Differential Regulation of Leukocyte Function-Associated Antigen-1/Intercellular Adhesion Molecules-1–Dependent Adhesion and Aggregation in HL-60 Cells

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Activation of integrin and organization of cytoskeletal proteins are highly regulated in cell adhesion and aggregation. The interaction of leukocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecules-1 (ICAM-1) mediates cell adhesion and aggregation, which facilitate leukocyte trafficking to inflamed tissues and augment effector functions. We investigated how LFA-1/ICAM-1–mediated adhesion and aggregation are regulated in HL-60 cells induced to differentiate into neutrophils by retinoic acid (RA). Uninduced HL-60 cells did not bind to ICAM-1 even with stimulation by 12-0-tetradecanoyl phorbol-13-acetate, although they express LFA-1 on the cell surface. When cultured with RA for 24 hours, HL-60 cells were able to adhere to ICAM-1 constitutively. The induction of adhesion did not accompany any change in surface density of LFA-1, indicating that the avidity of LFA-1 was in creased. The change in its avidity required de novo synthesis of proteins. Although ICAM-1 was intensely expressed on RA-induced HL-60 cells, these cells did not show any cellular aggregation. The HL-60 cells transfected with the active form of Ras (Val12) exhibited LFA-1/ICAM-1–dependent aggregation by RA stimulation without change in the avidity of LFA-1. In these Ras-transfectants, a cytoskeletal protein, paxillin, was tyrosine-phosphorylated, and the level of F-actin increased. Transforming growth factor (TGF)β, as well as cytochalasin D, prevented both the tyrosine phosphorylation of paxillin and the aggregation without any effects on the avidity of LFA-1. Thus, an increase in the avidity of LFA-1 was not sufficient for the induction of aggregation, which required activation of Ras and reorganization of cytoskeletal proteins. These results suggest that distinct regulatory mechanisms control LFA-1/ICAM-1–dependent adhesion and aggregation in HL-60 cells differentiating into neutrophils.

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a regulatory system for α1 integrin-mediated adhesion to fibronectin in PMNs.\(^{42,43}\)

We show here that RA induces LFA-1/ICAM-1-dependent adhesion in HL-60 cells, which requires de novo protein synthesis, but does not require an intact actin cytoskeleton. The high avidity state of LFA-1 is not sufficient for the induction of aggregation in RA-stimulated HL-60 cells. The activated Ras induces LFA-1/ICAM-1-dependent aggregation. The LFA-1/ICAM-1-dependent adhesion and aggregation are also differentiated in their sensitivity to the inhibitory effects of cytochalasin D and TGFβ.

**MATERIALS AND METHODS**

**Cells.** HL-60 cells were suspended with RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (FCS) (Sigma Chemical Co, St Louis, MO). For the adhesion experiments, growing cells were subcultured at a density of 2 \(\times 10^5\) cells/mL and treated with RA (1 \(\mu\)mol/L; Sigma) in the presence or absence of actinomycin D (0.1 \(\mu\)g/mL; Sigma), cycloheximide (2 \(\mu\)g/mL; Sigma), TGFβ (10 ng/mL; Genzyme, Cambridge, MA), cytochalasin D (1 \(\mu\)mol; Sigma), and wortmannin (10\(^{-7}\) mol/L; Wako Chemical Co, Osaka, Japan) for the indicated times.

**Purification and coating of ICAM-1.** ICAM-1 was immunofinity purified using anti-ICAM-1 antibody (RR1/1) Sepharose from mouse IgG-Sepharose–precleared cell lysates prepared from JY cells (\(1 \times 10^6\)) with lysis buffer (1% Triton X-100, 10 mmol/L Tris pH8.0, 150 mmol/L NaCl, 2 \(\mu\)g/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L iodoacetamide).\(^{12}\) The column was washed with 10 mmol/L Tris HCl, pH8.0 containing 150 mmol/L NaCl, and 0.1% Triton X-100, followed by 50 mmol/L triethylamine, pH10.0 containing 0.15 mol/L NaCl and 1% octyl glucopyranoside. ICAM-1 was eluted with 50 mmol/L triethylamine, pH12.5, 0.15 mol/L NaCl, 1% octyl glucopyranoside, neutralized immediately with 2 mol/L Tris HCl, pH6.0, and stored at -80°C. Purity was more than 95%, and the concentration of proteins was at 10 to 40 \(\mu\)g/mL.

