

## Induction of Collagenase Production in U937 Cells by Phorbol Ester and Partial Purification of the Induced Enzyme

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**ABSTRACT:** The U937 cell line is a monoblast-like cell line that can be induced to differentiate when treated with phorbol ester or a variety of other agents. Collagenase was detected in the media of U937 cell cultures after treatment with phorbol myristate acetate (PMA) at concentrations of 5 ng/mL or greater. In general, no collagenase was detected in the media of untreated cells. The induced collagenase cleaved native type I collagen into the  $3/4$  and  $1/4$ -length fragments and showed the inhibition by ethylenediaminetetraacetic acid characteristic of the action of mammalian collagenases. Collagenase activity could be detected in the media of treated cells 12–18 h after the addition of PMA. Secretion of collagenase continued for 2–3 days after PMA addition. The production of collagenase by PMA-treated U937 cells was inhibited by actinomycin D and cycloheximide, suggesting that the induction of the enzyme is the result of de novo synthesis. The collagenase secreted by U937 cells induced with PMA has been purified 12-fold by using DEAE-Sephacel followed by wheat germ agglutinin-agarose chromatography. The apparent molecular mass of this U937 collagenase, determined by gel filtration chromatography on the partially purified enzyme, was 29–36 kilodaltons.

Mammalian collagenases are specific metalloenzymes that cleave native, triple-helical collagen at a single site. Collagenases are required for the initiation of collagen breakdown since native collagen is resistant to attack by other proteolytic enzymes. After cleavage by collagenase, the collagen fragments denature under physiological conditions and are susceptible to degradation by other proteases (Harris & Vater, 1982; Harper, 1980).

Macrophages appear to play a significant role in wound healing (Leibovich & Ross, 1975) and other situations in which collagen breakdown and connective tissue remodeling occur. Collagenase synthesis by macrophages from a number of sources has been demonstrated. These include guinea pig peritoneal macrophages stimulated in vitro with endotoxin (Wahl et al., 1974), peritoneal macrophages from mice injected with thioglycolate broth (Werb & Gordon, 1975), human (Senior et al., 1972; Welgus et al., 1985a) and rabbit (Robertson et al., 1973; Horwitz & Crystal, 1976; Mainardi et al., 1980) alveolar macrophages, and human peripheral blood monocytes (Louie et al., 1984). Mammalian collagenases have been isolated or partially purified from cultured human skin fibroblasts (Stricklin et al., 1977), rabbit (Werb & Reynolds, 1976; Vater et al., 1981) and pig (Cawston & Tyler, 1979) synovial fibroblasts, human polymorphonuclear leukocytes (Murphy et al., 1982; Christner et al., 1982; Callaway et al., 1986), rat uterine cells (Roswit et al., 1983), and mouse bone cultures (Sakamoto et al., 1978). The relationship between the enzymes produced by the different cell types is not yet well understood. Macrophages and polymorphonuclear leukocytes are derived from the same stem cell. However, collagenase produced by human alveolar macrophages has been shown to have immunological cross-reactivity to the human skin fibroblast enzyme (Welgus et al., 1985a) while a monoclonal antibody raised to the enzyme from polymorphonuclear leukocytes did not recognize human skin fibroblast collagenase (Hasty et al., 1984).

The U937 cell line is a human monoblast-like cell line with characteristics of an immature monocyte (Sundstrom & Nilsson, 1976; Nilsson et al., 1981). Treatment of U937 cells with a number of agents induces differentiation and the acquisition of features of monocyte-macrophages (Harris & Ralph, 1985). These agents include phorbol ester (Nilsson et al., 1981; Gidlund et al., 1981; Ralph et al., 1982; Hattori et al., 1983), conditioned medium from mixed lymphocyte culture (Larrick et al., 1980), retinoic acid (Olsson & Breitman, 1982), 1,25-dihydroxyvitamin D<sub>3</sub> (Rigby et al., 1984), and interferons (Hattori et al., 1983; Guyre et al., 1983). U937 cells treated with phorbol ester show increased adherence and a monocyte-macrophage-like morphology (Nilsson et al., 1981; Hattori et al., 1983). Increased performance of macrophage functions such as phagocytosis, expression of Fc receptors, and direct and antibody-dependent cellular cytotoxicity is also seen (Ralph et al., 1982).

We have been interested in isolating the macrophage collagenase that cleaves interstitial collagen (types I, II, and III) and chose to study phorbol ester induced U937 cells as a source for this enzyme. Phorbol ester has been shown to stimulate collagenase production by rabbit synovial (Brinckerhoff et al., 1979; Aggeler et al., 1984a,b) and human skin fibroblasts (Clark et al., 1985; Salo et al., 1985) and by human peripheral blood monocytes (Louie et al., 1984). We have found that U937 cells treated with phorbol ester secrete a collagenase that degrades type I collagen and provide a convenient source for this enzyme. This report describes some of the characteristics of this phorbol ester induced collagenase production, a partial purification of the secreted collagenase, and the determination of the apparent molecular weight of the partially purified enzyme. To our knowledge, the only enrichment and physical characterization of a macrophage collagenase reported so far is the work done on a type V collagenase from rabbit alveolar macrophages (Mainardi et al., 1984).

### EXPERIMENTAL PROCEDURES

**Materials.** Phorbol 12-myristate 13-acetate (PMA)<sup>1</sup> was

obtained from Sigma Chemical Co. 4-*O*-Methylphorbol 12-myristate 13-acetate (MPMA) was obtained from P-L Biochemicals. [ $^3\text{H}$ ]Collagen (*N*-[*propionate*-2,3- $^3\text{H}$ ]propionylcollagen, rat tail tendon, type I) and ENHANCE were purchased from New England Nuclear. Trypsin [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated] and deoxyribonuclease I were purchased from Worthington. Ribonuclease A and wheat germ agglutinin-agarose were obtained from Sigma and DEAE-Sephacel from Pharmacia. Media and antibiotics were obtained from Irvine Scientific, fetal calf serum from Hyclone and lactalbumin hydrolysate from Sigma.

