Phorbol Ester-Induced Promyelocytic Leukemia Cell Adhesion to Marrow Stromal Cells Involves Fibronectin Specific α5β1 Integrin Receptors

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The human promyelocytic cell line NB4 exhibited a weak adhesion capacity for bone marrow-derived stromal cells and their extracellular matrices (5-15% of adherent cells). Adhesion was enhanced by pulse-treatment of cells with phorbol-ester (PMA 10⁻⁷ M). Adhesion was induced within minutes, was fibronectin-specific, and affected up to 100% of the treated cells. This biological response to PMA resulted from the activation of protein kinase C (PKC), since PKC inhibitors (staurosporine, sphingosine, CGP 41251, and calphostin C) prevented the phenomenon. Phenotypical analysis of integrin receptor expression (particularly FN receptors VLA-4 and VLA-5) at the membrane of untreated or PMA-treated cells revealed that PMA induced no significant modification of the level of expression of these receptors. However, inhibition studies carried out with anti-VLA monoclonal antibodies demonstrated that the FN-specific adhesion triggered by PKC involved the α5β1 FN-specific receptors (VLA-5). We showed that the binding of NB4 cells to fibronectin was RGD-dependent. PMA-induced adhesion was not correlated to phosphorylation of the VLA-5 receptor. These findings may partially explain the malignant behaviour of these cells: The loss of their capacity to adhere to stromal cells may arrest differentiation and explain the large number of leukemic cells in the circulation.

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The adhesion assay.

washed three times in serum-free RPMI medium before
row cell culture (Lanotte et al., 1981). Stromal cells
adherent layer derived from normal human bone mar­

before analysis,

M) for 60 min at 37°C. Cells were then washed twice
incubated in culture medium (10
6
was 48 h. Cells were washed in RPMI medium and

Exponential cell growth was obtained by culturing cells
medium supplement was furnished by IBF (France).

2
France). AIIB

HY, in a humidified 5% C02/air incubator, at 37°C.

was grown in RPMI medium with
A 23187, PGE1, PGE2, PGF1,0, PGF2,0, PGA1, PGD2,
IBMX, isoproterenol, 8-CPT-cAMP, 8-CI-cAMP, W7,
RGDS, and RGES peptides were all purchased from Sigma.
Phorbols were prepared as 2 mM stock solutions
in dimethylsulfoxide (DMSO) and diluted in RPMI before
each experiment. Cholera toxin (CT) was furnished by the Institut Mérieux. (Lyon, France), CGP 41251
was given by Ciba-Geigy (Basel, Switzerland). Calphos­
tin C and microcystin were gifts from S.O. Doskeland (Bergen, Norway). Calphostin C was photoactivated by
fluorescent light as described by Bruns et al. (1991).
Monoclonal antibodies (MoAbs) against β1 (K20), α2
(G19), α4 (HP2/1), α5 (SAM1), and α6 (GoH3) chains of
integrins were provided by Immunotech (Luminy,
France). AIIB2 anti-β1 rat monoclonal antibody was
used for inhibition of adhesion studies. Ulitrosy HY
medium supplement was furnished by IFB (France)
and BCA reagents by Pierce (Netherlands).

MATERIALS AND METHODS

Chemicals and reagents

Fibronectin was purified from human plasma
(Sigma-Chimie or IMEDEX, Lyon, France). Collagens
(I, III, IV, and V), laminin, staurosporine, sphingosine,
PMA, PDBu, PMA-methyl-ether, phorbol, 4α-phorbol,
A 23187, PGE1, PGE2, PGF1,0, PGF2,0, PGA1, PGD2,
IBMX, isoproterenol, 8-CPT-cAMP, 8-CI-cAMP, W7,
RGDS, and RGES peptides were all purchased from Sigma. Phorbols were prepared as 2 mM stock solutions
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used for inhibition of adhesion studies. Ulitrosy HY
medium supplement was furnished by IFB (France)
and BCA reagents by Pierce (Netherlands).

Cell culture and cell treatment with PMA

The promyelocytic cell line NB4 (Lanotte et al., 1991)
grew in RPMI medium with 1% (v/v) Ulitrosy HY,
in a humidified 5% CO2/air incubator, at 37°C. Exponential cell growth was obtained by culturing cells
between 5.104 and 5.105 cells per ml. The doubling time
was 48 h. Cells were washed in RPMI medium and
incubated in culture medium (107/ml) with PMA (10−7
M) for 60 min at 37°C. Cells were then washed twice
before analysis.

