β 1 Integrins mediate adherent phenotype of human erythroblastic cell lines after phorbol 12-myristate 13-acetate induction

Annie MOLLA,*† Rolande BERTHIER,* Agnes CHAPEL,* Annie SCHWEITZER* and Annie ANDRIEUX*†

*Institut National de la Sante et de la Recherche Medicale Unite 217, Laboratoire d'Hematologie, Departement de Biologie Moleculaire et Structurale, Centre d'Etudes Nucleaires, Grenoble, France and †Department of Stomatology, University of California, San Francisco, CA 94143-0512, U.S.A.

We investigated the effects of phorbol ester (phorbol 12-myristate 13-acetate; PMA) treatment on the adhesive behaviour of three erythroleukaemia cell lines: HEL, LAMA-84 and AP217. In the three cell lines PMA induced an increase in expression of a megakaryocytic marker: α IIB β 3 integrin, but did not promote activation of this receptor. Indeed, an antibody specific for the activated form of α IIB β 3 failed to react with the three cell lines. PMA induction led to different adhesive phenotypes depending on the cell line; in fact LAMA-84 and HEL cells became adherent while AP217 cells remained non-adherent. By studying cell surface receptors we found that the major difference between the adherent and the non-adherent cells was the expression of β 1

integrins. After PMA induction, β 1 integrin expression was totally abolished in AP217 cells and the amount of β 1 mRNA was reduced preventing new synthesis of the subunit. In HEL and LAMA-84 cells, PMA treatment did not alter the overall level of β 1 integrin but induced a new pattern of α -subunit expression: up-regulation of α 2 and α v subunits and down-regulation of α 4 and α 5 subunits. Function-perturbing antibodies against β 1, α 4, α 5, α v and α 2 reduced adhesion of HEL cells to fibronectin or collagen, whereas antibodies against β 3 or α v β 3 did not. Our results favour the involvement of β 1 integrins in PMA-induced adhesion of erythroleukaemia cells.

INTRODUCTION

In response to a variety of stimuli, the haematopoietic stem cells located in the bone marrow are able to proliferate and differentiate to produce all types of mature blood cells. During this process, changes occur in the adhesive properties of the cells [1]. In pathological situations such as leukaemia where the process of proliferation is abnormal, the adhesive properties of leukaemia cells may play a role in the progression and phenotypic properties of the disease [2,3].

Through second messenger activation, protein kinase C plays a key role in signal transduction and in the modulation of gene expression linked to proliferative and differentiation events [4,5]. Protein kinase C activators, such as phorbol esters, induce differentiation of immature haematopoietic cells [6]. Upon exposure to phorbol 12-myristate 13-acetate (PMA), T-cells are activated [7], whereas promyelocytic leukaemia cell lines lose their myeloblast marker genes and acquire a series of monocyte/macrophage properties [8,9]. On platelets, phorbol esters stimulate fibrinogen binding [10] and phosphorylation of the focal adhesion kinase via a protein kinase C-mediated pathway [11]. In erythroleukaemia cell lines, such as HEL, phorbol esters induce the surface expression of platelet glycoprotein α IIB β 3 and increase the ploidy of the cells [12,13]. HEL cells normally grow in suspension, but PMA induces the organization of the actomyosin system of these cells by promoting the formation of prominent actin stress fibres and focal adhesion sites, thus leading to an adherent phenotype [14].

Integrins are adhesive receptors that are concentrated in focal contacts and are likely to play a role in the attachment and

spreading of HEL cells. These heterodimeric glycoproteins, composed of non-covalently associated subunits, form a critical link between the extracellular matrix and the cytoskeleton. At present 16 α and eight β subunits have been described, but only 20 different combinations have been identified [15,16]. HEL cells express several integrin receptors, including the fibronectin receptor (α 5 β 1), the platelet glycoprotein α IIB β 3 and the α subunit of the vitronectin receptor (α v). By immunofluorescence detection on HEL cells, Ylänne et al. [17] showed that α IIB, β 3, α 5 and β 1 localize to focal contacts within 2 h of PMA treatment. After 24 h in culture, α IIB immunoreactivity disappears from focal adhesion sites and there are variations in other integrin subunits, depending on the matrix used. They also showed that HEL cells deposit thrombospondin on to the culture substratum and express the integrin subunits α v and β 3, which might bind thrombospondin. On the basis of these observations, Ylänne et al. have postulated that HEL adherence under PMA treatment is mediated first by the activation of the α IIB β 3 integrin complex and later by the α v β 3 complex.