PMA and MPMA were dissolved in dimethyl sulfoxide and stored as 0.1–1 mg/mL stock solutions at  $-20^\circ\text{C}$ . The stock solutions were diluted with media just before use. Dimethyl sulfoxide was present in the media at concentrations of 0.1% or less.

**Growth and Treatment of Cells.** The U937 cell line was obtained from American Type Culture Collection (CRL 1592). The cells were grown in RPMI-1640 containing 20% fetal calf serum, 4 mM glutamine, 200 units/mL penicillin G, and 200  $\mu\text{g}/\text{mL}$  streptomycin. Cultures were maintained at  $37^\circ\text{C}$  in the presence of 5%  $\text{CO}_2$ . For inductions with phorbol esters the cells were washed once with serum-free media consisting of RPMI-1640 containing 0.2% lactalbumin hydrolysate, 4 mM glutamine, 200 units/mL penicillin G, and 200  $\mu\text{g}/\text{mL}$  streptomycin. The cells were resuspended in serum-free media ( $5 \times 10^7$  cells/25 mL), placed in 150-cm $^2$  tissue culture flasks, and incubated for 1–3 h. Nonadherent cells were poured off, serum-free medium containing PMA or MPMA (5–100 ng/mL) was added, and the cells were incubated for 1–72 h at  $37^\circ\text{C}$ .

For assays of intracellular collagenase activity, lysis buffer (50 mM Tris-HCl, pH 7.5/200 mM NaCl/15 mM  $\text{CaCl}_2$ /0.1% Triton X-100) was added to the U937 cell pellet, and the cells were lysed with 10 strokes of a type B Dounce homogenizer. The lysate was centrifuged at 1800g for 15 min to remove nuclei. A portion of the cytosolic fraction was treated with 1  $\mu\text{g}/\text{mL}$  trypsin for 20 min at room temperature; the reaction was stopped by the addition of phenylmethanesulfonyl fluoride to 2 mM. Both trypsin-treated and untreated portions of the extract were assayed for collagenase activity.

**Collagenase Assays.** Levels of active collagenase present in conditioned media were determined by incubation of samples with 3–6.5  $\mu\text{g}/\text{mL}$  [ $^3\text{H}$ ]collagen in the presence of 40–50 mM Tris-HCl, pH 7.5, and 5% glycerol except where noted otherwise. In order to avoid precipitation of calcium with phosphate from the medium, no calcium was added to the reaction mixture. In some experiments *N*-ethylmaleimide was added to the assay mixtures to activate any latent collagenase that might be present. In general, the assay samples were incubated at  $25^\circ\text{C}$  for 3–4.5 h. In some cases, noted in the text and figure legends, 20–22-h incubation times were used. The reactions were stopped by addition of EDTA to 25 mM. The reaction mixtures were dried in a speed-vac concentrator (Savant), dissolved in sample buffer (80 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 100 mM dithiothreitol, 15% glycerol, and 0.006% bromphenol blue), and electrophoresed on SDS-polyacrylamide gels [10% acrylamide and 0.13% bis(acrylamide); Maniatis et al., 1982]. The gels were treated with ENHANCE for fluorography. XAR-5 film (Kodak) was

exposed to the gels for 16–20 h, and the autoradiograms were scanned with a densitometer (Helena). The extent of reaction was calculated from the areas under the  $\alpha$ -1(I) $^A$  and  $\alpha$ -1(I) peaks.

Collagenase present in partially purified samples was assayed in 20 mM Tris-HCl/5 mM  $\text{CaCl}_2$ , pH 7.5. The assay procedure was the same as that described earlier.

A unit of enzyme activity is defined as the amount of collagenase required to cleave 1  $\mu\text{g}$  of collagen in 1 h. In assays on crude media, cleavage of collagen continued for approximately 9 h. When assays with long incubation times (20–22 h) were done, the results are presented as the total amount of collagen cleaved or the percent of the collagen substrate cleaved. Approximate values for the number of collagenase units present were calculated by using a 9-h cutting time.

**Enzyme Purification.** One and one-half liters of conditioned media was collected from  $7.5 \times 10^8$  cells treated with 50 ng/mL PMA in serum-free media for 48 h. The medium was centrifuged at 2500 rpm for 10 min and then filtered through a 0.45- $\mu\text{m}$  filter. Phenylmethanesulfonyl fluoride and sodium azide were added to final concentrations of 1 mM and 0.02%, respectively. The following steps were carried out at  $4^\circ\text{C}$  except where noted otherwise. The conditioned medium was concentrated 30-fold by ultrafiltration with a YM30 membrane (Amicon). The concentrated medium was diluted 8-fold with 20 mM Tris-HCl/25 mM NaCl, pH 7.5, and reconstituted to the original volume. This step was repeated twice to remove the PMA, and the sample was then concentrated to 30 mL.  $\text{MgCl}_2$  was added to a final concentration of 10 mM, and the sample was treated with deoxyribonuclease I (1  $\mu\text{g}/\text{mL}$ ) and ribonuclease A (1  $\mu\text{g}/\text{mL}$ ) for 1 h at  $37^\circ\text{C}$ . Phenylmethanesulfonyl fluoride (1 mM, final concentration), soybean trypsin inhibitor, aprotinin, and leupeptin (1  $\mu\text{g}/\text{mL}$  each, final concentration) were added. The sample was dialyzed against 2 L of 20 mM Tris-HCl/25 mM NaCl/0.05% Brij-35, pH 8.2, overnight and then chromatographed on a DEAE-Sephacel column (1.6  $\times$  6.5 cm) equilibrated with the same buffer. The column was eluted with 144 mL of the starting buffer followed by the same buffer with 250 mM NaCl (172 mL) and then buffer with 1 M NaCl (38 mL). The flow rate was 15 mL/h; 3.2-mL fractions were collected.

The column fractions (1–9) containing the bulk of the collagenase activity were pooled, concentrated to 5 mL by ultrafiltration using a YM30 membrane, and dialyzed overnight against 1 L of 20 mM Tris/1 mM  $\text{CaCl}_2$ /0.5 M NaCl/0.05% Brij-35, pH 7.8. This sample was applied to a wheat germ agglutinin-agarose column (0.3  $\times$  40 cm) equilibrated with the same buffer. The column was eluted with 37 mL of the starting buffer followed by 27 mL of the same buffer containing 250 mM *N*-acetyl-D-glucosamine. The flow rate was 1 mL/h; 0.9-mL fractions were collected. Fractions 25–31 (pool I) and 32–42 (pool II) were combined, and each pool was concentrated to 2 mL by ultrafiltration.

