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Research Article

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12-o-Tetradecanoyl-phorbol-13-acetate–differentiated U937 Cells Express a Macrophage-like Profile of Neutral Proteinases

High Levels of Secreted Collagenase and Collagenase Inhibitor Accompany Low Levels of Intracellular Elastase and Cathepsin G

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Abstract

Human monocytic tumor cells of the U937 cell line contain substantial quantities of two neutrophil neutral proteinases, elastase and cathepsin G, raising the question of whether their presence reflects an expression of transformation or whether normal monocytes undergo a developmental stage in which they produce certain neutrophil proteinases. To address this issue, we examined U937 cells for production of collagenase, since human alveolar macrophages release fibroblast-like collagenase, an enzyme that is distinct from neutrophil collagenase. Using an immunoassay that utilized antibody to skin fibroblast collagenase, we found that U937 cells secreted barely detectable quantities of enzyme, 10–12 ng/10^6 cells per 24 h, under basal conditions. Upon incubation with 10 nM 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA), however, collagenase release increased 200-fold, comparable to the amount secreted by phorbol-stimulated human fibroblasts. Metabolic labeling and immunoprecipitation confirmed the enhanced synthesis of U937 cell collagenase upon TPA exposure. This enzyme activity further resembled fibroblast collagenase and differed from neutrophil collagenase by exhibiting preferential cleavage of monomeric type III collagen relative to type I. As previously observed with human alveolar macrophages, U937 cells also released a protein identical to the collagenase inhibitor produced by human skin fibroblasts, a molecule not associated with neutrophils. Release of this inhibitor increased 10-fold with TPA exposure.

In contrast to collagenase and collagense inhibitor, TPA-treated U937 cells contained only 10–15% as much elastase and cathepsin G activities as control cells. Thus, TPA-induced differentiation modified the presence of these enzymes in the direction of their content in normal monocytes. Since the neutral proteinase profile of undifferentiated U937 cells resembles that of neutrophils and changes markedly after cellular differentiation to one that is characteristic of monocytes, these data suggest that neutrophilic proteinases may be produced by normal monocytes during the early stages of their differentiation.

Introduction

U937 cells are a continuous line of human cells of committed monocytic origin. They have attracted considerable attention because of widespread interest in mononuclear phagocytes and because they can be grown readily in culture and are susceptible to manipulations that result in their differentiation and acquisition of phenotypic characteristics of mature monocytes (1–3). We and others have reported that U937 cells contain substantial amounts of neutrophil elastase and cathepsin G, enzymes previously found only in polymorphonuclear leukocytes (4–6). These findings raised the question of whether U937 cells depict the proteinase profile of normal immature monocytes, which may have a developmental stage involving the production of certain neutrophil proteinases, or whether these cells have an anomalous profile of proteinases. Since human alveolar macrophages secrete a collagenase and a collagenase inhibitor that are immunologically and functionally indistinguishable from the collagenase and collagenase inhibitor released by human fibroblasts (7), and distinct from any such proteins contained within neutrophils, we thought it would be of interest to examine U937 cells for the production of collagenolytic activity.

We have observed that U937 cells in their basal state produced barely detectable amounts of collagenase, but after exposure to 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA)[1] and subsequent cellular differentiation, there was a 200-fold increase in the secretion of this enzyme. By kinetic and immunologic criteria, the collagenase activity appeared to be identical to the collagenase elaborated by human alveolar macrophages, and unrelated to neutrophil collagenase. U937 cells also released the collagenase inhibitor typically produced by fibroblasts (8, 9) and macrophages (7, 10), a protein not associated with neutrophils. Whereas U937 cells produced essentially no collagenase in the basal state, the collagenase inhibitor was released by undifferentiated cells in significant amounts, but the levels of inhibitor nevertheless increased 10-fold upon exposure of the cells to TPA. Concomitant with the effects of TPA upon collagenase and collagenase inhibitor expression, there were marked reductions in cellular elastase and cathepsin G activities. These findings indicate that differentiation of U937 cells is accompanied by a shift from a neutrophilic to a monocytic profile of neutral proteinases.

