Secretion of 72 kDa type IV collagenase/gelatinase by cultured human lipocytes

Analysis of gene expression, protein synthesis and proteinase activity

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The matrix metalloproteinases play an important role in matrix degradation, but there is limited information about this family of enzymes in either normal or diseased human liver. In this study, we have examined the synthesis of a 72 kDa type IV collagenase/gelatinase by human hepatic lipocytes in primary culture. Hepatic lipocytes were isolated from wedges of normal human donor liver by Pronase/collagenase perfusion, purified by density-gradient centrifugation, and established in primary culture on uncoated plastic. By Northern-blot analysis, the total RNA extracted from cultured human lipocytes was found to contain 3.4 kb mRNA for the 72 kDa type IV collagenase/gelatinase. Low levels of expression of this mRNA were observed in freshly isolated lipocytes but expression increased with the duration of lipocyte culture. Using anti-human 72 kDa type IV collagenase/gelatinase IgG, synthesized enzyme was immunolocalized to monensin-treated human lipocyte cultures. *De novo* synthesis and secretion of 72 kDa type IV collagenase/gelatinase were confirmed by immunoprecipitation of radiolabelled enzyme from medium obtained from [³⁵S]methionine-treated cells. Activity of the secreted enzyme was demonstrated by gelatin-zymography and by degradation of soluble, radiolabelled [¹⁴C]gelatin. The enzyme was released both in active and latent pro-enzyme forms and its inhibition profile was that of a metalloproteinase. These studies indicate that cultured human hepatic lipocytes express the gene for the 72 kDa type IV collagenase/gelatinase, and secrete this enzyme, particularly in prolonged primary culture. As this enzyme exhibits degradative activity against basement membrane collagen, its release by activated hepatic lipocytes in the space of Disse could lead to disruption of the normal subendothelial liver matrix. It is suggested that this enzyme may have an important role in human liver injury and fibrosis.

INTRODUCTION

Hepatic lipocytes (fat-storing or Ito cells) are mesenchymal liver cells, located in the subendothelial space of Disse. In normal liver, they contain multiple cytoplasmic vitamin A storage vacuoles and exhibit long stellate processes that encircle the hepatic sinusoid [1,2].

Current evidence points to lipocytes as the primary source of extracellular matrix in liver injury. In diseased human and animal liver, lipocytes proliferate in close morphological association with areas of injury and fibrosis [3–9] and adopt an activated or myofibroblast-like appearance [10]. *In situ* hybridization studies demonstrate that in animal models and human liver disease, mRNA transcripts for matrix proteins (collagens I and III) are confined exclusively to these cells [11–13]. These observations, suggesting an important role for activated lipocytes in liver fibrosis, have been supported by studies of cultured lipocytes. Under conditions which promote activation (see below), lipocytes secrete collagens [14], laminin [15], proteoglycans [16,17] and fibronectin [18].

Identifying the stimuli which provoke lipocyte activation is a major focus of current studies. Although soluble factors including cytokines [19–23] and retinoids [24,25] play a role, cell–matrix interactions may be equally important [26]. The normal subendothelial space of Disse contains a basement-membrane-like matrix comprised of type IV collagen [27,28], laminin [15] and

proteoglycans [16]. When hepatic lipocytes are cultured on a model of this basement-membrane-like matrix (derived from the Engelbreth–Holm–Swarm murine sarcoma; EHS matrix) they are non-proliferative and synthesize small quantities of matrix proteins [26]. In contrast, cells grown on uncoated plastic or type I collagen become activated with the increasing duration of culture [26]. These studies imply that the normal liver matrix maintains hepatic lipocytes in a quiescent non-fibrogenic phenotype, and conversely, that disruption of this cell–matrix interaction is associated with development of a proliferative fibrogenic phenotype.

