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ORIGINAL ARTICLE

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Immunohistochemical identification of Ito cells and their myofibroblastic transformation in adult human liver

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Abstract To identify Ito cells in normal and pathological adult human livers, immunohistochemical studies were performed by the avidin-biotin-peroxidase complex method using monoclonal antibodies for α-smooth muscle actin (ASMA), desmin, and vimentin. Fifty one needle biopsies, 7 surgically resected specimens, and 5 autopsy specimens were studied. In the normal adult liver vascular smooth muscle cells and pericytes, together with perisinusoidal cells with thin cytoplasmic processes were positive for ASMA. These latter cells formed a loose and discontinuous layer along the sinusoidal walls. Immunoelectron microscopy showed that the ASMA-positive perisinusoidal cells were Ito cells containing fat droplets. The other sinusoidal lining cells were negative for ASMA. In chronic liver disease, ASMA-positive Ito cells showed an increase in number, size, and the intensity of immunostaining in areas of piecemeal necrosis), and formed a continuous cellular network. These cells were dendritic in shape with irregularly elongated cytoplasmic processes and contained an increased amount of microfilaments, in association with loss of the characteristic fat droplets. Thus, their ultrastructural features corresponded to those of myofibroblastic cells. Ito cells showed no staining for desmin in both normal and pathological livers. These results indicate that immunohistochemistry using an anti-ASMA antibody is a sensitive and reliable method for the identification of both normal and transformed Ito cells in adult human livers.

Key words Adult human liver · Ito cell
Myofibroblast · Immunocytochemistry
α-Smooth muscle actin

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Introduction

Hepatic Ito cells have three major physiological functions: vitamin A storage in their cytoplasmic fat droplets, production of extracellular matrix in the space of Disse, and regulation of the sinusoidal microcirculation (Ito and Shibasaki 1968; Wake 1980, 1988). However, after the liver cells are injured by any agent, the Ito cells in the affected sites promptly proliferate and show myofibroblastic transformation (Gressner 1991; Ramadori 1991). These transformed cells are extensively involved in the production of extracellular matrix. Recent molecular biology studies have detected mRNA for type I, III, and IV collagen as well as fibronectin and laminin in the total RNA extracted from Ito cells (Greenwel et al. 1991; Friedman et al. 1992) and myofibroblastic Ito cells at 12 days of culture show stronger expression of mRNA for collagen type III than quiescent Ito cells at 4 days of culture (Friedman et al. 1992). These studies have suggested that transformed Ito cells play a central role in hepatic fibrosis. In rat and mouse livers, Ito cells are consistently positive for desmin, which is one of the intermediate filament proteins (Yokoi et al. 1984; Burt et al. 1986; Tsutsumi et al. 1987). Therefore, they can be easily identified in vivo or in vitro by immunohistochemistry using a monoclonal antibody against desmin. Using this convenient and highly reproducible method, the importance of Ito cells in experimental liver fibrosis has been clarified (Ogawa et al. 1986; Ballardini et al. 1988; Yokoi et al. 1988). However, various studies (Ballardini et al. 1988; Nouchi et al. 1991; Schmitt-Gräff et al. 1991; Yamaoka et al. 1993; Enzan et al. 1994) have shown that Ito cells in the adult human liver are negative for desmin. This has caused considerable difficulty in identifying Ito cells in patients with liver disease (Enzan et al. 1992). To solve this problem, we utilized a monoclonal antibody directed against α-smooth muscle actin (ASMA). In the present study, we demonstrated that ASMA is a useful phenotypic marker for both normal and transformed Ito cells in the adult human liver.

Materials and methods

Needle biopsies and surgically resected liver tissues were obtained from 56 patients with chronic liver disease (CLD), including 11 with chronic persistent hepatitis, 26 with moderate chronic aggressive hepatitis (CAH-2A), 14 with severe chronic aggressive hepatitis (CAH-2B), and 5 with liver cirrhosis. Two CAH-2A patients and 3 cirrhosis patients also had hepatocellular carcinoma. There were 36 men and 20 women aged 23–74 years. Four of them were seropositive for hepatitis B surface antigen and 52 were positive for hepatitis C antibody.

Normal control liver tissue with no morphological abnormalities was obtained from 5 adult autopsy patients as well as from the peritumour regions of the surgical specimens from 2 patients with hepatic haemangiomas. All the control patients had normal liver function tests. Informed consent was obtained from each patient and/or the family members.

Part of each liver specimen was routinely prepared for light microscopy. Diagnoses were made on the basis of the histological findings and the clinical information. Chronic hepatitis was classified as proposed by the European Association for the Study of the Liver (Groote et al. 1968).

Small portions of 51 needle biopsies and 7 surgically resected liver specimens were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 h at 4° C and post-fixed in 1.5% osmium tetroxide in PB for 2 h at 4° C, followed by dehydration and embedding in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM 100S electron microscope.