The protein profiles for all of the columns were determined by reading the absorbance at 280 nm. Protein levels in the concentrated starting material, DEAE-Sephacel pool, and wheat germ agglutinin-agarose pools were determined from the amino acid composition. Samples were heated in 5.7 N hydrochloric acid at  $110^\circ\text{C}$  for 24 h in vacuo and then analyzed on a Beckman 6300 amino acid analyzer. The protein bands in SDS-polyacrylamide gels were visualized by silver staining using a Bio-Rad silver stain kit.

## RESULTS

**Induction of Collagenase Production by U937 Cells—Dose Response.** When U937 cells ( $5 \times 10^7$  cells) in serum-free

<sup>1</sup> Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; Brij-35, poly(oxyethylene) 23-lauryl ether; EDTA, ethylenediaminetetraacetic acid; MPMA, 4-*O*-methylphorbol 12-myristate 13-acetate; NEM, *N*-ethylmaleimide; PMA, phorbol 12-myristate 13-acetate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

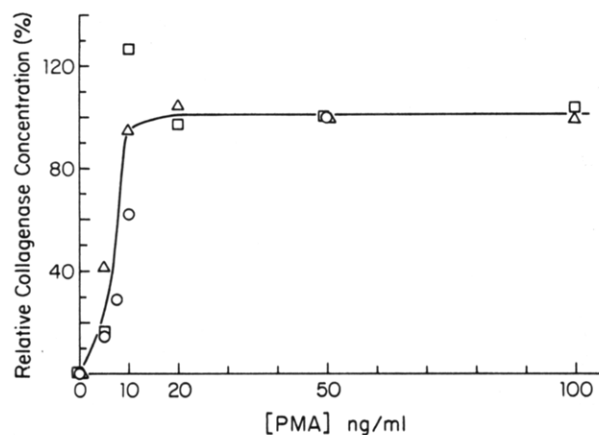


FIGURE 1: Levels of collagenase activity in conditioned media from PMA-treated U937 cells as a function of PMA concentration. Levels of collagenase activity observed with 50 ng/mL PMA were set at 100%; enzyme levels observed at other PMA concentrations were calculated as a percent of the value observed with 50 ng/mL PMA. Results of three experiments are shown: (1) Cells were treated with 0 and 5–50 ng/mL PMA, and samples of conditioned media were assayed by using an incubation time of 4.5 h. The level of collagenase activity in the conditioned media from cells treated with 50 ng/mL PMA was 0.3 unit/mL (○). (2) Cells were treated with 0 and 5–100 ng/mL PMA, and samples of media were assayed using an incubation time of 3 h. The level of collagenase activity obtained from cells treated with 50 ng/mL PMA was 0.7 unit/mL (□). (3) Cells were treated with 0 and 5–100 ng/mL PMA, and samples of media were incubated with collagen for 20 h in the absence of Tris buffer and glycerol. In this experiment, 1.4  $\mu$ g of collagen was cleaved in 20 h with 1 mL of medium from cells treated with 50 ng/mL PMA. This level of cutting in 20 h corresponds to approximately 0.15  $\mu$ g/h (Δ).

media (25 mL) were placed in tissue culture flasks (150 cm<sup>2</sup>), approximately half of the cells adhered to the flask. The U937 cells selected by adherence were treated with varying amounts of PMA in serum-free media, and the conditioned media were assayed for collagenase activity after 24 h of treatment. The results of three experiments are presented in Figure 1. Collagenase activity could be detected in the media of cells treated with 5 ng/mL PMA, and the collagenase levels rose as the PMA concentration was increased from 5 to 10 ng/mL. No collagenase activity was detected in the media of cells treated with serum-free media alone. In two of the experiments, maximal induction was achieved by treatment with 10 ng/mL PMA. In the third experiment, collagenase levels in media of cells treated with 10 ng/mL PMA were 60% of the levels in media of cells treated with 50 ng/mL PMA. In this experiment, we did not use enough PMA concentrations greater than 10 ng/mL to determine the amount of PMA required for maximal induction of collagenase. The collagenase levels observed in the conditioned media in these three experiments varied from approximately 0.15 to 0.7 unit/mL for cells treated with 50 ng/mL PMA. The collagenase detected in the media cleaved collagen into the approximately  $3/4$ - and  $1/4$ -length fragments (see Figure 2A) seen with other mammalian collagenases (Harper, 1980) and showed the inhibition by EDTA characteristic of mammalian collagenases (Harris & Vater, 1982).

In two dose-response experiments, the U937 cells were also treated with varying amounts of methylphorbol myristate acetate (5–100 ng/mL), a phorbol ester derivative with little tumor-promoting activity (Hecker, 1978). These experiments were designed to determine whether PMA is specifically required for the induction of collagenase. In the experiment in which the collagenase level in media from cells treated with PMA (50 ng/mL) for 24 h was 0.7 unit/mL, no collagenase activity (less than 0.02 unit/mL) could be detected in media