Differentiated functions of hepatocytes are also dependent on interactions with the normal liver matrix. When isolated hepatocytes are plated on basement-membrane-like matrices they maintain specific gene functions e.g. albumin or specific cytochrome *P*-450 isoenzyme expression, for prolonged periods [29–32]. Cells cultured on type I collagen progressively deteriorate, suggesting that impaired hepatocellular function in liver injury may in part be attributable to changes in the surrounding matrix. These studies indicate that the integrity of the normal liver matrix is fundamental to normal liver function. Thus degradation of this matrix could be a critical event in the pathogenesis of liver injury and fibrosis.

Lipocytes are an attractive candidate cell type for matrix proteinase release, based on their close proximity to the subendothelial matrix, as well as their important role in matrix

Abbreviations used: DMEM, Dulbecco's modification of Eagle's medium; Poly(A)⁺-RNA, polyadenylated RNA; FCS, fetal calf serum; PMA, phorbol myristate acetate; PBS, phosphate-buffered saline; APMA, aminophenylmercuric acetate; PDGF, platelet-derived growth factor; TGF- β , transforming growth factor- β ; PMSF, phenylmethanesulphonyl fluoride.

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production. In an earlier study [33] we described release by rat hepatic lipocytes of a matrix metalloproteinase with degradative activity against native type IV collagen and denatured interstitial collagens (gelatin) that corresponds to the 72 kDa type IV collagenase/gelatinase released by rabbit bone cultures [34] and transformed bronchial epithelial cells [35]. Studies of rat 72 kDa type IV collagenase/gelatinase are limited by concerns over their relevance to human disease and by the limited availability of appropriate molecular probes and antibodies for this species. Recently, we have developed a technique to isolate and culture lipocytes from normal human liver.

In the present study we have used a cDNA probe and IgG antibody to human 72 kDa type IV collagenase/gelatinase to examine synthesis of this enzyme by cultured human lipocytes. We report that these cells contain mRNA for 72 kDa type IV collagenase/gelatinase, and that this enzyme is synthesized *de novo* and secreted by human lipocytes. These studies point to the possibility that this enzyme has an important role in liver injury and fibrosis in man.

MATERIALS AND METHODS

Human lipocyte preparation and culture

Human liver tissue was obtained (via the UCSF Liver Transplantation Service or SRI International, Menlow Park, CA, U.S.A.) from donor organs originally intended for orthotopic transplantation, but subsequently deemed unsuitable for various technical reasons. Donor liver was perfused *in situ* with University of Wisconsin solution [36], transported and stored at 4 °C, and used for lipocyte preparation within 48 h of removal, as previously described [36].

In brief, a 400–600 g liver wedge was initially perfused at 60 ml/min with L15 salts [36] (containing 2 units of heparin/ml) at 37 °C into a vessel opening on the cut surface of the liver. Once clear of residual blood, the liver was perfused with 400 ml of Pronase solution [0.175 % (w/v); Boehringer–Mannheim Chemicals, Indianapolis, IN, U.S.A.] in 50 % Ham's/50 % Dulbecco's modification of Eagle's medium (DMEM; Flow Laboratories, Irvine, CA, U.S.A.) for 15–20 min. Recirculation was achieved by manual transfer of effluent into the perfusate reservoir. The liver was then perfused with 400 ml of collagenase solution [0.02 % (w/v); Boehringer–Mannheim] in 50 % Ham's/50 % DMEM for 30 min. The softened liver was disrupted manually and the crude cell suspension agitated at 37 °C

in a shaking water-bath at 250 rev./min for 30 min in 100 ml of 50 % Ham's/50 % DMEM containing 0.04 % (w/v) Pronase and 0.0004 % (w/v) DNAase (Boehringer–Mannheim). Debris was removed by filtration through sterile cotton gauze and the cell suspension was washed 4-fold by centrifugation (500 *g* × 5 min).

