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STELLATE CELLS (VITAMIN A-STORING CELLS) ARE THE PRIMARY SITE OF COLLAGEN SYNTHESIS IN NON-PARENCHYMAL CELLS IN THE LIVER

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ABSTRACT

A cell fraction enriched with lipid droplet-containing cells (perisinusoidal stellate cells, vitamin A-storing cells, or fat-storing cells), and freed from contamination of parenchymal cells, was prepared by low speed centrifugation followed by Percoll density gradient centrifugation. Cells in this fraction were found to take up and store the largest amount of vitamin A per cell among the liver cells including parenchymal cells, when [^{3}H]retinol was injected through the portal vein of rats. Analysis of collagen production by this fraction as well as another non-parenchymal cell fraction (rich in Kupffer cells and endothelial cells) in culture has shown that only the stellate cell-rich fraction produced collagen which was composed of type I (88.2%), type III (10.4%) and membranous type (1.4%). There was no indication of collagen production in other non-parenchymal cell fractions under the experimental conditions employed. These results indicate that, among non-parenchymal cells, lipid droplet-containing cells or stellate cells are primarily responsible for collagen production in the liver.

Hepatic fibrosis is one of the most vexatious diseases in human beings. A characteristic change in hepatic fibrosis is the deposition of connective tissue components in the liver (15). However, species of cells responsible for collagen metabolism in the liver under pathological conditions are still obscure. Hepatic parenchymal cell (hepatocyte) clones (5, 8) as well as parenchymal cells in primary culture (4, 7, 9) have been reported to be capable of synthesizing collagen. The stellate cells (13), which exist in the perisinusoidal space and store vitamin A as lipid droplets in the cytoplasm (17, 18), are also suggested to participate in collagen metabolism in the liver (18), and produce type III collagen (11). However, investigation has not been sufficient enough to identify what cell type or types are responsible for collagen production among non-parenchymal cells and what types of collagen are produced by these cells.

In order to elucidate collagen metabolism and its regulatory mechanism in the liver, we tried to isolate and fractionate non-parenchymal cells with special reference to stellate cells and examined collagen synthesis in the isolated cell fractions. Type analysis of the collagen synthesized was also performed.

MATERIALS AND METHODS

Cell Isolation and Culture

Liver cells were isolated from adult male rats, Wistar strain (body weight, 260-460 g). The liver

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This is paper No. 11 in a series of studies on the molecular mechanisms of hepatic fibrosis.

was perfused through the portal vein *in situ*, first with Ca^{2+}- and Mg^{2+}-free Hanks' solution containing 0.5 mM ethyleneglycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), then with 0.05% *Clostridium histolyticum* collagenase (Sigma, Type I) and 0.005% soybean trypsin inhibitor (Sigma, Type I-S) in Hanks' solution for 15 min at 37°C (5, 7). The liver was carefully excised from the animal, sliced with razor blades, suspended in Dulbecco's modified Eagle's medium (DMEM) and filtered through four layers of cheesecloth. The cell suspension was centrifuged at 50 *g* for 1 min to precipitate viable parenchymal cells. The supernatant was centrifuged further at 50 *g* for 3 min. The pellet from the second centrifugation, which contained dead and viable hepatocytes, was discarded (5, 7). The supernatant of the second run was centrifuged three times at 600 *g* for 5 min and the precipitated cells were suspended in DMEM. The cell suspension or density marker beads (Pharmacia) in DMEM (17.5 ml each) was mixed with 24 ml of Percoll solution (Percoll: ten times concentrated Ham's F-12=9 : 1, v/v) and centrifuged at 20,000 *g* for 30 min at 4°C using a Tomy Angle head rotor to obtain fractions S1+S2 (ϱ=1.02–1.07), S3 (ϱ= 1.07–1.08) and S4 (ϱ=1.08–1.14). Density of Percoll solution was determined using the colored density marker beads. Twenty grams of the fraction S1+S2 were mixed with 5 ml of DMEM, and further fractionated into S1 and S2 fractions by centrifugation. Cells in fractions S1–S4 were separately washed with DMEM and collected by centrifugation at 600 *g* for 5 min. This procedure was repeated twice. Cell number was counted with a hemocytometer and cell viability was determined by trypan blue exclusion. Cells were seeded onto 60 mm-Falcon plastic dishes ($1–20\times10^6$ cells/dish for cells in fractions S2 and S3 in 5 ml of DMEM containing 10% fetal bovine serum) and cultured under 5% CO_2-air at 37°C. The medium was replaced one day after inoculation, and then every other days.

