curred rhythmically and because reserpine, a drug known to block the rhythm of melatonin synthesis in the pineal gland, also blocked disk shedding (*5*). It is now known, however, that an intact pineal is not necessary for rhythmic disk shedding in rats or for light-evoked shedding in frogs (*14*). In addition, many experiments including studies of in vitro disk shedding (*7, 15*) strongly suggest that the principal features of the shedding control system are intrinsic to the eye (*16*). A role for methoxyindoles in the regulation of shedding remains tenable, however, because such compounds are actively synthesized within the retina (*2, 3, 17*).

Note added in proof: Subsequent to the acceptance of this report for publication we found that melatonin in a medium containing ascorbic acid, but lacking DMSO, significantly activated shedding at concentrations of 0.05 to 0.5 μ*M*.

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24 September 1982

Collagen Formation by the Hepatocyte in Primary Monolayer Culture and in vivo

Abstract. *Immunohistochemical techniques were used to confirm biochemical evidence that parenchymal cells isolated from adult rat liver and maintained in nonreplicating monolayer culture for 2 days synthesized type IV basement membrane collagen. On continued incubation in serum-free medium, the hepatocytes also synthesized the interstitial collagens, types I and III. Consistent with these results in culture, type IV collagen was localized to the hepatocytes in slices of pathologic rat liver. Hence collagen formation is a previously unrecognized function of the hepatocyte that may be important in the pathogenesis of liver fibrosis or cirrhosis.*

A fundamental, but as yet unanswered, question about the pathogenesis of liver fibrosis is which type of cell in the liver is responsible for production of collagen, the major protein component of extracellular matrix (*1*). Although it is widely assumed that in the liver, as in most other tissues, collagen is elaborated exclusively by fibroblasts (*2*), or possibly by resting fibroblast precursors (*3*), we have provided evidence that the liver parenchymal cell also synthesizes collagen (*4–6*).

We have shown that the hepatocyte contains a substantial portion of total rat liver prolyl hydroxylase, a key enzyme

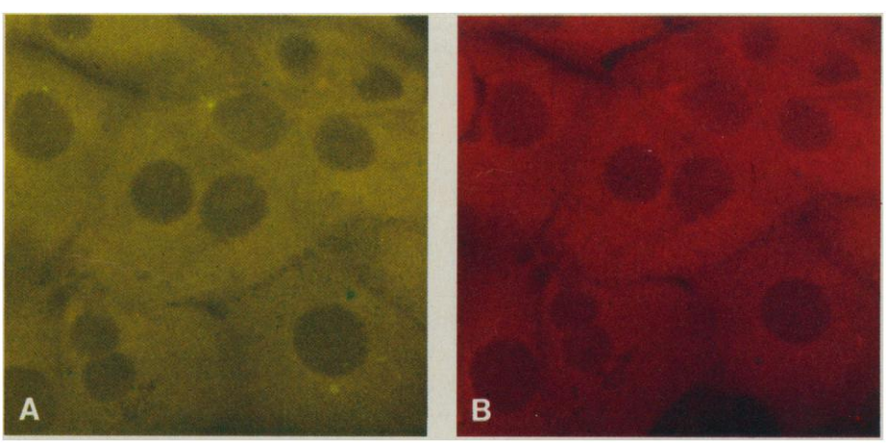

Fig. 1. (A) The presence of type IV collagen in 8-day-old rat hepatocytes in primary monolayer cultures was identified by indirect immunofluorescence. The cultures were reacted with rabbit antibodies to type IV collagen and then with fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobulin (*9*). Exposure of parallel cultures to antibodies against type I and type III collagen (*17*) also resulted in positive staining (not shown). (B) The same cells stained with antibodies to albumin, then with rhodamine-conjugated immunoglobulin against the first antibodies. Hepatocyte cultures were prepared from livers of male Sprague-Dawley rats by perfusion in situ with 0.03 percent crude bacterial collagenase (type I, Sigma) (*5*). The hepatocytes were suspended in culture medium and plated at a density of 3.5×10^6 cells in 60-mm plastic culture dishes (*5*). Highly purified, undenatured collagens were used as antigens in the rabbits to prepare the specific antibodies to collagen types I, III, and IV (*9, 17*). The collagens were prepared by a series of salt fractionations followed by selective chromatography on DEAE-cellulose (*11*). Contaminating antibodies reacting to other types of collagens were removed by cross absorption and coupling the absorbed antiserums to immobilized purified antigen, washing, and then recovering type-specific antibodies by elution (*17*).