To assess directly the involvement of specific integrin receptors in PMA-induced adhesion of erythroleukaemia cell lines, we have made use of two unique reagents developed in this laboratory: a monoclonal antibody AC7 which is specific for the activated state of α IIB β 3 [18] and an erythromegakaryocytic cell line, AP217 which behaves in many respects like HEL but does not adhere after PMA induction. We found that although cell surface expression of α IIB β 3 integrin increased after PMA treatment of HEL cells, the monoclonal AC7 was unable to recognize it. Thus, since this integrin receptor reaches the cell surface in a non-activated conformation, the initial adhesion

Abbreviations used: FCS, fetal calf serum; GAPDH, glyceraldehyde-phosphate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; VLA, very late antigen.

† To whom correspondence should be addressed at: CNRS URA 1815, Laboratoire d'étude des systèmes adhésifs cellulaires, U.J.F. Cernoy, BP 53X 38041 Grenoble Cedex, France.

cannot be accounted for by $\alpha\text{IIb}\beta 3$. In addition, $\alpha\text{IIb}\beta 3$ cell surface expression increased in the cell line AP217, which remains non-adherent upon PMA treatment. Therefore we have investigated and quantified the expression of alternative adhesion receptors in three erythroleukaemia cell lines (HEL, LAMA-84 and AP217) following PMA treatment. Expression of the $\beta 1$ integrin was completely abolished in AP217 but remained high in the two adherent cell lines. Furthermore, antibodies against $\beta 1$ integrins inhibited HEL cell adhesion to fibronectin whereas antibodies against $\beta 3$ integrins were ineffective. Our results strongly suggest that $\beta 1$ integrins are involved in PMA-induced adhesion of erythroleukaemia cells.

MATERIALS AND METHODS

Cells

HEL is an erythromegakaryocytic cell line developed from a patient who contracted leukaemia after treatment for a solid tumour [19]. LAMA-84 and AP217 were both established from peripheral blood of patients with chronic myeloid leukaemia in acute phase [20]. AP217 is a new cell line established in our laboratory in January 1992; cells have been continuously growing from this date with a doubling time of 20 h [21]. LAMA-84 and AP217 have morphological features of undifferentiated blast cells, but like HEL they have immunological characteristics of the megakaryocytic lineage. Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS) and antibiotics. For differentiation experiments, growing cells were subcultured at a density of 0.5×10^5 cells/ml, and PMA (100 nM) was added to the medium. The treatment was stopped by washing cells twice with PBS.

Antibodies and peptides

B2A is an anti- $\beta 3$ monoclonal antibody raised against purified human $\beta 3$ [22,23], and AC7 is elicited against a synthetic peptide located within the RGD (Arg-Gly-Asp) binding region of $\beta 3$ [24]. AC7 recognizes only the activated form of the integrin $\alpha\text{IIb}\beta 3$. Monoclonal B2A recognizes the integrin $\beta 3$ subunit and inhibits platelet aggregation [25] as well as the adhesion of platelets to fibronectin. A concentration of 3 $\mu\text{g}/\text{ml}$ of B2A abolished 50% of the fibronectin binding to thrombin-activated platelets (platelets at $1 \times 10^8/\text{ml}$ and fibronectin at 20 $\mu\text{g}/\text{ml}$; D. Gulino, personal communication).

Function-perturbing antibodies were obtained from the following sources: mouse anti-(chicken $\alpha\text{v}\beta 3$) (LM609) from D. Cheresch (Scripps Research Foundation, La Jolla, CA, U.S.A.); mouse anti-(human $\beta 1$) (A2BII) and mouse anti-(human $\alpha 5$) (B2GII) from C. Damsky (Department of Stomatology, UCSF, CA, U.S.A.); mouse anti-(human $\alpha 2$) (VM1) from V. Morhenn (SRI, Menlo Park, CA, U.S.A.); mouse anti- αv (L230) from R. Pytela (Lung Biology Center, UCSF, CA, U.S.A.) and mouse anti-(human $\alpha 4$) (L25) from Becton Dickinson (Bedford, MA, U.S.A.). Antibodies used in indirect immunofluorescence are commercially available. Mouse monoclonal antibodies to the $\beta 1$ subunit (clone K20), to the αv subunit (clone AMF7), to $\alpha 2\beta 1$ (clone Gi9), to the $\alpha 4$ subunit (clone HP2/1), to the $\alpha 5$ subunit (clone SAM1), to CD45 (clone ARB 12), to the thrombospondin receptor (clone FA6.152) and to the integrin $\alpha\text{IIb}\beta 3$ (clone P2), and rat monoclonal antibody to the integrin $\alpha 6$ subunit (clone G0H3), were obtained from Immunotech (Marseille, France).

Peptides were obtained by solid-phase synthesis using an Applied Biosystem synthesizer. The peptides were more than 95% homogeneous when analysed by HPLC, and the amino

acid composition of each peptide was consistent with its amino acid sequence.