Purified ICAM-1 was adsorbed to polystyrene microtitier plate (Limbro-Flow, McClean, VA) wells by addition of 2 \(\mu\)L of detergent solubilized protein to 100 \(\mu\)L of 20 mmol/L Tris HCl, pH 8.0, 0.15 mol/L NaCl. After a 90-minute incubation at room temperature, the plates were incubated for 30 minutes at room temperature in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and were then washed with assay media. The amount of ICAM-1 for coating was chosen to give a saturated cell binding. In this coating condition, site density was about 1,200 sites/\(\mu\)m² quantified using \(^{125}\)I-labeled RR1/1 (2 \(\mu\)Ci/\(\mu\)g) at a final concentration of 20
μg/mL, the number of which was comparable with that of the previous report.12

Cell adhesion assays. Adhesion assays with coated ICAM-1 were performed as described.13 Cells were labeled with 2',7'-bis-(2-carboxyethyl)-5 (and -6) carboxyfluorescein (BCECF; Molecular Probes, Inc, Eugene, OR) and suspended with RPMI1640, 10 mmol/L Hepes, pH7.4, 5% FCS. For inhibition, coated wells or labeled cells were incubated with 20 μg/mL of the appropriate antibodies for 30 minutes at room temperature before assays. Cells were transferred into coated wells at 5 x 10⁵/well, and then incubated at 37°C for 30 minutes. Nonadherent cells were removed with four 21-gauge needle aspirations. Bound cells were quantitated in the 96-well plate using a fluorescence concentration analyzer (IDEXX Corp. Westbrook, ME). Coating and adhesion assays with fibronectin were performed exactly as described.13

Antibodies. The following antibodies were used for immunoprecipitation and immunoblotting:13 ¹²⁵I-labeled monoclonal antiphosphotyrosine PY-20 (ICN Radiochemicals, Irvine, CA), monoclonal anti-Ras antibody (NC-RAS004), monoclonal antialtaic antibody (Chemicon Int Inc, Temecula, CA), monoclonal antialtaic antibody (Chemicon), monoclonal antialtaic antibody (BIOHIT, Helsinki, Finland), monoclonal antivinculin (BIOHIT), monoclonal antipaxilin antibody (Affiniti, Nottingham, UK), polyclonal anti-Fgr antibody (Upstate Biotechnology, Inc, Lake Placid, NY), and peroxidase-linked antimouse IgG (F(ab)2 fragments (Amersham Co, Arlington Heights, IL).

Anti-integrin-blocking monoclonal antibodies directed to α2, αL, αM were purchased from the Becton Dickinson (San Jose, CA). Monoclonal anti–ICAM-1 antibody (RR1/1),14 anti–LFA-1 antibody (TS1/22),15 and anti–VLA-4 (SG7.3)16 was used as blocking antibodies.

Flow cytometric analysis. Fluorescein isothiocyanate (FITC)-conjugated goat antimouse Ig F(ab) fragments (Cappel, Durham, NC) were used as a second antibody. Hanks’ balanced salt solution (HBSS) containing 3% fetal bovine serum (FBS), 0.1% sodium azide, and 10 mmol/L HEPES was used as a staining medium. HL-60 cells were stained and analyzed as described.15

Transfection of HL-60 cells with an activated Val-12 Ha-Ras expression plasmid. The Ha-ras Val-12 gene is expressed from a metallothionein promoter. HL-60 cells (4 x 10⁵) were transfected with 10 μg of plasmid DNAs by electroporation.16 The transfected cells were diluted and were plated 2 x 10⁶ per well onto 96-well
microplates. Two days after transfection, G418 (GIBCO) was added at 1 mg/mL of the final concentration, and subclones were isolated after 4 to 6 weeks of G418 treatment and were propagated in the presence of G418.

Determination of the cellular content of F-actin. The cellular content of F-actin was analyzed by staining with tetramethylrhodamine isothiocyanate (TRITC) phalloidin, as described. The HL-60 cells or Ras-transfectants were fixed with 9% or 12% polyacrylamide/sodium dodecyl sulfate gel. The transfer of the proteins to a polyvinylidene difluoride (PVDF) membrane and blotting with monoclonal antibodies plus peroxidase-linked antimouse IgG F(ab)2 fragments and an enhanced chemiluminescence (ECL) system (Amersham) were also used for immunoblotting analysis.

