March 14, 1997

Human Macrophage Differentiation Involves an Interaction between Integrins and Fibronectin

Amale Laouar, Cynthia B.H. Chubb, Frank Collart, and
Eliezer Huberman

Center for Mechanistic Biology and Biotechnology
Argonne National Laboratory
9700 South Cass Avenue
Argonne, Illinois 60439-4833

Address all correspondence to Eliezer Huberman, Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, Illinois 60439-4833. Tel: (630) 252-3821. Fax: (630) 252-3387. email: elih@anl.gov.

Work supported by the U.S. Department of Energy, Office of Health and Environmental Research, under Contract W-31-109-ENG-38.

Abstract

We have examined the role of integrins and extracellular matrix (ECM) proteins in macrophage differentiation of 1) human HL-60 myeloid leukemia cells induced by phorbol 12-myristate 13-acetate (PMA) and 2) human peripheral blood monocytes induced by either PMA or macrophage-colony stimulating factor (M-CSF). Increased β_1 integrin and fibronectin (FN) gene expression was observed in PMA-treated HL-60 cells and PMA- or M-CSF-treated monocytes, even at a time preceding the manifestation of macrophage markers. Treated HL-60 cells and monocytes also released and deposited FN on the culture dishes. An HL-60 cell variant, HL-525, which is deficient in protein kinase C β (PKCβ) and resistant to PMA-induced differentiation, failed to express FN after PMA treatment. Restoration of PKCβ resulted in PMA-induced FN gene expression and macrophage differentiation. The macrophage phenotype induced in HL-60 cells or monocytes was attenuated by anti-β_1 integrin or anti-FN MAbs. We suggest that macrophage differentiation involves activation of PKC and expression of specific integrins and ECM proteins. The stimulated cells, through their integrins, attach and spread on these substrates by binding to the deposited ECM proteins. This attachment and spreading in turn, through integrin signaling, leads to the macrophage phenotype.

Introduction

The extracellular matrix [1](ECM) is an intricate assembly of proteins that includes collagen, laminin, and fibronectin, among others (1). Cells interact with these proteins and with each other via specific receptors located on their surface. A major class of these receptors is the integrins, each of which is composed of two distinct α and β transmembrane glycoprotein subunits that are noncovalently linked (2). The integrins are classified into families of receptors. For example, the β_1 integrin family, also called very late after activation antigens, is implicated in cell adhesion to the ECM (2); a member of this family, $\alpha_4\beta_1$, is also involved in cell-cell interactions (3). The integrins bind to specific recognition sites on ECM proteins. One of these sites, which is present on fibronectin (FN), is composed of an Arg-Gly-Asp-Ser (RGDS) motif (4). Synthetic peptides containing RGDS have been found to promote cell adhesion when immobilized on suitable substrates and to inhibit cell adhesion to tissue culture dishes precoated with FN (5).

Macrophages adhere to, spread on, and engulf foreign particles, including microorganisms; adherence and spreading on foreign substrates (including tissue culture dishes) are considered to be hallmarks of macrophages. A number of ECM proteins have been shown to enhance the maturation of blood monocytes into macrophages (6–8). It is thus conceivable that adhesion molecules not only define macrophage characteristics but also participate in the process that leads to macrophage differentiation. We report here the role that specific adhesion molecules, FN in

---

[1]Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; FN, fibronectin; GAPDH, glyeraldehyde-3-phosphate dehydrogenase; GPRP, Gly-Pro-Arg-Pro; M-CSF, Macrophage-colony stimulating factor; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RA, all *trans*-retinoic acid; RGDS, Arg-Gly-Asp-Ser; RT, reverse transcriptase.

particular, play in macrophage differentiation of human leukemia cells and peripheral blood monocytes.

Results

Manifestation of integrins and fibronectin during human macrophage differentiation. To define the role specific integrins and FN play in macrophage differentiation, we initially examined their manifestation on the surface of viable human HL-60 myeloid leukemia cells and on human peripheral blood monocytes during differentiation induced by either PMA or M-CSF. Untreated HL-60 cells, whether cultured for 4 h or 2 d, exhibited some β_1 integrin immunostaining and little or no FN immunostaining (Fig. 1). Treatment of these cells with 3 nM PMA for 4 h caused the majority of the cells to display more intense immunostaining for both antigens. Prolonging this treatment to 2 d, which causes the HL-60 cells to acquire a macrophage phenotype (9), resulted in a marked increase in β_1 integrin immunostaining and a lesser increase in FN immunostaining scattered throughout the surface of the cells (Fig. 1). In contrast, treatment of HL-60 cells for up to 2 d with 240 U/ml M-CSF, which does not evoke a macrophage phenotype in these cells (10), failed to cause an increase in either β_1 integrin or FN immunostaining.

We also examined the immunostaining pattern of the α_5 antigen, which associates with $\beta 1$ integrin and binds FN through its RGDS motif (2). The pattern and intensity of α_5 immunostaining in untreated and PMA-treated HL-60 cells corresponded to that of β_1 immunostaining.

Because integrins belonging to other families may also be involved in macrophage differentiation, we examined the immunostaining pattern of $\alpha_v\beta_3$ integrin, which, similar to $\alpha_5\beta_1$, binds FN

through its RGDS motif (2, 11). For comparison, we included a MAb to the β_4 integrin, which binds to laminin but not to FN (12). Untreated HL-60 cells exhibited little or no immunostaining with the $\alpha_v\beta_3$ MAb. After treatment for 2 d with PMA, 50% of the cells reacted with the MAb; however, the immunofluorescence was less intense than that observed in PMA-treated cells reacting with the anti-β_1 MAb. In contrast, untreated HL-60 cells showed reactivity with the anti-β_4 MAb, but the reactivity decreased markedly after PMA treatment.

Freshly isolated peripheral blood monocytes exhibited weak immunostaining for β_1 integrin and FN. After two days in culture, a fraction of the cells displayed some immunostaining for these antigens (Fig. 2). Treatment of the isolated monocytes with either 3 nM PMA or 240 U/ml M-CSF for 4 h caused a majority of cells to display β_1 integrin and FN immunostaining, which became intense after 2 d (Fig. 2). These 2 d treated cells also exhibited a typical macrophage cell spreading (Fig. 2).

A noteworthy observation was the abundance of speckled FN immunostaining in the intercellular spaces of the dishes containing activated monocytes or HL-60 macrophages, but not in those containing control cells (Figs. 1 and 2). Limited, scattered intercellular β_1 integrin immunostaining was also observed, but this staining was, in large part, restricted to the vicinity of the adherent macrophages (Figs. 1 and 2). Little or no immunostaining with the isotypic control antibodies was observed in untreated cells or in differentiated monocytes or HL-60 cells.

