Collagen Measured in Primary Cultures of Normal Rat Hepatocytes Derives from Lipocytes within the Monolayer

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Abstract

The cellular origin of hepatic collagen is under active investigation. Several recent studies using cells in primary culture suggest that hepatocytes are the source of much of the collagen in normal rat liver. In view of other data indicating that lipocytes produce substantial amounts of this protein, we have reexamined collagen biosynthesis in hepatocyte cultures that have been carefully characterized with respect to the presence of lipocytes. We find that routinely prepared hepatocyte isolates contain, by number, ~ 10% lipocytes. Lipocytes in early culture are difficult to visualize by phase-contrast microscopy but after 4 d proliferate and eventually replace the parenchymal cells. The size of the lipocyte subpopulation in these cultures correlates positively with collagen production. Similarly, removal of lipocytes by further processing of the initial hepatocyte isolate significantly reduces collagen production. Moreover, the only cells within hepatocyte cultures that display type I collagen by immunohistochemistry are lipocytes. We conclude that lipocytes are the principal source of collagen in primary hepatocyte cultures. The findings indicate also that these cells are the previously described "fibroblasts" that appear in relatively long-term hepatocyte cultures.

Introduction

Hepatic fibrosis is a serious form of liver disease, characterized by the accumulation of collagen within the perisinusoidal space of Disse. The cellular origin of this subendothelial material remains uncertain. Cell culture studies, which provide the most direct means to examine individual liver cell populations, have been used to characterize hepatic collagen biosynthesis in vitro; several such studies implicate hepatocytes as an important source of collagen within the liver (1–6). While collagen production typically is low in early culture, it represents as much as 8% of newly synthesized protein in cultures maintained for 96 h after plating (3, 4, 6). The delayed increase in synthesis is accompanied by a switch in the type of collagen produced, from type IV ("basement membrane") to type I ("fibrillar") collagen (4, 6). The basis for these changes in collagen expression is unclear. Many investigators conclude that hepatocytes possess a latent capacity for synthesis of type I collagen that is unmasked in culture. It is also possible that hepatocyte isolates contain a subpopulation of mesenchymal cells that expands in culture, contributing substantially to collagen production. Outgrowth of "fibroblasts" is frequently observed in long-term epithelial cultures (7, 8).

Although the routine preparation of hepatocytes after collagenase perfusion of the liver yields isolates that are free of most mesenchymal elements (9, 10), the presence of lipocytes has not been evaluated in detail. This newly characterized perisinusoidal cell produces large amounts of collagen in pure primary culture (11–13), with type I being the predominant subspecies (11). Lipocytes are also the principal source of other extracellular matrix proteins in normal rat liver (14–16) and can be identified in culture by immunostaining with an antibody to the matrix glycoprotein laminin (14). In this report, we localize and quantitate lipocytes within hepatocyte cultures, and demonstrate that collagen production in these cultures is directly related to the size of the lipocyte subpopulation. We propose that the bulk of the collagen synthesized in primary hepatocyte cultures derives from this specialized mesenchymal cell.

Methods

Materials

[*methyl*-^{3}H]Thymidine (91 Ci/mmol) and L-[2,3,4,5-^{3}H]proline (108 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL; L-[U-^{14}C]glutamic acid (225 mCi/mmol) and L-[U-^{14}C]aspartic acid (180 mCi/mmol) were obtained from ICN Radiochemicals, Inc., Irvine, CA. Activated charcoal, disodium EDTA, hydroxyurea, normal rabbit IgG, ovalbumin, PMSF, sodium citrate, and Stractan (grade II) were purchased from Sigma Chemical Co., St. Louis, MO. Bacterial collagenase (type I) was from Worthington Biochemicals (Cooper Biomedical, Inc., Malvern PA), and bovine pancreatic DNase (type I, 50,000 Dornase U/mg) was from Calbiochem-Behring Corp., San Diego, CA. Ham's F-12 medium, DME, and calf serum were purchased from Flow Laboratories, Inc., McLean, VA. Eagle's MEM without calcium (MEM-E)[1] was prepared in the laboratory using amino acids obtained from Sigma Chemical Co., St. Louis, MO. Polyclonal rabbit anti-chicken desmin and rhodamine-conjugated sheep anti-rabbit Igs were from Dako Corp., Santa Barbara, CA; affinity-purified antibodies to laminin and type I collagen were prepared in this laboratory (14, 17).

Procedures

CELL ISOLATION AND CULTURE

Sprague-Dawley rats (200–250 g) were maintained with free access to food and water before experimentation. The portal vein was cannu-

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1. *Abbreviations used in this paper:* CE, centrifugal elutriation; CE/DG, CE plus density-gradient centrifugation; LSC, low-speed centrifugation; MEM-E, Eagle's MEM without calcium.

