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Research Article

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Lipocytes from Normal Rat Liver Release a Neutral Metalloproteinase that Degrades Basement Membrane (Type IV) Collagen

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Abstract

We report a proteinase that degrades basement-membrane (type IV) collagen and is produced by the liver. Its cellular source is lipocytes (fat-storing or Ito cells). Lipocytes were isolated from normal rat liver and established in primary culture. The cells synthesize and secrete a neutral proteinase, which by gelatin-substrate gel electrophoresis and gel filtration chromatography, has a molecular mass of 65,000 D. The enzyme is secreted in latent form and is activated by *p*-aminophenylmercuric acetate but not by trypsin. Enzyme activity in the presence of EDTA is restored selectively by zinc and is unaffected by serine-protease inhibitors. In assays with radio-labeled soluble substrates, it degrades native type IV (basement membrane) collagen but not interstitial collagen types I or V and exhibits no activity against laminin or casein. At temperatures causing partial denaturation of soluble collagen in vitro, it rapidly degrades types I and V. Thus, it is both a type IV collagenase and gelatinase. The enzyme may play a role in initiating breakdown of the subendothelial matrix in the Disse space as well as augmenting the effects of collagenases that attack native interstitial collagen.

Introduction

Basement membrane is a specialized form of extracellular matrix underlying the epithelium and endothelium of parenchymal tissues with important biologic effects on adjacent cells. It consists of a complex of type IV collagen, large glycoproteins (laminin, entactin), and proteoglycan (1, 2). Introduced into culture as the substratum, matrix of this type exerts major effects on the morphology and cell-specific function of mesenchymal and epithelial cells, including those in the liver (3–6). In vivo alteration or replacement of the basement membrane may be an important cause of epithelial dysfunction. Prominent among the effects of inflammation in the liver is deposition of a neomatrix in place of the normal basement membrane-like matrix in the subendothelial (Disse) space. This

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change, termed capillarization (7), correlates with clinically manifest liver disease (8).

Collagenase activity is generally increased during the early stage of liver injury (9, 10), suggesting that matrix degradation plays a role in the fibrosing process. These findings have stimulated new interest in mechanisms of matrix turnover, both normal and pathologic. Polymorphonuclear leukocytes contain collagenases and other proteinases that may be released at sites of inflammation (11). A number of mesenchymal cell types also secrete matrix proteinases, suggesting possible sources of these enzymes within parenchymal tissues. Included are metalloproteinases with gelatinase specificity with activity in vitro against denatured collagen. Limited digestion (by a specific collagenase) may cause unfolding sufficient to render the native collagen helix susceptible to gelatinase, which completes the degradation process.

The role of matrix proteinases originating within liver is poorly understood. Most studies have been performed with whole-liver homogenate, and the results are widely divergent (9, 10, 12–16). Although studies with isolated, cultured cells are reported (17–22), those of nonparenchymal cells have used mixed isolates containing sinusoidal endothelial cells, Kupffer cells, and lipocytes in indeterminate proportions. Precise information as to cellular sources of matrix proteinases in the liver is limited.

Lipocytes have attracted particular attention, because they are within the space of Disse, positioned to exert major effects on matrix metabolism. Virtually pure isolates can be prepared from normal rat liver and established in culture (23) where they elaborate relatively large amounts of matrix proteins (24–26). This work demonstrates that, in addition, they mediate matrix turnover, producing a neutral metalloproteinase with activity against native type IV (basement-membrane) collagen as well as activity against partially denatured collagen I and V.

Methods

Materials

Pronase was obtained from Calbiochem-Behring Corp. (La Jolla, CA) and collagenase (type I) from Worthington Biochemical Corp. (Freehold, NJ). Arabinogalactan (Larex-Lo) was purchased from Larex International (Tacoma, WA). Calf and horse serum were purchased from Flow Laboratories, Inc. (McLean, VA). Acetic anhydride (CFA 339; 106 mCi/mmol) and Amplify were obtained from Amersham Corp. (Arlington Heights, IL), and Bolton-Hunter reagent (NEX 120) from Dupont/New England Nuclear, Inc. (Boston, MA). Sephacryl S200 Superfine and S1000 Superfine were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ). Lactoperoxidase (from bovine milk), gelatin (type III, from calf skin), casein, *p*-aminophenyl mercuric acetate (APMA),¹ and soybean trypsin inhibitor (type I-S) were purchased

1. Abbreviations used in this paper: APMA, *p*-aminophenyl mercuric acetate; EHS, Engelbreth-Holm-Swarm; NEM, *N*-ethylmaleimide.