Adhesion assays

In the absence of added matrix, cells (1×10^6 in 1 ml) were incubated in RPMI 1640 supplemented with 10% FCS in the presence or absence of 100 nM PMA. At various times, unattached cells were removed by washing with PBS and attached cells were scraped off the culture dish with a rubber policeman into PBS. Both populations were counted. Assays were run in duplicate.

For cells cultured on fibronectin or collagen, wells of 96-well plates (Corning, Inc.) were incubated overnight at 4 °C with 100 μl of fibronectin (GIBCO-BRL, Gaithersburg, MD, U.S.A.) or collagen (Vitrogen 100, Celtrix, Santa Clara, CA, U.S.A.) at 1 $\mu\text{g}/\text{ml}$. Negative control wells were incubated with 0.2% BSA. Wells were washed with PBS and blocked with 100 μl of 0.2% BSA in PBS for 1 h, followed by three washes with PBS. HEL cells, grown for 72 h in the presence of 100 nM of PMA, were harvested with 0.05% trypsin/0.02% versene and washed in serum-free Dulbecco's modified Eagle's medium containing 0.2% soybean trypsin inhibitor (Sigma Chemical Co.). Cells were plated at a density of 1.5×10^4 in 100 μl in the presence of PMA (100 nM). Some assays were run in the presence of competitors: GRGDSP and GRGESP peptides at 100 nM, anti- $\alpha\text{v}\beta 3$ (LM609, dilution 1:10 of ascites), anti- $\alpha 4$ (L25, 100 $\mu\text{g}/\text{ml}$), anti- $\alpha 5$ (B2GII, hybridoma supernatant), anti- $\beta 1$ (A2BII, hybridoma supernatant), anti- $\beta 3$ (B2A, 100 $\mu\text{g}/\text{ml}$), anti- $\alpha 2$ (VM1, dilution 1:200 of ascites) and anti- αv (L230, hybridoma supernatant). To achieve an even distribution of cells, plates were spun at 3500 g for 10 s and incubated at 37 °C for 30 min. After incubation, plates were shaken on an orbital shaker (300 rev./min for 30 s) then washed three times with PBS. At the end of the wash, no cells remained adherent to the BSA-coated wells. Cell adherence was quantified using a protein quantification kit, BCA (Pierce, Rockford, IL, U.S.A.), by adding 100 μl of BCA reagent to each well, incubating for 1 h at 37 °C and then reading on a plate reader (Molecular Devices Corp., Menlo Park, CA, U.S.A.) at 562 nm.

Indirect immunofluorescence and flow cytometry

Cells were grown in the presence of 100 nM PMA for various times. Adherent cells (HEL, LAMA-84) were washed twice in PBS and were scraped off with a rubber policeman, suspended in PBS and counted. Non-adherent cells (AP217) were washed three times in PBS before counting. Surface expression of individual integrins was evaluated by flow cytometry after staining of 500 000 cells with integrin-subunit-specific antibodies followed by fluorescein isothiocyanate-conjugated secondary antibody (anti-mouse IgG from Jackson Immunoresearch Laboratories, Inc., West Grove, PA, U.S.A.). Commercially available anti-integrin antibodies were used at the dilution suggested by the manufacturer, otherwise anti- $\beta 3$ and anti- $\alpha\text{IIb}\beta 3$ antibodies (B2A and AC7) were used at 4 $\mu\text{g}/\text{ml}$. Antibody not binding specifically was removed by washing with phosphate buffer. Non-specific staining was assessed by using non-immune mouse IgG as primary antibody. Routinely, 10 000 cells were analysed in a Becton Dickinson flow cytometer.

RNA purification and amplification procedure

Cytoplasmic RNA was isolated according to Gough's method [25]. With this procedure, $4 \pm 0.5 \mu\text{g}$ of cytoplasmic RNA per 10^6

cells was obtained. Contaminating DNA was eliminated by digestion with Promega RNase-free DNase according to the manufacturer's instructions. mRNA (corresponding to 2 µg of total RNA) was reverse-transcribed to prepare first-strand cDNA. The reaction was performed for 2 h at 37 °C with 100 units of reverse transcriptase (GIBCO-BRL, Gaithersburg, MD, U.S.A.) and two 3' oligonucleotides chosen from within human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and integrin $\beta 1$ sequences. In a control experiment the enzyme was omitted. Part of the cDNA (corresponding to 300 ng of RNA) was amplified using the following primer sets: either 5'-GACCTCTACTACCTTATGGAC-3' and 5'-GGCATCGAAACCA-CCTTCTGG-3' to amplify base pair 533 to 865 from human $\beta 1$ integrin, or 5'-CTCAAGATCATCAGCAATGC-3' and 5'-AGCTTCCCGTTCAGCTCAG-3' to amplify base pair 490 to 743 from human GAPDH. Amplification steps were carried out with a Techne terminal cycler, using the following program: 1 min at 94 °C, 1 min at 50 °C, 1.5 min at 72 °C during 22 cycles and one final extension step at 72 °C for 4 min. Amplification products were subjected to electrophoresis and analysed by Southern blotting.