in collagen formation (*4*). Primary nonproliferating hepatocytes maintained in culture in a serum-free medium synthesized proteins that were sensitive to collagenase and were rich in hydroxyproline (*5*). The proteins were resistant to pepsin, were soluble in acetic acid, and migrated like collagen standards on chromatography (*5*). The rates of collagen synthesis by the cultured hepatocytes increased dramatically after the first 3 days in culture (reaching values as high as 6 percent of total protein synthesis by day 8), particularly if the hepatocytes were derived from regenerated liver (*6*). Despite an increase in the rate of collagen synthesis in 8-day-old cultures (as much as 500 times the rate in 1-day-old cultures), rates of DNA synthesis did not increase significantly (*5*). Moreover, synthesis of albumin and fibrinogen continued unabated (*5*). These findings make it unlikely that contamination of the hepatocytes with a replicating population of nonparenchymal cells could account for the collagen production by these cultures. Although others have reported that clones of epithelial cells derived from liver synthesize collagen (*7*), the relevance of this observation to the liver in vivo may be questioned because such cells are many generations descended from the original cell source. Our findings of collagen formation in nonreplicating primary cultures of rat hepatocytes have also been directly confirmed (*8*).

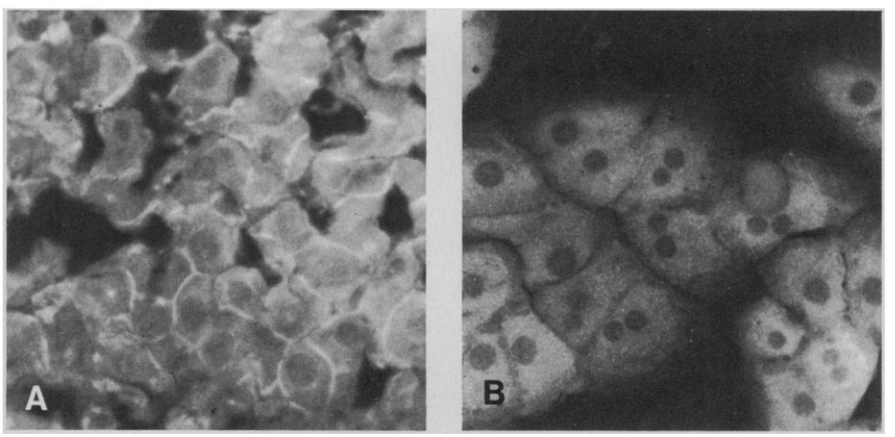

Fig. 2. (A) A 4-μm section of pathologic rat liver specimen (after experimental bile duct ligation) stained with antibody to type IV collagen. (B) Four-day-old primary cultures of hepatocytes isolated from regenerated rat liver and stained with antibody to type IV collagen. Positive reactivity was not observed when the pathologic liver slice (A) and the 4-day-old monolayer culture prepared from regenerated liver (B) were exposed to antibodies to collagen types I and III.