In Vivo *Uptake of [^{3}H] Retinol by Liver Cells and Its Tissue Distribution*

To examine the incorporation and distribution of vitamin A in liver parenchymal and non-parenchymal cells and also in various organs, 200 μCi of [^{3}H]vitamin A_1 (all-trans-[1-^{3}H(N)]-retinol; 14.3 Ci/mmol; New England Nuclear) was injected in the portal vein in two rats under anesthesia. The rats were sacrificed 90 min and 6 days after the injection, and tissue samples were obtained for the determination of radioactivity. Liver cells were fractionated by the procedure described above.

About 20 mg each of the tissue samples (liver, spleen, lung, intestine, skin, brain, kidney and heart) were sonicated with 1 ml distilled water. The feces at indicated time periods were also treated similarly. The sonicates (0.1 ml) was mixed with 2 ml of 50% ethanol. After adding 10 ml of ACS-II solution to this mixture, the radioactivity was counted by a Beckman LS-9000 liquid scintillation spectrometer. In case of cell suspensions, 0.1 ml each of fractions S1–S4 was diluted with 0.9 ml of distilled water, sonicated, mixed with ethanol and the radioactivity was counted as described above.

Electron Microscopy

For identification of the stellate cells, retinyl palmitate (Chocola A, Eisai, Tokyo; 3×10^5 IU/kg body weight) was subcutaneously injected to rats 2 weeks before sacrifice and liver cells were fractionated as described above.

One milliliter each of cell suspensions (10^5–10^6 cells/ml) of fractions S1–S4 was placed in a plastic beem capsule and centrifuged at 5,000 *g* for 5 min. After removal of the supernatant, the cell pellet was fixed with 2% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4, containing 1% sucrose for 2 h and post-fixed in 2% osmic acid in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C. The fixed cells were dehydrated in graded series of ethanol and embedded in Epon 812. Thick section was stained with 1% toluidine blue containing 1% borax and examined under a light microscope. Thin section was contrasted with 7% uranyl acetate and 0.4% lead citrate and examined in a JEOL 100 CX electron microscope at 100 kV.

Fluorescence Microscopy

Cell suspensions of fractions S1–S4 were examined under a Nikon FL fluorescence microscope (excitation filter VO, barrier filter O) to monitor vitamin A-containing cells which generate autofluorescence. Color photomicrographs were taken using an Ecktachrome ASA 400 (Kodak) film.

Labeling of Cells

Cells were preincubated in a 2 ml of DMEM containing 0.1 mM sodium ascorbate and 0.5 mM β-aminopropionitrile fumarate for 30 min at 37°C, incubated for 18 h in the presence of 100 μCi of

[^{3}H]proline (New England Nuclear; 20.0 Ci/mmol) per dish under 5% CO_2-air at 37°C.

Collagen Assay with Bacterial Collagenase and Type Analysis

Labeled proteins were prepared from combined mixtures of the medium and cell layer of fractions S2 and S3 in primary culture (day 2 and day 8), and of fraction S2 in subculture (day 10 and day 11). Radioactive collagen was assayed by the bacterial collagenase digestion method as described previously (8). For the preparation of labeled collagen from the cell layer and medium, carrier type I and type III collagens from the rat skin (final concentration; 200 μg/ml each) and protease inhibitors (phenylmethanesulfonylfluoride, 1 mM; *N*-ethylmaleimide, 1 mM; ethylenediaminetetraacetic acid, 25 mM) were added, and collagen was precipitated by adding ammonium sulfate (176 mg/ml) at 4°C.