Southern blotting

PCR products were run on 1.5% Sea plaque agarose gel (FMC bioproducts, Rockland, ME, U.S.A.) and electrophoretically transferred to a Hybond N⁺ membrane (Amersham, Arlington Heights, IL, U.S.A.). Amplified fragments were detected with the Amersham rapid hybridization system using ³²P-labelled cDNA probes. The integrin $\beta 1$ probe corresponded to a fragment of the human cDNA from positions 607 to 865 (Genbank, HUMFNRB). GAPDH was detected by the *Nsi*I-*Bst*XI digestion fragment of the rat GAPDH cDNA corresponding to the sequence from nucleotides 515 to 703 (Genbank, RATGAPDH). Both probes were labelled by incorporation of [α -³²P]dCTP using a random priming kit (Boehringer, Mannheim, Germany). After extensive washes, the membrane was autoradiographed and the radioactive spots were counted for 10 min in the absence of scintillator liquid (Cerenkov counting).

RESULTS

In response to PMA, HEL and LAMA-84 cells adhered but AP217 cells did not

HEL and LAMA-84 phenotypes display characteristics of both the erythroid and the megakaryocytic lineage [19,20].

AP217 is a new cell line established from the blood of a patient with chronic myeloid leukaemia in the acute phase. Having been established now for about three years, AP217 line continues to proliferate rapidly and exhibits a stable phenotype [21]. This cell line is negative for lymphocyte markers, but spontaneously expresses immature granulo-monocytic (CD33, CD11b) as well as platelet-megakaryocytic and erythroid markers. AP217 erythroid markers are represented by β and γ globins, EPO-receptors, transcription factor GATA-1 and glycophorin A. Platelet GPIIb RNA and GPIIbIIIa were expressed by 24% of the cells together with c-mpl receptor for thrombopoietin. C-mpl or cellular proto-oncogene of the myelo-proliferative leukaemia virus is a specific megakaryocytic growth factor that was cloned recently [26]. The megakaryocytic characteristics of this cell line are also strengthened by the increased ploidization of the cells after PMA induction (30% of the cells being 8N).

HEL, LAMA-84 and AP217 normally grow in suspension, but when they were cultured in the presence of PMA, HEL and LAMA-84 exhibited an adherent phenotype while AP217 did not

Figure 1 Adhesion of (a) HEL cells and (b) LAMA-84 (○) and AP217 (□) cells

Cells (1×10^6) were grown in the presence of 100 nM PMA. The percentage of adherent cells among the whole population is expressed as a function of the time of incubation [either hours in (a) or days in (b)].

(Figure 1). The kinetics of adhesion were different for the two adherent cell lines, being faster for HEL than for LAMA-84. After 3 h of induction, 95% of the whole HEL cell population was already attached to the plastic; the cells then spread, and 75% of the cells remained adherent for several days (Figure 1a). After 24 h of induction, 62% of the whole LAMA-84 cell population was adherent and after 48 h 75% of the cells stuck to the plastic (Figure 1b). In contrast, even after 3 days of PMA induction, AP217 cells did not attach (Figure 1b). Under our assay conditions and in the absence of PMA, the percentage of adherent cells in the three cell lines was below the level of detection (data not shown).

PMA induced megakaryocytic differentiation of HEL, LAMA-84 and AP217 but did not 'activate' α IIb β 3 integrin

PMA is known to initiate the differentiation of the erythromegakaryocytic cells to the megakaryocytic lineage, and integrin α IIb β 3 is one of the most specific markers for this cell lineage. Using monoclonal antibodies, we determined the expression of this marker at the cell surface of the three cell lines, cultured with and without PMA for 72 h. As determined by flow cytometry, the percentage of α IIb β 3-positive cells increased from 54.3% to 90.6% for LAMA-84, from 34.1% to 89.2% for HEL and from 23.4% to 77% for AP217 (Table 1). The percentage of β 3-positive cells increased in similar proportions (Table 1). The

Table 1 Detection of a megakaryocytic marker by flow cytometry analysis

Assays were performed on the three cell lines: LAMA-84, HEL and AP217. Cells were grown for 72 h in either the presence of PMA (+PMA) or in its absence (–PMA). The expression of cell surface antigens was quantified by indirect immunofluorescence. 10 000 cells were analysed by flow cytometry, and the percentage of positive events is reported. $\alpha\text{IIb}\beta 3^*$ is the activated form of $\alpha\text{IIb}\beta 3$ integrin and is detected by the monoclonal antibody AC7. Data are averages of two independent experiments.