Deparaffinized and rehydrated sections of formalin-fixed, paraffin-embedded liver tissues were incubated in 10% normal rabbit serum (Seikagaku Kougyou Corporation, Tokyo) for 30 min. Subsequently, they were incubated for 1 h at room temperature with a 1:50 dilution of monoclonal antibodies for ASMA (anti-ASMA), desmin and vimentin (all antibodies from DAKO, Glostrup, Denmark). After rinsing with 0.01 M phosphate-buffered saline (PBS, pH 7.2), the sections were incubated for 1 h at room temperature with a 1:200 dilution of biotinylated rabbit anti-mouse IgG $F(ab')_2$ fragment (DAKO) instead of biotinylated anti-mouse IgG (Vectastain, Vector Laboratories, Burlingame, Calif., USA) in order to enhance penetration of the immunoreagent. After blocking of endogenous peroxidase with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and rinsing with PBS, the sections were incubated for 45 min at room temperature in the avidin biotin complex (ABC) solution (Vectastain kit, Vector Laboratories).

Reaction products were then visualized by incubation in 0.05 M TRIS buffer (pH 7.6), with 0.5% 3,3'-diaminobenzidine tetrachloride and 0.1% hydrogen peroxide for 2 min at room temperature. After rinsing with tap water, the sections were counterstained with haematoxylin and mounted in Eukitt (Kindler, Freiburg, Germany).

Liver tissues from 56 CLD patients and the two hepatic haemangioma patients were fixed by immersion in periodate-lysine-paraformaldehyde solution for 18 h at 4° C. Then, sections 50 μm thick were cut on a vibratome and washed in cold PBS, followed by incubation for 18 h at 4° C in PBS containing a 1:50 dilution of mouse anti-ASMA. The subsequent procedures were performed as described above for immunohistochemistry, except for additional pre-fixation of the vibratome sections in 0.5% glutaraldehyde for 10 min at room temperature after incubation in the ABC solution and omission of the blocking of endogenous peroxidase. After immunostaining and rinsing with PBS, the sections were post-fixed in 2.5% osmium tetroxide in PB for 1 h at 4° C. Small fragments of the sections were then routinely processed and embedded in epoxy resin, followed by the procedures described above for electron microscopic examination.

As a negative control for immunostaining, sections were incubated in normal mouse serum at the same concentration as that of the primary antibodies.