These results indicate that acquisition of a macrophage phenotype in monocytes and HL-60

leukemia cells is associated with the cell surface manifestation of specific integrins and FN, with the latter being released and deposited on the culture dishes. Moreover, integrin and FN immunostaining was detected within 4 h after treatment with the inducer, a time frame which precedes the expression of the macrophage phenotype.

To provide evidence that protein kinase C (PKC) is involved not only in PMA- and M-CSF-induced macrophage differentiation (9, 13), but also in the increased levels of the β_1 integrin and FN antigens, we examined the effect of H-7, an inhibitor of PKC, PKA, and PKG and HA-1004 (an inhibitor of PKA and PKG but not PKC) (14, 15). In treated HL-60 cells and monocytes, 20 μM H-7 (but not 100 μM HA-1004) decreased by about 70% the number of cells exhibiting macrophage markers, as well as the β_1 integrin and FN immunostaining. In control HL-60 cells or monocytes, treatment with H-7 or HA-1004 failed to evoke such markers or to affect β_1 integrin and FN immunostaining.

Inhibition of PMA-induced macrophage differentiation in HL-60 cells. We tested the RGDS peptide and MAbs to FN and FN-binding integrins for the ability to alter macrophage differentiation in HL-60 cells. We assumed that such MAbs or the peptide would block the interaction of these integrins with the FN deposited on the culture dishes by the PMA-treated HL-60 cells, with its RGDS motif in particular. If this interaction is essential for PMA-induced macrophage differentiation, then the presence of these antibodies or the RGDS peptide should inhibit such a differentiation in the HL-60 cells. Our results indicated that the anti-FN, anti-β_1, and anti-α_5 MAbs and the RGDS peptide attenuated the manifestation of PMA-evoked

macrophage markers such as cell adherence and spreading, staining for non-specific esterase activity, and phagocytosis (Table 1). Moreover, the anti-FN, anti-β_1 integrin, and anti-α_5 integrin antibodies and the exogenous RGDS peptide diminished the PMA-evoked reduction in the number of cells (Table 1), which to a large degree is due to apoptosis (16). We also tested the MAbs to the $\alpha_v\beta_3$ and β_4 integrins for their ability to inhibit cell adherence and spreading, and phagocytosis. The anti-$\alpha_v\beta_3$ MAb caused a reduction of 25–30% in the expression of these markers in the PMA-treated cells. In contrast, the anti-β_4 MAb failed to affect these markers in such treated cells. Control antibodies and the control peptide Gly-Pro-Arg-Pro (GPRP) had no effect on the expression of the PMA-induced markers or on the reduction in the number of cells (Table 1). The MAbs or the peptides also had little or no effect on the replication or expression of maturation markers in control HL-60 cells (Table 1). These results indicate that PMA-induced macrophage differentiation in HL-60 cells involves an interaction between appropriate cell surface integrins and the RGDS motif present on FN, which is released and deposited on the surface of the culture dishes by the induced HL-60 cells.

Restoration of FN immunostaining in PMA-treated HL-525 cells. We studied cells from two HL-60 cell variants, HL-525 and HL-534 (17). Unlike the parental cells, these cells show diminished PKC β gene expression and are resistant to PMA-induced macrophage differentiation (17, 18). Relative to the parental HL-60 cells, HL-525 cells have a strikingly higher intensity of β_1 and α_5 integrin immunofluorescence, and HL-534 cells have a much lower, barely visible, immunostaining for these antigens. In both variants, treatment with 30 nM PMA for up to 2 d caused a limited increase in β_1 and α_5 immunostaining. Untreated HL-525 and HL-534 cells or

such cells treated with PMA failed to show FN immunostaining. Untreated and PMA-treated cells also failed to react with the anti-$\alpha_v\beta_3$ and anti-β_4 MAbs.

We have previously demonstrated that reestablishment of a functional PKCβ in HL-525 cells (via transfection with vectors containing PKCβ cDNA) restored their susceptibility to PMA-induced differentiation (9). The analysis of two PMA-treated transfectants (HL-525/β_1-C and HL-525/β_1F) revealed that the same fraction of cells that differentiated also regained FN immunostaining patterns and intensities like those of the corresponding PMA-treated HL-60 cells (Table 2). Since these transfected cells have a tendency to revert to the PMA-resistant phenotype (9), a sizable fraction of the transfected HL-525 cells were not susceptible to PMA-induced differentiation and FN immunostaining. No restoration of FN immunostaining was observed in HL-525 cells transfected with the control vector (Table 2). Another way to restore PKCβ gene expression and PMA susceptibility HL-525 cells is by pretreatment with all *trans*-retinoic acid (RA (19). Treatment with RA followed by PMA caused FN immunostaining patterns and intensities similar to those of PMA-treated HL-60 cells (Table 2). Treatment of HL-525 cells with either RA or PMA alone failed to induce FN immunostaining or macrophage differentiation (Table 2). These results implicate PKCβ in PMA-induced manifestation of FN in the HL-60 cell system.

Restoration of susceptibility to macrophage differentiation in HL-525 cells by exogenous FN. We cultured HL-525 and HL-534 cells on dishes precoated with human FN, which resulted in a time-dependent increase in markers such as adherence and spreading, non-specific esterase

activity, and phagocytosis in HL-525 cells, but not in HL-534 cells. After two days, more than 65% of the HL-525 cells had acquired a macrophage phenotype (Fig. 3 and Table 3). PMA treatment of HL-525 cells cultured on FN-precoated dishes caused a further increase in this percentage, to about 85%. No significant cell attachment and spreading was detected on dishes precoated with bovine serum albumin (BSA), polylysine, or other ECM proteins such as laminin, type I collagen, or type IV collagen.

To substantiate the involvement of β_1 integrins and RGDS in FN-induced macrophage differentiation of HL-525 cells, we tested the effect of the anti-β_1 integrin MAb and exogenous RGDS peptide. For comparison, we included the anti-FN MAb. Our results indicated HL-525 macrophage differentiation on human FN-precoated dishes was inhibited in the presence of anti-FN, anti-β_1 integrin MAb, and exogenous RGDS, but not in the presence of control reagents (Table 3). These results further support our supposition that acquisition of a macrophage phenotype involves the binding of specific integrins to the appropriate recognition site on ECM proteins such as FN.

Unlike PMA-induced differentiation in HL-60 cells, FN-induced macrophage differentiation of HL-525 cells was not affected by either the presence of 50 μM H-7 or 200 μM HA-1004. H-7 and HA-1004 at higher doses evoked up to 30% cell death, as determined by trypan blue staining. These results imply that PKC does not play a critical role in the steps leading to HL-525 macrophage differentiation that follow the interaction between specific integrins and FN.