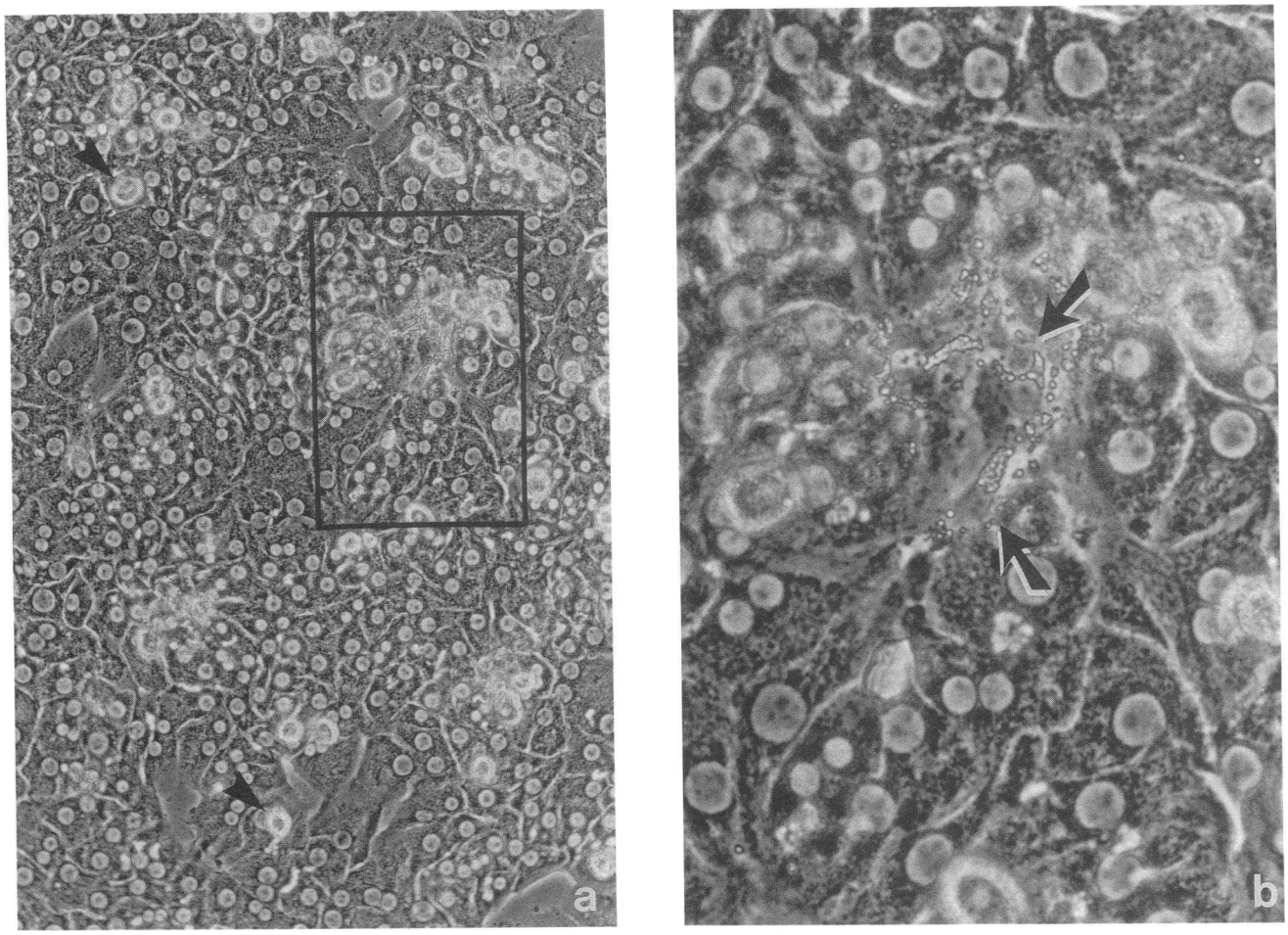


Figure 1. Fresh hepatocyte culture prepared by CE, at 24 h after plating. A low-power phase-contrast view (*a*) demonstrates lipocytes (*highlighted rectangle*) within the monolayer. Their small, refractile lipid droplets can be distinguished from nonviable hepatocytes and cellular debris (*arrowheads*). Higher magnification of the highlighted area (*b*) reveals lipid vesicles outlining the nuclei of two lipocytes (*arrows*). This field contains an estimated five lipocytes. *a*, × 50; *b*, × 150.

lated and collagenase perfusion was carried out at 35°C for 12–15 min, as described (9). The grossly digested liver was removed and suspended in 50 cm^3 of culture medium (1:1 mixture of Ham's F-12 and DME) containing 2.5 mg DNase. The suspension was incubated in a shaking water bath (37°C, 200 rpm) for 10 min, filtered through sterile cotton gauze, supplemented with an additional 2.5 mg DNase, and purified according to one of the following protocols.

Low-speed centrifugation (LSC). Hepatocytes were pelleted from the liver cell suspension by centrifugation at 50 *g* for 1 min. The supernatant, containing nonparenchymal cells, was removed and discarded. The pellet was washed four times by suspending the cells in 50 ml of MEM-E and centrifuging the suspension as above. The final isolate was resuspended in medium 199 (9) supplemented with 4 μU/ml insulin, 100 U/ml penicillin, 10^{-6} M corticosterone, and 5% calf serum.

Centrifugal elutriation (CE). Hepatocytes were isolated from the collagenase digest by two cycles of centrifugal elutriation in a Beckman J2-21 centrifuge and JE6-B rotor (Beckman Instruments, Inc., Palo Alto, CA), as described (9). The harvest from the second pass (flow rates > 62 ml/min) contained > 90% single cells. These cells were suspended in plating medium as described above.

CE/density-gradient centrifugation (CE/DG). The crude collagenase digest was first subjected to centrifugal elutriation, as above. Purified single hepatocytes (second pass, 2,400 rpm, > 62 ml/min) were layered on a discontinuous gradient of Stractan (17, 22, and 30%) prepared as described (18), and centrifuged at 13,000 *g* for 20 min at 4°C. Parenchymal cells were harvested from the 22–30% interface (d = 1.125), washed with MEM-E, and suspended in plating medium.

