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Hepatic lipocytes: The principal collagen-producing cells of normal rat liver

(hepatocytes/sinusoidal endothelium/vitamin A/liver cell culture)

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ABSTRACT **Hepatic lipocytes were isolated from normal rat liver and established in culture. A virtually pure isolate was obtained by fractionating enzymatically digested liver on a discontinuous gradient of arabinogalactan. Isolated cells displayed prominent rough endoplasmic reticulum and typical cytoplasmic droplets containing vitamin A. Lipocytes in primary culture were shown by immunofluorescence to secrete collagen types I, III, and IV and also laminin. Immunoassay of culture media showed that lipocytes during the first week in culture secrete type I (72.1–86.2% of total measured soluble collagen), type III (2.6–7.2%), and type IV (11.2–29.7%) collagens. Five percent of total secreted protein was collagen compared with 0.2% in similarly cultured hepatocytes and 1.7% in sinusoidal endothelial cells, as measured by the production of peptide-bound [^{3}H]hydroxyproline in cells incubated with [^{3}H]proline. The calculated amount of collagen synthesized by lipocytes per μg of cellular DNA was 10-fold greater than that produced by hepatocytes and over 20-fold greater than that produced by endothelial cells. The findings indicate that collagen synthesis and secretion are specialized functions of hepatic lipocytes, and that, in cells from normal liver, this represents production primarily of type I collagen. The phenotypic resemblance of these cells to fibroblasts supports the hypothesis that lipocytes are a major source of collagen in pathologic fibrosis and may be precursors of the fibroblast-like cells observed in liver injury.**

The cellular sources of collagen in normal or fibrotic liver has been a subject of speculation. Among the resident cells in normal liver, hepatocytes, sinusoidal endothelial cells, and lipocytes appear to synthesize collagen (1–3). Hepatic lipocytes (Ito cells, fat-storing cells) are stellate cells that are located in the perisinusoidal space (4). They are the predominant hepatic storage site for vitamin A (5), and are about as numerous as Kupffer cells (6, 7). On morphologic grounds, lipocytes have been associated with collagen fibers in rats that have CCl_4-induced liver fibrosis (3). However, such data do not establish that lipocytes actually synthesize collagen.

In this study, we report the isolation and primary culture of lipocytes from normal rat liver. We have characterized collagen synthesis by these cells with respect to the type and the amounts of collagen released, and we have compared lipocyte collagen synthesis to that of cultured hepatocytes and sinusoidal endothelial cells. The data indicate that lipocytes are the predominant collagen-producing cells of normal rat liver.

MATERIALS AND METHODS

***In Vivo* Labeling of Lipocytes and Other Nonparenchymal Cells.** Young male Sprague–Dawley rats, weighing 350–400 g and fed ad lib, were used. The vitamin A content of hepatic lipocytes was increased by daily subcutaneous injection of rats with retinyl acetate (Sigma) (the total dose was 500–750 kIU). Sinusoidal endothelial cells were labeled by intravenous injection of fluorescently tagged acetoacetylated low density lipoprotein and identified by fluorescence microscopy, as previously described (2). To identify Kupffer cells, formalin-fixed *Staphylococcus aureus* (Zymed Laboratories, Burlingame, CA) were labeled with fluorescein isothiocyanate (FITC) (8) and were injected intravenously in a 1-ml suspension (27 mg of staphylococci) 10 min prior to liver perfusion. Uptake of staphylococci is an exclusive property of Kupffer cells in rat liver as shown by the localization of organisms to cells displaying the Kupffer cell marker, endogenous peroxidase (unpublished results). Vitamin A within lipocytes was seen as rapidly fading yellow-green fluorescence when viewed with filters designed to detect FITC (9).

Liver Dispersion and Cell Isolation. Details of the method will be reported elsewhere. Briefly, the liver was digested enzymatically with Pronase and collagenase, as described by Knook *et al.* (6). Nonparenchymal cells were harvested by centrifugation of the digest and suspended in 25 ml of Eagle's minimal essential medium without calcium or magnesium (MEM-E) containing deoxyribonuclease (10 μg/ml) (Sigma). One quarter (6.25 ml) of the cell suspension was pipetted on top of each of the four Stractan gradients. Stractan (arabinogalactan, Sigma) was prepared as described (10). Each tube contained a discontinuous gradient consisting of 1.5 ml each of 6, 8, 12, and 20% Stractan (densities of 1.0210, 1.0298, 1.0476, and 1.0829, respectively). The gradients were centrifuged at 20,000 rpm for 30 min at 25°C in a Beckman SW-40 rotor at 25°C.