Induction of FN-induced HL-60 cell attachment in the presence of $MnCl_2$. No significant cell adherence and spreading was observed on any of the precoated dishes when untreated HL-60 cells were used, which we attribute to a low level of inactive FN-binding integrins (20). To test for this possibility, we incubated HL-60 cells on FN-precoated dishes in the absence and presence of $MnCl_2$, which activates integrin binding to FN (21, 22). For comparison we included HL-525 and HL-534 cells. After 1 h in the absence of $MnCl_2$, the attachment of HL-60 and HL-534 cells was <1% and that of HL-525 cells about 60%. Incubation for 1 h with 1 mM $MnCl_2$ resulted in about 5% attachment of HL-534 cells, which exhibit little or no β_1 and α_5 antigens; 40% attachment of HL-60 cells, which exhibit higher levels of these FN-binding integrins; and >90% attachment of HL-525 cells, which exhibit an abundance of these integrins. Incubation of HL-60, HL525, and HL-534 cells with $MnCl_2$ for longer time periods (up to 24 h) resulted in cytotoxicity. These results implicate the need for both the presence and activation of FN-binding integrins in HL-60 cell attachment to FN.

Expression of the β_1 integrin and FN genes. We analyzed the steady-state levels of β_1 integrin and FN mRNA in HL-60 and HL-525 cells. The β_1 integrin mRNA level of untreated HL-525 cells was higher than that of untreated HL-60 cells. PMA treatment of HL-60 cells increased the mRNA level in a time-dependent manner, with the steady-state level of β_1 integrin mRNA already elevated about twofold at 4 h. An attempt to assess FN mRNA by standard hybridization of a FN probe to total RNA or polyA-enriched RNA yielded inconsistent results. To circumvent this problem, we introduced semiquantitative RT-PCR analysis. Using this approach with three different sets of primers (including one that codes for RGDS), we observed FN-specific

amplification products in untreated HL-60 cells and elevated levels 4 h after PMA treatment (Fig. 4). These products were either absent or detected at low levels in untreated and PMA-treated HL-525 cells (Fig. 4). In contrast, PMA-induced FN-specific amplification products were detected in HL-525 cells pretreated with RA or transfected with PKCβ cDNA, but not in HL-525 cells treated with RA alone or cells transfected with the control vector (Fig. 4). These results implicate gene expression as a major contributing factor in determining the level of β_1 integrins and FN in HL-60 and HL-525 cells.

Inhibition of macrophage differentiation in monocytes by anti-β_1 integrin or anti-FN MAb. To assure that the involvement of an interaction between FN and β_1 integrins is not restricted to macrophage differentiation in the HL-60 cell system, we included peripheral blood monocytes in our studies. These cells, which are at a maturation stage close to macrophages, can acquire a macrophage phenotype after treatment with either PMA or M-CSF (23, 24). Our results indicated that after 2 d in culture, about 25% of the untreated adherent monocytes exhibited the spreading typical of a macrophage. Two days of treatment of monocytes with either PMA or M-CSF caused more than a twofold increase in the percentage of these macrophages. This treatment, however, failed to markedly alter the fraction of cells exhibiting phagocytosis (Table 4), because most macrophage markers also characterize monocytes. The anti-FN and anti-β_1 integrin MAbs were each able to reduce the fraction of spread cells in the PMA- and the M-CSF-treated monocytes; anti-FN MAb also reduced the fraction of spread cells in the control (Table 4). These two MAbs were each also able to reduce the fraction of cells exhibiting phagocytosis in both the untreated and the treated monocytes (Table 4). These results implicate an interaction

between β_1 integrins and FN in PMA or M-CSF-induced macrophage differentiation in monocytes.

Discussion

We propose that induction and interaction of cellular receptors such as integrins with specific ECM proteins (which are secreted by the cells and deposited on foreign substrates) are steps in a process that brings about macrophage differentiation in appropriate progenitor cells. We focused our attention on the β_1 integrin family of receptors and fibronectin, because these adhesion proteins have been reported to be present in blood macrophages (25,26). We used HL-60 cells and peripheral blood monocytes for these studies because they can acquire a macrophage phenotype upon treatment with specific stimuli (9,23,24,27).

In our initial experiments, we determined the cell surface level of β_1 integrin and FN antigens in HL-60 cells and monocytes by immunostaining with specific MAbs. PMA treatment of HL-60 cells and PMA or M-CSF treatment of monocytes resulted in an increased level of these antigens in a time-dependent manner. Moreover, the levels of FN and β_1 integrin antigens were already increased at 4 h after treatment with the inducers, a time frame that precedes the acquisition of the mature phenotype (23, 28–30). In the case of FN, increased expression was associated with its secretion and deposition on the dishes.

Additional support for an association between macrophage differentiation and FN came from our studies with HL-525 cells, which are deficient in PKCβ and resistant to PMA-induced macrophage differentiation (18). These cells, whether treated with the macrophage inducer or not, failed to exhibit FN. Yet, FN immunostaining was found after restoration of PMA-induced macrophage differentiation by either transfection with PKCβ cDNA (9) or by pretreatment with

RA, which also increases the expression of the PKCβ gene (19). Analysis of FN and β_1 integrin RNA steady-state levels by RT-PCR and Northern blotting, respectively, implicated gene expression as a major contributing factor in determining the level of these antigens in HL-60 and HL-525 cells. In this context, note that β_1 integrin RNA transcripts were reported in M-CSF-treated human monocytes and in a number of normal and leukemic human blood cells, including HL-60 cells (26, 31).

Proof that increased levels of FN and appropriate FN binding integrins are intimately involved in the induction process itself came from our two experimental approaches. In one approach, we tested the ability of anti-β_1-integrin and anti-FN MAbs to block macrophage differentiation in HL-60 cells and monocytes. We assumed that these molecules would prevent the interaction of β_1 integrins with the deposited FN. If this interaction is involved in macrophage differentiation, then the presence of either antibody should inhibit such differentiation. Our studies indicate that indeed both the anti-β_1-integrin and anti-FN MAbs were each able to attenuate macrophage differentiation in monocyte and HL-60 cells. Our results also showed such an attenuation in HL-60 cells when either anti-α_5 integrin MAb or RGDS peptide [the $\alpha_5\beta_1$ integrin binding site on FN (4)] was used. Because other integrins might also be involved in the interaction with FN (2), we included a MAb to $\alpha_v\beta_3$, an integrin that is present in cultured human macrophages (31) and that also binds FN through its RGDS motif (11). This antibody, which had limited reactivity with PMA-treated HL-60 cells, inhibited the expression of macrophage markers in these treated cells, but to a lesser degree than the anti-β_1 or α_5 MAbs. These results indicate that induction of macrophage differentiation involves the production and interaction of FN binding integrins

with FN, which is produced, released, and deposited on suitable substrates, most likely with its RGDS motif.

