Autocrine Regulation of Macrophage Differentiation and 92-kDa Gelatinase Production by Tumor Necrosis Factor-α via α5β1 Integrin in HL-60 Cells*

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Tumor necrosis factor-α (TNF-α) gene is one of the early response genes induced by phorbol 12-myristate 13-acetate (PMA) in human HL-60 myeloid leukemia cells. In the present study, we examined the role of the TNF-α autocrine loop in PMA-induced macrophage differentiation and gene expression of 92- and 72-kDa gelatinases (MMP-9 and MMP-2). In HL-60 cells, PMA inhibited cell proliferation and induced cell adhesion and spreading, expression of surface maturation marker OKM1 and phagocytic activity, as well as the expression of both gelatinases, which all characterize the macrophage phenotype. In contrast, TNF-α alone was only effective in inhibiting cell proliferation. Blocking the endogenous TNF-α activity with neutralizing anti-TNF-α antibodies abolished all these PMA-induced events with the exception of MMP-2 gene expression. Since fibronectin (FN)-mediated cell adhesion and spreading are prerequisite for both macrophage differentiation and MMP-9 gene expression in HL-60 cells, we hypothesized that TNF-α might be involved in modulating the expression of either the FN or its integrin receptor genes. Whereas PMA substantially enhanced the steady state mRNA and protein levels of both FN and α5β1 integrins, TNF-α alone had little effect on the expression of these genes. However, anti-TNF-α antibodies blocked PMA-induced augmentation of both α5 and β1 integrin gene expression without affecting the expression of the FN gene. Our results suggest that TNF-α may regulate macrophage differentiation and critical matrix-degrading activities of myeloid progenitor cells in an autocrine manner by augmenting surface levels of the α5β1 integrin, thus promoting interactions with the extracellular matrix, a key event for maturation and migration of these cells during inflammation.

1 Tumor necrosis factor-α (TNF-α), an inflammatory cytokine primarily produced by activated macrophages, participates in a wide range of immunological processes which could be either beneficial or detrimental to the body (1). In addition to being a mediator of activated macrophage function, TNF-α is known to be a feedback modulator of macrophage differentiation of myeloid progenitor cells. The effects of TNF-α on proliferation and differentiation of myeloid progenitor cells are bidirectional, depending on the differentiation state and potential of the target cells as well as the hematopoietic growth factors used to promote their differentiation. Whereas TNF-α acts synergistically with macrophage-colony stimulating factor (M-CSF) to stimulate proliferation of bone marrow cells differentiating toward the macrophage lineage (2), the cytokine inhibits the proliferation and promotes macrophage differentiation of bone marrow progenitor cells in the presence of stem cell factor or granulocyte-macrophage-colony stimulating factor (G-CSF) (3–5); TNF-α exerts these effects in both an autocrine and paracrine manner. In addition, TNF-α has been shown to be identical to a differentiation-inducing factor produced by mitogen-stimulated peripheral blood monocytes and leukemic cell lines that is capable of inducing monocyte-like characteristics in a number of myeloid cell lines (6). However, how TNF-α-induced gene expression contributes to macrophage differentiation of myeloid progenitor cells remains poorly characterized.

The 92-kDa (MMP-9) and 72-kDa (MMP-2) gelatinases, which belong to the matrix metalloproteinase family, are the key proteinases governing the degradation of basement membrane (7). Both MMP-9 and MMP-2 cleave basement membrane collagen types IV and V as well as different types of gelatin (8–11). Although the two proteinases share structural and catalytic similarities, their gene expression is differentially regulated, partly due to the distinct structure of the regulatory elements and promoters in their genes (12–14). Both MMP-9 and MMP-2 are produced by human macrophages, and their proteolytic activities are thought to be necessary for various functions of monocytes and macrophages such as extravasation, migration, and tissue remodeling during chronic inflammatory conditions (15–18). Although a number of previous studies have shown that the production of these MMPs is markedly up-regulated during macrophage differentiation, the regulatory mechanisms mediating this event remain to be elucidated.