Lipocytes were separated from other non-parenchymal liver cells by arabinogalactan (Larex-Lo, Larex Int., Tacoma, WA, U.S.A.) density-gradient centrifugation, as described previously for rat lipocytes [37]. Human lipocytes were recovered from the < 6 % or the 6–8 % arabinogalactan interfaces and cultured in medium 199 [38] supplemented with 10 % (w/v) calf and 10 % (w/v) horse serum (Flow Laboratories, Irvine, CA, U.S.A.), on uncoated plastic 35 mm dishes (Lux, Miles Scientific, Naperville, IL, U.S.A.). Media were changed daily.

Lipocytes (> 90 % pure) were identified by phase-contrast microscopy (Fig. 1), and by endogenous vitamin A auto-fluorescence and expression of desmin as described previously [36]. The results presented in this paper originate from six different human lipocyte preparations.

Human gingival fibroblast preparation and culture

Human gingival fibroblasts were prepared and maintained in culture as described by Heath *et al.* [39]. Before extraction of mRNA, cells were stimulated for 24 h with 3 % (w/v) partially purified cytokines [40].

Preparation of cDNA for human 72 kDa type IV collagenase/gelatinase

A 1.5 kb human 72 kDa type IV collagenase/gelatinase probe comprising nucleotides 76–1576 in the sequence described by Collier *et al.* [35] was obtained from a human gingival fibroblast cDNA library. The cDNA was generated from poly(A)⁺ RNA selected by oligo(dT)–cellulose chromatography after isolation of RNA by the guanidinium isothiocyanate/CsCl centrifugation method [41] from semi-confluent gingival fibroblasts that had been stimulated in DMEM/1 % fetal calf serum (FCS)/0.2 % lactalbumin hydrolysate/10 ng of phorbol myristate acetate (PMA)/ml and 3 % partially purified cytokines [40] for 24 h. cDNA synthesized using an Amersham kit was blunt-ended using T4 polymerase and then ligated to purpose-built oligonucleotide linkers. These linkers (12-mer and 16-mer), when annealed, resulted in a 3' overhang of 4 bp. The ligated cDNA was then loaded on to a 5–20 % potassium acetate gradient (containing 10 µg of ethidium bromide/ml) and centrifuged at 100 000 *g* for 20 h. Fractions, free of linkers and corresponding to > 1 kb in size, were pooled, concentrated by ethanol precipitation, and ligated to a modified SP64 vector (kindly provided by Dr. Spencer Emtage, Celltech Ltd., Slough, U.K.) that was cut with *Bst*X1 to produce a 5' overhang of 4 bp complementary to that generated by the cDNA-linker ligation. Recombined plasmids were diluted and transformed in library-efficient DH5 cells. Colony lifts on to Hybond-N were alkali-denatured, neutralized and fixed, followed by screening with an oligonucleotide probe corresponding to nucleotides 280–301 in the Collier *et al.* [35] sequence. Plasmid DNA was purified from positive clones and sequenced by the dideoxy-chain-termination method [42] using the double-stranded plasmid DNA as a template and oligonucleotide primers built to both the SP6 promoter and to a region on the 3' side of the pSP64 multiple cloning site. Identification of the 1.5 kb 72 kDa type IV collagenase/gelatinase cDNA was confirmed by sequencing the first 100 nucleotides at either end of the insert, and restriction analysis before its isolation for use as a probe.

RNA extraction and Northern blotting

Total RNA was extracted from either freshly isolated or

Fig. 1. Phase-contrast micrograph of human lipocytes in culture

Lipocytes were isolated and purified from normal human liver as described in the Materials and methods section and established in primary culture on uncoated plastic. A phase-contrast micrograph of cultured human lipocytes on day 7 of culture is shown.