Methods

Reagents. Dibutyryladenosine 3′5′-cyclic monophosphate (cAMP), TPA, formyl-methionyl-leucyl-phenylalanine (fMLP), succinyl-alanyl-alanyl-alanine-*p*-nitroanilide, succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide, and bovine pancreatic trypsin were obtained from Sigma

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1. *Abbreviations used in this paper:* cAMP, dibutyryladenosine 3′5′-cyclic monophosphate; ELISA(s), enzyme linked immunosorbent assay(s); fMLP, formyl-methionyl-leucyl-phenylalanine; TPA, 12-*o*-tetradecanoyl-phorbol-13-acetate; vitamin D_3, 1,25-dihydroxyvitamin D_3.

Chemical Co., St. Louis, MO. Purified human neutrophil elastase and cathepsin G were kindly provided by Dr. Edward J. Campbell, and 1,25-dihydroxyvitamin D_3 (vitamin D_3) by Dr. Steven L. Teitelbaum, both at The Jewish Hospital of St. Louis, MO. Human collagen types I (placenta), II (cartilage), and III (placenta) were generously supplied by Dr. Robert E. Burgeson, Shriner's Hospital, Portland, OR. [^{3}H]leucine, 122.5 Ci/mmol, was obtained from Amersham Corp., Boston, MA; [^{35}S]cysteine, 600 Ci/mmol, was obtained from New England Nuclear, Boston, MA. Culture media and tissue culture supplements were from the Basic Cancer Center, Washington University Medical Center, St. Louis, MO.

Cell culture. U937 cells, obtained from Dr. Hillel Koren, Duke University Medical Center, Durham, NC, were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine, as previously described (4). According to the experimental protocol, cell culture medium included either TPA (1.6×10^{-10}–1.6×10^{-6} M), cAMP (10^{-3} M) or vitamin D_3 (10^{-10}–10^{-6} M). TPA was added to culture medium immediately before use from a 1.6×10^{-3} M TPA in dimethyl sulfoxide stock solution that was stored at −20°C. cAMP was dissolved into culture medium directly. Vitamin D_3 was solubilized in 95% ethanol. At the conclusion of the incubation period, cell-conditioned medium was collected by centrifuging cultures, 200 *g* for 10 min, and removing the supernatant. In the case of adherent cells, as occurred with TPA exposure, cells were collected by scraping after a 10-min period on ice with cold phosphate-buffered saline, pH 7.5. To prepare cell extracts, cells were adjusted to 5×10^7/ml, then subjected to three cycles of freeze-thawing (dry ice–methanol) and sonification on ice (2-min cycles at 0.5 setting; Dismembranator; Fisher Scientific Co., Pittsburgh, PA).

Cell-conditioned medium. For studies of collagenase and collagenase inhibitor production using TPA as a differentiating agent, conditioned media were collected for time periods up to 72 h. When cAMP was used to induce differentiation, U937 cells were exposed to this agent for 72 h, after which the conditioned medium was removed and replaced with fresh medium for another 72 h. In studies employing vitamin D_3 for differentiation, culture medium was changed daily for 6 d. After collection, all conditioned media samples were adjusted to 0.05 M Tris, pH 7.5, containing 0.01 M $CaCl_2$, and then analyzed for collagenase and collagenase inhibitor.

Assay procedures. Elastase activity was measured by the solubilization of ^{14}C-labeled elastin (11). Cathepsin G activity was determined using succinyl-alanyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide as substrate and by monitoring absorbance at 410 nm (5, 12). Purified human leukocyte elastase (13) and cathepsin G (14, 15) were used as standards for these assays. Collagenase and collagenase inhibitor levels were quantified by enzyme-linked immunosorbent assays (ELISAs) (9, 16). The collagenase ELISA uses polyclonal antibody directed against human skin fibroblast collagenase that does not cross-react with the collagenolytic enzyme produced by human polymorphonuclear leukocytes (16). Functional collagenase activity was monitored by the solubilization of reconstituted guinea pig skin type I collagen fibrils which had been biosynthetically labeled with [^{14}C]glycine (17). Since collagenase is secreted as a zymogen (18, 19) and the conditioned medium contained 10% fetal calf serum, enzyme activation required exposure to large amounts of trypsin. Accordingly, trypsin titrations were performed using a range of concentrations from 0.5 to 50 μg of trypsin/100 μl of conditioned medium to ensure maximal procollagenase activation. Generally, 10–20 μg of trypsin per sample of conditioned medium incubated at room temperature for 10 min produced optimal collagenase activity. The action of trypsin was stopped by the addition of a fivefold molar excess of soybean trypsin inhibitor.

