**Activation of β<sub>1</sub> Integrin Fibronectin Receptors on HL60 Cells After Granulocytic Differentiation**

By John F. Bohnsack and JuKay Chang

Phorbol esters upregulate the functional affinity of β<sub>1</sub> integrin receptors for fibronectin on human neutrophils and other leukocytes. We investigated the ability of phorbol myristate acetate (PMA) to stimulate the human promyelocytic cell line HL-60 to adhere to fibronectin, either in its undifferentiated state (HL60) or after dimethylsulfoxide-induced differentiation along the granulocytic pathway (dHL60). PMA stimulated little adherence of undifferentiated HL60 to fibronectin or to the 120-kD chymotryptic cell-binding domain (CBD) of fibronectin. In contrast, PMA stimulated dHL60 cells to rapidly adhere to both fibronectin- and to CBD-coated plastic. PMA-stimulated dHL60 adherence to fibronectin was largely mediated by both α<sub>5</sub>β<sub>1</sub> and α<sub>6</sub>β<sub>1</sub>, whereas PMA-stimulated dHL60 adherence to CBD was largely mediated by α<sub>5</sub>β<sub>4</sub>. There was little contribution from β<sub>2</sub> integrins to PMA-stimulated dHL60 adherence to fibronectin or CBD. The inability of undifferentiated HL60 to adhere to fibronectin and CBD did not result from lack of expression of α<sub>5</sub>β<sub>1</sub> or α<sub>6</sub>β<sub>1</sub>, because HL60 and dHL60 express similar amounts of both α<sub>5</sub>β<sub>1</sub> and α<sub>6</sub>β<sub>1</sub> on their surface. In addition, 1 mmol/L Mn<sup>2+</sup> induced similar amounts of α<sub>5</sub>β<sub>1</sub>-dependent adherence of both HL60 and dHL60, showing that α<sub>5</sub>β<sub>1</sub> on undifferentiated HL60 is capable of binding to its ligand. These data suggest that activation of protein kinase C cannot functionally upregulate these β<sub>1</sub> integrins on undifferentiated HL60 cells. The development of PMA-stimulated β<sub>1</sub>-dependent adherence after granulocytic differentiation of HL60 cells suggests that the differentiated HL60 cell is a useful model for investigating functional coupling of protein kinase C to β<sub>1</sub> integrin in myeloid cells.

© 1994 by The American Society of Hematology.

---

**Materials and Methods**

**Reagents.** Fibronectin was purified from human plasma by gelatin-Sepharose chromatography as previously described. Gelatin (type II from swine skin) was purchased from Fisher Scientific (Pittsburgh, PA). The 120-kD chymotrypsin-derived cell-binding domain (CBD) of fibronectin was purchased from Telios (La Jolla, CA). PMA, trans-retinoic acid, di-octanoylglycerol (di-C<sub>8</sub>), and dimethylsulfoxide (DMSO) were purchased from Sigma (St Louis, MO).

---

LEUKOCYTES COME into contact with a variety of extracellular matrix macromolecules, including fibronectin, during extravasation into sites of inflammation. Fibronectin is a widely distributed connective tissue glycoprotein that contributes to the structure of connective tissue through its ability to bind to other extracellular matrix macromolecules and to act as a substrate to which connective tissue cells adhere through specific cell surface receptors called integrins. Integrins are a family of heterodimers consisting of an α chain paired with a β chain that mediate cell-cell and cell-protein adhesion. Multiple α chains associate with a common β chain, and integrins are classified according to their common β subunit.

We recently showed that human polymorphonuclear leukocytes (PMNs) express a β<sub>1</sub> integrin receptor for fibronectin, the α<sub>5</sub>β<sub>1</sub>. Although numerous reports had shown that monocytes and lymphocytes also express several β<sub>1</sub> integrin receptors for fibronectin, including α<sub>5</sub>β<sub>1</sub>, α<sub>4</sub>β<sub>1</sub>, and α<sub>6</sub>β<sub>1</sub>, the presence of the α<sub>5</sub>β<sub>1</sub> on PMNs was not previously appreciated because the receptor is expressed at low density on the PMN surface. It is hypothesized that lymphocyte, monocyte, and PMN α<sub>5</sub>β<sub>1</sub> mediate adherence-dependent functions, such as migration, in the extracellular space, but the actual in vivo function of this receptor on leukocytes has not been elucidated.