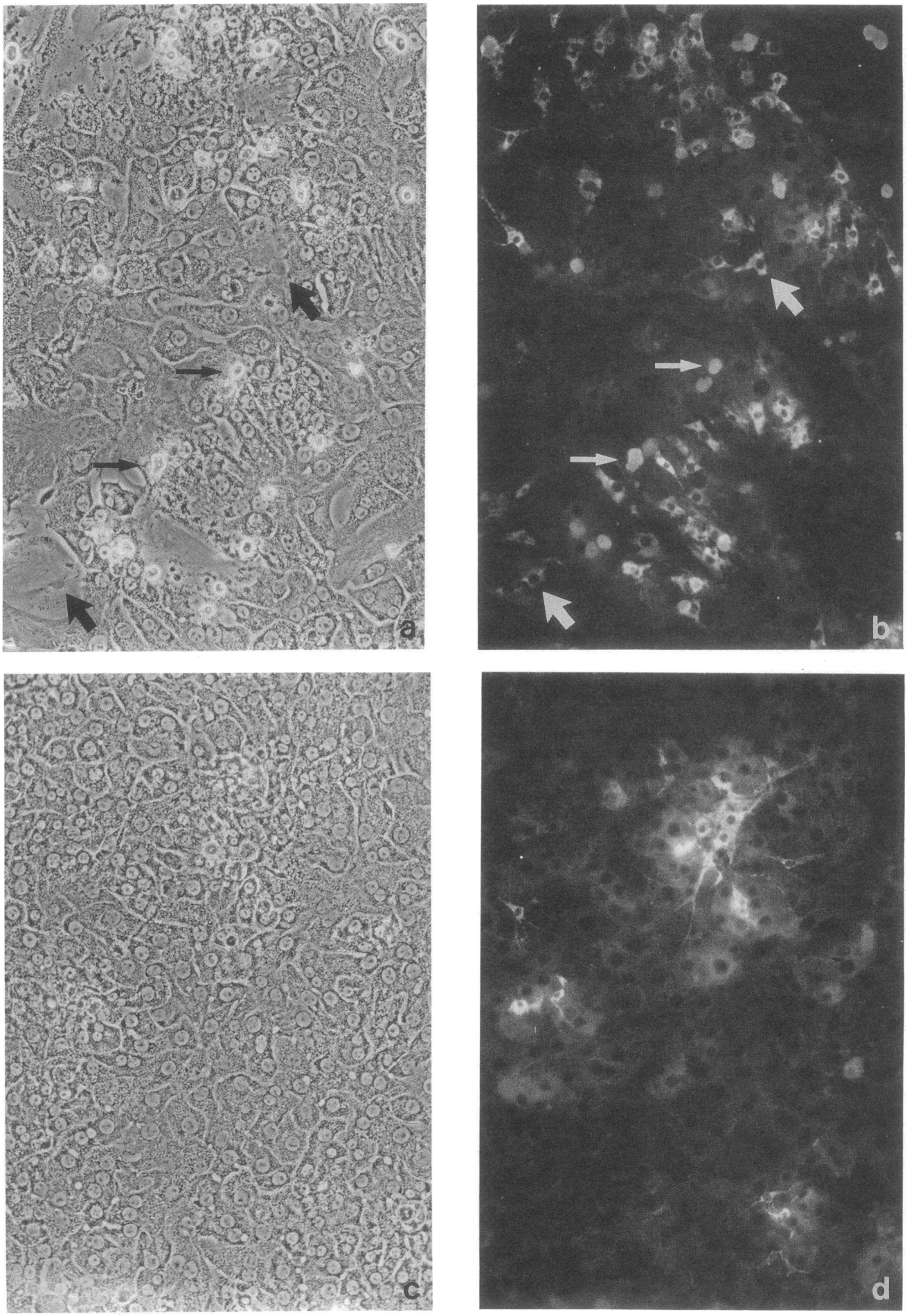
All cells were plated on type I collagen-coated tissue culture plastic (60 mm; Lux Nunc, Inc., Naperville, IL) unless otherwise specified, and maintained in a 37°C/5% CO_2 incubator for the duration of study. Culture medium was renewed daily.

IMMUNOHISTOCHEMISTRY

Cultures were washed three times with PBS. Cells were fixed with 0.5% paraformaldehyde (pH 7.4) for 15 min at room temperature, quenched with 0.1 M glycine, 0.05 M Tris (pH 7.4) for 15 min, permeabilized with ice-cold methanol for 10 min, and air dried. After rehydration with PBS, specimens were incubated for 60 min at room temperature with 100 μg/ml of affinity-purified antilaminin or antidesmin, diluted in PBS containing 2% ovalbumin. Specimens were then washed thoroughly with PBS, and a rhodamine-conjugated secondary antibody was added that was previously diluted in PBS containing 2% ovalbumin. After a 50-min incubation, culture plates were again washed with PBS, mounted with Gelvatol (Monsanto Co., Indian Orchard, MA) (15% solution in PBS), and viewed with a Nikon Diaphot photomicroscope fitted with epifluorescence (Nikon, Inc., Tokyo, Japan).

QUANTITATION OF LIPOCYTES

Lipocytes within hepatocyte cultures were identified immunohistochemically and counted. The number of lipocytes seen under fluorescent microscopy was divided by the total number of cells counted in the same field using phase-contrast illumination. 300–5,000 cells were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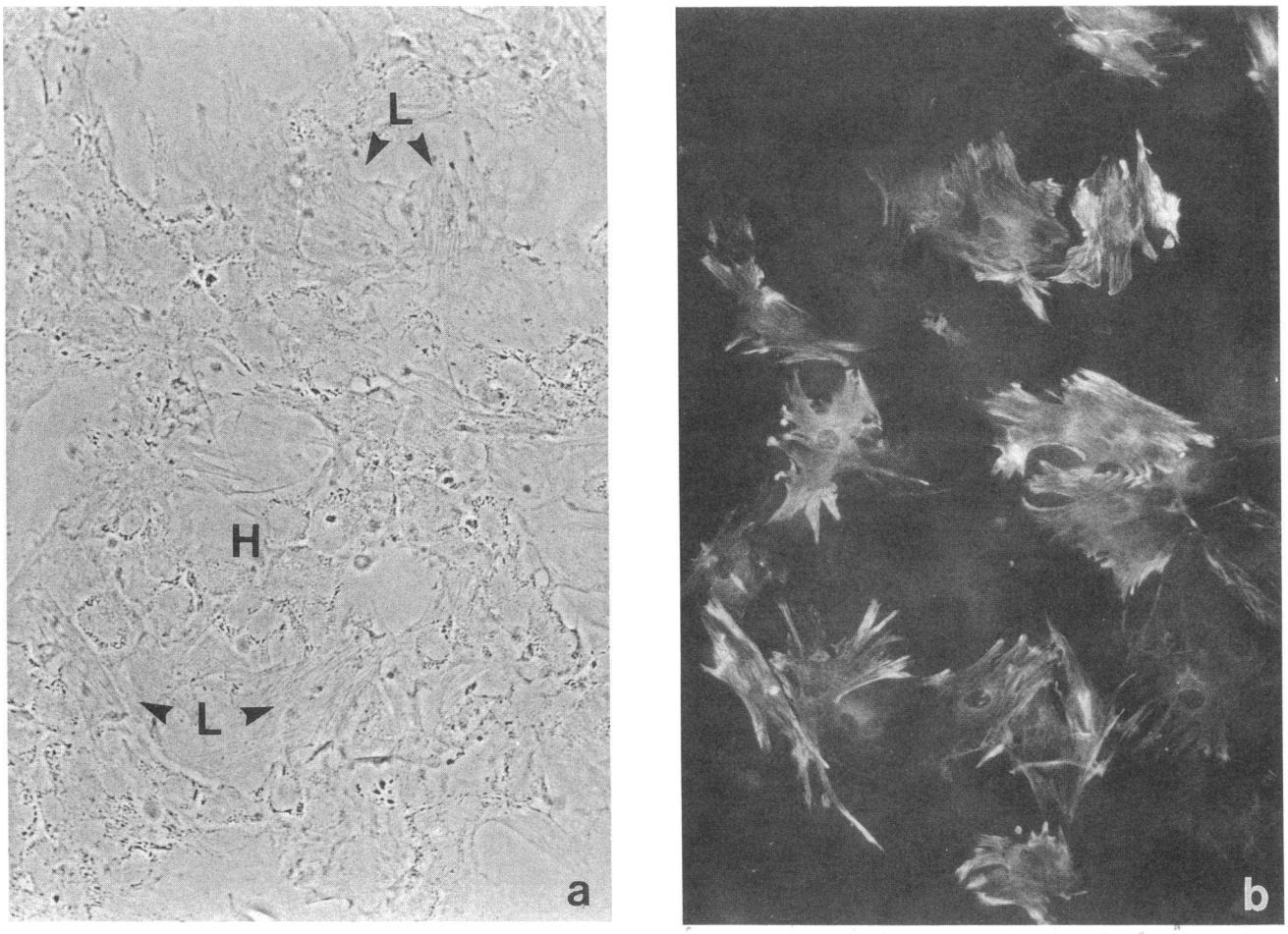