DNA content was determined as described by Burton (20). Total protein synthesis was measured by exposing cultures to serum-free medium containing [^{3}H]leucine, 100 μCi/ml, for 6 h followed by precipitation in cold 20% trichloroacetic acid. Cell numbers were quantified by a particle counter (Sysmex Microcellcounter CC 130; Tao Medical Electronics, Carson, CA) and protein concentrations of the cell extracts were determined spectrophotometrically (21).

Chemotaxis was determined using modified Boyden chambers and a double micropore membrane system (5 μm overlaying 0.45 μm) as previously described for monocyte chemotaxis (22). Accordingly, 2.5×10^6 cells/ml were added to the upper compartment, fMLP was added to the lower compartment, and the chamber incubated for 2 h at 37°C. The number of cells migrating to the interface between the membranes was determined by light microscopy, × 400.

Metabolic labeling and immunoprecipitation of collagenase and collagenase inhibitor. Synthesis of collagenase and collagenase inhibitor were demonstrated by metabolic labeling and immunoprecipitation. Cells were carefully washed several times with leucine-free media (collagenase) or cysteine-free media (collagenase inhibitor) and then cultured overnight in the same media containing either [^{3}H]leucine or [^{35}S]cysteine (50–100 μCi/ml of medium) alone or with TPA, 10^{-8} M. For immunoprecipitations performed in the presence of 10% fetal calf serum, the serum was dialyzed overnight against 0.05 M Tris, pH 7.5, containing 0.01 M $CaCl_2$ before being added to culture medium. Aliquots of the pulsed conditioned media, 400 μl, were mixed with an equal volume of immune buffer composed of phosphate-buffered saline (pH 7.4) containing 0.02% (wt/vol) sodium azide, 0.2% (vol/vol) Triton X-100, 1 mM phenylmethylsulfonylfluoride and 0.1% (wt/vol) bovine serum albumin to which 90 μl of 10% (wt/vol) IgGsorb (The Enzyme Center, Inc., Malden, MA) were added. The mixtures were held on ice for 10 min and then centrifuged at 10,000 *g* for 10 min. 6 μl of either anticollagenase IgG (18, 19) or anticollagenase inhibitor IgG (8, 9) were added to the supernatants for 1 h at 30°C and then overnight at 4°C. Immune complexes were separated by the addition of 60 μl of 10% (wt/vol) IgGsorb and incubation for 30 min at room temperature. The IgGsorb pellets were washed with immune buffer and then resuspended in electrophoresis sample buffer (0.065 M Tris-HCl, pH 6.8, containing 2.4% [wt/vol] sodium dodecyl sulfate and 10% [wt/vol] glycerol) and incubated for 10 min at 60°C. β-mercaptoethanol was added to a final concentration of 2% (vol/vol) and then the samples were boiled for 3–4 min. Autoradiography was performed by subjecting the samples to polyacrylamide gel electrophoresis (23), after which the slab gels were dried and then exposed to XAR-5 X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70°C.

Kinetic studies of U937 collagenase activity. U937 cell-conditioned medium was tested for collagenolytic activity against monomeric collagen types I, II, and III in solution at 25°C. Values for the Michaelis constant (K_m) and relative rates of catalysis against these collagen types were determined by incubating reaction mixtures containing increasing concentrations of substrate with trypsin-activated conditioned medium. The TC^A digestion products were then quantified by densitometric scanning of stained polyacrylamide slab gels (24) and the data plotted according to the method of Lineweaver and Burk.