In another type of experiment, we used HL-525 cells, which do not express the PKCβ and FN genes. We have shown that when these cells are either transfected with PKCβ cDNA (9) or pretreated with RA (19), they regain PKCβ expression and susceptibility to PMA-induced differentiation, as well as the expression of FN. Also, culturing HL-525 cells (which express a higher level of the $\alpha_5\beta_1$ integrin than HL-60 cells) on FN-precoated dishes caused their differentiation to macrophages even in the absence of PMA. No such differentiation was observed with dishes precoated with other ECM proteins such as laminin or type I or type IV collagens. The FN-induced differentiation was also inhibited by the anti-FN and anti-β_1 integrin MAbs and by RGDS. These studies further implicate FN and its interaction with suitable integrins in the attainment of a macrophage phenotype.

Our results are also consistent with the involvement of PKC in the manifestation of FN, but not in the steps that follow the interaction of FN with its receptor molecules. This conclusion is based on the following: a) PMA, an activator of PKC, induced the expression of the FN gene in both the HL-60 cells and monocytes; b) transfection of the HL-525 cells with PKCβ cDNA or pretreatment with RA (an inducer of PKCβ) restored the PMA-evoked manifestation of FN and macrophage differentiation; c) H-7, but not HA-1004, prevented the appearance of FN and macrophage differentiation in HL-60 cells or monocytes; and d) neither H-7 nor HA-1004 inhibited FN-induced macrophage differentiation in HL-525 cells.

We propose that macrophage differentiation in human progenitor cells induced by PMA or M-CSF involves the activation of PKC, phosphorylation of a variety of proteins, and induction of appropriate signal transduction steps (9, 32-34). These events lead to the expression of genes that code for the appropriate integrins including $\alpha_5\beta_1$ and ECM proteins, including FN, with the latter being produced, released, and deposited on foreign substrates. The stimulated cells, through their activated integrins, will attach and spread on these substrates by binding to specific motifs on the deposited FN and related ECM proteins. This attachment and spreading would in turn through integrin signaling lead to the activation of pertinent tyrosine kinase(s) and the expression of genes involved in the manifestation of the macrophage phenotype (35-40).

Materials and Methods

Reagents. Isotypic controls (IgG_1, IgG_{2a}, and IgG_{2b}), murine monoclonal antibodies (MAbs) to human fibronectin (FN-15, IgG_1), an α-naphthyl acetate esterase assay kit, RGDS and GPRP peptides, RA, and Ficoll-Hypaque were purchased from Sigma Chemical Co. (St. Louis, MO). MAbs to the human β_1 chain integrin, CD_{29} (K20, IgG_{2a}); and human α_5 chain integrin, $CD_{49}e$ (SAM1, IgG_{2b}) were purchased from Immunotech (Westbrook, ME). Indocarcyanine-conjugated anti-murine goat immunoglobulin ($CY3^{TM}$) was purchased from Jackson Immunoresearch Lab, Inc., (West Grove, PA), and the MAb to the human $\alpha_v\beta_3$ integrin, CD_{51}/CD_{61} MAb ($23C_G$, IgG_1) from Pharmingen (San Diego, CA). Anti-human β_4 integrin MAb was kindly provided by Dr. S. Kennel (Oak Ridge National Laboratory). M-CSF was purchased from Biosource International (Camarilo, CA), and plates precoated with either mouse laminin, collagen type I, or collagen type IV from Becton Dickinson (Bedford, MA).

Cells and Cell Culture. The human myeloid HL-60 leukemia cell line was originally obtained from R.C. Gallo (National Cancer Institute). The HL-525 and HL-534 cells (17, 18), as well as the HL-525 cells transfected with either $PKC\beta_1$ cDNA (HL-525/βI-C and HL-525/βI-F) or control vector (HL-525/neo), were established in our laboratory (9). Monocytes were obtained from heparinized whole human venous blood and separated by Ficoll-Hypaque density gradient (1.077 g/ml) centrifugation as previously described (41). The cells were incubated in tissue culture plates with RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum (Intergen Co., NY), 100 μg/ml penicillin-streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere containing 8% CO_2 at 37°C.

Differentiation markers. To determine the percentage of adherent and spread cells, we inoculated (2.5–5) × 10^5 cells in 0.5 ml of medium into each well of a 24-well tissue culture plate in the presence or absence of PMA or M-CSF. For some experiments, we precoated the surface of the wells with either FN or BSA by overnight incubation at room temperature with 0.5 ml of phosphate buffered saline (PBS) solution containing 20 μg protein/ml. The nonspecific sites were blocked with 1% BSA in PBS for 30 min. The wells were then washed with 3 mM $MnCl_2$, inoculated with the cells, and incubated for different time intervals. The percentage of cell attachment and cell spreading was determined as previously described (42). The fraction of attached cells was up to 20% higher than the percentage of attached cells with apparent spreading. Nonspecific esterase activity was determined by using the α-naphthyl acetate esterase assay kit as described by the manufacturer. Phagocytosis was determined as described previously (9) by the ability of cells to ingest sterilized and opsonized 1.7-μm (diameter) Fluoresbrite beads (Polysciences Inc., Warrington, PA). Cells were considered positive if they engulfed ≥ 20 beads per cell. To examine the blocking effect of MAbs or peptides on differentiation induction, we incubated the cells with the dialyzed MAb or the peptide for 20 min prior to, as well as during, the 2-d treatment with the inducers.

Immunofluorescence. The immunostaining procedures were carried out at 4°C using either 96-microwell plates or tissue culture chamber slides (Nunc, Inc., Naperville, IL). The cells were washed twice with PBSA (PBS containing 1% BSA and 0.1% NaN_3) and were incubated for 45 min with the appropriate primary MAb under saturating conditions. The cells were then washed again twice with PBSA and incubated for an additional 45 min with the secondary antibody

CY3™. After an additional wash with PBSA, the slides were mounted with phosphate-buffered Gelvatol™ (Becton Dickinson, Sunnyvale, CA). Fluorescence was examined using a MAC Digital Confocal microscope.

RT-PCR Analysis. RNA was purified by centrifugation through a CsCl cushion as previously described (43). cDNA was synthesized from total cellular RNA using SuperScript™ II reverse transcriptase (GibcoBRL) and the conditions recommended by the supplier. The reverse transcriptase (RT) reaction used 2 μg of total RNA and either 100 ng of oligo(dT) primer or 2 pmol of a gene-specific primer. Polymerase chain reaction (PCR) amplification used the *Tfl* polymerase (Stratagene) and conditions recommended by the supplier. The FN template primers, F1F/F2R (nucleotides [nt] 3945–3966 and 4325–4346; 396-bp product) and F5F/F6R (nt 3981–4001; 746-bp product) were derived from the human sequence (Genbank accession number X02761). The combination of primer F1F and F6R resulted in a 782-bp PCR product. The template primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), G1F/G2R (nt 713–734; 715-bp product), were derived from the human sequence (Genbank accession number X01677). One set of cycle parameters were used for all primers (denaturation at 94°C, 50; annealing at 63°C, 1 min; extension at 73°C, 1 min) with the total number of cycles (25–40) tailored to the specific primer pair. For all reactions, various amounts of the RT reaction were used to insure correspondence between the amount of amplified product and the input cDNA. For the FN amplification reactions, at least three independent primers pairs were used for each set of reverse transcriptase products to validate the amplification pattern.