Adhesion to human stromal cells and their
extracellular matrices

Stromal cells were obtained by trypsinization of the
adherent layer derived from normal human bone mar­
row cell culture (Lanotte et al., 1981). Stromal cells
were cultured in 24-well plates (3.104/well) for 7 days at
37°C, until they became confluent. Cultures were then
washed three times in serum-free RPMI medium before
the adhesion assay.

ECM were prepared from confluent stromal cell cul­
tures in 24-well plates. Cells were washed once in phos­
phate-buffered saline (PBS) then, 0.5 ml aliquots of
0.5% (w/v) Triton X100 in PBS were added to each well
as described by Vlodawsky et al. (1980). Plates were
incubated under slow agitation for 1 h at room temper­
ature. Wells were then washed five times with PBS
before use.

NB4 cells (2.106/ml) were incubated with 1 µCi/ml of
125I-5-Andodeoxyuridine (sp. act. 2,000 Ci/mmol; Amer­
sham-France) for 4 h at 37°C. They were then washed
three times in RPMI and resuspended at 1.6 x 106/ml
in RPMI supplemented with 1% Ultroser HY and 1%
BSA, with or without 10−7 M PMA. In each well, 300 µ
aliquots of radiolabeled-cell suspensions were added
containing either stromal cells or ECM and incubated
for 2 h at 37°C. Radioactivity in each well, evaluated
using a gamma counter (LKB), represented the total
cpm. Nonadherent cells were discarded and wells were
washed three times with RPMI medium. Adherent cells
were solubilized with a 0.5 M NaOH, 1% SDS solution
and counted.

Adhesion assay to coated surfaces

Culture plate wells (96 well plates) were coated for
3–4 h at 37°C with 50 µl of PBS containing 50 µg/ml of
either human fibronectin or other substrata (bovine
serum albumin, BSA; human laminin, human collagen).
Wells were then washed with PBS and 200 µl of a
3% (w/v) BSA solution was added to each well to satu­
rate nonspecific adsorption sites on the plastic surface.
Plates were incubated for 1 h at 37°C then washed three
times in PBS.

Exponentially growing NB4 cells were pelleted,
washed once in fresh medium, and resuspended at 107
cells/ml, in medium supplemented with Ulitrosy HY.
To each well were added 100 µl aliquots of cell sus­
ension; cells were allowed to adhere to the substrata for 2
h at 37°C. Unattached cells were subsequently removed
by washing three times with PBS. Adherence of cells
was quantified according to the method of Tuszinski
and Murphy (1990), based on the colorimetric evalua­
tion of cellular proteins in each well. Briefly, 200 µl of
bicinchoninic acid (BCA) reagent were added to each
well and the plates were incubated for 30 min at 60°C.
The optical density was then read at 570 nm.

To determine the inhibition of adhesion to fibro­
nectin, NB4 cells were preincubated in suspension either
with protein kinase C (PKC) inhibitors, with synthetic
peptides or with antibodies for 30–60 min at 37°C; cells
were then added to fibronectin-coated wells. When cells
were simultaneously treated with PMA and a PKC
inhibitor, the incubation with the PKC inhibitor pre­
ceeded by 30 min the PMA treatment and by 45 min the
addition of cells to FN-coated wells. For the experi­
ments with calphostin C, the incubation was carried
out for 2 h in an incubator with a fluorescent light
source to photoactivate calphostin C. Data in adhesion
assays represent triplicate measurements from at least
two separate experiments. Adherent cells are expressed
as the percent of the total cell number in the assay.
Inhibition of adhesion is expressed as a percentage of
the control (maximal adhesion).
Radiolabeling and integrin immunoprecipitation experiments

NB4 cells were grown in RPMI with 2% HY. For $^{125}$I labeling of membrane proteins, 30.10⁶ cells were incubated for 20 min at room temperature with 1 mCi Na $^{125}$I (Amersham) using the iodogen method. They were then washed three times and lysed. To measure phosphorylation, 30.10⁶ cells were washed three times in phosphate-free medium. Cells were resuspended at 2.10⁶/ml in the same medium with 2% HY and incubated with 1 mCi $^{32}$P-orthophosphate (Amersham) for 2 h at 37°C. DMSO (1/10 diluted) or PMA (2.10⁻⁷ M) were added for 20 min at the end of the incubation time. Thereafter cells were washed and lysed.