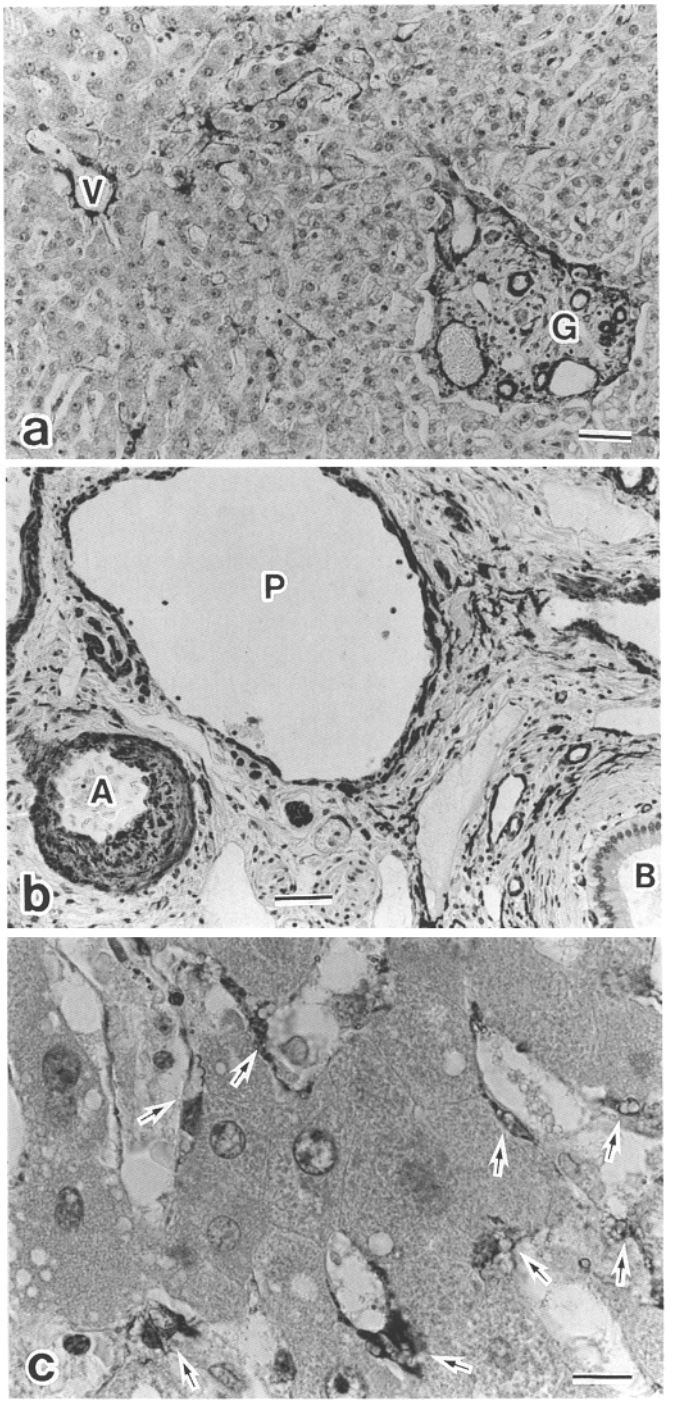

Fig. 1a–c α-Smooth muscle actin (ASMA)-positive cells in an autopsy specimen of adult human liver without any morphological abnormalities. **a** Positive cells can be seen in the portal tract (*G*), perivenular space (*V*), and liver lobule. *Bar* = 50 μm, × 140. **b** In the portal tract, smooth muscle cells of the interlobular artery (*A*) and portal vein (*P*) as well as the pericytes are strongly stained. Most of the fibroblasts are negative, although a few spindle-shaped stromal cells are weakly positive. *B*: septal bile duct. *Bar* = 10 μm, × 700. **c** Within the liver lobules, ASMA-positive cells form discontinuous single strands along the sinusoidal walls and contain some vacuoles (*arrows*). *Bar* = 10 μm, × 700

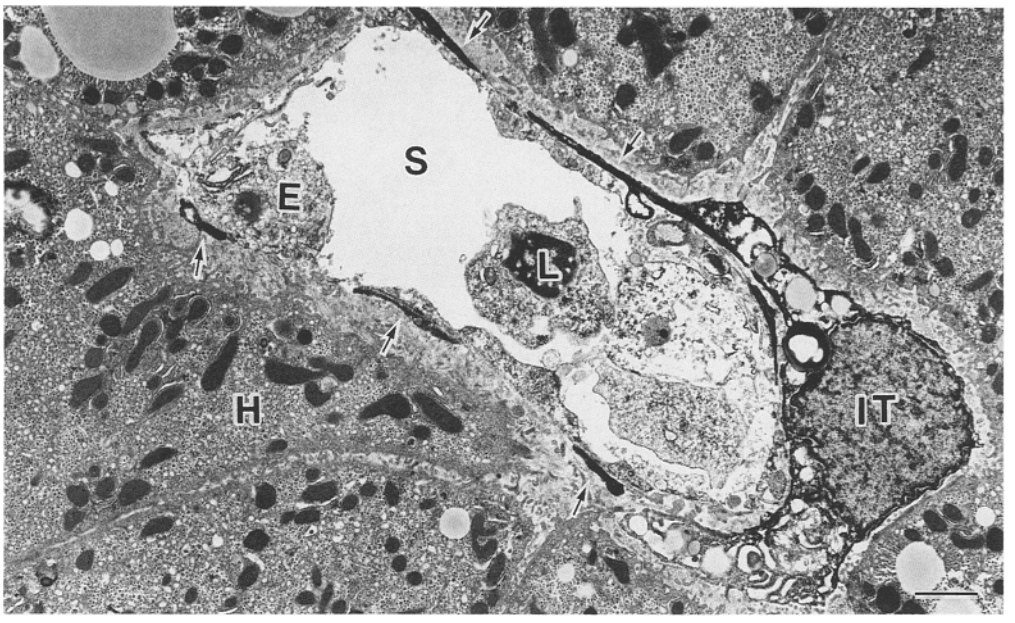

Fig. 2 Immunoelectron micrograph of an ASMA-positive Ito cell (*IT*) in normal adult human liver. The cell contains several fat droplets and its cytoplasmic processes (*arrows*) are intensely labelled. In contrast, a sinusoidal endothelial cell (*E*) and a lymphocyte (*L*) in the sinusoid (*S*) are negative for ASMA. *H*: hepatocyte. *Bar* = 2 μm, × 3700