References

1. Mosher, DF, Sottile, J, Wu, C, and McDonald, JA: Assembly of extracellular Matrix. Curr. Opin. Cell Biol. 4: 810–818 1992.

2. Hynes, RO: Integrin: Versatility, modulation and signaling in cell adhesion, Cell 69: 11–25, 1992.

3. Lobb, RR, and Hemler, ME: The pathophysiologic role of α_4 integrins in vivo. J. Clin. Invest. 94: 1722–1728, 1994.

4. Chen, Q, Kinch, MS, Lin, TH, Burridge, K, and Juliano, K: Integrin-mediated cell adhesion activates mitogen-activated protein kinase. J. Biol. Chem. 269: 26602–26605, 1994.

5. Hershkoviz, R, Alon, R, Gilat, D, and Lider, O: Activated T lymphocytes and macrophages secrete fibronectin which strongly supports cell adhesion. Cellular Immunology 141: 352–361, 1992.

6. Kaplan, G, and Gaudernack, G: *In vitro* differentiation of human monocytes. J. Exp. Med. 156: 1101–1104, 1982.

7. Bohnsack, JF, Chang, J, Zhou, X, and Yednock, TA: Mechanisms of β_1 integrin-dependent adherence of granulocytic HL-60 to fibronectin. J. Leuk. Biol. 37: 592–599, 1995.

8. Gudewicz, PW, Frewin, MB, Heinel, LA, and Minnear, FL: Priming of human monocyte superoxide production and arachidonic acid metabolism by adherence to collagen- and basement membrane-coated surfaces. J. Leuk. Biol. 55: 423–429, 1994.

9. Tonetti, DA, Henning-Chubb, C, Yamanishi, DT, and Huberman, E: Protein kinase C-β is required for macrophage differentiation of human HL-60 leukemia cells. J. Biol. Chem. 269: 23230–23235, 1994.

10. Vassiliadis, S, and Guilbert, LJ: Immune interferon induces early and late events in differentiation of HL-60 cells that are part of the same maturation program. Exp. Hematol. 19: 250–256, 1991.

11. Yamada, KM: Fibronectins: structure, functions and receptors. Curr. Opin. Cell Biol. 1: 956–963, 1989.

12. Mainiero, F, Pepe, A, Wary, KK, Spinardi, L, Mohammadi, M, Schlessinger, J, and Giancotti, FG: Signal transduction by the alpha 6 beta 4 integrin: distinct beta 4 subunit sites mediate recruitment of Shc/Grb2 and association with the cytoskeleton of hemidesmosomes. EMBO J. 14: 4470–4481, 1995.

13. Imamura, K, Dianoux, A, Nakamura, T, and Kufe, D: Colony-stimulating factor 1 activates protein kinase C in human monocytes. EMBO J. 9: 2423–2429, 1990.

14. Hidaka, H, Inagaki, M, Kawamoto, S, and Sasaki, Y: Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23: 5036–5041, 1984.

15. Asano, T, and Hidaka, H: Mechanism of action of a novel antivasopasm drug. J. Pharmacol. Exp. Ther. 231: 141–145, 1986.

16. Ohta, H, Sweeney, EA, Masamune, A, Yatomi, Y, Hakomori, S, and Igarashi, Y: Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. Cancer Res. 55: 691–697, 1995.

17. Homma, Y, Henning-Chubb, CB, and Huberman, E: Translocation of protein kinase C in human leukemia cells susceptible or resistant to differentiation induced by phorbol 12 myristate 13-acetate. Proc. Natl. Acad. Sci. USA, 83: 7316–7319, 1986.

18. Tonetti, DA, Horio, M, Collart, FR, and Huberman, E: Protein kinase C beta gene

expression is associated with susceptibility of human promyelocytic leukemia cells to phorbol ester-induced differentiation. Cell Growth Differen. 3: 739–745, 1992.

19. Kuender, DY, Mizobuchi, T, Kharbanda, SM, Datta, R, Huberman, E, Kufe, DW, and Stone, RM: All-trans retinoic acid reverses phorbol ester resistance in a human myeloid leukemia cell line. Blood 83: 490–496, 1994.

20. Van de Water, L, Aronson, D, and Braman, V: Alteration of fibronectin receptors (integrins) in phorbol ester-treated human promonocytic leukemia cells. Cancer Res. 48: 5730–5737, 1988.

21. Bohnsack, JF, and Chang, J: Activation of β_1-integrin fibronectin receptors on HL-60 cells after granulocytic differentiation. Blood 83: 543–552, 1994.

22. Sigurdson, SL, and Lwebuga-Mukasa, JS: Divalent cation-dependent regulation of rat alveolar epithelial cell adhesion and spreading. Exper. Cell Res. 213: 71–79, 1994.

23. Becker, S, Warren, MK, and Haskill, S: Colony-stimulating factor-induced monocyte survival and differentiation into macrophages in serum-free cultures. J. Immunol. 139: 3703–3709, 1987.

24. Meenakshi, T, Ross, FP, Martin, J, and Teitelbaum, SL: 1,25-dihydroxyvitamin D3 and macrophage colony-stimulating factor-1 synergistically phosphorylate talin. J. Cell Biochem. 53: 145–155, 1993.

25. Alitalo, K, Hovi, T, and Vaheri, A: Fibronectin is produced by human macrophages. J. Exp. Med. 151: 602–613, 1980.

26. Janiak, M, Sawicki, G, and Janowska-Wieczorek, A: Expression of β_1 integrin mRNAs in human leukemic blasts. Leukemia 8: 1544–1550, 1994.

27. Murao, S, Gemmell, MA, Callaham, MF, Anderson, NL, and Huberman, E: The control of macrophage cell differentiation in human promyelocytic (HL-60) leukemia

cells by 1,25-dihydroxyvitamin D3 and phorbol 12 -myristate 13 -acetate. Cancer Res. 43: 4989–4996, 1983.

28. Huberman, E, Weeks, C, Herrmann, A, Callaham, M, and Slaga, T: Alterations in polyamine levels induced by phorbol diesters and other agents that promote differentiation in human promyelocytic leukemia cells. Proc. Natl. Acad. Sci. USA, 78: 1062–1066, 1981.

29. Hirata, T, Bitterman, PB, Mornex, J-F, and Crystal, RG: Expression of the transferrin receptor gene during the process of mononuclear phagocyte maturation. J. Immunol. 136: 1339–1345, 1986.

