Collagenase production by adult rat hepatocytes in primary culture¹

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ABSTRACT

Adult rat hepatocytes were evaluated after 8 days of primary culture for their ability to produce a latent collagenase which could be activated by limited proteolysis with trypsin. The activated collagenase, like other vertebrate interstitial collagenases, cleaved native collagen in solution at neutral pH at the specific site of three-quarter distance from the N-terminus of the collagen molecule and was inactivated by the presence of EDTA, cysteine or serum. The molecular weights of the hepatocyte collagenase in latent and active forms were estimated to be 73,000 and 45,000, respectively. In early periods (2-6 days) of culture, only a trace level of collagenase activity was detected.

Immunohistochemical investigations using anti-rat albumin antibody showed that 98% of the cells remaining after 8 days of culture retained the ability to produce albumin, indicating that the hepatocytes had acquired an ability to produce collagenase during the period of 8 days of primary culture under the experimental conditions employed.

The elucidation of the mechanism of liver connective tissue metabolism has been of keen importance in terms of pathophysiology of hepatic fibrosis caused by a variety of stimuli. In this connection, attempts to detect a collagenase, a key enzyme of collagen degradation, and its producing cell(s) in normal and experimentally diseased livers have been reported (4, 12, 13). Fujiwara *et al.* first reported the presence of an active collagenase in the Kupffer cells isolated from normal rat liver (4). Okazaki and Maruyama (12) demonstrated an increased collagenolytic activity in CCl₄-treated rat livers, using the

collagen gel lysis method with the tissue slices. Increased levels of total and latent collagenase activities were also demonstrated in the liver homogenate of mice infected with *Schistosoma mansoni* (13). In the latter two cases, however, the cell(s) responsible for the elevated production of the enzyme remained unclear.

Recently, we have demonstrated that normal adult rat hepatocytes acquire the ability to produce interstitial proteoglycans (11) and collagens (7) during primary culture. This paper describes the production of collagenase by the cells under similar experimental conditions.

The hepatocytes, isolated from adult male rats, Wistar strain, by the *Cl. histolyticum* collagenase perfusion method followed by low speed centrifugation (8), were plated onto the 100-mm dishes (5×10^6 viable cells/dish) in Supplemented Williams' Medium E containing 5% fetal calf serum (GIBCO) as reported (7, 8). The cells

¹ This is paper 8 on a series on the molecular mechanisms of hepatic fibrosis.

Abbreviations: Supplemented Williams' Medium E: Williams' medium E supplemented with penicillin (50 mg/l), streptomycin (50 mg/l) and with Fungizone (0.25 mg/l); TPCK: L-1-tosylamide-2-phenylethyl-chloromethylketone

were cultured for two days under 5% CO₂-air at 37°C by replacing the medium after 24 h incubation, and then cultured in serum-free Supplemented Williams' Medium E for 8 days, by replacing the medium every two days.

The harvested media were concentrated in the presence of 0.02% NaN₃ by ultrafiltration through a Diaflo UM-10 membrane, dialyzed at 4°C against 0.05 M Tris-HCl buffer, pH 7.6, containing 0.15 M NaCl, 5 mM CaCl₂ and 0.02% NaN₃ (Tris-NaCl-CaCl₂ buffer) and evaluated for collagenase activity as reported previously (14). Fifty microliters of ¹⁴C-labeled collagen (1,300 cpm/100 µg) were incubated at 35°C with 150 µl of enzyme before and after activation by limited proteolysis with trypsin. Activation of the enzyme was carried out as follows: 80–130 µl of enzyme solution was preincubated with 10 µg of TPCK-trypsin/10 µl of 1 mM HCl for 10 min at 37°C, and the tryptic activity was blocked by adding 10 µl (100 µg) of soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO, U.S.A.) followed by 10 min incubation at room temperature.

In order to characterize hepatocyte collagenase, the concentrated day-8-culture medium (1.2 ml) showing the enzyme activity was applied to a Sephadex G-200 superfine column (1.2 × 110 cm) equilibrated with Tris-NaCl-CaCl₂ buffer and effluent fractions were assayed for collagenase activity. Collagen degradation products were examined by SDS-polyacrylamide slab gel (6%) electrophoresis at pH 8.0 followed by fluorography (3). For this purpose, ¹⁴C-labeled collagen was incubated at 22°C for predetermined periods of time and the reaction was stopped by adding 5 µl of 0.4 M EDTA (final 20 mM), 3 µl of 0.1 M phenylmethylsulfonylfluoride in isopropanol (final 6 mM) and 5 µl of 10% SDS (final 1%). The resulting solution was heated at 80°C for 10 min and subjected to electrophoresis.

DNA content was determined according to the method of Burton (2).

The isolated hepatocytes formed a polygonal structure resembling that in the liver, when cultured in Supplemented Williams' Medium E for 2 days. DNA contents per dish at days 0, 2 and 8 were 50 µg (5.2 × 10⁶ cells), 42 µg and 11 µg, respectively and the viability of the hepatocytes in the initial period of culture was approximately 80% with a contamination of less than 3% of non-hepatocyte cells (small cells) which were mainly erythrocytes (about 70% of the small cells). The relative population of

hepatocytes to small cells did not change until after 8 days of culture, although the cell numbers were gradually decreased. Immunohistochemical stainings of the cultured cells using anti-rat albumin antibody followed by hematoxylin-eosin stainings of the identical cell specimens (9) showed that the ratios of albumin-forming cells to the total remaining cells at day 2 and day 8 were 100% (157 cells/157 cells counted) and 98% (78 cells/79 cells counted), respectively (Fig. 1).