Human HL-60 myeloid leukemia cells retain the ability to differentiate along the monocyte, macrophage, or granulocyte pathway (19). This cell line, therefore, serves as a useful model system for studying the critical cellular events involved in these differentiation processes. Phorbol 12-myristate 13-acetate (PMA) induces HL-60 cells to differentiate toward the macrophage lineage (20–22), and the TNF-α gene is one of the early response genes induced by PMA during this process (23). In this study, we explored the role of TNF-α as an autocrine regulator in PMA-induced HL-60 differentiation. We examined four characteristic macrophage markers induced by PMA: (i) inhibition of cell replication; (ii) cell adhesion and spreading;
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(iii) manifestation of the surface maturation marker OKM1 (CD11b); and (iv) phagocytic activity. Although TNF-α was as effective as PMA in inhibiting cell replication, it could not induce the other three differentiation markers. However, neutralizing anti-TNF-α antibodies inhibited all four PMA-induced macrophage markers, indicating that TNF-α is an autocrine factor critical for PMA-induced macrophage differentiation in HL-60 cells. In addition, treatment with anti-TNF-α antibodies abolished PMA-induced MMP-9 gene expression without affecting the PMA-induced expression of MMP-2, suggesting that TNF-α does not mediate all PMA-induced gene expression in HL-60 cells. We demonstrate herein that one of the mechanisms whereby TNF-α modulates PMA-induced macrophage differentiation and MMP-9 gene expression is through augmenting the gene expression of the surface adhesion molecule α5β1 integrin. Our results suggest that TNF-α may act in an autocrine manner to enhance macrophage differentiation and matrix-degrading capability via promoting interactions of myeloid progenitor cells with extracellular matrix proteins.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate (PMA) was purchased from Chemicals for Cancer Research (Eden Prairie, MN). Tumor necrosis factor-α (TNF-α) (>1.0 × 10^9 units/mg) was purchased from Boehringer Mannheim. Gelatin and a mouse mononuclear antibody (mAb) to human CD11b (OKM1) (IgG3) and mAb to human FN (FN-15, IgG3), which was diazylated before use, were purchased from Sigma. Mouse mAbs to human β2 (K20, IgG2a) and α5 (SAM1, IgG2a) integrin were obtained from Immunotech (Westbrook, ME). Both mononuclear (mouse) and polyclonal (goat) anti-TNF-α neutralizing antibodies were purchased from R & D Systems (Minneapolis, MN) and were found equally effective in our study. Mouse IgG3 and goat IgG were also purchased from R & D Systems and used as a control. The experiments presented in this study were conducted by using polyclonal anti-TNF-α antibodies and goat IgG.

Cells and Cell Culture—The human HL-60 myeloid leukemia cell line was originally obtained from R. C. Gallo (National Cancer Institute). The cells were cultured and maintained in Petri dishes in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 15% heat-inactivated fetal bovine serum (Intergen Co., Purchase, NY), penicillin (100 units/ml), streptomycin (100 μg/ml), and 1-glutamine (2 mM) (Life Technologies, Inc.) in a humidified atmosphere containing 8% CO2 at 37 °C. All treatments were carried out in tissue culture dishes or cluster plates in serum-supplemented RPMI 1640 medium.

Differentiation Markers—To determine the differentiation markers, HL-60 cells were seeded at 2–4 × 10^5 cells/ml and treated for 24 h with 3 mM PMA or 10 ng/ml (>10^6 units/ml) TNF-α in the presence or absence of 10 μg/ml preimmune IgG or anti-TNF-α antibodies. The number of cells was determined by hemocytometer chamber counting; the percentage of cell adhesion and spreading was determined as described previously (24), and the percentage of cells exhibiting a cell surface maturation antigen OKM1 was determined by indirect immunofluorescent staining with the OKM1 mAb.

To examine the phagocytic activity, the cells (7 × 10^4 cells/well) were plated in eight-well Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) and treated as described above. After treatment, the cells were incubated for 18 h with 2 × 10^5 sterile and opsonized 1.27-μm (diameter) Fluoresbrite beads (Polysciences, Inc., Warrington, PA) (25). Thereafter, the medium was rapidly aspirated, and the cells fixed with 4% paraformaldehyde in PBS for 20 min prior to permeabilization for 20 min with 10 μg/ml I, α-lysophosphatidylcholine (Sigma). The cells were then stained for 10 min at room temperature with 0.1 μg/ml 4,6-diamidino-2-phenylindole (Boehringer Mannheim), followed by three washes in PBS and a 15-min incubation in hydroethidine working solution (Polysciences, Inc.). Following three final washes in PBS, the cells were mounted with phosphate-buffered gelatol (Becton Dickinson, Sunnyvale, CA) and analyzed by a Leitz Ortholux microscope. The cells were considered positive if they engulfed ≥20 beads/cell.