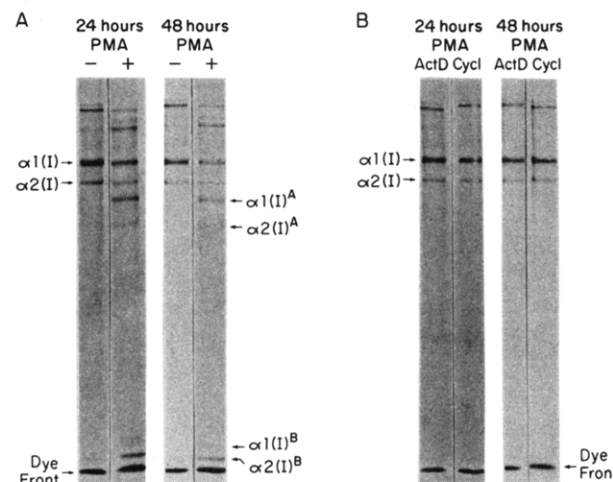
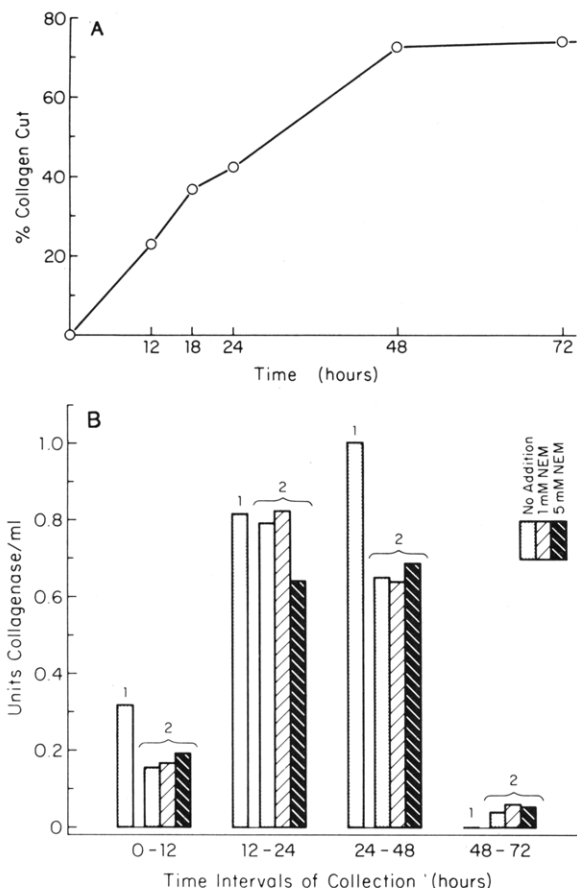


FIGURE 2: Autoradiogram of an SDS-polyacrylamide gel of [<sup>3</sup>H]-collagen cutting assays. (A) Conditioned media were harvested from U937 cells treated with 100 ng/mL PMA or serum-free media for 24 or 48 h. Samples of the conditioned media (100  $\mu$ L) were incubated with [<sup>3</sup>H]collagen for 22 h at 25 °C. The assay mixtures were electrophoresed on an SDS-polyacrylamide gel to separate intact collagen [ $\alpha$ -1(I) and  $\alpha$ -2(I) bands] from cleaved collagen [ $\alpha$ -1(I)<sup>A</sup>,  $\alpha$ -2(I)<sup>A</sup>,  $\alpha$ -1(I)<sup>B</sup>, and  $\alpha$ -2(I)<sup>B</sup> bands]. (B) Conditioned media were harvested from U937 cells treated with actinomycin D (ActD) and cycloheximide (Cycl) concomitantly with 100 ng/mL PMA. Media samples were assayed for collagenase activity as described in (A).

from MPMA-treated cells. In the experiment in which treatment with PMA (10 and 50 ng/mL) led to production of collagenase at levels of 0.2 and 0.3 unit/mL, treatment with MPMA (50 ng/mL) for 24 h yielded a collagenase concentration of 0.06 unit/mL. No significant collagenase activity was detected in media from cells treated with lower doses of MPMA.

**Time Course.** In order to measure the time course of collagenase production, either parallel cultures of U937 cells were treated with 50 ng/mL PMA and the conditioned media harvested from individual flasks at varying times, or aliquots of conditioned media were removed from duplicate cultures at varying times. In three experiments, collagenase activity could be detected in the culture media after 12 (Figure 3A,B) or 18 h (data not shown) of incubation with PMA. No activity was detected in the media of cells treated for less than 12 h. The increase in the collagenase level between 24 and 48 h and the plateau observed after 48 h, shown in Figure 3A, are representative of three experiments. A substantial decrease in the rate of accumulation of collagenase after 24 h and loss of collagenase activity between 48 and 72 h have each been observed in one experiment. In the latter case substantial nonspecific protease activity could be detected in the media. The collagenase levels observed in the conditioned media from U937 cells treated with PMA for 24 and 48 h were 3–17 units/10<sup>7</sup> cells and 10–26 units/10<sup>7</sup> cells, respectively. In general, no collagenase was detected in cells treated only with serum-free media; however, in occasional experiments very low levels of collagenase activity (estimated to be 0.02 unit/mL) could be detected.

We next asked whether the plateau in collagenase accumulation generally observed after 48 h is due to the cessation of collagenase secretion or to a balance between collagenase secretion and degradation or inactivation. U937 cells were treated with 50 ng/mL PMA, and the medium was replaced with fresh PMA-containing medium after 12, 24, and 48 h (see Figure 3B). Collagenase was detected in media collected after 12, 24, and 48 h, but little or no collagenase was detected in media collected after 72 h. This pattern of collagenase

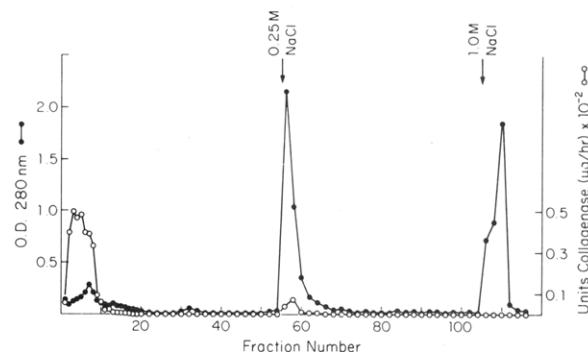


**FIGURE 3:** Time course of collagenase production by PMA-treated U937 cells. (A) U937 cells were treated with 100 ng/mL PMA, and conditioned media were removed for assay at the indicated times. The media harvested at 12–72 h were from individual flasks set up in parallel. Samples of conditioned media were incubated with [ $^3$ H]-collagen, in the absence of Tris buffer and glycerol, for 22 h. In assays of crude media, collagenase does not continue to cleave collagen for this length of time. The data are therefore expressed as percent of collagen cleaved. The levels of collagen cleavage observed correspond to approximately 0.3 (12 h), 0.5 (18 h), 0.6 (24 h), 1.05 (48 h), and 1.1 units/mL (72 h). (B) U937 cells were treated with 50 ng/mL PMA, and the medium was replaced after 12, 24, and 48 h with fresh medium containing PMA. Media from two flasks of cells were collected at 12, 24, 48, and 72 h. Collagenase assays were performed in the standard assay buffer on both samples from each time point. Sample 2 from each time point was also assayed in the presence of 1 or 5 mM NEM. Incubation times for these assays were 4–4½ h.

accumulation suggests that the plateau observed after 48 h in the experiment shown in Figure 3A could be the result of the termination of collagenase secretion.