Stoichiometry of collagenase inhibitor activity. Collagenase inhibitor activity could not be quantified by direct assay of serum-free conditioned media because of the high levels of collagenase concomitantly secreted after TPA exposure and the tendency for zymogen activation under serum-free conditions. Therefore serum-containing TPA-conditioned media were employed, thus preventing proteolytic activation of procollagenase and subsequent binding to inhibitor. In order to assay such media for inhibitory activity, however, collagenase inhibitor had to be separated from both procollagenase and α_2-macroglobulin. To accomplish this, 500 ml of TPA-conditioned medium was subjected to 0–60% ammonium sulfate precipitation, resuspended in 0.01 M Tris, pH 7.5, containing 0.0001 M $CaCl_2$, and dialyzed against several changes of this buffer. The 0–60% fraction was then applied to a 10 × 2.5-cm column of carboxymethylcellulose (CM 52; Whatman Inc., Clifton, NJ). Collagenase inhibitor and α_2-macroglobulin do not bind to this matrix while procollagenase adheres tightly (8, 18). The fall-through fractions were pooled, dialyzed against 0.05 M Tris, pH 7.5, and applied to a 10 × 2.5-cm column of phosphocellulose (P-ll; Whatman, Inc.). Collagenase inhibitor binds avidly to this matrix, whereas α_2-macroglobulin falls through (8). The bound inhibitor was eluted with 0.5 M $(NH_4)_2SO_4$, dialyzed against 0.005 M Tris, pH 7.5, and lyophilized for concentration. This partially purified inhibitor preparation, free of procollagenase and α_2-macroglobulin, was then assayed for capacity to inhibit pure fibroblast collagenase. The molar stoichiometry of inhibition was quantified by

calculating moles of collagenase inhibited per mole of immunoreactive protein, the latter determined using an ELISA for collagenase inhibitor (9).

Results

Collagenase and collagenase inhibitor. U937 cells were placed in culture for 72 h either in the presence or absence of TPA, and the conditioned media was analyzed for collagenase and collagenase inhibitor by ELISAs for each protein (9, 16). As shown in Fig. 1 *A*, U937 cells in their basal state released barely detectable quantities of collagenase, ~3 ng/μg DNA/72 h (10–12 ng/10^6 cells per d). After the addition of TPA to culture medium for 72 h, however, collagenase secretion increased dramatically, to ~600 ng/μg DNA/72 h, a level in excess of 200-fold that of basal levels. Such quantities equal or surpass the collagenase production of phorbol-stimulated normal human macrophages (7) and fibroblasts (25). The dose response to TPA paralleled the acquisition by U937 cells of other monocytic markers of differentiation (26); maximal collagenase expression was reached at 10^{-8} M TPA.

Whereas U937 cells produced essentially no collagenase in the basal state, the collagenase inhibitory protein was released by undifferentiated cells in significant amounts (Fig. 1 *B*), ~12 ng/μg DNA per 72 h (50 ng/10^6 cells per d), levels which are 30–50% of the basal secretory rates of normal alveolar macrophages in culture (7). Nevertheless, upon exposure to TPA, inhibitor production was augmented 10-fold, reaching levels (120 ng/μg DNA per 72 h; 500 ng/10^6 cells per d) comparable to phorbol-stimulated macrophages (7) although less than the amount secreted by stimulated fibroblasts (2–3 μg/10^6 cells per d; [25]). The dose response of collagenase inhibitor production to TPA mirrored that of collagenase, also reaching maximum values at 10^{-8} M concentration of the differentiating agent.

The time course of TPA's effect upon U937 collagenase and collagenase inhibitor production is shown in Fig. 2. An increase in the amounts of both proteins secreted into cell-conditioned media was apparent by 8–16 h after the start of TPA exposure. As also shown in Fig. 1, release of inhibitor by U937 cells in their basal state exceeded that of collagenase. However, after cellular differentiation by TPA, both the fold-stimulation and

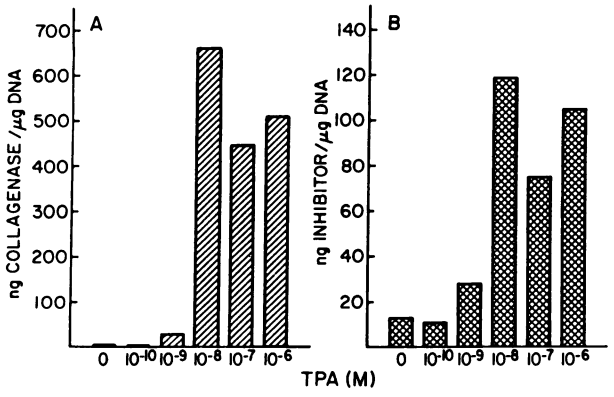

Figure 1. Dose response of U937 cell collagenase (*A*) and collagenase inhibitor (*B*) production after exposure to TPA. U937 cells (~5 × 10^5/2 ml of medium) were incubated in culture medium containing various concentrations of TPA for 72 h. Collagenase and collagenase inhibitor in the cell-conditioned media were then quantified by ELISA. The actual TPA concentration is 1.6 times the molarity shown in the figure (e.g., 1.6 × 10^{-8} M, etc.).