Antibody	Antigen	Detection (% of positive events)					
		LAMA-84		HEL		AP217	
		–PMA	+PMA	–PMA	+PMA	–PMA	+PMA
P2	$\alpha\text{IIb}\beta 3$	54.3	90.6	34.1	89.2	23.4	77.0
B2A	$\beta 3$	42.2	86.1	36.5	88.6	20.5	75.2
AC7	$\alpha\text{IIb}\beta 3^*$	0.6	2.5	1.4	2.0	1.8	1.0

Table 2 Detection of cell surface receptors by flow cytometry analysis

Assays were performed on three cell lines: LAMA-84, HEL and AP217. Cells were either incubated for 72 h in the presence of PMA (+PMA) or in its absence (–PMA). The expression of cell surface receptors was quantified by using specific monoclonal antibodies as indicated in the Table and an indirect immunofluorescence technique. Cells were analysed by flow cytometry. Data indicated in the Table represent the percentage of positive events when 10 000 cells are analysed. Data are the averages of two independent experiments.

Antibody	Antigen	Detection (% of positive events)					
		LAMA-84		HEL		AP217	
		–PMA	+PMA	–PMA	+PMA	–PMA	+PMA
K20	$\beta 1$	89.1	83.8	88.4	86.7	81.2	4.3
HP2/1	$\alpha 4$	89.3	31.0	93.5	66.7	91.4	0.1
SAM1	$\alpha 5$	80.4	50.2	54.5	18.5	52.8	0.1
GoH3	$\alpha 6$	0	0	0	0	0	0
Gi9	$\alpha 2\beta 1$	2.3	28.2	0.9	75.1	0.1	0.7
AMF7	αv	10.4	23.6	5.9	56.7	0.8	0.1
FA6.152	CD36	5.7	2.4	92.6	14.5	38.7	31.0
ARB 12	CD45	95.0	95.9	92.5	91.4	89.4	89.9

monoclonal antibody AC7, which recognizes the activated form of $\alpha\text{IIb}\beta 3$ at the platelet surface, did not react with either PMA-treated or untreated cells. Therefore, after PMA activation the three cell lines are clearly committed to the megakaryocytic lineage, as indicated by the increase of the specific marker $\alpha\text{IIb}\beta 3$. However, this platelet integrin is not expressed in the active form, and the three cell lines exhibit similar amounts of $\alpha\text{IIb}\beta 3$, which does not correlate with the two different adhesive phenotypes observed.

PMA induced different patterns of integrin expression on the non-adhesive cells AP217 versus the adhesive cells HEL and LAMA-84

In order to explain the two different adhesive phenotypes, we investigated the cell surface expression of another group of adhesive receptors belonging to the integrin family, the VLA (very late antigen) subgroup ($\beta 1$ integrins). Using the monoclonal antibody K20, we quantified the presence of the $\beta 1$ integrin subunit at the surface of the cells (Table 2). In the absence of PMA, all three cell lines expressed similar high levels of $\beta 1$ integrin. Under PMA stimulation for 72 h, LAMA-84 and HEL

Figure 2 Kinetics of integrin cell surface expression

HEL (closed symbols) or AP217 (open symbol) cells were grown without PMA (time 0) or in the presence of PMA for various times. Cell surface expression of integrin $\alpha 2$ (■), αv (▼) or $\beta 1$ (●) was determined with specific monoclonal antibodies and quantified by flow cytometry analysis.

still expressed high levels of $\beta 1$, but the level of this receptor decreased dramatically in AP217 cells (81.2% to 4.3%). To identify which α subunits are associated with the $\beta 1$ subunit we quantified the expression of $\alpha 6$, $\alpha 4$ and $\alpha 5$ subunits by flow cytometry using several monoclonal antibodies. Without PMA treatment, the three cell lines did not express any detectable $\alpha 6$ subunit but all of them expressed $\alpha 4$ and $\alpha 5$ (Table 2). After 72 h of PMA induction, the three cell lines still did not express $\alpha 6$ and we observed a variable decrease in expression of $\alpha 5$ and $\alpha 4$ subunits (Table 2) which was drastic in AP217; indeed both $\alpha 4$ and $\alpha 5$ became virtually undetectable. Indirect immunofluorescence on permeabilized AP217 cells verified that there was no accumulation of these subunits in the cytoplasm due to the lack of $\beta 1$ subunit (data not shown). Since the level of expression of $\beta 1$ was constant in treated or untreated LAMA-84 and HEL cells, whereas the level of expression of $\alpha 4$ and $\alpha 5$ was decreased, we looked for other α subunits that might be up-regulated by PMA. $\alpha 2\beta 1$ integrin was not expressed in untreated LAMA-84 and HEL cell lines, but after PMA treatment its expression