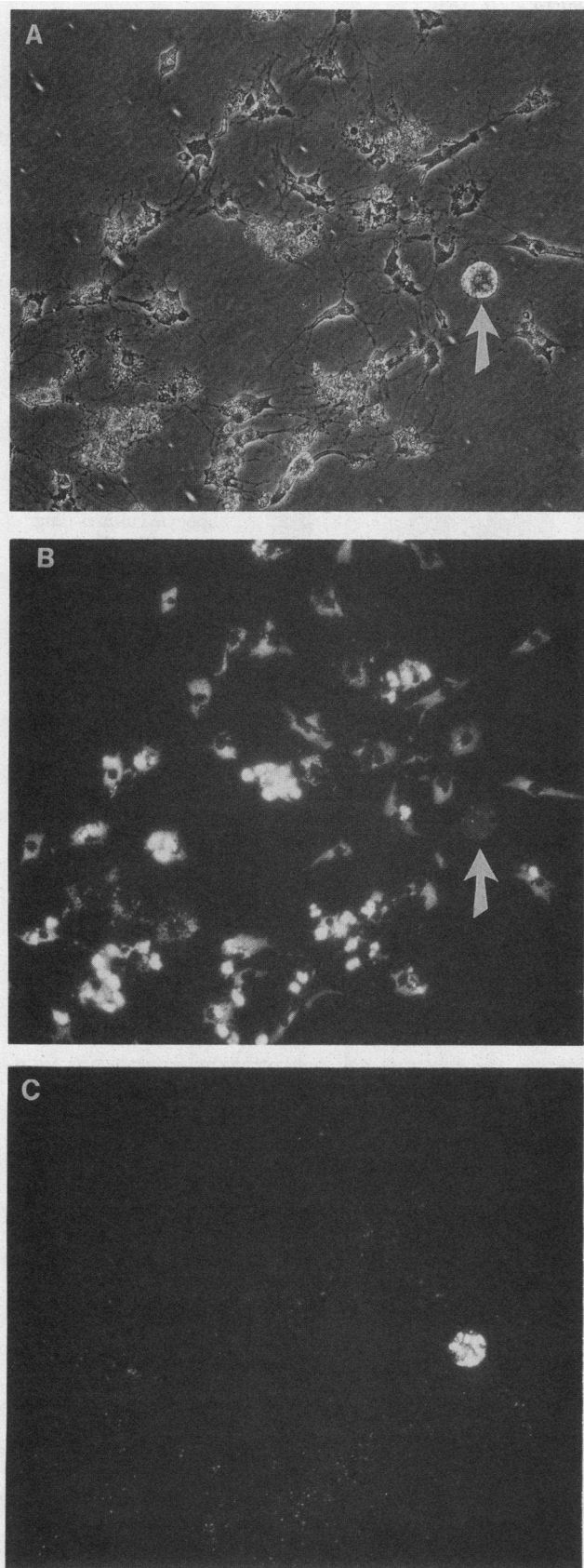


Figure 1. Identification of lipocytes and Kupffer cells in culture. Lipocytes were isolated and purified from normal rat liver, as described in Methods. The cells were cultured for 3 d and then incubated for 30 min with heat-killed, FITC-labeled *Staphylococci*, which are ingested by Kupffer cells only (23). After washes to remove free *Staph-*

from Sigma Chemical Co. (St. Louis, MO), and TPCK-trypsin from Cooper Biomedical, Inc. (Malvern, PA). Ecolume was obtained from ICN Radiochemicals, Inc. (Irvine, CA).

Cell isolation and culture

Lipocytes and Kupffer cells were isolated from normal rat liver (male Sprague Dawley rats, 450–600 g) by a combination of pronase and collagenase perfusion and purified by arabinogalactan density gradient centrifugation as described (23), with modifications. Primary lipocyte cultures were prepared from cells that were buoyant on 6% arabinogalactan and from cells at the interface between 6 and 8% arabinogalactan. Cells from the 8%/12% and 12%/15% arabinogalactan interfaces were pooled for purification of Kupffer cells by centrifugal elutriation (27, 28). Primary cultures of lipocytes and Kupffer cells were plated in 35-mm plastic tissue culture plates and maintained in medium 199 (28) supplemented with 10% calf and 10% horse serum, 4 mU/ml insulin, 10^{-6} M corticosterone and 100 U/ml penicillin. Media were changed daily.

Cell purity was assessed after 48–72 h in primary culture, by phase-contrast microscopy, intrinsic vitamin A fluorescence to identify lipocytes (23), and uptake of FITC-conjugated *Staphylococci* to identify Kupffer cells (23) (Fig. 1). Lipocyte cultures prepared from cells that were buoyant on 6% arabinogalactan were highly purified, containing < 0.1% Kupffer cells as contaminants. Lipocyte cultures prepared from cells at the 6–8% Stractan interface contained < 2% Kupffer cells as contaminants. Kupffer cell cultures were ~ 90% pure, with lipocytes as the principal contaminant.

Preparation of matrix-protein substrates

Acid-soluble type I collagen was extracted and purified from rat skin and radiolabeled with [14 C]acetic anhydride according to the method of Cawston and Barrett (29). Radiolabeled type I collagen (5 mg/ml in 0.05 M acetic acid) was stored at -20°C . Before use, this was diluted to 2 mg/ml in 0.05 M acetic acid and dialyzed, at 4°C , against 0.05 M Tris, pH 7.6, 0.2 M NaCl, 0.02% NaN_3 . Type V collagen was extracted and purified from pepsin-treated human placental amniotic membrane by the method of Burgeson et al. (30), with the exception that it was precipitated by dialysis against distilled water as described by Madri and Furthmayr (31). This preparation, which contained a small amount of residual type I collagen, was iodinated using the Bolton-Hunter reagent by the method of Roll et al. (32), and stored at 4°C in 0.05 M acetic acid. Type IV collagen was extracted from Engelbreth-Holm-Swarm (EHS) murine sarcoma by the method of Kleinman et al. (33). Type IV collagen was purified by differential salt precipitation and DEAE-cellulose chromatography and iodinated as described by Yurchenco and Furthmayr (34), with the exception that, for final purification, iodinated type IV collagen was dialyzed against 2 M guanidine, 0.05 M Tris, pH 7.4, 2 mM DTT, 5 mM EDTA, 2 mM *N*-ethylmaleimide (NEM), 0.1 mM PMSF and chromatographed, in this buffer, on a Sephacryl S1000 superfine column (1×95 cm). Storage of iodinated type IV collagen was at 4°C in the same buffer. Immediately before use, radiolabeled type IV and type V collagens were dialyzed against 0.05 M Tris, pH 7.6, 0.2 M NaCl, 10 mM CaCl_2 , 0.02% NaN_3 , and 0.05% Brij 35. Laminin was prepared from murine EHS sarcoma as described (25).

Assessment of released proteinase activities

Lipocytes or Kupffer cells, cultured for 72 h, were washed to remove serum and incubated in serum-free medium 199. Media were har-

ylcocci, cultures were examined by phase-contrast microscopy (Fig. 1 A), and by fluorescence microscopy under either UV (365 nm) excitation (Fig. 1 B) or blue (495 nm) excitation (Fig. 1 C). The intrinsic fluorescence of the vitamin A-containing lipocytes is evident in Fig. 1 B. Blue excitation for FITC shows that one cell in the field is a Kupffer cell (Fig. 1 C). The same cell in Fig. 1, A and B is indicated by an arrow.

vested after 48 h and clarified by centrifugation before analysis of released proteinase activities.

