Evidence for the production of type I collagen by adult rat hepatocytes in primary culture: immunohistochemical observations

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ABSTRACT

The production of type I collagen by adult rat hepatocytes in primary culture was confirmed immunohistochemically, using antibodies to rat albumin and type I collagen.

The isolated hepatocytes formed a hepatic cord-like structure, when cultured for 2 days, and showed positive reaction with anti-albumin antibody, but not with anti-type I collagen antibody. After 8 days of culture, however, the cells turned to show positive reactions with anti-type I collagen antibody as well as anti-albumin antibody. The results indicate that hepatocytes have acquired an ability to produce type I collagen during a period of 8 days in primary culture (cell differentiation), preserving the capability for albumin production.

Collagen is the major component of connective tissue. Although the synthesis of collagen has been studied primarily in cells originated from mesenchyme, its production has been demonstrated in a variety of non-mesenchymal cell systems (1) and recent studies have revealed that collagen is a family of proteins composed of at least five genetically distinct molecules (1).

Normal human liver contains relatively equal amounts of type I, III and IV (basement membrane) collagens. In cirrhotic livers, however, the amount of collagen of all these types increases and type I collagen becomes predominant at the later stage of the disease (9). Recently, the production of interstitial types of collagen and glycosaminoglycan by established cell lines originated from rat hepatocytes was reported (4, 7, 10). We could also demonstrate biochemically that even adult rat hepatocytes began to produce type I collagen during an 8-day-period of primary culture, although little collagen was produced by the cells in an early stage (second day) of culture (2).

To confirm whether cells responsible for collagen production are albumin-producing hepatocytes or non-hepatocyte cells which might be contaminated in the isolated hepatocyte fractions, we investigated albumin- and type I collagen-producing cells immunohistochemically using a cultured hepatocyte system.

Hepatocytes were isolated from adult male rats, Wistar strain, by the collagenase perfusion method followed by low speed centrifugation (3, 8). The cells were inoculated onto slide glass plates which were placed in 150 mm Falcon plastic culture dishes with Supplemented Williams’ Medium E containing 10% fetal calf serum (2). Fibroblasts isolated from the abdominal skin of the same rat by treatment with 0.05 % bacterial collagenase (Type I, Sigma Chemical Co., St. Louis, MO, U.S.A. )-0.1% trypsin (DIFCO Laboratories, Detroit, MI,
U.S.A.), were plated as described above. On the second and 8th days of primary culture, the slide glass plates, on which the cells were cultured, were taken out, rinsed with several changes of 0.11 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4 (Tris-buffered saline), and stored in 100% alcohol at −70°C until used.

Rabbit antibody to rat type I collagen was purified by affinity chromatography using Sepharose 4B gels coupled with type I, II or III collagen. Anti-serum to type I collagen was successively passed over columns of type II and III collagens and finally adsorbed to and eluted from an antigen (type I collagen) column, as reported previously (5). The anti-type I collagen antibody thus prepared showed a reciprocal titer of 1/512, and no cross-reaction with other types of collagen and albumin by the passive hemagglutination and hemagglutination inhibition assays. Rabbit antibody against rat serum albumin (Cappel Laboratories, PA, U.S.A.) was purified by affinity chromatography using CH-Sepharose coupled with rat serum albumin after passing through a column of CH-Sepharose coupled with fetal calf serum used for the cell culture.