Table 3 Adhesion of HEL cells on purified substrates

HEL cells were grown for 72 h in the presence of 100 nM PMA and plated on fibronectin (FN) or collagen (CO) in the absence of serum and in the presence of PMA (100 nM). Adhesion was measured as described in the Materials and methods section. Non-specific adhesion on BSA was subtracted. Results were expressed as percentage of control, which was adhesion of HEL to substrates in the absence of competitor. Competitors were as follows: Peptides: GRGDSP and GRGESP (100 μ M); antibodies: anti- β 1 (A2B1, hybridoma supernatant), anti- β 3 (B2A, 100 μ g/ml), anti- α v β 3 (LM609, 1:10 dilution of ascites), anti- α 5 (B2G11, hybridoma supernatant), anti- α 4 (L25, 100 μ g/ml), anti- α v (L230, hybridoma supernatant) and anti- α 2 (VM1, dilution 1:200 of ascites).

Substrate	Competitors	Adhesion (%)
FN	GRGDSP peptide	19 $\pm$ 1
FN	GRGESP peptide	83 $\pm$ 3
FN	Anti- β 1	38 $\pm$ 8
FN	Anti- β 3	100
FN	Anti- α v β 3	100
FN	Anti- α 5	56 $\pm$ 5
FN	Anti- α 4	89.5 $\pm$ 4.5
FN	Anti- α 4 + anti- α 5	35 $\pm$ 5
FN	Anti- α v	100
FN	Anti- α 4 + anti- α 5 + anti- α v	22 $\pm$ 2
CO	Anti- β 1	0
CO	Anti- α 2	0

increased (Table 2). In the same manner, PMA treatment resulted in an increased expression of α v subunit in LAMA-84 and HEL cells. PMA did not induce expression of either α 2 β 1 integrin or α v subunit in AP217 cells. To support our observation that changes in adhesive behaviour of HEL cells may involve α 2 and α v subunits, we studied the kinetics of cell surface expression of these subunits. As shown in Figure 2, the up-regulation of α 2 and α v was rapid; indeed, after 4 h of PMA induction newly synthesized integrins were already detected at the cell surface.

The matrix molecule thrombospondin may also be involved in the adhesive properties of erythromegakaryocytic leukaemia cell lines; therefore we examined the expression of thrombospondin receptor, CD36 [27], in the three cell lines (Table 2). LAMA-84 cells, with or without induction by PMA, did not express CD36. HEL cells without induction were highly positive for CD36, and PMA induced a drastic down-regulation of this receptor (92.6 % to 14.5 %). The expression of CD36 in AP217 cells was stable, with about 35 % of the cells being positive regardless of PMA treatment. Therefore, the surface expression of CD36 receptor does not correlate with the different adhesive properties we have observed for the three cell lines. Another surface antigen, CD45 [28] which is a tyrosine phosphatase, remained constant at the surface of the three cell lines (Table 2). Thus, erythroleukaemia cell membranes are not completely remodelled upon PMA treatment.

After PMA induction, HEL cells used β 1 and not β 3 integrins to adhere to fibronectin

We showed that after 72 h of induction by PMA, HEL cells expressed α 4 β 1, α 5 β 1, α 2 β 1, α 1Ib β 3 and α v subunit on the cell surface. To verify that the adhesion of HEL is integrin mediated, we performed adhesion assays in the presence of competitors. As indicated in Table 3, the adhesion of HEL to fibronectin was RGD-dependent, confirming the involvement of integrin receptors. Indeed, GRGDSP peptide reduced the adhesion of HEL to fibronectin by 82 % whereas negative control peptide GRGESP did not. To assess the roles of specific integrins, we performed adhesion assays on fibronectin in the presence of

Figure 3 Detection of β 1 cDNA in amplification products

cDNAs were synthesized from 300 ng of total RNA by reverse transcription. They were simultaneously amplified with β 1 integrin- and GAPDH-specific primers and hybridized with specific probes. Relative intensities (RI) indicated in the Figure are values calculated for β 1 when GAPDH signal (GA) equals 100 and are the average of two independent experiments. Lane 1, AP217 cells in the absence of reverse transcriptase; lane 2, untreated AP217 cells; lane 3, AP217 cells induced by PMA for 72 h.

various blocking antibodies. We first used antibodies against the β subunits; antibody against β 1 integrin [29] reduced the adhesion of HEL to fibronectin by 70 % whereas antibodies against β 3 or α v β 3 [30] did not (Table 3). GRGDSP peptide and antibodies against β 1 and β 3 produced the same level of inhibition (80 %, 70 % and no inhibition respectively) when we used HEL cells which had not been induced by PMA before the adhesion assay was performed (data not shown). These results indicated that β 3-containing integrins are not involved in adhesion of HEL cells to fibronectin after short (30 min) or long (72 h) PMA induction. We then used specific antibodies against the different α subunits expressed by HEL cells. Antibody against α 5 blocked 50 % of the adhesion to fibronectin whereas anti- α 4 antibody blocked only 15 %. Using a mixture of the two antibodies (α 5 plus α 4) gave us 70 % inhibition of the adhesion just as when we used β 1 blocking antibody. This suggested that α 4 β 1 and α 5 β 1 are the main VLA receptors for fibronectin on HEL cells. An anti- α v antibody [31] alone did not block adhesion, but if we added this antibody to the mixture of α 5 and α 4 antibodies we blocked 80 % of the adhesion, as we did with GRGDSP peptide.