Indirect Immunofluorescence—The cells (7 × 10^4 cells/well) were seeded in eight-well Lab-Tek chamber slides and treated for 24 h with 3 mM PMA in the absence or presence of 10 μg/ml preimmune IgG or anti-TNF-α antibodies. After treatment, the cells were rinsed with PBS and incubated for 30 min at room temperature with a blocking solution containing 1% bovine serum albumin and 1% normal goat serum (Sigma) in PBS, followed by a 2-h incubation with the appropriate primary mAb at saturating concentrations. The cells were then washed twice with PBS and incubated for an additional 45 min with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). After three more washes with PBS, the slides were mounted with phosphate-buffered gelatol. Fluorescence was examined using the Micro-Tome Mac Digital Confocal microscope described above.

Gelatin Zymography—The cells (1.5 × 10^6 cells/ml) were treated in serum-supplemented RPMI 1640 medium as indicated in the figure legends. After treatment, the cells were replaced with serum-free medium containing the appropriate antibodies and incubated for an additional 24 h prior to collection of conditioned media. Gelatin zymography analysis was performed on 7.5% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma) as described previously (26). Gelatinolytic activity was visualized as clear zones with Coomassie Brilliant Blue R-250 staining.

RNA Isolation and Northern Analysis—Total RNA was purified by centrifugation through a cesium chloride cushion as described by Chirgwin et al. (27). Northern blot analysis was performed as described previously (28). Hybridizations were performed with radiolabeled probes at 60 °C for 18–24 h in 0.5 M sodium phosphate, pH 7.2, 2 mM EDTA, 1% bovine serum albumin, 7% SDS. The blots were washed at room temperature for 30 min in 1× SSC (1 M sodium chloride, 1× EDTA), 0.1% SDS, and once in 0.1× SSPE, 0.1% SDS, followed by autoradiographing in the dark at ~80 °C. Human cDNA probes for MMP-9 was kindly provided by Dr. W. Stetler-Stevenson, National Institutes of Health, Bethesda. Human cDNA probes for MMP-2, TNF-α, and GAPDH were obtained from American Type Culture Collection (Rockville, MD), and those for α5 and β1 integrins were from Life Technologies, Inc. The mRNA level of a specific gene relative to that of GAPDH was determined by using a HP ScanJet 4c Scanner (Hewlett-Packard).

RT-PCR Analysis—Total RNA was purified as described above. cDNA was synthesized from total cellular RNA using SuperScript™ II reverse transcriptase (Life Technologies, Inc.) under the conditions recommended by the supplier. The reverse transcriptase reaction used 1–2 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 Tli polymerase (Stratagene) under conditions recommended by the supplier. The FN template primers, F1F/F2R (nucleotides (nt) 3945–3966 and 4325–4346; 396-bp product) and F3P/F6R (nt 3981–4001 and 4706–4727; 746 bp product) were derived from the human sequence (GenBank™ accession number X01867). One set of cycle parameters was used for all primers (denaturation at 94 °C, 50 s; 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 RNA samples and the RT reaction were used to ensure correspondence between the amount of amplification product and the input of RNA samples. For the RT amplification reactions, at least three independent primer pairs were used for reverse transcriptase reaction to validate the amplification pattern.

RESULTS

Autocrine Regulation of PMA-induced Macrophage Differentiation by TNF-α—To assess the role TNF-α plays in regulating macrophage differentiation induced by PMA in HL-60 cells, we first examined its effect on PMA-induced cell adhesion and spreading, which are the hallmarks of macrophage differentiation (19). Whereas more than 90% HL-60 cells exhibited cell adhesion and spreading after a 24-h PMA treatment, TNF-α-treated cells remained in suspension and displayed no apparent morphological changes as compared with the untreated HL-60 cells (Table I and Fig. 1, a–c). Blocking the endogenous TNF-α activities by neutralizing anti-TNF-α antibodies prevented PMA-induced cell adhesion and spreading (Table I and Fig. 1d), indicating that PMA-induced cell adhesion and spreading in these cells involve the autocrine TNF-α loop.