Substrate gel analysis. Proteinase activities in crude, unconcentrated, Kupffer cell or lipocyte-conditioned media were visualized by gelatin-substrate SDS-PAGE, as described by Herron et al. (35). For some experiments, casein (1 mg/ml in distilled water) or laminin (1 mg/ml in 0.2 M ammonium bicarbonate), rather than gelatin, were incorporated as substrates in SDS-polyacrylamide gels.

Quantitative analysis of gelatin-degrading activity. Kupffer cell or lipocyte-conditioned media were dialyzed against sample buffer (0.05 M Tris, pH 7.6, 0.2 M NaCl, 10 mM CaCl₂, and 0.02% NaN₃) for quantitative assay of gelatin-degrading activity by the method of Harris and Krane (36), with modifications. Rat skin [¹⁴C]type I collagen, (2 mg/ml in 0.05 M Tris, pH 7.6, 0.2 M NaCl, and 0.02% NaN₃) was heat denatured at 60°C for 20 min, immediately before use, to form [¹⁴C]-gelatin. 50 µl of this preparation were incubated, in triplicate, with 100 µl of sample for 16 h at 37°C in microcentrifuge tubes. At the end of the incubation, 50 µl of cold gelatin (3 mg/ml) were added and nondegraded substrate precipitated by addition of 50 µl of 100% (wt/vol) TCA. Tubes were placed on ice for 30 min, microcentrifuged at 4°C for 15 min, and 200 µl of supernatant counted in 5 ml Ecolume by scintillation spectrometry. Samples were assayed nonactivated or, where indicated, after treatment with APMA (1 mM × 60 min at 37°C) or TPCK-trypsin (1–100 µg/ml for 60 min at 37°C) followed by addition of a fivefold molar excess of soybean trypsin inhibitor (STI). Controls contained sample buffer, either alone or with addition of either APMA or trypsin/STI.

By this method, gelatin degradation was linear over 16 h ($r = 0.999$) and the coefficient of variation for 10 replicate samples was 4.3%. 1 mU of gelatinase activity is defined as degradation of 1 ng of gelatin per minute. Data were expressed per microgram cellular DNA, measured by a fluorometric technique (37).

SDS-PAGE

SDS-PAGE was performed on vertical slab gels according to the method of Laemmli (38). Gels were stained with 0.1% Coomassie blue in 50% methanol, 10% acetic acid (vol/vol), destained in 5% methanol, 7.5% acetic acid (vol/vol), and washed in distilled water. Gels containing ¹⁴C-labeled substrates were soaked in Amplify for 20 min before drying. For autoradiography, dried gels were placed on Kodak X-Omat S film with a screen for 24–72 h at –70°C.

Results

Substrate gel analysis of lipocyte-conditioned media. When analyzed by gelatin-substrate SDS-PAGE, the medium from hepatic lipocytes (fat-storing or Ito cells) contained a single band of gelatin-degrading activity, M_r 65 kD (Fig. 2 A, lane 1). This activity was present in unconcentrated crude media from pure lipocyte cultures (Fig. 2 A) (obtained by culturing cells that were buoyant on 6% arabinogalactan) and was the only gelatin-degrading activity both in those cultures and in cultures containing < 2% Kupffer cells as contaminants (not shown). Its release was markedly decreased in the presence of cycloheximide (Fig. 2 A, lane 2) and thus requires ongoing protein synthesis. Kupffer cell cultures (~ 90% pure, with lipocytes as contaminants) released a higher molecular weight gelatin-degrading activity, M_r 95 kD (Fig. 2 B). Relatively low levels of the 65-kD activity also were detectable in media obtained from these cultures, consistent with the degree of lipocyte contamination (Fig. 2 B). Thus, the activity migrating at 65 kD appears to be a product mainly, if not exclusively, of lipocytes. Its substrate range was studied with gels that incorporated laminin or casein in place of gelatin, and no activity was detected (Fig. 2 C). The activity was inhibited by EDTA

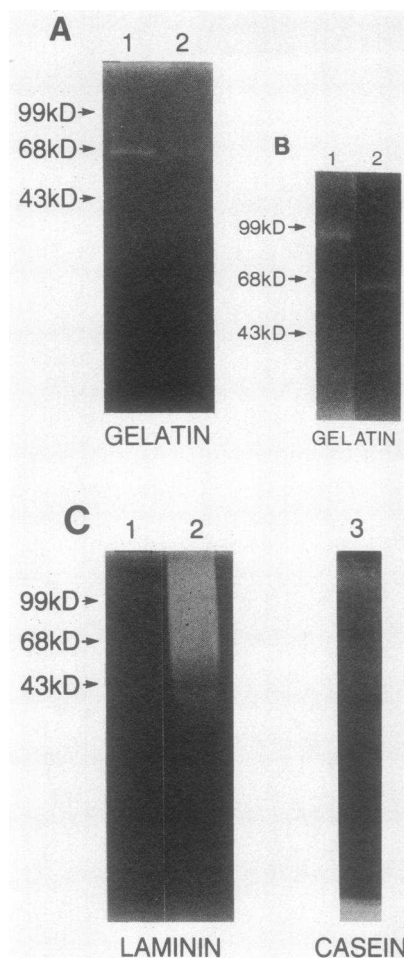


Figure 2. Substrate-gel analysis of culture media. Primary lipocyte cultures (2–3 d old) were maintained in serum-free media for 48 h. Conditioned media were harvested and analyzed by gelatin-substrate SDS-PAGE as described in Methods. (A) Pure lipocytes were cultured in the absence (lane 1) or presence (lane 2) of cycloheximide (5 µg/ml). (B) Conditioned medium from Kupffer cells (lane 1) was analyzed in parallel with medium from lipocyte cultures (lane 2). (C) Conditioned media were electrophoresed on gels containing laminin (lanes 1 and 2) or casein (lane 3) as substrate, and otherwise treated as before; lanes 1 and 3, lipocyte-conditioned medium; lane 2, PMA-stimulated human neutrophil-conditioned medium, used as a positive control (degradation of casein by this medium, not

shown, was similar to that of laminin). Gelatin-degrading activity in these lipocyte-conditioned media was verified in parallel studies (not shown).

added to the substrate gel incubation buffer, but was unaffected by either PMSF or NEM (Fig. 3). These results indicate that the gelatin-degrading activity is a metalloproteinase.