To assess the role of α 2 β 1 at the surface of PMA-treated HEL cells we performed adhesion assays on collagen type 1. Antibodies against β 1 or α 2 subunits completely blocked the adhesion (Table 3), indicating that α 2 β 1 integrin is the main receptor for collagen on HEL cells.

AP217 cells were still non-adherent in these adhesion assays (data not shown).

PMA induces a down-regulation of β 1 mRNA level in AP217 cells

To find out whether the absence of β 1 integrin cell surface expression in AP217 cells was caused by a failure in the maturation process of the protein or by a down-regulation at the gene transcription level, we quantified β 1 mRNA in these cells. Using PCR methodology under non-saturating conditions (21 cycles, 300 ng of RNA), the β 1 mRNA was transcribed and amplified from total RNA preparations. DNase treatment of RNA excluded possible contamination by genomic DNA [32]. As expected, no signal was obtained when the reverse transcriptase was omitted (Figure 3, lane 1), thus confirming the absence of contaminating DNA. Amplification of GAPDH mRNA was used to monitor the integrity of RNA samples and to quantify the relative intensity of the radioactive spots obtained after electrophoretic transfer and hybridization with specific probes. Experiments were carried out on untreated AP217 cells and on AP217 cells grown in the presence of PMA for 72 h (Figure 3, lanes 2 and 3 respectively). After amplification and hybridization, a β 1 mRNA relative intensity of 69 was obtained

for untreated cells and this value decreased to 20 for PMA-treated cells. The level of cell surface expression of $\beta 1$ integrin protein on AP217 decreased from 81 % to 20 % within 48 h of PMA treatment and then remained stable (around 4 %) at 72 h and 120 h (Figure 2b). Therefore the low level of expression of $\beta 1$ integrin subunit is probably due either to a decrease in transcription of $\beta 1$ mRNA or to a higher turnover rate of that message.

DISCUSSION

Differentiation of haematopoietic cells in bone marrow or in lymphoid organs and their transport to the bloodstream depends on their adherence to extracellular matrix [33]. Erythroblastic cells (such as HEL and LAMA-84) treated with PMA become adherent and committed to the megakaryocytic lineage [12,14]. Phorbol esters are thus convenient tools to promote *in vitro* commitment and were used in this work to study the relationship between adhesion receptors and megakaryocytopoiesis. We have established a novel erythromegakaryocytic cell line (AP217) which, when exposed to PMA, behaved mostly like the HEL cell line but did not become adherent. This has given us the opportunity to identify components that might be involved in PMA-induced adhesion. During normal megakaryocytic differentiation, several markers appear at the cell surface. The $\alpha \text{IIb}\beta 3$ integrin is expressed in immature megakaryocytes, being one of the most specific, and the $\alpha 6$ integrin is a marker of mature megakaryocytes [34]. In the three cell lines studied (LAMA-84, HEL and AP217), phorbol esters induced cell surface expression of the platelet integrin $\alpha \text{IIb}\beta 3$; however, the integrin subunit $\alpha 6$ was never detected at the cell surface. Conversely, the phosphotyrosine phosphatase receptor (CD45), which is expressed on early megakaryocytes and is absent on mature megakaryocytes and platelets [35], was still present after PMA treatment. Therefore, PMA did induce differentiation of these leukaemia cell lines towards the megakaryocytic lineage but did not seem to promote maturation.

It has been suggested that the adherent phenotype of HEL which appears after PMA treatment is partly mediated by the activation of the $\alpha \text{IIb}\beta 3$ integrin at the cell surface [17]. We used a monoclonal antibody (AC7) specific to the activated state of $\alpha \text{IIb}\beta 3$ [18] to test this hypothesis. We found that the integrin $\alpha \text{IIb}\beta 3$ was not present on the surface of the cells in the high-affinity conformational state; nevertheless, we cannot exclude the possible existence of multiple functional forms of the integrin $\alpha \text{IIb}\beta 3$ that may not be recognized by AC7. However, when taken together with the observation that AP217 cells also expressed integrin $\alpha \text{IIb}\beta 3$ at high level and were non-adherent, it is likely that the integrin $\alpha \text{IIb}\beta 3$ is not playing a significant role in the PMA-induced adhesion of erythromegakaryocytic cell lines.

