of guinea pig platelets with an IC50 of 60 and 80 nmol/L, respectively (data not shown).

Thus, α5β1 but not α4β1, αvβ3, or αIIbb3 mediates the adherence of unstimulated MK to immobilized FN.

MK did not adhere to immobilized FGN unless they were first activated with thrombin. Bitistatin and MK0852 inhibited the adherence of activated MK to FGN, with an IC50 of 30 and 200 nmol/L, respectively, as shown in Fig 5. The anti-α4
antibody, HP2/1, did not inhibit binding (data not shown). The anti-α5 antibody, SAM-1, did not inhibit binding, and SAM-1 in combination with bitistatin or MK0852 did not cause a greater degree of inhibition over that caused by bitistatin or MK0852 alone (data not shown). Therefore, αIIbb3 mediates the binding of activated MK to FGN, as is true for platelets. However, when thrombin-activated MK were allowed to adhere to FN, concentrations of bitistatin (50 nmol/L) and MK0852 (100 nmol/L) near their IC50 for FGN binding only inhibited FN binding by about 12% (Fig 6). The α5-blocking antibody, SAM-1, at a concentration above its IC50 for inhibiting FN binding by unstimulated MK (2 μg/mL), also showed much less inhibition of activated MK (24%; Fig 6). However, when activated MK were pretreated with either SAM-1 plus bitistatin or SAM-1 plus MK0852, the inhibition of adherence to FN was found to be additive (Fig 6). The binding of activated MK to FN was not blocked by HP2/1 or LM609 either alone or in combination with SAM-1 or bitistatin. A previous study demonstrated that the adherence of thrombopoietin (TPO)-activated MK to FN was mediated by αIIbb3 and to a lesser extent by α5β1 but did not determine whether the effects of inhibiting these integrins was additive.

To determine whether unstimulated MK would bind soluble FN with the same integrin that they use to adhere to immobilized FN, we incubated guinea pig MK with human plasma FN in solution. Figure 7A demonstrates that soluble human plasma FN can bind to the surface of guinea pig MK and that endogenous guinea pig FN was not detected by the antihuman FN antibody. Endogenous FN in guinea pig MK was detected with an antibody that we had previously shown to react with FN in this species, as shown in Fig 7B. Endogenous FN was distributed throughout the MK cytoplasm. Control MK incubated with nonimmune normal IgG are shown in Fig 7C.

To study the inhibition of binding of soluble FN, MK were incubated with human FN in the presence and absence of integrin blocking agents. After the incubation of MK with human FN, immunoprecipitated FN that had bound to the MK surface was analyzed by Western blotting using the antihuman FN antibody. Figure 8 shows a representative of four experiments in which MK were incubated with MK0852 (lane 1),
The study demonstrated that α5β1 is primarily responsible for the adherence of resting MK to FN based on the inhibition of binding by SAM-1, an MoAb, and EMF10, a disintegrin, at concentrations that have been shown to block the activity of this integrin in other cells. The binding of MK to FN induced the formation of pseudopods that contained α-actinin, consistent with the ability of ligand-interactions to induce cytoskeletal changes. The binding of resting MK to FN was not mediated by αIIbβ3, because neither bitistatin nor MK0852 inhibited the binding at concentrations of 1 and 10 μmol/L, respectively.

These concentrations are considerably higher than those shown to block binding to these integrins in other cells.16–19 MoAbs that block α4 activity, HP2/1, or αvβ3 activity, LM609, did not affect the adhesion of MK to FN, suggesting that neither integrin is involved.

The role of α5β1 in the binding of MK to FN is supported by the demonstration that MoAb 8A2 markedly increased the number of MK that were attached to FN, and this binding was only inhibited by the α5 blocking antibody. MoAb 8A2 has been shown to bind to the β1 subunit of integrins, and the antibody amplifies integrin activity.15,25 Therefore, although α5β1 is in an active state in resting MK, its activity can be further heightened.