Cell Culture. After centrifugation, lipocytes were recovered from the interface between 6% Stractan and medium. The cells were washed, and 1–2 × 10^6 cells per dish were plated in 35-mm tissue culture dishes (Lux, Miles Scientific, Naperville, IL) in 1 ml of medium 199 (2) (GIBCO) supplemented with calf and horse serum (Flow Laboratories), each at 10% (vol/vol). Lipocyte viability was assessed by measuring trypan blue exclusion (11). Medium was replaced every 24 hr. For endothelial cell isolation the cells at the interfaces between 8–12% Stractan and 12–20% Stractan were pooled. Endothelial cells were then separated from Kupffer cells and erythrocytes by centrifugal elutriation and plated on plain plastic 35-mm Petri dishes, as described (2). Hepatocytes were isolated from male Sprague–Dawley rats (12), purified by centrifugal elutriation, and cultured at a density of 2 × 10^6 cells per 35-mm plate.

Morphologic and Immunohistologic Methods. Affinity-purified antibodies to murine collagen types I, III, and IV were prepared as described (13, 14). Antibodies to laminin and type V collagen were the gift of Heinz Furthmayr and Joseph

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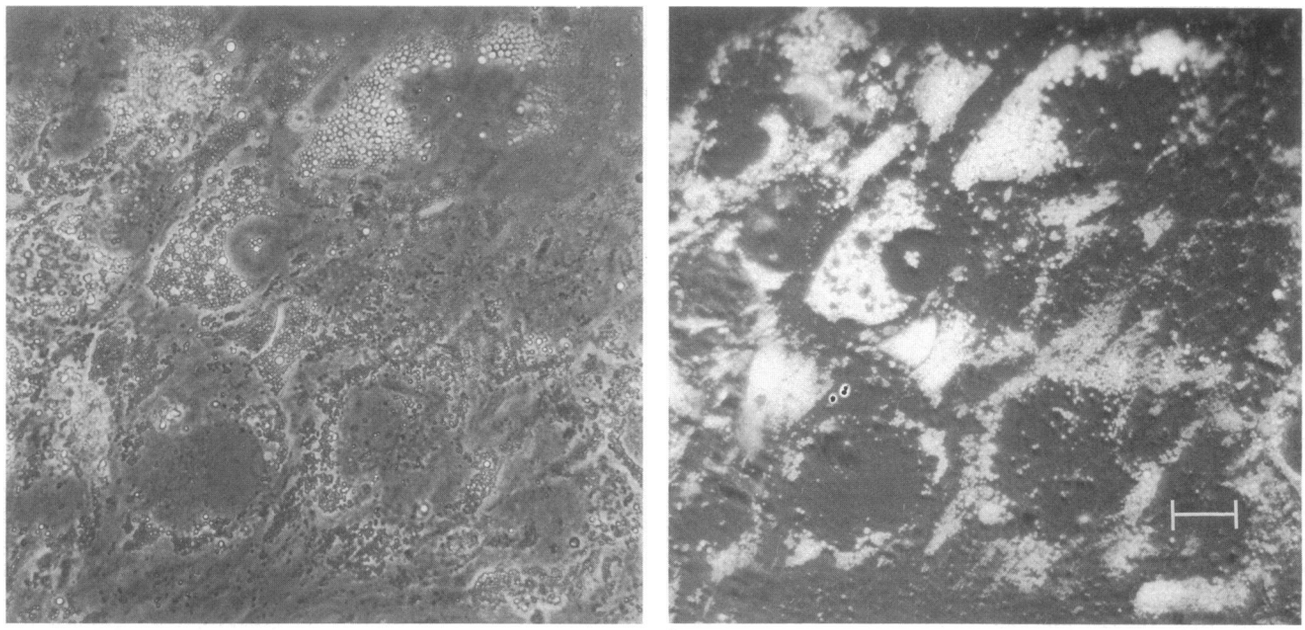

FIG. 1. Phase-contrast (*a*) and fluorescence (*b*) micrographs of a lipocyte culture (day 4 of incubation). A prominent feature of lipocytes under phase-contrast microscopy (*a*) is abundant refractile intracellular droplets. When the same field is viewed by epi-illumination (*b*), the droplets are fluorescent due to their vitamin A content. (Bar = 20 μm.)