Collagenase activity in these samples was measured in the presence or absence of *N*-ethylmaleimide. The NEM was included to activate any latent collagenase that might be present. The level of collagenase activity in the presence of NEM in samples collected 24 and 48 h after the addition of PMA was 0.8–1.1 times the level of activity in the absence of NEM (See Figure 3B). NEM appeared to cause a small stimulation of collagenase activity in the samples collected 12 (1.25-fold) and 72 h after the addition of PMA. However, the level of collagenase activity at 72 h is too low for accurate quantitation. In another experiment in which cells were treated with 100 ng/mL PMA and media harvested after 24 or 48 h of treatment, collagenase activity determined in the presence of NEM was the same as that observed in the absence of NEM.

In order to determine whether the continuous presence of PMA is required for collagenase production, another exper-



**FIGURE 4:** DEAE-Sephacel chromatography of conditioned medium from PMA-treated U937 cells. Collagenase assays on fractions 1–13 were done with 1, 3, and 10  $\mu\text{L}$  of each fraction, and assays on fractions 54–60 were done with 25 and 50  $\mu\text{L}$  of each fraction. Collagenase assays on all other fractions were done with 90  $\mu\text{L}$ .

iment was done in which PMA-containing media were replaced after 24 or 48 h either with media containing PMA or with serum-free media. Conditioned media collected after 24 h of PMA treatment contained slightly more collagenase activity than media collected after 48 h of PMA treatment [2.5  $\mu\text{g}$  of collagen cut (20 h) $^{-1}$  mL $^{-1}$  and 2.1  $\mu\text{g}$  of collagen cut (20 h) $^{-1}$  mL $^{-1}$ , respectively]. Significant amounts of collagenase activity were also seen in the media collected after 72 h of PMA treatment [1.4  $\mu\text{g}$  of collagen cut (20 h) $^{-1}$  mL $^{-1}$ ]. The amount of collagenase secreted by cells that were switched into serum-free media was slightly lower at 48 h [1.8  $\mu\text{g}$  of collagen cut (20 h) $^{-1}$  mL $^{-1}$ ] and 72 h [1.1  $\mu\text{g}$  of collagen cut (20 h) $^{-1}$  mL $^{-1}$ ] than the amount secreted by cells treated continuously with PMA. Once the U937 cells were stimulated to produce collagenase, the removal of PMA had only a small effect on the level of collagenase synthesis.

For the measurement of intracellular collagenase levels, extracts were prepared from U937 cells treated with 10–100 ng/mL PMA, or serum-free media, for 24–48 h. No collagenase activity was detected in untreated extracts or in extracts that had been treated with trypsin in order to activate latent enzyme which might be present.

We tested whether transcription and translation are required for phorbol ester induced collagenase production by treating U937 cells with actinomycin D (5 ng/mL) or cycloheximide (1 ng/mL) concomitantly with PMA. No collagenase activity was detected in the conditioned media under these conditions (see Figure 2B).

**Purification of Collagenase Produced by PMA-Treated U937 Cells.** Conditioned medium was collected from U937 cells that had been treated with 50 ng/mL phorbol ester for 48 h. The medium was concentrated and the buffer exchanged by ultrafiltration. The concentrated sample was treated with deoxyribonuclease I and ribonuclease A and then chromatographed on a DEAE-Sephacel column (see Figure 4) equilibrated with a low-salt buffer (20 mM Tris/25 mM NaCl/0.05% Brij-35, pH 8.2). Bound proteins were eluted with 0.25 M NaCl and then with 1 M NaCl in the same buffer. Twenty-eight percent of the collagenase activity applied to the column eluted with the unbound material (Table I), and an additional 1% eluted with the 0.25 M NaCl wash.

The unbound material (fractions 1–9) was chromatographed on a wheat germ agglutinin-agarose column. Most of the protein did not bind or bound only weakly to the column. Collagenase eluted as a broad peak behind the major protein peak (Figure 5). Approximately half of the active fractions contained substantial amounts of protein; only those fractions with low protein levels were pooled. These fractions were combined into two pools (fractions 25–31, pool I, and 32–42,

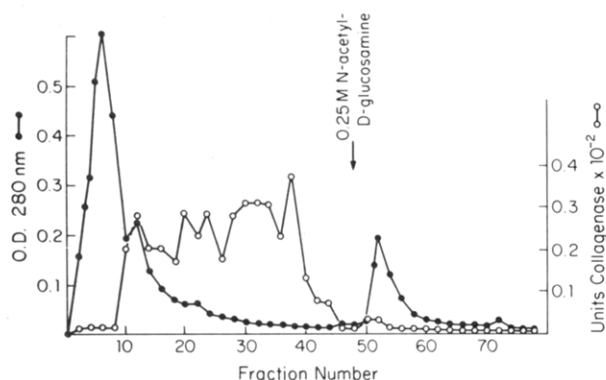


FIGURE 5: Wheat germ agglutinin-agarose column on collagenase enriched by DEAE-Sephacel chromatography. The unbound material from DEAE-Sephacel chromatography, which contained most of the recovered collagenase activity, was chromatographed on a wheat germ agglutinin-agarose column. Collagenase assays were done with 2  $\mu$ L of each fraction.

Table I: Enrichment of Collagenase from U937 Cell Media

	protein (mg)	units	sp act. <sup>a</sup>	yield (%)	purifica- tion (x-fold)
concentrated medium	8.7	1920	221		
DEAE-Sephacel	1.1	540	491	28	2
wheat germ agglutinin-aga- rose	0.1	260	2600	13.5	12

<sup>a</sup>Specific activity is defined as micrograms of collagen cut per hour per milligram of protein at 25 °C.

pool II) for comparison of the protein species present. The assays on individual column fractions indicated that all of the activity applied to the column was recovered; 60% of this activity was in fractions 25–42.