The binding of soluble human FN to resting MK was also demonstrated, and α5β1 was the primary integrin involved. There were patchy accumulations of human FN on the MK surface, similar to those we detected when endogenous FN was released from thrombin-treated MK.7 There was no evidence of intracellular human FN in these experiments, indicating that exogenous FN was not taken up by guinea pig MK. This is consistent with previous reports that indicate that FN in MK differs from plasma FN and that plasma FN is not taken up by MK.26 α5β1 was identified as the integrin involved in the binding of soluble FN to MK by evidence that the binding was inhibited by SAM-1 and EMF10 but not by anti-αIIbβ3 blocking agents (bitistatin and MK0852) or by the anti-α4 antibody (HP2/1). Thus, both the binding of soluble FN to MK and the binding of MK to immobilized FN are mediated primarily by α5β1.

Resting MK did not adhere to FGN, but MK exposed to thrombin did bind to FGN. The adherence of activated MK was mediated by αIIbβ3 based on the demonstration that bitistatin and MK0852 inhibited adhesion at concentrations that can inhibit αIIbβ3 binding in other cells and that can inhibit platelet aggregation. α4β1 and αvβ1 are not involved in the binding of MK to FGN, because HP2/1 and LM609 did not inhibit the binding of activated MK to FGN. SAM-1, the anti-α5 antibody, did not inhibit the binding, and SAM-1 in combination with bitistatin or MK0852 did not cause a greater degree of inhibition of binding than that caused when only these anti-αIIbβ3 agents were used alone. Thus, although αIIbβ3 is expressed in MK, it can mediate the binding of MK to FGN only if it is activated.
and it appears to be the primary integrin involved in the binding of MK to FGN.

In contrast, the emergence of an activated αIIbβ3 integrin influences the role of α5β1 in binding activated MK to FN. The inhibitory effects of the anti-α5 antibody, SAM-1, and the anti-αIIbβ3 agents, bitistatin and MK0852, are less than half that demonstrated against resting MK. However, combinations of SAM-1 with bitistatin or with MK0852 are additive. This indicates that both integrins must be blocked to inhibit the adhesion of activated MK to FN; therefore, both are involved in this activity.

Binding to FGN via αIIbβ3 is a criteria for activated platelets. By analogy, the absence of binding of freshly isolated guinea pig MK to FGN suggests that they are in a resting state. Although MK can be isolated from bone marrow and remain in a resting state, MK grown in culture in the presence of growth factors would be expected to bind to FGN, because TPO can activate αIIbβ3 in MK.24

The demonstration that α5β1 mediates MK binding to FN has several physiological implications in megakaryopoiesis and platelet production. Bone marrow matrix is rich in FN, and FN-α5β1 interactions have a major role in the growth and differentiation of erythrocytes9 and granulocytes.10 α5β1 regulates the adhesion and migration of their precursors and the release of reticulocytes into the circulation27 and, therefore, may also mediate MK maturation.

The binding of FN to α5β1 has also been shown to be an essential step in the formation of assembled FN by fibroblasts, whereas other integrins usually are not involved in this process.28 Assembled FN is fibrillar, is usually cross-linked, and is insoluble to deoxycholate. Assembled FN is considerably more active than unassembled FN in cellular processes such as adhesion or migration.26 The demonstration that FN is bound primarily to α5β1 on the MK surface indicates that MK may assemble a more biologically active form of FN and thereby regulate the interaction between MK and other bone marrow cells and matrix.

Thrombin-induced amplification of αIIbβ3 activity in MK most likely is due to inside-out signaling, a mechanism in which the interaction of agonists or growth factors with a nonintegrin receptor initiates intracellular signaling that results in the activation of an integrin on the cell surface. Inside-out signaling has been extensively studied in platelets in which αIIbβ3 is activated in response to agonists such as thrombin, most likely due to conformational changes.29

Inside-out signaling has been demonstrated in MK derived from bone marrow stem cells and in M07e cells, a megakaryocytic cell line that expresses c-MPL and MK markers and responds to TPO.30,31 The activity of α4β1 and α5β1 is low in M07e and CD34+ cells.32 However, interleukin-3 (IL-3), stem cell factor (SCF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) can activate α5β1 and α4β1 in M07e cells12,33 and TPO can activate α5β1.24,31 TPO also enhanced αIIbβ3-dependent adhesion of MK derived from CD34+ cells by inside-out signaling.24

The studies by Cui et al31 and Levesque et al32 have shown that growth factor-induced activation of integrins and the resultant adhesion of hematopoietic cells to ECM is transient. However, the brief attachment may be sufficient to modulate the response of MK to external stimuli.

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