Labeled cells were resuspended in 500 μl of ice-cold lysis buffer containing 10 mM triethanolamine, 150 mM NaCl, 1% NP40, 5 mM Mg²⁺, 2 mM sodium metavanadate, 1 mM PMSF, 20 μg/ml leupeptin, 1 mM TLCK, 0.2 mM trifluoperazin, 1 μM microcystin at pH 7.8, for 45 min at 0–4°C. Cell lysates were spun down at 13,500 rpm for 15 min at 4°C and supernatants were precleared by incubating them with 100 μl of protein G-Sepharose precoated with normal mouse serum overnight at 4°C. The precleared lysates were then incubated with 100 μl of protein G-Sepharose coated with anti-α5 antibody for 2 h at 4°C. Beads were then pelleted and washed six times in lysis buffer. Immunoprecipitates were solubilized with 100 μl of SDS-sample buffer for 5 min at 100°C. Samples were electrophoresed on 7.5% SDS-PAGE according to Laemmli (1970).

Cell sorter analysis

NB4 cells were pretreated or not with 10⁻⁷ M PMA for 1 h at 37°C. Cells (2.10⁶/ml) were centrifuged and resuspended in PBS with 0.5% BSA, at 4°C. Subsequent steps were performed at this temperature; 50 μl aliquots of NB4 suspensions were added to 20 μl of MoAbs (previously mentioned) in Eppendorf tubes. After a 30-min incubation, cells were washed twice with a BSA-containing PBS solution, by centrifugation at 2,700 rpm for 5 min. Cell pellets were resuspended in 100 μl of a 1/30 dilution of a FITC-labeled goat antimouse antibody (Nordic Immunology) and incubated for 30 min. After two washings, cells were resuspended in 1 ml PBS and analyzed by flow cytometry on an Ortho 50 H flow cytometer.

RESULTS AND DISCUSSION

PMA modulates leukemic cell adhesion to marrow stromal cells and ECM

It is well established that hemopoietic progenitors, immature myeloblastic or promyelocytic cells are tightly associated with marrow stromal cells in the marrow as well as to in vitro cultured stromal cells (Dexter et al., 1977a; Coulombel et al., 1988, reviewed by Storok-Storb, 1988). By contrast, leukemic cells at the same stages show narrow egress that could be favoured by loose interactions with stromal cells. In this work, using NB4 leukemic cells in an in vitro assay, we found that only 10% and 7% of these cells adhered to stromal cells and to extracellular matrices, respectively (Table 1). We wondered whether these low scores reflected some constitutive or functional adhesion defects. PMA, a potent activator of the PKC intracellular messenger effector system, added to the NB4 cell culture rapidly raised the percent adherent cells to 33% on stromal cells and 38% on ECM (Table 1). The percentages of adherent cells remained relatively low (no more than 38%). This may be explained by the fact that cells adhere to a specific substrate, which is the limiting parameter in the assay. Indeed, we found that nonadherent cells from the first exposure attached to a similar stromal cell layer in a second assay, with the same proportions, thus demonstrating that all PMA-treated cells gained the capacity to adhere to microenvironmental cells (not shown). These data posed the question of the mechanism responsible for PMA-induced leukemic cell adhesion and the biochemical features of the receptor-ligand complex which is the ultimate target of the PMA signal.

PMA-induced adhesion of leukemic cells to ECM is fibronectin-specific and involves the activation of PKC

We tested the ability of nontreated or PMA-treated cells to adhere to different substrata that are known components of the ECM: laminin, fibronectin, collagens I, III, IV, and V. We observed that nontreated cells did not adhere to collagens IV and V and presented only a weak adhesion to laminin, collagen I, collagen III, and fibronectin (Fig. 1). Controls with noncoated or BSA-coated plastic surfaces were negative. When cells were incubated with PMA, adhesion to FN was enhanced by 5–8-fold when compared to control without PMA. This response was restricted to FN: adhesion to other substrata was not enhanced by PMA treatment. NB4 cell suspension cultures consisted of spherical, nonadherent cells; 2 h after PMA treatment about 50% of the adherent cell population showed cytoplasmic elongation (pseudopodia). Prolonged incubation in the presence of PMA (3 h or more) increased this fraction and resulted in the spreading of cells on the substrata.