Madri (Department of Pathology, Yale University). Rat procollagens I and III were isolated by the method of Smith *et al.* (15), and purity was assessed by $NaDodSO_4$/PAGE (16). Antisera were raised in rabbits and titered by hemagglutination inhibition or immunodiffusion. For immunofluorescent studies of extracellular matrix proteins, cells in plastic dishes were fixed with 0.5% paraformaldehyde for 1 hr, were incubated in 0.25% bovine testicular hyaluronidase (Sigma) for 30 min, and incubated with antisera for immunofluorescence labeling (17).

For electron microscopy, freshly isolated lipocyte pellets or cultured cells on dishes were fixed, dehydrated, and embedded in Epox 812 (Fullam, Schenectady, NY).

Quantitation of Collagen Production. Type-specific collagen synthesis by cells in culture was assessed in culture medium by an ELISA (18). Cells established in culture on plain plastic were incubated in medium containing ascorbate (50 μg/ml), β-aminopropionitrile (100 μg/ml) and 20% (vol/vol) serum. Assays were performed using antibodies to collagen and an avidin-biotin-peroxidase complex as described by Madri and Barwick (19). Fresh medium served as a control. Final data are represented as ng of collagen per μg of DNA (20).

Collagen synthesis was estimated also from the incorporation of [^{3}H]proline into peptide-bound [^{3}H]hydroxyproline. Five-day-old lipocyte cultures, 3-day-old sinusoidal endothelial cells, or 2-day-old hepatocytes were incubated for 24 hr in Medium 199 with [2,3,4,5-^{3}H]proline (50 μCi/ml; 1 Ci = 37 GBq; Amersham) and fresh ascorbate (50 μg/ml) and with or without serum (see above). Cells and media were harvested separately into 0.5 M acetic acid containing protease inhibitors (21), then dialyzed against distilled water, and hydrolyzed in 6 M HCl for amino acid analysis (22). The individual fractions were suspended in Aquasol (New England Nuclear) and the radioactivity was quantitated with a counting efficiency of 33%. Final data are expressed as dpm of tritiated hydroxyproline or tritiated proline per μg of DNA (20).

RESULTS

Isolation and Culture of Lipocytes. Each of four interfaces of the discontinuous gradient of Stractan after centrifugation yielded a population of nonparenchymal cells. The cellular composition of individual isolates was reproducible, as assessed by fluorescence microscopy. The top of the gradient contained 2–4 $\times$ 10^6 lipocytes that were >95% pure. This yield represented 80% of the total number of lipocytes recovered from the gradient and 20% of those in the crude starting digest.

Lipocytes obtained from the top of the gradient adhered to uncoated plastic culture dishes with an efficiency of 59.5 $\pm$ 8.9% (n = 4) when the number of attached cells was measured 24 hr after plating. This time point was chosen because lipocytes are relatively buoyant and, therefore, settle slowly. After 24 hr in culture lipocytes attached to the substratum were mostly spherical but flattened, thereafter, and were stellate in appearance after 48 hr (Figs. 1 and 2). By