Mitogen-activated protein kinase (MAP kinase) activities. Parental and Ras-transfected HL-60 cells were lyzed and the lysates were immunoprecipitated with the affinity-purified rabbit polyclonal anti-ERK 1 (extracellular signal-regulated kinases) or anti-ERK 2 antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). MAP kinases were determined in immunoprecipitates in a reaction buffer containing 1 μCi(y-32P)ATP, 20 μmol ATP, and 1.5 mg/mL myelin basic protein (MBP; Sigma) for 20 minutes at 30°C, as described previously.

RESULTS

Differential regulation of avidity of LFA-1 and VLA-4 in HL-60 cells. As shown in Fig 1A, HL-60 cells express LFA-1 and VLA-4, but they did not express other β2 (αM, αX) and β1 (α1, α2, α3, α5, α6) integrins (data not shown). TPA is a potent activator for adhesion through VLA-4 and LFA-1. When stimulated with TPA for 30 minutes HL-60 cells adhered to fibronectin (FN) in a VLA-4-dependent manner, but could not adhere to ICAM-1, which is a ligand for LFA-1 (Fig 1B and C), despite the fact that the expression levels of VLA-4 and LFA-1 were comparable. Thus, these results indicate that LFA-1 on HL-60 is in the low avidity state even with stimulation and suggest that an avidity of LFA-1 could be regulated differentially from that of VLA-4.

RA-induced LFA-1/ICAM-1 adhesion. When HL-60 cells were cultured in the presence of 1 μmol/L RA for 24 hours, they became adhered to ICAM-1 constitutively without TPA (Fig 2A). The adhesion was completely dependent on LFA-1/ICAM-1 in RA-treated HL-60 cells because antibodies to both LFA-1 and ICAM-1 blocked adhesion, whereas the antibody to CR3 did not (Fig 2B). These data showed that RA induced LFA-1/ICAM-1-dependent adhesion of HL-60 cells without any stimuli.

RA stimulation did not augment the expression of LFA-1 (Fig 2C). This result indicated that an increase in the avidity of LFA-1, rather than the expression on the cell surface, accounted for adhesion to ICAM-1.

The induction of adhesion by RA requires transcriptional and translational activities, but does not require actin-based cytoskeleton. Adhesion of HL-60 cells to ICAM-1 was examined following the incubation with RA in the presence of 0.1 μg/mL of actinomycin D or 2 μg/mL of cycloheximide for 24 hours. The adhesion was completely blocked by actinomycin D or cycloheximide (Fig 3). The concentration of these drugs used in our experiments did not exert toxic effects on cell adhesion because the induction of VLA-4-mediated adhesion to FN by TPA was not inhibited in HL-60 cells pretreated with the same doses (data not shown). Thus, de novo synthesis of proteins was required for the induction of LFA-1/ICAM-1-dependent adhesion by RA.

HL-60 cells were also incubated with RA in the presence of 1 μmol/L of cytochalasin D or 10 ng/mL of TGFβ for 24 hours. These agents were maintained in the assay during the subsequent 30-minute cell adhesion assay. The induction of LFA-1/ICAM-1-dependent adhesion by RA was not inhibited by cytochalasin D or TGFβ (Fig 3).