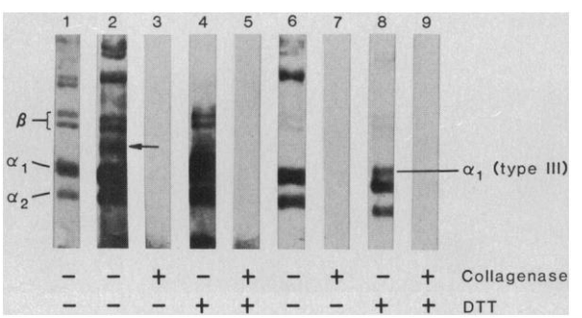

Fig. 3. Fluorogram of rat hepatocyte collagens produced on the 6th day of monolayer culture. (Lane 1) Radioactive collagen standards. (Lanes 2 and 3) Acetic acid–soluble collagen extracted from the hepatocyte layer before reduction with dithiothreitol (*DTT*), and (lanes 4 and 5) after reduction. (Lanes 6 and 7) Collagen isolated from the culture medium before delayed reduction with DTT, and (lanes 8 and 9) after reduction with DTT. Radioactive collagen was prepared from 6-day-old hepatocyte cultures after incubation for 24 hours with fresh ascorbate (0.1 m*M*), β-aminopropionitrile (100 mg/ml), and [^{3}H]proline and [^{3}H]glycine (100 μCi of each per plate). The cells and medium were gently scraped into tubes placed on ice, and the plates were washed with tris buffer (0.05*M*, *p*H 7.2) containing 20 m*M* EDTA, 8 m*M* *N*-ethylmaleimide, and 1 m*M* phenylmethylsulfonyl fluoride (all chemicals from Sigma). Soluble materials were separated from the cell-associated material by centrifugation, and the collagen in the pellet was extracted with 0.5*M* acetic acid in the cold. The extract was dialyzed against 0.5*M* acetic acid before treatment with pepsin (0.1 mg/liter, 6 hours, 4°C). The reaction mixture was then neutralized and dialyzed again against 0.5*M* acetic acid in the cold. The material was divided into two equal portions, one of which was treated with bacterial collagenase (*6*) before polyacrylamide slab gel electrophoresis (*18*) with 3 percent stacking gels and 5 percent separating gels and urea (0.5*M*) in the running buffer. Collagen in the soluble medium fraction was precipitated by the addition of 4.5*M* NaCl before dialysis, pepsinization, and collagenase treatment as descibed above for the cell-associated collagen.

We now report the use of immunohistochemistry to confirm that hepatocytes produce collagen and to determine which types of collagen are formed in culture. Hepatocytes, isolated from the livers of normal rats or from animals subjected 4 days earlier to a two-thirds partial hepatectomy, were maintained as monolayers on glass cover slips (*4*). After 2 days of incubation, when the rates of collagen synthesis were low (0.01 to 0.1 percent) (*5*), and at various ages in culture thereafter up to 8 days, when collagen synthesis had risen to as high as 6 percent (*6*), the culture medium was removed, and the cell layers were washed with saline twice and air-dried. The fixed monolayers were exposed to antibodies specifically directed against the α_1 chain of type IV collagen, previously described as C chain (*9*), the major component of basement membrane collagen. In addition, the hepatocytes were exposed to antibodies specific to collagen types I and III, the major interstitial forms of collagen (*10*). The identical cells were simultaneously stained with antibodies to rat albumin or to rat fibrinogen.

In testing 2-day-old cultures with the antibodies to collagen, we found positive staining of the hepatocytes for type IV collagen, but negative staining for type I or type III collagens. The same cells exhibited positive staining when they were exposed simultaneously to antibodies to albumin or to fibrinogen (not shown). This establishes that the cell producing type IV collagen is the hepatocyte. Positive staining of the hepatocytes with antibodies to type IV collagen (Figs. 1A and 2B), albumin (Fig. 1B), or fibrinogen (not shown) persisted throughout 8 days of culture. Moreover, in cultures older than 4 days, positive staining for type III and type I collagens in addition to type IV was observed in the cells that stained positively for albumin or fibrinogen (not shown). The three collagen phenotypes appeared in the same sequence regardless of whether they were prepared from normal (not shown) or from regenerated liver (Fig. 2B). In all cultures that demonstrated positive reaction for collagens type IV, III, or I, the staining uniformly involved all of the hepatocytes, thus excluding the existence of a subpopulation of collagen-producing liver cells.