When collagenase activity was assayed after concentration of the cultured medium, only a trace level of the enzyme activity was detected throughout the culture period up to 8 days. After treatment with trypsin, however, a marked increase in the enzyme activity was observed with the day 8 medium (Table 1), suggesting that collagenase was produced in a latent form by the cells after 6 days of culture.

Sephadex G-200 chromatography of the day 8 culture medium showed that the latent collagenase was eluted out at a fraction corresponding to a molecular weight of approximately 73,000. Rechromatography of the enzyme fraction after trypsin treatment indicated that the molecular weight of the activated enzyme was about 45,000.

Fig. 2 shows a typical electrophoretogram of the reaction products of type I collagen incubated with the activated enzyme at 22°C. Hepatocyte collagenase, like other vertebrate collagenases (5, 10), cleaved native collagen in solution at the specific site, forming α^A and α^B fragments (lanes 2–4). The appearance of further degrading products in the case of crude enzyme (day 8 medium, lanes 8–10) suggests that the hepatocytes produce some other neutral protease(s) in addition to collagenase. Hepatocyte collagenase was inactivated by the presence of EDTA, cysteine and human serum, but not by phenylmethylsulfonylfluoride and *N*-ethylmaleimide (Table 2), indicating that the enzyme is a metallo-protease, like other vertebrate collagenases (5, 10).

Although the hepatocytes employed in this study were isolated by the perfusion of rat liver with *Cl. histolyticum* collagenase, the enzyme activity described above could not be attributed to the exogenous collagenase for the following reasons: firstly, some exogenous enzyme(s) are known to be retained in the isolated hepatocytes. However, these enzymes are inactivated and degraded during the first few days in culture (6); secondly, the isolated cells were preincubated for two days with serum containing α₂-macroglobulin, an inhibitor common to proteases

Fig. 1 Indirect immunofluorescent staining of rat hepatocytes in primary culture with anti-rat albumin antibodies. The cells on the 2nd day (a, b) and 8th day (c, d) of the culture were stained with anti-rat albumin antibodies (a, c) and then stained with hematoxylin-eosin (b, d). The arrowheads indicate the identical points in a and b or in c and d, which are the nuclei of dead cells showing strong autofluorescence.

Table 1 *Collagenase Production by Rat Hepatocytes in Primary Culture*

Cultured period	DNA/dish μg	Collagenase activity			
		Before activation	After activation		
		mU/dish	mU/ 10^6 cells ¹	mU/dish	mU/ 10^6 cells ¹
Days 2-4	17.0	1.1	0.6	3.8	2.1
Days 4-6	13.9	2.1	1.5	2.8	1.9
Days 6-8	10.8	2.7	2.4	418	371

Culture media from 32 dishes were harvested on days 4, 6 and 8 and concentrated to about 2.5 ml, separately. Aliquots of 80 μl (days 2-4 and days 4-6) or 10 μl (days 6-8) of the media were assayed for collagenase activity before and after activation with trypsin (see text).

¹ Cell numbers were estimated from DNA contents in dishes, assuming the amount of DNA/ 10^6 cells to be 9.6 μg .

including collagenase (1); thirdly, only a trace level of collagenolytic activity was detected until after 6 days of culture, and finally, the degradation products of collagen observed in this study were characteristic of vertebrate collagenases.

Collagenase production by the hepatocyte was

first demonstrated in this study using a cell culture system. Although hepatocytes produced only a trace amount of the enzyme at early periods of primary culture, the cells began to produce a markedly elevated amount of the enzyme after 6 days of culture when the cells

Fig. 2 Fluorography of SDS-polyacrylamide gel electrophoretogram of ^{14}C -labeled collagen incubated with hepatocyte collagenase. Fifty microliters of ^{14}C -labeled collagen (580 cpm/45 μg) were incubated at 22°C with partially purified hepatocyte collagenase (20 mU) for 0(1), 1(2), 2(3) and 4(4) days or with crude enzyme (concentrated medium (280 mU)) for 0(8), 1(9) and 2(10) days after activation with trypsin treatment. The reaction mixture was incubated with partially purified enzyme for 4 days in the presence of 20 mM EDTA (5) or with the enzyme for 4 days before trypsin treatment (7). The reaction products with purified tadpole collagenase are also shown as reference (6).

Table 2 *Effects of Various Reagents on Rat Hepatocyte Collagenase*

Reagent	Concentration mM	Collagenase activity munits	Inhibition %
Buffer alone	—	45	—
EDTA	10	6	87
PMSF	5	41	9
<i>N</i> -Ethylmaleimide	5	36	20
Cysteine	5	22	51
Serum	5 ¹	7	84

Partially purified hepatocyte collagenase (140 μl) was preincubated with 10 μl of each reagent for 10 min at room temperature and enzyme reaction was performed by incubating with 50 μl of ^{14}C -labeled collagen substrate solution (1,300 cpm/100 μg) at pH 7.6 and 35°C for 24 h.

¹ volume percent. PMSF: phenylmethylsulfonylfluoride

also produced interstitial proteoglycans (11) and collagens (7). Essentially all cells that survived (98%) were albumin-producing cells (hepatocytes). These results strongly suggest that the

hepatocyte can be a candidate cell responsible for collagenase production, thus participating in the metabolism of liver connective tissue under certain pathological conditions.

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