To confirm further the role of TNF-α in PMA-induced HL-60
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Manifestation of differentiation markers in HL-60 cells treated for 24 h with 10 ng/ml TNF-α or 3 nM PMA in the presence or absence of 10 μg/ml anti-TNF-α antibodies or preimmune IgG

The results shown represent the mean ± S.D. of 3–6 independent experiments performed.

| Treatment          | Cell number<sup>a</sup> | Cell adhesion and spreading<sup>b</sup> | Cells reacting with OKM<sup>1</sup> | Phagocytizing cells<sup>d</sup> |
|--------------------|-------------------------|----------------------------------------|----------------------------------|-------------------------------|
| Control            | × 10<sup>5</sup>/ml     | <0.1                                   | ≤5                               | ≤5                            |
| TNF-α              | 2.6 ± 0.6               | <0.1                                   | ≤5                               | ≤5                            |
| TNF-α + IgG        | 2.7 ± 0.3               | <0.1                                   | ≤5                               | ≤5                            |
| TNF-α + anti-TNF-α | 5.5 ± 0.3               | <0.1                                   | ≤5                               | ≤5                            |
| PMA                | 2.5 ± 0.4               | 92 ± 5                                 | 85 ± 11                           | 89 ± 5                         |
| PMA + IgG          | 2.8 ± 0.2               | 91 ± 7                                 | 86 ± 8                           | 87 ± 11                        |
| PMA + anti-TNF-α   | 4.9 ± 0.3               | 13 ± 4                                 | ≤5                               | 24 ± 6                         |

<sup>a</sup> HL-60 cells were plated at 2 × 10<sup>5</sup> cells/ml and treated for 24 h with respective reagents as indicated. Number of the viable cells was determined by trypan blue exclusion and hemacytometer chamber counting. Each treatment was done in triplicate.

<sup>b</sup> The percentage of adherent and spread cells relative to untreated cells, which is <0.1%.

<sup>c</sup> OKM1 activity is expressed as the percentage of cells reacting with the OKM1 monoclonal antibody as determined by indirect immunofluorescent staining.

<sup>d</sup> The percentage of cells engulfing ≥20 beads/cell (range 20–80 beads/cell), which was determined from confocal microscope images as described under “Experimental Procedures.”

Fig. 1. Inhibitory effect of anti-TNF-α antibodies on PMA-induced cell adhesion and spreading in HL-60 cells. The cells (2 × 10<sup>5</sup> cells/ml) were seeded in plastic tissue culture dishes and treated with 10 ng/ml TNF-α or 3 nM PMA for 24 h in the absence or presence of 10 μg/ml neutralizing anti-TNF-α antibodies. a, untreated cells; b, TNF-α; c, PMA; d, PMA + anti-TNF-α. × 320. Note that TNF-α reduced cell number but did not affect cell morphology. Treatment with anti-TNF-α antibodies abolished PMA-induced cell adhesion and spreading and resumed cell proliferation inhibited by PMA.

differentiation, we analyzed three additional differentiation markers; the results are summarized in Table I. TNF-α at 10 ng/ml was as potent as PMA at 3 nM in inhibiting HL-60 cell proliferation at 24 h after treatment. Neutralizing anti-TNF-α antibodies (10 μg/ml) were able to largely reverse both the PMA- and the TNF-α-induced growth inhibition. In addition, treatment with PMA resulted in approximately 85% HL-60 cells exhibiting reactivity with the OKM1 mAb, which is characteristic of mature human monocytes, macrophages, and granulocytes (29). Addition of anti-TNF-α antibodies, but not the IgG control, to PMA-treated cells reduced cell reactivity with the OKM1 mAb to 5% or less, which is similar to the basal level observed in untreated or TNF-α-treated cells. Similarly, anti-TNF-α antibodies caused a more than 70% reduction in PMA-induced phagocytic activity, a primary function of mature macrophages, whereas exogenous TNF-α had no effect compared with the untreated cells. Based on these results, we conclude that TNF-α is an autocrine regulator of PMA-induced macrophage differentiation in HL-60 cells but in itself it is insufficient to induce a macrophage phenotype. Thus, additional factor(s), which have yet to be identified, are required to cooperate with TNF-α to generate sufficient signals for macrophage differentiation in HL-60 cells.