Regulated leukocyte adherence to fibronectin through modulation of α<sub>5</sub>β<sub>1</sub> affinity may be an important component of leukocyte migration through connective tissue. Unactivated PMNs do not adhere strongly to fibronectin in vitro, but phorbol myristate acetate (PMA) and the chemoattractant C5a stimulate rapid PMN adhesion to fibronectin, and α<sub>5</sub>β<sub>1</sub> contributes to the forces mediating activated PMN adherence to fibronectin. Phorbol esters and other ligands have also been shown to stimulate lymphocytes to increase the functional affinity of their cell-surface α<sub>5</sub>β<sub>1</sub> for fibronectin.6,7 The mechanism by which the affinity of α<sub>5</sub>β<sub>1</sub> for fibronectin is altered is not clear, but the effect of varying concentrations of divalent cations, such as Mn<sup>2+</sup>, on the affinity of purified α<sub>5</sub>β<sub>1</sub> for its ligand<sup>2</sup> suggests that alterations in the structure of the receptor may account for changes in the functional affinity of the receptor. The ability of phorbol esters to increase α<sub>5</sub>β<sub>1</sub> functional affinity also implicates a role for protein kinase C (PKC) in its regulation.

The human promyelocytic cell line HL-60 has been used in a number of studies to characterize the regulation of expression of different antigens and functions of myeloid cells during differentiation. In their undifferentiated state, HL-60 cells morphologically resemble promyelocytes, but can be differentiated in vitro to resemble mature PMNs. HL60 cells have been described to acquire receptors for fibronectin during their differentiation along the granulocytic pathway. Rapid cellular responses to PMA stimulation, such as superoxide generation and actin polymerization, have also been reported to develop in HL60 cells after differentiation towards a PMN-like cell. The acquisition of fibronectin receptors and PMA-responsiveness after granulocytic differentiation suggested that the HL60 cell could serve as a useful model for studying mechanisms regulating the functional affinity of α<sub>5</sub>β<sub>1</sub> in myeloid cells.

---

From the Departments of Pediatrics and Pathology, University of Utah School of Medicine, Salt Lake City.

Submitted May 17, 1993; accepted September 22, 1993.

Supported by Public Health Service Grant No. AI26733 and The Thrasher Research Fund.

Address reprint requests to John F. Bohnsack, MD, Department of Pediatrics, Room 2A152 MC, University of Utah School of Medicine, 50 N Medical Dr, Salt Lake City, UT 84132.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0006-4971/94/8302-0020$3.00/0


543
HL60 and dHL60 were suspended at 5.5 × 10⁶ cells/mL in 135 mM NaCl, 0.5% HSA. In experiments with Mn²⁺, the cells were washed once at 37°C, washed twice, and resuspended at
106/mL in HBSS++/0.5% HSA (Cutter Biologicals, Berkeley, CA). Cells were counted in a gamma counter. Adherent cells were lysed with 1% Triton X-100, scraped off the plate with a cotton tip swab, and counted. MoAbs were incubated with the cells for 10 minutes at 37°C before and during the adherence assay. Staurosporine was prepared as a 2 mmol/L stock solution in DMSO and was present during the entire adhesion assay.

Staurosporine was purchased from Boehringer Mannheim (Indianapolis, IN).

Monoclonal antibodies (MoAbs) Rat anti-α, MoAb 16 and rat anti-β, MoAb 13 were gifts from Dr Steve Akiyama (National Institutes of Health, Bethesda, MD). Mouse anti-α, MoAb HP2/1 was purchased from AMAC (Westbrook, ME). Mouse anti-β, MoAb IB4 was a gift from Dr Samuel Wright (Rockefeller University, New York, NY). Mouse MoAb 60.1 directed against the α subunit (CD11b) of CD11b/CD18 (αβ2) was a gift from Dr Patrick Beatty (University of Utah, Salt Lake City, UT). W6/32 is a mouse MoAb directed against a common determinant of class I HLA. All MoAbs were purified as previously described. Purified rat IgG was purchased from Calbiochem (La Jolla, CA). Mouse antihuman transferrin receptor MoAb was purchased from Boehringer Mannheim (Indianapolis, IN).