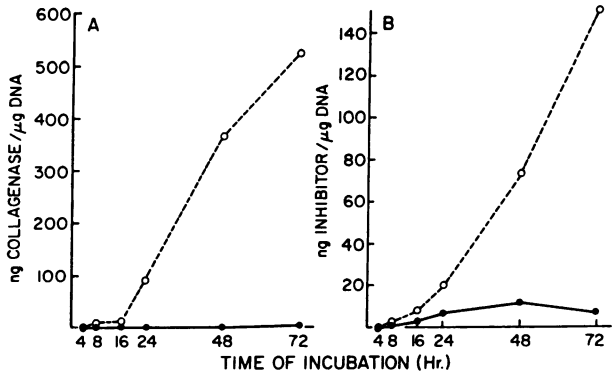

Figure 2. Time course of U937 cell collagenase and collagenase inhibitor production after exposure to TPA. U937 cells (~7.5 × 10^6/30 ml of medium) were incubated in culture medium, with or without TPA, 1.6 × 10^{-8} M. At various time points, 1 ml of conditioned medium was removed and concentrations of collagenase and collagenase inhibitor determined by ELISA. (○) Conditioned media containing TPA; (●) conditioned media without TPA.

absolute amounts of enzyme produced significantly surpassed the analogous values for inhibitor. Hence, it was possible to detect collagenase activity after TPA. Consistent with observations in normal macrophages (7), HL-60 cells (10), and fibroblasts (25), virtually all of the collagenase and inhibitor produced by U937 cells was secreted into culture medium; there was no evidence for significant intracellular storage of either protein (data not shown).

Both the collagenase and collagenase inhibitor proteins released by TPA-differentiated U937 cells appear to be immunologically and functionally equivalent to their counterparts produced by alveolar macrophages and skin fibroblasts. Using specific antibody preparations directed at each fibroblast protein, metabolic labeling and immunoprecipitation studies were performed on U937 cells in their basal state and after exposure to TPA. As shown in Fig. 3, each specific antibody precipitated a protein species of identical electrophoretic mobility to either pure procollagenase (Fig. 3 *A*, lanes *2* and *5*) or pure collagenase inhibitor (Fig. 3 *B*, lanes *1* and *2*). The doublet nature of the immunoprecipitated procollagenase (18, 19) and the characteristically broad staining pattern of the inhibitor (8, 9) were readily apparent. Furthermore, comparison of metabolically labeled conditioned media samples from U937 cells in their basal state with those obtained after the induction of cellular differentiation (Fig. 3 *A*, lane *1* vs. lane *2* and lane *4* vs. lane *5*; Fig. 3 *B*, lane *1* vs. lane *2*) confirms the effects of TPA shown by ELISA (Figs. 1 and 2) and also demonstrates that the collagenase and inhibitor secreted after TPA exposure represent newly synthesized proteins.

U937 cell-conditioned media contained collagenolytic activity that cleaved type I, II, and III collagens in a manner characteristic of mammalian collagenases. At 25°C, only a single three-fourths/one-fourth cleavage was observed, producing typical three-fourths–length TC^A and one-fourth–length TC^B fragments (not shown). As shown in Table I, the affinity of U937 collagenase for human collagen types I–III was similar, K_m = 0.6–1.9 μM, values indistinguishable from those displayed by human alveolar macrophage and skin fibroblast collagenases (7, 24). Analysis of the relative susceptibilities of these monomeric collagens to proteolytic attack revealed preferential degradation of type III collagen relative to type I, and the extremely low rate