Ras induces LFA-1/ICAM-1-dependent aggregation in RA-treated HL-60 cells. Activated Val-12 Ras (Ras with valine at residue 12) was ligated to the inducible metallothio-nein promoter and was introduced into HL-60 cells. We selected the clones (no. 20 and 28), the basal expressions of which were twofold to threefold more than those in the parental HL-60 cells and increased fivefold more 24 hours after stimulation with Cd2+ (Fig 4A). We confirmed that the
activity of Map kinases increased in these Ras-transfectants (Fig 4B). The clone, which expressed only the neomycin-resistant gene and did not show the increased levels of Ras protein even if exposed to Cd^2+, was selected and used as a negative control (Fig 4A). Although parental HL-60 cells and the negative control clone did not aggregate due to RA stimulation, Ras-transfected clones (no. 28 and 20) aggregated drastically 48 hours after RA stimulation (Fig 4C). Cellular aggregation of Ras-transfectants by RA was inhibited by the anti-LFA-1 or anti-ICAM-1 antibody (Fig 4C). RA induced the expression of ICAM-1 in the parental and Ras-transfected HL-60 cells to a similar degree (Fig 5A). The expression level of LFA-1 did not change on RA stimulation and was similar in both cells (Fig 5A). Furthermore, the LFA-1/ICAM-1-dependent adhesion of Ras-transfectants was not augmented compared with that of parental HL-60 cells (Figs 1B and 5B). These data showed that activated Ras induced LFA-1/ICAM-1-dependent aggregation without any effect on the expression of LFA-1/ICAM-1 and the avidity of LFA-1.
Inhibition of aggregation of Ras-transfectants by cytochalasin D and TGFβ. Cytochalasin D and TGFβ, which had no effects on LFA-1/ICAM-1-dependent adhesion, prevented the formation of cellular aggregation (Fig 6). Cytochalasin D and TGFβ did not influence the expression of ICAM-1 (data not shown). Thus, in contrast to the adhesion, the aggregation was sensitive to treatment with cytochalasin D and TGFβ. As expected, actinomycin D or cycloheximide, the agents that inhibited the activation of LFA-1 as shown in Fig 3, prevented LFA-1/ICAM-1-dependent aggregation of Ras-transfectants by RA (Fig 6). Wortmannin did not affect the aggregation (Fig 6).

The effects of Ras on polymerization of actin and the expressions of contractile proteins. Filamentous actin (F-actin) levels have been measured by phalloidin staining. The F-actin content was increased in Ras-transfectants in a Cd²⁺-dependent manner (Fig 7A). RA stimulation had little effect on the F-actin content in parental and Ras-transfected HL-60 cells (data not shown). Paxillin was not expressed in unstimulated cells and was induced equally in parental and Ras-transfected HL-60 cells after RA stimulation (Fig 7B). The representative cytoskeletal proteins, actin, actinin, talin, and vinculin, which were expressed constitutively at similar levels, were unchanged after RA stimulation in parental cells and Ras-transfectants.

β2 and cytoskeleton-dependent tyrosine phosphorylation of paxillin in Ras-transfectants by RA stimulation. To understand the molecular mechanisms of cellular aggregation induced in Ras-transfectants, we investigated the tyrosine phosphorylation of paxillin after RA stimulation because its phosphorylation causes β2 integrin-dependent cytoskeletal reorganization.²⁵,²⁶ The lysates from nontreated or RA-treated parental or Ras-transfected HL-60 cells were immunoprecipitated with the monoclonal antipaxillin antibody, and the precipitates were analyzed by immunoblotting using the ¹²⁵I-labeled antiphosphotyrosine antibody (PY-20). Paxillin was tyrosine-phosphorylated in Ras-transfectants, but not in parental cells, by RA stimulation (Fig 8A). On the other hand, Fgr, the tyrosine phosphorylation of which was induced on RA stimulation (unpublished data), was tyrosine-phosphorylated to a similar extent in both parental and Ras-transfected HL-60 cells by RA stimulation (Fig 8A).

The tyrosine phosphorylation of paxillin in Ras-transfectants was not induced when the cells were incubated with RA in the presence of the anti-β2 antibody, cytochalasin D, or TGFβ (Fig 8B), all of which prevented the aggregation (Fig 6). The anti-β2 antibody is a blocking antibody and inhibited a ligation of β2 to its ligands, which led to preven-

Fig 5. (A) Expressions of ICAM-1 (a) or LFA-1 (b) in the parental or Ras-transfected HL-60 cells after RA stimulation. Parental HL-60 cells and Ras-transfectants (no. 28) were cultured with 1 µmol/L of RA for 48 hours. These cells were stained with anti-ICAM-1 (a) or anti-LFA-1 (b) followed by FITC-labeled antimeg IgG F(ab')₂ fragments (parent: ---) (Ras: ---). They were also stained with FITC-labeled antimeg IgG F(ab')₂ fragments alone (parent: ---) (Ras, ---) as a negative control. (B) Adhesion to ICAM-1 of Ras-transfectants cultured with RA. Ras-transfectants (no. 28) were cultured with 1 µmol/L of RA for 0, 24 and 48 hours. After washing, the binding to ICAM-1 or BSA was measured as in Fig 1.
tion of the tyrosine phosphorylation of paxillin. But anti-LFA-1, anti-ICAM-1 or anti-CR3 alone could not inhibit completely the tyrosine phosphorylation of paxillin. The use of a mixture of these antibodies inhibited it as well as the anti-β2 antibody. These data demonstrated that the tyrosine phosphorylation of paxillin was observed in LFA-I/ICAM-1-mediated aggregation and CR3-mediated spreading on plastic plates. Thus, activated Ras induced the β2 integrin-dependent tyrosine phosphorylation of paxillin.