Quantitative analysis of gelatin-degrading activity released by lipocytes. Gelatin-degrading activity (Table I) was present predominantly as latent proenzyme that was activated by APMA. The measured activity from pure lipocyte cultures (Table I) varied three to fivefold for nonactivated media and up to 20-fold for media activated with APMA. The data from cultures containing Kupffer cells as a minor contaminant (< 2% by cell number) varied to the same extent, suggesting that the presence of Kupffer cells did not account for the variation.

Gel filtration of lipocyte-conditioned medium. Lipocyte-conditioned medium was chromatographed on a column of Sephacryl S200, with analysis of individual fractions by gelatin-substrate SDS-PAGE and by assay (after treatment with APMA) for gelatin-degrading activity using [¹⁴C]gelatin as substrate. As shown in Fig. 4, the peak of activity migrated at 65 kD. By gelatin substrate SDS-PAGE, the only activity detectable within this peak was the 65-kD proteinase. In other experiments, in which larger volumes of lipocyte-conditioned media were pooled and concentrated, the results of column chromatography were similar. Recovery of gelatin-degrading

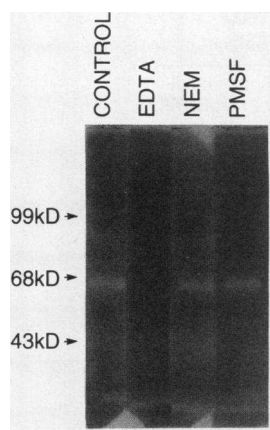


Figure 3. Characterization by substrate-gel of the proteinase activity in lipocyte-conditioned culture media. Media were prepared and harvested as described in Fig. 2. Replicate samples from a single lipocyte preparation were electrophoresed in four lanes of the same gelatin substrate gel. After electrophoresis, individual lanes were excised and incubated in 0.05 M Tris, pH 8.0, 5 mM CaCl₂, 0.02% azide containing no proteinase inhibitors (control), EDTA (10 mM), NEM (2 mM) or PMSF (1 mM) before being fixed and stained.

activity from the column was 100%. The data suggest that lipocytes produce a single proteinase species of 65 kD.

Characterization and substrate specificity of partially purified 65-kD proteinase. Fractions containing the 65-kD proteinase from the Sephacryl S200 column were pooled for analysis. The recovered enzyme was almost entirely in a latent form (Table II). It was activated > 70-fold by APMA but < 2-fold by trypsin. It was completely inhibited by EDTA, but not by PMSF or NEM (Table III), confirming the results with crude culture media. To assess the metal requirement of the enzyme, EDTA inhibition and its reversibility was examined in detail. The enzyme was markedly sensitive to EDTA, even in the presence of 5 mM calcium (Table IV). Activity was restored by zinc at a molar ratio of 2:1 with respect to EDTA. At a higher molar ratio, the activation was markedly diminished. Partial activation resulted from addition of manganese or copper. The APMA-activated enzyme was completely inhibited also by DTT.

The APMA-activated proteinase was incubated with individual radiolabeled matrix protein substrates, with analysis of reaction products by SDS-PAGE and autoradiography. Degra-

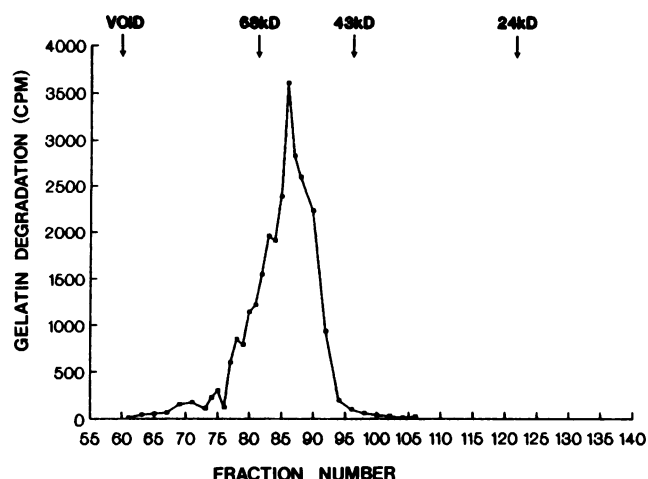


Figure 4. Gel chromatography of lipocyte gelatinase. Serum-free, lipocyte-conditioned media, collected between 2 and 6 d of primary culture were pooled (total volume 125 ml), concentrated 12.5-fold with polyethylene glycol (PEG, 40,000) and dialyzed against column buffer (0.05 M Tris, pH 7.6, containing 0.2 M NaCl, 10 mM CaCl₂, 0.02% NaN₃, and 0.05% Brij 35). This material was chromatographed at 4°C on a Sephacryl S200 superfine column (90 × 2.4 cm) at a flow rate of 13.75 ml/min, with collection of 2.75-ml fractions. Individual fractions were assayed (after activation with APMA) for gelatinase activity, as in Table I. The Sephacryl S200 column was calibrated with BSA (68 kD), ovalbumin (43 kD), and chymotrypsinogen (24 kD).

dation of [¹⁴C]gelatin at 37°C is illustrated in Fig. 5. Relatively short-term incubation yielded multiple products of intermediate molecular weight. With increasing duration of incubation, the proportion of very low molecular weight products increased, indicating secondary cleavage of initial degradation products.