Figure 3. Hepatocyte culture prepared by CE 8 d after plating. The culture was fixed and stained with antidesmin. Phase-contrast (*a*) and fluorescent (*b*) views represent the same microscopic field. After 1 wk in culture, lipocytes (*L*) have flattened and spread and are identifiable by phase-contrast microscopy between groups of hepatocytes (*H*). Immunostaining with antidesmin confirms the dual population of cells within the monolayer. × 50.

counted for each determination in random microscopic fields. Duplicate cell counts varied by no more than 15%.

THYMIDINE INCORPORATION

At various time points in culture, hepatocytes were incubated for 24 h in culture medium containing 10 μCi/ml [^{3}H]thymidine. Control plates were labeled with the same concentration of [^{3}H]thymidine, plus 10 mM hydroxyurea. A pair of unlabeled plates was reserved for chemical quantitation of DNA. At the end of the labeling period, plates were rinsed three times in PBS, immersed in ice-cold 5% TCA for 15 min, and dehydrated in 95% ethanol. Thymidine incorporation into DNA was measured using the method of McGowan et al. (19). Results from experimental plates were corrected for those measured in control plates, and expressed as disintegrations per minute per microgram of cellular DNA per day. DNA was quantitated by a fluorometric method (20).

AUTORADIOGRAPHY

Cells labeled with [^{3}H]thymidine were fixed and dehydrated as described above. Kodak NTB-2 emulsion was diluted 1:1 with distilled water and heated to 42°C for 1 h. Culture plates, from which the sides had been trimmed away, were dipped in the emulsion, air dried, and exposed for 3 d. Plates were developed using Kodax GBX Developer and Fixer (Eastman Kodak Co., Rochester, NY) according to package directions, counterstained with hematoxylin, and viewed with a Nikon Diaphot microscope (Nikon, Inc.).

METABOLIC LABELING AND PROCESSING OF HEPATOCYTE CULTURES FOR AMINO ACID ANALYSIS

Hepatocytes were incubated for 24 h with culture medium containing 50 μCi/ml L-[2,3,4,5-^{3}H]proline and 50 μg/ml ascorbate. At the end of the labeling period, culture medium and cell layers were harvested separately and supplemented with protease inhibitors at 4°C (5 mM EDTA, 0.5 mM PMSF, 0.25 M acetic acid final concentration). Culture medium was cleared of cellular debris by centrifugation; cell layers were disrupted by sonication. An aliquot of the sonicated cell suspension was removed for DNA assay. Each sample was dialyzed against distilled water for 48 h at 4°C to remove unincorporated amino acids and low molecular weight peptides; dialysates were then hydrolyzed in 6 N HCl (110°C, 20 h) and evaporated to dryness under a stream of

Figure 2. Lipocytes in hepatocyte cultures prepared by two different methods. Hepatocytes prepared by LSC and CE were stained with antilaminin on day 2 of culture. Paired phase-contrast and fluorescent views illustrate the same microscopic field. In cultures prepared by LSC (*a* and *b*), immunofluorescent staining reveals lipocytes (*large arrows*) that are scattered throughout the hepatocyte monolayer, although only a small proportion of these cells is visible under phase-contrast. The bright fluorescence of lipocytes is distinct from nonspecific fluorescence, which is associated with nonviable cells (*small arrows*). In cultures of isolates prepared by CE (*c* and *d*), lipocytes are fewer in number and frequently in clusters. These cells are not apparent in the phase-contrast view. × 50.

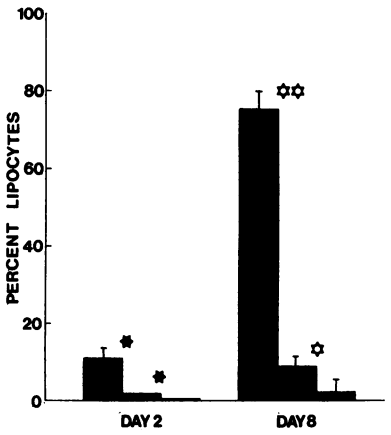

Figure 4. Quantitation of lipocytes in hepatocyte cultures. Lipocytes were counted in three types of hepatocyte cultures as described in Methods, at 2 and 8 d after plating. The relative proportion of lipocytes in each culture is illustrated. Bars represent mean±SEM (n = 3). *Left bar* of each group, LSC; *middle bar*, CE; *right bar*, CE/DG. ✱$P < 0.05$ vs. CE/DG; ✲$P < 0.05$, ✲✲$P < 0.01$ vs. day 2.