Sample cell specimens, pretreated with acetone for 10 min, were incubated with anti-albumin antibody in a moist chamber for 60 min at room temperature, rinsed in several changes of Tris-buffered saline, then incubated with fluorescein-isothiocyanate-labeled anti-rabbit IgG goat IgG (MBL Co., Nagoya), diluted 1:16 with the buffer, for 60 min. After rinse in the buffer, the specimens were incubated with anti-type I collagen antibody for 60 min, and then with biotinylated-anti-rabbit IgG goat IgG (Vector Laboratories, CA, U.S.A.), diluted 1:16 with the buffer, for 60 min, as described above, and finally incubated with Rhodamine 600-avidin D (Vector Laboratories, CA, U.S.A.), diluted 1:16 with the buffer, for 30 min, then mounted in an 80% glycerol in the same buffer. The stained samples were observed with a Nikon FL fluorescence microscope. As a control, another set of cultured cells on slide glasses was incubated with rabbit IgG (absorbance at 280 nm = 0.5) immunized with Freund's complete adjuvant alone. We also stained rat and human fibroblasts, and rat hepatocytes co-cultured with rat fibroblasts to examine the specificity of the antibodies for immunofluorescent stainings. Rat fibroblasts were stained only with anti-type I collagen antibody (Table 1). Hepatocytes and fibroblasts co-cultured for 2 days were stained with anti-albumin and anti-type I collagen antibodies, respectively. However, human fibroblasts were stained neither with anti-rat albumin nor with anti-rat type I collagen antibodies. These results indicate that the antibodies to albumin and collagen used in this study are highly specific and the collagen produced by the fibroblasts does not deposit on the hepatocytes co-cultured. The sample cell specimens, which had been stained with antibodies to albumin and type I collagen and examined by fluorescence microscopy, were subjected to hematoxylin-eosin staining and examined by light microscopy as reported pre-

<table>
<thead>
<tr>
<th>Product</th>
<th>Hepatocyte</th>
<th>Frequency</th>
<th>Dermal fibroblast</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2nd day</td>
<td>8th day&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2nd day&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>132/135</td>
<td>74/83</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Type I collagen</td>
<td>nd</td>
<td>64/83</td>
<td>79/85</td>
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Observed by indirect immunofluorescent double stainings, using antibodies to rat albumin and type I collagen. Total cell numbers to be examined were counted after hematoxylin-eosin staining (denominator) and numbers of cells producing albumin and type I collagen were estimated from the cells showing positive reactions with corresponding antibodies (numerator).

<sup>1</sup> The number of cells producing both type I collagen and albumin was 61.

<sup>2</sup> Albumin- and type I collagen-producing cells were surveyed with different cultured dishes.

nd: not detected
Previously (6), the isolated hepatocytes formed a hepatic cord-like structure, when cultured for 2 days, and showed positive reaction with anti-albumin antibody, but not with anti-type I collagen antibody (Fig. 1, a, b and c). After 8 days of culture, however, the cells turned to show positive reactions with anti-type I collagen antibody as well as anti-albumin antibody (Fig. 1, d, e and f), although the cell numbers were decreased and various sizes of polygonal cells lied scattered. When frequencies of albumin-producing and type I collagen-producing cells were examined in day-2 and day-8 cells, using the photos of immunofluorescent double stainings and hematoxylin-eosin stainings (Fig. 1), 89% of the cells remaining after 8 days of culture was albumin-producing and 77% was type I collagen-producing (Table 1). The cells simultaneously producing albumin and type I collagen were 73% of total cells counted, indicating that the collagen was produced by hepatocytes in this culture system, but not by non-albumin-producing cells which might have been present in the isolated cell preparation and rapidly grown during the culture. Day-2-cells showed that 98% of the cells was albumin-producing with no type I collagen production (Table 1).

Analysis of the collagen production during primary culture of hepatocytes has revealed that total collagen synthesis, in which type I collagen is predominant, increased 7 to 9 times on the 8th day, regardless of the addition of aphidicolin, an inhibitor of DNA polymerase α, or not (2). Moreover, albumin-production per cell increased after 8 days of culture (unpublished data).

The results described above provide a strong line of evidence for the production of interstitial collagen by hepatocytes. The cells have acquired an ability to produce collagen under certain conditions such as primary culture (cell differentiation). This suggests that the hepatocyte may participate in liver connective tissue metabolism under certain pathological conditions. Studies along this line are now in progress.

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