Degradation of collagen substrates was examined with collagen types I, IV, and V. Soluble native rat skin [¹⁴C]type I

Table I. Release of Degradative Activity against [¹⁴C]Gelatin by Cultured Lipocytes

	Gelatinase activity	
	Mean	Range
	mU/μg DNA	
Nonactivated	3.9	(1.3–8.1)
APMA-activated	12.5	(2.3–47.6)
	<i>P</i> < 0.05	

Primary cultures of lipocytes were maintained for 72 h after plating and then transferred to serum-free media for 48 h. Unconcentrated conditioned media were harvested and dialyzed against 0.05 M Tris-HCl, pH 7.6, 0.2 M NaCl, 10 mM CaCl₂, and 0.02% azide. Gelatinase activity was assayed with heat-denatured (60°C × 20 min) rat skin [¹⁴C]type I collagen as substrate (350 cpm/μg sp act) as described in Methods, with or without activation (1 mM APMA for 60 min at 37°C). 1 mU of gelatinase activity is defined as degradation of 1 ng of gelatin/min. The measured radioactivity averaged 427 cpm for nonactivated and 1,775 cpm for APMA-activated samples. Statistical comparisons were made between nonactivated and APMA-activated samples using the Wilcoxon rank sum test for paired data (*n* = 5).

Table II. Latent Proteinase Activation

Sample	Gelatinase activity
	% of maximal activity
Nonactivated	1
+APMA	100
+Trypsin (0.01 μg/ml, 60 min)	3
+Trypsin (0.10 μg/ml, 60 min)	2
+Trypsin (1.00 μg/μl, 60 min)	5
+Trypsin (1.00 μg/μl, 20 min)	2
+Trypsin (1.00 μg/μl, 5 min)	1

Pooled column fractions containing the 65-kD proteinase were assayed for gelatinase activity as described in the legend to Table I. Samples were assayed before or after exposure to APMA (1 mM for 60 min at 37°C) or trypsin at the indicated concentration and time, at 20°C. A fivefold molar excess of soybean trypsin inhibitor was added to trypsin-treated samples before addition of the [¹⁴C]gelatin substrate. Higher concentrations of trypsin (10 and 100 μg/ml, respectively) or incubation at 37°C failed to further activate the enzyme (data not shown). The activity of the native sample was 0.6 and for the APMA-treated sample was 42.9 mU/100 μl.

Table III. Inhibition of APMA-activated Proteinase

Sample	Gelatinase activity % of control
Control	100
+EDTA (10 mM)	0
+NEM (2 mM)	100
+PMSF vehicle (isopropanol)	100
+PMSF (1 mM)	94

Pooled column fractions containing the 65-kD proteinase were assayed for gelatinase activity as described in the legend to Table I. Paired samples were assayed after exposure to APMA: the nonactivated samples in this experiment had no detectable activity; after APMA (control), the activity was 2.5–3.3 mU/100 μ l.

collagen was not degraded by the 65-kD proteinase at 25°C (Fig. 6 A), in contrast to [14 C]gelatin (Fig. 6 B). Similar results were obtained with soluble native human amniotic 125 I-type I collagen as substrate (Fig. 7). However, at 32 and 37°C (temperatures at which thermal denaturation of soluble type I collagen occurs, [16]), degradation was evident (Fig. 7). In addition, the substrate became susceptible to trypsin, indicating unfolding of the helix. Similarly, native human amniotic 125 I-type V collagen was not degraded at 25°C but was attacked by both the 65-kD proteinase and trypsin at 32 and 37°C (Fig. 7). (Thermal denaturation of soluble type V collagen is initiated at 30°C with a half-maximal temperature [T_m] of 33°C [39].) These results indicate that the proteinase is inactive against native collagen types I and V, but that it will attack interstitial collagens that have been partially denatured.

In contrast to these results, the proteinase cleaved native murine 125 I-type IV (basement membrane) collagen in a time-dependent, EDTA-inhibitable manner at 30°C (Figs. 8 and 9). (Thermal denaturation of soluble type IV collagen is initiated at 33°C, with a T_m of 40°C [40].) This is seen as disappearance

Table IV. Reversibility of EDTA Inhibition of Lipocyte Proteinase

Proteinase sample	Added metal	Activity % control
APMA-activated	—	100
+5 mM EDTA	—	1
+0.5 mM EDTA	—	2
+0.1 mM EDTA	—	5
+0.01 mM EDTA	—	14
+0.1 mM EDTA	Zn (0.11 mM)	41
+0.1 mM EDTA	Zn (0.20 mM)	92
+0.1 mM EDTA	Zn (0.50 mM)	30
+0.1 mM EDTA	Mn (0.20 mM)	52
+0.1 mM EDTA	Cu (0.20 mM)	32
+0.1 mM EDTA	Mg (0.2 mM)	6
+0.1 mM EDTA	Ca (0.2 mM)	7

APMA, 1 mM; proteinase samples were activated with incubation at 37°C for 60 min.

The enzyme preparation was the peak fraction from gel chromatography (see Fig. 4), in a buffer containing 5 mM calcium.

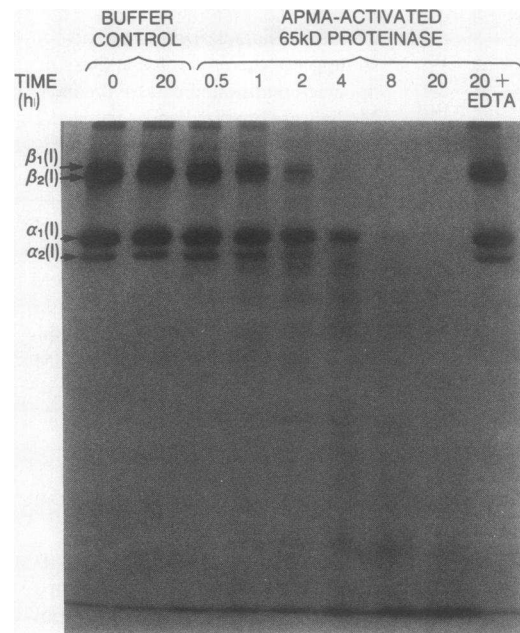


Figure 5. Analysis of [14 C]gelatin degradation. Rat skin [14 C]type I collagen was heat-denatured (60°C \times 20 min) to form [14 C]gelatin and incubated, at 37°C, with APMA-treated buffer solution or APMA-activated pooled column fractions containing the 65-kD proteinase (fractions 76–90, see Fig. 4). Each reaction contained 100 μ g of [14 C]gelatin (specific activity, 350 cpm/ μ g) and 5.5 mU of gelatinase activity in a final volume of 200 μ l. At the end of the reaction, 50 μ l were mixed with sample buffer (38) containing 2-mercaptoethanol and electrophoresed on a 7.5% polyacrylamide gel. An autoradiogram of the gel is shown.