10–14-day-old human lipocyte cultures and from human gingival fibroblast cultures by lysis in guanidinium isothiocyanate (Fluka Chemicals, Glossop, U.K.). RNA was purified by the method of Chomczynski & Saachi [43] and stored in 0.5% SDS at -70°C . Expression of mRNA for 72 kDa type IV collagenase/gelatinase was analysed by Northern blotting. Samples of total RNA were subjected to electrophoresis on a 1% denaturing agarose gel, transferred to a nylon membrane (Hybond, Amersham International, Aylesbury, Bucks., U.K.) and hybridized with the 1.5 kb cDNA probe for human 72 kDa type IV collagenase/gelatinase, labelled with [^{32}P]ATP by random priming using the Klenow reagent (Amersham International). Following hybridization, membranes were washed at 70°C in 0.1% SDS/ $0.1 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M-NaCl}/0.015 \text{ M-sodium citrate}$), dried and exposed to Kodak X-Omat S film for 24–72 h at -70°C .

Immunostaining for type IV collagenase/gelatinase

Primary human lipocytes (7–14 days old), cultured on plastic, were washed three times in medium 199 and cultured in medium 199 containing 10% (w/v) calf/10% (w/v) horse serum and $5 \mu\text{M}$ -monensin (Sigma Chemical Co., Poole, Dorset, U.K.) for 4 h. Monensin was added to cultures to inhibit protein secretion from the Golgi apparatus, thus enhancing intracellular accumulation. Lipocytes were washed three times in phosphate-buffered saline (PBS), pH 7.4, before fixation and after each stage of the following protocol. Cells were fixed in freshly generated 4% (v/v) paraformaldehyde/PBS for 5 min, permeabilized with 0.1% Triton X-100 (for 5 min), and incubated with $50 \mu\text{g}$ of polyclonal sheep anti-human 72 kDa type IV collagenase/gelatinase IgG/ml [44] for 30 min. Controls were incubated in $50 \mu\text{g}$ of non-immune sheep IgG/ml (Sigma Chemical Co., Poole, Dorset, U.K.). This was followed by incubation for 30 min in fluorescein isothiocyanate-conjugated donkey anti-sheep antibody (Sigma Chemical Co., Poole, Dorset, U.K.) diluted 1:100 in PBS. Slides were mounted in Citifluor glycerol/PBS solution (City University, London, U.K.), and visualized using a Zeiss photomicroscope. Photographs were taken on Kodak Ektachrome film rated at 400 ASA.

Immunoprecipitation of type IV collagenase/gelatinase

Human lipocytes (7–14 days old) in primary culture on plastic were washed three times and then pre-incubated overnight in serum-free, methionine-free medium 199 containing 0.5% BSA. Approx. 10^7 cells were labelled for 5 h in serum-free, methionine-free medium 199 containing $250 \mu\text{Ci}$ of [^{35}S]methionine/ml (Amersham). Medium was harvested on ice and phenylmethane sulphonyl fluoride (PMSF) (final concentration, 2 mM) was added to inhibit degradation of secreted proteins. An aliquot (1 ml) of medium was clarified by centrifugation, concentrated 4-fold, and $250 \mu\text{l}$ was incubated overnight at 4°C with $250 \mu\text{l}$ of immunoprecipitation buffer [0.05 M-Tris , pH 8.6, 0.4 M-NaCl , 5 mM-EDTA with 1% (v/v) Nonidet P-40] and $50 \mu\text{l}$ of 2.5 mg of polyclonal sheep anti-(human 72 kDa type IV collagenase/gelatinase) IgG/ml (or in controls, non-immune sheep IgG). Immune complexes were precipitated by incubation with $25 \mu\text{l}$ of Pansorbin (Calbiochem, La Jolla, CA, U.S.A.) at room temperature for 90 min on a rocking platform, followed by centrifugation at $12000 g$ for 3 min. The pellet was washed twice by dissolving in $300 \mu\text{l}$ of immunoprecipitation buffer, and micro-centrifuging at $12000 g$ for 3 min through a 34% sucrose cushion. For each sample the pellet was boiled for 10 min in $100 \mu\text{l}$ of $2 \times \text{SDS/PAGE}$ buffer containing β -mercaptoethanol, and subjected to electrophoresis on a 10% SDS/polyacrylamide gel according to the method of Laemmli [45]. Following electro-

phoresis, gels were washed briefly in Enhance (Dupont-NEN, Stevenage, Herts., U.K.) and dried. For autoradiography, dried gels were placed on Kodak X-Omat S film with an intensifying screen for 14 days at -70°C .