The enzyme preparation obtained after DEAE-Sephacel and wheat germ agglutinin-agarose chromatography had a specific activity of 2600 units/mg (see Table I). In a pilot experiment, a pool of conditioned media from U937 cells treated with a variety of inducers was taken through the purification procedure described earlier. This experiment yielded collagenase with a specific activity of 9000 units/mg (data not shown), but we did not have enough purified material for characterization.

The proteins present at each stage of the purification, presented in Figures 4 and 5 and Table I, were analyzed by SDS-polyacrylamide gel electrophoresis. The gel shown in Figure 6 indicates that a number of protein species were separated from collagenase during the DEAE-Sephacel chromatography. The use of the wheat germ agglutinin-agarose column led to the enrichment of a set of proteins with apparent molecular masses of 45–62 kilodaltons. Essentially the same set of proteins were present in the two wheat germ agglutinin-agarose pools. A major, diffuse protein band running between the 21 and 31 kDa standards was present in both pools but did not clearly correspond to a band in the DEAE-Sephacel pool. When a sample of wheat germ agglutinin was analyzed by SDS-polyacrylamide gel electrophoresis, a diffuse band in the same molecular weight range was observed. It therefore appears likely that this band represents wheat germ agglutinin that had leached from the column. This material was removed by chromatography of the sample on an *N*-acetyl-D-glucosamine-agarose column. However, our recovery of enzyme activity was low (20 units) after concentration of the active fractions from this column.

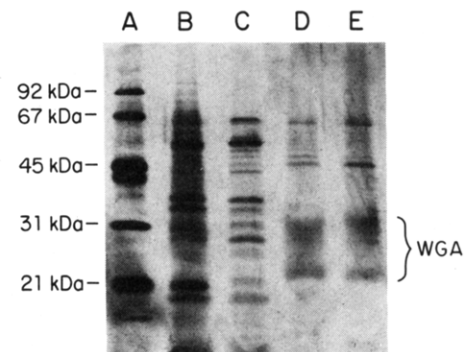


FIGURE 6: SDS-polyacrylamide gel on samples from various stages of the purification. (A) Standards: phosphorylase *a* (92 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa). (B) Conditioned medium after concentration, deoxyribonuclease and ribonuclease treatment, and dialysis. (C) DEAE-Sephacel pool, fractions 1–9. (D) Wheat germ agglutinin-agarose pool I, fractions 25–31. (E) Wheat germ agglutinin-agarose pool II, fractions 32–42.

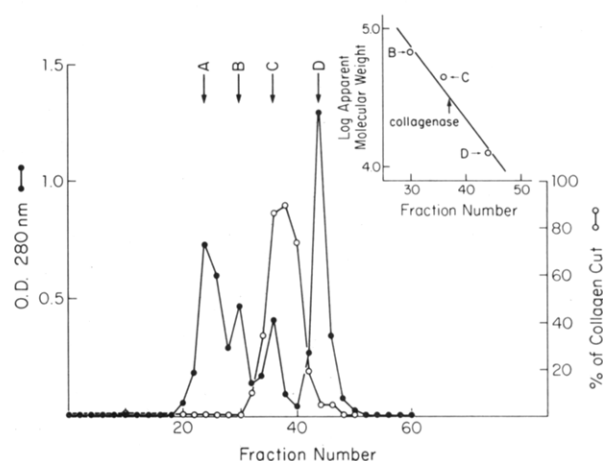


FIGURE 7: Sephacryl S-200 chromatography of partially purified U937 collagenase. A portion of concentrated pool I (0.5 mL) from the wheat germ agglutinin-agarose column was mixed with 1.5 mL of a mixture of protein standards (phosphorylase *a*, bovine serum albumin, ovalbumin, cytochrome *c*, 5 mg/mL each, and blue dextran) in 50 mM Tris-HCl/1 M NaCl/0.05% Brij, pH 7.5. This mixture was chromatographed on a Sephacryl S-200 column (2.2  $\times$  80 cm) which was eluted with same buffer. The flow rate was 5 mL/h; 3.5-mL fractions were collected. (A) Blue dextran and phosphorylase *a*, (B) bovine serum albumin (67 kDa), (C) ovalbumin (45 kDa), and (D) cytochrome *c* (12.5 kDa). Collagenase assays were done on 45  $\mu$ L of each fraction.

A portion of pool I from the wheat germ agglutinin-agarose column was chromatographed on a Sephacryl S-200 column (see Figure 7). The activity eluted as a somewhat broad peak with an apparent molecular mass of 29–36 kDa.

## DISCUSSION

The concentration of phorbol ester required for induction of collagenase production by U937 cells is comparable to concentrations required for induction of collagenase in fibroblasts. U937 cells secrete collagenase after treatment with PMA at concentrations of 5 ng/mL or greater; maximal collagenase production was observed with 10 ng/mL PMA (16 nM) in two experiments. High levels of collagenase secretion have been reported for rabbit synovial fibroblasts treated with 16–160 nM PMA (Aggeler et al., 1984b). Collagenase synthesis in human skin fibroblasts has been shown to be maximally stimulated by 10 nM PMA (Clark et al., 1985; Salo et al., 1985).

The phorbol ester concentrations that induce collagenase synthesis in U937 cells are also similar to concentrations that



have been used to induce some macrophage-like characteristics. Treatment of U937 cells with 6–30 nM PMA resulted in the appearance of a monocyte-macrophage morphology (Hattori et al., 1983). U937 cells treated with 16–30 nM PMA displayed ADCC activity against erythrocytes (Gidlund et al., 1981; Hattori et al., 1983). Higher concentrations of PMA are required for stimulation of direct and antibody-dependent killing of tumor cells by U937 cells (Ralph et al., 1982).