of the alpha-1-(IV) and alpha-2-(IV) bands, together with the appearance of cleavage products of M_r 125 and 92 kD (Fig. 8). Degradation was detectable also at 25°C with formation of the same molecular size cleavage products, and was inhibited by EDTA (Figs. 8 and 9). At 37°C, (conditions that initiate thermal denaturation of soluble type IV collagen) degradation of the 125 I-type IV collagen substrate was accelerated and yielded products of different molecular size (Fig. 9). The sensitivity of native 125 I-type IV collagen to trypsin (10 μ g/ml \times 20 h) was also temperature dependent; limited cleavage was observed at 25 and 30°C, consistent with previous reports of the sensitivity of the native molecule to proteolytic digestion (33, 40–42). By contrast, trypsin-mediated degradation was extensive at 37°C. These results indicate that type IV collagen in its native conformation is degraded by the 65-kD proteinase. The additional and more extensive degradation at 37°C presumably reflects thermal denaturation of the substrate.

Discussion

Hepatic lipocytes (fat-storing, stellate, perisinusoidal, or Ito cells) are located in the subendothelial space of Disse. In normal liver, they exhibit long cellular processes that encircle the sinusoid (43). They also store vitamin A esters in vacuoles (43, 44). In models of acute liver injury, lipocyte proliferation is prominent in areas of necrosis (45–50) where deposition of new matrix proteins is occurring (51). This morphologic evidence suggests an important role for lipocytes in liver fibrosis and is supported by findings from cell culture. Lipocytes, ob-

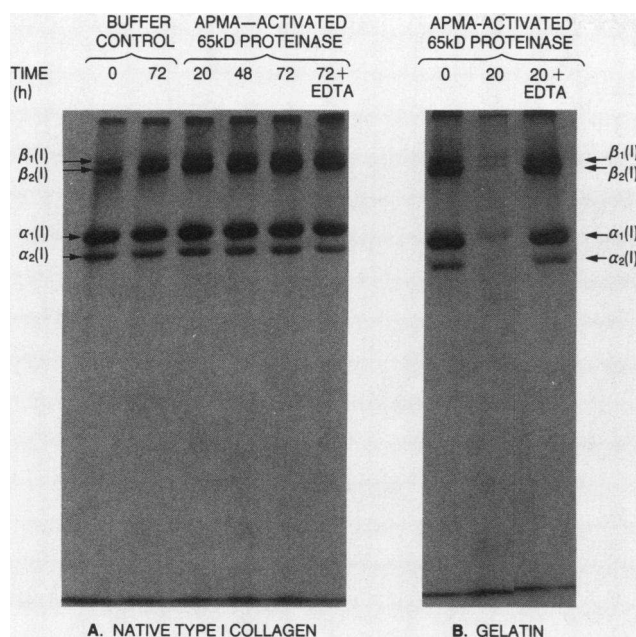


Figure 6. Analysis of type I collagen degradation. Pooled column fractions, processed and activated as in Fig. 5, were incubated at 25°C, with either native rat skin [14 C]type I collagen (Fig. 6 A) or the same substrate that had been heat-denatured (60°C \times 20 min) (Fig. 6 B). Reactions with native [14 C]type I collagen were performed in the presence of 0.25 M glucose to inhibit fibril formation. For both substrates each reaction contained 5.5 mU of gelatinase activity and 100 μ g of [14 C]gelatin or [14 C]type I collagen in a final volume of 200 μ l. At the end of the reaction, samples were analyzed as in Fig. 5. Buffer controls for the gelatin substrate (not shown) were identical to those shown in Fig. 5. An autoradiogram is shown.

tained from normal rat liver and studied in primary culture, are a major source of liver matrix proteins including collagens (24), laminin (25), and proteoglycans (26, 52) and fibronectin (53).

These results indicate that lipocytes may be involved in matrix turnover as well as its synthesis. Assignment of the 65-kD proteinase to lipocytes is based on the fact that the cultures are > 99% pure. We cannot exclude that Kupffer cells also produce this proteinase in small amounts. However, their principal product, as visualized on gelatin substrate gels, is a proteinase of 95 kD, which is similar in size and possibly related to enzymes secreted by alveolar macrophages (54) and neutrophils (55–58).

Two aspects of the experimental results bear on the regulation of proteinase activity. The first is that the proteinase secreted by lipocytes is almost entirely latent, as is typical of neutral metalloproteinases. The question of proteinase activation *in vivo* is an important one and still not settled. Stromelysins may have this role (59) and are secreted by mesenchymal cells (60–63). However, we found no activity of this type (as judged by casein degradation) in the medium from lipocyte cultures. It may be produced *in situ* by a second cell type. Also, membrane-associated proteinases have been described in rat liver (64). The second is batch-to-batch variation in proteinase secretion by the cultured lipocytes. We were unable to relate this to cell density, the age of the culture, the presence of Kupffer cells, or the assay itself. Within a single batch of cells, variation in the assay was acceptably small (< 10%), indicating its reproducibility. One possibility is that culturing *per se* (e.g., contact with plastic) induces varying degrees of lipocyte stimulation. Proteinase production by other cells is modulated by extracellular matrix (65). Studies of the regulation of lipocyte