Assessment of released proteinase activities

Human hepatic lipocytes were initially maintained in primary culture on plastic in medium 199 containing 10% (w/v) calf/10% (w/v) horse serum for 7–14 days. To collect medium for analysis, lipocytes were washed three times and pre-incubated for 4 h in serum-free medium 199 (which was discarded) and then incubated in fresh serum-free medium 199 for 24 h. For some experiments cells were cultured in serum-free medium containing cycloheximide ($5 \mu\text{g}/\text{ml}$). Serum-free lipocyte media were harvested after 24 h, clarified by centrifugation, and stored frozen at -20°C before analysis of released proteinase activities. Some samples were lyophilized for transportation to the U.K., reconstituted to their original volume and then analysed. This had no detectable effect on enzymic activity.

Zymography. Proteinase activities in crude unconcentrated human lipocyte media were visualized by gelatin zymography [46].

Quantitative analysis of gelatin-degrading activity. Human lipocyte media were dialysed against sample buffer (0.05 M-Tris , pH 7.6, 0.2 M-NaCl , 10 mM-CaCl_2 , 0.02% NaN_3), for the quantitative assay of gelatin-degrading activity by the method of Harris & Krane [47], with modifications as described previously [33]. Samples were assayed either non-activated or following activation with aminophenylmercuric acetate (APMA) (at a concentration of 1 mM for 60 min at 37°C). For some experiments reactions were performed in the presence of proteinase inhibitors (as described in the Results section).

RESULTS

Detection of mRNA for type IV collagenase/gelatinase

By Northern-blot analysis, total RNA from cultured human lipocytes was found to contain a single hybridization signal of 3.4 kb when probed with cDNA for 72 kDa type IV collagenase/gelatinase (Fig. 2, lanes 2 and 3 and Fig. 3, lanes 1 and 2). This signal was detectable after washing under stringent conditions (0.1% SDS/ $0.1 \times \text{SSC}$ at 70°C), indicating that binding was specific. The estimated molecular size of 3.4 kb, corresponds to that previously reported for human 72 kDa type IV collagenase/gelatinase mRNA in transformed human bronchial epithelial cells [35], and is identical in size to the mRNA detected in stimulated human gingival fibroblasts using the same cDNA probe (Fig. 2, lane 1). In total RNA prepared from freshly isolated human lipocytes, mRNA for 72 kDa type IV collagenase/gelatinase was barely detectable (Fig. 3), but the concentration had increased markedly, in the same lipocyte preparation, by day 12 in primary culture (Fig. 3).

Synthesis of 72 kDa type IV collagenase/gelatinase by human hepatic lipocytes

(a) Immunolocalization. The 72 kDa type IV collagenase/gelatinase was immunolocalized in monensin-treated primary cultures of human hepatic lipocytes. Immunofluorescent staining was concentrated in the perinuclear region of the cytoplasm, consistent with the distribution of the Golgi (Fig. 4a). Lipocytes incubated with non-immune sheep IgG, as controls, demonstrated no significant immunofluorescent staining (Fig. 4b). Cells not pretreated with monensin had a markedly diminished, but detectable, cytoplasmic immunofluorescence (results not shown).