The precipitate was suspended in 0.5 M acetic acid and treated with one-tenth volume of pepsin (Boehringer Mannheim; 100 μg/ml of 0.5 M acetic acid) for 6 h at 6–8°C. After addition of one-tenth volume of 1 M Tris-HCl buffer, pH 7.6, the resulting solution was titrated to pH 8.0 with 2 M NaOH to inactivate pepsin (8).

Collagen types were analyzed by polyacrylamide slab gel electrophoresis in sodium dodecyl sulfate (SDS). Samples containing approximately 2×10^4 cpm of non-dialyzable radioactive material, before or after collagenase digestion, were mixed with 5 μl of 0.5 M Tris-HCl buffer, pH 6.8, 10 μl of 1% SDS, 15 μl of H_2O and 10 μl of bromophenol blue-glycerol solution, with or without 10 μl of 0.1 M dithiothreitol, and heated at 80°C for 10 min. Electrophoresis was performed by using a 3% stacking gel, pH 6.8, and a 4.5% separating gel, pH 8.8. For fluorography, the slab gel was dried after incubation with 1.1 M sodium salicylate for 1 h (2). The dried gel was exposed to a preflashed Kodak X-ray film at −70°C (14) for 3–21 days. Relative contents of collagen components were determined by densitometry of the developed film (8).

RESULTS AND DISCUSSION

Fractionation and Identification of Isolated Non-parenchymal Cells

Fractions containing non-parenchymal cells were prepared after removal of parenchymal cells by low speed centrifugation. Non-parenchymal cells were further fractionated by Percoll density gradient centrifugation, and fractions S1–S4 were prepared as described (5, 7). Fraction S1 contained cell debris. Fraction S2 (yield, 4.5×10^4 to 1.5×10^5 cells/g wet weight liver; viability, $87.4\pm3.9\%$) contained small cells, some of which contained lipid droplets. Some cells in the fraction S2 emanated rapidly fading green autofluorescence of vitamin A under a fluorescence microscope. Fraction S3 contained endothelial-like and macrophage-like cells. Few lipid-containing cells were observed in this fraction. Fraction S4 contained only erythrocytes (5, 7).

Distribution of stellate cells in fractions S1–S4 was examined by counting the number of cells carrying lipid droplets in the cytoplasm in the total number of cells retaining the nucleus in a thick section. As high as $70.7\pm2.2\%$ of cells in the fraction S2, round in shape when observed by fixation immediately after isolation and precipitation in the beem capsule (Fig. 1), contained lipid droplets in the cytoplasm. The nuclei in these cells were indented by the lipid droplets in the cytoplasm (Fig. 1).

Histochemical observations suggest that vitamin A is concentrated in these lipid droplets (17). The ultrastructure of the cytoplasm of the isolated cells was quite similar to that of cells *in vivo* (*cf.* 18). A cell fraction showing similar characteristics was recently isolated by centrifugal elutriation (12), although cell activity in terms of collagen synthesis was not shown.

Cells in fractions S2 and S3 proliferated in DMEM containing 10% fetal bovine serum. Morphology of the cultured cells was examined by a phase contrast microscope. Cells in the fraction S2 showed a fibroblastic, but flat shape with well-developed processes (Fig. 2A). No epithelial-like cells were observed in this fraction and production of serum albumin, which is a typical function of liver parenchymal cells, was not detected (9). Cells in the fraction S3 were round or oval (Fig. 2B). Larger cells (L in Fig. 2B) were quite similar to the macrophages and smaller cells (S in Fig. 2B) were identified as endothelial cells by their morphology.