We have been able to detect collagenase in the media of PMA-treated U937 cells after 12–18 h of incubation, but we have not detected collagenase activity using shorter incubation times. Collagenase secretion continued for 2–3 days after PMA addition. The delayed appearance of collagenase, the duration of secretion, and the observed inhibition of collagenase production by actinomycin D and cycloheximide suggest that PMA stimulates collagenase synthesis rather than release of stored enzyme. Our inability to detect intracellular enzyme is also consistent with this hypothesis, although incomplete activation of intracellular enzyme cannot be ruled out. While preparation of this paper was in progress, Welgus et al. reported, in abstract form, the secretion of collagenase by PMA-treated U937 cells (Welgus et al., 1985b). They report that metabolic labeling and immunoprecipitation experiments indicate that secretion of collagenase after PMA treatment is the result of new synthesis. It has also been reported that collagenase appears in the media of rabbit synovial fibroblasts between 6 and 12 h after PMA addition. Increased levels of messenger RNA have been detected in the PMA-treated cells, indicating that PMA induces de novo synthesis of collagenase in these cells (Brinckerhoff et al., 1982; Aggeler et al., 1984a).

The time of appearance of collagenase in the media of U937 cells is similar to the time at which some macrophage-like characteristics appear. Morphological changes are observed in U937 cells within 1 day after PMA addition (Hattori et al., 1983). ADCC activity against erythrocytes has been detected after 1 day of treatment with PMA (Gidlund et al., 1981; Hattori et al., 1983).

It is not clear why collagenase secretion by PMA-treated U937 cells is shut off after 2–3 days. Several features of induced U937 cells, including ADCC against erythrocytes (Gidlund et al., 1981; Hattori et al., 1983), are present 5 days after PMA addition. One change that appears to correlate with collagenase production is the detachment of adherent U937 cells beginning 24 h after PMA addition (Nilsson et al., 1981). Variable patterns of collagenase secretion have been reported for macrophages. Collagenase secretion peaks 2–3 days after endotoxin addition to guinea pig peritoneal macrophages (Wahl et al., 1974) while secretion of collagenase by mouse peritoneal macrophages, stimulated in vivo with thioglycolate broth, can be detected after 7 days of culture (Werb & Gordon, 1975).

A variety of agents that stimulate monocytes were tested for their ability to induce collagenase secretion by U937 cells. When U937 cells were treated with concanavalin A (20  $\mu\text{g}/\text{mL}$ ), *Escherichia coli* endotoxin (B6, 0.3  $\mu\text{g}/\text{mL}$ ), interleukin 1 (1 unit/mL), or latex beads (0.1%), collagenase activity could be detected in the media (M. Gilmore-Hebert, unpublished results). These results as well as the dose-response and time course data suggest that the collagenase secretion by PMA-treated U937 cells is likely to be the result of PMA-induced differentiation.

Although methylphorbol myristate acetate has little tumor-promoting activity (Hecker, 1978), it is not a completely inactive phorbol ester derivative. MPMA has been reported to stimulate cytolysis of erythrocytes by guinea pig peritoneal

macrophages (Keisari & Pick, 1981). Approximately 100-fold higher concentrations of MPMA than PMA were required for a similar degree of stimulation. MPMA induced variable levels of collagenase synthesis in U937 cells. Higher doses of MPMA than PMA are required for induction, but the MPMA concentration needed for maximal induction of collagenase has not been determined.

We have detected collagenase activity in conditioned media from PMA-treated U937 cells in the absence of treatments that have been shown to activate latent collagenases (Stricklin et al., 1983). Active collagenase has also been detected in conditioned media from human peripheral blood monocytes (Louie et al., 1984). This is in contrast to the situation observed with human skin and rabbit synovial fibroblasts where collagenase is only found in a latent form (Bauer et al., 1975; Aggeler et al., 1984a). Treatment with *N*-ethylmaleimide has been shown to activate collagenase from human peripheral blood monocytes (Louie et al., 1984) and human polymorphonuclear leukocytes (Uitto et al., 1980; Callaway et al., 1986). Treatment with NEM had little effect on the collagenase activity we measured in conditioned media from PMA-treated U937 cells. These data suggest that little or no latent collagenase is generally present in the U937-conditioned media at the time it is harvested. However, we did not do sufficient studies with other activating agents such as trypsin or organomercurials (Stricklin et al., 1983) to determine whether additional activation could be achieved with these agents. In one experiment, we observed latent collagenase in the media of PMA-treated U937 cells grown by Karyon Technology, Inc., in alginate beads. This latent enzyme could be activated by trypsin. It seems likely that the U937 collagenase is secreted as a latent enzyme which was activated in the media in most of our experiments.

We were interested in purifying collagenase from U937 cells in order to characterize it further and have achieved partial purification. The U937 collagenase appears either to be destabilized by certain kinds of chromatography or to bind irreversibly to certain resins. Chromatography on DEAE-Sephacel in very low ionic strength buffer, on chromatofocusing resins, or on a Mono S (FPLC cation-exchange) column led to low yields of recovered enzyme and little purification. Significant losses of enzyme activity have also occurred during concentration of partially purified samples. This instability of the enzyme during purification has also been reported for collagenase from other cell sources (Werb & Reynolds, 1975; Cawston & Tyler, 1979). As was reported for pig synovial collagenase (Cawston & Tyler, 1979), we found that the nonionic detergent Brij-35 increased the stability of the U937 collagenase and therefore included it in the chromatography buffers. Despite the use of detergent the yield of enzyme recovered from the DEAE-Sephacel column was low. However, this step was useful in removing a number of protein species, oligonucleotides, and proteases.

We have had consistently high yields of enzyme activity using wheat germ agglutinin-agarose chromatography. This may be a generally useful step for purification of collagenase since it has also been used successfully for the purification of collagenase from polymorphonuclear leukocytes (Callaway et al., 1986). The collagenase from U937 cells also bound to pea agglutinin-agarose. The elution profile was similar to that observed with the wheat germ agglutinin-agarose. The binding of collagenase to these resins suggests that it is a glycoprotein containing an asparagine-linked complex carbohydrate moiety.