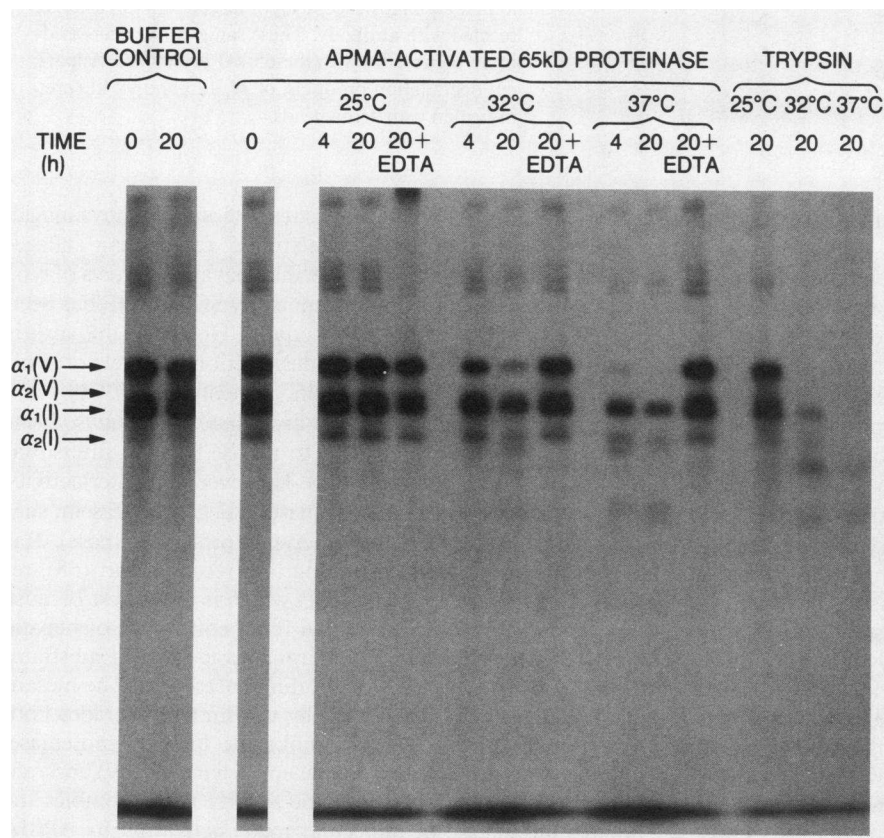


Figure 7. Analysis of human type I and type V collagen degradation. Concentrated, APMA-activated, pooled column fractions containing the 65-kD proteinase (fractions 76–90, see Fig. 4) were incubated at 25, 32, or 37°C. The substrate was a mixture of radioiodinated type I and type V collagens from human placenta. The reaction (final volume, 225 μ l) contained 53.7 mU of gelatinase activity and a total of 90,000 cpm of labeled substrate. Parallel incubations with trypsin (10 μ g/ml) were carried out. At each time point, 20- μ l aliquots were electrophoresed on a 6% polyacrylamide gel for autoradiography.

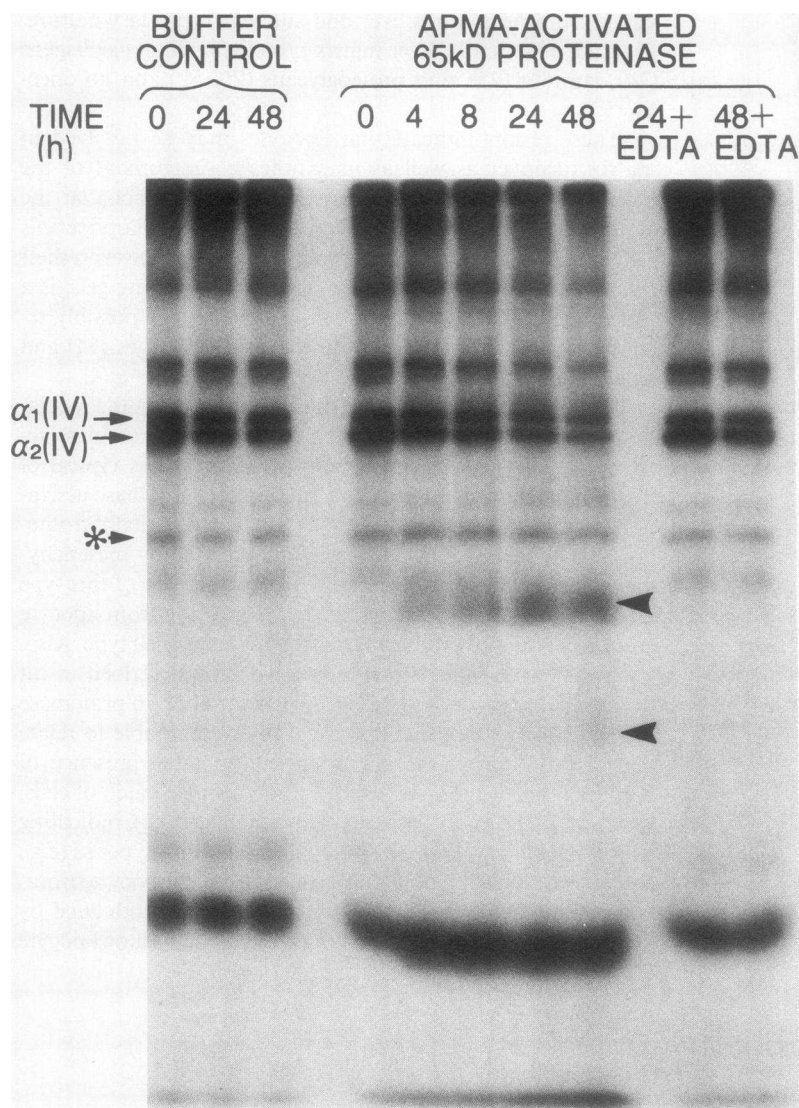


Figure 8. Analysis of type IV collagen degradation. Concentrated, APMA-activated, pooled column fractions containing the 65-kD proteinase were incubated at 30°C, in a reaction containing 257 mU of gelatinase activity and 80,000 cpm of iodinated type IV collagen in a final volume of 140 μ l. At the indicated time points, 15- μ l aliquots were electrophoresed on a 6% gel for autoradiography. In preliminary studies the substrate was evaluated by immunoblot with an affinity purified antibody to type IV collagen (4). All iodinated bands except that marked with an asterisk reacted with antibody. These same bands decreased upon incubation with the 65-kD proteinase. Apparent degradation products, of M_r 125 and 92 kD are indicated with arrowheads.

proteinase production will be an important goal of further studies.