Uptake of [^{3}H]Retinol by the Liver Cells

When [^{3}H]retinol was injected via portal vein, the largest amount of the labeled retinol was taken up by the liver within 90 min after injection, although the labeled material was detected in all organs examined (Table 1). The radioactivity of the retinol in the liver did not change until 6 days after the injection. The high content of [^{3}H]retinol in liver can not be explained by intrahepatic blood, because the liver was perfused before

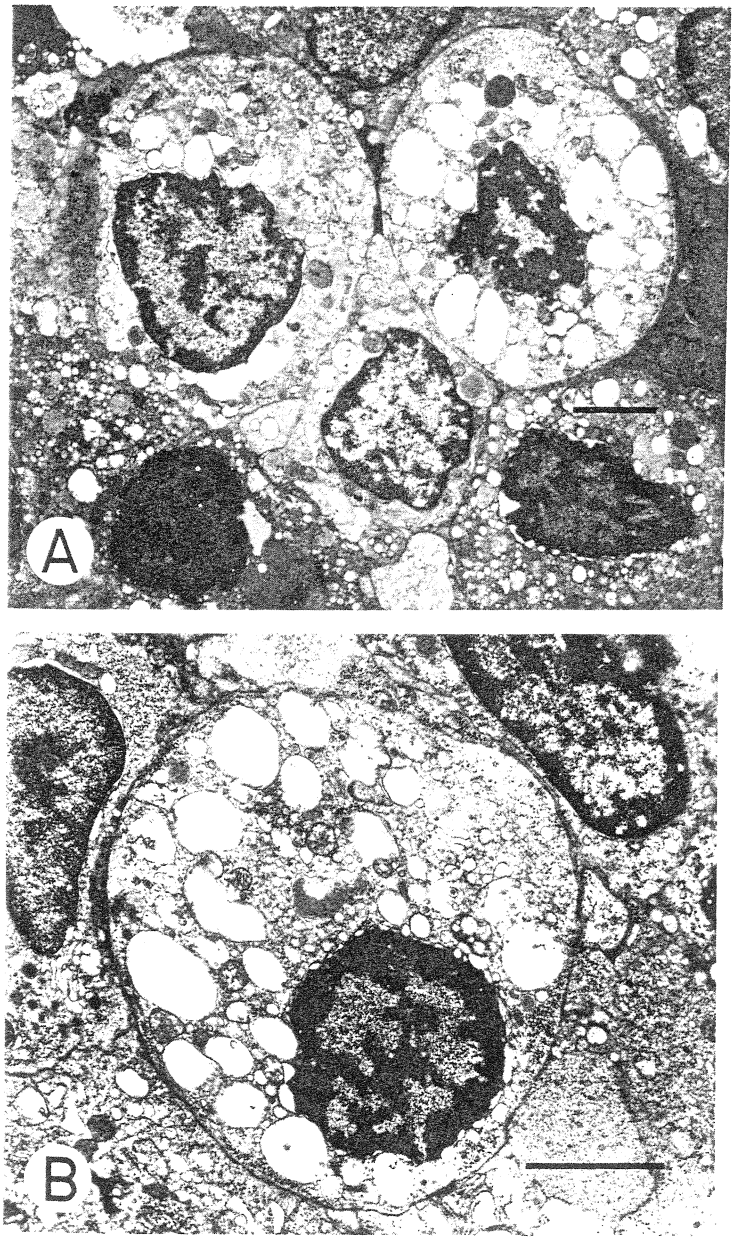

Fig. 1 Electron micrographs of isolated perisinusoidal stellate cells from the liver of male Wistar rat. The characteristic vitamin A lipid droplets are seen in the cytoplasm (A). Nucleus is relatively large and indented by the lipid droplets; other organelles are also compressed by the many large and small lipid droplets (B). The bar represents 3 μm.