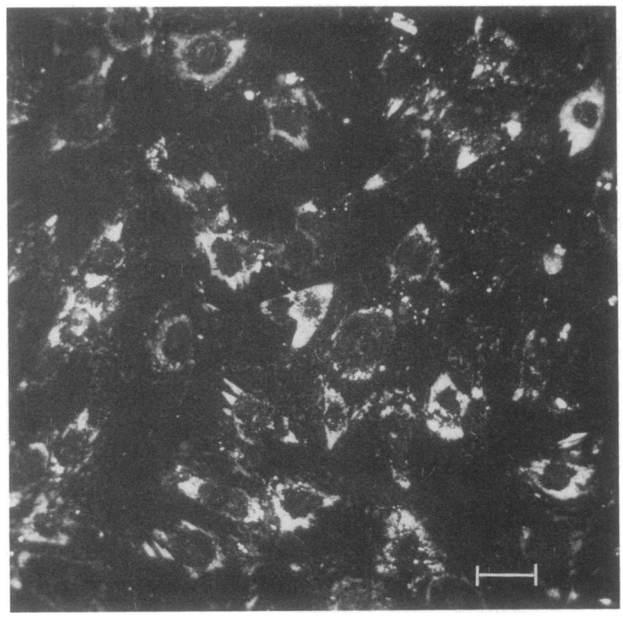

FIG. 2. Fluorescence micrograph of lipocytes after one week in primary culture. (Bar = 80 μm.)

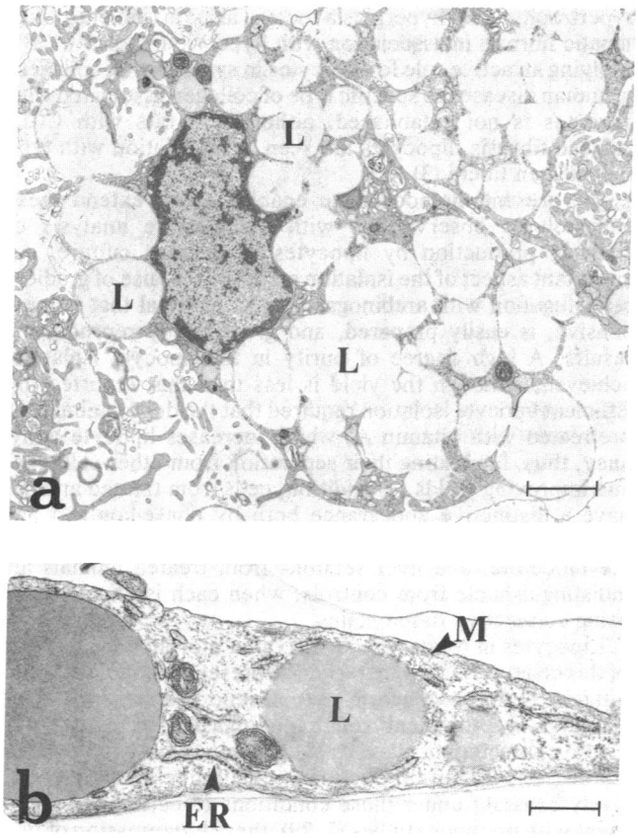

FIG. 3. (*a*) Electron micrograph of a fresh lipocyte isolate with abundant vitamin A-containing droplets (L). (Bar = 2.0 μm.) (*b*) Electron micrograph of a cultured lipocyte showing typical ultrastructure. L, lipid (vitamin A); M, microtubule; ER, endoplasmic reticulum. (Bar = 0.8 μm.)

fluorescence and phase-contrast microscopy, all cells exhibited typical vitamin A fluorescence for a period of at least 21 days. The ultrastructural features of lipocytes examined immediately following isolation (Fig. 3*a*) and in primary culture (Fig. 3*b*) included prominent lipid droplets, well-developed rough endoplasmic reticulum, and microtubules, but virtually no lysosomes.

The attachment, phase-contrast morphology, and survival of lipocytes on plain glass or glass coverslips coated with collagen types I, III, IV, or V, fibronectin or laminin were indistinguishable from those of cells on uncoated plastic. Therefore, cells were routinely plated on uncoated plastic.

Collagen Production by Lipocytes. Cell-associated collagen was characterized qualitatively by immunofluorescence, with type-specific antibodies. Lipocytes bound antibodies to collagen types I, III, and IV, and to laminin, but not to type V collagen. Labeling for type I collagen was increased after pretreatment of fixed cells with hyaluronidase, suggesting that the interstitial collagens may be partially masked by hyaluronic acid and/or proteoglycan. There were distinctive patterns for the individual collagen types. Types I and III collagen and procollagen were in the form of coarse fibrils whereas the labeling pattern for type IV collagen and laminin resembled a fine meshwork (Fig. 4).