nitrogen. Hydrolysates were resuspended in 0.2 N sodium citrate, decolorized with activated charcoal, and then chromatographed on a Beckman model 119 amino acid analyzer (AA-15 resin, 56 × 0.9 cm, 53.5°C). Amino acids were eluted with 0.2 N citrate buffer (Beckman Instruments, Inc.), pH 3.42, at a flow rate of 70 ml/h. Radioactivity was quantitated in column fractions by scintillation counting in Ecolite (WestChem, San Diego, CA), with an efficiency of 33%. The identity of radiolabeled amino acids in biological samples was confirmed by comparing their elution profiles to those of amino acid standards (hydroxyproline, aspartate, glutamate, and proline); samples and standards were chromatographed at pH 2.92 as well as 3.42 to achieve separation of hydroxyproline and aspartate (21). Glutamate and aspartate, which can be generated from proline in hepatocyte cultures (22), accounted for < 1 and < 10% of measured counts attributed to proline and hydroxyproline, respectively.

QUANTITATION OF COLLAGEN SYNTHESIS

Net collagen production by hepatocyte cultures was expressed as disintegrations per minute [^{3}H]hydroxyproline incorporated per microgram of cellular DNA. Relative collagen synthesis (as a percentage of total protein) was calculated using a modification of the formula of Diegelmann and Peterkofsky (11, 23).

Results

Yield, purity, and viability of hepatocytes prepared by three different methods. The yield of cells prepared by conventional LSC was 2 × 10^8, of which an estimated 60% were single hepatocytes; the remainder of the population consisted of doublets or triplets. The yield after CE was 8 × 10^7 hepatocytes, all single cells. Approximately 6 × 10^7 cells were recovered after purification by CE/DG. Cells from each preparation were plated at a density of 2 × 10^5/cm^2. Viability of cell cultures, as assessed by morphology and trypan blue exclusion, was > 95% (10). Purity of cultures was first estimated using phase-contrast microscopy at 2 h after plating, when cells had begun to attach to the culture surface. No change was noted over the first 48 h of culture, and purity was recorded quantitatively on day 2 (see below).

Morphology and immunohistochemistry. Fresh hepatocyte cultures viewed under phase-contrast microscopy contained occasional amorphous deposits, on or within an otherwise homogeneous monolayer. Close inspection revealed some of these to be collections of refractile lipid droplets (Fig. 1), which, under ultraviolet illumination, displayed a rapidly fading yellow-green fluorescence. This fluorescence, due to vitamin A ester storage, is characteristic of hepatic lipocytes (24), and could be enhanced by the administration of retinyl acetate (300,000 U/kg) to experimental animals ~ 1 wk before collagenase perfusion (data not shown). Vitamin A loading was not employed for any of the experiments in this report.

Lipocytes were also identified in hepatocyte cultures by immunohistochemistry. Antibodies to both laminin, an extracellular matrix protein, and desmin, an intermediate filament protein, were used as specific markers for lipocytes in hepatocyte cultures (14, 25). In early cultures, lipocytes stained most intensely with antilaminin (Fig. 2, *b* and *d*). In cultures older than 4 d, localization of lipocytes with antilaminin was difficult because of diffuse laminin fluorescence throughout the extracellular matrix. Antidesmin continued to highlight individual cells at day 8 (Fig. 3 *b*), and was superior as a marker for lipocytes in older cultures.

Quantitation of the lipocyte subpopulation. In cultures prepared by LSC and maintained for 2 d, immunostaining with antilaminin revealed lipocytes scattered throughout the monolayer (Fig. 2 *a* and *b*). By contrast, cultures of similar age prepared by CE contained only occasional clusters of lipocytes (Fig. 2 *c* and *d*), and those prepared by CE/DG had almost none. At 8 d after plating, the morphology of the cultures had changed substantially. While all of the cells had flattened and

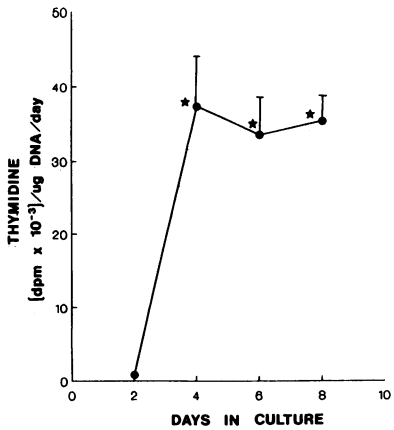

Figure 5. Thymidine incorporation. Graph illustrates the amount of [^{3}H]thymidine incorporated daily into DNA by cultures prepared with elutriated cells (CE). Values represent mean±SEM (n = 3), corrected for nonreplicative DNA synthesis (see Methods). *$P < 0.005$ vs. day 2, by t test.

Table I. Proline Incorporation by Hepatocyte Cultures

Day	[^{3}H]Proline		
	LSC*	CE*‡	CE/DG*‡
	dpm × 10^{-3}/μg DNA/24 h		
2	494±33	518±68	414±39
4	469±36	540±107	380±15
6	302±21	517±71	351±57
8	336±63	468±64	316±71
10	456±26	400±85	334±31

Incorporation of radiolabeled proline into nondialyzable material was measured by amino acid analysis (see Methods). Data represent mean±SEM (n = 3) for cells and medium combined. Statistical comparisons were made using one-tailed t test.
* $P > 0.05$, day 10 vs. day 2.
‡ $P > 0.05$, vs. LSC.