Fig. 2. Northern-blot analysis of total RNA from human hepatic lipocytes and human gingival fibroblasts

Total RNA was extracted from cultured human lipocytes (lanes 2 and 3) or cultured human gingival fibroblasts (lane 1) as described in the Materials and methods section. Samples of total RNA were analysed by Northern blotting. After electrophoresis on a 1% denaturing agarose gel, RNA samples were transferred to a nylon membrane and hybridized with a radiolabelled 1.5 kb cDNA probe for human 72 kDa type IV collagenase/gelatinase. After stringent washing (see the Materials and methods section) specific hybridization signals were visualized by autoradiography. Lane 1, 10 μ g of total RNA from human gingival fibroblasts. Lane 2 (10 μ g) and lane 3 (5 μ g) contain total RNA from human hepatic lipocytes.

Fig. 3. Northern-blot analysis of total RNA from freshly isolated and 12-day-old cultured human hepatic lipocytes

Total RNA from cultured human lipocytes was extracted and blotted as described in the Materials and methods section and the legend to Fig. 2. After hybridization with a radiolabelled 1.5 kb cDNA probe for human 72 kDa type IV collagenase/gelatinase the membrane was washed stringently and hybridization signals were visualized by autoradiography. Lane 1, 10 μ g of RNA from freshly isolated lipocytes; lane 2, 10 μ g of RNA from the same preparation of lipocytes after 12 days in primary culture.

(b) Immunoprecipitation. Synthesis *de novo* and release of 72 kDa type IV collagenase/gelatinase into the culture media by human lipocytes were demonstrated by immunoprecipitation. Culture media obtained from human lipocytes incubated with [35 S]methionine contained a single band of radiolabelled protein,

Fig. 4. Immunolocalization of the 72 kDa type IV collagenase/gelatinase

Lipocytes were isolated and purified from normal human liver as described in the Materials and methods section and established in primary culture. Cultures were exposed to monensin (final concentration, 5 μ M) for 4 h, washed and fixed with 4% (v/v) paraformaldehyde/PBS for 5 min. Fixed cells were immunostained using 50 μ g of sheep anti-(human 72 kDa type IV collagenase/gelatinase) IgG/ml (a) or, in controls, non-immune sheep IgG (b) as described in the Materials and methods section. 72 kDa type IV collagenase/gelatinase was immunolocalized to the cytoplasm of cultured lipocytes, with granular staining concentrated in the perinuclear region, consistent with the distribution of the Golgi.

of molecular mass 72 kDa, when immunoprecipitated with sheep anti-(human 72 kDa type IV collagenase/gelatinase) IgG (Fig. 5). No detectable radiolabelled protein at this molecular mass was found in media immunoprecipitated with non-immune sheep IgG.

Release of type IV collagenase/gelatinase activity by human lipocytes

(a) Zymography. When analysed by gelatin zymography, media from human hepatic lipocytes contained a single band of gelatin-degrading activity, of molecular mass 65 kDa (Fig. 6). This co-migrated with a well-characterized major band of gelatin-degrading activity released by cultured rat hepatic lipocytes [33] (Fig. 6). No gelatin-degrading activity was detected in cell-free blank control media and release of the activity of the 65 kDa protein was markedly inhibited by incubation of lipocyte cultures in media containing cycloheximide at a concentration of 5 μ g/ml (Fig. 6). Similar results were found in four consecutive human lipocyte preparations.

(b) Quantitative analysis of [14 C]gelatin degradation. Unconcentrated media from human lipocyte cultures contained degradative activity against [14 C]gelatin (Table 1). Detectable gelatinase activity was markedly increased if samples were pre-treated with APMA (1 mM at 37 $^{\circ}$ C for 60 min), indicating that more than 50% of the released proteinase was in a latent proenzyme form. APMA-activated proteinase in human lipocyte culture media was completely inhibited by EDTA, but only