Dissociated Regulation of PMA-induced Gene Expression of 92- and 72-kDa Gelatinases by TNF-α—The induction of matrix-degrading proteinases such as the 92-kDa (MMP-9) and 72-kDa (MMP-2) gelatinases is associated with macrophage differentiation (15–17). PMA treatment (3 nM) resulted in the induction of both MMP-2 and MMP-9 gene expression (Fig. 2, A and B), with the level of MMP-2 steady state mRNA being much lower than that of the MMP-9 steady state mRNA (3 days’ exposure of the autoradiograph film for MMP-2 versus 6-h exposure for MMP-9). Accordingly, MMP-2 secretion was barely detectable by gelatin zymogram (data not shown), whereas secretion of MMP-9 proenzyme was abundant (Fig. 2C). Compared with PMA, TNF-α (10 ng/ml) only weakly induced MMP-2 and MMP-9 gene expression as well as MMP-9 proenzyme secretion. Anti-TNF-α antibodies abolished PMA-induced MMP-9 but not MMP-2 gene expression (Fig. 2, A and B). The effect of the antibody treatment on secretion of the MMP-9 proenzyme (Fig. 2C) mirrored that on its mRNA. These findings suggest that while PMA-induced MMP-9 gene expression is mediated through the TNF-α autocrine loop, PMA-induced MMP-2 gene expression is independent of the endogenous TNF-α activity.

TNF-α Autocrine Loop Is Essential but Not Sufficient for PMA-induced Gene Expression of α5β1 Integrin—Since we found previously that both PMA-induced macrophage differentiation<sup>2</sup> and MMP-9 gene expression (30) required FN-mediated cell adhesion and spreading, and anti-TNF-α antibodies blocked this process, we suspected that TNF-α might be the intermediary that controls PMA-induced gene expression of FN or its surface integrin receptors. To confirm this hypothesis, we examined the expression of the FN and α5β1 integrin genes in PMA- and TNF-α-treated HL-60 cells, and we determined the effect of anti-TNF-α antibodies on these events. We did not

<sup>2</sup> A. Laouar, C. B. H. Chubb, F. R. Collart, and E. Huberman, manuscript in preparation.
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including the α5β1 integrin in this study because this integrin was not involved in FN-mediated cell adhesion and spreading induced by PMA or M-CSF in both HL-60 and human peripheral blood monocytes. Expression of both α5 and β1 integrin genes was examined by Northern blotting analysis. The FN gene expression was assayed by reverse transcriptase-polymerase chain reaction (RT-PCR), because its expression was induced in low abundance and Northern blotting analysis yielded inconsistent results. Three sets of FN primers were tested, and the results shown in Fig. 3A represent amplification of a single 365-bp FN fragment. Various amounts of RNA samples and the RT reaction were used to ensure that amplification of the 365-bp FN fragment corresponded to the input of RNA samples. PMA (3 nM) induced a 5-fold increase in the level of FN steady state mRNA (Fig. 3A), which was followed by cell surface display and extracellular deposition of the FN protein (Fig. 4). Similarly, PMA also enhanced the steady state level of β1 integrin mRNA by 4-fold and α5 integrin mRNA by 6-fold (Fig. 3B), with concomitant increases in surface expression of the respective protein (Fig. 4). TNF-α alone failed to affect expression of FN or α5 or β1 integrin gene. Blocking the endogenous TNF-α activity with anti-TNF-α antibodies had little or no effect on PMA-induced FN steady state mRNA and protein levels (Fig. 3A and Fig. 4), demonstrating that PMA-induced FN expression requires neither the autocrine TNF-α loop nor cell adhesion and spreading. In contrast, treatment with anti-TNF-α antibodies resulted in a substantial reduction in PMA-induced β1 and α5 gene expression (Fig. 3B and Fig. 4). Accordingly, surface levels of both β1 and α5 proteins stimulated by PMA were substantially inhibited by anti-TNF-α antibodies (Fig. 4). Taken together, our results have shown that TNF-α is an autocrine mediator for PMA-induced augmentation of α5β1 integrin gene expression, but TNF-α alone is insufficient and necessitates additional factor(s) to stimulate α5β1 gene expression. Our findings were further supported by the temporal sequence of expression of the FN, TNF-α, and α5β1 integrin genes induced by PMA. As shown in Fig. 5A, PMA induced an early and transient expression of the TNF-α gene. Peak induction of the TNF-α steady state mRNA was observed at 2 h after PMA treatment, which was maintained for up to 4 h. The TNF-α steady state mRNA levels then dropped markedly at 8 h and became nearly undetectable at 24 h after treatment. The induction of TNF-α gene expression was followed by aug-