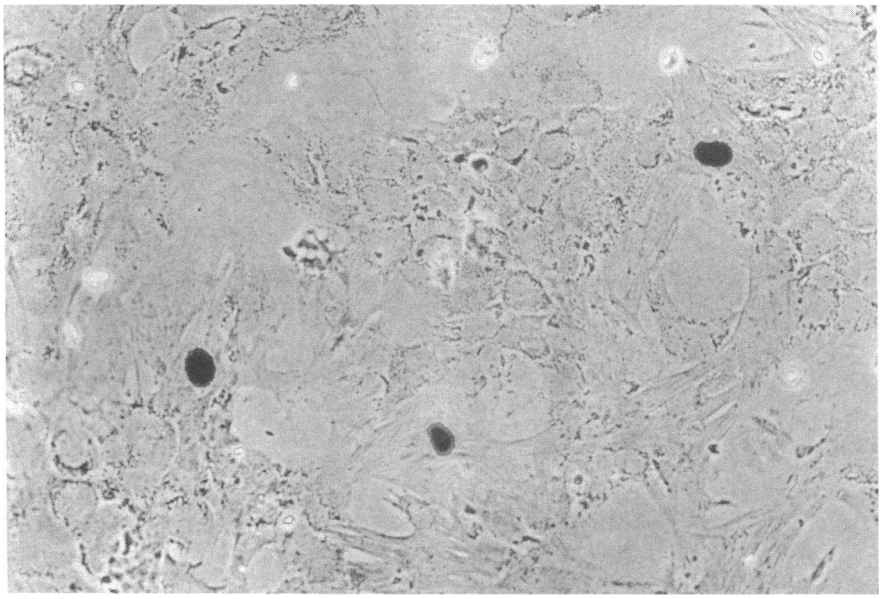

Figure 6. Nuclear labeling of hepatocyte cultures. Autoradiogram of a 6-d-old hepatocyte culture, prepared by CE, incubated with [^{3}H]thymidine for 24 h, fixed, overlaid with photographic emulsion, and exposed for 3 d. The field shows three labeled nuclei, all in lipocytes, identified as described in Fig. 4.

spread on the culture surface, lipocytes were distinguishable by their faint nuclei and fibrillar cytoplasm. Identified by staining with antidesmin, they represented a larger fraction of all cultures on day 8 than on day 2; however, the initial differences among preparations (LSC, CE, or CE/DG) persisted. The change in the lipocyte population is illustrated in Fig. 3 for cells purified initially by CE. By direct counting of immunohistochemically stained monolayers, lipocytes on day 2 constituted 11% of cultures prepared by LSC, 2% of those prepared by CE, and < 1% of those prepared by CE/DG. By 8 d after plating, the proportion of lipocytes had increased in each of the cultures, reaching 75% for those prepared by LSC. Changes were significant in cultures prepared by LSC and CE, but not for those prepared by CE/DG (Fig. 4).

Proliferation of lipocytes within hepatocyte cultures was documented by incorporation of [^{3}H]thymidine into DNA. Replicative DNA synthesis increased sharply in elutriated cells after a lag phase of ~ 48 h (Fig. 5). Autoradiography of parallel cultures demonstrated that [^{3}H]thymidine incorporation was predominantly nuclear (Fig. 6) and confined to a subpopulation of cells that exhibited the characteristic appearance of lipocytes. Of cells identifiable morphologically as hepatocytes, < 1% were labeled. That the majority of replicative DNA synthesis takes place in lipocytes was confirmed by comparing [^{3}H]thymidine incorporation in hepatocyte cultures purified by CE and those purified by CE/DG (Fig. 7). On day 4, as DNA synthesis accelerated maximally, cultures containing a small proportion of lipocytes (CE) incorporated five times more [^{3}H]thymidine into DNA than those with negligible contamination (CE/DG).

Collagen production. Total protein synthesis in cultures incubated with radioactive proline was similar regardless of the method of cell preparation (Table I). In all cultures, only a small amount of collagen was synthesized at early time points (Fig. 8). Collagen production, however, correlated with the presence of lipocytes. After 4 d collagen synthesis in cultures prepared by LSC rose progressively, reaching eight times the original value by day 10. Similar results were obtained when culture medium and cell layers were analyzed individually (data not shown). Relative collagen synthesis increased in the same fashion, representing < 1% of total protein synthesis on day 2, and rising to 9.7% of newly synthesized peptides by day 10 (Fig. 9). This pattern has been described by others (3, 4, 6). Cultures prepared by CE displayed a blunted rise in collagen

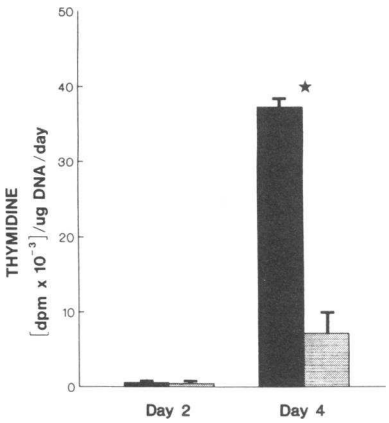

Figure 7. Thymidine incorporation in hepatocyte cultures prepared by two different methods. *Solid bars,* CE; *shaded bars,* CE/DG. Replicative DNA synthesis is measured as in Fig. 5. *P < 0.0005 vs. CE/DG.

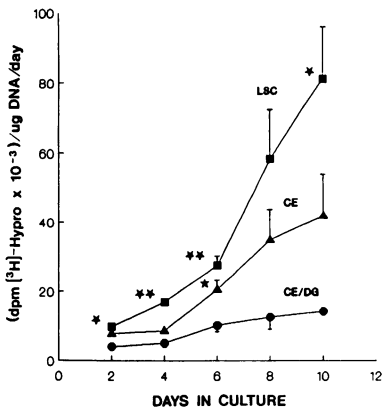

Figure 8. Collagen production. Graph illustrates the incorporation of radioactive proline into peptide-bound [^{3}H]hydroxyproline by hepatocyte cultures prepared by three different methods. ■, LSC; ▲, CE; ●, CE/DG. Points represent mean±SEM for three cell preparations, each assayed in duplicate. *P < 0.05; **P < 0.01 vs. CE/DG by t test.

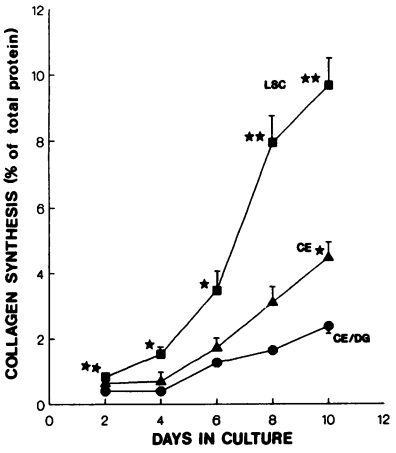

Figure 9. Relative collagen synthesis. The graph presents the data of Fig. 8, with collagen production calculated as a percentage of total protein synthesis (11, 23). Abbreviations and symbols as in Fig. 8.

synthesis after day 4; both absolute and relative collagen synthesis were increased in 10-d-old cultures, but to a lesser extent than in those prepared by LSC (Figs. 8 and 9). In striking contrast to these findings was the profile exhibited by CE/DG-purified hepatocytes: collagen production accounted for < 2.5% of total protein synthesis over the entire period of observation (Fig. 9).