We wondered if other substances acting on the different signal transduction pathways could restore a physiological state of adhesion to FN in the same manner as PMA. The cAMP elevating agents (CT, IBMX, isoproterenol, PGA1 and PGE2) and the cAMP analogs (8-CPT-cAMP and 8-Cl-cAMP) did not modify the adhesion of NB4 cells (Table 2). Other prostaglandins such as PGF1α, PGF2α, PGA1, and PGD2 were also ineffective in inducing adhesion. PMA (10⁻⁷ M), PDBu (10⁻⁶ M) (previously mentioned) in Eppendorf tubes. After a 30-min incubation, cells were washed twice with a BSA-containing PBS solution, by centrifugation at 2,700 rpm for 5 min. Cell pellets were resuspended in 100 μl of a 1/30 dilution of a FITC-labeled goat antimouse antibody (Nordic Immunology) and incubated for 30 min. After two washings, cells were resuspended in 1 ml PBS and analyzed by flow cytometry on an Ortho 50 H flow cytometer.

| TABLE 1. NB4 cell adhesion to human marrow stromal and their ECM¹ |
|-----------------|-----------------|-----------------|
|                 | NB4 adherent cells (%) of total cpm |
|                 | Stromal cells    | ECM             |
|                 | 10.0 ± 1.5       | 7.0 ± 1.5       |
| PMA             | 33.5 ± 2         | 38.0 ± 2        |

¹Radio-labeled NB4 cells treated or not with PMA (10⁻⁷M) were added to stromal cells or ECM preparations and allowed to adhere for 2 h at 37°C. Results are expressed as a percentage of total cpm in each well. Each value represents the mean of quadruplicate assays and standard deviations.
Fig. 1. Adhesion of NB4 cells to different substrata. Substrata were prepared by coating tissue culture plates for 4 h at 37°C with 2.5 μg/well of the indicated proteins and saturating nonspecific binding sites with BSA (see Materials and Methods). Adherent cells were then counted using the 5-(loxyl)2-tryizolr-2-oxazolyl-betanin (BTA) assay. A. Adhesion of NB4 cells to different substrata coated with fibronectin (50 μg/ml). B. Adhesion of NB4 cells to different substrata coated with the indicated proteins at 37°C. The number of adherent cells was determined by the BCA assay (the curve is calibrated using the linear correlation between the absorbance at 570 nm and the number of adherent cells observed in this assay, not shown). B. Plates were coated with fibronectin (50 μg/ml solution). NB4 cells were treated with PMA (10^{-7} M) and added to the wells. Plates were immediately centrifuged for 1 min at 1,200 rpm and incubated at 37°C for various periods of time (5, 15, 30, 60, 90, 120, 240, and 360 min of incubation). Reaction was stopped by removing nonattached cells as described in Materials and Methods. Adherent cells were measured using the BCA assay.

Fig. 2. Dose-response and kinetic of adhesion of PMA-treated cells to fibronectin-coated wells. A. NB4 cells (10^6/ml) treated with different concentrations of PMA (10^{-10} M-10^{-6} M) were allowed to attach to fibronectin-coated plates for 2 h at 37°C. The number of adherent cells was determined by the BCA assay (the curve is calibrated using the linear correlation between the absorbance at 570 nm and the number of attached cells observed in this assay, not shown). B. Plates were coated with fibronectin (50 μg/ml solution). NB4 cells were treated with PMA (10^{-7} M) and added to the wells. Plates were immediately centrifuged for 1 min at 1,200 rpm and incubated at 37°C for various periods of time (5, 15, 30, 60, 90, 120, 240, and 360 min of incubation). Reaction was stopped by removing nonattached cells as described in Materials and Methods. Adherent cells were measured using the BCA assay.

and this was not due to cell death. In addition, the induction of adhesion by PMA was blocked when plates were incubated at 4°C (not shown). These data suggested an energy-dependent process in adhesion.

As PMA is a potent activator of PKC, we wondered whether the induction of adhesion could be inhibited by specific PKC inhibitors. Cells were either preincubated for 30 min with staurosporine (10^{-8} M to 10^{-6} M) or sphingosine (10^{-7} M to 10^{-4} M) before addition of PMA (10^{-7} M). PMA-induced NB4 cell adhesion was completely inhibited when plates were incubated at 4°C (not shown). These data suggested an energy-dependent process in adhesion.