**DISCUSSION**

In this study, we demonstrated that LFA-1/ICAM-1-dependent adhesion and aggregation of HL-60 cells induced to differentiate into neutrophils required distinct regulatory processes, an increase in the avidity of LFA-1, and Ras-dependent cytoskeletal reorganization, respectively. RA induced the high avidity state of LFA-1. The fact that modulation of the avidity required de novo protein synthesis suggests that the existence of protein components keeps
LFA-1 in the high avidity state. It has been suggested that TPA-induced LFA-1-mediated adhesion is dependent on the actin cytoskeleton. TPA was presumed to increase the avidity of LFA-1 for its ligand by promoting the interaction of LFA-1 with cytoskeletal proteins. However, no direct evidence has shown that the association of LFA-1 with cytoskeletal proteins caused the high avidity state of LFA-1. In our system, RA-induced LFA-1/ICAM-1-dependent adhesion of HL-60 cells was resistant to the broad-range concentration (0.1 to 2 μmol/L) of cytochalasin D and C3 toxin, a specific inhibitor of Rho. This indicates that the integrity of the actin cytoskeleton is not essential for the activation of LFA-1 in RA-treated HL-60 cells. Therefore, the RA-induced molecule(s) that controls the high avidity state of LFA-1 might not be directly associated with the actin cytoskeleton. Interestingly, this molecule(s) seems to be different from the one necessary for the activation of β1 integrin because β1 integrin-dependent adhesion to fibronectin was induced by TPA in HL-60 cells without accompanying β2 integrin (LFA-1)-dependent adhesion. This result
suggests that molecular mechanisms that control β1- and β2-dependent adhesion could be different.

The activation of LFA-1 was not sufficient for the induction of aggregation in RA-treated HL-60 cells. We showed that the activated Ras induced LFA-1/ICAM-1-dependent aggregation of HL-60 cells. Ras was found to be involved in integrin-mediated signal transduction, suggesting its roles in post-cell-adhesion events.30 Our previous report described that Ras was not activated in RA-treated HL-60 cells.51 It seems that Ras activation is an important process for induction of aggregation. Recent studies showed that LFA-1/ICAM-1-mediated aggregation was dependent on the actin cytoskeleton and tyrosine phosphorylation in cell-to-cell contact sites,50 and that the cytoskeletal protein, paxillin, was tyrosine-phosphorylated on stimulation through β2 integrin and present in adhesion sites in neutrophils.52,56 Consistent with these studies, on the induction of aggregation, paxillin was tyrosine-phosphorylated, and the F-actin content increased in Ras-transfected HL-60 cells. Ras was demonstrated to sequentially activate the assembly of specific cytoskeletal proteins at the plasma membrane to trigger the aggregation process.

Our results demonstrated that TGFB selectively inhibited LFA-1/ICAM-1-dependent aggregation of RA-treated HL-60 cells without affecting the expression and the avidity of LFA-1. Recently, it was reported that TGFB caused a downregulation of some of the Src family kinases as the mechanism for its action on the suppression of cell growth, suggesting that tyrosine kinases are target molecules of TGFB for its suppressive effects.53 TGFB prevented β2-dependent tyrosine phosphorylation of paxillin. However, TGFB did not suppress the activities of Fgr and Lyn, which were predominantly expressed by RA-treated HL-60 cells (data not shown). Thus, tyrosine kinases responsible for tyrosine phosphorylation of paxillin remains to be elucidated. It is well known that when PMN aggregates, a LFA-1-mediated signal can trigger generation of toxic products such as hydrogen peroxide.53,54 Therefore, the function of TGFB observed here may be implicated in the physiological protection system against tissue injury in the inflammatory site. Identification of the tyrosine kinases involved in paxillin phosphorylation and clarification of the inhibitory mechanism by TGFB are important to understand the regulation of aggregation and immune responses.

In conclusion, our experimental system provides a new opportunity to dissect molecular mechanisms of cell adhesion and aggregation, as well as development of a new strategy to regulate immune responses.

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