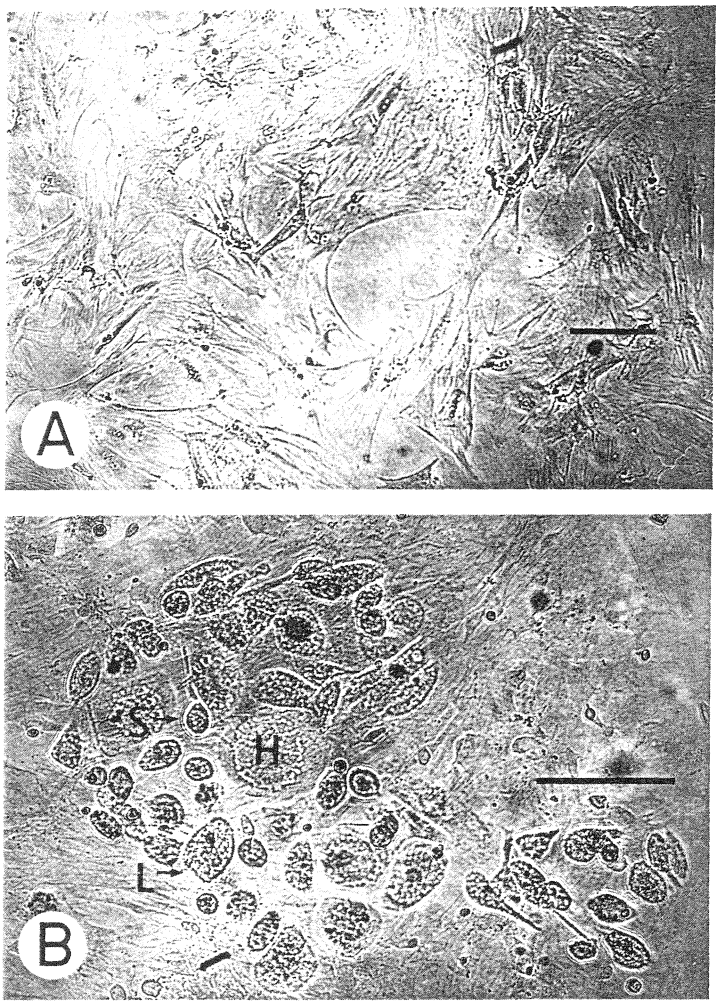

Fig. 2 Phase contrast photomicrographs of cultured liver cells. A: Fibroblast-like cells in the fraction S2 after 14 days of primary culture. B: Cells in the fraction S3 after 14 days of primary culture. No fibroblast-like cells are seen. Round cells (L) are macrophage-like cells, and smaller oval cells (S) are endothelial cells. A large round cell (H) is a degenerated parenchymal cell. The bar represents 20 μm.

sampling and retinol content in the blood was low (Table 1). These results were consistent with the report that the main storage site of vitamin A in mammals is the liver (3).

In order to examine the distribution of retinol in the liver, radioactivity per cell was determined after cell fractionation (Table 2). A major part of radioactivity was recovered in the parenchymal cell fraction 90 min and even 6 days after injection. However, specific activity of [^{3}H]retinol (per cell) was the highest in the stellate cell-rich fraction S2, both 90 min and 6 days after injection. These results strongly support earlier morphological observations (10, 17) that the stellate cell is the storage site of vitamin A in the liver, and are not inconsistent with a recent report on the retinol transfer from hepatocytes to stellate cells (1).

Fraction S3, composed mainly of Kupffer cells and endothelial cells, also contained [^{3}H]retinol. These cells may participate in vitamin A metabolism, although the cells do not store vitamin A as lipid droplets in the cytoplasm (data not shown).