Secretion of soluble collagen was quantitated by ELISA (Table 1). Lipocytes secreted predominantly type I collagen; production of collagens type III and type IV was detectable but minor. Secretion of collagen, qualitatively as well as quantitatively, was stable for at least 7 days in primary culture. In similar studies with unconcentrated medium from cultured hepatocytes and sinusoidal endothelial cells, there was no detectable type I collagen.

Newly synthesized collagen from cells incubated with tritiated proline was measured as peptide-bound tritiated hydroxyproline. Five percent of the total protein secreted by lipocytes was collagen (Table 2); this result was unaffected by the presence or absence of serum in the incubation medium (data not shown). From parallel studies with hepatocytes and sinusoidal endothelial cells in primary culture, it was found that the ratio of collagen to noncollagen protein synthesis was 20- to 30-fold greater for lipocytes than for hepatocytes and approximately 3-fold greater than for endothelial cells. More-

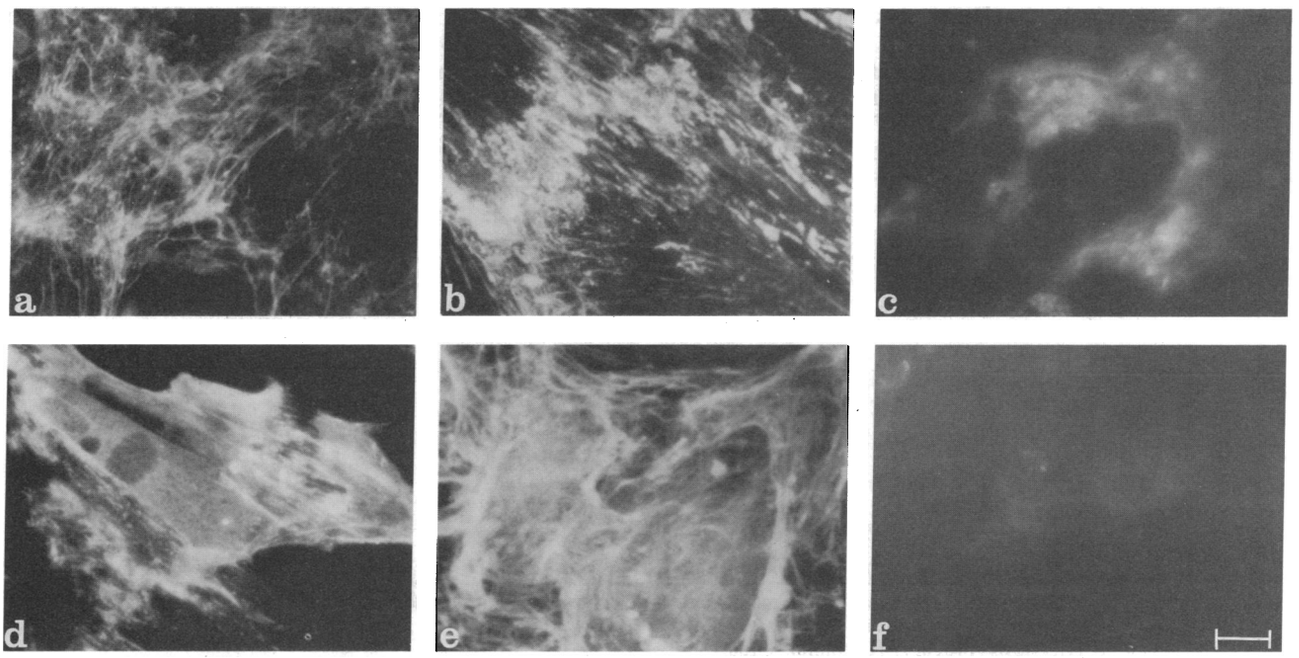

FIG. 4. Immunofluorescence micrographs of 7-day-old lipocytes in primary culture. Cells were fixed and incubated with antibodies to the following proteins: (*a*) type I collagen, (*b*) type IV collagen, (*c*) type III collagen, (*d*) laminin, (*e*) type I procollagen, and (*f*) control culture, nonimmune rabbit IgG. (Bar = 20 μm.)