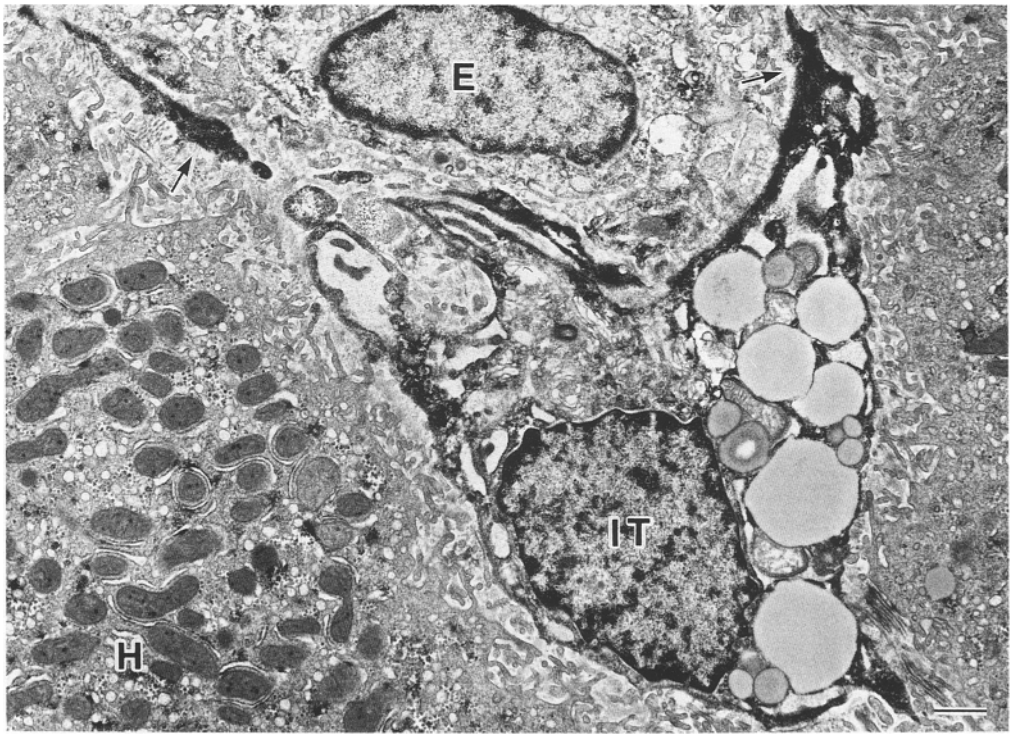

Fig. 3 A higher magnification of an ASMA-positive Ito cell (*IT*) in normal adult human liver. A considerable part of the cytoplasm is filled with fat droplets. Reaction products are predominantly deposited in the peripheral cytoplasm and the cell processes (*arrows*). *E*: sinusoidal endothelial cell, *H*: hepatocyte. *Bar* = 1 μm, × 6600

Results

In normal adult human liver ASMA-positive cells were found not only in the portal tracts and perivenular spaces, but also around the hepatic veins and within the liver lobules (Fig. 1a). In the portal tracts, smooth muscle cells of both the interlobular arteries and the portal vein branches were intensely stained (Fig. 1b). The pericytes around the venules and capillaries were also positive for ASMA. Vascular smooth muscle cells showed the strongest staining among the ASMA-positive cells in the liver, so the immunostaining of these cells was used as a positive control. In addition, a few spindle-shaped stromal cells were positive for ASMA. They were located around the bile ducts and scattered freely in the connective tissue of the portal tracts. However, the fibroblasts at these sites were usually negative for ASMA. The central veins and the hepatic veins were surrounded by one to several layers of ASMA-positive cells, but neither the endothelial cells nor the biliary epithelial cells were positive for ASMA.