Cell culture. The HL60 cell line was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in RPMI containing 10 mM/L HEPES and 10% fetal calf serum. Cells were routinely split 1:3 at a density between 1 and 2 × 10⁶/mL. Cells were induced to differentiate by addition of 1.25% DMSO or 1 μmol/L retinoic acid to freshly split cells. Both DMSO-differentiated HL60 (dHL60) and retinoic acid-differentiated HL60 (rHL60) cells became smaller and developed band and segmented nuclei as expected. In addition, both agents induced HL60 cells to produce superoxide in response to stimulation with phorbol myristate acetate and to express cell surface CD11b (αM) (see Results), both markers of granulocytic differentiation.

Adhesion assay. Adherence of 111In-labeled HL60 or differentiated HL60 cells to protein-coated tissue culture wells was performed as previously described for human PMNs. Cells were removed from culture, washed twice in Hanks' Buffered Salt Solution (HBSS), and suspended at 2 to 4 × 10⁶ cells/mL in HBSS containing 0.5% human serum albumin (HSA) for injection (HBSS/0.5% HSA; Cutter Biologicals, Berkeley, CA). Cells were then labeled with 111In-oxine (0.5 μCi/1 × 10⁶ cells) for 15 minutes at 37°C, washed twice, and resuspended at 5 × 10⁶/mL in HBSS/0.5% HSA. In experiments with Mn²⁺, the cells were washed once with 1 mmol/L EDTA in HBSS without Ca²⁺ or Mg²⁺ (HBSS⁻), and resuspended in HBSS⁻ containing 1 mmol/L Mn²⁺. Cells (225 μL) were incubated in protein-coated 16-mm plastic tissue culture wells (Nunc, Naperville, IN) in the presence or absence of agonists for varying lengths of time at 37°C. PMA and di-C8 were stored as stock solutions of 100 μmol/mL and 300 mmol/L, respectively, in ethanol, and diluted in buffer just before addition to the adhesion assay. The final concentration of ethanol never exceeded 0.1%, a concentration that was shown not to affect adhesion. Nonadherent cells were removed by aspiration and a single wash with HBSS and counted in a gamma counter. Adherent cells were lysed with 1 mol/L NH₄OH, scraped off the plate with a cotton tip swab, and counted. MoAbs were incubated with the cells for 10 minutes at room temperature before and during the adherence assay. Staurosporine was prepared as a 2 mmol/L stock solution in DMSO and diluted in DMSO just before addition to the cells. The final concentration of DMSO was 0.25%, a concentration of DMSO that alone did not affect unstimulated or stimulated adhesion. Staurosporine was incubated with the cells for 10 minutes at 37°C before the assay and was present during the entire adhesion assay.

Tissue culture plates were coated with 500 μL fibronectin, gelatin, or the 120-kD chymotrypsin-derived CBD of fibronectin at 50 μg/mL in HBSS overnight, aspirated, and washed just before the experiment. Preliminary experiments showed that HL60 cells exhibited a high basal background adhesion to the 120-kD fragment similar to that seen with uncoated tissue culture plastic. This was quenched by incubating the 120-kD-coated plates with gelatin at 50 μg/mL.

Flow cytometry. Washed HL60, dHL60, or rHL60 cells (5 × 10⁶ in HBSS¹⁺/0.5% HSA) were incubated with saturating concentrations of MoAb (or rat IgG as a negative control) for 30 minutes on ice, washed, incubated with fluoresceinated goat antimouse or goat antirat antibody for 30 minutes on ice, then washed again, and fixed with 1% paraformaldehyde in phosphate-buffered saline, pH 7.4. Labeled cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

Immunoprecipitation of phosphorylated transferrin receptor. HL60 and dHL60 were suspended at 5.5 × 10⁶ cells/mL in 135
A

![Graph]

B

![Graph]

Fig 2. Time course of acquisition of PMA-stimulated HL60 adherence to fibronectin and superoxide production in culture after the addition of DMSO. (A) PMA-stimulated adherence of HL60 cells developed by 24 hours in culture with DMSO, reaching its peak between 48 and 96 hours. (B) PMA-stimulated superoxide production was not detectable by 24 hours, reaching its peak between 72 and 96 hours after the addition of DMSO. Results represent the mean ± SEM from at least three experiments.