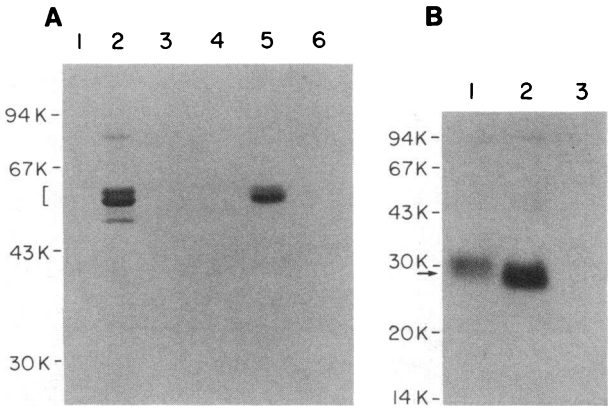

Figure 3. Immunoprecipitation of metabolically labeled U937 cell-derived collagenase and collagenase inhibitor. U937 cells were incubated in serum-containing medium with or without TPA (1.6×10^{-8} M) for 48 h. The cells were then exposed to fresh serum-free or serum-containing medium containing either [^{3}H]leucine (collagenase) or [^{35}S]cysteine (inhibitor) for 24 h and the medium processed as detailed under Methods. (*A*) Collagenase: lane *1*, untreated cells, serum-free, plus specific collagenase antibody; lane *2*, TPA-treated cells, serum-free, plus specific collagenase antibody; lane *3*, TPA-treated cells, serum-free, plus preimmune antibody; lane *4*, untreated cells, serum-containing, plus specific collagenase antibody; lane *5*, TPA-treated cells, serum-containing, plus specific collagenase antibody; lane *6*, TPA-treated cells, serum-containing, plus preimmune antibody. The bracket denote the migration position of pure human skin fibroblast procollagenase doublet (molecular weights of 60,000 and 55,000). The doublet protein bands present in lane *2* of relative molecular weight, ~52,000 and 47,000, represent active collagenase, probably activated by other proteinases in serum-free medium (18, 19). (*B*) Collagenase inhibitor: lane *1*, untreated cells, serum-containing, plus specific inhibitor antibody; lane *2*, TPA-treated cells, serum-containing, plus specific inhibitor antibody; lane *3*, TPA-treated cells, serum-containing, plus preimmune antibody. The arrow denotes the migration position of pure human skin fibroblast collagenase inhibitor.

of type II cleavage, features also exhibited by alveolar macrophage and skin fibroblast enzymes. Selective cleavage of type I collagen monomers as compared with type III, a distinguishing feature of neutrophil collagenase activity (27), was never observed in U937 conditioned media or cellular extracts. The functional activity of the U937 inhibitory protein likewise paralleled that of its macrophage and fibroblast counterparts (7–9). Using the ELISA to quantify the amount of collagenase inhibitor present in U937 conditioned media, an ~1:1 molar stoichiometry of inhibition was observed between the inhibitor and exogenously added pure fibroblast collagenase (see Methods; data not shown).

In contrast to the effects observed with TPA, the use of cAMP (1) and vitamin D_3 (3, 28, 29) as differentiating agents resulted in only inconsistent and modest stimulation of U937 collagenase and collagenase inhibitor expression. The addition of cAMP (1 mM) to culture media in several experiments caused a variable increase in the secretion of both collagenase and inhibitor proteins, but in every case the magnitude of stimulation even after 6 d of conditioning was always <20% of that observed with TPA. Exposure to vitamin D_3 (10^{-7} M) stimulated release of collagenase in only one of four experiments, and to only 15% of the levels produced with TPA; collagenase inhibitor secretion was not affected whatsoever. Both cAMP and vitamin D_3 diminished the proliferative capacity of treated U937 cells (data not shown).

Elastase and cathepsin G. Cell extracts prepared from undifferentiated cells and from cells exposed to TPA (10^{-8} M) for 72 h were examined for elastase and cathepsin G activities. As shown in Table II, both enzyme activities of the TPA-treated cells decreased to 10–15% of the levels found in the basal state. These observations were not due to the development of an associated inhibitor, as the cell extracts exhibited no inhibition against pure neutrophil elastase or cathepsin G. Moreover, immunostaining demonstrated a marked diminution in immunoreactive elastase and cathepsin G within permeabilized cells (Senior, R. M., D. Burnett, and N. L. Connolly, unpublished observations). Vitamin D_3 and cAMP each caused only modest decreases in the cellular activities of elastase and cathepsin as compared with TPA (Table II).