Fig. 2. Effect of anti-TNF-α antibodies on PMA-induced MMP-2 and MMP-9 gene expression in HL-60 cells. The cells (4 × 10⁶ cells/ml) were either untreated (Control) or treated with 10 ng/ml TNF-α or 3 nM PMA in the absence or presence of 10 μg/ml preimmune IgG or anti-TNF-α antibodies in serum-supplemented RPMI 1640 medium. At 24 h, cells were harvested for RNA isolation. RNA samples (20 μg/lane) were resolved on a 1.2% agarose gel containing 2.2 M formaldehyde as described under “Experimental Procedures.” The blots were hybridized to radiolabeled cDNAs for MMP-2 (A) or MMP-9 (B) and autoradiographed for 3 days or 6 h, respectively. Hybridization to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to demonstrate equal loading of the RNA samples. C, cells were treated as above. After incubation for 24 h, cells were replaced with serum-free medium and incubated for additional 24 h prior to collection of conditioned media for analysis of gelatinolytic activity of MMP-9 by gelatin zymogram as described under “Experimental Procedures.” The gelatinolytic activity of MMP-9 proenzyme is visualized as a clear band against a dark background.

Fig. 3. Effect of anti-TNF-α antibodies on α5β1 integrin and fibronectin gene expression in PMA- or TNF-α-treated HL-60 cells. The cells were either untreated (Control) or treated for 24 h with 10 ng/ml TNF-α or 3 nM PMA in the absence or presence of 10 μg/ml preimmune IgG or anti-TNF-α antibodies. After incubation, the cells were harvested for RNA isolation. A, FN mRNA levels were analyzed by RT-PCR as described under “Experimental Procedures.” A single 365-bp fragment was detected. Amplification of GAPDH mRNA was used as a quantitative control for RNA samples and amplification efficacy. Various amounts of RNA samples and the RT reaction were used to ensure that the amount of the amplification product corresponded to that of RNA samples used. The results were reproducible with RNA samples isolated from three independent treatments. B, RNA samples (20 μg/lane) were resolved on a 1.2% agarose gel containing 2.2 M formaldehyde and subject to Northern blot analysis as described. The blot was hybridized sequentially to radiolabeled cDNAs for β1 and α5 integrins. GAPDH hybridization was used to demonstrate the equal loading of RNA samples.
ment expression of the $\beta_1$ and $\alpha_5$ genes, which started at 4–8 h after addition of PMA and steadily increased thereafter for up to 24 h (Fig. 5A). On the other hand, induction of the FN gene expression occurred within 30 min after addition of PMA, and the FN steady state mRNA levels increased steadily for at least 24 h after PMA treatment (Fig. 5B). Therefore, induction of FN gene expression occurs before that of TNF-$\alpha$, which is consistent with our conclusion that PMA-induced FN expression is independent of the autocrine TNF-$\alpha$ activities.