Within the liver lobules, ASMA-positive spindle cells

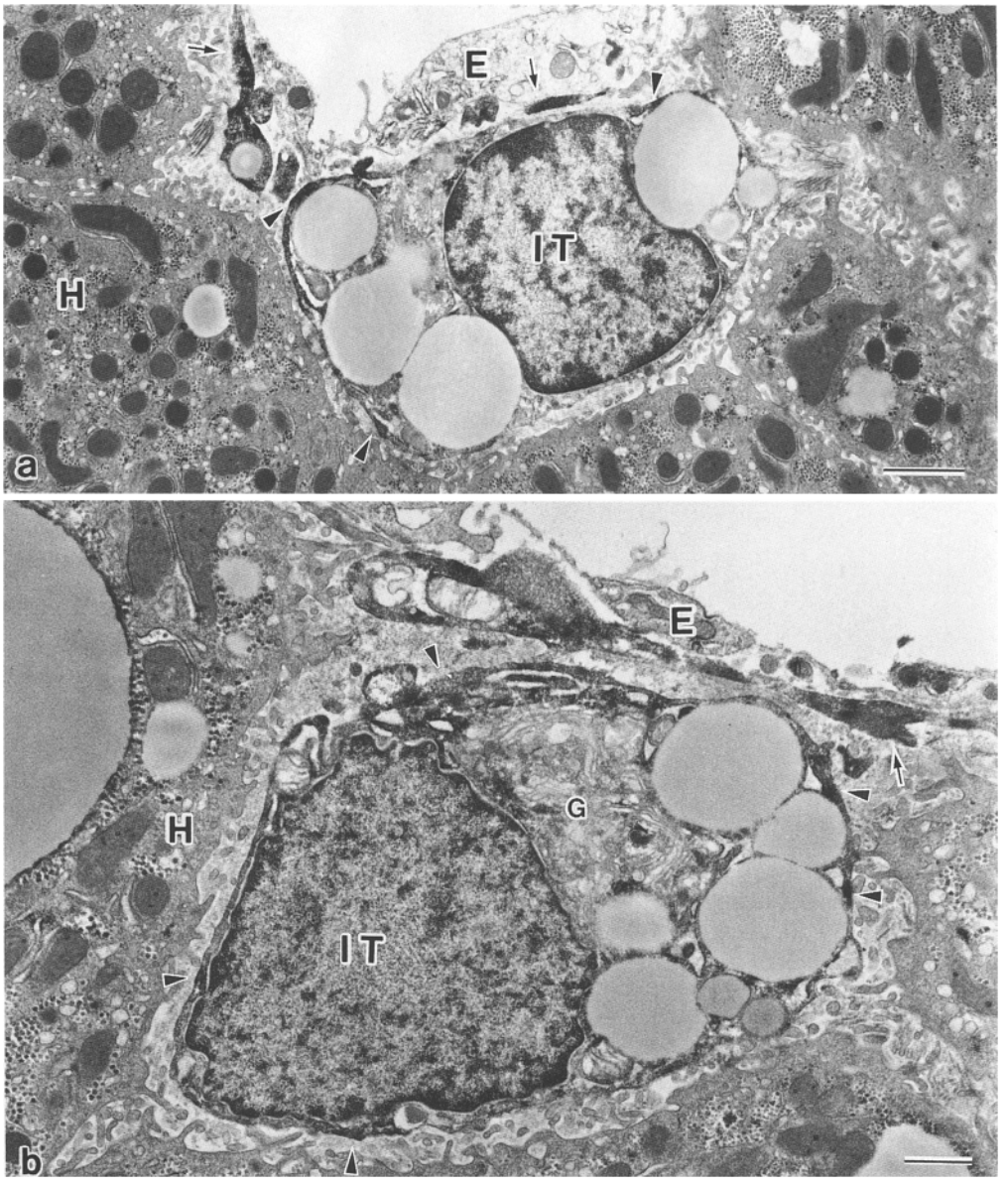

Fig. 4a, b ASMA-positive Ito cells in the interhepatocytic recesses of normal adult human liver. Fat droplets occupy the major part of the cytoplasm (**a** and **b**) and immunostaining for ASMA is localized to small peripheral areas (*arrowheads*). *E*: sinusoidal endothelial cell, *G*: Golgi complex, *H*: hepatocyte, *IT*: Ito cell, *arrows*: ASMA-positive cytoplasmic processes of Ito cells. **a** *Bar* = 2 μm, × 5300, **b** *Bar* = 1 μm, × 8300

with long, thin cytoplasmic processes were seen along the sinusoidal walls. They appeared to be located in the subendothelial spaces and formed a loose, discontinuous network with an uneven distribution. The cytoplasm of these cells contained a few small vacuoles (Fig. 1c). Immunoelectron microscopy demonstrated that the perisinusoidal ASMA-positive cells were exclusively Ito cells, containing a few characteristic fat droplets (0.5 to 3.5 μm in diameter) (Fig. 2). The other types of sinusoidal lining cells, such as sinusoidal endothelial cells and Kupffer cells, as well as the liver parenchymal cells, were all negative for ASMA. The ASMA-positive Ito cells showed more marked deposition of reaction products in the peripheral cytoplasm than in the central cytoplasm (Fig. 3). The Golgi complex and centriole were frequently located in the latter portion. Some of the ASMA-positive Ito cells were filled with fat droplets and they showed immunostaining for ASMA only in a narrow rim of perikaryonic cytoplasm (Fig. 4). The cytoplasmic processes were usually 0.2 to 2 μm wide and contained numerous microfilaments along with very few other organelles. These processes were always strongly labelled.

The antibody for desmin failed to stain any type of sinusoidal lining cells, including the Ito cells (data not shown), and only the vascular smooth muscle cells were positive. However, they were less frequently and less intensely stained, when compared with the reaction for ASMA. Almost all of the mesenchymal cells, especially the endothelial cells of the portal tracts, were positive for vimentin (data not shown).

In cases of chronic liver disease, in the areas of piecemeal necrosis (PMN), ASMA-positive perisinusoidal cells were increased in number and formed a continuous

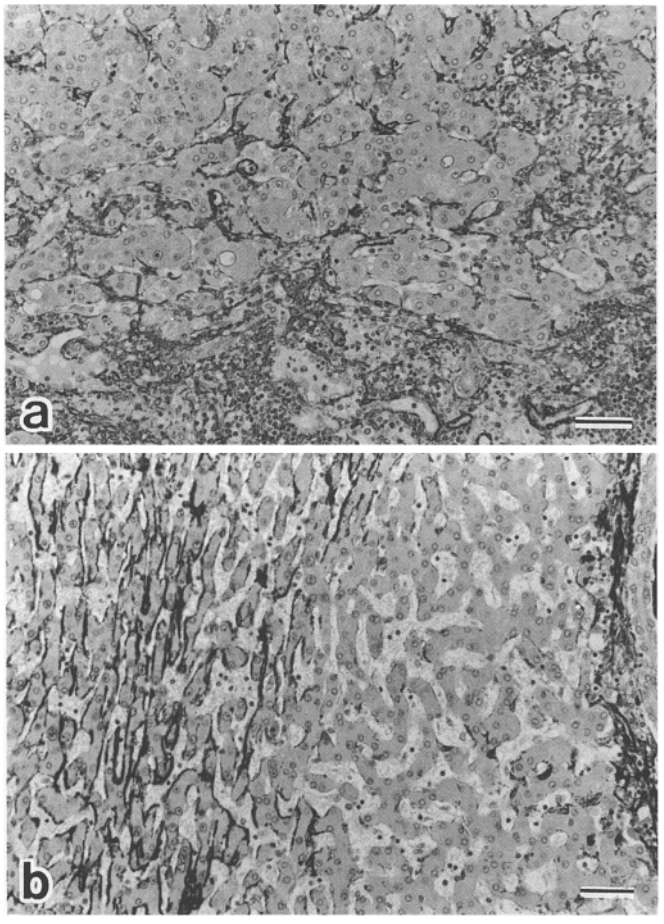

Fig. 5a, b ASMA-positive Ito cells in a region of PMN in a patient with CAH-2B (**a**) and in a region of liver cell atrophy adjacent to a hepatocellular carcinoma (**b**). The Ito cells form a continuous network in both cases. *Bar* = 50 μm, × 140