Chemotaxis to fMLP. Although vitamin D_3 and cAMP exerted only inconsistent and small effects on collagenase and collagenase inhibitor production, both agents caused a marked increase in the chemotactic responsiveness of U937 cells to fMLP, consistent with the observations of others ([1], Table III). The chemotactic responsiveness of TPA-treated cells was difficult to assess because exposure to this agent caused the cells to become tightly adherent, requiring vigorous manipulation in order to harvest them for use in chemotaxis assays, thus possibly causing cellular injury. In any event, it is clear that the maturational

Table I. Comparison of the Collagen Specificity of U937 and Skin Fibroblast Collagenases

Enzyme source	Collagen type	K_m	Relative rate of cleavage*
		μM	
U937	I	0.6	27.8
	II	1.1	1.0
	III	1.9	426
			k_{cat} (h^{-1})§
Skin Fibroblast‡	I	0.8	53.4
	II	2.1	1.0
	III	1.4	565

* From Lineweaver-Burk plots (see Methods). The rate of human type II cleavage by U937-conditioned medium has been arbitrarily set equal to 1.0.

‡ Values shown are actual catalytic rates from Welgus, et al. (24).

§ k_{cat}, catalytic rate.

Table II. Effects of Cellular Differentiation on Elastase and Cathepsin G Activities of U937 Cell Extracts

	Enzyme activity, percent of control undifferentiated cells	
Differentiating agent	Elastase	Cathepsin G
None	100	100
TPA	12	14
cAMP	82	75
Vitamin D_3	79	51

Results are means of a minimum of two separate studies done in duplicate. Elastase and cathepsin G activities of cell extracts were determined as described under Methods.

Table III. Effects of Cellular Differentiation on Chemotactic Responsiveness of U937 Cells

Differentiating agent	Migrating cells per high power field
None	2
cAMP	154
Vitamin D_3	71

Results are means of a minimum of two separate studies done in triplicate. Chemotaxis to fMLP was performed as described under Methods. Results shown are the maximal responses observed, which occurred at 10^{-8} M fMLP.

changes induced by various differentiating agents are quite distinct as regards the capacity for alteration of the neutral proteinase profile of U937 cells and for promotion of responsiveness to chemotactic stimuli (Table IV).

Discussion

Recently, we and others reported that U937 cells in their basal undifferentiated state contain substantial amounts of neutrophil elastase and cathepsin G, two serine neutral proteinases previously associated only with neutrophils (4–6). These findings raised the question of whether U937 cells depict the proteinase profile of normal immature monocytes, which may undergo an early developmental stage involving the production of certain neutrophil proteinases, or whether these enzymes are an expression of transformation. The data in the present report indicate that undifferentiated U937 cells produce negligible amounts of collagenase, but differentiation induced by TPA results in a dramatic increase in collagenase production and the collagenase released by differentiated cells is immunologically and functionally identical to alveolar macrophage collagenase and thus distinct from the collagenolytic enzyme found in neutrophils. Macrophage and neutrophil collagenases are readily distinguished by their opposite selectivities against monomeric type I and III collagens (27), by their lack of cross-reactivity to monoclonal and polyclonal antibody preparations (30), and by the fact that neutrophil collagenase is stored within granules (31) while the macrophage enzyme is secreted without significant intracellular storage (7). Likewise, the collagenase inhibitor elaborated by undifferentiated U937 cells, whose expression is markedly increased by exposure to TPA, is a proven secretory product of alveolar macrophages (7) and is neither contained in nor released from neutrophils (8). Thus, with respect to proteins that regulate collagen turnover, U937 cells after TPA exposure accurately reflect what has been observed with normal mature mononuclear phagocytes.

Table IV. Effects of Various Differentiating Agents on U937 Neutral Proteinase Production and Chemotactic Responsiveness

Differentiating agent	Cathepsin G activity	Elastase activity	Collagenase	Collagenase inhibitor	Chemotaxis to fMLP
None	++++	++++	(+)	+	0
TPA	(+)	(+)	++++	++++	ND
cAMP	++	++	+	++	++++
Vitamin D_3	++	++	+	+	++++

+ to ++++, denote increasing levels. (+), denotes barely detectable levels. ND, not determined.