To determine which receptors might mediate the adherent phenotype of these cells, we quantified the cell surface expression of other integrin subunits. A comparison between cell surface receptors expressed in the two adherent cell lines (LAMA-84 and HEL) and in the non-adherent cell line (AP217) supported the involvement of $\beta 1$ integrins in the adhesive phenotype. After PMA treatment, the $\beta 1$ integrin subunit disappeared from the AP217 cell surface and its synthesis was dramatically decreased due either to an inhibition at the gene transcription level or to an increased RNA turnover. Conversely, in LAMA-84 and HEL cells, the $\beta 1$ integrin subunit was still present regardless of PMA treatment. In these two cell lines, PMA treatment was followed by a down-regulation of $\alpha 4$ and $\alpha 5$ and an up-regulation of $\alpha 2$ and $\alpha \nu$ subunits. HEL and LAMA-84 cells without PMA

treatment express $\beta 1$, $\alpha 4$ and $\alpha 5$ and are non-adherent. Since we know that $\alpha 4\beta 1$ and $\alpha 5\beta 1$ might exist in multiple conformations [36,37], we propose that PMA can modify the activated state of $\alpha 5\beta 1$ or $\alpha 4\beta 1$ integrin and promote an initial adhesion signal. After the initial adhesion, $\alpha 2\beta 1$ and $\alpha \nu$ integrins, which appear at the cell surface within 4 h of PMA treatment, may account for the spreading of the cells. Burget et al. [38] have also reported that erythroid cells (like K562) committed to the megakaryocytic lineage express the $\alpha 2$ integrin subunit and that they adhere to collagen. Moreover, $\alpha 2\beta 1$ was reported to be a functional integrin receptor in platelets [39]. Since AP217 cells do not express any $\beta 1$ integrins and remain non-adherent, a direct correlation between the adhesive phenotype of HEL and LAMA-84 cells and the expression of the VLA integrins subunits ($\beta 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha \nu$) can be made. Furthermore, we confirmed the role of these receptors by using function-perturbing antibodies. Antibodies against $\beta 1$, $\alpha 4$, $\alpha 5$, $\alpha \nu$ and $\alpha 2$ reduced adhesion of HEL to fibronectin or collagen, whereas antibodies against $\beta 3$ or $\alpha \nu\beta 3$ did not, implicating $\beta 1$ integrins, but not $\beta 3$ integrins, in the adhesion of erythroleukaemia cells.

In the three erythromegakaryocytic cell lines, PMA profoundly modified the expression of integrins at the cell surface, and both up- and down-regulation was observed. Interestingly, in other systems, enhancement of adherence by phorbol esters did not always involve up-regulation of receptor expression; in T-lymphocytes, adherence to collagen and fibronectin was mediated by $\alpha 2\beta 1$ and $\alpha 5\beta 1$ and PMA increased only the functionality of these structures [40]. Danilov and Juliano [41] have also reported that the marked enhancement of Chinese hamster ovary cell adhesion to fibronectin, following phorbol ester treatment, is not due to changes in the cell surface expression or in the affinity of the fibronectin receptor. Also, phorbol esters stimulate RNA transcription of the fibronectin receptor in K 562 cells, but the newly synthesized integrin is poorly functional due to altered glycosylation [42].

This work was performed in the absence of added matrix, since we know that the matrix itself can modify cell surface receptor expression and localization. We have not identified the ligands involved in the adhesion process, but we found that serum was essential. Cells probably do secrete matrix proteins, like fibronectin or collagen, that may play a role in the adhesion. Thrombospondin has been suspected of being a major extracellular matrix molecule involved in the adhesion of erythroleukaemia cell lines. However, we did not find any correlation between the expression of the thrombospondin receptor, CD36, at the cell surface and the adhesive phenotype since this expression remained high in the non-adherent AP217 cells.

If PMA treatment of HEL and LAMA-84 cells promoted an integrin shift at the cell surface from $\alpha 4$ and $\alpha 5$ to $\alpha 2$ and $\alpha \nu$, PMA was unable to transduce the same signal in AP217 cells. The failure may be due to the lack of some protein kinase C isoenzymes [13], or to cell molecule regulators [43,44], or to modifications of cytoskeletal components. It is noteworthy that PMA treatment resulted in down-regulation of $\beta 1$ integrin expression at the gene transcription level. The question that arises is whether $\beta 1$ integrin down-regulation is a consequence of reduced adhesion or the primary event. Since AP217 cells do not express $\beta 1$ integrin after PMA induction, they should prove to be a very useful tool in studying the specific signalling pathway associated with the $\beta 1$ integrin receptor.

In conclusion, in this study we have observed that PMA induces profound modifications in integrin cell surface expression of three erythromegakaryocytic cell lines, and that changes in adhesive behaviour appear to be mediated by $\beta 1$ integrins and not through $\alpha \text{IIb}\beta 3$ activation.

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