TABLE 2. Effects of various compounds triggering the different signal transduction pathways on the FN-adhesion of NB4 cells

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Molarity</th>
<th>Induction of cell adhesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP-elevating agents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CT</td>
<td>10^{-8}</td>
<td>-</td>
</tr>
<tr>
<td>IBMX</td>
<td>10^{-6}</td>
<td>-</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10^{-5}</td>
<td>-</td>
</tr>
<tr>
<td>PGE1</td>
<td>10^{-4}</td>
<td>-</td>
</tr>
<tr>
<td>PGE2</td>
<td>10^{-3}</td>
<td>-</td>
</tr>
<tr>
<td>cAMP analogs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-CPT-cAMP</td>
<td>10^{-4}</td>
<td>-</td>
</tr>
<tr>
<td>8-Cl-cAMP</td>
<td>10^{-4}</td>
<td>-</td>
</tr>
<tr>
<td>phorbols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>10^{-7}</td>
<td>+++</td>
</tr>
<tr>
<td>PDBu</td>
<td>10^{-6}</td>
<td>+++</td>
</tr>
<tr>
<td>PMA-Methyl-Ether</td>
<td>2.10^{-5}</td>
<td>+++</td>
</tr>
<tr>
<td>Phorbol</td>
<td>2.10^{-6}</td>
<td>-</td>
</tr>
<tr>
<td>4α-Phorbol</td>
<td>2.10^{-5}</td>
<td>-</td>
</tr>
</tbody>
</table>

1Highest concentration tested.
2Compared to control without reagent (i.e., 5-15% of adherent cells/(-): 0-15%; (+): 15-30%; (++): 30-70%; (+++): 70-100%).

M), and PMA-methyl-ether (2.10^{-6} M) induced an increase in the adhesion of NB4 cells to FN (from 5-15% to 70-100% adherent cells). However PMA was the most potent phorbol to increase adhesion. Nontumor promotor phorbols (phorbol and 4α-phorbol) were inefficient.

NB4 cells were incubated for 30 min with increasing doses of PMA (10^{-10} M to 10^{-6} M), at 37°C (Fig. 2A). Maximal adhesion was obtained with 10^{-7} M PMA (90-100% of adherent cells). PMA-induced NB4 cell adhesion to FN followed a rapid kinetic. A maximal adhesion was obtained 15 min after addition of PMA (Fig. 2B) and persisted for at least 360 min. Moreover, we found (data not shown) that a pulse of PMA, followed by washing off PMA, was enough to fully induce adhesion to FN. It suggests that adhesion of NB4 cells could account for a direct modification of the adhesion effector complex, rather than a transcriptional regulation. Adhesion to FN-coated surface was inhibited by blocking the membrane remodeling: adhesion decreased by 66% in the presence of 10 mM sodium azide and this was not due to cell death. In addition, the induction of adhesion by PMA was blocked when plates were incubated at 4°C (not shown). These data suggested an energy-dependent process in adhesion.

As PMA is a potent activator of PKC, we wondered whether the induction of adhesion could be inhibited by specific PKC inhibitors. Cells were either preincubated for 30 min with staurosporine (10^{-8} M to 10^{-6} M) or sphingosine (10^{-7} M to 10^{-4} M) before addition of PMA (10^{-7} M) or co-incubated with PMA and CGP41251 (10^{-10} M to 10^{-5} M) or calphostin C (5.10^{-9} M to 10^{-6} M). All these compounds inhibited adhesion in a dose-dependent manner (Table 3). CGP 41251 and calphostin C showed higher specificity for PKC compared to staurosporine or sphingosine (Meyer et al., 1989; Bruns et al., 1991). W7, a specific inhibitor of calmodulin-dependent protein kinase did not modulate the NB4 cell adhesion to FN. All these compounds had no effect on cell viability for the 2-h incubation time, even at the
TABLE 3. Effects of different protein kinase inhibitors on the PMA-induced adhesion of NB4 cells to FN

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Molarity (M)</th>
<th>% of inhibition of adhesion</th>
<th>IC50 (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stauroporine</td>
<td>10^-8</td>
<td>0</td>
<td>1.65.10^-7</td>
</tr>
<tr>
<td></td>
<td>10^-7</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^-6</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Sphingosine</td>
<td>10^-8</td>
<td>0</td>
<td>6.3.10^-8</td>
</tr>
<tr>
<td></td>
<td>10^-7</td>
<td>0</td>
<td>8.6.10^-8</td>
</tr>
<tr>
<td>CGP41251</td>
<td>10^-9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^-8</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Calphostin C</td>
<td>2.10^-8</td>
<td>8</td>
<td>3.6.10^-7</td>
</tr>
<tr>
<td></td>
<td>10^-7</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>W7</td>
<td>10^-7 to 5.10^-12</td>
<td>69</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: Cells were either preincubated with serial dilutions of an inhibitory compound (stauroporine or sphingosine) before the addition of PMA, or simultaneously incubated with an inhibitory compound (CGP 41251, Calphostin C or W7) and PMA. Adhesion was analyzed by flow cytometry.