**DISCUSSION**

In this study, we have shown that a TNF-$\alpha$ autocrine loop is required, but not sufficient, for macrophage differentiation induced by PMA in HL-60 cells. Consistent with our finding, several previous studies have established TNF-$\alpha$ as a competence factor which primes the progenitor cells at early stages of macrophage differentiation. TNF-$\alpha$ acts in concert with other hematopoietic growth factors to inhibit cell proliferation and promote maturation, and this action may be achieved in either an autocrine or paracrine fashion. TNF-$\alpha$ was found to be expressed in all colonies of bone marrow progenitor cells induced to differentiate toward the macrophage lineage by M-CSF or GM-CSF, regardless of the differentiation stages, suggesting an important role of this molecule during macrophage differentiation (31). Blocking the endogenous TNF-$\alpha$ activity during GM-CSF-induced macrophage differentiation in bone marrow progenitor cells resulted in increased cell proliferation, suggesting the involvement of an autocrine mechanism in which TNF-$\alpha$ expression signals the onset of differentiation and the cessation of proliferation (4); there seemed to be a time window during which the differentiating cells were responsive to the endogenous production of TNF-$\alpha$, since blocking TNF-$\alpha$ expression was effective on day 3 of differentiation but not on other days. Similarly, in a study using neonatal cord blood-derived stem cells (5), it has been found that there is a window of sensitivity related to the priming effects of TNF-$\alpha$; stem cells pretreated with TNF-$\alpha$ for up to 3 days responded to the differentiating effects of GM-CSF, and after 5 days of TNF-$\alpha$ pretreatment, GM-CSF was unable to promote maturation. Although the cellular events triggered by TNF-$\alpha$ during macrophage differentiation in these progenitor cells remain to be clarified, our findings in HL-60 cells provide hints that one of these events may be the stimulation of gene expression of surface adhesion molecules such as $\alpha_5\beta_1$ integrin, thus promoting interactions of immature cells with the marrow microenvironment. In accordance with this hypothesis, synthesis and surface expression of the $\alpha_5\beta_1$ integrin are stimulated by M-CSF, and mature monocytes constitutively express an abundance of this integrin (33), suggesting that appearance of this integrin is an event accompanying macrophage differentiation. Indeed, we found that blocking the endogenous TNF-$\alpha$ activity with neutralizing anti-TNF-$\alpha$ antibodies failed to affect PMA- or M-CSF-induced cell adhesion and maturation in human peripheral blood monocytes, suggesting that TNF-$\alpha$ acts at differentiation stages preceding the monocyctic maturation.

During macrophage differentiation, one of the major changes in gene expression is the induction of matrix metalloproteinases (MMPs) such as 92- and 72-kDa gelatinases (MMP-9 and MMP-2, respectively) (15–17). Production of these enzymes is thought to be critical for extravasation and migration of mono-

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3 B. Xie and E. Huberman, unpublished results.
cytes and macrophages through the extracellular matrix (18). We have shown in a separate report (30) that differentiation-associated MMP-9 gene expression in HL-60 cells and in human peripheral blood monocytes requires FN-mediated cell adhesion and spreading; signaling of the FN-induced MMP-9 gene expression is channeled through the $a_\beta_1$ integrin receptor and apparently does not involve the $a_\beta_1$ receptor. Dependence of PMA-induced MMP-9 gene expression on FN/integrin-mediated cell adhesion and spreading is further confirmed by our finding in this study; treatment with neutralizing anti-TNF-a antibodies causes down-regulation of $a_\beta_1$ integrin gene expression, resulting in substantial inhibition of cell adhesion and spreading as well as of MMP-9 gene expression. The autocrine regulation of TNF-a is not restricted to the MMP-9 gene expression. In U937 cells, PMA-induced gene expression of interstitial collagenase (MMP-1) is also significantly reduced by anti-TNF-a antibodies (34), suggesting that TNF-a is a key molecule in controlling the critical matrix-degrading activities during macrophage differentiation. The MMP whose expression escapes the control of TNF-a is MMP-2, providing another example of dissociated regulation of this MMP and other members of the MMP family. It is intriguing that PMA induces MMP-2 gene expression in HL-60 (this study) and U937 cells (16), although its promoter does not contain an AP-1 site (12). Since we noted an 8-h lag phase for PMA-induced MMP-2 gene expression, it is possible that induction of MMP-2 is not directly mediated by PMA but rather by factors induced by PMA.

In summary, we have shown in the present study that TNF-a acts as a feedback regulator of PMA-induced macrophage differentiation in HL-60 cells. The cytokine also plays a role in controlling the gene expression of matrix-degrading proteases such as MMP-9 during PMA-induced macrophage differentiation. Our findings suggest that during inflammatory responses, TNF-a may cooperate with other hematopoietic factors to promote maturation of myeloid progenitor cells and their migration through the extracellular matrix by modulating the gene expression of integrins, the key cell surface receptors for matrix macromolecules.

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