Table 1 *Distribution of [^{3}H]Retinol in Various Rat Organs, Serum, and Feces after Intraportal Injection*

Organ	Radioactivity (dpm×10^{-5}/g wet weight)	
	90 min	6 days
Liver	206.6±3.7	190.8±0.7
Spleen	13.8±0.4	28.9±0.1
Lung	28.4±0.6	4.5±0.1
Intestine	ND	1.9±0.1
Skin	0.6±0.1	1.4±0.1
Brain	ND	1.3±0.1
Kidney	6.0±0.1	1.0±0.1
Heart	ND	0.9±0.1
Serum[a]	5.8±0.1	1.4±0.1
Feces	ND	0.4±0.1

Animals were sacrificed 90 min or 6 days after injection of 200 μCi of [^{3}H]retinol through the portal vein. Radioactivity was counted as described under Materials and Methods. The values are the mean of two assays ± deviation. ND: not determined. [a]Expressed as dpm/ml

Collagen Synthesis by Non-parenchymal Cells

In order to elucidate cell type or types responsible for collagen metabolism among non-parenchymal cells in the liver, collagen production by proliferative cells from fractions S2 and S3 was analyzed using a cell culture system.

Cells in the fraction S2 were found to produce collagen on day 8 in primary culture, although collagen production was not indicated at an earlier stage of culture (day 2). Capability of collagen production by cells was retained in the secondary culture (Table 3), suggesting that the stellate cell is a candidate cell responsible for collagen production. Cells in the fraction S3, composed mainly of macrophages and endothelial cells, produced little collagen either on day 2 or day 8 in primary culture under the conditions employed.

Types of collagen produced by cells in the fraction S2 in secondary culture were analysed by fluorography after SDS-polyacrylamide slab gel electrophoresis under non-reducing and reducing conditions (Fig. 3). Type I collagen is the major component synthesized (Fig. 3a). Minor components include type III collagen which remained at the γ-region under non-reducing condition (Fig. 3a), but migrated to the α1-region after reduction (Fig. 3b), and membranous collagen which remained slightly below the origin under non-reducing condition (Fig. 3a), but migrated to a region slightly lower than the β-region after reduction (Fig. 3b). All these bands were susceptible to purified bacterial collagenase (Fig. 3, c and d). Quantitation of these collagen bands by densitometry indicated that the percentage of type I, type III and membranous collagens was 88.2%, 10.4% and 1.4%, which is consistent with an observation on collagen types in human alcoholic liver cirrhosis (16).

General Discussion

Our previous studies on collagen metabolism in the liver have shown that hepatic parenchymal cells produce collagen *in vivo* (5) and *in vitro* (4, 6–9). In this study, we have investigated the cell type responsible for collagen production among non-parenchymal cells in the liver, and demonstrated that a cell fraction rich in lipid droplet-containing cells (stellate cells) is the candidate for collagen production. Neither Kupffer cells nor endothelial cells produced collagen under the conditions employed. Thus, both parenchymal

Table 2 *Distribution of [^{3}H]Retinol in Various Liver Cell Fractions*

Cell fraction	Radioactivity / Fraction (dpm×10^{-3})		Cell number×10^{-6} / Fraction		Radioactivity / 10^6 Cells (dpm×10^{-3})	
	90 min	6 days	90 min	6 days	90 min	6 days
Non-parenchymal cells (Density: g/ml)						
S1 (1.02–1.04)	805 ± 10	658 ± 36	ND	ND	–	–
S2 (1.04–1.07)	1,070 ± 8	503 ± 10	1.3	3.4	823± 6	148±3
S3 (1.07–1.08)	86 ± 9	109 ± 2	0.11	4.2	782±82	22±1
S4 (1.08–1.14)	36 ± 4	70 ± 5	ND	ND	–	–
Parenchymal cells	17,044±980	6,828±232	240	110	71± 4	62±2

Animals were sacrificed 90 min or 6 days after injection of 200 μCi of [^{3}H]retinol, and liver cells were separated by low-speed centrifugation followed by Percoll density gradient centrifugation. The values are the mean of two determinations ± deviation. ND: not determined