mmol/L NaCl, 4.5 mmol/L KCl, 1.3 mmol/L MgCl₂, 1.5 mmol/L CaCl₂, 10 mmol/L HEPES, pH 7.45, containing 1 mg/mL glucose and 1% HSA (HEPES buffer) and labeled for 2 hours at 37°C with 0.5 mCi of ³²P (orthophosphoric acid) per 10⁷ cells. The cells were then pelleted, resuspended at 5.5 × 10⁶/mL in the HEPES buffer, divided into equal aliquots, and stimulated with PMA at varying concentrations. After stimulation with PMA for 15 minutes at 37°C, the cells were pelleted rapidly (5 minutes at 1,000g) and the pellet was lysed for 30 minutes on ice with 1% NP40 in 10 mmol/L Tris, 145 mmol/L NaCl, 20 mmol/L NaF, 1 mmol/L Na₃VO₃, 2.5 mmol/L NaH₂PO₄, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L DFP, 2 mmol/L iodoacetamide, pH 7.45 (lysis buffer). The lysate was centrifuged at 12,000g for 20 minutes at 4°C, the pellet was discarded, and the supernatant was precleared by incubation with a mixture of protein G sepharose and Sepharose 4B overnight at 4°C. The transferrin receptor was immunoprecipitated from the precleared lysates by mixing 5 µg of antitransferrin MoAb and 20 µL of protein G sepharose for 2 hours at 4°C, after which the beads were washed exhaustively with lysis buffer. The beads were boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer under reducing conditions, subjected to electrophoresis on a 7% SDS gel, and analyzed by autoradiography, as previously described.¹⁵

RESULTS

We initially determined whether HL60 cells or HL60 cells that had been cultured in the presence of 1.25% DMSO for 3 to 4 days (dHL60) adhered in short-term assays to fibronectin- or gelatin-coated tissue culture dishes in the presence or absence of PMA at 10 ng/mL. Undifferentiated HL60 cells exhibited little adhesion to fibronectin, even after stimulation with PMA (Fig 1). Basal adherence of dHL60 cells to fibronectin was increased compared with the basal adherence of HL60 cells to fibronectin, but, in contrast to the lack of PMA-stimulated HL60 adherence to fibronectin, PMA stimulated a fourfold to fivefold increase in dHL60 adherence to fibronectin (Fig 1). Only very small percentages of undifferentiated HL-60 cells or DMSO-differentiated HL60 cells (dHL60) adhered to gelatin after 15 minutes, even when PMA was added. The lack of PMA-stimulated dHL60 adherence to gelatin-coated plastic contrasts with the rapid and marked PMA-stimulated adherence of normal human PMNs to gelatin.¹⁹ Thus, DMSO treatment of HL60 cells resulted in the ability of the dHL60 to rapidly adhere to fibronectin, but not gelatin, in response to stimulation with PMA.

The ability of PMA to maximally stimulate adherence of DMSO-treated HL60 cells to adhere to fibronectin required 3 to 4 days exposure to DMSO, although PMA-stimulated DMSO-treated HL60 cells showed increased adherence to fibronectin after as little as 24 hours of culture with DMSO (Fig 2A). PMA-stimulated superoxide production, an index of granulocytic differentiation, was undetectable 24 hours after the addition of DMSO to the culture but also peaked after 3 to 4 days of exposure to DMSO (Fig 2B). Subsequent studies were performed with HL60 cells that had been exposed to DMSO for 3 to 4 days.

The percentage of HL60 cells adherent to fibronectin was not markedly increased by incubation for up to 60 minutes with PMA at 10 ng/mL (Fig 3). Higher concentrations of PMA (up to 100 ng/mL) also failed to induce more pronounced adhesion of HL60 cells to fibronectin (not shown).

Thus, the undifferentiated HL60 cells were almost completely unable to increase their adhesion to fibronectin in response to PMA in short-term adherence assays. In contrast, PMA stimulated rapid dHL60 adherence to fibronectin, with a detectable increase in adherence occurring after 5 minutes and peak levels of adherence occurring between 15 and 30 minutes after the addition of PMA. No further increase or reversal of stimulated dHL60 adherence was seen at 60 minutes. Unstimulated dHL60 adherence to fibronectin gradually increased over the course of 60 minutes (Fig 3).