network (Fig. 5a). These ASMA-positive perisinusoidal cells appeared to be larger and more irregular than those in the normal adult livers, and the extent of these changes was in parallel with the level of inflammatory activity. In the congested areas and in peritumour atrophic liver tissue (Fig. 5b), the perisinusoidal cells showed increased staining for ASMA, despite the absence of any recognizable hepatocyte necrosis at these locations. Electron microscopy showed an increase of microfilaments which were mainly found in the peripheral cytoplasm and arranged in parallel with the long axis of the cell. Subplasmalemmal dense areas were also frequently seen (Fig. 6). Immunoelectron microscopy showed that the ASMA-positive Ito cells in the areas of PMN contained few or no fat droplets, and there was a corresponding increase of microfilaments. Reaction products for ASMA were deposited predominantly on the microfilaments (Fig. 7). The ASMA-positive transformed Ito cells featured irregular cytoplasmic processes up to 30 μm long and showed an elongated and dendritic configuration (Fig. 8). In marked contrast to these significant changes of the Ito cells in the regions of PMN, the Ito cells located away from the sites of liver cell damage showed similar immunohistochemical and ultrastructural features to those in the normal adult liver.

The desmin or vimentin staining of Ito cells in the regions of PMN was similar to that in normal adult liver.

In addition, the control sections constantly displayed a negative reaction.

Discussion

The use of an antibody directed against ASMA, an actin isoform typically found in smooth muscle cells (Skalli et al. 1986, 1987, 1989), allows the identification of Ito

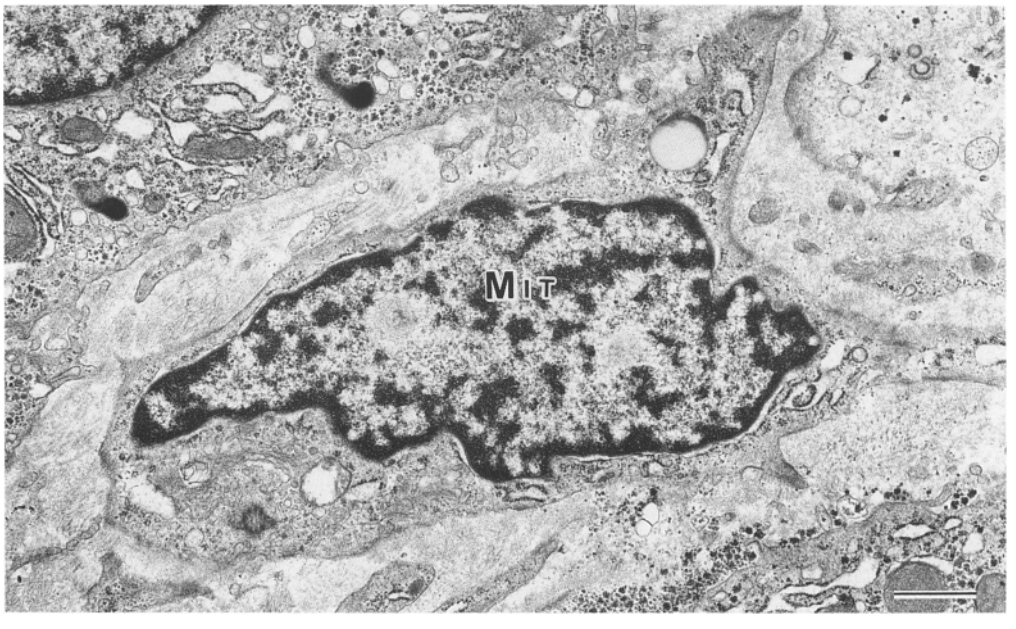

Fig. 6 Electron micrograph of a myofibroblastic Ito cell in a region of PMN. The cell shows the loss of its characteristic fat droplets and a concomitant increase in microfilaments, especially in the subplasmalemmal region. *Bar* = 1 μm, × 11000