Table 1. Type-specific collagen production by lipocytes in primary culture

Collagen type	Relative secretion†		
	Day 3	Day 5	Day 7
I	72.1 ± 7.1	86.2 ± 10.1	63.1 ± 11.5
III	6.9 ± 2.4	2.6 ± 1.1	7.2 ± 1.1
IV	21.0 ± 4.7	11.2 ± 9.0	29.7 ± 10.9

The mean amounts of total collagen secreted were (ng of collagen per μg of DNA per 24 hr): day 3, 811.2; day 5, 2567.7; and day 7, 1476.7.

†Type-specific collagen production is shown as a percent of total collagen in the culture medium on culture days 3, 5, and 7. The combined amounts of collagen types I, III, and IV were assigned as 100% of total collagen measured for each culture period. For each time period, cells were incubated for 24 hr in medium containing β-aminopropionitrile and ascorbate. Samples were frozen at −70°C until analysis. Each value represents mean ± SD for three separate lipocyte preparations.

over, total collagen synthesis (cpm [^{3}H]hydroxyproline per μg of DNA), was up to 10 times greater for lipocytes than for hepatocytes and 20 times greater than for sinusoidal endothelial cells labeled under identical conditions (Fig. 5). To ensure that the measured [^{3}H]hydroxyproline from cultured hepatocytes was not artifactually low (due to dilution of labeled proline by endogenous unlabeled amino acid), we measured the specific activity of the [^{3}H]prolyl-tRNA pool, by the method of Airhart *et al.* (25). Under the conditions of these experiments, the specific activity of prolyl-tRNA reached 90% of that in the medium within 15 min after introduction of [^{3}H]proline. Thus, the observed isotope incorporation accurately reflects the synthesis of collagen by hepatocytes.

DISCUSSION

Hepatic lipocytes have attracted interest recently because of evidence, primarily morphologic, suggesting that these cells synthesize collagen in hepatic fibrosis. In human alcoholic fibrosis, lipocytes are associated with increased local collagen accumulation both at the periphery of the hepatic lobule (26) and within the Disse space (27). Lipocyte hypertrophy and hyperplasia occur also in patients with hepatic fibrosis in association with hypervitaminosis A (28), implying an active role for lipocytes in synthesizing collagen. In human disease the specific type of collagen associated with lipocytes is not established, although in rats with CCl_4-induced fibrosis, lipocytes are seen in association with type III collagen fibers (3).

The present methods have enabled us to extend these morphologic observations with quantitative analysis of collagen production by lipocytes in primary culture. An important aspect of the isolation method is the use of gradient centrifugation with arabinogalactan, a material that is inexpensive, is easily prepared, and gives highly reproducible results. A high degree of purity in the lipocyte isolate is achieved, although the yield is less than that reported (6). Efficient lipocyte isolation required that the donor animals be pretreated with vitamin A, which increases lipocyte buoyancy, thus, facilitating their separation from other cell types and improving yields. In addition, cells from treated animals have a distinctive appearance both by phase-contrast and fluorescence microscopy (Fig. 1). The amounts of vitamin A are moderate, and liver sections from treated animals are indistinguishable from controls, when each is examined by using connective tissue stains.

Lipocytes in primary culture exhibit morphologic features of this cell type *in vivo*, namely, the presence of rapidly fading vitamin A fluorescence in cytoplasmic droplets, a stellate shape, and prominent rough endoplasmic reticulum (4). These characteristics are stable for several days in culture. Likewise, collagen production is qualitatively and quantitatively constant under these conditions of culture. In agreement with previous studies (3, 29), there is expression of type III collagen on the cell surface, as detected by immunofluorescence. In the culture medium, however, ≈70% of the total measured collagen is type I. Thus, we characterize these cells as secretors predominantly of type I collagen in normal liver. Whether this characterization is relevant also to fibrogenesis in liver injury is a matter of conjecture. It is possible that, under specific kinds of stimuli, lipocytes undergo a shift in their collagen phenotype, becoming producers of type III collagen. Numerous examples of "type-switching" have been documented, notably in cells main-

Table 2. Incorporation of [^{3}H]proline into collagen and noncollagen protein by lipocytes, endothelial cells and hepatocytes in pure primary culture