Fig. 5. Immunoprecipitation of the 72 kDa type IV collagenase/gelatinase

Human lipocytes in primary culture were pre-incubated overnight in serum-free, methionine-free, medium 199. Approx. 10⁷ cells were then incubated for 5 h in the same medium containing 250 µCi/ml of [³⁵S]methionine. Samples of medium were harvested and immunoprecipitation was undertaken with either sheep anti-(human 72 kDa type IV collagenase/gelatinase) IgG or, in controls, non-immune sheep IgG as described in the Materials and methods section. Precipitated radiolabelled proteins were visualized following SDS/PAGE by autoradiography. Human lipocytes secreted a 72 kDa radiolabelled protein that was immunoprecipitated with anti-(human 72 kDa type IV collagenase/gelatinase) IgG (lane 2), but not by non-immune sheep IgG (lane 1).

Table 1. Release of degradative activity against [¹⁴C]gelatin by cultured human hepatic lipocytes; activation by an organomercurial (APMA) and effect of proteinase inhibitors

Samples of unconcentrated serum-free media harvested from human lipocyte cultures were dialysed against 0.05 M-Tris/HCl, pH 7.6, 0.2 M-NaCl, 10 mM-CaCl₂ and 0.02% azide. Gelatin-degrading activity was assayed using heat-denatured (60 °C, 20 min) rat skin ¹⁴C-labelled type I collagen as the substrate (specific radioactivity 377 c.p.m./µg) as described in the Materials and methods section, with or without activation (1 mM-APMA for 60 min at 37 °C). For some experiments proteinase inhibitors were added to APMA-activated samples as shown. Percentage activity results (*) for PMSF-treated samples were calculated by comparison with the appropriate solvent control (propan-2-ol). One m-unit of gelatinase activity was defined as degradation of 1 ng of gelatin/min. The measured radioactivity averaged 2122 c.p.m. for non-activated and 4424 c.p.m. for APMA-activated samples. Abbreviation: NEM, N-ethylmaleimide.

	[¹⁴ C]Gelatin degradation (m-units)	Activity (%)
Non-activated	6.2	
APMA-activated	13.0	100
+ EDTA (10 mM)	< 0.1	< 0.8
+ NEM (2 mM)	12.5	95.4
+ PMSF solvent (isopropanol)	12.1	(100)*
+ PMSF (1 mM)	10.9	89.9*

minimally inhibited by thiol or serine proteinase inhibitors (N-ethylmaleimide or PMSF respectively; Table 1).

DISCUSSION

The family of matrix metalloproteinases consists of at least eight closely related genes which encode for enzymes involved in degradation of matrix proteins [48,49]. Type IV collagenase/gelatinase (72 kDa) is one member of this family of enzymes, which exhibits degradative activity against type IV and denatured collagens and is secreted by cells of mesenchymal origin [33,34] and by transformed [35] or neoplastic cells [50].

In liver, the role of metalloproteinases in normal matrix turnover or pathological fibrosis is uncertain. The types of metalloproteinases secreted, their cellular origins and relative activities are poorly defined [51]. Thus little is known about metalloproteinase gene expression and its regulation in liver. We have reported previously the release of 72 kDa type IV collagenase/gelatinase by purified rat hepatic lipocytes [33]. Extension of these observations in rat tissue to man has been made possible by our ability to prepare primary cultures of hepatic lipocytes from human liver, and by the development of cDNA probes and IgG antibodies to human 72 kDa type IV collagenase/gelatinase. Our studies demonstrate that cultured human lipocytes express the gene for 72 kDa type IV collagenase/gelatinase, synthesize immunoreactive enzyme *de novo* and release gelatin-degrading metalloproteinase activity into culture media.

mRNA for 72 kDa type IV collagenase/gelatinase was detected in total RNA extracted from 12-day-old primary lipocyte cultures, but was barely detectable in total RNA prepared from freshly isolated lipocytes. This indicates that expression of the gene for the 72 kDa type IV collagenase/gelatinase by lipocytes is minimal in normal human liver. Earlier studies have shown that rat hepatic lipocytes in culture become activated with time, particularly on exposure to plastic [14,26,37,52]. Our studies suggest that this well-characterized phenotypic activation is also associated with expression of the gene for 72 kDa type IV