30. Jungi TW, and Hafner, S: Quantitative assessment of Fc receptor expression and function during *in vitro* differentiation of human monocytes to macrophages. Immunology 58: 131–137, 1986.

31. De Nichilo MO, and Burns, GF: Granulocyte-macrophage and macrophage colony-stimulating factors differentially regulate alpha v integrin expression on cultured human macrophages. Proc. Natl. Acad. Sci. USA, 90: 2517–2521, 1993.

32. Anderson, NL, Gemmell, MA, Cousseus, PM, Murao, S, and Huberman, E: Specific protein phosphorylation in human promyelocytic HL-60 leukemia cells susceptible or resistant to induction of cell differentiation by phorbol 12 -myristate 13-acetate. Cancer Res. 45: 4955–4962, 1985.

33. Seger, R, and Krebs, EG: The MAPK signaling cascade. FASEB J. 9: 726–735, 1995.

34. Posada, J, and Cooper, JA: Requirements for phosphorylation of MAP kinase during meiosis in *Xenopus* oocytes. Science 255: 212–215, 1992.

35. Haskill, S, Johnson, C, Eierman, D, Becker, S, and Warren, K: Adherence induces selective mRNA expression of monocyte mediators and proto-oncogenes. J. Immunol.,

140: 1690–1694, 1988.

36. Haskill, S, Beg, AA, Tompkins, SM, Morris, JS, Yurochko, AD, Sampson-Johannes, A, Mondal, K, Ralph, P, and Baldwin, ASJ: Characterization of an immediate-early gene induced in adherent monocytes that encodes IκB-like activity. Cell, 65: 1281–1289, 1991.

37. Yurochko, AD, Liu, DY, Eierman, D, and Haskill, S: Integrins as a primary signal transduction molecule regulating monocyte immediate-early gene induction. Proc. Natl. Acad. Sci. USA, 89: 9034–9038, 1992.

38. Lin, TH, Yurochko, A., Kornberg, L, Morris, J, Walker, JJ, Haskill, S, and Juliano, RL: The role of protein tyrosine phosphorylation in integrin-mediated gene induction in monocytes. J. Cell Biol., 126: 1585–1593, 1994.

39. Lin, TH, Rosales, C, Mondal, K, Bolen, JB, Haskill, S, and Juliano, RL: Integrin-mediated tyrosine phosphorylation and cytokine message induction in a monocytic cell line: A possible signaling role for the Syk tyrosine kinase. J. Biol. Chem., 270: 16189–16197, 1995.

40. Fan, S-T, Mackman, N, Cui, M-Z, and Edgington, TS: Integrin regulation of an inflammatory effector gene. J. Immunol., 154: 3266–3274, 1995.

41. Laouar, A, Wietzerbin, J, and Bauvois, B: Divergent regulation of cell surface protease expression in HL-60 cells differentiated into macrophages with granulocyte macrophage colony stimulating factor or neutrophilis with retinoic acid. Int. Immunol. 5: 965–973, 1993.

42. Bauvois, B, Rouillard, D, Sanceau, J, and Wietzerbin, J: INF-gamma and transforming growth factor - β_1 differently regulate fibronectin and laminin receptors of human differentiation monocytic cells. J. of Immunol. 148: 3912–3919, 1992.

43. Chirgwin, JM, Przybyla, AE, MacDonald, AE, and Rutter, W.J: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294–5299, 1979.

Table 1. Inhibition of PMA-induced macrophage differentiation in HL-60 cells by anti-FN, anti-β_1 integrin, or anti-α_5 integrin MAbs or by RGDS

	Number of cells (10^5/ml)		Adherent and spread cells (%)		Cells showing nonspecific esterase activity (%)		Phagocytizing cells (%)	
Inhibitor	–	+PMA	–	+PMA	–	+PMA	–	+PMA
Control	8.0±0.3	2.6±0.3	≤1	91±7	≤3	68±5	≤3	83±9
Antibodies								
Anti-FN	7.3±0.5	4.4±0.3	≤1	17±7	3±2	18±2	8±4	27±11
Anti-β_1	7.6±0.1	3.8±0.3	≤1	35±4	≤3	34±3	5±1	33±5
Anti-α_5	7.2±0.2	4.1±0.3	≤1	32±7	NT	NT	4±1	38±6
IgG	7.5±0.4	2.5±0.4	≤1	87±4	≤3	65±6	3±1	84±11
Peptides								
RGDS	7.1±0.4	3.5±0.1	≤1	45±10	≤3	40±5	≤3	42±5
GPRP	7.3±0.2	2.9±0.2	≤1	88±8	≤3	76±1	≤3	73±8

The cells were incubated for 2 d with 3 nM PMA in the presence or in the absence of the antibodies or peptides: 70 μg/ml anti-FN, anti-β_1 or anti-α_5 MoAb (or isotypic control MAb, IgG) or 700 μg/ml RGDS peptide (or control peptide, GPRP). The results represent the mean ± SD of 3–8 independent experiments. NT, not tested.

Table 2. Association between acquisition of a PMA-induced macrophage phenotype and induction of FN immunostaining in HL-60 and HL-525 cells

Treatment		Adherent and spread cells (%)	Cells reactive with anti-FN MAb (%)	IgG* (%)
HL-60				
2 d control		<1	<1	<1
2 d PMA		87±5	77±4	7±4
HL-525				
2 d control		<1	<1	3±1
2 d PMA		<1	<1	4±2
HL-525/^{NEO}				
2 d control		<1	<1	5±3
2 d PMA		<1	<1	3±1
HL-525/β_1-C				
2 d control		<1	<1	<1
2 d PMA		32±7	28±9	7±5
HL-525/β_1-F				
2 d control		<1	<1	8±3
2 d PMA		45±7	40±9	5±2
HL-525				
3 d	2 d			
control	control	<1	<1	2±1
ATRA	control	<1	<1	4±3
control	PMA	<1	4±2	9±5
ATRA	PMA	62±10	57±3	11±2

HL-60 cells were treated with 3 nM PMA while HL-525 cells and the transfectant with 30 nM PMA. The concentration of RA was 1 μM. The results represent the means ± SD of 2-6 independent experiments.

*IgG column represents results obtained with mouse IgG_2 β antibody, which was used as a control for the anti-FN MAb.