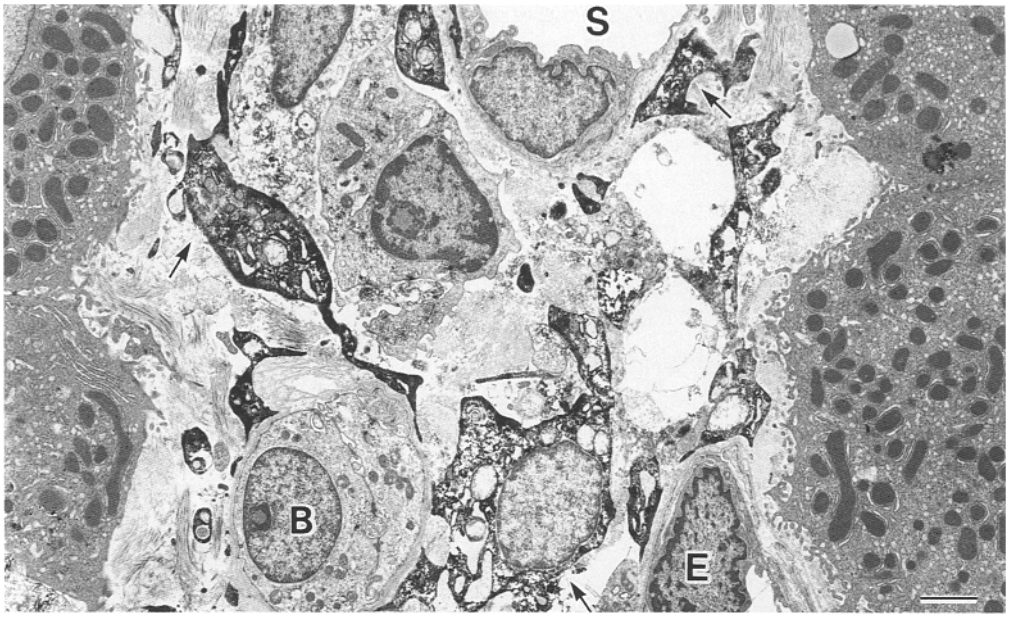

Fig. 7 Immunoelectron micrograph of ASMA-positive myofibroblastic Ito cells (*arrows*) in an area of piecemeal necrosis. They are intermingled with various infiltrating inflammatory cells and newly synthesized collagen fibrils. *Bar* = 2 μm, × 3700

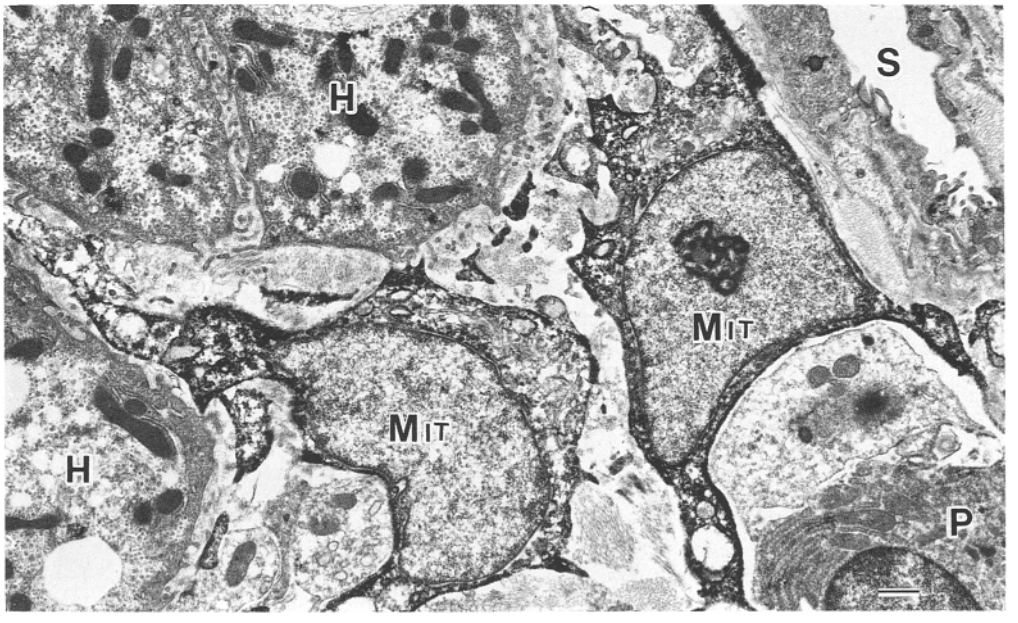

Fig. 8 A higher magnification of myofibroblastic Ito cells (*MIT*) with a dendritic and elongated configuration. *H*: hepatocyte, *P*: plasma cell, *S*: sinusoid. *Bar* = 1 μm, × 5500

cells in the adult human liver, based on the fact that these cells belong to the same lineage as pericytes (Wake 1988) which are always positive for ASMA (Toccanier-Pelte et al. 1987; Skalli et al. 1989; Kapanci et al. 1992). In this study, we demonstrated consistently the presence of ASMA-positive perisinusoidal cells in normal adult human liver. The morphology and location of these ASMA-positive perisinusoidal cells was easily recognizable by light microscopy, but the cell type could not be identified precisely. However, immunoelectron microscopic examination revealed clearly that only the Ito cells were positive for ASMA among all types of sinusoidal lining cells. The Ito cells contained a few characteristic fat droplets (0.5 to 3.5 μm in diameter) and their morphological features corresponded all to those of the ASMA-positive perisinusoidal cells containing vacuoles seen at the light microscopic level. Nouchi et al. (1991), Schmitt-Gräff et al. (1991), and Yamaoka et al. (1993) have recently reported that the perisinusoidal cells of adult human livers show staining for ASMA. However, Nouchi et al. (1991) only investigated diseased livers. In the normal adult human liver, Schmitt-Gräff et al. (1991) rarely found ASMA-positive perisinusoidal cells (Ito cells). However, Yamaoka et al. (1993) detected these cells in a diffuse distribution throughout the lobule. Both studies used the same anti-ASMA antibody (Yamaoka et al. 1993), but there were some methodological differences as described in Table 1. Although the cause of the