To verify that simultaneous formation of multiple forms of collagen by the hepatocyte represented de novo synthesis, we exposed 6-day-old cultures for 24 hours to medium containing radioactive proline and glycine. The culture medium was separated from the hepatocytes; in each fraction, the noncollagenous pro-

teins were digested with pepsin, and the collagenous proteins were extracted with acetic acid, separated by electrophoresis on polyacrylamide slab gels, and visualized by fluorography. Three cell-associated collagens were identified: (i) the typical α_1 and α_2 chains of type I collagen (lane 2 in Fig. 3); (ii) a high molecular weight collagen (above the β region), which was reduced to a lower molecular weight by treatment with dithiothreitol (DTT) (lane 4 in Fig. 3), corresponding to the α_1 chain of type III collagen; and (iii) two collagenase-sensitive, reducible radioactive bands observed in the γ region (top of the lane) on lanes 2 and 6 and an additional band migrating between α_1 (type I) and the β region (lane 2 in Fig. 3, arrow). These three bands are consistent with pepsin-generated fragments of the α_1 chain of type IV collagen (*11*). Type V collagen, if present, would also migrate near the region indicated by the arrow (lane 2). However, it is unlikely that this band (arrow, lane 2) represents type V collagen because when cultures were stained with antibodies specific to type V, only trace reactivity was observed. Moreover, attempts to isolate type V collagen from the cultures with selective salt fractionation procedures have thus far been unsuccessful. The medium from hepatocyte cultures contained type I and type III collagens (lanes 6 and 8 in Fig. 3) and also faint, reducible bands in the γ region (lane 6) consistent with pepsin-generated fragments of the α_1 chain of type IV collagen. Although the hepatocyte cultures were incubated in the presence of β-aminopropionitrile during the period of exposure to radioactive proline and glycine, there is still some evidence for cross-linked collagen in the β region in lanes 2, 4, 6, and 8 (Fig. 3).

These results provide evidence that mature hepatocytes in culture are capable of simultaneously synthesizing at least three types of collagen. That the collagen initially formed by the hepatocyte in culture is a component of basement membrane implies physiologic or pathophysiologic importance. A complete and thickened basement membrane in the sinusoid adjacent to the hepatocyte has been observed in normal regenerated liver and in cirrhosis (*12*). However, the relevance of our findings with cultured hepatocytes to the liver in vivo has been questioned (*1, 13*) in that many types of cells may produce collagen in culture (*14*). To demonstrate that the hepatocyte produces collagen in vivo as it does in culture, we stained slices of pathologic rat liver with the antibodies to collagen. The hepatocytes as well as the adjacent sinusoids uniformly stained positively for type IV collagen (Fig. 2A). Only under pathologic conditions, such as experimental bile duct ligation, does the hepatocyte produce and deposit type IV collagen. This phenomenon cannot be observed under normal physiologic conditions. No staining of the hepatocytes was observed with antibodies to type I or type III collagen. We conclude that among the normal reparative processes in the liver is the production of basement membrane collagen by the hepatocyte.

The conditions used to isolate and maintain the hepatocyte in culture may evoke its collagen-synthesizing potential and thus simulate some of the conditions of liver injury in vivo (*1*). Our study demonstrates that under culture conditions that permit expression of numerous specialized functions of the adult liver, such as albumin or fibrinogen formation or drug-mediated induction of mature forms of cytochrome P-450 (*15*), hepatocytes also sequentially recapitulate the time course of phenotypic expression of collagens associated with liver injury in vivo (*16*). Hence, the sequential appearance of basement membrane collagen, type I collagen, and type III collagen from hepatocytes in culture implies that these cells in vivo may be important contributors to the fibrotic process.

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Specific Reading Disability: Identification of an Inherited Form Through Linkage Analysis

Abstract. *Linkage analysis in families with apparent autosomal dominant reading disability produced a lod score of 3.241. Since the traditionally accepted significance level for linkage is a lod score of 3.0, these results strongly suggest that a gene playing a major etiologic role in one form of reading disability is on chromosome 15.*

Specific reading disability is diagnosed in an individual with severe reading and spelling problems in the absence of neurological, intellectual, emotional, or environmental handicap. Although multiple etiologies are likely within this broadly defined group of affected individuals, a strongly positive family history is frequently reported by educators working with affected children. This suggests that many cases may be primarily genetic in origin. The lack of precise diagnostic criteria and laboratory tests have restricted the ability of investigators to