Table 3. Inhibition of human FN-induced macrophage differentiation in HL-525 cells by anti-FN or anti-β_1 integrin MAb or by RGDS

	Number of cells (10^5/ml)		Adherent and spread cells (%)		Cells showing nonspecific esterase activity (%)		Phagocytizing cells (%)	
Inhibitor	BSA	FN	BSA	FN	BSA	FN	BSA	FN
Control	8.2±0.5	3.3±0.6	≤1	70±10	2±1	65±1	2±1	76±4
Antibodies								
Anti-FN	7.3±0.7	4.0±0.7	3±1	29±3	2±1	22±12	4±1	25±16
Anti-β_1	7.5±0.9	3.9±0.2	3±2	34±2	4±1	35±13	5±3	26±12
IgG	7.8±0.4	3.4±0.3	3±1	73±9	2±1	62±7	3±2	84±7
Peptides								
RGDS	7.8±0.3	4.3±0.5	2±1	23±12	3±1	15±10	3±2	30±15
GPRP	7.7±0.3	3.2±0.1	≤1	70±12	≤1	60±15	3±3	78±5

Cells were incubated for 2 d on the BSA- or FN-coated wells in the presence or in the absence of the antibodies or peptides: 70 μg/ml anti-FN or anti-β_1 MAb (or isotypic control, IgG) or 700 μg/ml RGDS peptide (or control peptide, GPRP). The results are the mean ± SD of 3-5 independent experiments.

Table 4. Inhibition of PMA- or M-CSF-induced macrophage differentiation in peripheral blood monocytes by either anti-FN or anti-β_1 integrin MAb

	Adherent and spread cells (%)			Phagocytizing cells (%)		
	–	PMA	M-CSF	–	PMA	M-CSF
Control	25±10	70±14	53±6	60±5	75±5	64±3
Antibodies						
Anti-FN	8±2	15±7	26±3	41±5	39±4	33±2
Anti-β_1	15±7	30±12	19±3	43±7	35±2	46±5
IgG	25±12	66±14	56±4	63±9	73±3	60±8

The monocytes obtained from three individuals were either treated or untreated for 2 d with 3 nM PMA or 240 U/ml M-CSF in the presence or in the absence of antibodies or peptides: 70 μg/ml anti-FN or anti-β_1 MAb (or isotypic control, IgG). The results are the mean ± SD 3-5 independent experiments.