Two hypotheses could account for the inability of undifferentiated HL60 to adhere to fibronectin: either undifferentiated HL60 lack the appropriate adherence receptors or the receptors cannot be appropriately activated. To test these hypotheses, we first examined the integrin receptors responsible for mediating PMA-stimulated dHL60 adherence to fibronectin. The anti-β₃ MoAb 13 almost completely blocked PMA-stimulated dHL60 adherence to fibronectin (Fig 4A), indicating that a β₃ integrin or integrins, plays a major role in PMA-stimulated dHL60 adherence to fibronectin. In contrast, the anti-β₁ MoAb IB4 inhibited only a small percentage of PMA-stimulated dHL60 adherence to fibronectin (Fig 4A). The receptors mediating adhesion of dHL60 cells to fibronectin differ...
from those mediating normal human PMN adhesion to fibronectin because PMA-stimulated adhesion of normal human PMNs to fibronectin is largely $\beta_2$ dependent.\(^3\) PMA-stimulated PMN adhesion to gelatin is also largely $\beta_2$ dependent,\(^19\) but, as noted above, PMA does not stimulate dHL60 adherence to gelatin. These findings indicate that the $\beta_2$-dependent adherence of dHL60 cells is quite weak compared with the $\beta_2$-dependent adhesion of normal PMNs. As is also the case for PMNs,\(^3\) the combination of the two MoAbs directed against the $\beta_2$ and $\beta_2$ subunits completely blocked PMA-stimulated dHL60 adherence to fibronectin (Fig 4A). The $\beta_2$ integrin participating in stimulated dHL60 adhesion to fibronectin appeared to be largely CD11b/CD18 ($\alpha_{4}\beta_2$) because the combination of anti-$\alpha_{4}$ MoAb 60.1 with the anti-$\beta_2$ MoAb 13 also completely blocked PMA-stimulated dHL60 adherence to fibronectin (3% ± 0.5%, $n = 3$).

Experiments with MoAbs directed against $\alpha$ subunits of the $\beta_2$ integrins showed that stimulated $\beta_2$-dependent adherence to fibronectin is mediated partly by $\alpha_4\beta_1$, and partly by $\alpha_4\beta_1$ (Fig 4B). The combination of the two MoAbs directed against these two $\alpha$ subunits inhibited almost the same amount of adherence as the anti-$\beta_2$ MoAb, with the remaining adherence being blocked by anti-$\beta_2$ MoAb (Fig 4B). The combination of either anti-$\alpha$ MoAb with anti-$\beta_2$ MoAb did not completely inhibit adherence (Fig 4B), providing further evidence that $\alpha_4\beta_1$, $\alpha_4\beta_1$, and $\alpha_4\beta_1$ all contribute to stimulated dHL60 adherence to fibronectin.

To more selectively study the function of the $\alpha_4\beta_1$, we examined the ability of HL60 and dHL60 to adhere to the chymotryptic-derived 120-kD fragment of fibronectin that contains the RGD-binding site for $\alpha_4\beta_1$. As shown in Fig 5, HL60 cells did not adhere to the 120-kD fragment even after stimulation with PMA, whereas PMA stimulated a significant increase in dHL60 adhesion to the 120-kD fragment. As expected, PMA-stimulated dHL60 adherence to the 120-kD fibronectin fragment was almost completely inhibited by the anti-$\alpha_4$ MoAb 16 (Fig 5). The small amount of inhibition caused by the anti-$\beta_2$ MoAb also implied a minor role for a $\beta_2$ integrin or integrins (Fig 5).