Table 1 α-Smooth muscle actin-positive Ito cells in the normal adult human liver (*ABC* avidin biotin complex; *ASMA* α-smooth muscle actin; *PLP* periodate-lysine paraformaldehyde)

Author	Materials		Methods	Findings
	Liver specimens	Diagnosis of "normal" liver		
Schmitt-Gräff et al. (1991)	Six liver specimens: no description of the source	Histological features correlated with clinical information	Immunohistochemistry cryostat sections (immunofluorescence) formalin-fixed and paraffin-embedded sections (ABC method)	Positive cells were rare: periportal zone +/− intermediate zone +/− perivenular zone +/−
Yamaoka et al. (1993)	Four liver specimens: obtained at surgery ($n=3$) from two patients with suspected gallbladder carcinoma and one patient with insulinoma, and at laparoscopy ($n=1$) from one patient with a minimal increase of transaminase	Histological features	Immunohistochemistry cryostat sections (ABC method)	Diffuse distribution throughout the lobule: much less in the periportal zone than in the pericentral and intermediate zones
Present study	Seven liver specimens: specimens from autopsied adult patients ($n=5$) and the peritumoral portions of resected hemangiomas ($n=2$)	Histological features correlated with clinical information	Immunohistochemistry formalin-fixed and paraffin-embedded sections (ABC method)	Formation of a loose, discontinuous cellular network along the sinusoidal wall, uneven distribution in the lobule
			Immunoelectron microscopy vibratome sections of PLP-fixed sections (ABC method)	Ito cells containing several fat droplets were positive for ASMA.

marked discrepancy in the number of ASMA-positive Ito cells detected in these earlier studies remains unknown, our present study shows a loose, discontinuous network with an uneven distribution of ASMA-positive Ito cells. We demonstrated by immunoelectron microscopy that Ito cells containing several fat droplets and even those filled with droplets were positive for ASMA. By immunohistochemical studies, it may be difficult to detect Ito cells with immunostaining for ASMA in only a small portion of the cytoplasm, as shown in Fig. 4. In summary, the Ito cells in normal adult human livers are positive for ASMA, even though they show no myofibroblastic transformation.

Myofibroblastic transformation of the Ito cells in areas of PMN was characterized ultrastructurally by a decrease or loss of fat droplets and a significant increase of microfilaments. Our immunohistochemical study of perisinusoidal cells in the areas of PMN showed an increase in the intensity of immunostaining for ASMA and an increase in the number of positive cells. These cells were swollen, elongated, and formed a continuous network along the sinusoidal walls. These hyperplastic and hypertrophic ASMA-positive perisinusoidal cells in the areas of PMN appeared to correspond to Ito cells that had undergone myofibroblastic transformation (Gressner 1991; Ramadori 1991). The strong labelling of the peripheral cytoplasm and cytoplasmic processes rich in microfilaments suggests that these microfilaments were chiefly composed of ASMA filaments. Schmitt-Gräff et al. (1991) used immunoelectron microscopy with immunogold staining, and demonstrated intense labelling for ASMA of both the microfilament bundles and the cytoplasmic processes of stromal cells in areas of focal nodular hyperplasia. However, the demonstration of only a small part of these ASMA-positive stromal cells made it difficult to identify the nature of these cells. For the identification of Ito cells, it is necessary to demonstrate their topographical relationship to adjacent structures, especially to the liver parenchymal cells and sinusoids, as well as the ultrastructural features of the whole cell. The ABC method is suitable for this purpose, although the strong deposition of reaction products partly obscures the ultrastructural details.

Until now, the morphological features of myofibroblastic Ito cells have been only described ultrastructurally in various liver diseases, such as chronic hypervitaminosis A (Hruban et al. 1974; Farrel et al. 1977), nodular hyperplasia (Callea et al. 1982), alcoholic perivenular fibrosis (Nakano et al. 1982), and cirrhosis (Lamouliatte et al. 1985). Our study showed that myofibroblastic Ito cells were identifiable, even at the light microscopic level, by increased labelling for ASMA and the formation of continuous networks along the sinusoidal walls. They were usually seen in areas of hepatocyte necrosis and

postnecrotic fibrosis. Moreover, our findings suggested that liver cell atrophy and degeneration due to chronic congestion and tumour compression may induce the myofibroblastic transformation of Ito cells.

In conclusion, immunohistochemistry using anti-ASMA can be used for the identification of both normal and myofibroblastic Ito cells in the adult human liver. Thus, the myofibroblastic transformation of Ito cells may be a quantitative rather than qualitative change.

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