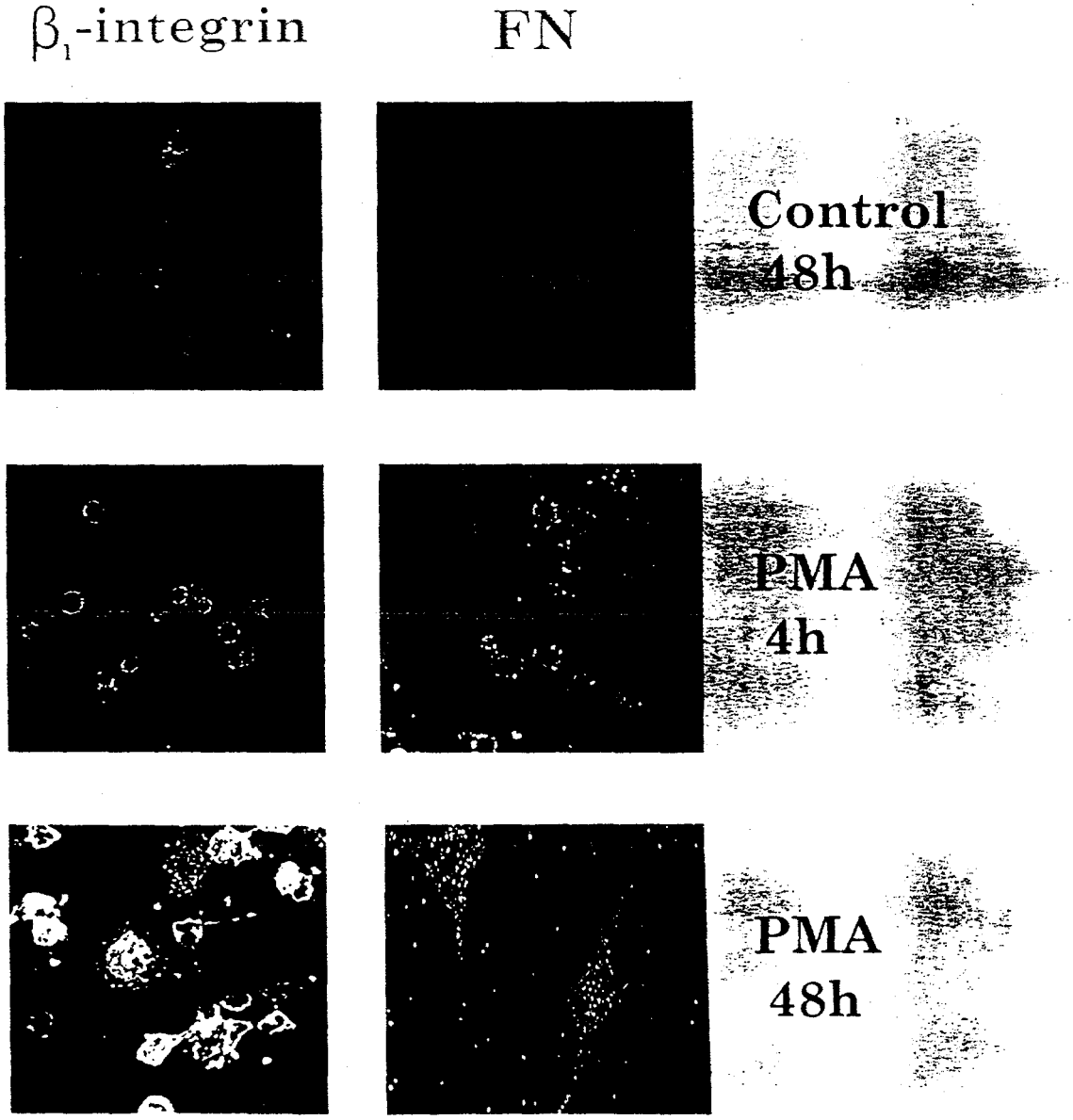
Figure Legends

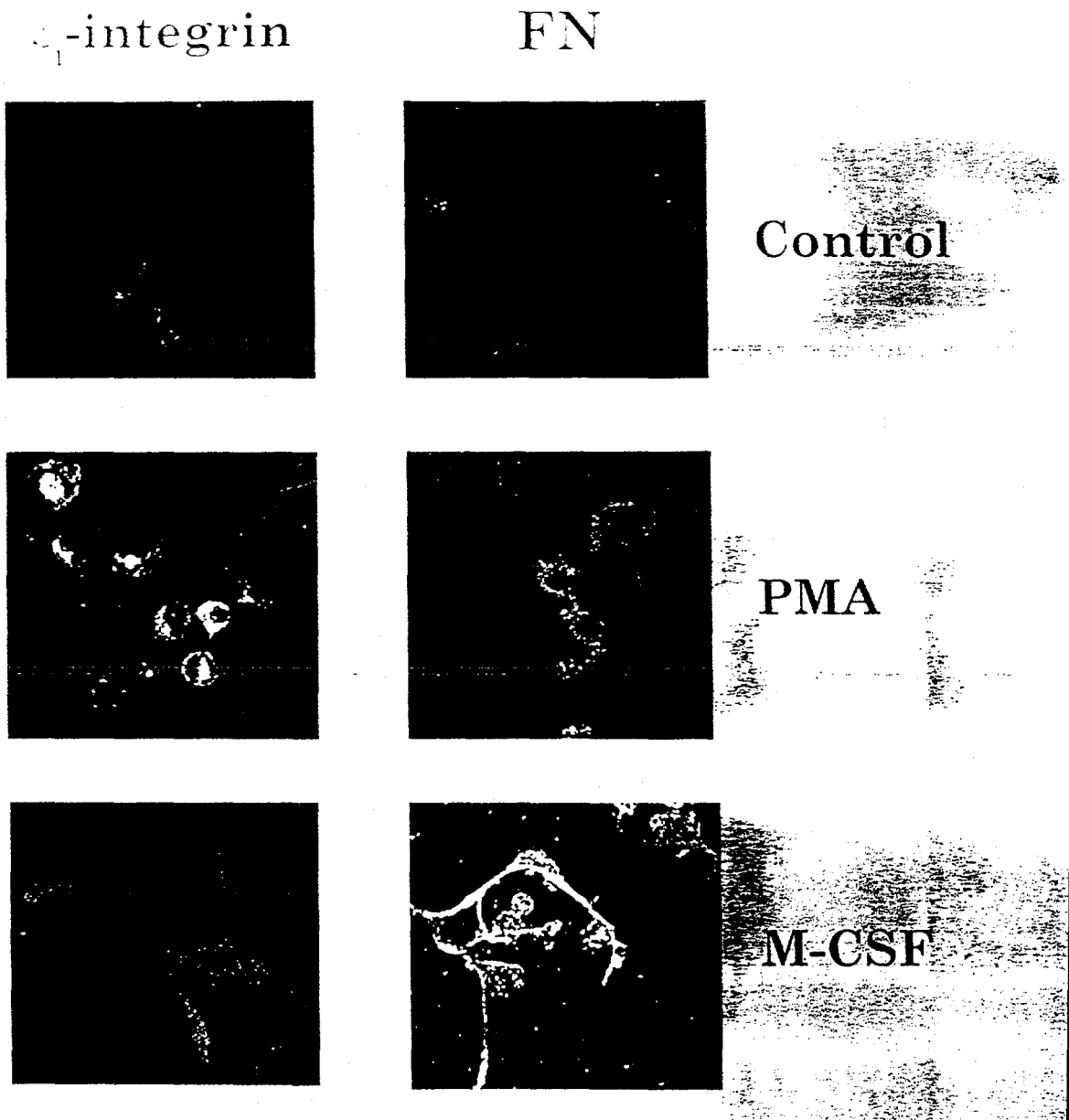
Figure 1 Manifestation of the β_1 integrin and FN antigens on the surface of untreated and PMA-treated HL-60 cells. Cells were either incubated in the absence or the presence of 3 nM PMA and were then reacted with the MAbs.

Figure 2 Manifestation of the β_1 integrin and FN antigens on the surface of untreated or either PMA or M-CSF-treated monocytes. Cells were either incubated for 48 h in the absence or the presence of 3 nM PMA or 240 U/ml M-CSF.

Figure 3 Attachment and spreading of HL-525 cells incubated in tissue culture dishes precoated for 48 h with either BSA (A) or FN (B). The pattern of FN (C) on the precoated dishes in the absence of cells was visualized after immunostaining with anti-FN MAb.

Figure 4 RT-PCR analysis of FN mRNA levels in PMA-treated HL-60 cells (A), RA/PMA-treated HL-525 cells (B), or PKC β-transfected HL-525 cells (C). HL-60 cells were treated with 3 nM PMA; HL-525 cells with 30 nM PMA and/or 1 μM RA; and PKC β-transfected HL-525 cells were treated with 30 nM PMA.

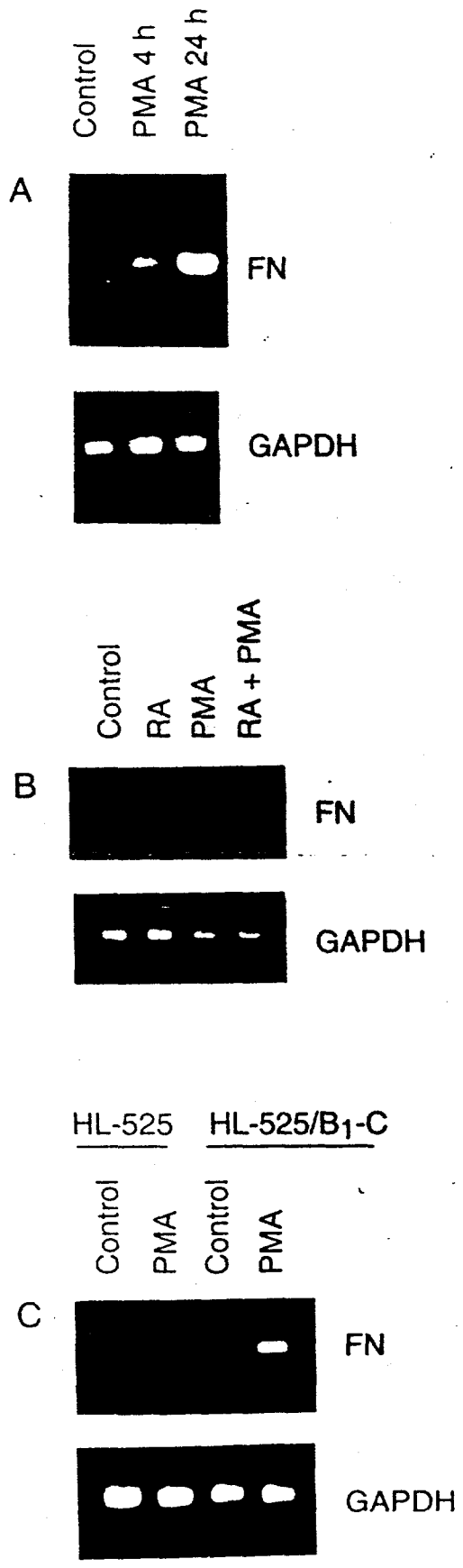

β1-integrin
FN
Control
48h
PMA
4h
PMA
48h


α_1-integrin
FN
Control
PMA
M-CSF

A

B

C


Control
PMA 4 h
PMA 24 h
A
FN
GAPDH
Control
RA
PMA
RA + PMA
B
FN
GAPDH
HL-525
HL-525/B1-C
Control
PMA
Control
PMA
C
FN
GAPDH