These experiments showed that PMA-stimulated dHL60 adherence to fibronectin is mostly mediated by $\alpha_4\beta_1$ and $\alpha_4\beta_1$. We therefore examined the cell surface expression of these integrin subunits on HL60 and dHL60 cells by flow cytometry. We found that the amount of $\alpha_4$, $\alpha_4$, and $\beta_2$ is approximately the same on HL60 and dHL60; in fact, the fluorescence intensity of each of these integrin subunits is slightly less on the dHL60 than on the HL60 (Fig 6). These data indicate that the inability of the HL60 to adhere to fibronectin is not due to a lack of expression of $\alpha_4\beta_1$ or $\alpha_4\beta_1$, and that increased surface expression of $\alpha_4\beta_1$ or $\alpha_4\beta_1$ on dHL60 does not account for the increase in dHL60 adhesion to fibronectin after PMA stimulation. DMSO-induced differentiation also resulted in an increase in the amount of $\alpha_4$ (CD11b) on the surface of the dHL60 (Fig 6), a finding consistent with differentiation of the HL60 cell along the granulocytic pathway.\(^21\)

The relatively small contribution of $\beta_2$ integrins to stimulated dHL60 adherence suggested the possibility that the DMSO used to differentiate the dHL60 inhibited $\beta_2$ function. To address this possibility, we examined the ability of PMA to stimulate adherence to fibronectin in HL60 cells induced to differentiate along the granulocytic pathway by incubation with $10^{-4}$ mol/L retinoic acid for 4 days (rHL60), conditions previously described to cause granulocytic differentiation of HL60 cells.\(^22\) As shown in Fig 7A, PMA stimulated rapid adhesion of rHL60 cells to fibronectin, and this adhesion was almost entirely $\beta_2$ dependent because the adhesion was largely inhibited by MoAb 13. Flow cytometry showed similar amounts of $\alpha_4\beta_1$ and $\alpha_4\beta_1$ on the surface of dHL60 and rHL60 (data not shown), and surface expression of $\alpha_4\beta_1$ was also upregulated on rHL60 compared with HL60, with a mean 2.2-fold increase in $\alpha_4\beta_1$ relative to that on HL60 ($n = 3$ experiments), compared
ACTIVATION OF $\beta_1$ INTEGRINS ON HL60 CELLS

Figure 4. PMA-stimulated dHL60 adherence to fibronectin is largely $\beta_1$ dependent, and involves at least two $\beta_1$ integrins. (A) PMA-stimulated dHL60 adherence to fibronectin is partly blocked by anti-$\beta_2$ MoAb IB4, but is largely blocked by anti-$\beta_1$ MoAb 13. Rat IgG and control MoAb W6/32 did not affect PMA-stimulated dHL60 adherence to fibronectin. (B) PMA-stimulated dHL60 adherence to fibronectin is partly blocked by anti-$\alpha_5$ MoAb 16 and by anti-$\alpha_4$ MoAb HP2/1. The combination of MoAb 16 and HP2/1 almost completely blocks stimulated adherence, with the remainder being blocked by the addition of anti-$\beta_2$ MoAb IB4. Complete inhibition of PMA-stimulated adherence required the simultaneous presence of MoAbs directed against $\alpha_4$, $\alpha_5$, and $\beta_2$. Results represent the mean ± SD of the number of determinations shown in parentheses. *$P$ < .05, compared with PMA alone, by Student’s t-test.

with a mean 3.2-fold increase on dHL60 (n = 4 experiments). Differentiation of HL60 cells with DMSO for 6 days also did not change the relative contribution of $\beta_2$ integrins to stimulated adherence as the anti-$\beta_2$ MoAb 13 almost completely inhibited PMA-stimulated adherence of day-6 dHL60 to fibronectin (Fig 7B).

The presence of $\alpha_4\beta_1$ or $\alpha_5\beta_1$ on both HL60 and dHL60 suggested that the $\beta_1$ integrins on HL60 cells either lack the capacity to adhere to their ligands or that they cannot be induced to do so. Substitution of Mn$^{2+}$ for Ca$^{2+}$ and Mg$^{2+}$ has been shown to increase the affinity of the $\alpha_5\beta_1$ for fibronectin.\textsuperscript{8} We therefore determined the ability of HL60 $\alpha_5\beta_1$ to mediate cellular adhesion to the 120-kD CBD of fibronectin in the presence of 1 mmol/L Mn$^{2+}$. As shown in Fig 8, the presence of 1 mmol/L Mn$^{2+}$ caused a marked increase in both HL60 and dHL60 adherence to the 120-kD fragment. Also, the Mn$^{2+}$-induced adherence of the HL60 cells to the 120-kD fragment was approximately equal to the magnitude of Mn$^{2+}$-mediated dHL60 adherence. Adhesion of both HL60 and dHL60 was largely inhibited by anti-$\alpha_5$ MoAb, indicating that the adherence is mediated by $\alpha_5\beta_1$ (Fig 8). These data show that the $\alpha_5\beta_1$ on HL60 cells has the capacity to bind to the CBD of fibronectin and that the inability of undifferentiated HL60 to adhere to the 120-kD fragment results from an inability to upregulate $\alpha_5\beta_1$ in response to PMA stimulation.