Fig. 6. Substrate gel analysis of cell-culture media

Human lipocytes in primary culture were washed three times, pre-incubated for 4 h and then cultured for 24 h in fresh serum-free medium 199. Media were harvested and analysed by gelatin zymography as described in the Materials and methods section. Media from one preparation of human lipocytes (lanes 2–4) were compared with a concentrated (positive control) sample from mixed cultures of rat lipocytes and Kupffer cells (lane 1). A single band of gelatin-degrading activity was detected in human lipocyte media (lane 2). Secretion of this activity was inhibited for lipocytes cultured in the presence of cycloheximide, 5 µg/ml (lane 3) and no activity was present in cell-free blank medium (lane 4).

collagenase/gelatinase in cultured human lipocytes. In addition to contact with plastic, lipocyte activation may occur in these cultures because of exposure to cytokines, which promote lipocyte proliferation via induction of platelet-derived growth factor (PDGF) receptor expression [19]. Other cytokines, e.g. transforming growth factor- β (TGF- β) [53], may also be involved in modulating type IV collagenase/gelatinase gene expression in hepatic lipocytes. The relative importance of these stimulatory cytokines *in vivo* has yet to be determined.

These studies of gene expression were complemented by immunolocalization and immunoprecipitation studies demonstrating that cultured human lipocytes synthesize and secrete 72 kDa type IV collagenase/gelatinase. In further experiments we have demonstrated, by two techniques, that the released proteinase has degradative activity against gelatin. First, by gelatin zymography [a technique which reveals latent proteinase activities and separates inhibitors (e.g. tissue inhibitor of metalloproteinases) from enzymes], a single band showing gelatin-degrading activity and of appropriate molecular mass was detected in culture media obtained from human lipocytes (Fig. 6). Secondly, degradative activity was also demonstrated against soluble [14 C]gelatin. Although detectable in untreated samples, the activity was markedly increased following activation with APMA, indicating that the type IV collagenase/gelatinase was released predominantly in the proenzyme form. The mechanism of activation of this enzyme *in vivo* is currently unknown. The APMA-activated enzyme was completely inhibited by EDTA, but not by thiol or serine proteinase inhibitors. Together these results indicate that the gelatin-degrading enzyme released by cultured human lipocytes is a metalloproteinase with properties identical to those previously reported for 72 kDa type IV collagenase/gelatinase [34,35].

The physiological role of 72 kDa type IV collagenase/gelatinase is uncertain. In normal liver our results indicate that expression of this gene by lipocytes is low, consistent with minimal turnover of the extracellular matrix. This low level of expression may reflect a 'housekeeping' function for lipocytes in matrix remodelling in the space of Disse. The proenzyme form of 72 kDa type IV collagenase/gelatinase is also a normal component of human plasma [54]; however, the significance of this observation is unclear. It seems more likely that the enzyme's activities are most significant within local tissues rather than as a systemic mediator, particularly during inflammation and repair. The effects of the active enzyme include disruption of basement membranes, leading to secondary effects on cell function. In diseased liver, secretion of this enzyme by activated lipocytes may be particularly important as the normal subendothelial matrix, comprising of type IV collagen, proteoglycans and laminin, maintains differentiated functions of hepatocytes [29–32] and also maintains hepatic lipocytes in a quiescent non-fibrogenic phenotype [26]. Degradation of the normal liver matrix may disturb hepatocyte function and further promote activation of lipocytes to a proliferating and fibrogenic phenotype. The ability to degrade normal subendothelial matrix may also facilitate migration of lipocytes through the space of Disse to sites of liver injury. It is suggested that secretion of 72 kDa type IV collagenase/gelatinase by activated hepatic lipocytes may contribute significantly to the pathogenesis of liver fibrosis in man.

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