Several early studies of liver proteinases focused almost exclusively on interstitial collagenase, using type I collagen as substrate (17, 18), and none clearly identified cellular sources. Activity attributed to hepatocytes could have derived from lipocytes in view of recent evidence that the latter cell type is present within hepatocyte cultures constituting up to 10% of the total cell number (66). A second methodological concern is that, although most studies were designed to detect interstitial collagenases, many used assay conditions that fail to distinguish collagenase from gelatinase activity and must be reevaluated in light of the present evidence that lipocytes release a gelatin-degrading neutral metalloproteinase. The methods commonly employed to measure collagenase activity (reconstituted and diffuse fibril assays, or methods that use soluble type I collagen as substrate [16]) have notoriously high values for nonspecific (e.g., trypsin-mediated) degradation of the substrate. The temperature at which reactions are performed is particularly important (67). Type I collagen in solution is susceptible to nonspecific proteolysis > 30°C, whereas the corresponding temperature for fibril assays is 35–37°C. Thus,

assays carried out with soluble substrate and at 'physiologic' temperatures may measure a gelatinase, rather than collagenase. A demonstration of the specific cleavage products of type I collagen (TCA and TCB fragments) by SDS-PAGE has been assumed to verify collagenase activity. However, this also is subject to misinterpretation, as degradation products of similar size may occur with nonspecific proteolysis (16).

Granuloma cells derived from *Schistosoma mansoni*-infested murine liver were found to release both an interstitial collagenase and a gelatinase (22). However, the latter activity differed from the lipocyte proteinase in degrading casein, suggesting that it may be a stromelysin (proteoglycanase). Rat kidney mesangial cells, according to a recent report (68), release a neutral metalloproteinase, which is of interest because these mesenchymal cells are the renal equivalent of hepatic lipocytes. Although the molecular mass and range of substrates for the two enzymes are similar, differences exist. The mesangial cell proteinase is activated by trypsin and degrades both casein and type V collagen, unlike the lipocyte proteinase. Oncogene-transformed bronchial epithelial cells (69) and rabbit bone cultures (70) secrete an activity that resembles the lipocyte proteinase in molecular mass, activation by APMA

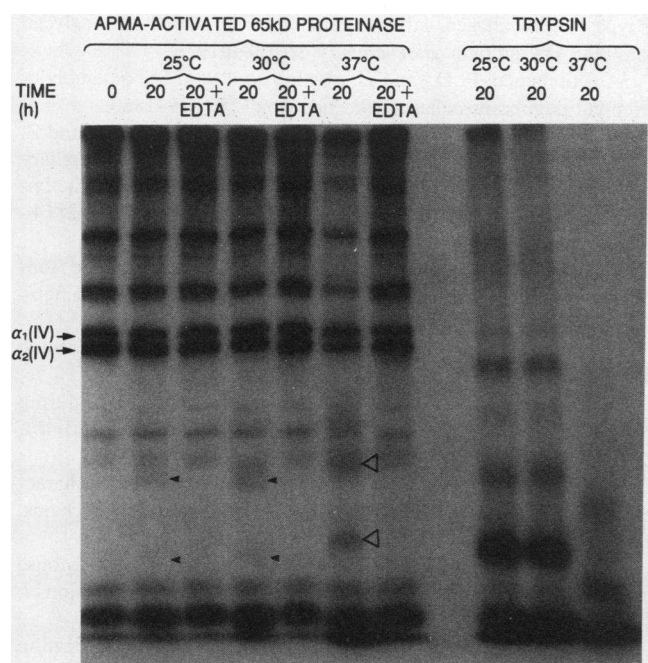


Figure 9. Concentrated, APMA-activated, column fractions containing the 65-kD proteinase or trypsin ($10 \mu\text{g}/\mu\text{l}$) were incubated at 25, 30, and 37°C with native soluble ^{125}I -type IV collagen. The initial reaction mixture contained 36 mU of gelatinase activity and 31,500 cpm of labeled substrate in a final volume of $210 \mu\text{l}$. At each time point, $30\text{-}\mu\text{l}$ aliquots were reduced and electrophoresed on a 5% SDS-polyacrylamide gel for autoradiography. Reaction products obtained at 25 and 30°C are marked with small arrowheads. Different molecular size products, obtained at 37°C , are marked with large arrowheads.

but not by trypsin, and sensitivity to sulfhydryl reduction (70). The range of substrates degraded also is essentially identical. A minor difference is the fact that rabbit bone gelatinase appeared to have low activity against casein and type V collagen, whereas lipocyte proteinase did not degrade casein and attacked type V collagen only at temperatures (32 and 37°C) associated with partial denaturation.

Proteinases with similar substrate specificity have been shown to attack intact basement membranes (71, 72). The lipocyte proteinase, with activity against native basement membrane (type IV) collagen, may be involved in the initiation of matrix degradation in the liver. In current models of the basement membrane, the principal collagen is type IV, which forms the core of the complex (34). Recent work from this and other laboratories has demonstrated the importance of a basement membrane-like complex in the maintenance of differentiated function of hepatocytes (4–6). These studies have shown, moreover, that the entire matrix complex is required: individual purified matrix proteins (type IV collagen, laminin, fibronectin) fail to sustain the expression of hepatocyte-specific functions (4). These data support the concept that the quaternary structure of the subendothelial matrix is critical to its biologic role. Thus, degradation of a single component, such as type IV collagen, may alter the matrix structure sufficiently to have pathologic effects. Also, matrix production by lipocytes is regulated by the extracellular matrix itself: lipocytes cultured on a basement membrane-like substratum are strikingly more quiescent than are cells on individual matrix

proteins or on plastic (3). Alteration of the matrix may cause activation of lipocytes to a proliferating and fibrogenic mode. Such a phenomenon could contribute to the progression of fibrosis that occurs in the apparent absence of the original pathogenic factor (73).

In conclusion, these studies describe a mechanism for the degradation of basement-membrane matrix, mediated by hepatic lipocytes. They point the way to examining production of this proteinase in the intact liver, in order to define its role in hepatic inflammation and fibrosis.

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