The ability of PMA to stimulate dHL60 adherence to fibronectin suggested a role for PKC in the intracellular pathway leading to enhanced adherence because phorbol esters are known to directly activate PKC.\textsuperscript{23} To further test this hypothesis, we determined whether the addition of an endogenous PKC activator, di-C$_8$,\textsuperscript{24} would also stimulate dHL60 adherence to fibronectin. As shown in Fig 9, di-C$_8$ stimulated rapid adhesion of dHL60 cells to fibronectin in a concentration-dependent manner (Fig 9). Preincubation of dHL60 with staurosporine inhibited both PMA- and di-C$_8$-
stimulated adherence to fibronectin (Fig 10). The di-Ca++-stimulated dHL60 adherence to fibronectin was also almost completely inhibited by anti-β1 MoAb (Table 1). Thus, activation of PKC in dHL60 results in cellular adhesion to fibronectin that is largely β1 dependent.

The inability of PMA to stimulate HL60 adherence suggested that PMA does not activate PKC in HL60 cells. To test this hypothesis, we determined the ability of PMA to stimulate phosphorylation of the transferrin receptor in HL60 cells, as previously described by others.25 As shown in Fig 11, PMA stimulated phosphorylation of the transferrin receptor in both HL60 and dHL60 cells in a concentration-dependent manner, confirming that PMA activates PKC in HL60 cells.

DISCUSSION

The ability of phorbol esters to induce upregulation of β1 integrins in a variety of cell types, including PMNs,3,19,26 macrophages,11 lymphocytes,27 and fibroblasts,27 suggests that increased functional activity of cell surface β1 integrins is an inevitable consequence of PKC activation by phorbol ester. Thus, it was surprising to find that PMA did not upregulate the functional activity of αβ1 and αβ1 on HL60 to the same degree that it did on dHL60 cells, despite the fact that the two cells expressed similar amounts of the two integrins, and that HL60 cells possess a PKC activity that is increased after PMA stimulation,25 as we have confirmed here. The inability of the undifferentiated HL60 to adhere to fibronectin after PMA stimulation implies that HL60 cells have an interruption in the PKC-dependent pathway that regulates increased functional activity of the β1 integrins. Other PMA-stimulated cellular responses have been reported to be absent in undifferentiated HL60 cells and to develop during DMSO-induced differentiation, including actin polymerization,12,28 superoxide production,11 adherence to endothelial cells,21 and activation of the Na+/H+ antiporter.29 These data indicate that the functional coupling of PKC to certain cellular responses may be acquired during DMSO-induced HL60 differentiation and also suggest that this model of cellular differentiation will be useful in determining the intracellular pathways mediating PKC-activated β1-dependent adherence in human myeloid cells.

Changes in the functional activity of the αβ1 integrin after cellular differentiation have been previously reported by others. Symington et al.30 showed that long-term (>38 hours) treatment of the erythroleukemia cell line K562 with PMA increased the surface expression of αβ1, but resulted in decreased adhesion of the K562 cells to fibronectin. These investigators reported a change in glycosylation of the α and
ACTIVATION OF \( \beta_1 \) INTEGRINS ON HL60 CELLS