TABLE 4. NB4 cell surface expression of VLA integrin subunits analyzed by flow cytometry

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Integrin subunit specificity</th>
<th>Positive cells (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 20</td>
<td>β1</td>
<td>85.5</td>
</tr>
<tr>
<td>GI 9</td>
<td>α2</td>
<td>2.5</td>
</tr>
<tr>
<td>HP2/1</td>
<td>α4</td>
<td>84.1</td>
</tr>
<tr>
<td>SAM 1</td>
<td>α5</td>
<td>85.0</td>
</tr>
<tr>
<td>GoH 3</td>
<td>α6</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Note: Untreated or PMA-treated cells were incubated with primary monoclonal antibodies directed against a specific integrin subunit, then with fluorescein isothiocyanate-labeled goat anti-mouse IgG. Results expressed as percentage of positive cells are representative values from two separate experiments.

PKC activation induces integrin-dependent leukemic cell adhesion

PMA-induced adhesion was also blocked when cells were incubated in medium with 1 mM EDTA (94% inhibition, not shown). This result supported the idea of a divergent cation-dependent mechanism resembling integrin-mediated adhesion. We examined the possibility of involvement of VLA-integrin receptors in PMA-induced adhesion. One hypothesis would be that PMA changes the expression pattern of receptors involved in recognition of ECM components and especially FN as it has been described for U937 cells (Ferreira et al., 1991). The expression of CD29, CDw49b, CDw49d, CW49c, and CDw49f molecules, which correspond to β1, α2, α4, α5, and α6 chains of integrins, was analyzed. CD29, CDw49d, and CDw49c (β1, α4, and α5) were found to be expressed on NB4 cells (Table 4); other antigens (α2 and α6) were not detected. The results showed that α4β1 (VLA-4) and α5β1 (VLA-5) receptors are constitutively expressed on NB4 cells and that PMA did not modulate either the percentage of positive cells or the level of membrane expression of these integrins (Fig. 3). The absence of expression of other integrins (α2β1, α5β1) persists after PMA treatment. This suggests that PMA-induced adhesion could be explained rather by a structural modification and/or activation of a FN-specific integrin receptor, than by an increase in its membrane expression.

We determined whether the expressed receptors (α4β1 and α5β1) were involved in PMA-induced adhesion. GI9 (anti-α2) and HP2/1 (anti-α4) MoAbs did not affect NB4 adhesion to FN (Fig. 4). A slight inhibitory effect (13% inhibition) was observed with GoH3 (anti-α6) MoAb. By contrast, AIIB2 (anti-β1) MoAb and SAM1 (anti-α5) MoAbs inhibited attachment of NB4 cells to FN (87% and 80% inhibition, respectively). This inhibitory effect was not significantly increased (90%) when the anti-β1 MoAb was simultaneously added to the anti-α5 MoAb. These results support the conclusion that adhesion of NB4 cells to FN observed after PMA treatment was mediated by the receptor α5β1 (VLA-5) and that the other FN-specific receptor VLA-4 was not involved in this interaction. The primary cell binding site of FN has been assigned to the sequence Arg-Gly-Asp (RGD) localized within the central binding domain of the molecule and is recognized with high affinity by the α5β1 integrin (Hynes, 1987; Ruoslahti, 1987). Competition experiments demonstrated that the synthetic RGD peptide inhibited NB4 cells adhesion to FN with an ID50 of ~ 5.8 mM (Fig. 5). The control peptide RGES did not prevent cell adhesion. It confirmed that PMA-induced adhesion to FN is mediated by the α5β1 integrin, a conclusion that is in agreement with previous work showing that VLA-5 is a specific FN-receptor involving the RGD site in a number of cell systems (Ruoslahti and Pierschbacher, 1987; Ruoslahti et al., 1988).