In established (8-d-old) cultures containing < 15% lipocytes, relative collagen synthesis and lipocyte number were linearly related (Fig. 10). Collagen synthesis reached a plateau in cultures that were predominantly lipocytes. There was no clear correlation between lipocyte number and collagen synthesis in cultures < 4 d old (not shown).

Immunolocalization of collagen within hepatocyte cultures. Hepatocytes prepared by CE were cultured on uncoated plastic dishes in the presence of 50 μg/ml ascorbate. 8 d after plating, the cultures were examined immunohistochemically for the presence of type I collagen. As illustrated in Fig. 11, fluorescence was concentrated over lipocytes, with little or none over hepatocytes.

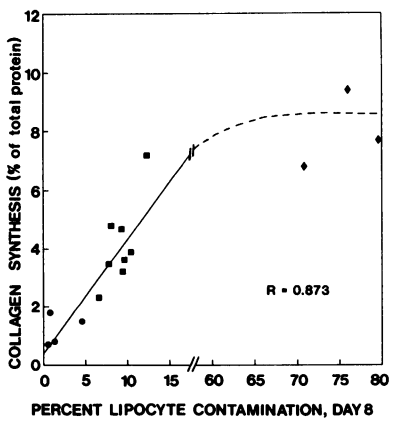

Figure 10. Correlation between collagen synthesis and the proportion of lipocytes in hepatocyte cultures. Cultures with varying levels of lipocytes (prepared by LSC, CE, or CE/DG) were examined on day 8 of incubation. Each point represents a single culture. The amount of collagen produced is expressed as a percentage of total protein synthesis and is plotted against the proportion of lipocytes in the culture. For cultures with < 15% lipocytes, the relationship is linear ($R = 0.873$). ♦, LSC; ■, CE; ●, CE/DG.

Discussion

The cellular source(s) of collagen in liver must be identified to elucidate the pathogenesis of hepatic fibrosis. With methods now available for isolating and culturing the principal cell types from normal liver, this area is receiving new attention. Several reports indicate that hepatocytes produce collagen in culture (1–6), exhibiting a delayed acceleration in collagen synthesis after several days in vitro (3, 4, 6). This phenomenon has been attributed to "culture-adaptation," which is characteristic of hepatocytes (26, 27) and accounts for culture-related changes in collagen production in other types of cells (28–30). The present results suggest that the exponential rise in collagen synthesis observed in hepatocyte cultures is not the result of an alteration in hepatocyte phenotype, but instead is attributable to a growing subpopulation of hepatic lipocytes.

Lipocytes, also known as Ito cells or stellate cells, exhibit the properties of smooth muscle cells in normal liver (31) and probably represent vascular pericytes (32). In inflammation they acquire fibroblastic features, losing their lipid droplets and gaining abundant rough endoplasmic reticulum (33). In culture, they demonstrate the ability to secrete abundant amounts of collagen (11–13, 34), particularly type I collagen, which is typical of hepatic fibrosis in vivo (35, 36). Unless meticulous purification methods are employed, lipocytes appear as contaminants of hepatocyte cultures, and, as shown in the experiments above, their presence interferes with the assessment of collagen synthesis by hepatocytes in culture.

Crude liver cell suspensions purified by LSC contain large numbers of lipocytes, although they are essentially free of endothelial and Kupffer cells (9, 10). This may reflect the fact that lipocytes in vivo reside in close apposition to hepatocytes, beneath the sinusoidal endothelium. Collagenase digestion may fail to separate lipocytes from hepatocytes, if the two are strongly adherent, moreover, hepatocyte doublets and triplets, which are frequently obtained in fresh isolates, may enclose lipocytes. Centrifugal elutriation removes most of the potentially contaminating lipocytes by separating single hepatocytes from larger aggregates. Even greater purity is obtained when elutriated isolates are separated by density-gradient centrifugation. Dislodged from hepatocytes by the force of centrifugation, the more buoyant lipocytes migrate toward the top of the gradient, leaving a hepatocyte fraction which is > 99% homogeneous.

As hepatocyte cultures are maintained over time, the proportion of lipocytes increases. This change occurs regardless of the initial method of purification, but is most pronounced in isolates prepared by LSC. The shift toward mesenchymal predominance reflects both lipocyte proliferation and hepatocyte loss; the former is documented by [^{3}H]thymidine incorporation and nuclear labeling, while the latter is apparent from the net decrease in total DNA per plate that occurs over the initial week of culture (data not shown). Outgrowth of lipocytes in hepatocyte cultures is accompanied by a marked increase in collagen synthesis. When analyzed directly, relative collagen production correlates positively with the size of the lipocyte subpopulation, achieving a linear relationship in established cultures at 8 d of age (Fig. 9). Immunohistochemical staining provides supportive evidence that collagen derives from lipocytes.

Collagen production may be influenced either positively or negatively by soluble factors within the culture medium.