The U937 collagenase chromatographs on a Sephacryl S-200 column as a somewhat broad peak which elutes just

after ovalbumin. The molecular weight of this enzyme as determined by gel filtration appears to be similar to that of fibroblast collagenase. The human skin fibroblast enzyme was shown to elute as a peak of approximately 42 000 daltons when chromatographed on a Sephadex G-100 column (Stricklin et al., 1977). The reported molecular mass of rabbit synovial collagenase, as determined by gel filtration on a Sephadex G-100 column, is 32 000–35 000 daltons (Werb & Reynolds, 1975). Collagenase from polymorphonuclear leukocytes appears to be somewhat larger; the apparent molecular mass as determined by Sephacryl S-200 chromatography was 65 000–75 000 daltons (Callaway et al., 1986). The observation that the U937 collagenase is similar in size to the fibroblast enzyme but different from the enzyme from polymorphonuclear leukocytes is consistent with the immunological data indicating that the fibroblast and polymorphonuclear leukocyte enzymes are different (Hasty et al., 1984) and that the macrophage collagenase is fibroblast-like (Welgus et al., 1985a).

In conclusion, we have demonstrated the secretion of collagenase by U937 cells in response to treatment with PMA. U937 cells appear to provide both a good system for study of the regulation of collagenase production in macrophages and a good source for the isolation of macrophage collagenase. We have achieved enrichment of collagenase from U937 cells, but further work remains on the purification. It will be of interest to compare the properties of macrophage collagenase with collagenase from other sources when pure macrophage collagenase is available.

#### ADDED IN PROOF

While this paper was in proof, Welgus et al. (1986) published a study on the production of neutral proteases by PMA-treated U937 cells. This paper included results similar to ours on the dose response and time course of PMA-induced collagenase production.

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**Registry No.** PMA, 16561-29-8; collagenase, 9001-12-1.

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## Use of Lectin Affinity Chromatography for the Purification of Collagenase from Human Polymorphonuclear Leukocytes

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**ABSTRACT:** Polymorphonuclear leukocytes (PMNLs) store collagenase in an inactive form in secretory granules. The enzyme can be activated in vitro by limited proteolysis or by sulfhydryl-modifying agents such as *N*-ethylmaleimide (NEM). We have enriched NEM-activated collagenase 820-fold using granule isolation, gel filtration, and wheat germ agglutinin (WGA)-agarose chromatography. The use of WGA-agarose resulted in a 55-fold enrichment of collagenase in a single step with very little loss of activity. The chromatographic behavior of collagenase on other lectin matrices was explored and gave information about the type of complex asparagine-linked oligosaccharide found on collagenase isolated from PMNLs.

Mammalian collagenase (EC 3.4.24.7) cleaves native triple-helical collagen (types I, II, and III) between residues 772 and 773 to produce  $3/4$ - and  $1/4$ -length fragments. This cleavage site in type I collagen is a Gly-Ile bond in the  $\alpha$ -1 chain and a Gly-Leu bond in the  $\alpha$ -2 chain [for review, see Harper (1980)]. The triple-helical conformation of the cleavage fragments is unstable at physiological temperatures and upon unfolding becomes susceptible to general proteolysis. This implies that the initial cleavage of collagen by collagenase is the rate-limiting step in collagen degradation.

Collagenases are metalloenzymes that require both zinc and calcium for enzymatic activity. Mammalian collagenases capable of cleaving collagen types I, II, and III have been isolated from human fibroblasts (Stricklin et al., 1977, 1978; Wilhelm et al., 1984), rat uterus (Roswit et al., 1983), rabbit synovial fibroblast cells (Vater et al., 1981), and mouse bone cultures (Sakamoto et al., 1978). The collagenases from these sources have apparent molecular masses ranging from 45 to 65 kDa<sup>1</sup> and are synthesized in an inactive form. The process of activation of the procollagenase zymogen and the presence of both specific and nonspecific collagenase inhibitors results in precise regulation of enzyme activity (Cawston et al., 1983; Macartney & Tschesche, 1983b,c; Stricklin & Welgus, 1983; Stricklin et al., 1983; Vater et al., 1983; Welgus & Stricklin, 1983).

Human polymorphonuclear leukocytes (PMNLs, or neutrophils)<sup>1</sup> store collagenase in secretory granules as an enzymatically inactive molecule (Lazarus et al., 1968; Murphy et

al., 1982; Macartney & Tschesche, 1983a; Hasty et al., 1984). This enzyme can be activated by mild proteolytic digestion or by compounds that modify sulfhydryl residues such as NEM (Murphy et al., 1980; Macartney & Tschesche, 1983a; Hasty et al., 1984; Weiss et al., 1985). Attempts have been made to isolate this enzyme from PMNLs by using conventional chromatographic procedures (Uitto et al., 1980; Christner et al., 1982; Murphy et al., 1982; Macartney & Tschesche, 1983a). These preparations had relatively low specific activities when compared to collagenases purified from other sources (Harris & Vater, 1982). This report describes the use of lectin chromatography to rapidly achieve a high degree of purification of the enzyme and provide information about its carbohydrate structure.

### EXPERIMENTAL PROCEDURES

#### Materials

All radiochemicals and related material were obtained from New England Nuclear. The protein molecular weight standards and protein staining kit were purchased from Bio-Rad. Miracloth was from Calbiochem, and Cytodex beads were from Pharmacia. The SDS was from Bethesda Research Laboratories, and all other reagents were purchased from Sigma Chemical Co.

#### Methods

Except for the isolation of PMNLs from the cellular components in blood and the collagenase assay, all procedures were performed at 4 °C. The pH of all buffers was adjusted while the solution was at 4 °C.

*Isolation of Polymorphonuclear Leukocytes (PMNLs).* Leukopheresis was performed on 2 units of human blood (from Hemacare, Van Nuys, CA) to enrich for white blood cells (recovery was about 10<sup>10</sup> cells). Each of these leukopheresis concentrates was subsequently subjected to discontinuous density gradient centrifugation on Ficoll-Hypaque to isolate the PMNLs (English & Anderson, 1974).

<sup>1</sup> Abbreviations: Brij-35, poly(oxyethylene) 23-lauryl ether; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; E-PHA, erythroagglutinating phytohemagglutinin; kDa, kilodalton; L-PHA, leukoagglutinating phytohemagglutinin; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PMNL, polymorphonuclear leukocyte; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; WGA, wheat germ agglutinin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.