Our data regarding elastase and cathepsin G activities also support the notion that U937 cells undergo differentiation in a manner that is consistent with normal mononuclear phagocyte development. Exposure to TPA and subsequent differentiation is accompanied by a profound decrease in the intracellular content of these serine proteinases (Table II), so that the cells become much more like normal monocytes, which have very low levels of these two enzymes (4, 5). Differentiation with lymphokine-rich media has also been reported to markedly reduce the cathepsin G activity of U937 cells (6), a finding consistent with our observations using TPA. It is noteworthy that elastase and cathepsin G are the principal proteinase constituents of neutrophil azurophilic granules and that normal immature bone marrow monocytes have been shown to undergo an early developmental stage in which they transiently contain such granules (32). The positive effects of TPA-induced differentiation on U937 collagenase and collagenase inhibitor expression and the negative effects upon elastase and cathepsin G activities all suggest that differentiated U937 cells possess a neutral proteinase profile characteristic of mature monocytes/macrophages. Thus it appears plausible that the expression of neutrophil proteinases is a feature of normal mononuclear phagocyte development, however, this question can be answered conclusively only by examination of normal immature monocytes. Of note, a recent report (33) providing evidence in support of this concept demonstrates that human peripheral blood monocytes contain serine proteinase elastase activity that is replaced by metalloproteinase elastase activity when the cells are allowed to undergo in vitro maturation into macrophages.

An interesting finding of this study relates to the spectrum of maturational changes that differentiating agents can induce in U937 cells. The data presented suggest that effects on neutral proteinases and chemotactic responsiveness during maturation are not necessarily linked, and that individual agents may rather selectively promote the induction of certain properties of mature monocytes in U937 cells. Thus, vitamin D_3 and cAMP did not consistently augment the synthesis of collagenase or collagenase inhibitor proteins or substantially decrease the intracellular elastase or cathepsin G activities (Table II), yet each caused a dramatic increase in the chemotactic responsiveness of the cells to fMLP, a differentiation feature due to acquisition of fMLP receptors on the cell surface (1). While a part of TPA's pronounced effect on U937 cell collagenase and inhibitor expression may reflect the stimulatory action of this agent on the release of such proteins, as has been observed in mature fibroblasts (25) and macrophages (7), rather than a direct modulation of the differentiation process, the marked diminution in observed elastase and cathepsin G activities would seem to militate against this argument. Rather, the disparate effects of cAMP and TPA upon the neutral proteinase profile of U937 cells may be better explained by the biochemical mechanisms underlying their ability to function as differentiating agents. Both induce the activation of protein kinases (34, 35), but the protein kinase C activated by phorbol ester binding to the cell surface membrane is distinct from cAMP-dependent protein kinase. Thus, a different set of cellular proteins is ultimately phosphorylated by each agent, presumably mediating the expression of separate pathways of cellular development. In any event, it seems clear from the effects of vitamin D_3 and cAMP upon U937 cells that the maturational

changes associated with neutral proteinase production are separable from other features of differentiation, such as chemotactic responsiveness. From these findings one must conclude that caution is necessary in generalizing about the differentiating effects of an agent based upon a particular cell function or feature.

It may be significant that U937 cells in their basal undifferentiated state release collagenase inhibitor in amounts almost comparable to the amounts secreted by normal alveolar macrophages, but release essentially no collagenase. In this regard it is of interest to compare them with cells from another human leukocytic stem cell line, HL-60. HL-60 cells do not express either collagenase or collagenase inhibitor in the basal state; however, when induced to differentiate along a monocytic lineage by phorbol esters or vitamin D_3, HL-60 cells secrete large amounts of collagenase inhibitor but still fail to produce collagenase (10). Since HL-60 cells are more immature than U937, having the capacity to develop into either myelocytic or monocytic cells, whereas U937 are committed on the monocytic pathway, it appears that the onset of production of collagenase inhibitor precedes collagenase production in the developing mononuclear phagocyte.

Mononuclear phagocytes have been implicated in the remodeling of extracellular matrix. Among their activities potentially involved in such remodeling is production of collagenase, the enzyme that initiates the cleavage of native collagen (36) and of inhibitory molecules that specifically block this enzyme's catalytic function. A number of studies have demonstrated the release of collagenase activity from animal mononuclear phagocytes (37, 38) and recently these observations have been extended to human peripheral blood monocytes (39) and alveolar macrophages (7) in culture. The production of both collagenase and collagenase inhibitor proteins by alveolar macrophages (7) and by TPA-differentiated U937 cells provide conclusive evidence that human mononuclear phagocytes can directly modulate the turnover of collagen. The expression of collagenase and collagenase inhibitor by mononuclear phagocytes and its implications for the pathogenesis of disease states are currently under investigation.

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