	Incorporation, dpm × 10^{-3} per μg of DNA*		Relative incorporation†, %	Collagen synthesis, % of total protein‡
	[^{3}H]Hydroxyproline	[^{3}H]Proline		
Lipocytes (n = 4)				
Cell	32.1 ± 12.0	534.1 ± 132.6	6.01	2.59
Medium	72.9 ± 12.6	408.3 ± 107.1	17.85	8.53
Total	105.0 ± 12.0	942.4 ± 161.1	11.12	5.00
Endothelial cells (n = 3)				
Cell	0.86 ± 0.12	98.1 ± 10.7	0.88	0.36
Medium	4.2 ± 0.56	25.0 ± 2.6	16.80	7.95
Total	5.0 ± 0.64	123.1 ± 13.0	4.06	1.72
Hepatocytes (n = 4)				
Cell	6.3 ± 2.1	1189.3 ± 589.0	0.53	0.22
Medium	5.4 ± 1.2	883.0 ± 255.0	0.61	0.25
Total	11.7 ± 3.3	2072.3 ± 832.3	0.56	0.23

*The values shown are mean ± SD; measurement of [^{3}H]proline incorporation by amino acid analysis was performed in duplicate for each culture.

†([^{3}H]Hydroxyproline/[^{3}H]proline) × 100.

‡Calculated according to the formula of Diegelmann (23): % of collagen = (dpm [^{3}H]hydroxyproline + x)/[(dpm [^{3}H]proline − x) 5.4 + (dpm [^{3}H]hydroxyproline + x)], where x = dpm [^{3}H]hydroxyproline/0.826 and 0.826 = ratio of hydroxyproline to proline residues found in rat type I collagen (24).

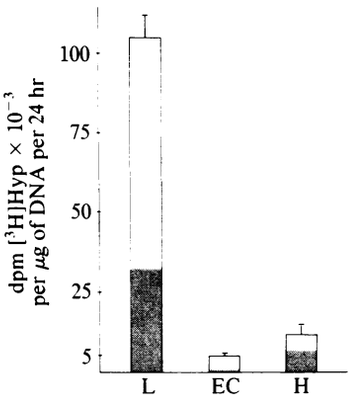

FIG. 5. Total collagen synthesis by primary cultures of lipocytes, endothelial cells, and hepatocytes from normal rat liver. Each bar shows the extent of incorporation of [^{3}H]proline into [^{3}H]hydroxyproline ([^{3}H]Hyp) in both cell layer (shaded region) and medium (open region). Data for total collagen synthesis is expressed as mean ± SD for either 3 (EC, endothelial cells) or 4 (L, lipocytes or H, hepatocytes) separate cultures.

tained in long term culture (30). Lipocytes appear to be, quantitatively, a far more important source of collagen in the liver than are parenchymal cells or sinusoidal endothelium, even if it is assumed that lipocytes, like Kupffer cells, are only one-sixth as numerous as hepatocytes (31, 32). This is contrary to the findings of Tseng *et al.*, who reported that collagen production by hepatocytes was substantially greater than that produced by mixed nonparenchymal cells (21). In that study, however, lipocytes were not specifically identified in the nonparenchymal cell isolate nor excluded from the hepatocyte fraction.

Current concepts of the pathogenesis of hepatic fibrosis include the hypothesis that lipocytes are specialized cells that in acute liver injury transform from vitamin A-storing cells to fibroblast-like cells containing prominent rough endoplasmic reticulum (26). The present study supports this by showing that lipocytes are specialized in collagen production. The amount of [^{3}H]proline incorporated into [^{3}H]hydroxyproline approximates that reported for normal skin fibroblasts in primary culture (33). Also, collagen represents five to ten percent of total protein synthesis by lipocytes, which is similar to fibroblasts (34), and two-thirds of the collagen synthesized is secreted into the medium (Fig. 5). On the other hand, lipocytes exhibit characteristics of smooth muscle cells as well. The production of both interstitial and basement membrane collagen is typical of smooth muscle, whereas fibroblasts synthesize only minute amounts of collagens other than type I (35). Also common to smooth muscle cells and lipocytes is the intermediate filament protein desmin (36), which is absent from fibroblasts (37). Thus, lipocytes may represent a unique cell type with properties of both fibroblasts and smooth muscle cells and are critically situated with respect to maintaining or altering the extracellular matrix in the subendothelial space.

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