PKC-dependent activation of α5β1 receptor is not due to its phosphorylation

Considering the kinetic of response to PMA (Fig. 2B), it was unlikely that PMA acted at the gene transcription level to promote adhesion, as it has already been described in other cell systems (Goldstein et al., 1990). Cell sorter analyses proved that adhesion did not result from a modulation of the expression of integrin receptors at the membrane surface (de novo synthesis or redistribution of cryptic receptors). The activation of an integrin-receptor complex constitutively expressed at the cell surface, as already described in other hemopoietic cells (Symington et al., 1989; Ylanne et al., 1990; Van Kooyk et al., 1991), seems more likely. We questioned whether stimulation of PKC by PMA resulted in an altered pattern of phosphorylation of the α5β1 receptor. A modulation of phosphorylation of integrins (β integrins or α6β1) induced by PMA treatment has already been observed in mononuclear cells and in macrophages by Chatila et al. (1989) and Shaw et al. (1990). As seen in Figure 6, two bands were observed after immunoprecipitation of iodinated cells, corresponding to the α5 chain (160 kDa) and the β1 chain (135 kDa) of the receptor but none of them were constitutively phosphorylated in NB4 cells and PMA treatment did not induce their phosphorylation. To definitively exclude the possibility of phosphatase activity in lysates, the highly potent phosphatase inhibitor microcystin (1 μM) was added to the panel of phosphatase inhibitors classically used (see methods). In conclusion, VLA-5 seems to be a very poor substrate for PKC since no phosphoryla-
Fig. 3. Flow cytometry analysis of the expression of β1, α4, and α5 integrin subunits on NB4 cell surface treated or not with PMA. NB4 cells were incubated for 30 min at 4°C with a primary antibody (K20, HP2/1 or SAMI), washed twice, then labeled with a FITC-conjugated goat antimouse IgG for 30 min at 30°C.

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CONCLUSIONS

Numerous reports have suggested that integrins are involved in cell migration and metastatic tumor invasion (Liotta, 1986). Moreover, the increased or decreased expression of integrins was found associated to the enhanced capacity of cells to become tumorigenic. In the hematopoietic system, the adhesive capacity of
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Fig. 4. Inhibition of PMA-treated NB4 cells adhesion to fibronectin by anti-integrin MoAbs. PMA-treated cells were preincubated for 30 min at 37°C with the following MoAbs (1/100 dilution) before their addition to fibronectin-coated wells: AII B2 (anti-α1 chain), G19 (anti-α2 chain), HP2/1 (anti-α4 chain), SAM1 (anti-α5 chain), and GoH3 (anti-α6 chain). Results are expressed as a percentage of adherent cells in controls (wells without MoAbs).

progenitor cells regulates proliferation and differentiation (Torok-Storb, 1988) and influences the response to growth factors (Gordon, 1988). Abnormal response to growth factors and maturation defects are features of leukemia generally associated to undue egress of immature cells from the marrow. In this work, we show that PKC activation can restore a physiological state of adhesion of promyelocytic cells to stromal cells or their ECM through a specific RGD-dependent binding to FN. This interaction resulted from a PKC driven intracellular mechanism that operated on the VLA-5 specific FN receptor. The increase in adhesion does not result from a modulation of the expression of VLA-5 receptors at the membrane surface or from an activation of this receptor consequent to its phosphorylation. In this work, we have defined a promising model to study the synergistic effects of FN-dependent adhesion and growth factor or maturation inducer signals on leukemic cell differentiation.

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Fig. 5. Inhibition of PMA-treated NB4 cell adhesion to FN by RGDS peptide. Cells were incubated with RGDS peptide (●). RGDS peptide (○) was used as a negative control. Adhesion on FN coated wells (coating concentration of 25 μg/ml) was carried out for 2 h at 37°C. Each point is the mean of triplicate experiments.

Fig. 6. Immunoprecipitation of α5β1 integrin. Surface iodinated NB4 cells (lanes 1, 2) or 32P-labeled untreated (lane 3) or PMA-treated (lane 4) cells were lysed and immunoprecipitated. Lysates (lane 1) were incubated with normal mouse serum coupled to protein G-Sepharose beads overnight, then with anti-α5-protein G-Sepharose beads. Bound proteins were solubilized by SDS-containing sample buffer and resolved by 7.5% SDS-PAGE and visualized by autoradiography.