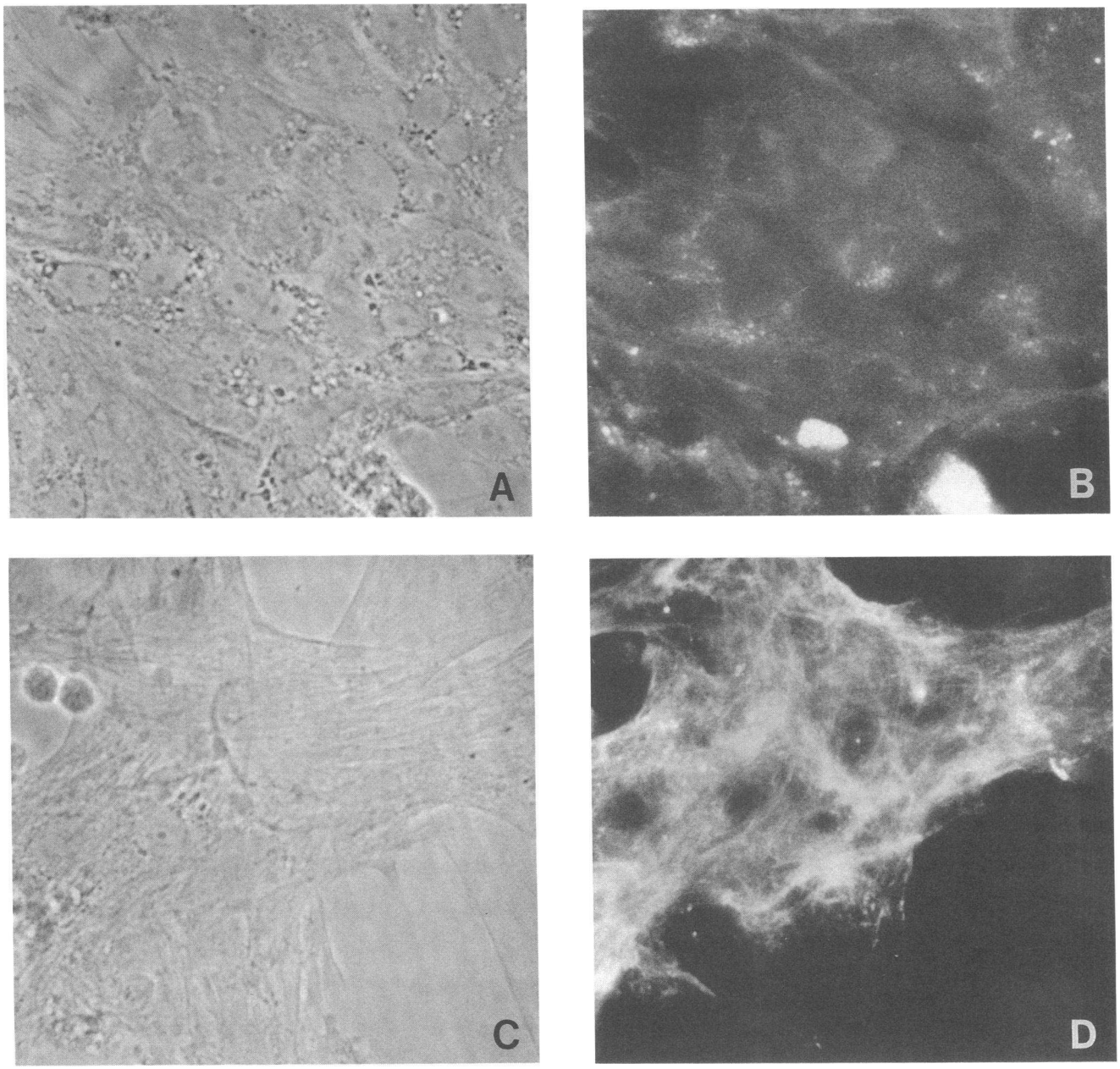

Figure 11. Type I collagen immunofluorescence in hepatocyte cultures. Hepatocytes, prepared by CE and plated on uncoated plastic dishes, were stained for type I collagen on day 8. (*A*) A group of hepatocytes, which display little or no specific fluorescence (*B*). Lipocytes within the same culture (*C*) exhibit bright fluorescence (*D*). × 400.

Serum and corticosterone, both used as medium supplements, have potentially opposing effects. Because serum is mitogenic for mesenchymal cells in culture (37), it may enhance collagen synthesis indirectly by promoting lipocyte growth, while glucocorticoids may suppress collagen production (5, 38, 39). The latter effect, however, requires a dose of corticosterone that exceeds that employed in the present experiments (10^{-6} M). Thus, the net effect of these culture medium additives should be a secondary increase in collagen production which follows lipocyte proliferation.

The cell culture substratum may be another important determinant of collagen synthesis. For comparison with previously published work, all studies were performed using a conventional support of plastic coated with a thin layer of rat-tail tendon (type I) collagen. However, both hepatocytes (26, 40) and lipocytes (Friedman, S. L., F. J. Roll, J. Boyles, D. M. Arenson, and D. M. Bissell, unpublished observations) exhibit phenotypic changes on this substratum, with lipocytes undergoing morphologic and functional "activation" to a proliferative, collagen-producing mode. This suggests that exposure to type I collagen stimulates lipocyte collagen production in vitro, and that maintenance of liver cells on such a substratum elicits cellular responses that are characteristic of hepatic inflammation in vivo. This phenomenon may be reflected in the present experiments, in which relative collagen synthesis on a type I collagen substratum is substantially greater on day 8 than on day 2, independent of lipocyte number (Figs. 4 and 8). That hepatocytes continue to synthesize only small amounts of collagen under these conditions suggests that their role in hepatic fibrogenesis is minor.

Additional factors regulating collagen production appear to be operative in these cultures. As lipocytes replace hepatocytes, relative collagen synthesis rises to 9% of total protein. This fraction equals that produced by pure cultures of lipocytes (11) or fibroblasts (41). If it is assumed from Table I that the remaining hepatocytes in mixed cultures continue to synthesize protein, the findings suggest that lipocytes within hepatocyte cultures produce larger amounts of collagen per cell than do lipocytes in pure culture. This implies relative stimulation in mixed cultures, which may be due to lack of feedback regulation by collagen protein (42), to interactions between hepatocytes and lipocytes (43, 44), or to other processes. At the same time, relative collagen synthesis does not exceed 9%, which suggests that factors controlling maximal collagen synthesis are present in both mixed and pure cultures.

Our experiments address the long-standing concern that hepatocyte cultures are contaminated by "fibroblasts," and illustrate that the cells previously judged to be fibroblasts, in fact represent lipocytes, a specific resident cell type of normal liver. Furthermore, we show that lipocytes are the principal source of newly synthesized collagen in hepatocyte cultures. While these results are consistent with qualitative evidence that hepatocytes produce collagen (45–50), they also indicate that in culture the relative contribution of hepatocytes to this process is minimal. Based on these studies, we conclude that the latent capacity for collagen production after liver injury resides in nonparenchymal cells, particularly lipocytes.

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