\( \beta \) subunits that was detectable as a shift in electrophoretic mobility of \( \alpha_\beta_1 \) on SDS-PAGE and proposed that this structural modification resulted in decreased functional activity of the receptor. We have not detected any size difference between the \( \alpha_3 \) and \( \beta_1 \) subunit immunoprecipitated from the undifferentiated and differentiated HL60 cells (unpublished observations), suggesting that this alteration does not account for the findings presented here. Keratinocytes undergoing differentiation in vitro have been described to lose the ability to adhere to fibronectin without loss of cell surface \( \alpha_\beta_1 \). Of interest, isolated \( \alpha_\beta_1 \) from differentiated keratinocytes showed a decreased ability to bind to fibronectin in the presence of \( 1 \) mmol/L Mn\(^{2+}\), suggesting an irreversible alteration in the structure of \( \alpha_\beta_1 \) on differentia-
ated keratinocytes. In contrast, our results show that the decreased functional activity of HL60 αβ₁ is not caused by an inability of HL60 αβ₁ to bind to fibronectin because 1 mmol/L Mn²⁺ induced equal αβ₁-mediated adherence of both HL60 and dHL60 to the 120-kD CBD of fibronectin.

It was recently reported that normal myeloid precursors isolated from human bone marrow express α₄β₁ and α₅β₁, but very little α₂β₁, α₅β₁, or α₆β₁, and that the surface expression of α₄β₁ and α₅β₁ decreases during normal granulocytic differentiation. The same report also indicated that human PMNs express a small amount of α₄β₁ on their surface. Thus, the presence of α₄β₁ on dHL60 cells is not inconsistent with the concept that these cells are differentiating along the granulocytic pathway. The pattern of decreased α₄β₁ and α₅β₁ integrin expression during normal maturation of granulocytes in the bone marrow suggests the possibility that α₄β₁ and α₅β₁ anchor myeloid precursors to bone marrow stromal cells and connective tissue. If the latter hypothesis is true, then the decreased functional activity of HL60 β₁ integrins may have contributed to the early release of this malignant cell into the circulation.

The function of PMN β₁ integrins in vivo has not been
delineated, but models of inflammation demonstrating $\beta_2$-independent accumulation of PMNs in the extravascular space suggests that there are roles for $\beta_2$-independent mechanisms of activated PMN adhesion in vivo, and illustrate the potential importance of understanding such mechanisms. The ability of PMA and CSAs to activate PMN $\alpha_\beta$ function suggests that PMN $\beta_1$ integrins are functionally coupled to signal transduction pathways important in PMN function. Furthermore, the presence of PMA-stimulated $\beta_1$-dependent adhesion to fibronectin in the PMN-like dHL60 cell is consistent with our hypothesis that agonist-induced $\beta_1$-dependent adhesion is a component of the adhesive repertoire of normal human PMNs. Whereas $\beta_1$ function appears to be important in stimulated PMN adhesion to extracellular matrix protein-coated surfaces in vitro, it is not clear that $\beta_2$ integrins are critical for the adhesive interactions required for movement through the three-dimensional matrix of the extravascular compartment. On the contrary, PMN migration in three-dimensional hydrated collagen gels has been reported to be $\beta_2$ independent. We hypothesize that the rather weak $\beta_1$-mediated interactions that result from the low density of $\beta_1$ receptors on the PMN surface are suited for the adhesive interactions necessary for PMN function in the extravascular compartment. The minimal contribution of the $\beta_2$ integrins to PMA-stimulated dHL60 adherence to fibronectin suggests that the DMSO-differentiated HL60 will be a useful experimental model in which to study the regulation of PMN $\alpha_\beta$ function.

### REFERENCES


### Table 1. di-Ca-Induced dHL60 Adherence is $\beta_1$ Dependent

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Agonist</th>
<th>Buffer</th>
<th>MoAb 13</th>
<th>Rat IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>11 ± 4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>di-Ca (100 μmol/L)</td>
<td>33 ± 8</td>
<td>10 ± 6*</td>
<td>32 ± 12</td>
<td></td>
</tr>
</tbody>
</table>

Values are the percentage of adherence ± SD to fibronectin measured after 15 minutes (n = 3 for all experiments). Abbreviation: ND, not determined.

* P < .01 compared with di-Ca alone by Student’s t-test.
32. Kerst JM, Sanders JB, Slaper-Cortenbach ICM, Doorakers MC, Hooibrink B, van Oers RHJ, von dem Borne AEGK, van der Schoot CE: $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are differentially expressed during myelopoiesis and mediate the adherence of human CD34+ cells to fibronectin in an activation dependent way. Blood 81:344, 1993
Activation of beta 1 integrin fibronectin receptors on HL60 cells after granulocytic differentiation

JF Bohnsack and J Chang