Table 3 *Biosynthesis of Collagen by Rat Liver Non-parenchymal Cells in Culture*

Cell fraction	Days in	Collagen (dpm×10^{-4}/dish)	Non-collagenous proteins (dpm×10^{-4}/dish)	Rate of collagen synthesis[a] (%)
	Primary culture			
S2	2	—[b]	27.2±0.7	0
S3	2	—[b]	25.3±0.1	0
S2	8	3.97±0.1	13.8±0.3	5.1±0.2
S3	8	—[b]	1.2±0.1	0
	Secondary culture			
S2	10	7.75±0.4	14.5±0.4	9.0±0.7
S2	12	8.03±0.1	12.4±0.2	10.7±0.3

Cells were cultured in medium containing [^{3}H]proline for 18 h and synthesis of proteins was determined with protease-free bacterial collagenase. The values are the mean of duplicate determinations ±deviation. [a]Calculated assuming that collagen contains imino acid 5.4 times higher than in other proteins. [b]Not detected

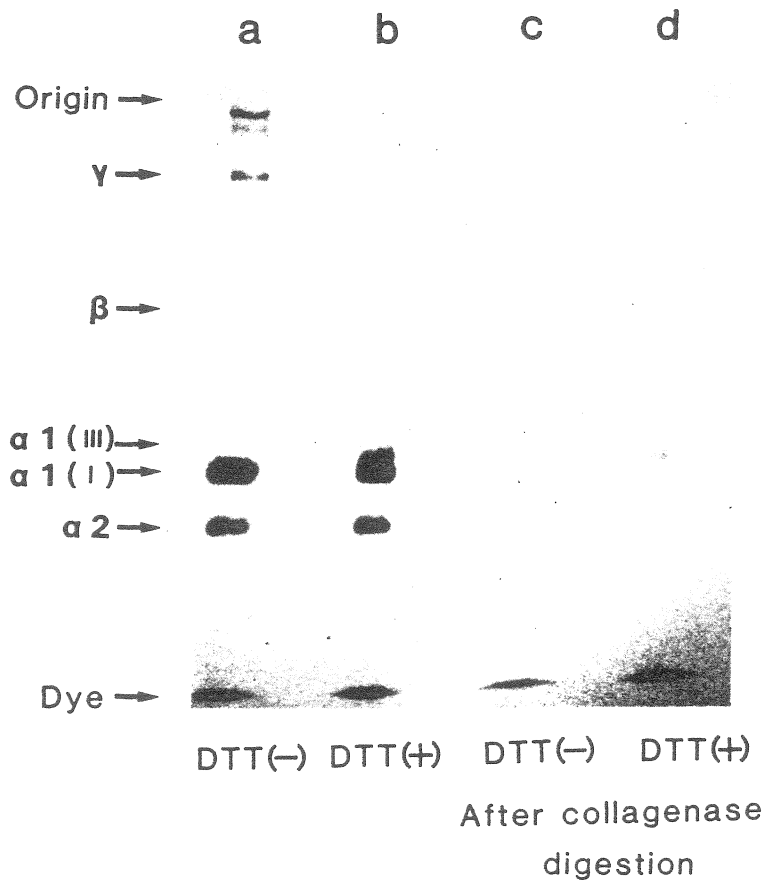

Fig. 3 Fluorescence autoradiograms of [^{3}H]proline-labeled proteins. Cells in the fraction S2 at confluency in the secondary passage were cultured in a medium containing [^{3}H]proline for 18 h. Collagenous proteins were partially purified from the cell layer and medium by pepsin digestion after precipitation with ammonium sulfate. SDS-Polyacrylamide slab gel electrophoresis was performed before (a, b) or after (c, d) the treatment with purified bacterial collagenase. b and d: Electrophoresed after reduction with dithiothreitol. Arrows indicate the migration positions of carrier rat collagen chains.

cells and stellate cells can produce collagen *in situ* under normal conditions. However, which cell type is primarily responsible for hepatic fibrosis under pathological conditions such as chronic hepatitis and liver cirrhosis remains to be further elucidated.

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